Evaluation of Microbial Electrolysis Cells in the treatment of domestic wastewater

Thesis submitted to Newcastle University for the degree of Doctor of Philosophy

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Abstract

Wastewater can be an energy source and not a problem. This study investigates whether rapidly emerging bioelectrochemical technologies can go beyond working in a laboratory under controlled temperatures with simple substrates and actually become a realistic option for a new generation of sustainable wastewater treatment plants.

The actual amount of energy available in the wastewater is established using a new methodology. The energy is found to be considerably higher than the previous measurement, or estimates based on the chemical oxygen demand with a domestic wastewater sample containing 17.8 kJ/gCOD and a mixed wastewater containing 28.7 kJ/gCOD.

With the energy content established the use of bioelectrochemical systems is examined comparing real wastewater to the 'model' substrate of acetate. The abundance of exoelectrogenic bacteria within the sample, and the acclimation of these systems is examined through the use of most probable number experiments. It is found that there may be as few as 10-20 exoelectrogens per 100 mL. The impact of temperature, substrate and inoculum source on performance and community structure is analysed using pyrosequencing. Substrate is found to have a critical role, with greater diversity in acetate fed systems than the wastewater fed ones, indicating that something other than complexity is driving diversity.

Laboratory scale microbial electrolysis cells are operated in batch mode fail when fed wastewater, whilst acetate fed reactors continue working, the reasons for this are examined. However a pilot scale, continuous flow microbial electrolysis cell is built and tested at a domestic wastewater treatment facility. Contrary to the laboratory reactors, this continues to operate after 3 months, and has achieved 70% electrical energy recovery, and an average 30% COD removal.

This study concludes that wastewater is a very complex but valuable resource, and that the biological systems required to extract this resource are equally complex. Through the work conducted here a greater understanding and confidence in the ability of these systems to treat wastewater sustainably has been gained.

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continual inspiration; my husband Oliver, whose moral support, guidance, motivation and good humour has been unfaltering throughout; and lastly I thank my son Ben and his beautiful smile.

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Chapter 1. Introduction

There is growing consensus that wastewater is a resource not a problem (Verstraete and Vlaeminck, 2011, Sutton et al., 2011, McCarty et al., 2011). The conventional treatment of wastewater removes its organic content via aerobic processes, termed activated sludge, this is energy expensive typically 3% of the electrical energy usage of many developed countries (Curtis, 2010). Not only is the energy in wastewater removed not recovered, we expend considerable energy in performing this removal.

In the UK the water sector energy use has increased 10% in the last 10 years (Water UK, 2012, Water UK, 2011), industrial electricity prices have increased by 69% since 2000 (National Statistics, 2011). If these trends continue the energy bill for the water sector will be vastly higher than for the current 9016 GWh (Water UK, 2012). With infrastructure requiring long term planning and capital investment, it is hard to see without drastic action how the necessary changes can be made. Technologies that require relatively simple modifications to the current infrastructure to become operational are more likely to be given a chance rather than those which require wholesale change. New technology should ideally fit reasonably well into the existing infrastructure, and as a minimum achieve similar loading rates per unit area to activated sludge of 0.4-1.2 kg BOD $m^{-3}d^{-1}$ (Grady, 1999). The high capital costs of change and the uncertainty of using a different technology, coupled with the regulation of both effluent quality and pricing structures, are an obstacle to change.

There are alternatives to this approach. Replacing the aerobic activated sludge process with an anaerobic process means the energy stored in the organic content of the wastewater is converted to methane (80% efficiency) which can be combusted to produce electricity (35% efficiency) (McCarty et al., 2011). Only around 30% of the total energy in the wastewater can be captured as electricity in anaerobic systems, although with heat exchange in the combustion process, or the use of non-combustion methods of conversion, this could be increased (McCarty et al., 2011).

The scientific challenges of creating an energy neutral or even energy positive wastewater treatment process are also substantial and complex. The process needed to replace activated sludge must:

- Extract and convert energy to a useable form at an efficiency that justifies the costs.
- Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
- Treat low strength domestic wastewater, which is problematic for anaerobic digestion technologies (Rittmann, 2001).
- Work at ambient, often low temperatures, again problematic for anaerobic digestion (Lettinga et al., 1999).
- Work continuously and reliably.

An innovative and relatively new approach to wastewater treatment is through the use of bioelectrochemical systems (BES), though the fuel cell technology lying behind this process is over 100 years old (Potter, 1911) (see appendix I for a history of development). Here wastewater is consumed in a battery like cell, redox reaction catalysed by bacteria pushing electrons around in an electrical circuit, thus creating electricity (Rabaey et al., 2007). In a microbial fuel cell (MFC) the electricity is captured directly (Logan, 2005), in a microbial electrolysis cell (MEC) the electricity is supplemented by an external source to make a product such as hydrogen or methane (Rozendal et al., 2006) or to perform a process such as reductive dechlorination (Aulenta et al., 2008) or de-salination (Mehanna et al., 2010). There are substantial losses within these systems (Logan et al., 2006), it is suggested they may reach a higher conversion efficiency of 44% (McCarty et al., 2011), the performance of MFCs to date has only reached around 1 tenth of that needed to be competitive with anaerobic digestion (Pham et al., 2006). With MECs the potential higher value (energetically or commercially) of the product formed or process completed means this technology is likely to be more viable and may be the driver of development (Foley et al., 2010).

As organic matter is degraded by bacteria it releases electrons (oxidation) providing energy for the cells. These electrons then pass to an electron acceptor (or reduced species), which is normally oxygen, nitrate or sulphate depending on their availability providing further energy for the cells (Rittmann, 2001).It has been shown that there is a group of organisms that are capable of passing electrons to materials (such as metal oxides) outside the cell, which are then transferred by that material to an electron acceptor. This process is termed electrogenesis, and the group of organisms are known as exoelectrogens (Logan, 2008). MFCs exploit this, providing the bacteria with a

surface to donate electrons to, and then using the principles of all electrochemical cells to transport these electrons and create current.

MFCs, like electrochemical cells usually have two compartments, the anode chamber containing organic matter to be degraded, and the cathode chamber containing an electron acceptor. In the anode chamber organic matter is degraded by bacteria producing electrons, the absence of a preferred electron acceptor such as oxygen, means these electrons pass into the anode material then through a wire to the cathode. The H^+ ions generated in this reaction pass through the membrane from the anode to cathode chamber. At the cathode the electrons, H^+ ions and a reduced species (typically oxygen) combine to form for example H_2O. Electrical current is generated in the wire as the electrons pass from one side to the other.

An MEC reactor is an adaptation of an MFC. In an MEC both the anode and cathode chamber are anaerobic. Rather than creating H_2O in the cathode chamber, the electrons and H^+ ions are combined to generate H_2 gas rather than electricity. The process of forming H_2 is however endothermic, i.e. it requires energy. It cannot happen spontaneously. The addition of a small amount of electricity (with acetate this is in theory 0.114 V, in practice <0.25 V), is required to generate the H_2 gas (Logan et al., 2008). This is substantially less energy than is required to produce H_2 through water electrolysis, typically 1.8-2.0 V. A schematic of an MEC is shown in Figure 1-1.

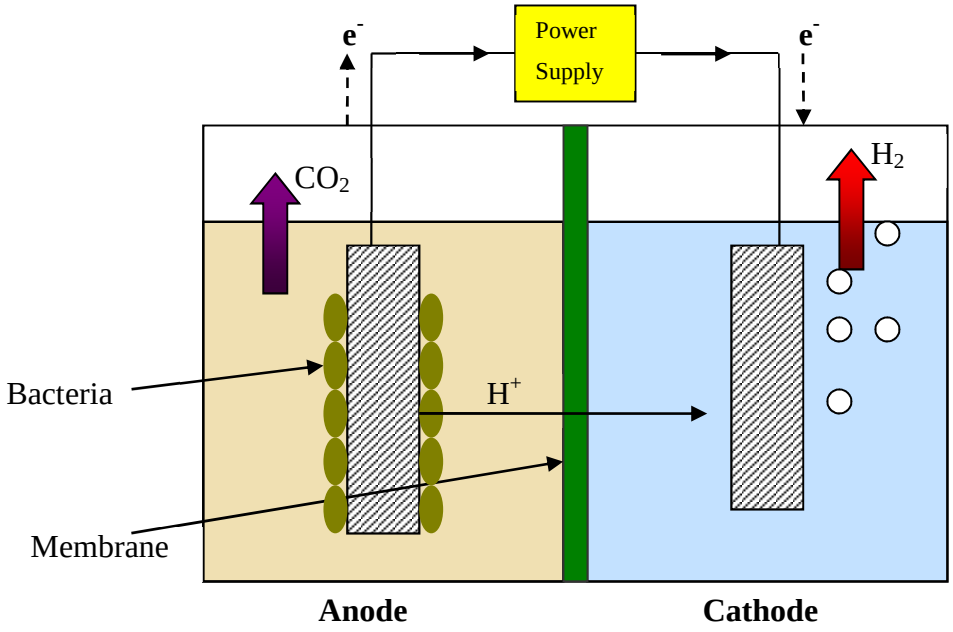

Figure 1-1 Generalised schematic of an MEC adapted from (Liu et al., 2005b) showing the flow of electrons and hydrogen ions and the function of the anode and cathode sections

The theoretical electrochemical energy gains or requirements of a MFC and MEC respectively will vary with temperatures, substrate free energy and ionic concentrations especially pH, as shown in appendix II. Even if it were possible to determine the potentials accurately in practice these theoretical values are not achieved. Energy is lost through all the transfer processes which take place to allow this reaction to happen. There are both electrochemical losses known as overpotentials caused by losses in redox reactions and transfer to the electrodes, losses in transfer of ions between the electrodes, limitations caused by transfer rates being different for different species, and on top of this there are losses caused by transfer of both electrons and ions in and out of the bacteria, losses to the bacteria themselves as they use energy, losses of electron transfer, and also losses by side or chain reactions occurring which do not advantage the fuel cell (Logan, 2008). This means that the energy gained in an MFC is less, and the energy input required in an MEC is more, than would theoretically be the case, represented in Figure 1.2.

In an MEC substantially more energy input than the theoretical is needed, in acetate fed systems these typically range from 0.4 V to 0.8 V with greater hydrogen gas production at higher voltages but less energy efficiency (Call and Logan, 2008). Glucose fed reactors have been shown to operate at applied voltages of 0.9 V (Selembo et al., 2009a), although far less work has been carried out on this substrate and its limits of applied voltage are undefined. In a larger scale system it is likely overpotentials (the difference between the theoretical potential at which the reaction occurs, and the observed potential of the electrode) will be increased and therefore the power input might be higher. In a pilot scale reactor fed on wine wastewater the input voltage of 0.9 V was used, although this performed less well than laboratory trials at a smaller laboratory scale on the same substrate, high over potentials being one of the suggested reasons (Cusick et al., 2011).

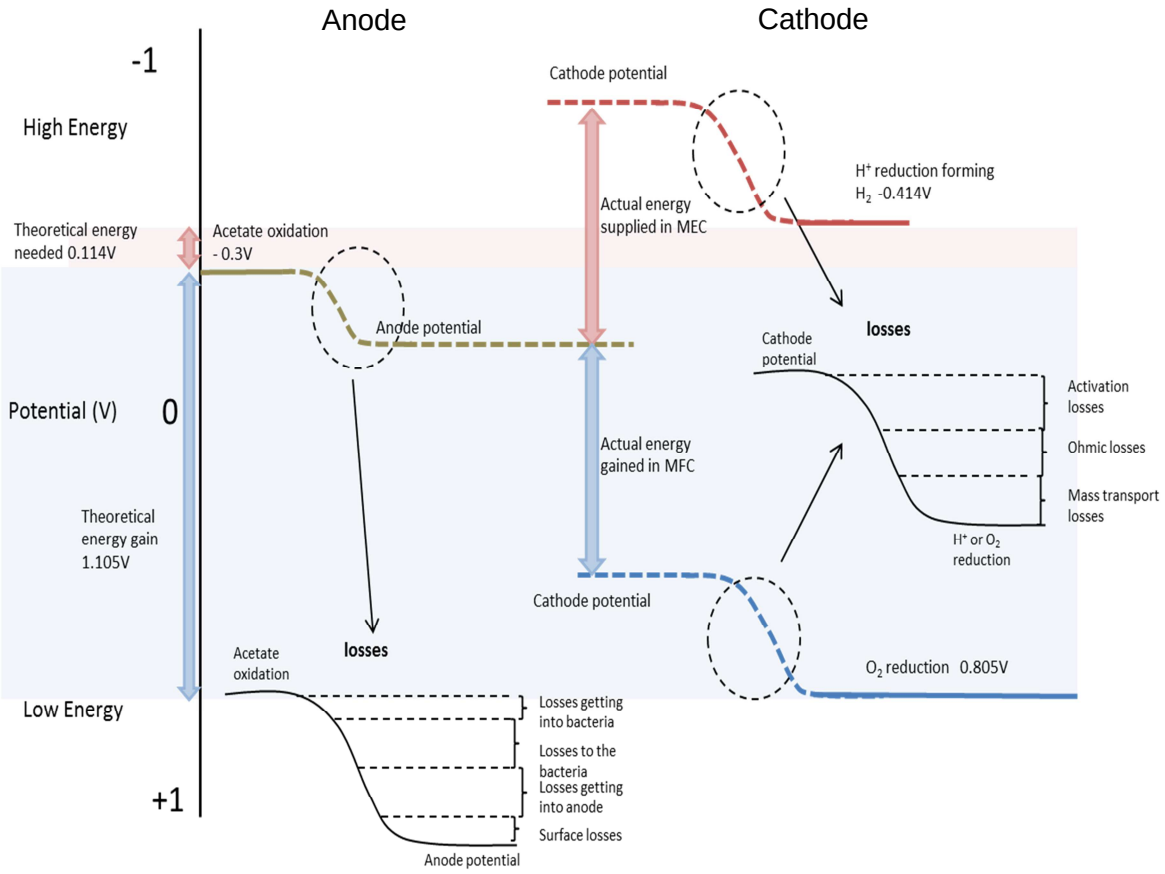

Figure 1-2 Representation of the energy losses within an MFC and MEC using acetate. Energy is shown as potential on the vertical axis, the green line shown the potential of the anode from the potential of acetate (solid line) to the actual anode potential (dotted line) which dependant on the losses. The reduction potential of the MFC and MEC cathode reactions is shown as the solid blue and red lines respectively, whereas the actual cathode potential is again shown in the dotted lines and is dependent on losses. The predicted total energy gain (MFC) and loss (MEC) is shown by the thick arrows and can be variable depending on these losses, but will always be less than that theoretically predicted as seen in the thick arrows at the vertical axis

Understanding the complexities of the electrochemistry of these systems is however only part of the challenge of understanding and ultimately manipulating BES technology. The microbiology of such systems plays a critical role in dictating their efficiency and their success or failure. The microbial community, which catalyses and enables the whole process to take place will also be affected by temperature, pH and substrates (Rittmann, 2001), it will vary with time and within the reactor, and the factors of competition, symbiosis and random assembly lead to a highly complex and unpredictable system.

BES systems run on electrochemical principles but rely on microbial communities. Therefore predicting their absolute function and output of energy, or indeed the input of energy needed, is at this stage in our understanding not possible. The empirical collection of this information is necessary in helping us identify not only if this technology is viable but also the areas that can and need to improved. Critically understanding the bacterial communities and the energy transfers within these systems lies at the heart of being able to manipulate and use this technology.

BES in general and MECs in particular have the potential to fulfil these needs of the wastewater industry (Foley et al., 2010). MECs are entirely anaerobic, eliminating the need for any aeration or complex membrane systems, meaning their engineering can be simple and 'retrofittable' within existing infrastructure. Although hydrogen production is focused on in this study, the flexibility of this process to make other high value products is an economic driver. However the key challenges to overcome are the scientific ones. An increasing body of work is amassing showing improved efficiencies and performance, however the vast majority of this is with simple substrates at warm temperatures (Rader and Logan, 2010, Call et al., 2009, Cheng et al., 2006b, Zhang et al., 2010). Evidence that BES work at low temperature is conflicting (Jadhav and Ghangrekar, 2009, Cheng et al., 2011), the only published study of a large scale 'hydrogen producing' MEC did not produce hydrogen (Cusick et al., 2011), and MECs studies using real wastewater as a substrate are limited, the longest documented study runs reactors for 7.6 days (Wagner et al., 2009).

1.1. Aim and objectives

The overall aim of this research is to understand if BES can be used as a sustainable method of wastewater treatment.

Much work has been and is being carried out fine tuning BES technologies within laboratories, testing new materials and moving towards greater output efficiencies, however large volumes of this work is conducted at warm temperatures and with simple artificial substrates (Hu et al., 2008, Logan et al., 2008, Selembo et al., 2009a, Tartakovsky et al., 2009). This research does not strive towards making such efficiencies, but answers the following fundamental questions of: can they work with

real wastewaters? and, can they work at realistic temperatures? this was addressed by completing the following objectives:

- Quantifying the amount of energy available in the wastewater
- Analysing the start-up and community development of MFC systems.
- Testing the operation and performance of MFC reactors at low temperatures
- Monitoring the performance of MEC reactors with wastewater substrate
- Building and testing a pilot scale MEC reactor run at a wastewater treatment site.

Chapter 2. Determination of the Internal Chemical Energy of Wastewater

Parts of this chapter have been published as Heidrich, E.S., Curtis T.P., and Dolfing J., Determination of the Internal Chemical Energy of Wastewater. Environmental Science & Technology, 2011. 45(2): p. 827-832.

The wastewater industry is facing a paradigm shift, learning to view domestic wastewater not as a waste stream which needs to be disposed of, but as a resource from which to generate energy. The extent of that resource is a strategically important question. However, the only previous published measurement of the internal chemical energy of wastewater measured 6.3 kJ/L, calculated to be 14.7 kJ/gCOD. It has long been assumed that the energy content in wastewater relates directly to chemical oxygen demand (COD). However there is no standard relationship between COD and energy content. In this study a new methodology of preparing samples for measuring the internal chemical energy in wastewater is developed, and an analysis made between this and the COD measurements taken. The mixed wastewater examined, using freeze drying of samples to minimise loss of volatiles, had 28.7 kJ/gCOD, whilst domestic wastewater tested had 17.8 kJ/gCOD nearly 20% higher than previously estimated. The size of the resource that wastewater presents is clearly both complex and variable, but is likely to be significantly greater than previously thought. A systematic evaluation into the energy contained in wastewaters is warranted.

2.1. Introduction

Every one of us produces at least around 40 $gBOD_5$ (biochemical oxygen demand consumed over 5 days), in waste every day, in richer countries this is likely to be nearer 80 $gBOD_5$,(Mara, 2004), equating to around 60-120 gCOD/person/day (Kiely, 1997). If there were 14.7 kJ/gCOD (Shizas and Bagley, 2004), the only previous published measurement of the energy value of wastewater, with 6.8 billion people in the world, 2.2 - 4.4 x 10^{18} joules of energy per year is available, or a continuous supply rate of 70 - 140 gigawatts of energy, the equivalent of burning 52 - 104 million tonnes of oil in a modern power station, or 12 - 24,000 of the world largest wind turbines working continuously. This estimation does not even include all the energy contained in our agricultural and industrial wastewater.

Despite the resource that wastewater represents, most developed countries spend substantial quantities of energy treating the wastewater so it can be released without harm to the environment, the US uses approximately 1.3% of its total electricity consumption doing so (Carns, 2005, Logan, 2008). The energy for wastewater treatment will be a particular burden in the urban areas of less well-off nations. Wastewater is typically viewed as a problem which we need to spend energy to solve, rather than a resource. If the energy contained in wastewater is harnessed, not only could it help the water industries become self-sufficient in energy or even net providers, but it could also be a modest source of energy in parts of the world which currently lack reliable and affordable energy supply.

Wastewater contains a largely uncharacterised and undefined mixture of compounds, including many organics, likely to range from small, simple chains through to more complex molecules. All organic compounds contain energy stored within their bonds. The energy that can be obtained from wastewater by different processes is varied, methane gas from anaerobic digestion, electricity from microbial fuel cells (MFCs), or hydrogen in the case of microbial electrolysis cells (MECs) (Logan, 2008) or a fermentation process (Davila-Vazquez et al., 2008). Large amounts of research is being undertaken in all of these areas but there has been very little work conducted in quantifying the amount of energy held in wastewater to start with.

The COD of wastewater has long been used as a relatively simple and reliable method of determining the 'strength' of waste, and by inference the energy contained within it. However there is no empirical formula for the determination of the energy content from the COD measurement. The only previous study to attempt to determine the energy content of raw municipal wastewater by experiment was conducted by Shizas and Bagley (2004) using a bomb calorimeter. Here a single grab sample of domestic wastewater from a treatment plant in Toronto was dried in an oven overnight at 103^{o}C before being analysed by bomb calorimetry. It was found that the domestic wastewater had a measured COD of 431 mg/L, and an energy value of 3.2 ± 0.1 kJ/g dry sample; with 1.98 g/L of solids this equates to 6.3 kJ/L. This interesting observation has led to the pioneering interpretation that wastewater contains 14.7 kJ/gCOD (Logan, 2008), which has been cited in the literature several times in particular with relation to microbial fuel cell work (Liao et al., 2006, Schroder, 2008, Logan, 2009). However the oven drying of samples will have driven off many volatile organic compounds, such as

methanol (boiling point 64.7 ^{o}C), ethanol (78.4 ^{o}C), and formic acid (101 ^{o}C). Moreover, the calculations were based on a single grab sample from one treatment plant, and using the COD measurement taken prior to drying, it is very likely that some of this COD will have also been lost before the energy determination was made. The work of Shizas and Bagley (Shizas and Bagley, 2004) provides a valuable starting point for the estimation of energy in wastewater, but given the volatile losses, and the measurement of the COD before these losses have occurred, this value must be an underestimation of the true internal chemical energy of wastewater.

The objectives of this study were to develop an improved methodology for measuring internal chemical energy, to better quantify the internal chemical energy of wastewaters, and to evaluate the relationship between internal chemical energy and COD.

2.2. Materials and methods

2.2.1. *Collection and storage of samples*

Two 24 hour composite samples of influent wastewater were taken, one from Cramlington Wastewater Treatment Plant, which deals with a mixed (i.e. industrial and domestic) wastewater, and the other from Hendon Treatment Plant, primarily treating domestic wastewater, both in the North East of England. Within two hours of collection, 3 L of sample was placed into the deep freeze at -80 ^{o}C, and a further 3 L was placed into an oven at 104 ^{o}C. A sample was stored in a refrigerator at 4 ^{o}C.

2.2.2. *Drying procedures*

After a period of around 48 hours in the oven at 104 ^{o}C the sample was fully dried. This was then ground into a powder using a pestle and mortar, and stored in four measured quantities of approximately 0.5 g in clean, dried sealed containers. The frozen samples were dried using a freeze dryer (Labconco Freezone, Labconco Corp. USA) which when used daily over a period of 4 weeks was capable of drying about 1.5 L of sample, each 20 hour drying period removing a few millilitres of liquid. The samples were stored at -80 ^{o}C between drying for 12 hours whilst the freeze dryer stabilised. This procedure was repeated until enough sample was dried to yield four 0.5 g samples. These were then ground and stored in the same way as the oven dried samples.

2.2.3. *Wastewater analysis*

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total organic carbon (TOC), inorganic carbon (IC), total carbon (TC) and chemical oxygen demand (COD) measurements were carried out in the two days after collection using the refrigerated samples. The methods described in Standard Methods for the Examination of Water and Wastewater (APHA, 1998) were used. TS was also measured using the freeze drying process. Further COD tests were carried out on rehydrated freeze dried and oven dried samples. All measurements were taken in triplicate.

2.2.4. *Energy content*

The energy content of the dried wastes was determined using an adiabatic bomb calorimeter, Gallenkamp Autobomb. The internal bomb was a stainless steel unit surrounded by a water jacket with a volume of 1900 mL, with a further cooling jacket outside with a flow of 300 mL/min. The system also included a mechanical stirrer, ignition unit and a digital thermometer accurate to 0.01 ^{o}C. The effective heat capacity of the system i.e. the heat required to cause a unit rise in temperature of the calorimeter was determined using triplicate samples of pure benzoic acid. This was used to calibrate the heat of combustion of the system components such as the wire and cotton, and the effective heat capacity of the bomb, its water jacket and thermometer. After this determination all of the components of the system were then kept constant throughout the tests. Four samples of benzoic acid were used on each time of operation of the bomb calorimeter to verify the technique.

The samples were dried, weighed to around 1 g, and compacted before combustion in the bomb. It was found that the samples did not fully combust, and therefore they were mixed in a 1:1 ratio with a combustion aid of benzoic acid, a method used by Shizas and Bagley (2004). The exact sample weight and the temperature rise in the surrounding water jacket was recorded and used to determine the energy content of each sample. All measurements including the benzoic acid standards were taken in a randomised order.

2.2.5. *Energy content calculations*

The bomb calorimeter measures the heat of combustion of the bomb's contents. When the bomb is ignited the contents including the fuse wire, cotton thread used to attach the sample to the fuse wire and the fuel, including any benzoic acid used is burnt, and this heat is absorbed by the bomb and its surrounding water jacket. In addition to the heat from the combustion, there is also heat created by the formation of nitric acid from the

nitrogen contained in the air inside the bomb. Moles of nitric acid formed are found by titration of the bombs contents with 0.1M NaOH. It is assumed that there is 57.8 kJ/mol of nitric acid; the oxidation state of the nitrogen is not taken into consideration as is standard practice (Rossini, 1956). The kilojoules contained in the sample are calculated in the following equation:

$$-\Delta U_{c,s} = ((V_w + B)(c_{p,w})(\Delta T) + (-\Delta U_{c,w}) + (-\Delta U_{c,c}) + (-\Delta U_{c,b})(m_b) - (Q_{f,n}\, mol_{nitric})) / m_s$$

Table 2-1 Definition of parameters in the equation above used to calculate energy of combustion

Term	Definition
$-\Delta U_{c,s}$	Energy of combustion at constant volume for sample (kJ/g)
$-\Delta U_{c,b}$	Energy of combustion at constant volume for benzoic acid = 26.42 kJ/g[a]
$-\Delta U_{c,w}$	Energy of combustion at constant volume for fuse wire = 0.013 kJ/g[b]
$-\Delta U_{c,c}$	Energy of combustion at constant volume for cotton = 0.082 kJ/g[b]
V_w	Volume of water = 1940 g[b]
B	Volume of water equivalent to the effect of the bomb container = 390 g[b]
$c_{p,w}$	Specific heat capacity of water = 0.00418/g/^oC[a]
ΔT	Temperature rise (^oC)
m_b	Mass of benzoic acid combusted (g)
m_s	Mass of sample combusted (g)
$Q_{f,n}$	Heat of formation of nitric acid = 57.8 kJ/mol[a]
mol_{nitric}	Moles of nitric acid formed (mol)

[a](Atkins, 2006)

[b]Determined in laboratory

2.2.6. *Measurement of volatile fatty acids*

The loss of known volatile fatty acids (VFA's) was measured for each drying technique using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Triplicate 20 mL samples of 50 ppm acetate solution were dried overnight in an oven at 104 ^oC, and in the freeze dryer. These were then re-hydrated with 20 mL of deionised water, and the VFAs measured.

2.2.7. *Measurement of anions*

The anion content of both wastewaters was measured in triplicate using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent.

2.2.8. *Measurement of volatile halocarbons*

Dried 20mg samples were rehydrated using 20 mL de-ionised water and, 20 mL wastewater samples were sealed within a sample jar, with the addition of 20 mg of salt (KCl). These were left for 24hrs at 30^{o}C, the headspace gas was then analysed using an Agilent 7890A GC split/split less injector linked to an Agilent 5975C MSD

2.2.9. *Statistical techniques*

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run two sample t-tests on the data. Before the tests were performed the data was checked for equal variance and normal distribution, validating the use of a two sample t-test.

2.3. Results

This paper uses an improved methodology: freeze drying the samples prior to using a bomb calorimeter. With this method only a few millilitres of liquid can be removed in a 24 hr operational period. Therefore drying enough wastewater to yield several grams of solids takes between 4 - 8 weeks. Although far more time consuming it is believed this is the best method available for drying the wastewater without raising its temperature and thus removing the volatiles.

Table 2-2 Measured wastewater parameters of the two different samples used in the energy analysis

	Cramlington	**Hendon**
COD	718.4 ± 9.7	576.2 ± 40.8
COD- oven dried	368.2 ± 12.3	324.0 ± 18.1
COD - freeze dried	587.1 ± 32.2	425.3 ± 16.5
Total solids - oven dried	1392 ± 35	1070 ± 60
Total solids - freeze dried	1597 ± 40	1130 ± 20
Total organic carbon	116.5	115.8
Total carbon	181.8 ± 2.3	196.4 ± 1.2
Inorganic carbon	65.3 ± 1.2	80.5 ± 0.1
Volatile solids (standard method)	953 ± 143	427 ± 20
Total Kjeldahl Nitrogen	92.4 ± 0.0	71.9 ± 4.3
Chloride (ppm)	391 ± 10.9	169.6 ± 17.2

Mean ± standard deviation (n=3), all values are in mg/L unless otherwise stated

Table 2-2 shows the differences between the two wastewaters, and the effects of the drying processes on the COD and solids recovery from these wastewaters. Oven drying reduces the measured COD from 718.4 mg/L in the original wet sample to 368.2 mg/L (49% loss) in the Cramlington wastewater and from 576.2 mg/L to 324.0 mg/L (44% loss) in the Hendon sample, whilst freeze drying gives losses of 18% and 26%. The freeze drying process captured 5-12% more mass than oven drying. This demonstrates that freeze drying is a more accurate method to determine the total amount of COD than oven drying. However, even freeze drying resulted in COD losses of 18-26%. This is probably due to the loss of the volatile fraction of the COD such as short chain fatty acids. This was confirmed using ion chromatography where oven dried samples contained 0.000 ppm acetate whereas freeze dried samples contained 1.8 ppm, compared to the original 54.5 ppm. Acetate is one of the smaller and therefore more volatile of the VFA's and is likely to represent some of the greatest losses.

Table 2-3 Measured internal energy content values given as both energy per litre and energy per gCOD using the post drying measurement of COD

	Cramlington		Hendon	
	Oven dried	**Freeze dried**	**Oven dried**	**Freeze dried**
kJ/L	8.3 ±1.8	16.8 ± 3.3	5.6 ±1.0	7.6 ± 0.9
kJ/gCOD	22.5 ± 4.8	28.7 ± 5.6	17.7 ± 3.2	17.8 ± 2.1

Mean of four measurements ± standard deviation
Values for kJ/gCOD are calculated from the COD measurement after drying and re-hydrating, and TS measurement for the given drying method.

The freeze drying method enabled a significantly greater proportion of the energy in the wastewater to be measured, over 50% more for Cramlington (p value 0.010), and 24% more for Hendon (p value 0.044). There are also significant differences between the two wastewaters, with the Cramlington waste being more energy rich (p value 0.019). The energy content per gram of oxidisable material measured i.e. kJ/gCOD is considerably higher for both wastewaters than previous estimates of around 14 kJ/gCOD, for the Cramlington wastewater this is even higher with the freeze dried sample.

The energy captured by the freeze drying process does not equate to all the energy available in the wastewater sample. Based on the percentage losses of measured COD from the original sample to the freeze dried sample (18% for Cramlington and 26% for

Hendon), the actual energy of the Cramlington wastewater could be as high as 20 kJ/L, and 10 kJ/L for the Hendon wastewater.

2.3.1. *Theoretical results - can internal chemical energy per gram COD be calculated from first principles?*

If we were able to evaluate the energy content of wastewater from the COD measurement, this would require an estimation of which organic compounds are present. With this, the internal chemical energy for each individual organic compound can be calculated on the basis of simple thermodynamic calculations as follows (thermodynamic values are taken from Atkins (2006)) based on the principle that 1 gram of COD equals 1/32 mol O_2, i.e. for every 1 mol O_2 there is 32 grams COD.

If we assume that the organic compound present is methane:

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \qquad (1 \text{ mol } CH_4 = 64 \text{ gCOD})$$

The overall enthalpy for the reaction can be calculated on the basis of Hess's Law, which states that the enthalpy of a reaction is equal to the sum of the enthalpy of formation ($\Delta_f H$) of all the products minus the sum of the enthalpy of formation of all the reactants. Using tabulated values for the enthalpy of formation the energy released in the above reaction with methane is as follows:

$$\begin{aligned}
\Delta_f H \text{ (kJ/mol)} &= \sum \Delta_f H \text{ products} - \sum \Delta_f H \text{ reactants} \\
&= 2(\Delta_f H\ H_2O) + \Delta_f H\ CO_2 - \Delta_f H\ CH_4 - 2(\Delta_f H\ O_2) \\
&= 2(-285.83 \text{ kJ/mol}) + -393.51 \text{ kJ/mol} - -74.81 \text{ kJ/mol} - 2(0 \text{ kJ/mol}) \\
&= -890.5 \text{ kJ/mol} \\
&= -890.5 \text{ kJ/mol} / 64 \text{ gCOD} \\
&= -13.9 \text{ kJ/gCOD}
\end{aligned}$$

Analogous calculations for a wide range of organic compounds show that the typical $\Delta_f H$ values of $C_aH_bO_c$ compounds fall within a fairly narrow range of 13-15 kJ/gCOD, with a few exceptions such as formic and oxalic acid with 15.7 kJ/gCOD, ethyne with 16.3 kJ/gCOD and methanol with 17.8 kJ/gCOD. (See Appendix III).

It could be concluded that 13.9 kJ/gCOD is the maximum amount of heat energy that can be gained from methanogenic wastewater treatment. Therefore from a relatively simple COD measurement the potential energy yield would be known. However biodegradation of organic content in wastewater does not necessarily lead to methanogenesis. Some waste streams can be used for biohydrogen production. Here 1 gCOD is equal to 1/16 mol H_2, ($2H_2 + O_2 \rightarrow 2H_2O$) therefore 1 mol H_2 equals 16 gCOD, giving an energy yield of 17.9 kJ/gCOD (286 kJ/mol H_2 / (16 gCOD / mol H_2)).

The simple $C_aH_bO_c$ compounds are not necessarily the only wastewater components, and other classes of compounds such as halocarbons can contain far more internal chemical energy per gCOD. The explanation to this can be supported by writing the equations that describe their degradation down as oxidations of the carbon moiety with reducing equivalents released as H_2, coupled to the oxidation of the H_2 to water. In highly substituted compounds such as organohalogens, less H_2 is potentially available. The oxidation reaction of H_2 to water becomes less important in the overall equation, the ratio of H:CO_2 decreases, increasing the overall value of kJ/gCOD. This is illustrated using methane and one of its halogenated equivalents trichloromethane (thermodynamic data taken from (Hanselmann, 1991)):

Methane

$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (-393.5 + 4(0)) - (-74.8 + 2(-285.8))$

$= 252.9$kJ/reaction

$$4H_2 + 2O_2 \rightarrow 4H_2O$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (4(-285.8)) - (0 + 2(0))$

$= -1143.2$ kJ/reaction

These two values are then added together to give the overall enthalpy of reaction to be -890.3 kJ/mol, this can then be divided by the COD to give -13.9 kJ/gCOD

Trichloromethane

$$CHCl_3 + 2H_2O \rightarrow CO_2 + 3HCl + H_2$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-393.5 + 3(-167.1) + 0) – (-103.1 + 2(-285.8))

= -220.1 kJ/mol

$$H_2 + \tfrac{1}{2} O_2 \rightarrow H_2O$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-285.8) - (0 + 0.5(0))

= -285kJ/mol

The total enthalpy of reaction is -505.9 kJ/mol, giving -31.6 kJ/gCOD.

It becomes clear how important the reducing equivalents of H_2 are in terms of energetic value, this is illustrated in Figure 2-1, (values given in Appendix III). As the number of substitutions of hydrogen increases, so does the value of energy per gram COD. The value of energy per gram of COD can vary far more widely than previously thought.

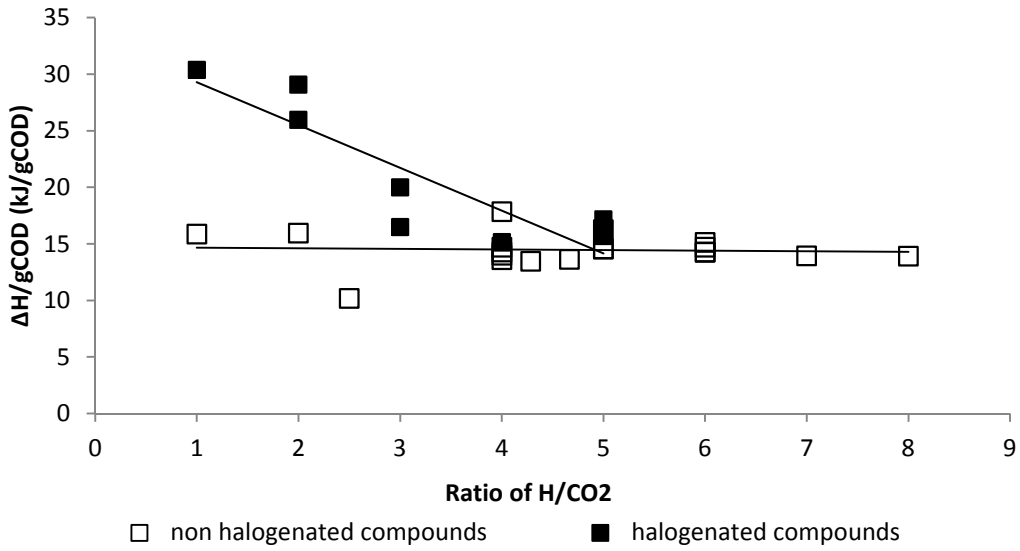

Figure 2-1 Energy content per gCOD of a variety of organic compounds plotted against their degree of oxidation

2.4. Discussion

The predicted energy gained from treatment of municipal wastewaters has been shown to be higher than the previous estimation. The domestic wastewater analysed in this paper has 20% more energy per litre than the estimation made by Shizas and Bagley (Shizas and Bagley, 2004). In addition to this, as the volatiles in their wastewater were not captured, it is likely their sample could have had an energy value around 35% higher, (based on the percentage losses between oven and freeze drying in this study) this would be 8.5 kJ/L. This has a significant impact on the development and implementation of technologies for the treatment of 'low strength' municipal wastewater which pose a greater challenge for the recovery of energy than concentrated waste. These waste streams are clearly richer in energy than previously thought.

The internal chemical energy of the wastewaters per gCOD was greater than expected by comparison to acetate (heat of combustion is 13.6 kJ/gram COD) or glucose (heat of combustion is 14.3 kJ/gram COD). From the data (Table 2-2) of the two wastewaters it can also be seen that the carbon oxidation state plays an important role in determining the energy present. Both samples have a very similar value of TOC (total organic carbon), yet very different COD values. This means that the Cramlington waste with the much higher COD has proportionally more reduction capacity and therefore chemical energy per carbon molecule than the Hendon wastewater. Another possible cause of these high values is that there are compounds within the wastewater that have an energy value, yet are not oxidised during a COD test, most notably urea, which contains 10.4 kJ/g (Atkins, 2006) when combusted, yet undergoes a hydrolysis reaction rather than an oxidation. This compound, which is certain to be present in domestic wastewater (and though it is assumed to hydrolyse in the sewer, a fraction may reach the wastewater treatment site), contributes to the overall energy of combustion of waste but not to the COD measurement, there are likely to be others compounds which do the same. Additionally there could be some compounds which have proportionally far greater energy content per gram of COD than glucose and acetate, such as organohalogens or other highly substituted compounds.

Although many simple halocarbons are no longer in use, some more complex ones are still common in many industrial processes for example as solvents and pesticides, and in the manufacture of in plastics, adhesives, sealants and paper pulp. Organic halocarbons also occur in natural systems. Chlorination treatment also introduces this halogen which

could then combine with other organics. It can be seen from the anion analysis (Table 2-2) that there is significant quantity of chloride ions in the wastewaters, with more in the Cramlington wastewater. This wastewater is likely to contain a more diverse range of organic compounds as this site takes in mixed wastes, some of which must have a high specific energy value and volatility, resulting in high energy wastewater. Volatile halocarbons, however, were not detected with the GC MS method described.

The energy values found in this study are also higher than that reported by Shizas and Bagley (2004). However the calculations in their paper were based on oven dried wastewater energy data, versus a COD measurement taken from the original wastewater sample, which in our study was found to be reduced by about 50% after oven drying. If the same calculation algorithms were used on the data in the present paper then the Cramlington and Hendon wastewaters would contain 11.6 kJ/gCOD and 9.9 kJ/gCOD respectively, while they actually contained at least 2.4 times higher (28.7 kJ/gCOD) and 1.8 times higher (17.8 kJ/gCOD), these calculations are shown in Appendix IV. Thus the energy reported per gCOD cited in the literature (Logan, 2008) based on the Shizas and Bagley paper (Shizas and Bagley, 2004) is probably a substantial underestimation. By comparison to the Hendon domestic wastewater the energy of their municipal wastewater could have had at least 26.4 kJ/gCOD, rather than the 14.7 kJ/gCOD reported.

Clearly not all the energy available in wastewater can be extracted in a useful form as no process is 100 % efficient. Ideally one would be able to measure or calculate the energy biologically available as kJ/gBOD, (although not suitable for anaerobic processes), this is not possible given the unknown and variable composition of wastewater. However knowing the potential energy available would give insight into the types of waste that might be in the waste stream which would also be of importance in the choice of treatment method. Some wastes which may be high in energy value, such as halogenated wastes may be unsuitable or unattractive to some treatment methods. For example one mole of trichloromethane at 506 kJ/mol would only yield 0.25 moles of methane equal to 222 kJ through methanogenic treatment, or one mole of H_2 equal to 286 kJ through biohydrogen production. Although these halogenated compounds are energy rich per gram of COD due to their lack of hydrogen, this actually makes them unattractive to terms of energy extraction for methane or hydrogen production, however

it may be possible to recover this energy using other treatment methods which may be able to capture electrons directly.

In microbial fuel cells (MFC's) the reaction taking place is essentially a combustion reaction, i.e. the organic compound is oxidized to carbon dioxide and water, the difference being that this reaction occurs not as combustion but as redox reactions in two half cells. Importantly, it is the free energy of the organics that determines the maximum electricity yield. This technology could theoretically capture more of the energy available in complex or halogenated compounds than for example methanogenic treatment.

The measurement of the internal or combustion energy of the wastewater and use of this as a basis for efficiency calculations will not necessarily yield all the information required to fully understand the energy flows in such systems. It can be observed using internal chemical energy data, a methanogenic process could in some cases be endothermic, the combustion energy of the methane product being higher than that of the starting substrate. This is the case with the conversion of one mole of acetate (13.6 kJ/gCOD) to one mole of methane (13.9 kJ/gCOD). In this scenario energy appears to have been created. It is actually the Gibbs free energy (the amount of energy that can be extracted from a process occurring at constant pressure) which should be examined for this and other reactions as this parameter informs us of the amount of energy available to organisms for the generation of biomass and an energy rich product. This is also the case for MFC's and MEC's where it is voltage which is measured which relates directly to Gibbs free energy. However without knowing the composition of wastewater, its Gibbs free energy content cannot be determined.

A consequential difference was found between the internal chemical energy measured on freeze dried samples as compared to oven dried samples. This difference was greater than the difference observed by measuring mass alone. This shows that there are significant losses of volatile compounds when a wastewater sample is dried at 104 ^{o}C and that in the case of the mixed wastewaters these volatiles can contain proportionally more energy per gCOD than the non-volatiles captured in both methods. It is shown that, although a clear improvement on the traditional oven drying method, the freeze drying method still results in significant loss of semi-volatiles such as acetate, so even

with the improved method we are still not capturing all the energy available in the wastewater.

Bomb Calorimetry remains the only method for measurement of internal chemical energy or calorific value, and for this method the material must be combustible i.e. dry. To give reasonably accurate results the temperature change in the bomb calorimeter must be in the region of 1 - 3 ^{o}C, usually a gram of substance will provide this. In our analyses this gram was half made up by the use of a combustion aid (benzoic acid) to ensure full combustion and the correct temperature rise. Had the proportion of wastewater to benzoic acid been decreased, making the drying process easier, it was feared that the uncertainty inherent to the introduction of the standard would overshadow the accuracy of the measurements of the samples. Although more challenging the methodology of freeze drying samples is an improvement on previous methods although it does not achieve the full capture of all volatiles. These results begin to get close to the true amount of energy in wastewater, and challenge the assumption that measured COD is equivalent to the amount of energy. Freeze drying, although far more time consuming, therefore should be the method of choice when completing such analysis in particular with complex wastes, despite its far greater time consumption rate unless or until new methods and equipment are developed to reduce the time burden using this principle. One such method could be the use of membranes, in particular through the use of reverse osmosis which would 'trap' molecules as small as salts and allow water to be removed. Such techniques may allow for more rapid, cost effective and efficient drying of samples, thus enabling more sampling to be undertaken.

It is clear from our data that the energy value of different wastewaters is variable, as would be expected; there is no standard relationship to measured COD. Values ranged from 17.7 kJ/gCOD to 28.7 kJ/gCOD, when measuring the COD remaining in the dried sample, however we cannot know how much compounds such as urea contribute to this. This means than a measurement of the amount of oxygen required to oxidise the organics within wastewater is not a simple representation of the amount of energy contained within that waste. This is particularly the case when dealing with mixed wastes, where the energy content is proportionally far greater per gCOD. It seems that 13 – 14 kJ/gCOD is the minimum energy content that could be found in wastewaters, however it may be significantly greater. Given the variability in the amount of energy

per gram COD it seems better to measure this energy directly rather than making an estimation, despite the fact that even with the better drying method there are still losses.

Given the huge amount of wastewater globally and the potential energy stored within it, it is important that this potential energy should be determined. With new technologies such as fuel cells being developed, the estimation of this resource is not as trivial as previously assumed. It has been shown that wastewaters can lie well outside the previously estimated values. A systematic review of the energy contained within different waste streams is needed. This paper examines two wastewaters from a reasonably similar geographical location and has found extremely diverse results. It is hoped that this methodology will be repeated and improved upon in terms of time taken, allowing the dissemination of multiple studies using different wastewaters building up a comprehensive and global picture of the energy available in wastewater. This would form the strategic foundation block to the establishment of new and existing technologies within the wastewater industry harnessing this valuable renewable energy source.

Chapter 3. How many exoelectrogens make a Bioelectrochemical System?

3.1. Introduction

The inoculation and subsequent acclimatisation of a bioelectrochemical system (BES) is fundamental to the operation of such systems (Logan and Regan, 2006, Rittmann, 2006). Yet the origin, abundance and physiology of these organisms is the area of greatest uncertainty in design (Oh et al., 2010).

The main goal of the inoculation and acclimatisation of a reactor is typically to 'get it going' as quickly as possible, typically the sources of seed includes: reactors already working in the lab (Jeremiasse et al., 2009, Cheng et al., 2009, Call and Logan, 2008); anaerobic sludge (Chae et al., 2010, Yang et al., 2009); return activated sludge (Torres et al., 2009); mixtures of sludges; or simply wastewater taken at various stages from the treatment plant (Kiely et al., 2011b, Wang et al., 2008). The source and volume of inoculum varies between studies. There is no consensus of how a BES reactor should be started up, or how long acclimatisation will take. This can lead to problems, highlighted by a pilot scale study where several attempts were made to acclimatise the reactor (Cusick et al., 2011).

The bacteria needed for microbial fuel cells to work are termed exoelectrogens (Logan, 2008) due to their ability to transfer electrons outside their cell. Three transfer mechanisms have been proposed.

Firstly electrons can be transferred through conduction with direct contact between the cytoplasmic membrane of the bacteria and the solid substrate being reduced, this mechanism has primarily been associated with the genera *Shewanella* and *Geobacter* (Myers and Myers, 1992, Mehta et al., 2005).

The second mechanism is an electron shuttle. Some bacteria are able to excrete compounds or shuttles into the electrolyte which are capable of transferring electron to an electrode. Rabaey *et al.,* (2005) found that *Psuedomonas aeruginosa* produced Pyocyanin, a mediator which was not only able to transfer electrons from this taxon to the anode of an MFC, but could also work for other species when introduced back into a mixed culture. Thus, a bacterium unable to transfer electrons itself, may become

exoelectrogenic due to the presence of a different shuttle producing bacteria. *Shewanella* species have been seen to do this with the production of riboflavins (von Canstein et al., 2008).

Thirdly electrons might also be transferred through conductive microscopic pili named nanowires which extend from the bacteria cell to other cells or any other electron acceptor (Reguera et al., 2005). *Geobacter* and *Shewanella* species have both been linked to this activity (Gorby et al., 2006). Putative nanowires have been observed using electron microscopy extending to a conductive surface. Conducting probe atomic force microscopy (Reguera et al., 2005) and conductive scanning tunnelling microscopy (Gorby et al., 2006) have been used to reveal that the pili which had previously been observed as attachment mechanisms for bacteria onto Fe oxides, were highly conductive.

It has been proposed that symbiotic relationships between different bacteria groups enhance the function of mixed cultures and improve process stability (Lovley, 2008), possibly by allowing inter-species electron transfer (Rabaey et al., 2005). Many of the exoelectrogens typically associated with BES's such as *Geobacter sulfurreducens* have limited metabolic diversity, and are only able to utilise the end products of fermentation (Caccavo Jr et al., 1994). A reactor fed with a waste requires bacteria which are able to digest the complex substrates, but may not necessarily be able to utilise the anode for respiration (Kiely et al., 2011c). The hydrolysis step within these food chains has been shown to be the rate limiting step with regard to the current production (Velasquez-Orta et al., 2011).

In general, growth in bacterial systems can be described through the equation $N_T = N_0 exp^{rt}$, where the number of bacteria present at a specific time period (N_T) is equal to the number of bacteria present at the start (N_0) multiplied by the exponential of the growth rate (r) over the time span (t). (Rittmann, 2001). With N_T known various other properties can be calculated such as specific activity and growth yield. However in MFCs these are not well understood (Logan, 2008), although growth rates have been defined for some of the key organisms involved in MFC reactions such as *Geobacter*, (Cord-Ruwisch et al., 1998). A cell yield of 0.07-0.22 g-COD-cell/g-COD-substrate has been calculated (Logan, 2008) from an early study by Rabaey et al. (2003) using total bacterial concentrations within the reactors determined turbidometrically and the total

COD removed during the experiments. Freguia et al. (2007) reported estimates of growth yields of -0.016 to 0.403 mol-C-biomass/mol-C-substrate, based on measurement of the substrate removal which was then used to calculate cell yield through a mass balance approach. Yield has been shown to drop with decreasing external resistance (Katuri et al., 2011).

However the value of N_T is complex and unknown. Although a body of research is growing identifying the functions of bacteria within working BES reactors, little is known of their abundance in a natural sample (N_0) and absolute number within a working system (N_T). Additionally the pattern of acclimatisation, the period is likely to be crucial in the community formation, also remains largely unexplored.

Using the acclimatisation period of reactors the aims of this study were to firstly identify the optimum level of inoculum needed to start a reactor with a view to identifying a protocol for the further experiments. Secondly to estimate the most probable number of exoelectrogens present in a sample of wastewater which can be used as a guide to the sequencing depth needed to find these organisms, and to determine N_0 for a reactor. Thirdly to define the growth rates (r) within MFC systems through examining the start-up phase. With these two factors quantified the N_T can be estimated, as can specific activity and yield. Finally by examining the pattern of acclimatisation on different substrates, key differences in community formation can be identified.

3.2. Method

3.2.1. *Reactor Set-up*

Double chamber tubular design MFC reactors (78 mL each chamber) were used, constructed in Perspex, with an internal diameter of 40mm and length of 60mm. The anode was a 2.5 cm^2 carbon felt (Olmec Advanced Materials Ltd, UK), the cathode a 2.5 cm^2 platinum coated titanium mesh with a surface area 8.13 cm^2 (Tishop.com, UK). The cation selective membrane between the reactor chambers was Nafion® 117 (DuPont, France), with an area of 12.6cm^2. The electrodes were positioned 1cm apart. The components of the reactor were cleaned before use and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK)

The cathode chamber was filled with 50 mM pH 7 phosphate buffer saturated with air for 20 minutes before being added into the reactors. Three different media were used:

1. Acetate solution with added nutrients (Call and Logan, 2008)
2. Starch solution with added nutrients (Call and Logan, 2008)
3. Primary settled wastewater (Cramlington WWTP, Northumbrian Water Ltd)

The quantities of starch and acetate in the nutrient solutions were balanced to give similar total chemical oxygen demand (TCOD) as the wastewater, and were autoclaved (121°C, 15 min) before use. The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into ¼ strength Ringers sterile dilutent (APHA, 1998). The contact time under UV was altered to give effective sterilisation as defined as colony free plates in triplicate at zero dilution. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see Appendix V).

The three medias were sparged under sterile conditions for 10 minutes using ultra high purity (UHP) nitrogen (99.998%), until the dissolved oxygen (DO) as measured on a DO probe Jenway 970 (Bibby Scientific Ltd, UK) reached zero.

3.2.2. *Inoculum*

Screened raw influent wastewater from Cramlington wastewater treatment plant (Northumbria, UK). This wastewater is a mixture of industrial and domestic origin. Samples were stored anaerobically at 4^{o}C and used within 24 hours of collection. The inoculum was also sparged with UPH nitrogen before use.

3.2.3. *Start –up and acclimatisation*

Duplicate reactors were inoculated with differing volumes of wastewater (1 mL, 10 mL, 25 mL and 50 mL). The anode compartment was then filled with the sterile substrates. Control ‘reactors’ (using no inoculum) were run during each test. An inverted 50ml syringe filled with UPH nitrogen was placed into the refilling port on top of the anode chamber to provide an anaerobic headspace. The cathode chamber once filled was left open allowing the diffusion of oxygen into the liquid. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor. A data logging

multimeter (Pico ADC-16, Pico Technologies, UK) was attached to record voltage output every 30 minutes. Reactors were allowed 800 hours at room temperature (20-25 ^{o}C) to show acclimatisation before the experiment was ended. With the acetate fed experiment a further set of reactors were run with lower dilutions of inocula, 0.01 mL, 0.1 mL and repeated 1mL with 25 mL as a positive control.

3.2.4. *Enumeration of bacteria*

The total number of aerobic culturable bacteria present in the wastewater samples used for inoculation was approximated using a spread plate method 9215C (APHA, 1998), with peptone based nutrient agar (Lab M Ltd, UK). Serial dilutions were undertaken into sterile ¼ strength ringers solution, with each dilution plated in triplicate. Plates were incubated at 37 ^{o}C for 48 hours. Anaerobic bacteria were enumerated using a basal salts media (Shelton and Tiedje, 1984) with 1 g/L of both yeast extract and glucose as a carbon source. The media was autoclaved (121 ^{o}C for 15 min) and sparged with sterile UHP nitrogen for 20 minutes. A volume of 9 mL was then added to sterilised Hungate tubes, 1 mL of wastewater was then added to five tubes, and dilutions made down to 10^{-12} with five replicates at each dilution. The headspace of the tubes was sparged with nitrogen, and the tubes incubated at 37 ^{o}C for two weeks. The number of bacteria was determined using the MPN methodology (APHA, 1998).

3.2.5. *Analytical methods*

TCOD of the medias and inocula were measured in duplicate according to standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) colorimetric reagent kit. Volatile fatty acids of the media and inocula were measured in duplicate using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. When the current of the cell had dropped to zero TCOD and VFA's of the cell were measured using the same method as inocula and media above.

3.2.6. *Most probable number (MPN) calculations*

With non-standard dilutions the pre-calculated MPN tables (APHA, 1998) cannot be used. The MNP is calculated through a series of iterations based on a Poisson and binomial distributions (Blodgett, 2005) using the following formula, solving λ for the concentration:

$$\sum_{j-1}^{k} \frac{g_j m_j}{1 - \exp\ (-\lambda m_j)} \ = \ \sum_{j-1}^{k} t_j m_j$$

K = the number of dilutions,

gj = the number of positive (or growth) tubes in the jth dilution,

mj = the amount of the original sample put in each tube in the jth dilution,

tj = denotes the number of tubes in the jth dilution

A probability is assigned to each possibility of the number of bacteria based on the outcome at each dilution, a positive outcome being voltage produced in by the reactor. The number with the highest probability is given as the MPN. Using the spreadsheet developed by Bloggett to make these iterative calculations, the most probable numbers of exoelectrogens per 100 mL of wastewater can be calculated (Garthright and Blodgett, 2003) using the inocula volumes, and the test outcome.

Thomas' simple formula which is based on the same principles as the full test, but a simpler algorithm to solve, can also be applied to the data set, this formula has been shown to have substantial agreement (Thomas, 1942). Using only the lowest dilution that doesn't have all positive tubes, the highest dilution with at least one positive tube and the dilutions in between the following calculation can be made:

$$MPN/100\ ml \ = \ \frac{no.positive\ tubes \ \times \ 100}{\sqrt{(ml\ sample\ in\ negative\ tubes) \times (ml\ sample\ in\ all\ tubes)}}$$

The confidence limits of this calculation at the 95% level can be calculated using Haldane's formula (Haldane, 1939):

m_1, m_2, m_3 ……. denotes inoculation amounts ranging from the largest to the smallest of the chosen dilutions

g_1, g_2, g_3 ……. denotes the number of positive tubes at the corresponding dilutions

$$T_1 = \exp(-MPN \times m_1)\ , T_2 = \exp(-MPN \times m_2) \ldots\ldots\ldots etc$$

$$B = [g_1 \times m_1 \times m_1 \times T_1/((T_1 - 1)^2)] + [g_2 \times m_2 \times m_2 \times T_2/((T_2 - 1)^2)] + [g_3], [\ g_4] \ldots. etc.$$

$$Standard\ Error\ of\ \log 10\ (MPN) = \ 1/\left(2.303 \times MPN \times (B^{0.5})\right)$$

95% confidence intervals are given by:

$$Log_{10}\ (MPN) \pm 1.96 \times Standard\ Error$$

3.2.1. *Growth rate, specific activity and yield calculations*

Growth rate of bacteria (μ) is classically calculated by quantifying the number of bacteria at two time intervals. In this experiment voltage is deemed to be a suitable proxy for exoelectrogenic bacteria, the rate of voltage rise being equivalent to the rate of growth. It is assumed that each bacterium is capable of donating an amount of electrons therefore an increasing number of electrons are donated to the circuit (i.e. the voltage increases at a constant resistance) as the absolute number of bacteria increases, (it does not represent an increasing ability to metabolise), i.e. voltage is deemed proportional to bacterial number. This can be from the growth rate expression:

$$N_T = N_0 e^{\mu t}$$

Where N_T is the number of bacteria at time t (in this case the voltage), N_0 is the number of bacteria (voltage) at time zero (t_0) and μ is the growth rate. Therefore growth can be defined as:

$$\mu = \frac{\ln N_t - \ln N_0}{(t - t_0)}$$

Specific activity (q), defined as moles electrons per cell per second can be calculated over the period of growth as follows:

$$q = \frac{I \times (t_1 - t_o)/F}{N_0}$$

Where I is the current in amps (coulombs/second) as calculate from the measured voltage V, and resistance R calculated through I=V/R, t_1-t_0 is representative of the time period of each measurement, (i.e. every 30 minutes, the total coulombs of charge within this period is therefore I multiplied by 30 minutes multiplied by 60 seconds) and F is Faradays constant of 96485 coulombs/mol e^-. The growth rate and starting MPN is used to calculate the number of cells at each time period N_T. This can be converted to moles of acetate per cell per second (1 mole acetate = 8 moles electrons), to give substrate utilisation (U).

Growth yield (Y) is the amount of biomass or cells produced by the bacteria per mass of degraded substrate measured in g-COD-cell/g-COD-substrate. Rather than use the total COD removed in the reactor, which would also involve COD digested via other routes only the g-COD substrate put to the circuit is used as calculated from the substrate utilisation above. The yield is calculated as follows:

$$Y = \frac{(N_T - N_0) \times W \times COD_{cell}}{\sum_{t0}^{t} U \times COD_{sub}}$$

Where the total cells produced over the growth period N_T-N_0 is multiplied by an estimation of the weight of cells W of 5.3 x 10^{-13} g-cell given in Logan (2008) and the estimation for anaerobically grown cells of the formula of $C_{4.9}H_{9.4}O_{2.9}N$ equating 1.25 g-COD/g-cell, (Rittmann, 2001). The sum of the substrate utilisation U as calculated above is multiplied by COD_{sub} the amount of COD per mole of substrate, 64 for acetate.

3.3. Results

3.3.1. *Number of bacteria in wastewater*

The spread plate counts of the wastewater, and anaerobic multiple tube count indicate there is 8.3 x 10^5 culturable aerobic bacteria, and 6.9 x 10^4 culturable anaerobic per ml of this wastewater, giving a rough estimate of the total bacteria per mL of wastewater to be 10^6. Although this method may over estimate numbers due to some bacteria being able to grow under both conditions, and underestimating numbers due to bacteria being intolerant to the media, the overall value calculated fits in with previous estimates (Tchobanoglous, 1991).

3.3.2. *Most probable number of exoelectrogens*

The number of positive outcomes of each test are shown in Table 3-1. From this the MPN can be calculated shown in Table 3-2. The MPN of exoelectrogens in an acetate fed reactor is 17 per ml of wastewater, this number drops to 1 per ml for a starch fed reactor and 0.6 per ml for a wastewater fed reactor. Superficially it appears that acetate metabolising exoelectrogens are quite rare organisms, starch metabolising exoelectrogens are even rarer and wastewater metabolising exoelectrogens are rarer still.

Table 3-1 The number of positive outcomes for each inocula size out of the total number of reactors run

Inocula size (mL)	50	25	10	1	0.1	0.01
Wastewater	2/2	2/2	0/2	1/2	-	-
Starch	2/2	2/2	1/2	0/2	-	-
Acetate	2/2	4/4	2/2	3/4	1/3	0/2

Table 3-2 The MPN in 1 ml of wastewater given by the two methods stated, numbers in brackets indicate the upper and lower bounds at 95% confidence. The probability of presence in wastewater is calculated from the total count of viable bacteria per 1 ml

Substrate	MPN calculation (Blodgett 2005)	MPN estimation (Thomas 1942)	Probability of presence in 1 ml of wastewater
Wastewater	0.6 (0.3-2.5)	0.8 (0.3-2.5)	6×10^{-7}
Starch	1.0 (0.3-3.2)	1.1 (0.3-4.0)	10^{-6}
Acetate	17.0 (5.5-52)	17.6 (6-51.5)	1.7×10^{-5}

An alternative explanation is that the lower MPNs, and therefore the probabilities of these organisms being present in 1 ml of wastewater, are the product of two or more events. In wastewater and starch there are long chain molecules present which undergo a series of steps in their breakdown. Each step is probably undertaken by different microorganisms. The electrons pass down this chain leading to the final step of donation to the electrode, represented by the acetate reactor. Thus the MPN of the wastewater and starch fed cells is the probable MPN of the acetate fed cells (the number of exoelectrogens) multiplied by the probability of each of the upstream steps. Here all of these steps are simplified into one probability step, however in reality this may be many steps the product of which is equal to 0.04 for wastewater and 0.06 for starch as shown in Figure 3.1.

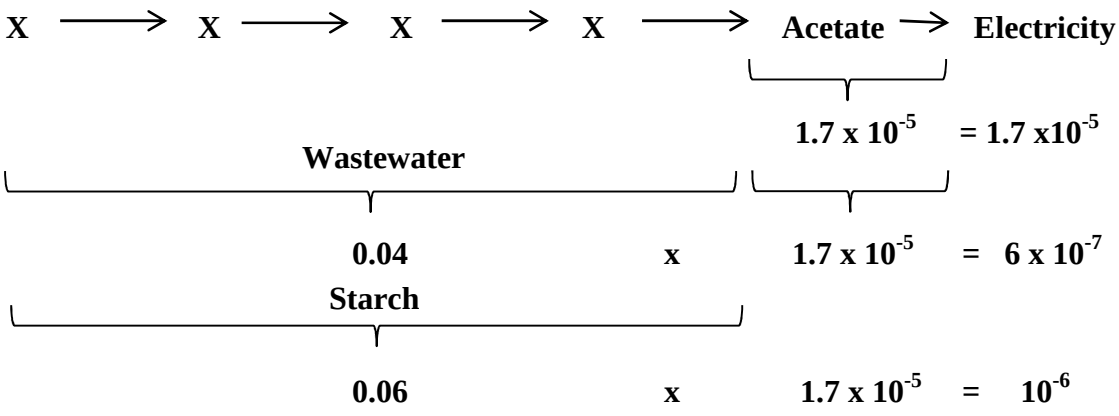

Figure 3-1 Estimated probabilities of numbers of bacteria present in the wastewater begin to produce a working MFC fed on three different substrates of acetate, starch and wastewater based on the numbers determined in the MPN method

3.3.3. *Growth rates*

The individual growth rates for the three different substrates are shown in Table 3-3. The rates were not significantly different (p=0.282 one way ANOVA), and showed agreement with other studies.

Table 3-3 Average growth rates for exoelectrogens fed on different substrates estimated using the rise in voltage measured in the acclimatising reactors

	Average growth rate
Wastewater fed community	0.028 h^{-1} ± 0.013
Starch fed community	0.023 h^{-1} ± 0.005
Acetate fed exoelectrogens	0.035 h^{-1} ± 0.020
Geobacter sulfurreducens (Cord-Ruwisch et al., 1998)	0.023 – 0.099 h^{-1}
Geobacter sulfurreducens (Esteve-Nunez et al., 2005)	0.04 – 0.09 h-1
Fermenting micro-organisms (Rittmann, 2001)	0.05 h-1

3.3.4. *Acclimatisation pattern*

Using an arbitrary value for N_0 (the starting number of bacteria per ml), the known growth rate and the time period over which the experiment was conducted, the pattern of acclimatisation can be modelled.

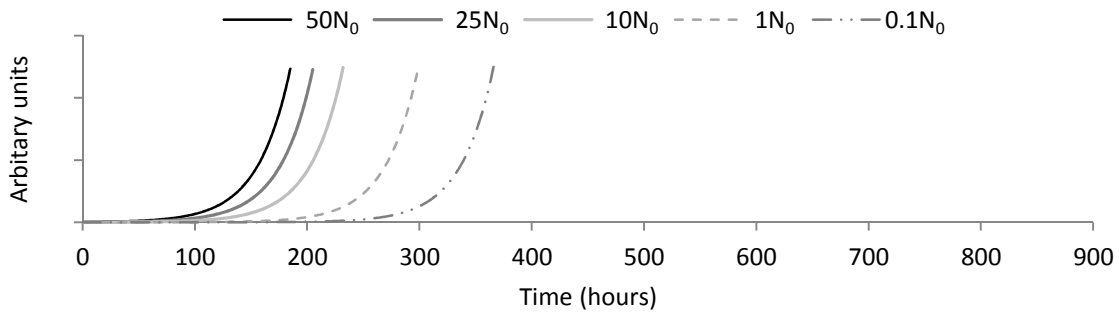

Figure 3-2 Model of the acclimatisation of reactors inoculated with varying amounts of bacteria as denoted by N_0 based on the formula $N_T = N_0 exp^{rt}$ where r the growth rate is the average growth rate determined experimentally of 0.03 hr^{-1} and t time is given on the bottom axis

The pattern of acclimatisation that occurred for the wastewater and starch fed did not follow the model. All reactors acclimatised at the approximate same time. If the growth rates and time are equal, mathematically this means that N_0 is similar for the different volumes of inocula.

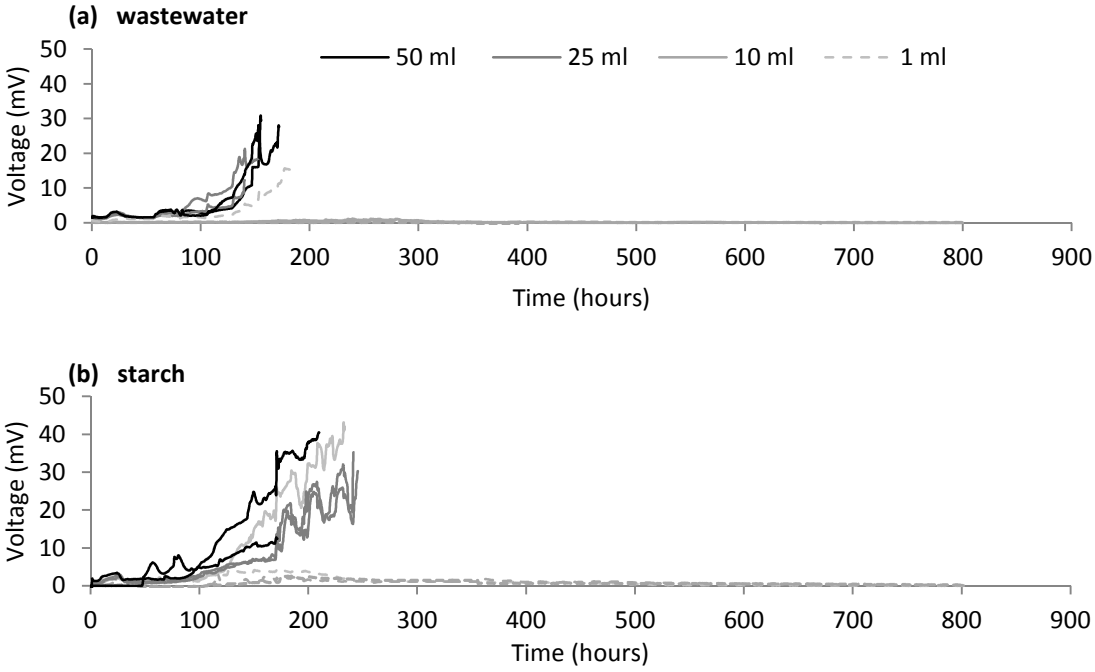

Figure 3-3 Pattern of acclimatisation of the wastewater (a) and starch (b) fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Superficially the pattern observed for the acetate fed reactors appears to follow the model pattern. However this is not the case as the lag time to acclimatisation is over extended with reducing amounts of inocula.

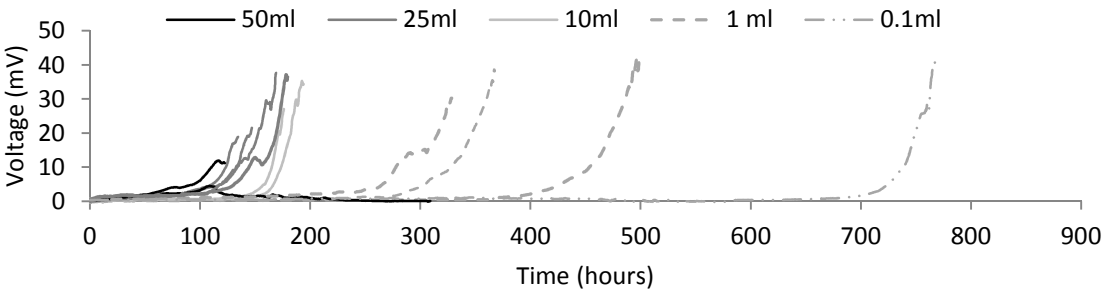

Figure 3-4 Acclimatisation of the acetate fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Using $N_T=N_0\exp^{rt}$ the calculated number of bacteria at the time the reactor inoculated with 0.1 ml (which must have contained at least one bacteria) reaches 10 mV would be 1.8×10^{11} bacteria, equivalent to the predicted number of bacteria in 1 kg of soil (Whitman et al., 1998), and 4×10^{7} times greater than the number of bacteria at 10 mV in the cell inoculated with 50 ml of wastewater (assuming an MPN of 1.7 per ml). This is clearly implausible, growth is not purely exponential, there is likely to be a lag phase with no growth. Yields calculated on the basis of these N_T and N_0 values both with (up to 8 g-COD cell/g-COD) and without (10^{-4} and 10^{-7} g-COD cell/g-COD) growth in the

lag phase give results discordant with the current literature, (these are shown in appendix VII).

3.4. Discussion

If the aim of acclimatising a reactor is to get it going, then it has been shown that a larger volume inoculum will give a quicker (in the case of acetate) and more likely (in the case of complex substrates) successful inoculation, although a proportion of the intended substrate may also be needed. As clear differences were observed between experiments, acclimatisation with the intended substrate is likely to be essential to successful operation. However, more importantly, these results also give insight into the abundance and distribution of exoelectrogenic and other crucial organisms, and to their community development within a reactor.

Discovering the number of exoelectrogenic bacteria per ml of wastewater is a strategically important question. It would inform us of the sequencing depth needed to identify these bacteria. By using the MPN methodology in a series of MFCs and aerobic and anaerobic culturing methods of the same wastewater, an estimation of this number has been gained. Acetate digesting exoelectrogens can be found at an estimated quantity of 17 per ml of wastewater, giving the probability of a bacterium in 1 ml of wastewater being an exoelectrogen as 1.7×10^{-5}, or put differently 0.0017% of the bacteria present in wastewater are exoelectrogenic. With 1000 sequencing reads there would be a reasonable chance of identifying only 1 or 2 exoelectrogens. When compared to the pyrosequencing carried out in chapter 4 a similar answer emerges. Two wastewater samples were analysed, and the total sequencing effort needed to capture 90% of all the sequences in the sample estimated using statistical algorithm as shown in Appendix X. Comparing the total number of *Geobacter* (the known exoelectrogen present in the wastewater samples) found in the sample to the estimated sampling effort, in one sample Geobacter represented an estimated 0.0012 % of the total bacteria, in the other this was lower at 0.00001 %. The two very different approached result in a similar estimation of the number of exoelectrogens present in wastewater. The use of further microbial techniques such as flow cytometry or QPCR would also help the verification of these results.

The number of acetate exoelectrogens is rare: 17 per ml. The number of starch or wastewater exoelectrogens is even lower at 1 per ml. It could be plausible that these are

even rarer organisms, however the likely explanation is that a chain of metabolism is occurring, this fits with the literature (Velasquez-Orta et al., 2011, Kiely et al., 2011c). The probability of achieving a working MFC fed on a complex substrate is therefore the probability of the exoelectrogenic step as identified above, multiplied by the probabilities of each of the upstream steps in the metabolic chain, and is therefore lower than the probability of forming with the acetate step alone. The MPN value is an approximation, yet even considering the upper and lower bounds of the calculation at 95% confidence, as shown in Table 3-2, this pattern is observed. Clearly however this is dependent on the inoculum used; with different inocula such as soil or sludge one would expect different results.

Growth rates, although intuitively demonstrated by the rise in voltage within an MFC, have not previously been calculated. It is an important value to know, especially when modelling such systems. This study calculated the average growth rate of 0.03 hr^{-1}, this value agrees with those documented in the literature from known exoelectrogenic bacteria. No statistical difference is found between reactors fed on acetate and more complex wastewaters, contrary to previous work (Velasquez-Orta et al., 2011) this study shows that the growth rate of exoelectrogens is likely to be the limiting factor.

The pattern of acclimatisation demonstrated within these reactors did not follow the expected pattern. Additionally the pattern observed in the acetate reactors is different to the pattern observed in the reactors fed with more complex substrates. Simple exponential growth does not appear to be happening in either system. The values of N_T within these systems are therefore questionable, as are the calculated yields and specific activities (see appendix VII).

The positive starch and wastewater fed reactors were fewer in number due to the reduced probabilities of the communities forming, but all acclimatised at approximately the same time regardless of the inoculum volume. The growth rates calculated were not statistically different between the different inocula, time was recorded accurately. Explaining this mathematically on the basis of $N_T = N_0 exp^{rt}$ this means either: N_0 is the same for the different inoculum sizes; the N_T of the reactors producing the same voltage is actually different; the rates as defined by voltage rise are not representative of growth rates; or the system may not be described by the equation $N_T = N_0 exp^{rt}$.

More of the acetate cells acclimatised leading to a higher MPN value, the pattern of acclimatisation here does show a clear link to inoculum size, however the size of the lag phase is far greater than would be predicted. Again the rates calculated were not statistically different between the different inoculum sizes and time was also recorded accurately. Here on the basis of $N_T = N_0 exp^{rt}$ either; N_0 is not linearly related to inoculum sizes, i.e. 50 mLs of wastewater contains more exoelectrogens than 50 times 1 ml; the N_T of the reactors producing the same voltage is actually different; there is a lag period before the growth rate starts which is also related, but not linear to, the inoculum size; or again the system is not described by $N_T = N_0 exp^{rt}$.

The MPN method and therefore N_0, is based on the following assumptions: bacteria are distributed randomly within the sample; they are separate, not clustered together; they do not repel each other; and every reactor whose inoculum contains even one viable organism will produce detectable growth or change and the reactors are independent (Blodgett, 2009). It seems likely that exoelectrogens will cluster, there function of passing electrons outside the cell may be used for passing electrons between cells when no external electron sink is available (Bretschger, 2010). In the sequencing data in chapter 1063 *Geobacter* are found in one wastewater sample and 4 in the other, also indicative of clustering. If clustering is occurring, the MPN is likely to be an underestimation as will be N_0 and N_T. This does not however explain the different patterns of acclimatisation observed between the substrates. Additionally the large upper and lower bounds given in the MPN calculations due to the relatively low sample size, could also lead to both under and over estimations of N_0 where the MPN is used.

The relationship of voltage with N_T could be more complex than assumed. Voltage generated from the electrode may be limited by properties relating to the anode itself rather than the bacteria on it, or may quickly reach saturation point of the biofilm, however then one would expect to observe the same pattern in all reactors.

Growth rates are assumed to be represented by the rising voltage measured across the reactors. This may not be the case if the bacterial population has to grow to a certain threshold level (at an unknown growth rate which may different for different inocula sizes) before any voltage is produced. Additionally an assumption is made that increasing voltage is caused by an increasing number of bacteria, not an increasing

capability of these bacteria to donate electrons, this may not be true. Again this does not account for the differences between substrates.

The period of acclimatisation is both highly complex and variable between substrates, yet does show a clear observable pattern, indicating an underlying mechanism. It seems likely that these systems are not described by $N_T = N_0 exp^{rt}$. Such deviations could be caused if the exoelectrogens present N_0 were able to induce electrogenic activity in other bacteria through the excretion of electron shuttles: $N_T > N_0 exp^{rt}$, and in addition a further growth equation of the 'induced' exoelectrogens would act to confuse the picture. In the case of the complex substrate systems something within the chain of metabolism which is unrelated to the bacteria quantity could be triggering the start of the acclimatisation, this causes the reactor to work or fail regardless of the number of exoelectrogens present at the start. In the acetate fed reactors a further factor related to the inoculum size could be causing the extended lag observed, such as the movement of the exoelectrogens to the anode surface.

The period of acclimatisation is not only complex, it is likely to be a period of high competition for resources and possible low efficiency for the exoelectrogens as seen from the low coulombic efficiencies and comparable COD removal in both the positive and negative reactors (see appendix VI).

If the aim of acclimatisation is to merely 'get the reactor going' this study has shown that using a large proportion of wastewater is best. The experiment has also demonstrated that the abundance of organisms needed to start an MFC is low within wastewater, and even lower when these systems are to be fed on complex substrates. The growth rates defined are similar to those observed for exoelectrogenic species in other environments, and are likely to be the limiting factor in MFC acclimatisation. The pattern of acclimatisation a fuel cell is complex and not explained solely by exponential growth. The clear differences between these systems demonstrate the vital importance of acclimatising a community for the eventual use of the reactor. A reactor fed on acetate is different to one fed on wastewater. By developing a greater understanding of this ecology and its development, the move towards more stable biological system can be made. Understanding the nature, abundance and location of these exoelectrogens is crucial.

Chapter 4. Can Microbial Fuel Cells operate at low temperature?

4.1. Introduction

Bioelectrochemical Systems (BES's) are being heralded as a new method of energy efficient wastewater treatment, yielding electrical energy or other products from the bacterial breakdown of organics in an electrochemical cell. For future application of this technology understanding the microbial ecology, community structure and relating this to performance is desirable (Parameswaran et al., 2010) . The majority of fuel cell research is carried out using acetate as a feed at 30^{o}C with the implicit assumption that this will translate into the treatment of real wastewaters at ambient temperatures. To use low strength high volume wastes like wastewater the bacterial communities within BES need to be able to digest complex and variable substrates and do so outside, which in the UK, Europe and many parts of the USA means at low temperatures. If the communities of bacteria able to perform this task do not occur naturally further work and investment into this area may be futile.

As noted above most BES studies are conducted in laboratories at a temperature of 30 ^{o}C (Call and Logan, 2008, Cheng and Logan, 2007a, Selembo et al., 2009b). Few ambient treatment plants will get this warm. Several studies investigating the performance of MFCs over temperatures between 20-30 ^{o}C have found that the maximum power output with acetate was reduced by 9% (Liu et al., 2005a) and 12% (Ahn and Logan, 2010) when the temperature was lowered from 30 ^{o}C to 20 ^{o}C and 23 ^{o}C respectively, using beer waste a 10% drop was seen at these temperatures (Wang et al., 2008). The reduction in performance was lower than predicted by biological process modelling, suggesting that bacterial growth at 32 ^{o}C is not optimal, or that other factors are more limiting (Liu et al., 2005a). Complex wastes were also treated by Ahn and Logan (2010), and it was found that temperature had a greater effect on these than the simple compounds.

Lower (below 20 ^{o}C) and more realistic temperatures have been even less well studied. Min et al (2008) found that at 15 ^{o}C no successful operation was achieved, after 200 hours of operation the experiment was stopped. Cheng et al. (2011) found at 15 ^{o}C start up took 210 hours but at 4 ^{o}C there was no appreciable power output after one month (720 hours) and the experiment was stopped. In the same study a reactor started at 30 ^{o}C was then dropped to 4 ^{o}C and power output was achieved, but around 60% lower than

that the higher temperature. Larrosa-Guerrero *et al*. (2010) operated reactors at 4 ^{o}C and 35 ^{o}C using a mixture of domestic and brewery wastewater, observing a decline in COD removal from 94% to 58% and power density from 174.0 mWm^{-3} to 15.1 mWm^{-3} at the lower temperature.

By contrast Jadhav and Ghangrekar (2009) operated an MFC's in a temperature range of 8-22 ^{o}C and found that the current and coulombic efficiencies were higher than that produced in the temperature range of 20-35 ^{o}C. However in this study temperatures were ambient not controlled and thus confounded by time. They inferred that a reduction in methanogenic bacterial activity at lower temperatures increased MFC performance, although the microbiology of the systems was not examined. Similar results were obtained by Catal *et al.* (2011), here the biofilm was examined using scanning electron microscopy and found to be thicker in the higher temperature reactors.

MFC systems are based on electrochemical and microbiological principles: temperature affects both. The electrochemical impacts of temperature can be calculated using the Nernst equation based on known free energies for substrates such as acetate, or estimated free energies if wastewater is used (Logan, 2008). In bacterial systems rates of reaction roughly double for every 10^{o}C rise in temperature (Rittmann, 2001). However, the actual behaviour of these complex systems at different temperatures and fed on different substrates remains an area of great uncertainty in this field of research.

An increasing number of studies into the microbial communities of BES using techniques such as restriction fragment length polymorphism (RFLP), clone libraries and denaturing gradient gel electrophoresis (DGGE) are adding to the knowledge base we have about these communities. There are advantages to these various techniques such as the high reproducibility and in the case of DGGE and RFLP the large number of samples than can be run (van Elsas and Boersma, 2011, Kirk et al., 2004). However all these techniques are limited in that only a small fraction, (in the case of DDGE estimated at 1-2 % (Macnaughton et al., 1999), of the species present are targeted in these studies, total diversity cannot be estimated from these limited results. Never the less it has been repeatedly shown that *Geobacter sulfurreducens* dominates in acetate fed reactors, although this can vary when reactors are inoculated with different media (Kiely et al., 2011c). As substrates become increasingly complex moving from VFA's

to carbohydrates to actual wastewater the dominant species become more varied (Kiely et al., 2011c). Some wastewater fed reactors were found to be dominated by *Betaproteobacteria* (Patil et al., 2009), although in other studies *Geobacter* still dominates (Cusick et al., 2010).

Most of the techniques that have been used are limited by their capacity to identify the most dominant species within the communities. Next generation sequencing (capable of sequencing to a far greater depth) has now been used in two MFC studies. Lee *et al*. (2010) used FLX Titanium pyrosequencing to sequence four samples of biofilm, triplicate samples were taken from an acetate fed reactor comparing this to a single sample taken from a glucose fed reactor. The profiles found in the samples were not significantly different. A further study by Parameswaran (2010) analysed the biofilm of two MFC reactors fed on ethanol examining the impact to the communities when methanogenesis was prevented in one, identifying the role of hydrogen scavengers.

The aim of this study was to determine if microbial fuel cells can work at low temperatures, and if the inocula affects this. By running reactors fed on both wastewater and acetate the relative importance of the final 'electrogenic' step, and the up- stream hydrolysis and fermentation steps can be evaluated. The impact of temperature, inoculum and substrate on the microbial communities and total diversity within these reactors was examined using next generation sequencing techniques.

4.2. Methods

4.2.1. *Experimental design*

The variables examined were: temperature (warm 26.5 ^{o}C and cold 7.5 ^{o}C); substrate (acetate and wastewater); and inoculum (Arctic soil and wastewater). Each set of conditions were run in parallel duplicate reactors and biofilm samples taken from each. The two series of experiments, acetate and wastewater, were conducted using the same 8 reactors under identical conditions, the two wastewater inoculum samples were used to seed the acetate (wastewater sample1) and wastewater fed (wastewater sample 2) experiments. This is represented in Figure 4-1.

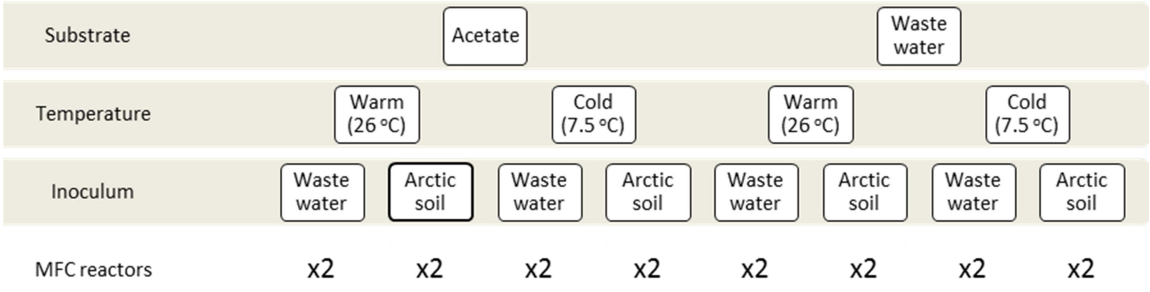

Figure 4-1 Illustration of the multi-tiered reactor conditions used

The warm temperature was chosen to represent the typical ambient laboratory temperatures of many MFC studies. The low temperature is the lowest sustained temperature of a wastewater treatment plant in the North of England (54°58'N, 01°36'W) experienced over a winter period (Northumbrian Water Ltd). The different substrates represent the most commonly used laboratory substrate acetate, and compared to wastewater. The two different inocula were the usual inoculum of wastewater, and Arctic soil (see below) which could potentially have more bacteria with low temperature, exoelectrogenic capability.

Wastewater typically contains 10^5 - 10^6 bacteria per mL (Tchobanoglous, 1991) soils can contain around 10^9 bacteria per gram (Whitman et al., 1998). Many soil environments are low in oxygen, and iron rich, favouring anaerobes and iron reducers and potentially therefore exoelectrogens. Arctic soils have been shown to have to be biologically active, accounting for around 6% of the total global methane sources (Ehhalt et al., 2001). (Hoj et al., 2005, Kotsyurbenko et al., 2004, Metje and Frenzel, 2005). Soil taken from Ny-Ålesund, in the Spitsbergen area of Norway has been shown to contain a wide range of methanogenic groups active at temperatures ranging from 1-25 °C (Hoj et al., 2005, Hoj et al., 2008).

4.2.2. *Reactor design and operation*

Eight identical double chamber tubular MFC reactors (78 mL each chamber) with an internal diameter of 40mm and length of 60mm were used. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (Tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor, and a multimeter to measure the voltage (Pico ADC-16, Pico Technology, UK). The membrane between the reactor

chambers was Nafion 117, with an area of 12.6cm^2. Reactors were sparged with 99.99% pure N2 in the anode chamber, and air in the cathode chamber for 15 minutes after every re-fill.

Four reactors were operated at a temperature of 26.5 ^{o}C in an incubator (Stuart Scientific SI 50, UK), the other four at 7.5 ^{o}C in a low temperature incubator (Sanyo MIR-254, (Sanyo Biomedical, USA). The temperature was logged continuously over the experiment using a EL-USB-1 temperature data logger (Lascar Electronics, UK). The reactors were inoculated and filled with substrate, replacing this every 5-6 days until a stable power generation was achieved. The reactors were then re-filled and three successive 3 day cycles were run logging the voltage over this time. Chemical oxygen demand (COD) removal during each batch was determined using standard methods (APHA, 1998) and Spectroquant ® test kits (Merck & Co. Inc., USA).

4.2.3. *Media and inocula*

Autoclaved acetate media (Call and Logan, 2008) containing 1 g/L sodium acetate was compared to wastewater taken from Cramlington wastewater treatment site (Northumbrian Water Ltd, UK) which was UV sterilised prior to use. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see appendix V). The cathode chamber was filled with 1M pH 7 phosphate buffer. The conductivity of the nutrient media, wastewater and the phosphate buffer was measured using an EC 300 (VWR Ltd, UK) and equalised for the temperatures of 7.5 ^{o}C and at 26.5 ^{o}C.

The wastewater inoculum was collected from Cramlington wastewater treatment plant, a Northumbrian Water site in the North of England, it was raw wastewater collected prior to any form of treatment, and is believed to be of mixed industrial and domestic, COD 0.7-0.8g/L. Once collected the sample was stored in a fridge at 4 ^{o}C within a closed container. The Arctic soil was collected from Ny-Ålesund, Spitsbergen in Svalbard. This was wrapped within three sealed bags and stored at 4 ^{o}C until used. The inocula of wastewater and soil were measured out to 5 mL or 5 g respectively before being added to the reactors. Samples of each inocula were preserved in a 50:50 in a mix of ethanol and autoclaved PBS pH7 in the freezer at -20 ^{o}C for microbial analysis.

4.2.4. *Microbiological techniques*

At the end of each experiment the anode was removed aseptically from the chamber using aseptic technique and preserved in a 50:50 mix of ethanol and autoclaved PBS pH7 and stored in a freezer at -20 ^{o}C. A 5 ml or 5 g sample of the original inocula was also taken and preserved in this way. The inocula samples were pelletized and the DNA then extracted. With the anode samples the bacteria that had dispersed into the liquid was pelletized and then added to the central section of the anode felt cut from the whole anode. The DNA was extracted by placing this sample into the beaded tube of a FastDNA Spin Kit for Soil (Qbiogene MP Biomedicals, UK). Extraction was completed according to the manufacturer's instructions. The samples were then pyrosequenced following amplification of the 16s rRNA gene fragments.

The primers used were F515 (GTGNCAGCMGCCGCGGTAA) and R926 (CCGYCAAT-TYMTTTRAGTTT). Each sample was labelled with a unique 8 base pairs (bp) barcode connected to a GA linker. Sequencing was completed from the Titanium A adaptor only forward from the F515, capturing the V4 region and most of the V5 region with a Titanium read of 400-500 bp. Triplicate PCR reactions were carried out using the Roche FastStart HiFi reaction kit (Roche Diagnostics Ltd., UK) and subject to the following optimised thermal cycles: initial denaturation at 95°C for 4 minutes; 23 cycles of denaturation at 95°C for 1 minutes; annealing at 55°C for 45 seconds; extension at 72°C for 1 minute; final extension at 78°C for 8 minutes. An automated thermal cycle Techne TC-5000 (Bibby Scientific, UK) was used.

The triplicate samples were then pooled and cleaned using QIAquick PCR Purification Kit (Qiagen, UK). The DNA concentration was quantified by UV-Vis spectrophotometry using a Nanodrop 2000 (Thermo scientific, USA). The individual samples were pooled to give equal concentrations of all reactor samples, and double concentration of the wastewater and arctic soil seed. Sequencing was carried out by the Centre for Genomic Research (University of Liverpool, UK) using the Roche 454 sequencing GS FLX Titanium Series.

4.2.5. *Data analysis*

The pyrosequencing data set was split according to the barcodes and unassigned sequences were removed[1]. The flowgram files were cleaned using a filtering algorithm Amplicon Noise (Quince et al., 2009) to give the filtered flowgram file. Filtering at a minimum flowgram length of 360 bp including the key and primer before first noisy signal, all flowgrams were then truncated to 360 bp. A pairwise distance matrix was then calculated using the Pyronoise algorithm (Quince et al., 2009). This uses an iterative Expectation-Maximization algorithm which constructs denoised sequences by clustering flowgrams using the initial hierarchical clusters generated in the previous step and the filtered flowgram file. The cut-off for initial clustering is set at 0.01 and the cluster size is 60, as recommended by Quince et al. (2009). The flowgrams can then be denoised.

PCR errors were then removed again using Seqnoise, generating a distance matrix using the Needleman-Wunsch algorithm for pairwise alignment. The optimal parameters used here were the cut-off for initial clustering of 0.08 and cluster size of 30. Chimera removal was completed using the Perseus algorithm (Quince et al., 2011) which for each sequence searches for the closest chimeric match using the other sequences as possible parents. (Quince et al., 2011). The sequences are then classified and the good classes filtered at a 50% probability of being chimeric, producing the final FASTA file which is denoised and chimera free ready for analysis in QIIME (Caporaso et al., 2010).

Using the QIIME pipeline tutorial the following analysis was completed: assigning taxonomy using Greengenes (http://greengenes.lbl.gov) at the 97% similarity level; creating an OTU table; classification using the RDP classifier; summary of taxonomic data from classification; generation of rarefaction data of the diversity in a reactor; calculation of the differences between the reactors; performing Principle Co-ordinates Analysis (PCoA); jackknifing and bootstrapping to understand uncertainty in beta diversity output; and generating Unweighted Pair Group Method with Arithmetic Mean (UPMGA) trees for hierarchical clustering of samples. The dissimilarity of the community structure between duplicates was examined using both a weighted (relative abundance) and unweighted (presence/absence) phylogenetic diversity metrics using

[1] *The analysis of the pyrosequencing data was carried out by Dr Matthew Wade, a Bioinformatics researcher within the School of Civil Engineering and Geosciences at Newcastle University.*

UniFrac, giving a distance matrix containing a dissimilarity value for each pairwise comparison. The raw OTU table generated was used to produce the species abundance pattern (with the log abundance normalised to the number of sequences in each sample) and the rank abundance curves, (where percentage abundance is used to normalise samples).

An estimate of the total diversity for each sample was calculated using the Bayesian approach as described in Quince et al. (2008), where the 'posterior distribution' of the taxa area curve is estimated, from the known distribution of the data gathered in the sequencing. Three distributions are modelled: log-normal; inverse Gaussian; and Sichel, and deviance information criterion (DIC) are used to compare the fit from each model. The lower the deviance or DIC values the better the model fit, those models within 6 of the best DIC value can be considered as a plausible fit. Using the fitted abundance distributions the sampling effort required to capture 90% of the taxa within that sample is estimated.

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run analysis of variance (ANOVA) tests on the experimental data, and t-tests on the distance matrix data for the sequences samples. Data were checked for normality prior to completing ANOVA, and if necessary the Box-Cox transformation was used.

The performance of the MFC reactors were analysed on the basis of three variables: % COD removal as measured; coulombic efficiency (CE); and power density (mW/m^2). The latter two variables were calculated using the measured COD and voltage within the cells, as described in Appendix VIII. Correlation of the community structure with these performance factors was done using BEST (Biological Environmental and Stepwise method) within Primer 6 (Primer-E Ltd. UK).

4.3. Results

4.3.1. *Cell acclimatisation*

All 16 reactors acclimatised and produced voltage. The acetate fed reactors showed a clear pattern of acclimatisation related to both temperature and inocula with the warm reactors acclimatising first, and the Arctic soil inoculated reactors starting first as shown in Figure 4-2. The cold wastewater inoculated reactors did not produce current until after around 800 hours, longer than the time allowed in previous studies (Cheng et al.

2011, Min et al (2008). The acclimatisation of the wastewater fed reactors was only affected by temperature: the warm reactors started producing current at day 1, the cold reactors at day 20. All duplicates behaved in a very similar way.

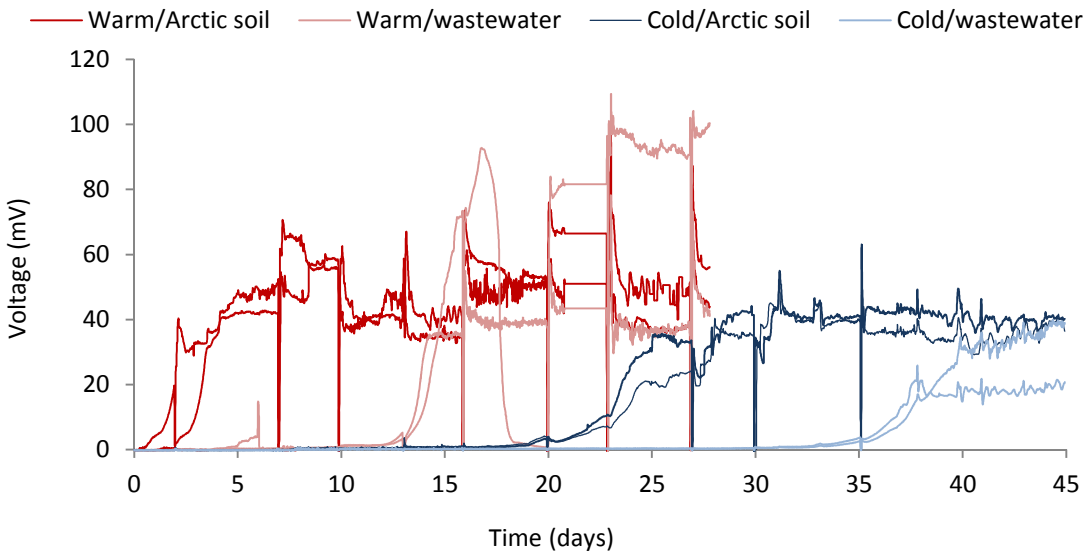

Figure 4-2 Acclimatisation of the acetate fed reactors inoculated with the two different inocula and run at warm (27.5 ^{o}C) and cold (7.5 ^{o}C) temperatures

4.3.2. *Cell performance*

Over the three batch runs, the reactor performance was variable especially within the warm reactors, as seen in Figure 4-3. The variation in performance was not a function of either the inocula or the substrate and the highest variation was seen between the duplicates.

Three measures of performance averaged for each reactor over the triplicate batches are shown in Figure 4-4. The coulombic efficiency is higher in the acetate fed reactors; and the COD removal is higher in the wastewater fed reactors. Power densities do not appear to vary with substrate, inoculum or temperature, however two individual reactors had considerably higher power densities than the others and their duplicates: acetate warm ww 2; and wastewater warm soil 1.

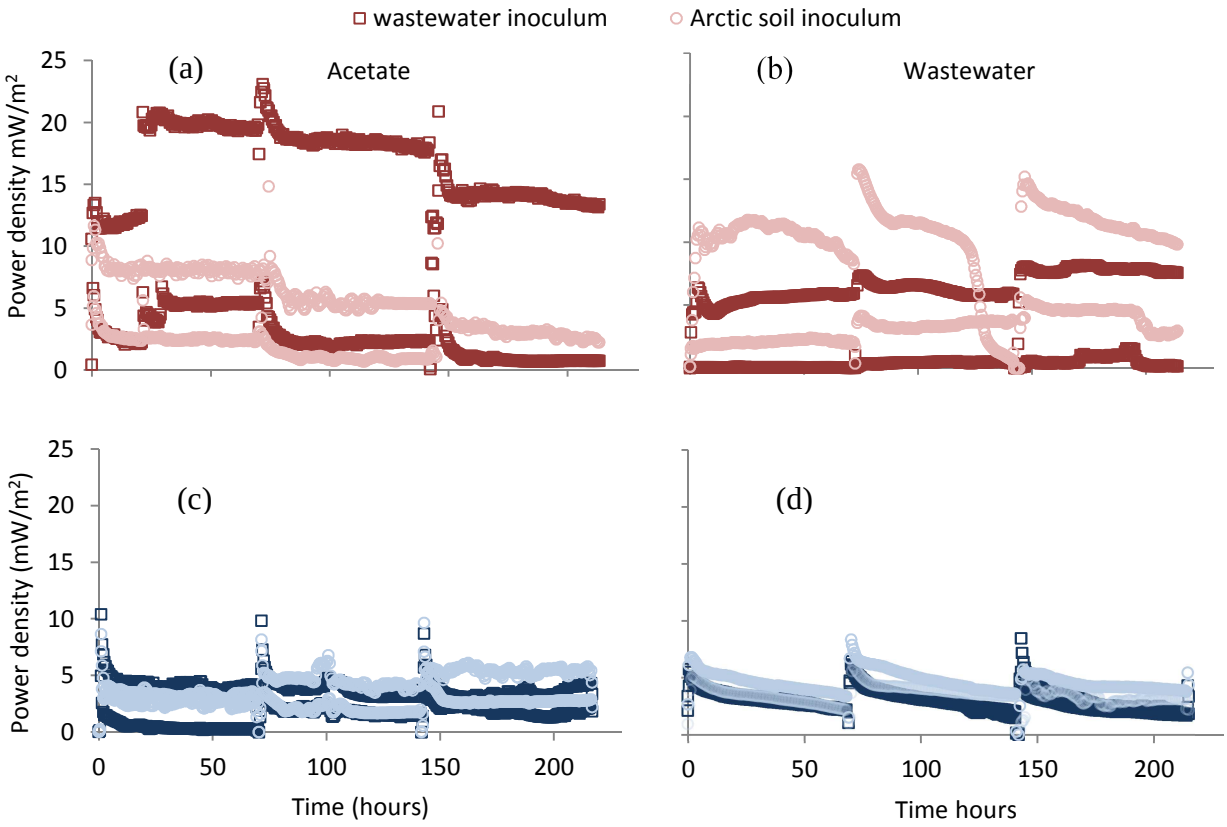

Figure 4-3 Power density plots showing the three consecutive batch runs for: (a) acetate fed reactors run at 27.5 °C, (b) wastewater fed reactor run at 27.5 °C (c) acetate fed reactor run at 7.5 °C (d) wastewater fed reactor run at 7.5 °C

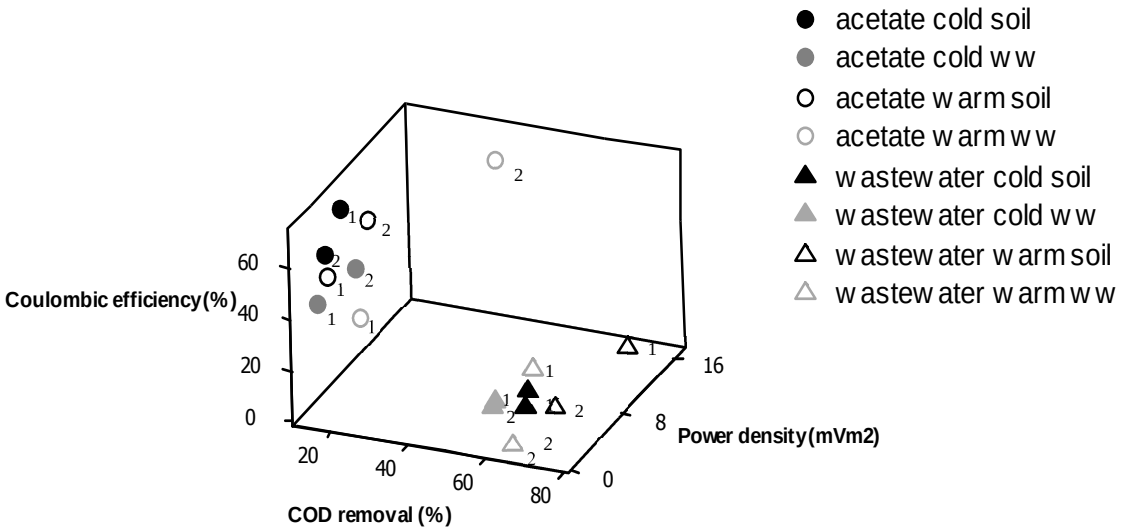

Figure 4-4 3D plot showing reactor performance in terms of Coulombic efficiency, COD removal and power density of the various reactor conditions, duplicates of each condition are labelled on the plot next to the symbols

By performing an ANOVA on the three performance indicators using the factors of feed, temperature and inocula a complex picture emerges. The power density results, i.e. the ability of the biofilm to put electrons to the circuit, were not normally distributed, when transformed, none of the performance factors analysed were significant (feed p = 0.746, inoculum p = 0.249, and temperature p = 0.147). For coulombic efficiency both inoculum (p=0.009) and feed (p=0.000) were significant yet temperature was not. The acetate fed reactors performing better (54.5%) than wastewater fed ones (12.3%), and the Arctic soil inoculated reactors performing better (37.4%) than the wastewater inoculated ones (29.4%). The reactors fed wastewater removed significantly more COD (62.1%), than the acetate reactors (19.4%) (p=0.000) the warm reactors also removed more (45.9%) than the cold ones (33.7%) (p=0.000), the type of inoculum was not significant. Two way ANOVA was performed between each interaction with each performance indicator. For CE the interaction between substrate and inoculum was significant (p = 0.057) with the inoculum having a much stronger effect with the acetate feed than the wastewater feed, and the Artic soil acetate fed reactors performing the best. The interaction between substrate and inoculum was also significant in the COD removal (p = 0.008), the Arctic soil inoculum having a higher COD removal in the wastewater fed reactors, but a lower COD removal in the acetate fed reactors than the wastewater inoculum. No other interactions were significant.

4.3.3. *Similarity of duplicate reactors*

It is seen in the data above that the duplicate reactors performance varied considerably, especially for the warm temperature reactors. Using the sequencing data a Unifrac dissimilarity matrix was plotted, using phylogenetic information the ‘distance’ between each sample is quantified and corresponds to the degree of similarity (Appendix IX). The values show that the duplicate reactors fed with acetate are indistinguishable (p=0.000). This was observed with both the weighted analysis which incorporates information on relative abundance of each OTU, and the unweighted analysis which is based on the presence or absence of each OTU. The wastewater fed duplicate reactors were typically different, with the exception of the hot Arctic soil inoculated reactors (p=0.000). The two wastewater inocula samples taken from the same treatment plant but at different plants were also indistinguishable (p=0.000). This pattern is also observed in Figure 4-5, where the acetate duplicates are paired, and appear to cluster on the basis of temperature. The wastewater fed reactor duplicates are not paired together and do not

cluster with temperature or inoculum. Further details of the bacteria groups present within these reactors can be found in Appendix XI.

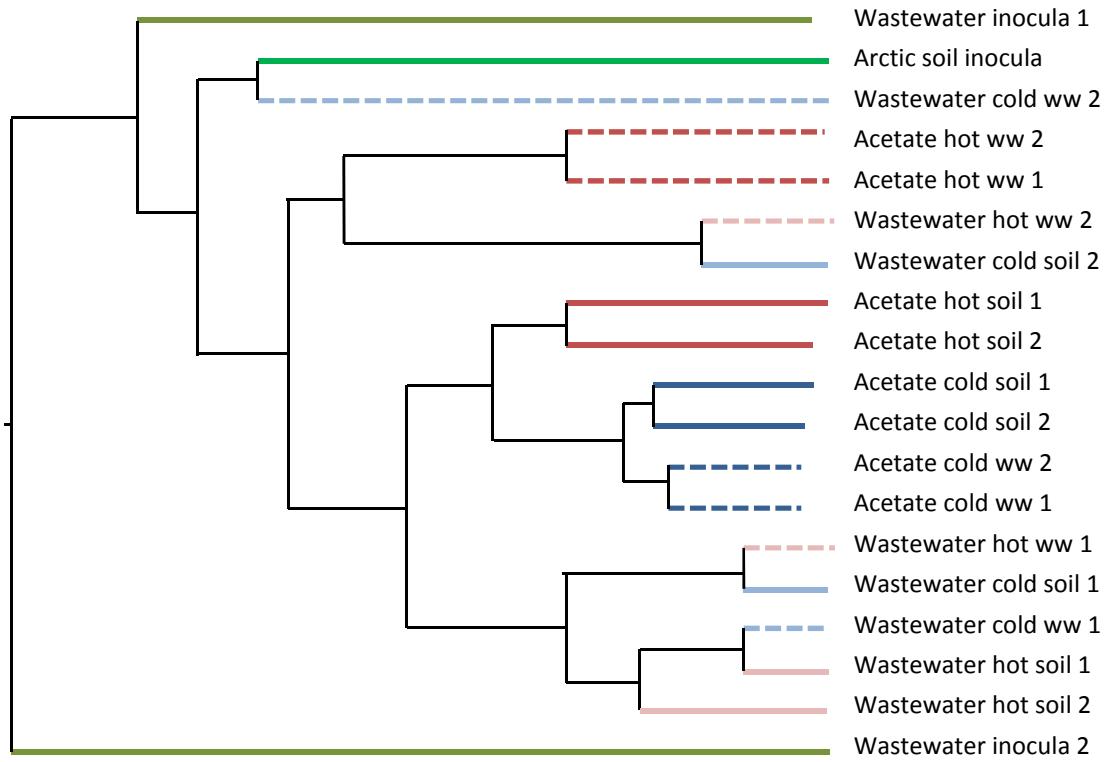

Figure 4-5 Dendrogram resulting from the UPMGA hierachical weighted clustering of samples, the length of lines is relative to the dissimilarity between samples, groupings of samples are denoted by the coloured end portion of the lines

4.3.4. *Microbial diversity*

In total 19 samples were analysed. The number of sequences per sample ranged from 8112 to 77436 with a total number of observations of 549178. The species abundance pattern plotted from the OTU table shows a large variation in the diversity of the samples shown in Figure 4-6. As expected the Arctic soil inoculum is the most diverse, followed by the wastewater inocula. The acetate fed reactors however are considerably more diverse that the wastewater fed reactors, the most diverse of these (acetate cold soil 2) has a similar diversity to the wastewater inoculum, and the least diverse (acetate warm ww 2, the reactor with the highest power density) is similar to the most diverse of the wastewater fed reactors.

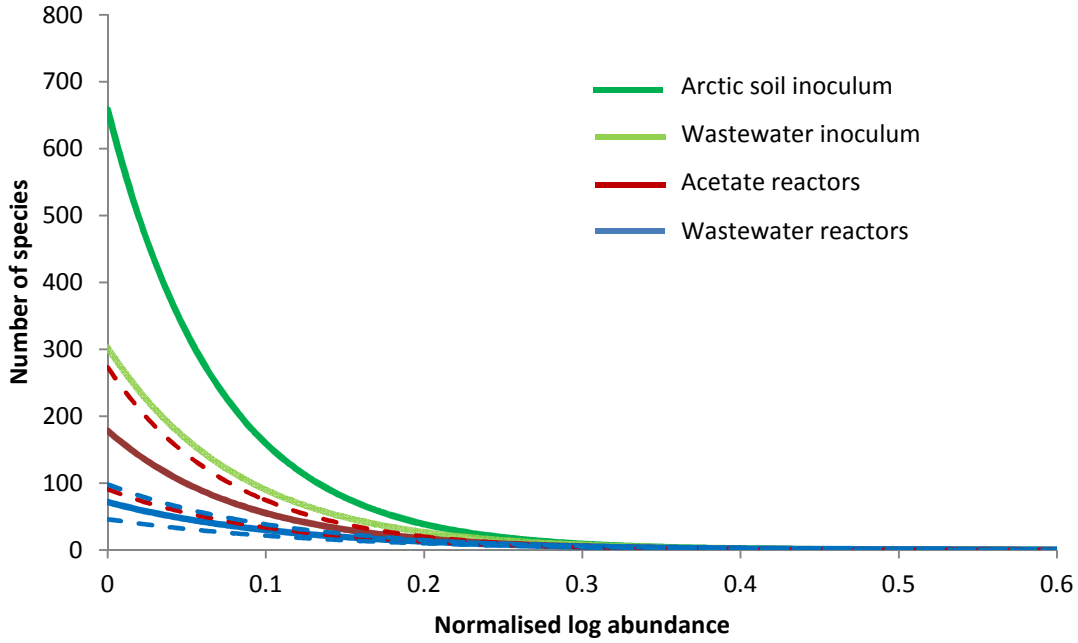

Figure 4-6 Species abundance pattern, the number of species is plotted against the log abundance normalised to the total number of observations for each sample. The plots for the acetate and wastewater fed reactors are averages of the eight reactors used, the highest and lowest within each substrate grouping are shown with the dashed lines. The wastewater inoculum line is an average of the two samples

The observation of the greater diversity in the acetate fed reactors is also seen in the total diversity estimates. A summary of these values is presented in Figure 4-7 where is clearly seen that for all the three distribution models the acetate fed cells have a higher predicted diversity, and that the acetate soil inoculated reactors have a higher total diversity than the wastewater inoculated ones. Performing a nested ANOVA on the Box Cox transformed total diversity estimates, shows that the acetate fed reactors have a statistically significantly higher diversity (log-normal $p = 0.001$; inverse Gaussian $p = 0.000$; and Sichel $p = 0.027$). Within the acetate fed reactors the Arctic soil inoculated reactors have a higher predicted diversity (log-normal $p = 0.006$; inverse Gaussian $p = 0.003$; and Sichel $p = 0.013$), the lower temperatures also give higher diversity (log-normal $p = 0.037$; inverse Gaussian $p = 0.012$; and Sichel $p = 0.029$). There is a strong interaction between the acetate feed and the inoculum type ($p = 0.024$) but not with temperature ($p = 0.156$) observed in both the log-normal and inverse Gaussian distributions. The full tables of diversity predictions, DIC values and estimate sampling requirements can be found in appendix X.

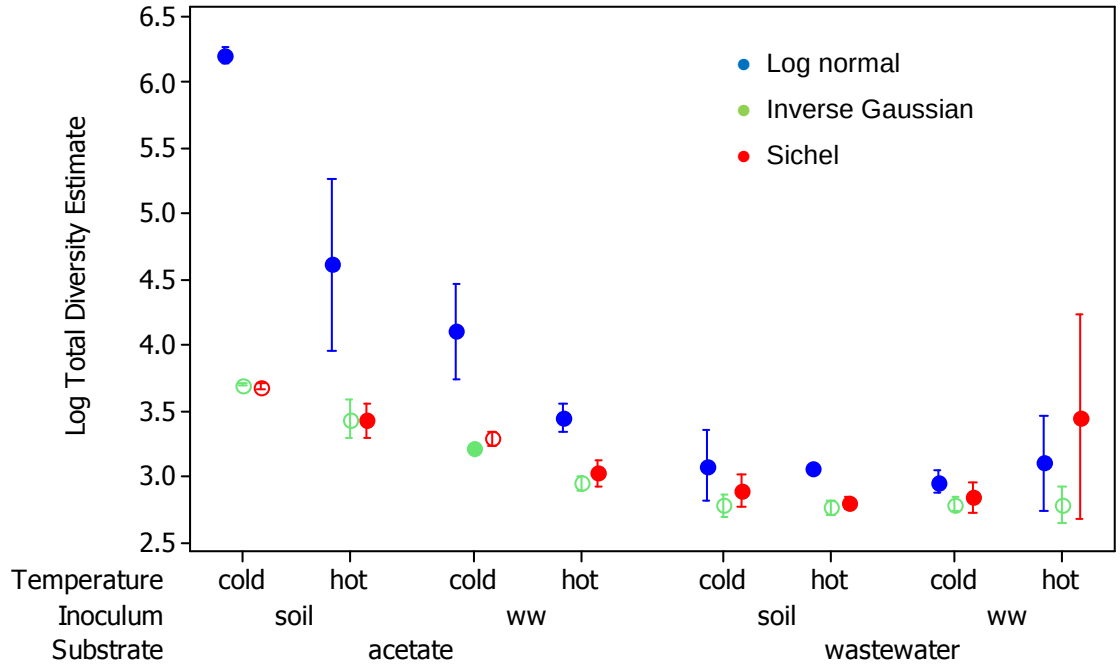

Figure 4-7 The estimates of total diversity for each set of reactor conditions, the three points within each sample are the mean of the duplicate samples modelled to log-normal, inverse Gaussian, and Sichel estimates, the best fit according to the DIC values is denoted by a closed circle, lines are one standard error of the mean

4.4. Discussion

All the reactor conditions tested produced current showing that MFCs can function at low temperatures, with real wastewaters and the bacteria required for them to do so can be found within the wastewater itself. This finding is of great significance to the industrial feasibility of MFC technology for wastewater treatment.

The power output produced by the MFCs was not significantly affected by either temperature feed or inoculum. Although some warm reactors achieved a power density much higher than the cold reactors, due to the variability between reactors this was not significant. The reasons for this variability, were not discovered, no statistical link could be made between the community structure and the power density. The higher coulombic efficiencies within the acetate fed reactors did not translate into higher power densities, only low amounts of COD was converted efficiently into power. Whereas in the wastewater fed reactors more COD was converted less efficiently producing a similar power. In terms of wastewater treatment, this high COD removal, albeit at low CE, is an advantage.

The lack of temperature effect seems at first to be unlikely. Based on the laws of thermodynamics, the free energy available in many chemical reactions decreases as temperature decreases. However in a fuel cell system the energy available is the difference in energy between two half reactions. As both the half cells are equally affected by temperature, the difference between them, or energy available does not decrease with lower temperatures (Appendix II). This is a simplification, many other factors such as dissociation constants and partial pressures of gases will affect the energy, additionally the metabolic activity of the bacteria also reduces with lower temperatures (Rittmann, 2001), however these do not appear to be having a significant impact although may be responsible for some of the variability in performance. On the basis of the results presented here, it can be asserted that low temperature systems have a similar level of energy available for both bacterial metabolism and electricity production as higher temperature systems.

The lack of temperature effect could be caused by the reactor design itself. The inherent inefficiencies and overpotentials within the reactors could be limiting the performance such that the temperature effect is not observed, i.e. all the reactors are working at the limit of their performance and warming them cannot result in improvements. If lower temperature reactors did prove to have slower microbial kinetics, as would be expected and as is indicated by the slower acclimatisation in the cold reactors this could be overcome through relatively simple engineering solutions such as increasing the size of the anode. An increase in the size of the anode would give a greater surface area for the biofilm to grow, and therefore more active bacteria to compensate for the slower metabolic rates.

A further counter intuitive result of this study it that the acetate fed cells have a higher microbial diversity than the wastewater fed cells. It would be assumed that in a wastewater fed systems that the complexity of the substrates available for metabolism, and different metabolic pathways would result in a higher diversity of bacteria, with different groups digesting different substrates at different times. With acetate fed reactors, the only metabolic pathway within a fuel cell should be the direct breakdown of acetate and donation of electrons to the electrode, the most efficient species should dominate theoretically leading to a much less diverse community. This is not seen to be

the case, with a higher diversity in the acetate fed cells being shown both by the species abundance pattern and by the analysis of all the total diversity estimates.

It is proposed that the diversity of the systems is determined not by the diversity of the metabolism within it, but by the overall energy available to the bacteria, and that the free energy available to bacteria in the acetate reactors is greater than in the wastewater reactors. This energy difference could be due to several reasons: acetate may have more free energy per g COD than wastewater; the free energy in acetate may be more accessible to the bacteria, i.e. it is easier to degrade than many of the compounds in wastewater; or that energy is lost during the metabolic chain, with acetate this chain is short, therefore the losses are low, within wastewater these chains are much longer and therefore the losses of energy are greater, this would also produce the coulombic efficiencies observed. The fact that there is no observed difference in the diversity between the warm and cold reactors is further evidence that the energy available in these is actually similar.

Results indicate that the energy flux within a microbial system is key to determining the ecology of that system. The total free energy available is likely to affect the balance of births and deaths of individual species, with greater energy resulting in more births i.e. greater abundance and therefore ultimately greater diversity. The free energy will also impact on the speciation rate (i.e. a greater number of births will ultimately lead to greater chances for speciation). This is counter to the theory that a diverse range of substrates available would provide a variety of different metabolic pathways for different organism to exploit, and therefore lead to a higher diversity.

If a quantitative link could be made between the free energy in a system and the diversity modelling of these complex biological ecologies, being able to understand such phenomena as acclimatisation, adaptation and functional redundancy, and ultimately therefore the manipulation of biological systems becomes a greater possibility (Curtis and Sloan, 2006). We are however still a long way from this in the plant and animal world ecologists have argued there is no single species/energy link (Clarke and Gaston, 2006) and even if it was the key parameter the free energy in wastewater systems cannot yet be reliably measured. Although it is evidenced here that free energy may be the key in determining diversity, a conclusive answer cannot be

given let alone a quantitative link on the basis of these results alone, further research is required.

A further effect on diversity is seen with the inoculum, which interacts with the substrate. The Arctic soil inocula has a greater diversity which seems to be carried forward into the acetate fed cells, a greater number of these species surviving within the reactors where energy may be plentiful. As the performance of the acetate and wastewater fed cells is similar despite the increased diversity of the acetate reactors, it could be concluded that this increased diversity is non-beneficial, or at least neutral to the performance of the reactor. Thus although wastewater reactors will always have lower coulombic efficiencies due to the losses within the metabolic chain, they may actually be more efficient at turning the energy available into wastewater digesting biomass and electricity.

The majority of fuel cell research is conducted at warm temperatures and with simple substrates. It has been shown in this research that reactor performance is not significantly affected by the temperature, neither is the diversity of the community developed. Inoculating reactors with cold adapted organisms does not have any benefit on the performance of the reactors. The substrate fed to the reactor again has little impact on the performance, however results in very different diversities.

It is generally assumed that an acetate fed reactor may represent the optimum conditions for an MFC, however this may not be the case. These findings suggest that wastewater feed has less available energy and therefore results in a more efficient biomass being formed. This has positive implications for the introduction of bioelectrochemical systems into wastewater treatment.

Chapter 5. Time taken until failure for MEC's fed on acetate compared to those fed on wastewater

5.1. Introduction

In 2005 a discovery was made that a microbial fuel cell could be turned into a microbial electrolysis cell adding a small supplement of electricity at the cathode to produce products such as hydrogen gas (Rozendal et al., 2006, Liu et al., 2005b). This new technology has spurned much excitement and research into increasing the performance and gas yield of such reactors (Wang et al., 2011b, Sleutels et al., 2011, Cheng and Logan, 2011). The aim of this research being to achieve a commercially viable and sustainable means of treating waste organics (Oh et al., 2010, Rittmann, 2008, Clauwaert et al., 2008).

Substantial steps have been taken towards enabling the implementation of this technology. Low cost and more robust alternatives to many of the materials used in an MEC have been discovered such as stainless steel (Call et al., 2009) and nickel (Selembo et al., 2009a) cathodes. Alternative membrane materials have been trialled successfully (Rozendal et al., 2008c), as well as not using a membrane at all (Clauwaert and Verstraete, 2009). Anodes with greater surface areas have been found (Call and Logan, 2008) as well as methods to enhance the performance of the carbon anodes (Cheng and Logan, 2007b). New cell architectures and configurations have also helped improve performance (Cheng and Logan, 2011, Wang et al., 2010). Such developments have seen the performance of these reactors increase from hydrogen production rates of 0.01-0.1 m^3H_2/m^3reactor/day (Liu et al., 2005b, Rozendal et al., 2006) to 17.8 m^3H_2/m^3reactor/day (Cheng and Logan, 2011), although the same rise in not seen in the electrical recoveries of these systems 169% (Rozendal et al., 2006) 533% (Liu et al., 2005b) in the initial studies to 115% (Cheng and Logan, 2011) due to the higher input voltages used. All of this research has used acetate as a model compound.

Research with complex substrates is more limited. The ability of MECs to digest complex substrates has been proved such as domestic wastewater (Ditzig et al., 2007), piggery wastewater (Jia et al., 2010), potato wastewater (Kiely et al., 2011a) and end products of fermentation (Wang et al., 2011a, Lalaurette et al., 2009). Limited research has been conducted into the long term performance of MFCs and MECs, deterioration in performance of an MFC after a year of operation has been attributed to the gas

diffusion cathode (Zhang et al., 2011). Marine MFCs used as batteries to power offshore monitoring devices have been monitored for up to a year (Reimers et al., 2001, Tender and Lowy, 2004) and 18 months (Lowy et al., 2006), power production was maintained over this period although in two studies it did deteriorate steadily (Lowy et al., 2006, Reimers et al., 2001), and in another there were occasional drops in the output (Tender and Lowy, 2004). Such studies may not directly translate to MFCs or MECs used for wastewater, in a marine environment the ionic concentrations, gradients and flows will be different, as will the bacteria.

By analysing all the published papers in the area of MECs up to October 2011 the limited scope of how well we understand the long term performance of these systems especially when fed on real wastewaters becomes clear, as seen in Figure 5-1.In 26% of papers the duration of the experiment was not given. In many other cases this time frame is not stated explicitly but can be inferred using the tables, graphs and other information given. In relatively few articles the durability is highlighted as a factor. Two research articles have however been published which indicate the technology might have long term applicability with experiments lasting 9 months (Lee and Rittmann, 2010) and 8 months (Jia et al., 2010) , both running on acetate. Although several other studies do state a decline in performance over time (Jeremiasse et al., 2009, Rozendal et al., 2008b, Lalaurette et al., 2009, Hu et al., 2009).

With acetate fed reactors, 73% of all MEC studies, the time scales mentioned range from 4 to 6480 hours, with 1159 as the average. However when wastewater is used, (only 10% of laboratory studies) the range is between 12 and 184 hours, with an average of 122.5 hours, this time of operation is significantly different ($p=0.000$, two sample T test). For other substrates such as VFA’s and glucose the average run time is 276 hours. This is shown in Figure 5-1, the studies with no time frame stated are not included in the graph. The explanation for this disparity is not evident in the literature, in one study acetate and piggery wastewater are compared directly with acetate reactors running for 8 months and the experiments with wastewater lasting just 12 hours, no reason for this experimental procedure is given (Jia et al., 2010). There is a clear gap in this area of research.

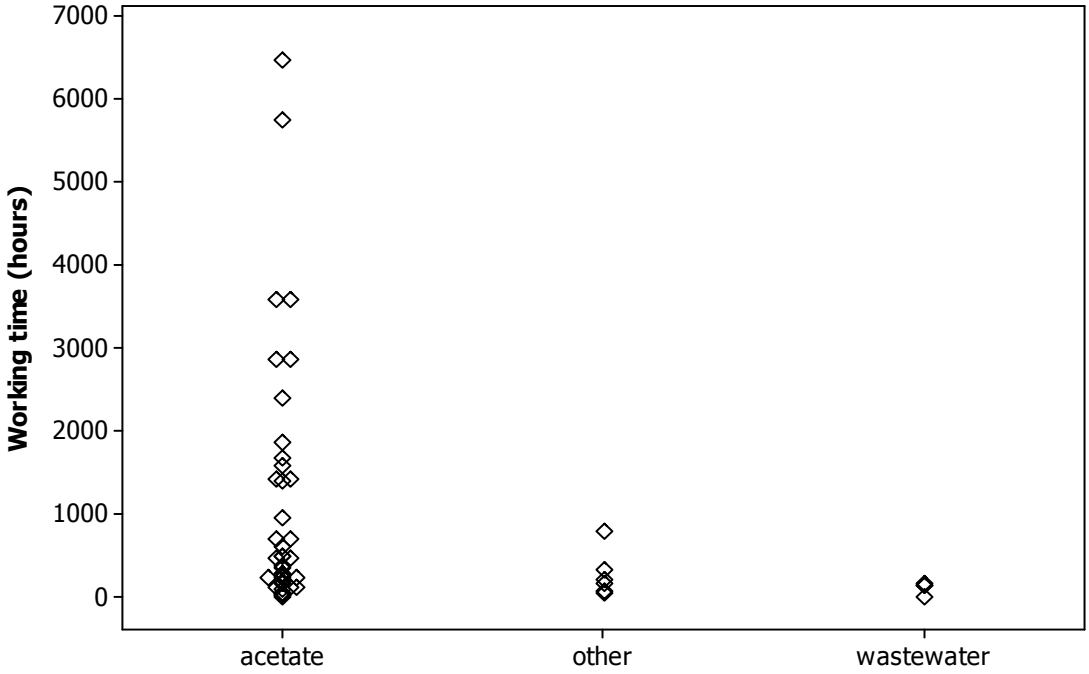

Figure 5-1 The working time of all MEC studies documented in the literature to date (Oct 2011), shown for the different substrates

If MECs are to be a viable and sustainable treatment option for the future then we need to gain an understanding of their long term performance with real wastewaters. Most of the research in MECs does not use real, or even complex artificial wastewaters, and most are run over a relatively short period of time. If this research is to translate into application, this relies on two key assumptions:

1. Real wastewaters containing mixture of simple and complex organic molecules will behave in the same way as acetate, a simple readily digestible molecule most frequently used in BES research. We know this not to be the case with anaerobic digestion (Rittmann, 2001).
2. A system that works at a particular efficiency for a short period of time will do for a long period of time. This is again unlikely as even with the clean technology of chemical fuel cells, long term durability tests have lasted around 4000 hours (166 days), although a couple of studies have extended this to 1.5 and even 3 years (Schmittinger and Vahidi, 2008). Failure is associated with blocked membranes, electrode deterioration and many other factors that may increase overpotentials. Biological systems have the added complexity of the behaviour of microorganisms.

Failure in laboratory batch fed wastewater reactors has been observed many times during preliminary laboratory testing. The aim of this research is to determine if wastewater fed MEC laboratory reactors are capable of operating over the same time periods as acetate fed reactors, and, if this is not the case, to identify the reasons why.

5.2. Method

5.2.1. *Reactor design and set up*

Double chamber MEC reactors (78 mL each chamber) were used which were of a tubular design, internal diameter of 40mm, length 60mm. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. The membrane between the reactor chambers was Nafion 117, with an area of 12.6cm^2. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 1 Ω resistor, 0.7 V supplied using a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong), and a multimeter to measure the voltage (Pico ADC-16), logged every 30 minutes onto a computer.

All reactors were cleaned and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK). The cathode media was 50 mM phosphate buffer, which was sparged with 99.99% pure N_2 for 10 minutes prior to being put into the reactors. The acetate based anode media used was that of Call and Logan (Call and Logan, 2008), during the tests where this was supplemented with protein, Aspargine was added to give an equivalent level of nitrogen to that measured in the real wastewater. The wastewater used was raw influent wastewater (post screens prior to primary sedimentation) from Cramlington wastewater treatment plant. The anode media was sparged for 10 minutes with N_2 prior to use. All reactors were initially acclimatised in MFC mode as per the method used in other studies (Call and Logan, 2008, Cheng and Logan, 2007a, Hu et al., 2008, Wagner et al., 2009), inoculated with 25 ml of raw wastewater and fed acetate media.

The gas produced by the cathode side was captured via a liquid displacement method in a 12 ml glass tube with a septa fitted to the top for sampling. The volume of this gas was measured by drawing it into a 5 ml gas tight syringe (SGE Analytical Science,

Australia). The anode gas was captured in an inverted 10 ml syringe placed into the top of the reactor and filled with the N_2 gas.

5.2.2. *Analytical procedures*

The following analysis was conducted in duplicate for both the effluent and influent of the cathode and anode liquids of each batch run. The chemical oxygen demand (COD) using standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) kit tubes. Volatile fatty acids (VFA's) were measured using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. The anion content using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The pH was measured using a pH probe (Jenway 3310, U.K.) and conductivity using an EC 300 probe (VWR Ltd, UK). The anode and cathode potential was measured using Ag/AgCl reference electrodes (BASI, U.K.) during each batch.

Hydrogen gas was measured on a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using triplicate injections of each sample, set against a three point calibration run once at the start of the measuring period and once at the end using standard calibration gases (Scientific and Technical Gases, U.K.). These gas measurements were verified using a Trace Ultra GC TCD with a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

GC-MS analysis of gaseous hydrocarbons, including halomethanes, was performed on a Agilent 7890A GC in split mode; injector at (280°C), linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software in full scan mode (10-150 amu/sec). A standard containing 100 ppm of three chloromethanes was injected (100ul headspace) followed by the reactor headspace samples (100ul) every 2 minutes. Separation was

performed on an Agilent fused silica capillary column (60m x 0.25mm i.d) coated with 0.25um dimethyl poly-siloxane (HP-5) phase. The GC remained at 30°C temperature for 90 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 20 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library if greater than 90% fit.

5.2.3. *Microbial analysis*

An assessment of the level of microbial activity occurring in the reactors was needed to give an understanding if failure was caused by a reduction or complete elimination of microbial activity, or conversely a competitive but non complementary microbial process. Methods involving the extraction and quantification of DNA from the anode biofilm were not suitable for this purpose as this would capture both the alive and active DNA and that DNA remaining on the biofilm from bacteria which were dead or inactive. Ribonucleic acid (RNA) is used within cells to convert DNA i.e. the genetic code into working proteins (Rittmann, 2001); it can therefore be used as a proxy for the amount of biological activity occurring in the cell (Milner et al., 2008, Low et al., 2000). As RNA is so susceptible to contamination and degradation, the simple and relatively quick approach of measuring the amount of nucleic acid extracted on a Nanodrop, and then comparing this directly to the amount of DNA extracted at the same time, would give the most reliable quantitative results.

Duplicate samples of anode material were taken for RNA and DNA extraction, from duplicate reactors sacrificed whilst working, and duplicate reactors after failure. The following procedure was carried out as quickly as possible inside a microbiological cabinet, to prevent the loss of RNA which readily breaks down if contaminated with RNases. All working areas and equipment was cleaned thoroughly with ethanol followed by RNase AWAY (Invitrogen Life Sciences, U.K.), including the anode cutting equipment which had also been washed with detergent and then heated to 240 ^{o}C for 4 hours in a furnace, prior to use. Each reactor at the point of sampling was taken into the microbiological cabinet maintaining the electrical circuit. The reactor was quickly dismantled and using a coring device duplicate 4mm diameter sections of the anode were cut and placed into a sterile RNase free 2 ml eppendorf, containing 1 ml of TRIzol Reagent (Invitrogen Invitrogen Life Sciences, U.K.), the sample was vortexed for 5 seconds to ensure complete submersion in the reagent, and then the samples frozen

at -80 ^{o}C. Duplicate cores were taken in the same way afterwards for DNA extraction and stored in 50:50 ethanol and phosphate buffer at -20 ^{o}C.

Extraction and clean-up of the RNA sample was then completed using a RNeasy Mini Kit (QIAGEN, Germany) as per the manufacturer's instructions. Once cleaned the samples were frozen at -20 ^{o}C. The DNA was extracted using a QBiogene FastDNA spin kit for soil (MP Biomedicals, U.K.) and also frozen in two samples at -20 ^{o}C. The quantity of nucleic acid present was then measured in duplicate on a Nanodrop Nanodrop 2000 (Thermo scientific, USA). The ratio of DNA to RNA could then be calculated for each sample.

5.2.4. *Experimental procedure*

Failure had been observed several times in these bench scale reactors used as MEC's when fed with wastewater. The purpose of these experiments was to determine if this failure was statistically significant, and if so to try and identify the particular cause. In total 12 wastewater fed reactors and 10 acetate fed reactors were used in this study, the materials and architecture of all the reactors were the same, and the same operating procedures observed throughout. The work was conducted at laboratory room temperatures of between 20-25 ^{o}C.

Initially 8 reactors were run, 4 of fed with acetate media and 4 with real wastewater. After each batch of 3-4 days the effluent was analysed for COD, VFA's, anions, pH and conductivity and the gas measured, the reactors were then refilled with N_2 sparged media to the anode and phosphate buffer to the cathode. Once having completed two batch runs producing gas, 2 reactors of each feed were sacrificed and the RNA and DNA were sampled, the remaining reactors were run and sampled as described until gas production ceased, or in the case of the acetate ones until they were stopped at 130 days.

A further experiment was conducted using 4 wastewater fed reactors to eliminate the possibility that a drop in pH in the wastewater fed reactors was causing failure. Duplicate reactors were run containing wastewater, and the same wastewater buffered to pH 7 using 50 mM phosphate buffer. All reactors were run in batch mode and samples as described above until gas production ceased. Examination as to whether the biofilm was damaged/killed during failure was gained by switching the failed MECs to MFC

mode (increased resistance and no external load), and refilling with UV sterilised wastewater (see Appendix V for details of this method).

Due to the observed drop in Cl^- ions prior to the point of failure, it was hypothesised that locally high levels of NH_4^+ at the anode, caused by the degradation of proteins present in the wastewater could be reacting with the chloride ions to form chloramines, which would then kill off the biofilm resulting in failure of the cell. This hypothesis was tested running 4 acetate fed reactors, by supplementing duplicate reactors with protein Aspargine at levels comparable to the wastewater levels as detected through the use of the TKN Standard Method 4500-Norg (APHA, 1998), comparing these to duplicate control reactors with no protein. Again sampling was carried out as above, in addition the effluent of the reactors was analysed for residual chlorine using the DPD test, Standard Methods 4500-Cl D, (APHA, 1998).

A further hypothesis to account for failure and the drop in chlorine was that the chlorination of organics, especially methane could be occurring in the reactors due to the potential of the anode. Under standard conditions, at pH 7 the required potential for chlorination of methane at a Cl concentration of 1 mM is 0.44 V, when considering that the reactors may have a pH slightly deviant from 7, and that the partial pressures of the methane and chloromethane produced would not be equivalent, it is conceivable that the anode potential needed for this reaction could be occurring in the reactors, producing chloromethanes and therefore removing the hydrogen ions from the system and eliminating H_2 production. Again 4 wastewater reactors were run in batch mode with the same analysis as described above, in addition both the anode and cathode gasses were captured and analysed for methane, hydrogen and chloromethane using the instruments and methods stated above. Duplicate reactors fed with acetate were run at the same time and subject to the same analysis. After failure reactors were again switched to MFC mode and the anode gas continued to be sampled.

5.2.5. *Calculations*

The reactor performance was evaluated in terms of the volume of hydrogen produced, and also the coulombic efficiency and electrical energy recovery. The definition of these two efficiencies can be found in section 6.2.5.

5.2.6. *Statistics*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

5.3. Results

5.3.1. *Time taken until failure*

The run time of the reactors is shown in Figure 5-2 as the amount of hydrogen produced at the end of each batch, the reactors terminated at 7 days for RNA sampling are not shown. It is seen that the Acetate fed reactors run for a longer period of time, including those supplemented with protein and produce more hydrogen than the wastewater reactors. The buffered wastewater reactors initially perform well, but then stop producing hydrogen after a short time period.

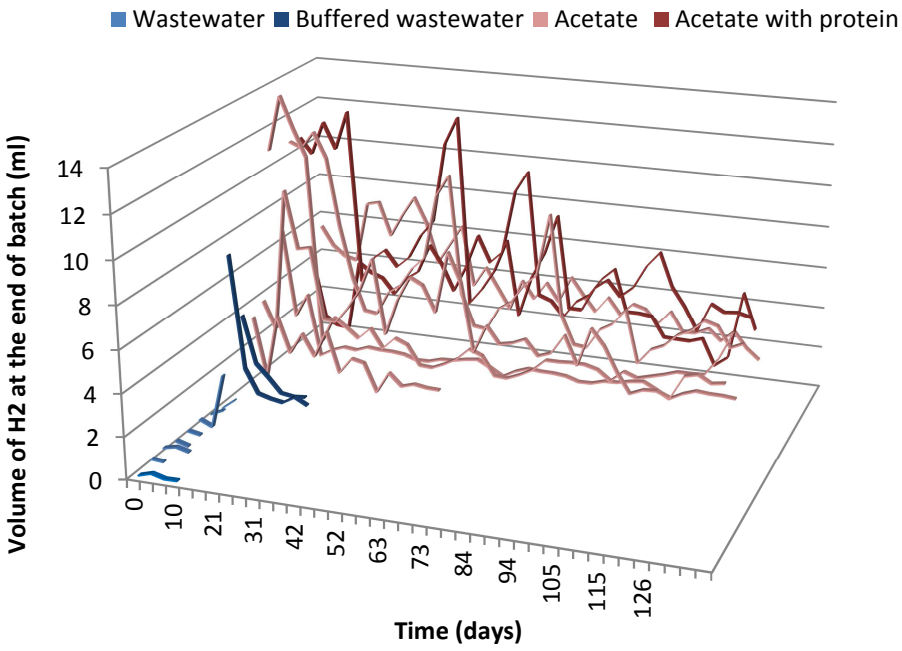

Figure 5-2 Graphic showing the working period of all reactors as indicated by the length on the line along the time axis, the volume of H_2 produced at the end of each batch is given on the y axis as an indication of reactor performance which is seen to be variable, where the line is discontinued this illustrates zero H_2 production and the reactor is deemed to have failed

All 10 of the reactors fed on wastewater failed within 7-17 days of operation, failure was determined by no measureable gas production at the cathode. Of the 8 acetate fed reactors one failed at 56 days, but the others remained functioning until the experiment was terminated after 130 days. With 130 days used as the minimum run time for the acetate fed reactors, the difference in time to failure is significant ($p=0.000$, two sample t-test) as shown graphically in Figure 5-2.

5.3.2. *Reactor performance*

The average performance data collected over the duration of different experiments is shown in Table 5-1. The acetate fed cells have a greater coulombic efficiency and electrical energy recovery. The COD removal is reasonably similar for all substrates, but higher for the buffered wastewater, although this does not translate into improved coulombic efficiency or energy recovery. In all cases there is a large degree of variation, as is seen by the standard deviations. This is also seen through the hydrogen production data in Figure 5-2, which is higher for the acetate fed reactors, but does deteriorate throughout the test period.

Table 5-1 Summary of reactor performance using three different parameters other than H_2 production for the experiments using different substrates, values are the average values of all the reactors run on the given substrate

	COD removal	Coulombic Efficiency	Electrical Energy Recovery
Wastewater	23.2% ± 12.2	7.5% ± 3.9	15.7% ± 20.1
Buffered wastewater	43.8% ± 7.8	3.7% ± 1.7	13.5% ± 16.6
Acetate	28.6% ± 11.5	10.9% ± 2.0	33.0% ± 15.1
Acetate with protein	32.3% ± 13.4	10.4% ± 3.6	35.1% ± 22.9

Values represent average of all the batch experiment run on the given substrates where hydrogen was produced, ± one standard deviation.

There is a reduced performance between the acetate fed reactors as compared to the wastewater ones of around 50 % if energy recovery is considered.

5.3.3. *Biological processes*

The average RNA: DNA ratio of the duplicate samples show that there is significant difference between the working and failed reactors at the 90% confidence interval ($p=0.068$ two-sampled t-test). This difference is more pronounced with the wastewater fed reactors, where the average ratio value for the working reactors is 11.5 compared to the failed reactors 3.9. The acetate working reactors have an average a ratio of 6.1, with the single failed cell being 4.2.

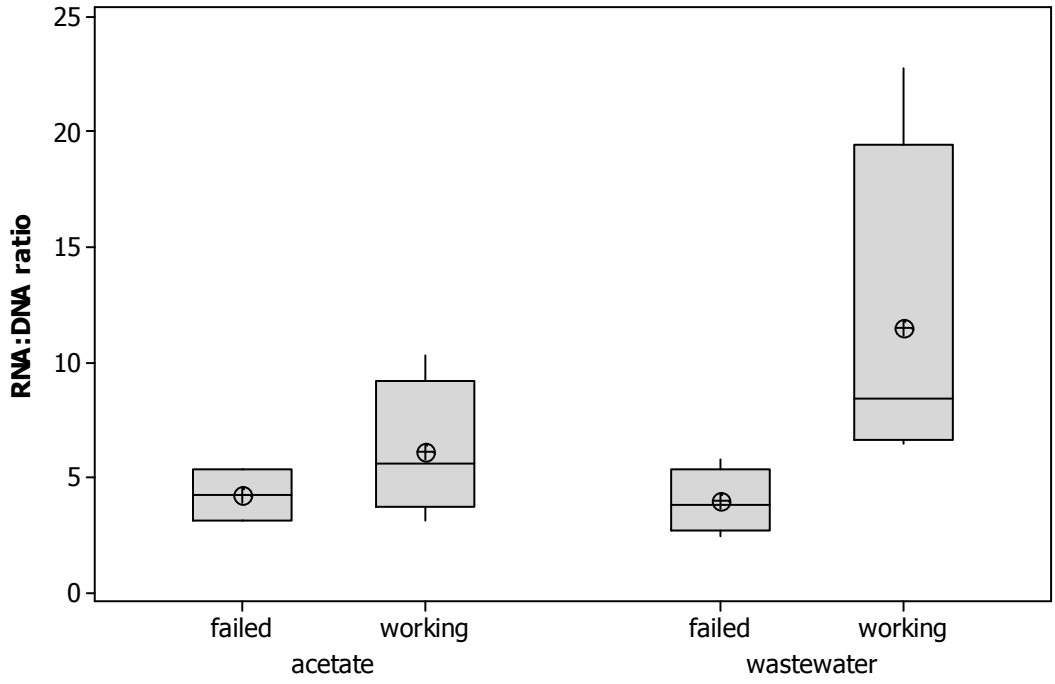

Figure 5-3 Box plot of the RNA: DNA ratios of failed and working reactors fed with both acetate and wastewater, the data represents a summary of the duplicate samples taken from duplicate reactors (i.e. four samples in total) with the central line representing the median and the mean given by the circle with cross

5.3.4. *Low pH*

In the wastewater fed reactors, which contained no additional buffering, it was observed that at around the point of failure there was a decline in the pH of the anode effluent from a starting value 6-6.5 to around 5.5. The acetate fed reactors, (the nutrient media containing 50mM pH 7 phosphate buffer) did not show any significant fall in pH during the full time period over which their function was monitored.

With the additional duplicate reactors fed on wastewater and buffered wastewater there was the same observed drop in pH with the non-buffered reactors. The buffered reactors kept a constant pH and initially performed better but then also failed within 17 days of operation. No significant difference in the run time between the buffered and non-buffered reactors ($p=0.306$, two sample t-test).

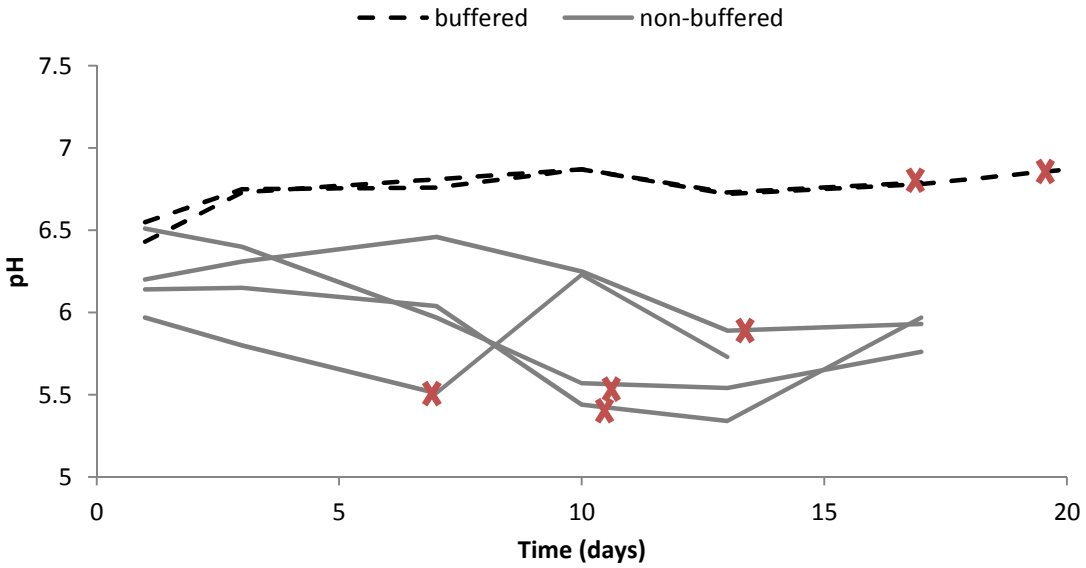

Figure 5-4 Measured pH of the wastewater reactor liquid during the course of the batch experiments, the point of failure is denoted by the red cross where gas production ceased

5.3.5. *Toxic build up within the reactors*

The full anion analysis of the cell effluent showed that there was a fall in chloride ions prior to failure of the wastewater reactors. Both the acetate media and the wastewater contained approximately 250-300 mg/L of chloride. During the course of each batch run with the acetate fed reactors, approximately 50 mg/L of the chloride would be taken up in the reactor, this remained relatively constant throughout the full time period the acetate reactors were operated for. However in the wastewater reactors, when working and producing hydrogen, the chloride removal in the cell was observed to be virtually complete prior to the reactor failure, i.e. 250-300 mg/L of chloride ions were being removed. The levels of chloride in the cathode compartment of these reactors remained the same as the original influent. After failure of the reactors when no hydrogen was produced, this chloride removal stopped. The only wastewater reactors that this drop was not observed in were the duplicate buffered wastewater reactors, here chloride removal remained constant at around 50-100 mg/L during each batch, the reactors did however also fail.

In the acetate reactors supplemented with protein the chloride removal remained roughly constant throughout the experiment at between 50-100 mg/L, and the reactors did not fail. No chloramines could be detected in the effluent of these reactors, disproving the hypothesis of chloramine formation. The performance of the protein

supplemented reactors in terms of electrical energy recovery was not significantly different to the non-supplemented ones (p=0.376, two sample t-test).

Further evidence that a toxic chlorine based product was not being formed was gained using four failed wastewater reactors, duplicate reactors were refilled with UV sterilised wastewater non sterile wastewater, put into MFC mode, i.e. increased resistance and no external load. With all four reactors biological activity started within 1 hour, and reached a level of current production as would be expected of a fully acclimatised MFC cell using the same cell materials. The electrogenic biofilm was capable of functioning. After one batch in MFC mode, the reactors were then all returned to MEC mode, where no gas was produced and the failed status continued. In MFC mode, the chloride removal was relatively constant again at around 50 mg/L.

5.3.6. *Formation of halogenated organics*

Analysis of the headspace gas for 4 wastewater fed reactors and 2 acetate fed did not show detectable levels of halogenated organics, levels were below 0.01% of the 10 ml headspace. This was the case for wastewater fed reactors before, during and after failure and for acetate fed reactors. The same observed drop in chlorides was seen in these reactors.

5.3.7. *Other factors*

The analysis of VFA's in the effluent of the reactors showed that in all cases for both acetate and wastewater there was some acetate remaining at the end of each batch. There was no acetate in the influent wastewater, but always a small amount 20-40 mg/L in the effluent of these reactors, this did not alter once the reactors had failed.

The conductivity for the wastewater was around 1.8 mS, the buffered wastewater was 6.3 mS, and the acetate media was 5.9 mS. The conductivity of the reactor effluent was on average 1.6 mS for the wastewater fed cells both before and after failure even when a drop in chloride ions was recorded, the average for the buffered wastewater cell effluent was 5.5 mS and again did not change after failure, the acetate cells also showed a slight drop in conductivity of the effluent to 5.2 mS.

The production of methane at the anode of the reactors was on average 0.002 ml for the wastewater reactors when working, after failure this increased slightly to 0.029 ml. The methane production remained relatively constant throughout the course of the

experiment and the slight rise after failure is not likely to represent a competitive biological process which is the cause of cell failure, as the average methane production in the acetate fed cells was always higher at 0.072 ml per batch, and also the converted MFC cells that functioned well, also produced on average 0.035 ml per batch.

The materials used in these reactors that could become degraded during use, i.e. the cathode and membrane, could be directly and successfully re-used in a new cell, the failure was not due to cathode degradation or membrane clogging. In addition, by increasing the applied voltage of the reactors from 0.7 V to 1.0 V immediately after failure, thus combating any increased overpotentials that could have built up during the short operation period, the reactors could not be revived and did not produce hydrogen. Failure was not therefore caused by the simple the deterioration of the cell components.

5.4. Discussion

Small laboratory scale wastewater fed reactors fail after a short period of time whereas acetate fed reactors do not. This is significant. The cause of this failure could not be identified during the course of this study. Relatively ‘simple’ explanations such as degradation of electrodes or membranes, a drop in conductivity, or lack of available VFA’s have been ruled out as possible causes of failure.

A further hypothesis that failure of the reactors is caused by a reduced or eliminated level of electrogenic activity in the reactors was also seen not to be the case. If true this hypothesis would result in the reduced DNA:RNA ratio observed and low current production. However once failure had occurred the reactors could be instantly ‘revived’ by switching them into MFC mode. The electrogenic bacteria were therefore present on the electrode and were capable of donating electrons.

The hypothesis that there is a competitive biological process occurring such as methanogenesis, as suggested in other studies (Cusick et al., 2011), has been shown not to be the case. The RNA to DNA ratio indicates a reduced biological activity in the failed wastewater cells, suggesting that the biofilm is less able to function and metabolise after failure. It is not likely that a non-complementary competing biological activity is taking over the reactor and eliminating the MEC process. It can be seen that there is greater activity in the wastewater reactors than the acetate reactors, this might be an indication of the greater and more multi-layered metabolism that has to occur in

these reactors when fed complex substrates. It is also observed that the failed acetate reactor did not differ significantly to the working ones, suggesting the reason for failure here was different to that for the wastewater reactors. Additionally the levels of methane generated in the wastewater reactors after was less than in the working acetate reactors. A competitive process such as methanogenesis is therefore unlikely to be the cause.

The hypothesis that a low pH was causing failure, either through altering the electrochemistry or affecting biological function is shown not to be correct. The simple experiment adding buffer to the wastewater also resulted in failure despite initial improvement in reactor performance, here the drop in chloride was not observed. The slightly lowered pH is likely to have a detrimental effect on the cell though. The pH measurement taken is of the whole of the liquid in the reactor, in reality the pH near the anode may be greater. Such a pH will impact on the microorganisms present and the electrochemical reactions within the cell, as pH is a logarithmic function of the concentration of H+ ions, then even a small change in this value has a large impact on the overall thermodynamic balance of the system as is calculated via the Nernst equation. Torres et al (2008) found that an increase in phosphate buffer in the anode media lead to a thicker biofilm and greater current generation in a microbial fuel cell due to the increased diffusion of H^+ out of the biofilm layer, thus making it more accessible to transport to the cathode. Although pH could be limiting the performance of non-buffered reactor it is not the cause of failure.

The formation of halomethanes such as chloromethane could potentially occur at the potentials within these reactors account for the loss of chloride and would cause failure as these compounds are toxic. This would fit the pattern of failure exhibited in the reactors as it would take some time for the levels of methane to build up which could then be converted to the halomethanes, this would ‘use up’ the H^+ ions in the anode section and H_2 would cease to be produced at the cathode. However no chloromethanes could be detected in the headspace gas of these reactors, (below 0.01%) either before or after failure, in fact no halogenated organics could be detected. Additionally the acetate fed cells did not fail when supplemented with protein, and most importantly the exoelectrogenic biofilm is able to work as an MFC after failure so has not been killed. It could be possible that the negative chlorine ions were simply temporarily attracted to the positive anode during the operation of the fuel cell, and therefore not measured in the bulk liquid of the cell. This would account for the observed ‘disappearance’ of the

chloride ions, but is not likely to affect the performance of the cell. The range of analysis carried out indicates that failure is not caused by a chlorine effect; the observed chlorine drop is simply co-incidental to the failure.

The problem of failure needs to be resolved. If MECs are to be a useable technology they need to function with real wastewater. Studying these systems when they are prone to sudden and rapid failure is difficult, therefore identifying the reasons for failure, solving them, and increasing efficiency becomes very challenging. This difficulty leads to acetate being used in most research as this does allow greater scope for experimentation. However it is clear that the processes operating in a reactor fed with real wastewater are different to those occurring in a reactor fed with acetate. The acetate research will not directly inform us of performance with wastewater.

The failure in wastewater fed, laboratory scale, batch fed reactors has been proved, but the reason not identified. Conversely, as part of this research, a larger scale MEC run in continuous mode at a wastewater treatment site fed on raw wastewater has worked producing almost pure hydrogen for a period of over 3 months, (see chapter 6). It is likely that something is occurring within the small batch reactors to prevent either the production of hydrogen ions at the anode, the transfer of these ions, or the hydrogen evolution reaction at the cathode. It may be the case that at this small scale and fed with batch mode that the system and in particular the microbial community involved is fragile and unable to adapt to change, and therefore a build-up of something at an undetectable level has catastrophic consequences. Further work is still needed to identify the cause of this failure, and therefore be able to take steps to resolve it. This can only be done by using real wastewater rather than simple artificial media. The long term performance of wastewater fed MECs is a research gap that must be filled.

Chapter 6. Production of hydrogen from domestic wastewater in a pilot scale microbial electrolysis cell

Addressing the need to recover energy from the treatment of wastewater the first working pilot scale demonstration of a wastewater fed microbial electrolysis cell is presented. A 120 litre (L) microbial electrolysis cell (MEC) was operated on a site in Northern England, using raw domestic wastewater to produce virtually pure hydrogen gas for a period of over 3 months. The volumetric loading rate was 0.14 kgCOD/m^3/day, just below the typical loading rates for activated sludge of 0.2-2 kgCOD/m^3/day, at an energetic cost of 2.3 kJ/gCOD, below the values for activated sludge 2.5-7.2 kJ/gCOD. The reactor produced an equivalent of 0.015 L H_2/L/day, and recovered around 70% of the electrical energy input, with a coulombic efficiency of 55-60%. Although the reactor did not reach the breakeven energy recovery of 100%, this value appears well within reach with improved hydrogen capture, and reactor design. Importantly for the first time a 'proof of concept' has been made, with a technology that is capable of energy capture using low strength domestic wastewaters at ambient temperatures.

6.1. Introduction

In an era of increasing energy costs and environmental awareness, wastewater treatment industries need to look at alternative treatment options to reduce their energy bills. It has been estimated that domestic wastewater alone may contain 7.6 kJ/L of energy, while stronger industrial wastewaters contain substantially more (Heidrich et al., 2011). There is an increasingly urgent need to recover some of this energy, or at the very least not expend additional energy on treatment; the activated sludge process uses 2.5-7.2 kJ/gCOD (Pant et al., 2011). Energy recovery could be achieved through anaerobic digestion to methane gas or microbial fuel cell technology directly to electricity; however life cycle assessment has shown that the production of a higher value product through the suite of bioelectrochemical systems (BES) may be the most viable solution (Foley et al., 2010). One such technology is the production of hydrogen in a microbial electrolysis cell (MEC) (Rozendal et al., 2006).

Since the MEC process was first reported (Rozendal et al., 2006, Liu et al., 2005b) MECs have emerged as a potential technology option for a new generation of wastewater treatment systems (Rozendal et al., 2008a). In an MEC bacteria use the

energy stored in the organic compounds of wastewater to metabolise and grow, donating electrons to an electrode (Rozendal et al., 2006). The electrons then travel in a circuit producing current and therefore electrical power; in an MEC these electrons are consumed at the cathode along with a supplement of electrical power. The H^+ ions also created by the breakdown of organics at the anode travel across the microbial fuel cell membrane to the cathode. Here they can combine to form H_2, however this process is endothermic requiring energy, so a supplement of electrical energy is added to the system to allow it to take place (Liu et al., 2005b).

Fuel cell technologies may offer a sustainable future for wastewater treatment, although there are still many hurdles to overcome. Progress is being made with new reactor design (Call and Logan, 2008, Rozendal et al., 2008b), improved materials (Cheng et al., 2006a, Cheng and Logan, 2008), greater understanding of the mechanisms involved (Aelterman et al., 2008, Clauwaert et al., 2008), and even improved understanding of the microbes that are at work in these systems (Holmes et al., 2004, Kim et al., 2004, Lovley, 2008, Rabaey et al., 2004). Most of this research is performed at laboratory scale, using simple substrates, often at a controlled warm temperature. Many problems have been overcome, such as validation of using multi electrode systems (Rader and Logan, 2010) and finding a low cost alternative to the platinum cathode (Zhang et al., 2010). Although of great value in improving our understanding of MEC's, these studies do not tell us about the challenges or even benefits of running such systems at a larger scale with real wastewaters in temperate climates. There is a need to demonstrate that these systems can work at a larger scale and under realistic conditions, elevating the technology from a laboratory curiosity into a practical solution to an industrial problem.

A pioneering study by Cusick et al (2011) published on the largest MEC reactor to date, a 1000 L pilot scale reactor at a winery in California. The reactor proved slow to start up with pH and temperature control being problematic. When these issues were corrected by heating to 31 ^{o}C and the addition of buffer and acetic acid, the reactor did improve in performance. The energy produced during the operation exceeded the input energy (heating not included), but this was primarily due to methane production (86%) with only trace amounts of hydrogen. Methane production was attributed to the reactor being membraneless allowing hydrogen produced at the cathode to be directly consumed by hydrogenotrophic methanogens within the reactor. The reactor performance tailed off at around 90 days, when the heating unit broke (Cusick et al., 2011). The study has

provided valuable insights into the operation of MECs: (i) the membraneless systems that work well at laboratory scale and when fed in batch mode may not be so good at larger scale and under continuous feed, and (ii) inoculation and start-up are important parameters.

Addressing the issue of a membrane is critical to reactor performance. Most laboratory scale membrane systems use Nafion 117 (Logan et al., 2006), an expensive and delicate proton exchange membrane (Logan et al., 2006); this would be both impractical and costly on a large scale. Also the high efficiencies published: 406% electrical energy recovery (the amount of electrical energy put in that is recovered, this can be higher that 100% as there is also substrate energy within the system) and 86% total energy efficiency (the amount of substrate and electrical energy recovered) (Call and Logan, 2008) are from membrane-less systems. The lack of membrane greatly reduces the resistance in the cell, improving the transmission of protons to the cathode. Membrane systems have lower efficiencies: 169% electrical energy recovery and 53% overall energy efficiency has been reported (Rozendal et al., 2006). These efficiencies are likely to decrease further with time as the membrane becomes fouled.

The issues of inoculation and start-up are poorly understood (Oh et al., 2010) Although the use of acetate is likely to reduce the acclimatisation period (Cusick et al., 2011). However the biological community needed for the degradation of complex substrates is thought to be different to that needed for acetate (Kiely et al., 2011c). A community of acetate degraders able to work at 30 ^{o}C is not likely to be the community needed to degrade wastewater at ambient UK temperatures. There is evidence in the literature that microbes exist that are able to digest wastewater (Ditzig et al., 2007) and operate at low temperatures (Lu et al., 2011). Like anaerobic digestion, however, it may well be that a long period of acclimatisation is needed and unavoidable to achieve a stable community (Rittmann, 2001).

If these start-up issues can be resolved, then the reactor in theory will function, however it would also need to reach a neutral or positive energy balance, i.e. recovering all the electrical energy input plus a substantial fraction of the substrate energy input.

To test whether these systems have a chance of achieving these goals under realistic conditions, a pilot scale 120 L reactor was placed on a wastewater treatment site in

North East England. This site takes in primarily domestic wastewater with an average Total COD of 450 mg/L. The reactor was built using low cost alternatives to the standard lab materials used for the cathode and membrane. The reactor was not heated, held inside a large unheated building, and run throughout a UK spring and summer (5-20 ^{o}C minimum and maximum temperatures) and is still in operation at the time of writing this paper. These operating conditions are likely therefore to represent close to a worst case scenario i.e. low concentration feed; non optimal components; no heating; and no additional supplement of acetate or buffering capacity after the initial acclimatisation period.

Working closely with partners at Northumbrian Water Ltd. the aim of this study was to establish reactor operation and to determine if a neutral or positive energy recovery is achievable. From that data we can evaluate if MEC technology is likely to be a viable treatment option for the future.

6.2. Methods

6.2.1. *Field Site*

The pilot scale reactor was set up and run at Howdon wastewater treatment site, situated near the city of Newcastle Upon-Tyne in the North East of England (54^{o}58'N, 01^{o}36'W). An average of 246500 m^{3} of domestic wastewater is treated daily, using 96 MWh; the activated sludge process uses around 60% of this. The wastewater used in the MEC was taken from the grit channels after primary screening, but before settling.

6.2.2. *MEC reactor*

The reactor was based on a cassette style design, with six identical cassettes being placed into a rectangular reactor with a total working volume of 120 L. The tank has a Perspex plate fitted over the liquid layer giving a small head room to the anode compartment of 2.2 L. Each of the cathode gas tubes from the cassettes projected above this Perspex sheet. The cassettes were set along alternate sides of the reactor to allow s-shaped flow, and once in place gave a final anode volume of 88 L.

Each cassette was constructed using 10 mm thick plastic sheeting and consisted of an internal cathode section 0.280 m by 0.200 m by 0.048 m deep, of a volume 2.6 L. The cathode material was stainless steel wire wool grade 1 (Merlin, UK), 20g was used in each cathode, giving a projected cathode surface area for each electrode of 0.056 m^{2}. A

0.8 m length of stainless steel wire was wound several times into the wire wool to make a firm electrical connection, and then to the outside of the cell. Each cathode electrical assembly had an internal resistance from the extremities of the wire wool to the end of the exposed wire of less than 2.75 Ω. The cathode was separated using a membrane wrapped around a plastic frame inserted into the electrode assembly on both sides. The membrane used was RhinoHide® (Entek Ltd, UK), a durable low cost microporous membrane traditionally used as a battery separator. The anode material was a sheet of carbon felt (Olmec Advanced Materials Ltd, UK), 0.2 m wide by 0.3m high and 10 mm thick. This was sandwiched between two sheets of stainless steel mesh acting a current collector. The anode assemblies were also connected by a 0.8 m length of stainless steel wire fed through the centre of the felt material, each electrode having an internal resistance less than 3.4 Ω.

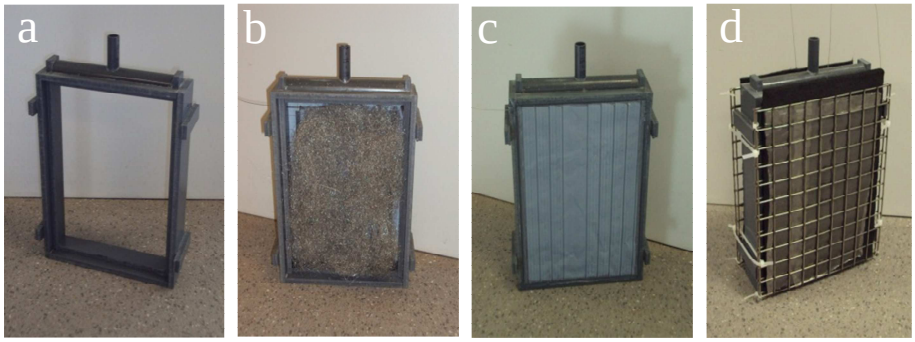

Figure 6-1 Photographs of the electrode assembly unit – a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane, d) anode with wire mesh current collector

The gas production from the anode compartment was captured from the ports in the Perspex lid, using 3mm ID PVC tubing (VWR Jencons, UK). The cathode gas was initially captured using 4mm annealed copper GC tubing connected to each cathode compartment using copper compression fittings, (Hamilton Gas Products Ltd, Northern Ireland), due to rapid corrosion this was later replaced with 3mm ID PVC tubing (VWR, UK). Both pipelines contained a gas sampling port.

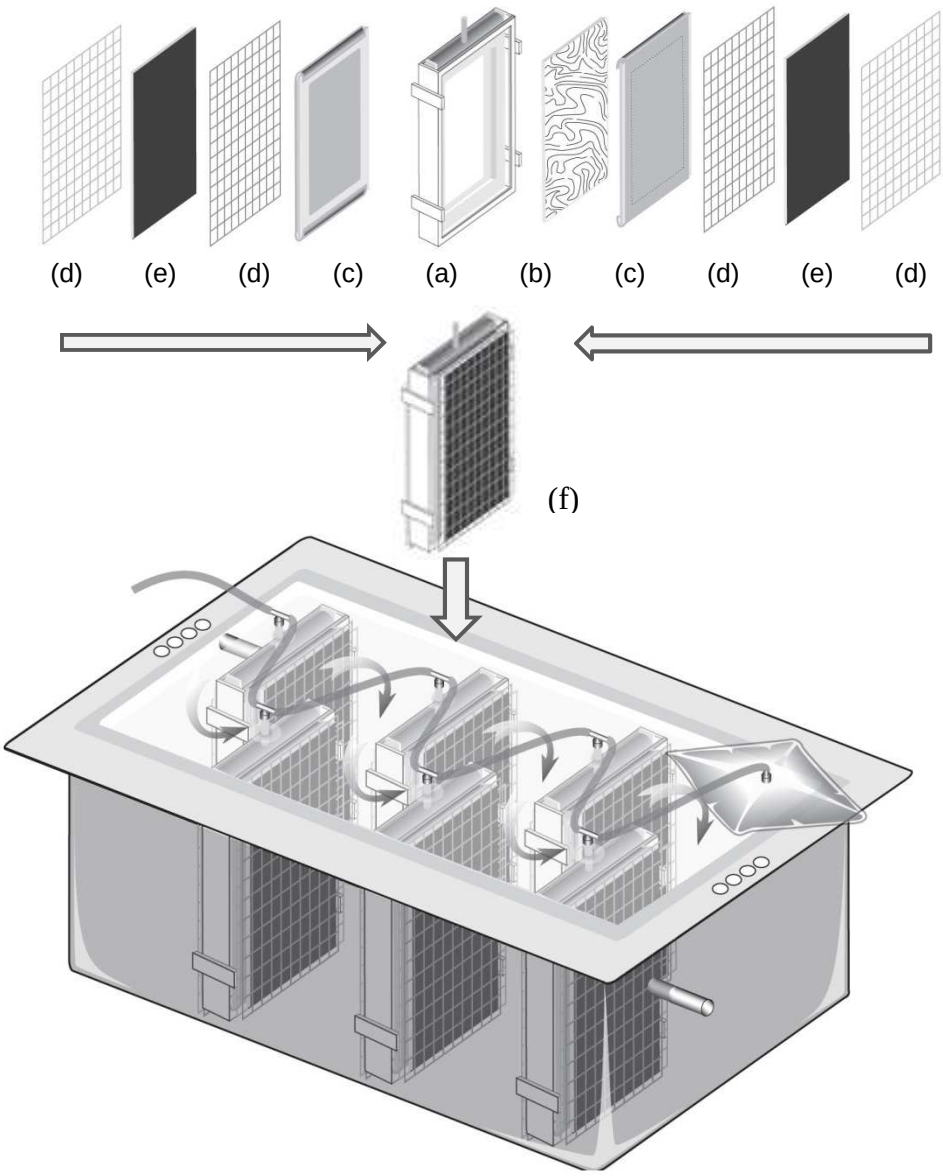

Figure 6-2 Schematic diagram of the reactor module components, a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane fixed around a PVC frame, d) stainless steel wire mesh, e) anode with wire mesh current collector. These component fit together to form a single module (f), six of these go into the reactor vessel where wastewater flows around them. Gas is collected through tubing into a gas bag

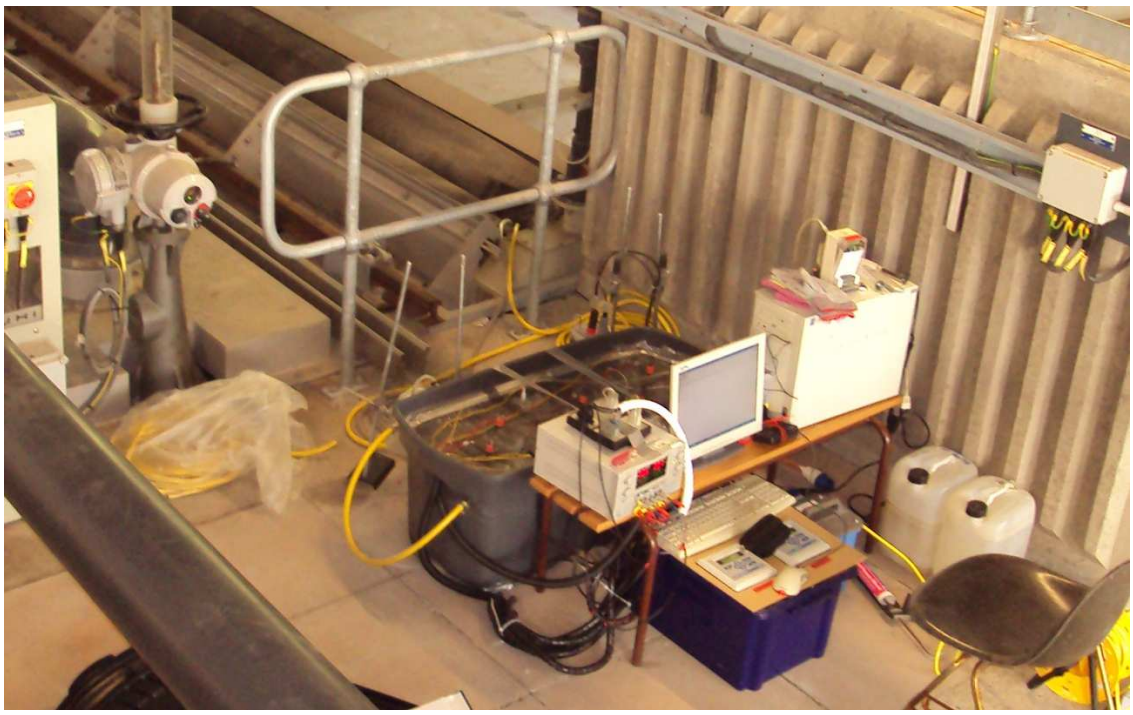

Figure 6-3 Photograph of the reactor in situ at Howden wastewater treatment site the grit lane where the influent was drawn from is seen in the top left hand corner of the picture

The reactor was situated on site in a large unheated building housing the grit channels, wastewater was pumped from the grit channels into a preliminary storage tank, providing some primary settling. During operation a peristaltic pump (Watson Marlow 520S, UK) was used to pump water into the storage tank, where it could then flow into and through the reactor, and back out to the grit channels via a smaller sampling tank at the end. These tanks were used for sampling and monitoring of the influent and effluent.

6.2.3. *Analytical procedures*

Power was provided to the electrodes using a PSM 2/2A power supply (Caltek Industrial Ltd, Hong Kong), the voltage of each cassette was monitored across a 0.1 Ω Multicomp Resistor (Farnell Ltd, UK) using a Pico AC-16 Data Logger (Pico Technology, UK), and recorded on a computer every 30 minutes.

In both the influent settling tank and the effluent tank the dissolved oxygen (DO) and pH were measured using pH and DO submersion probes (Broadley James Corporation, USA) connected to a pH DO transmitter (Model 30, Broadley James Corporation, USA), feeding an electrical output to a Pico EL 037 Converter and Pico EL 005 Enviromon Data Logger (Pico Technology, UK); these data were recorded onto the

computer every 30 minutes. Temperature was logged using 3 EL-USB-TC Thermocouple data logger (Lascar Electronics, UK) placed in the settling and effluent tanks and one placed in the reactor itself.

The gas pipelines were connected to optical gas bubble counters (made 'in-house' at Newcastle University), giving a measurement of gas volume. The operation of these counters failed after several weeks of operation. They were replaced with 1 L and then 5 L Tedlar gas bags (Sigma Aldrich, U.K.); the volume of gas was then measured by removal from the bags initially using a 100ml borosilicate gas tight syringe, and then using a larger 1 L glass tight syringe (both SGE Analytical Science, Australia). The sampling ports on each pipeline were initially used to take a sample of cathode gas 3 times a week, into a Labco Evacuated Exetainer (Labco Ltd, UK). Once gas production had risen to a higher volume, 2 L of the cathode gas was dispensed from the collecting gas bag into another 5L gas bag which was taken away for analysis. Anode gas was not measured volumetrically due to leakage but was sampled directly from the anode compartment into a 3 ml exetainers for compositional analysis.

Hydrogen gas was measured using a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using duplicate injections, set against a three point calibration. These gas measurements were verified using a Trace Ultra gas chromatograph (GC) with a thermal conduction detector (TCD) and a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements for anode and cathode gas were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

To ensure accuracy calibration standards used for the gas measurements were injected into a Labco evacuated exetainers in the laboratory at the same time (+/- 10 minutes) as the samples taken in the field. Tests carried out previously had indicated that these containers were not completely gas tight especially for hydrogen. This procedure did not have to be carried out for the cathode gas once operation had been switched to gas bags.

Liquid samples of the influent and effluent were taken 3 times a week. The total chemical oxygen demand (COD), and soluble chemical oxygen demand (SCOD) were measured in duplicate using standard methods (APHA, 1998) (Spectroquant ® test kits, Merck & Co. Inc., USA). Volatile Fatty Acids (VFA's) were determined using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The conductivity of the solution was measured using a conductivity meter, EC 300 (VWR Ltd, UK).

6.2.4. *Start up and operation*

The reactor was initially started up in batch mode, allowing all the oxygen, nitrates and sulphates within the wastewater to be consumed. Based on the lessons learnt from the previous pilot study, (Cusick et al., 2011), (Logan, B.E. personal communication),the wastewater was supplemented with acetate at a concentration of 0.5g/L. The applied voltage of 0.6 V was provided by a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong). The dosing was repeated and the reactor refilled after a 2 week period, during which time no gas production was observed.

6.2.5. *Efficiency calculations*

Four efficiency calculations are made in this study on the basis of the electrical and substrate energy used (Logan, 2008).

(i) Electrical energy recovery (η_E)- Energy recovery is the amount of electrical energy put into the reactor that is recovered as hydrogen.

The electrical energy input W_E is calculated as:

$$W_E = \sum_{1}^{n} (I\ E_{ps} \Delta t - I^2 R_{ex} \Delta t)$$

Where I is the current calculated for the circuit based on the measured voltage E and external resistor R_{ex} ($I=E/R_{ex}$), E_{ps} is the applied voltage of the power supply, this value is adjusted for the losses caused by the external resistor (I^2R_{ex}), which in reality are negligible. The time increment denoted by Δt represents the conversion of samples taken every 30 minutes into seconds. The data is summed for all 6 cells over the each batch cycle. The output of energy (W_{out}) is calculated from the measured moles of

hydrogen produced $N_{H2,}$ and the standard higher heating value of hydrogen of 285.83 kJ/mol ΔH_{H2}.

$$W_{out} = \Delta H_{H2}\, N_{H2}$$

The higher heating value is chosen over the lower heating value which takes into account the heat lost through the production of water vapour during burning. It is expected that this H_2 product would be used either as a commercial product for industry, or in a clean H_2 consuming fuel cell to create electricity, not for combustion. Methane could also be added to this value to further increase the quantity of output energy, but was not included for these same reasons.

Total Energy recovery (excluding pump requirements) can then be calculated as follows:

$$\eta_E = \frac{W_{out}}{W_E}$$

(ii) Total energy efficiency (η_{E+S}) the amount of input energy both electrical and substrate that is recovered as hydrogen.

The substrate energy (*Ws*) is calculate as

$$W_s = \Delta COD\, \Delta H_{ww/COD}$$

Where ΔCOD is the change in COD in grams, estimated as the difference in COD of the influent and effluent at the end of each batch. $\Delta H_{ww/COD}$ is the energy content per gCOD as measured on similar domestic wastewater of of 17.8 kJ/gCOD (Heidrich et al., 2011). Total energy efficiency is then calculated as:

$$\eta_{E+S} = \frac{W_{out}}{W_E + W_S}$$

(iii) Coulombic efficiency (CE) - the amount of hydrogen produced compared to the amount theoretically possible based on the current, or total charge passing through the cell.

Theoretical hydrogen production based on current (N_{CE}) is calculated as:

$$N_{CE} = \frac{\sum_1^n I\Delta t}{2F}$$

Where *I* is the current calculated from the measure voltage, Δt is the conversion of the time interval 30 minutes to 1 second to give coulombs per data sample, this is then summed over the 6 cells for the whole batch. Faradays constant (*F*) is 96485 coulombs/mol e^-, and is the moles of electrons per mole of hydrogen. Coulombic efficiency *CE* is then calculated as:

$$CE = \frac{N_{CE}}{N_{H2}}$$

(iv) Substrate efficiency - the amount of hydrogen produced compared to the amount theoretically possible based on substrate removed in the reactor.

Theoretical hydrogen production based on substrate removal *(N_S)* is calculated as:

$$N_S = 0.0625\, \Delta COD \Delta t$$

As 64 gCOD can be converted to 4 moles H_2, each g COD is equivalent to 0.0625 moles H_2. The change in COD is measured at the end of each batch, and used to calculate the total COD removed from the 88 L reactor over the duration of the sampling period based on a HRT of 1 day. Substrate efficiency is then calculated as:

$$S_E = \frac{N_S}{N_{H2}}$$

The (η_E) correlates directly to the coulombic efficiency (CE) by re-arrangement of their respective equations. It is assumed that the phrase $I^2 R_{ex} \Delta t$ in calculating W_E is negligible by comparison to the first term (this is observed to be the case in practice):

$$\eta_E = \frac{\Delta H_{H2} \times 1000}{2F \times E_{ps}}\, CE$$

This means halving the E_{ps} doubles the η_E if the CE can be maintained. An increase in CE at the same E_{ps} causes a linear increase in η_E.

6.2.6. *Statistical analysis*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

6.3. Results

6.3.1. *Reactor design and resistance limitations*

The internal resistance of a BES design is critical to its performance. Resistance is mainly caused by electrode overpotential and ohmic losses in the liquid, although there may also be losses in the bacterial transfer etc. as shown in Figure 1.2. These losses impact on the amount of energy that can be gained in and MFC and the amount for energy needed in an MEC, these effects are even greater in a scaled up system where losses become proportionally more significant (Rozendal et al., 2008a). Within the cell

designed the anode and cathode, although separated by a membrane, were relatively close together, with around 1cm distance between them, this will have minimised the ohmic losses within the liquid phase (i.e. the resistance in the movement of ions from the anode to cathode) which is especially important when using real wastewaters with no artificial increase in liquid conductivity.

However the electrode resistance with this design is high, with the cathode having a resistance of 2.8Ω and each anode sheet being 3.4Ω from the extremities of the electrode to the end of the connecting wire. With a total anode surface area for the whole reactor of 0.76 m^2 and a further 0.3 m^2 of cathode, these resistances will have a large impact in reducing the efficiency of the reactor performance. With a 0.6V load, as would be desirable based on laboratory studies (Call and Logan, 2008) this anode resistance would result in an approximate maximum current of 0.2A, increasing the load to 0.9 as needed with other wastewater studies (Kiely et al., 2011a, Cusick et al., 2011) would produce a maximum of 0.3A, and the 1.1V load used would result in around 0.4 A maximum current, assuming no other losses. This would give anode current densities of 0.3, 0.4 and 0.5 A/m^2 respectively, well below the target for BES of 10 A/m^2 which would enable similar treatment rates to activated sludge (Rozendal et al., 2008a), although current densities within MECs do tend to be lower than those of MFCs (Kiely et al., 2011a).

In reality there was greater resistance within the reactor than the electrode overpotentials alone. The current densities measured were 0.04, 0.1 and 0.3 A/m^2 at 0.7, 0.9 and 1.1V load added respectively. This means that the current density only increases by around 0.6 A/m^2/volt, far lower than two early MEC laboratory studies (1.3 A/m2/volt in (Liu et al., 2005b) and 1.78 A/m^2/volt in (Rozendal et al., 2006)). Additionally this shows that there is an inherent overpotential in the system also of over 0.6 volts as seen in Figure 6.4, over this voltage needs to be added to generate any current.

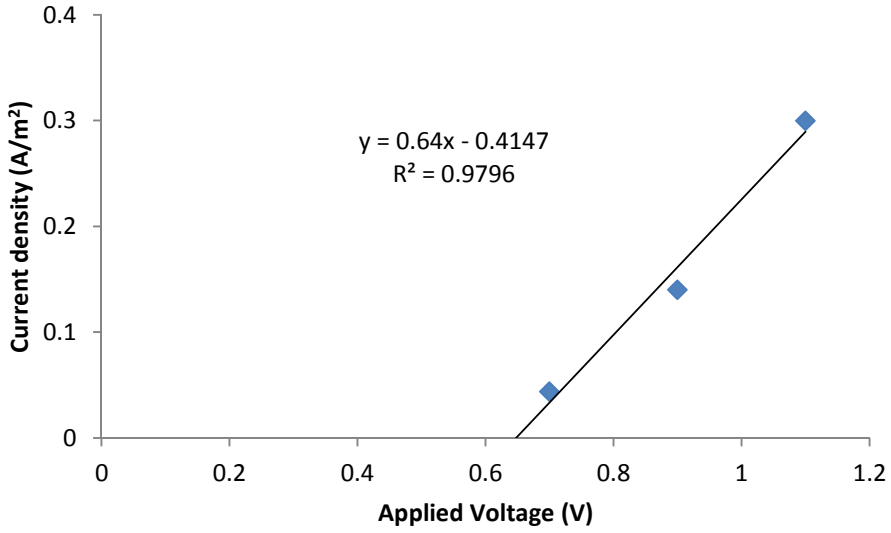

Figure 6-4 Current density as a function of applied voltage as measured in the pilot scale reactor after the initial two week acclimatisation period, showing the linear regression equation and R2 value. The intersect of the x-axis indicates the overpotential of the system

6.3.2. *Start-up and acclimatisation*

During the first 30 days of operation the reactor was run in batch mode with a supplement of 0.5 g/L of sodium acetate and an input voltage of 0.6 V. During this time there was no observed gas production and the current density was very low reaching 0.04 A/m^2 after the first two weeks. After this period wastewater was pumped through the reactor with a HRT of one day with no further addition of acetate. For the subsequent 10 days very little gas was produced and the current density remained at this very low level. At day 40 the input voltage was raised from 0.6 V to 0.9 V. The reactor was run with this input of voltage for the next 24 days; the average power density during this time reached 0.1 A/m^2. Gas production was low with an average of 9 mL/day, however once the gas lines had been flushed the purity of this gas (H_2) began to reach 100%. The electrical energy efficiency η_E was only 1 %. The voltage was then further increased to 1.1 V, and power densities rose and stabilised at 0.3 A/m^2. This led to a dramatic improvement in gas production, and the reactor entered its "working phase", the results of which are shown below. The start-up period took 64 days.

6.3.3. *Working performance of MEC reactor*

After the long start-up, and subsequent increase in the voltage to 1.1 volts, the MEC worked for the following 85 days, and continues to do so. The results presented here are for this period.

The volume of gas produced per day was highly variable. However the gas composition was consistent, hydrogen 100% ± 6.4, methane 1.8% ± 0.9. No trace of CO_2, N_2 or O_2 could be detected using the GC's or MIMS. H_2S could not be measured accurately however the MIMS did not detect any gas at this atomic weight and there was no detectable odour present. The daily H_2 production is shown in Figure 6-5. Production gradually increased during the first 30 days; after this the average production was around 1.2 L per day for the reactor, equivalent to 0.015 L-H_2/L/day.

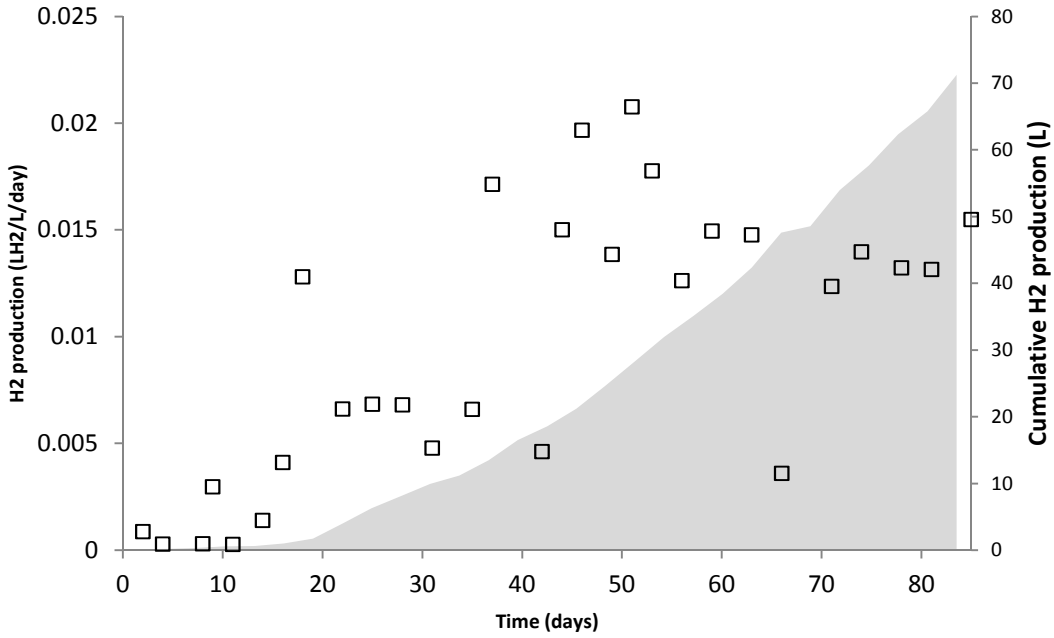

Figure 6-5 Hydrogen production during the working phase of reactor after the 64 day acclimatisation period, points showing the production rate at each time of sampling, and the area showing the cumulative production of the course of this period

The electrical energy recovery of the cell was quite variable as seen in Figure 6-6 (a), but did show an increasing trend and on occasion approached 100% (complete energy recovery) . The total energy efficiency (b) which gives the true performance of the cell was also variable, and considerably lower as both the electrical and substrate energy are considered as inputs. The energy efficiency shows an increasing trend reaching the 30 % level at the end of the study. The peak values are associated with very low COD removal measurements (making substrate energy input very low), and are not therefore likely to be representative of the true performance of the reactor. Coulombic efficiency (Fig. 5c) shows a similar trend to energy recovery (Fig. 5a), stabilising at around 55-60 % in the last 30 days.

The coulombic efficiency (CE) correlates with energy recovery (η_E) (R^2 = 0.998, Pearsons correlation). This correlation factor is calculated as N_E = 1.29 CE using the average input power voltage, this value is also seen in the data and is consistent over the course of the study. If the CE could remain at the 60% and the power input dropped to 0.9 volts 100% η_E would be achieved. Alternatively with this power input CE needs to reach 75% to achieve 100% η_E. The substrate efficiency (d), due to the highly variable influent and effluent COD values (as shown in Figure 6-7 can exceed 100%, and was often very low and even negative. The average substrate efficiency for whole the operational period is 10%.

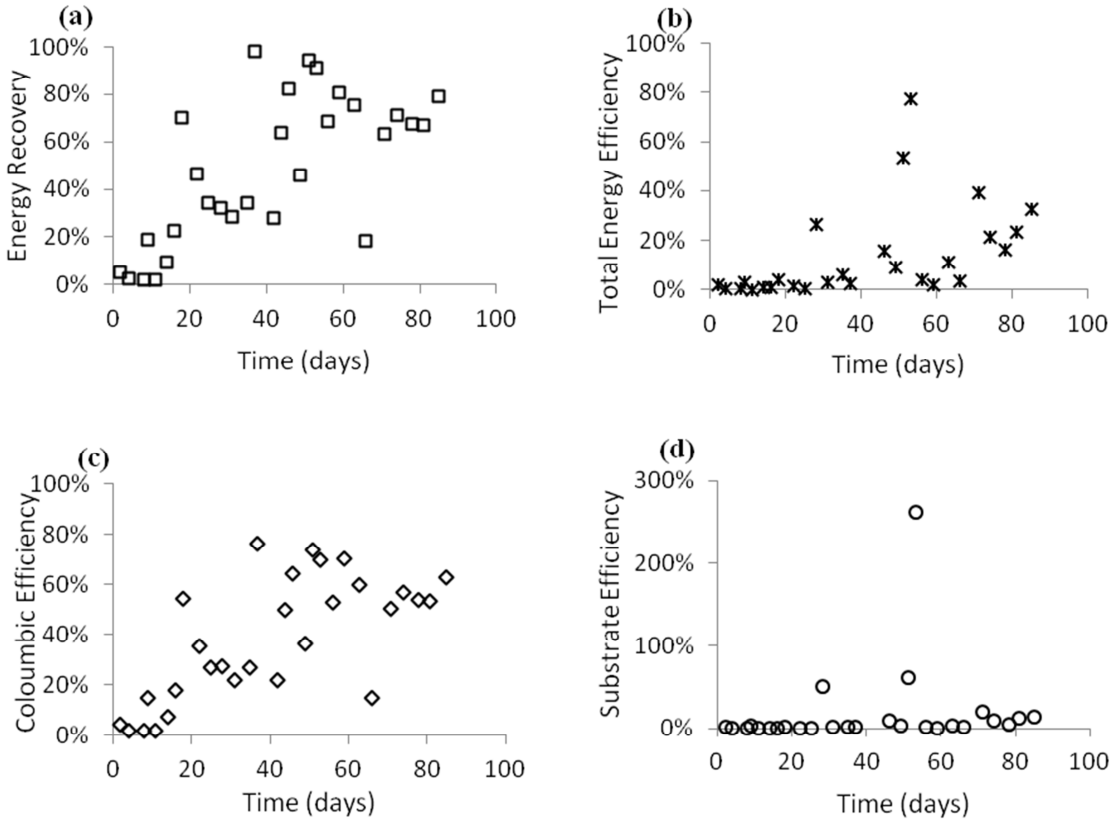

Figure 6-6 MEC reactor efficiencies over the 85 day working period a) electrical energy recovery b) total energy efficiency c) coulombic efficiency d) substrate efficiency

The levels of influent COD was highly variable which is likely to be one of the factors underlying the variation in performance. This factor was particularly the case at day 30 when the settling tank became full with sludge and influent COD was extremely high. This variability led to occasional negative values for % COD removal. The average removal of 33.7%, equates to 0.14 kgCOD/m^3/day, just below the range for activated sludge of 0.2-2 kgCOD/m^3/day (Grady, 1999). The COD effluent levels occasionally approached and dropped below the UK standard of 125 mg/l (EEC, 1991).

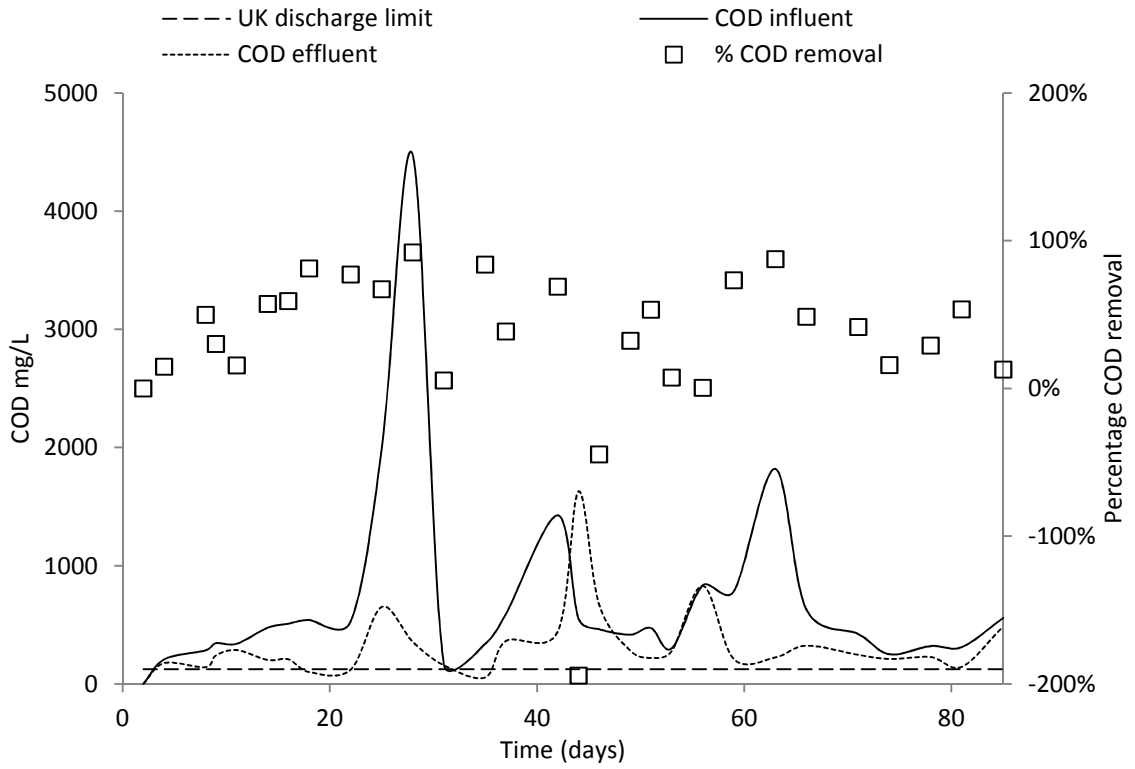

Figure 6-7 COD influent and effluent shown by the lines along with the UK discharge standard of 125 mg/l, percentage COD removal is also shown using the squares

Despite the variable influent COD and therefore variable performance, many of the other measured factors remained relatively constant throughout the operational period. The headspace of the anode compartment (2.2 L volume) contained elevated levels of CO_2 (1.9%) and low levels of CH_4 (0.4%), equivalent to 8.8 ml of CH_4, or 0.006 mg COD and 0.3 kJ. The gas production at the anode could not be measured quantitatively due to leakage. The daily production of methane at the cathode was 22 mL/day, equivalent to 0.014 mg COD, and 0.8 kJ of energy, approximately 5-6% of the amount of energy recovered as hydrogen.

The pH of the influent and effluent were continuously monitored, the influent was on average pH 7, the effluent pH 6.7, never dropping below pH 6. The DO of the influent was on average 4.2 mg/L and the effluent was 0 mg/L. The amount of VFA's dropped between the influent and the effluent, but there was frequently some acetic acid left in the effluent up to 45 mg/L, i.e. the available food source was not used up. This was confirmed by the average SCOD of the effluent of 115 mg/L. There was an average removal of 1.8 g/day of sulphate in the reactor, but never full depletion with the effluent containing 89.6 mg/L on average. The reactor removed an average of 0.2 g/day of chloride, although this value was highly variable. Fluoride and phosphate remained

relatively constant between the influent and effluent, nitrates were not present in either. There was no measured drop in conductivity between the influent and effluent.

The temperature of the influent wastewater varied considerably throughout the working period between June and September. The range of temperature was more stable within the reactor, and was on average 0.9 ^{o}C higher than the temperature of the influent. With a 88 L capacity and HRT of 1 day, this means 0.37 kJ/day of energy was lost to heat, equivalent to 20 mg COD, or 31 ml H_2. Temperature did not significantly influence energy recovery (p=0.678 influent, p=0.664 reactor, p=0.778 effluent, Pearson Correlation). Most of the fluctuation observed was diurnal and periods of the more extreme temperatures were short lived.

Table 6-1 Maximum, minimum and average temperature (^{o}C) of the influent, effluent and reactor ± 1 standard deviation which were continually logged over the experimental period

	Influent	Reactor	Effluent
Maximum	27.0 ± 2.3	21.0 ± 1.2	22.5 ± 1.6
Minimum	8.5 ± 2.3	13.5 ± 1.2	12.0 ± 1.6
Average	15.8 ± 2.3	16.6 ± 1.2	16.6 ± 1.6

The total material costs of the reactor, not including pumps, power supply and computing/recording instruments, was equivalent to £2344/m^3, of which the cathode and membrane combined represented less than 2%.

6.4. Discussion

This pilot scale reactor worked, producing almost pure hydrogen gas from raw influent domestic wastewater at U.K. ambient temperatures for a 3 month period and continues to do so. It is believed to be the first successful study of its kind, which brings the prospect of sustainable wastewater treatment and hydrogen production through the use of bioelectrochemical systems onto a new and exciting phase.

The reactor has removed on average 34% of COD, and occasionally reaching the UK discharge standard of 125 mgCOD/L, equating to a treatment rate of 0.14 kgCOD/m^3/day, just below the range for activated sludge. The reactor has performed this task using less energy than would be needed for aeration in a traditional activated sludge process. The electrical energy recovery on occasion nearly reached values of 100%, and was consistently around 70% during the later stages of the study. At this

level of performance (i.e. 70%) the energetic treatment costs were 2.3 kJ/gCOD, below the values for activated sludge of 2.5-7.2 kJ/gCOD (Pant et al., 2011). By implementing improvements to the reactor such as: increasing electrode surface areas; reducing the distance between electrodes; having a more efficient flow paths; consistent pumping; and improved materials, the η_E could be greater than 100%, making it a net energy producer. On the basis of this fairly large proof of concept study, energy neutral or even energy positive wastewater treatment is clearly a realistic goal.

The total energy recovery showed an increasing trend during the course of the study, levelling out at around 30%, with around a third of all energy both from the wastewater and from the power supply being recovered as hydrogen gas. Coulombic efficiencies of the reactor were high, levelling out at around 55-60 %, methane production accounts for an additional 3.5%. Other losses might be caused by some short circuiting in the reactor. It is likely therefore that a large proportion of the missing 40% of CE can be attributed to a loss of hydrogen gas from the system. Hydrogen is an extremely small molecule and is able to permeate most plastics, and is therefore likely to be leaking out of the reactor. In a tightly engineered system theoretically the coulombic efficiency could approach its maximum of 100%, resulting in an electrical energy recovery of 129%.

The substrate efficiency of the cell was considerably lower than the other efficiencies measured. This efficiency represents how much of the substrate is actually recovered as hydrogen, and gives an indication of how much substrate is used in the MEC process. Even if the 40% loss of hydrogen through leakage (as suggested by the CE of 60%) is accounted for in this calculation then the substrate efficiency would only increase from 10% to around 23%. Losses may be taken to suggest that substrate is being used in competitive oxidation processes, but only low levels of oxygen entered the cell with the influent. Sulphate reduction equated to about 3.6% of the total COD removal. Limited nitrates were available. Further losses can be accounted for by the probable build-up of sludge within the reactor as evidenced by the constant COD removal value throughout the study despite the increasing efficiency of the reactor, and that on three occasions a very high COD peak entered the reactor, on two of these occasions the peak of COD is not seen to leave the reactor see Figure 6-7.

Clearly the high resistance of the reactor means the overall efficiencies of the reactor will be low. The resistance observed is more problematic in this larger scale system than at the laboratory scale, and would also become increasingly challenging with further scale up. Improved reactor design is needed to overcome these problems. In a large scale system a considerable wire length is likely to be inevitable, resistance could be reduced through the use of a thicker wire, additionally resistance could be reduced in the electrode by improving the connection between the electrode, current collectors and wire. Further research into different materials and different configurations of materials would hopefully lead to improvements at a larger scale.

Further efficiency losses as identified above could be minimised by improving the engineering of the system. The two 'new' materials used in this study for the membrane and cathode have not been truly evaluated. More expensive alternatives such as Nafion membrane and a Pt coated cathode may prove to be worthwhile investments if performance increases greatly with their use. The biological MEC process works, and works relatively consistently for a period of at least three months. Although tested in realistic conditions, this was over a spring/summer period, survival over periods of sustained low temperature has yet to be confirmed.

The relationship between electrical energy recovery, electrical power input and coulombic efficiency has been defined however the prediction energy requirements for a larger scale MEC system may be difficult to make. Theoretical input voltages lie far from those needed in reality even for acetate fed cells, typically between 0.4-1.0 V compared to the 0.114 V theoretically needed (pH 7, 298 K) (Logan, 2008). A relatively small change in the electrical power input can have a large effect of the overall electrical energy recovery, yet if this value is not high enough to overcome the losses in the cell no hydrogen will be produced.

Undoubtedly there are many factors that require further investigation. Many of the inefficiencies could be overcome by improved engineering, but also a greater understanding of the biological processes (both working with and against the cell performance), community structure and ecology would allow for more confident design and manipulation.

The aim of this research was to determine if MEC technology could be a viable and alternative to the activated sludge process. The pilot scale reactor has worked producing hydrogen, with real wastewaters at ambient temperatures for over 3 months at a volumetric treatment rate just below that for activated sludge. A breakeven energy was not consistently achieved during the course of the study, yet is believed to be within reach with improved hydrogen capture and improved design to increase efficiencies. With this proof of concept now made we are a large step closer to using MEC technology for sustainable wastewater treatment.

Chapter 7. Conclusions

The overall aim of this research is to reach an understanding of whether microbial electrolysis cells could be a domestic wastewater treatment option.

I conclude that energy neutral or energy positive wastewater treatment should be possible. This research started by looking into how much energy is held intrinsically within the wastewater, and concluded that the amount of energy in the wastewater is substantial, more than previously thought, and more that the energy costs currently incurred in its treatment (18-29 kJ/gCOD vs. 2.5-7.2 kJ/gCOD in activated sludge treatment). Although this energy measured is internal chemical energy which is higher than the Gibbs Free Energy that would be available to microorganisms, with a biological system engineered for energy extraction from wastewater rather than an energy input, i.e. utilising other redox pathways rather than simple aerobic oxidation.

With the conclusion made that there is enough energy inherently contained in wastewater to treat it, the next question was to determine if Microbial Electrolysis Cells could meet this demand, replacing the high energy demanding activated sludge process with an energy yielding process. Parts of the thesis, in particular the low temperature work, suggested this might be possible yet other parts of the research did not such as the failure in MEC wastewater fed reactors. However by building and testing a pilot scale reactor on site at a wastewater treatment the most positive and conclusive evidence that this technology could work for real wastewater applications was gained. The reactor, even though it was a ‘first design’ using low cost alternatives to the optimum materials, and with many other problems such as non-optimised flow and hydrogen leakage and high resistance, it came reasonably close to its breakeven energy point. Even without breaking even it was more effective in terms of energy used per gCOD removed, and came close to the volumetric loading rates of the activated sludge process.

There is still much work to be done at this scale and larger to: understand the issues of scaling; economic feasibility; hydrogen capture and storage; design and materials; and optimisation. This work could then lead to retrofitting old activated sludge lanes with microbial electrolysis cells, radically changing the wastewater industry.

All the research conducted in this PhD has shown that the substrate acetate is not an adequate model of wastewater. This has been shown simply in terms of the energy available per gCOD, the acclimatisation and number of exoelectrogens able to digest these substrates, the diversity of the community fed with these substrates and their function within microbial electrolysis cells. The higher diversity estimates and complex acclimatisation pattern of acetate fed reactors suggest acetate may not be the optimum compound to use in BES's. Wastewater fed systems may have less free energy available, and therefore result in a more efficient biomass being formed. The lower coulombic efficiencies observed in wastewater fed reactors might be an inevitable result of electrons being lost within the longer chains of digestion, and not necessarily an indication of inefficient biomass.

The conclusion that temperature does not affect the performance of MFCs is surprising, although does correspond to some of the literature in this area (Catal et al., 2011, Jadhav and Ghangrekar, 2009). This suggests that there is a similar level of free energy available in systems run at different temperatures, and that low temperatures do not represent a disadvantage for BES. This is also observed in the pilot reactor, here low temperatures may be an advantage reducing methanogenic activity which proved fatal in the only other pilot scale MEC study to be published (run at 30 ^{o}C) (Cusick et al., 2011).

A further surprising conclusion was that inoculum did not have an effect on reactor performance, although the inoculum did interact with substrate to produce higher diversities within acetate fed reactors inoculated with high diversity soil. Exoelectrogenic bacteria were present naturally in all the wastewater inocula, and the Arctic soil inocula used throughout this research, albeit at low levels. The number or proportion of exoelectrogens was estimated to be 0.0017% using the very old methodology of MPNs, using the most recent next generation sequencing techniques and mathematical modelling algorithms, the estimates were 0.0012% and 0.00001% for two different wastewater samples. This therefore appears to be a reasonable good estimate of the rarity of such species.

BES reactors have been shown to work in challenging, real life, environments, and many observations have been made about the abundance and diversity of the organisms needed for the operation of these systems. This research has moved a substantial step

forward in proving that these technologies could be an energy efficient replacement of the activated sludge process. However we are still a long way from a deep and holistic understanding of the bacterial world operating within these systems, the energy requirements of these communities, their metabolic limits, their response to stress and ultimately their stability and function. Without this deep understanding we are reliant upon empirical data gathering, testing reactors in various environments until these limits are found. If we could model the free energy needs of the bacterial community, estimate the free energy available in the substrate, and calculate the efficiencies of the electrochemical cell, such systems could be modelled accurately and ultimately engineered to produce positive energy recovery.

Chapter 8. Perspectives on the use of MECs in the treatment of wastewater

This work has demonstrated a proof of concept of the use of MECs with domestic wastewater to produce hydrogen at the 100L scale over a 3 month time period. However this does not mean that they will be a viable wastewater treatment option. The work conducted in this research goes some way to confirming to technical feasibility of this technology in the treatment of domestic wastewaters, it does not however prove or suggest that this will be an economic viability, such an assertion is beyond the scope of this study.

There are many considerations which would need to be focused on in order to determine this economic viability for any technology to replace activated sludge treatment (AS), including those criteria stated in the introduction:

1. Extract and convert energy to a useable form at an efficiency that justifies the costs.
2. Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
3. Treat low strength domestic wastewater.
4. Work at ambient, often low temperatures.
5. Work continuously and reliably.

The detailed costing of this technology is beyond the scope of this thesis. It has been suggested that MEC technology may be an economically viable alternative to AS over other treatments such as anaerobic digestion (AD) or MFCs (Foley et al., 2010, Curtis, 2010) based on the reduction in aeration costs and the potential value of products produced. However to change the UK wastewater infrastructure would require exchanging the current AS process components for a system with higher capital costs (estimated at 0.4 €/kgCOD for an MEC compared to 0.1 €/kgCOD for AS, (Rozendal et al., 2008a)) aiming to recover the costs through the product generated. It is clear that even with low cost materials used in this research, and the idea of retrofitting the cells into existing infrastructure (Cha et al., 2010), the capital costs of filling tanks with complex electrode assemblies would be far higher than installing the aeration pipework. It would need to be ascertained whether the ‘payback’ in terms of reduction of the

energy costs and the products generated would equal the higher capital costs over the lifetime of the cells (which is again unknown at this stage).

The design life of typical wastewater treatment infrastructure is at least 25 years. MECs have not been tested over such time periods in even in the relatively clean conditions of laboratories. It is highly likely the many of the components of a typical MEC would not survive for long periods when handling real wastes, membranes for example are particularly problematical clogging over time (Zhang et al., 2011), yet membraneless are also problematic at large scale (Cusick et al., 2011). Even the estimates for a 5 year life span of electrodes and membranes used in the estimates above (Rozendal et al., 2008a) are untested under real conditions and may be unrealistic. The life span and maintenance requirements of BES will be a critical factor in determining if this technology can be used economically within the wastewater industry.

A further cost consideration is the labour costs associated with this new technology. The level of maintenance required in the MEC process is again unknown, but is likely to be higher than the AS, though may be compensated for by the reduction in sludge treatment which is a considerable fraction of the operational costs (Verstraete and Vlaeminck, 2011). The hydrogen or product produced may also require purification again the costs of this would need to be accounted for in identifying if the economic benefits of the product outweigh the costs.

The full economic costing of the MEC process versus other processes is complex, with many unknowns. It is likely to vary with: the scale and wastewater type of different treatment plants; water usage and availability; energy and material prices; and therefore inherently through time (McCarty et al., 2011). The ‘upgrading’ of AS plants with improved energy recovery from sludge AD, improved process control and greater levels of primary settling such as the Strass plant in Austria which generates 108% of its electricity use (Nowak et al., 2011) may prove to be more economically viable. The addition of AD onto the AS process is the route many UK water companies are taking including Northumbria Water Ltd who have one large sludge AD plant in operation and one under construction. However such a high degree energy recovery is exceptional, and many experts in the field question the concept of using the energy intensive process of AS to insolubalise waste organics to sludge which then can undergo energy recovery (Verstraete and Vlaeminck, 2011).

The treatment levels of the pilot MEC run were both low and variable, averaging only at 34%, the AS process can remove up to 95% of the COD (Tchobanoglous, 1991) although this is rarely the case as they are usually part of a treatment flow with pre-settling and post clarification removing a proportion of the COD (Grady, 1999). The MEC reactor demonstrated did on occasions remove the COD down to the discharge limit of 125 mgCOD/L (EEC, 1991) so operation at this level is possible. The ability to use domestic wastewaters is a clear advantage over AD which tends to be restricted to high strength industrial or farm wastes, or sludge generated by AD. Further work would be needed to demonstrate that this treatment could consistently reach discharge standards, and the electrical conductivity of the wastewater at these low strengths is sufficient for the cells to function.

Even if part of a treatment flow with pre-settling and post clarification it is likely that the MEC would need to improve treatment rates to encourage investment, additionally the more organics removed the higher the energy yield can be. Treatment rates could be improved by reducing electrode spacing; however this would have the knock on effect of reducing the volumetric loading rate. The MEC could therefore end up requiring the same unit space as trickling filters, and therefore not be a viable option either due to land restrictions or poor economic comparability to this low energy treatment option.
There is an increasing body of research demonstrating that BES technologies will work at ambient temperatures (Jadhav and Ghangrekar, 2009, Catal et al., 2011, Larrosa-Guerrero et al., 2010), added to by the work in this thesis. Further work may be required in demonstrating this with real wastewaters at a larger scale, and also in quantifying and overcoming the kinetic effect of the lower temperatures on bacterial metabolism.

Many challenges lie ahead with BES research both from a technological and economic perspective. Only through completing and importantly combining these research areas will we be able to reach an understanding as to whether the technology can be used in the wastewater treatment plants of the future.

Chapter 9. Recommendations for future research

This research set out to answer the question as to whether microbial electrolysis cells could be used for wastewater treatment. Most of this research has strengthened the case that they are, however many more research and application questions remain unanswered. Each piece of research described in this thesis could be developed further to give more conclusive answers:

Chapter 2: A comprehensive survey into the amount of energy contained within wastewater is warranted. In the research conducted two samples were tested from different wastewater treatment plants and the results showed a large difference in the energy content between the samples and with that which would be predicted. Discovering the energy in wastewater is fundamental to the study of bioelectrochemical systems, and other technologies which aim to yield energy from wastewater. If we are to evaluate the true potential of these technologies we need to know how much energy is actually encapsulated in domestic wastewater, enabling efficiencies to be calculated and therefore better solutions engineered.

Measuring internal energy by calorimetry is a standard method in the solid waste industry (Garg et al., 2007, Lupa et al., 2011), yet when applied to wastewater the problem arises that samples have to be dry, and even with the improved and extremely laborious freeze drying method used in this research 20-30% of the volatiles in wastewater were lost. With an improved and quicker method, such as the use of distillation or reverse osmosis, a comprehensive survey of wastewaters in the UK could be made. This would: facilitate decisions on where best to invest in new technologies; give an indication of which technologies might be more suitable for different wastewaters; inform of the efficiency of processes; and most importantly – make decision makers believe energy extraction from wastewaters is economically viable and worthwhile.

Chapter 3: With a more definitive answer to the number of bacteria present and their growth pattern, accurate assessments of specific activity and growth yields could be made. Accurate estimations of these values are needed for parameterising models of these systems. By redesigning these experiments, and the reactors used to minimise or at least quantify all losses, a mass balance could be made and these values determined.

However the most intriguing question arising from this work was the difference in the pattern of acclimatisation observed in the acetate fed cells and those with complex substrates. Although possible reasons for this difference were suggested, a conclusive answer was not found. By conducting further research scaling between acetate and starch in terms of substrate complexity, the step causing the change in response of acclimatisation could be found, which may give valuable insight into the development and ultimately the function of these communities. The use of other microbiological techniques such as flow cytometry and QPCR may also help in the accurate determination of these values.

Chapter 4: The finding that temperature and inoculum had little effect on reactor performance is significant to the eventual implementation of this technology. The high variability within the warmer reactors would however be worth investigating further, if all the warm reactors were able to work at the maximum level shown by some, temperature would be a significant factor. The reactor configuration used in these experiments may have been limiting factor, thus if repeated with a higher performing reactor design, the temperature effect may be observed.

The counterintuitive observation that acetate fed cells produced a higher diversity was of great interest in this work. Further research is needed to determine if it is energy that controls the diversity, not the complexity of the substrate. This could be examined by scaling through simple compounds with known and increasing free energies (e.g. from the ΔG of the reaction under standard conditions at pH 7: acetate 27.40 kJ/e^- eq, pyruvate 35.09 kJ/ e^- eq and glucose 41.35 kJ/e^- eq) and observing how diversity changes.

Chapter 5: The conclusion that laboratory wastewater fed reactors fail after a short period of time is contradicted by chapter 6 where the pilot MEC worked. Determining the reason for failure at the small scale is a priority for any further lab scale research studies. Other than scale, the two different factors in the lab based experiments compared to the pilot, are that feed is continuous not batch, and that the laboratory reactors are acclimatised as a MFCs. Research into these factors, and a solution to the failure is needed to achieve the working laboratory wastewater fed systems required for investigations into the use of this technology for wastewater treatment.

Chapter 6: The final part of this research gave the most conclusive answer as to whether MECs can work for wastewater treatment and will, when published, put the research of MECs onto a new platform. Much research is still needed into improving efficiencies and critically achieving the breakeven energy recovery, further scaling, different materials and design, and the economic feasibility of implementing this technology at scale. If the use of this technology is validated, research is needed into the strategic implications this will have on the wastewater treatment industry.

Further recommendations: The research described has increased our understanding of how BES can function in wastewater treatment. A more fundamental direction of research would be the use of BES in understanding the energetic laws and rules which underpin biological systems. Such rules would have huge impact on design in both the near and distant future (Curtis et al., 2003). BES offer the unique opportunity, effectively opening a window on the energy involved in biological reaction, as this energy is routed through an external circuit and can therefore be measured allowing energetic interactions to be unravelled.

By designing a biocalorimeter type BES reactor, where all energetic inputs and outputs are measured (with no leakage) this could be tested using simple substrates and monocultures, and simple laws developed. For example if a substrate chemically yields 'x' kilojoules of Gibbs free energy (ΔG), exactly how much of this can be accessed by bacteria at a set pH and temperature, what proportions go to growth and maintenance for the BES to be stable and what the energy transfer efficiency is. By then scaling to more complex substrates and mixed cultures insight could be gained on: the fermentation processes and on how and why some reaction routes may be favored over others; if the overall ΔG of a complex substrate adequate to model outcome or is more complexity required; and if the energy needs are similar amongst trophic layers.

Through manipulating the systems thermodynamic constraints (temperature, pressure, and ionic strength) to give predictable outcomes, the rules identified above could be verified. Knowledge would also be gained on which thresholds of energy can change community behavior, and how easily these can be manipulated, how much the bacteria can compensate for these changes. Additionally by taking the system to the energetic edge the real limits can be defined and compered to theoretical limits. Ultimately an understanding of how energy requirements of a community link to abundance and

diversity could be gained, and allow for these to be manipulated to increase system stability.

By using a BES in this novel way, the thermodynamic laws which underpin the microbial world may be discovered. The rules generated could be used to create a model allowing biotechnologies to be reliably engineered. The feasibility and efficiency of a bioprocess being modeled at the investment stage without relying on estimates from empirical data. This would have huge scope to promote change and development across the scientific and engineering community.

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Chapter 11. Appendices

11.1. Appendix I - History of microbial fuel cell technology

The concept of fuel cells, a device that can convert electrochemical energy into electricity is not new. The first working chemical fuel cell is attributed to Sir William Grove in 1839 (Lewis, 1966). Progress since then has been slow and sporadic. Although it was understood that the direct conversion of chemical energy to electrical energy was more efficient than combustion in a heat engine (where up to 80% of the energy in the fuel is lost through heat in the exhaust, friction, air turbulence and the heating up and movement of engine parts), historically the abundance of fuel meant that the simpler combustion engine took precedence. The main surge of work in fuel cells has been in the last 10-15 years as fossil fuel prices, and the need for cleaner and more efficient energy production has increased (Logan, 2008).

The first biologically catalysed fuel cell was made in 1911 by a Professor of Botany M.C. Potter at Newcastle University. He discovered that an electrical current could be produced using bacteria as the catalyst on the anode, with a glucose and yeast mixture under various conditions of temperature and concentration he produced a maximum of 0.3 to 0.5 volts (Potter, 1911). This work was added to by Barnet Cohen who built a small bacterial battery using a series of half cells. This work drew more attention to the area, however the major drawback of the system was highlighted, only a very low current is able to be produced and it is rapidly discharged. The use of mediators such as potassium ferrycyanide and benzoquinone did enable greater voltage to be produced however the current remained low (Cohen, 1930).

Del Duca et al. (1963) re-visited the idea and set up a working laboratory model built using urea as a fuel. Urea was broken down enzymically by urease to produce ammonia at the anode, which then reacted with an air cathode producing current. A conceptual design was put forward for a 20-Watt portable urea battery, containing 64 individual cells, however the battery life was only 2 weeks.

Karube et al.(1976), described how carbohydrates were broken down to hydrogen using a fixed matrix of fermentative bacteria, the hydrogen reacted in the electrochemical cell. These studies were the first to use a design very similar to those MFCs used today, but with a salt bridge rather than an artificial membrane. It was believed that the bacteria's

role was to break down the carbohydrate to make electrochemically active products, which were entirely responsible for the current generation. It was not seen that the bacteria themselves were creating the electrochemical current, through the donation of electrons, though this was almost certainly the case.

R. M. Allen and then H. P. Bennetto worked on microbial fuel cells throughout the 1980's at Kings College, London. They had the vision that fuels cells could be a solution to the poor sanitation and lack of electricity supply in the then termed 'third world'. A paper which was the culmination of this work was published in 1993, simply titled Microbial Fuel-Cells – Electricity Production from Carbohydrates, was the first to show an understanding of the mechanism at work (Allen and Bennetto, 1993), although electron transfer was still not understood. It was thought that electrons were extracted from the oxidation of carbohydrates; these would then become trapped within the bacteria, but would become available for transfer to the anode through the use of a chemical redox mediator. Chemical mediators such as ferricyanide were expensive, non-sustainable and toxic to the environment.

The breakthrough discovery was made in 1999 that chemical mediators where not needed in the cells (Kim et al., 1999). This critical discovery that MFCs do not require these mediators, and the ever increasing pressures to reduce pollution, has led to an explosion of research in this area.

In 2005 it was discovered that microbes could be used in an electrolysis cell (Rozendal et al., 2006, Liu et al., 2005b). Electrical energy input can be combined with the energy derived from the fuel by bacteria to drive electrolysis reactions making products which would otherwise require much larger inputs of energy, most notably hydrogen. Thus hydrogen can be produced at greater efficiencies than is the limit with fermentation, and in theory at around one tenth of the electrical energy input of water electrolysis.

11.2. Appendix II - Theoretical cell energetics

The basic reaction occurring in an MFC or MEC can be split into two half reactions, the anode reaction which is the catabolic breakdown of the organic substrate to produce electrons, and the cathode reaction which is the donation of these electrons. The quantity of energy released per electron transferred is dependent on the chemical properties of those compounds involved, and is given by the Gibbs free energy of the reaction or ΔG_r:

$$\Delta G_r = \Delta G_r^0 + RT \ln Q$$

Equation 1

Where ΔG_r is the Gibbs free energy of the reaction, ΔG_r^0 is the Gibbs free energy for the reaction under standard conditions (temperature of 298 K and chemical concentrations of 1M for liquids and 1 bar for gases) as tabulated (Atkins, 2006), R is the gas constant 8.31 J/mol-K, T is temperature, and Q is the reaction quotient i.e. the ratio of the activities of the products and the reactants.

The cell potential (E_{emf}) can be calculated from Gibbs free energy of each half reaction:

$$E_{emf}^0 = -\Delta G_r^0 / nF$$

Equation 2

Where n is the number of moles of electrons transferred and F is Faradays constant 96485 J/mol e^-.

Alternatively the potential can be calculated directly when the potential under standard conditions is known:

$$E_{emf} = {E_{emf}}^0 - \frac{RT}{nF} \ln Q$$

Equation 3

Using acetate as an example electron donor, the half-cell, and full reaction values are given for ΔG_r and E_{emf} in Table 11-1 under standard environmental conditions pH 7, 298 K:

Table 11-1 Calculated theoretical energies (as Gibbs free energy and Potential) of half-cell reactions occurring within BES fed with acetate

	Reaction	ΔG_r/ kJ/ e- eq	Potential E (V)
Anode/ donor	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$	27.40	-0.300 (-0.284)
Cathode /acceptor MFC	$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O$	-78.72	0.805 (0.816)
Overall MFC	$\frac{1}{8}CH_3COO^- + \frac{1}{4}O_2 \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}H_2O + \frac{1}{8}HCO_3^-$	-106.12	1.105 (1.100)
Cathode /acceptor MEC	$H^+ + e^- \rightarrow \frac{1}{2}H_2$	39.94	-0.414
Overall MEC	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{2}H_2 + \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^-$	12.54	-0.114 (-0.130)

Values for E_{emf} written in bracket are those calculated from the tabulated ΔG_r and E_{emf} values which vary slightly (Rittmann, 2001, Atkins, 2006).

From the equations above it can be seen that anode and cathode potentials vary with temperatures (T), substrates (ΔG_r^0 or E_{emf}^0) and ionic concentrations (Q), especially pH. These can be calculated as shown below (except in the case of wastewater). However in a real system they may vary from time to time, place to place, and even within the same reactor as substrates are utilised and H^+ ions produced:

Substrate

In an acetate fed MEC the theoretical anode potential (E_{An}) under standard biological conditions (i.e. pH 7, temperature 25 ^oC) would be -0.284 V and the for the hydrogen evolution reaction (again at pH 7) it is -0.414 V, giving a cell potential E_{emf} of -0.13V an additional 0.13V would need to be added, with glucose this difference is positive 0.015V, theoretically no energy would need to be added. With wastewater and its unknown composition and variability the theoretical anode potential cannot calculated,

the potential of a variety of compounds which may be found within wastewater are shown in Table 11-2.

Table 11-2 Known Gibbs free energy and potential values for a variety of compounds which may be present in wastewater

Substrate	ΔGr (kJ/mol e-)	E_{An} (V)	E_{emf} (V)
Methane	23.53	-0.244	-0.170
Acetate	27.40	-0.284	-0.130
Propionate	27.63	-0.286	-0.128
Ethanol	31.18	-0.323	-0.091
Protein	32.22	-0.334	-0.080
Lactate	32.29	-0.335	-0.079
Citrate	33.08	-0.343	-0.071
Methanol	36.84	-0.382	-0.032
Glycerol	38.88	-0.403	-0.011
Formate	39.19	-0.406	-0.008
Glucose	41.35	-0.429	0.015

ΔGr values from (Rittmann, 2001)

Temperature

Using acetate in an MFC as an example, with an acetate concentration of 0.12M (1 g/L of Na-acetate), bicarbonate concentration of 0.005M, at pH 7, and partial pressure of O_2 as 0.2, the potential, E_{emf} of the anode and cathode can be calculated through a range of temperatures from 0 to 30 ^{o}C:

Anode reaction

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O$$

Cathode reaction

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O$$

The potential under standard environmental conditions (E^0) for these reactions are 0.187V and 1.229V respectively. Using Equation 3 above:

Anode

$$E_{An} = {E_{An}}^0 - \frac{RT}{nF}\ln\frac{[CH_3COO^-]}{[HCO_3^-]^2[H^+]^9}$$

$$E_{An} = 0.187 - \frac{(8.31\,J/mol\,K)\,(T)}{(8)(\,96485\,C/mol)}\ln\frac{[0.012]}{[0.005]^2[10^{-7}]^9}$$

Cathode

$$E_{Ca} = E_{Ca}{}^{0} - \frac{RT}{nF}\ln\frac{[H_2O]}{[O_2]^{1/2}[H^+]^2}$$

$$E_{Ca} = 1.229 - \frac{(8.31\ J/mol\ K)\ (T)}{(2)(\ 96485\ C/mol)}\ \ln\frac{[1]}{[0.02]^{1/2}[10^{-7}]^2}$$

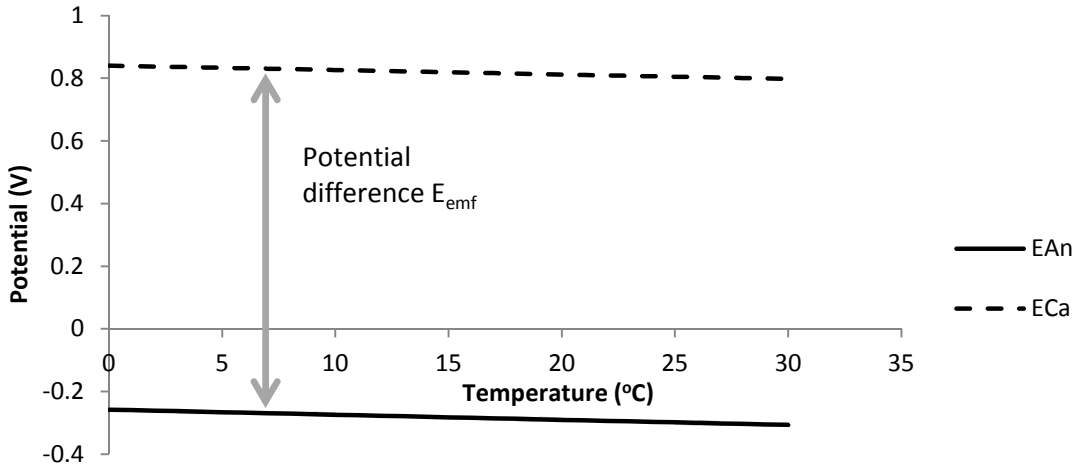


Figure 11-1 Calculated anode and cathode potential though a range of temperatures using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; pH 7; and partial pressure of O_2 as 0.2

The difference between the anode and cathode potential seen in Figure 11-1 varies only slightly from -1.098 V at 0 ^{o}C to -1.104 V at 30 ^{o}C. Theoretically therefore the energy available to be produced via a fuel cell is not greatly affected by temperature within the ranges given. This is however a simplistic approach to a system which, as stated previously is highly complex. As temperatures vary, so will many other factors including dissociation constants, partial pressures of gases and metabolic activity of the bacteria. It is therefore unlikely that the fuel cell will be able to generate as much current at lower temperatures as higher ones, yet it may not be as detrimentally affected by temperature as straight anaerobic digestion.

pH

The reaction co-efficient (Q) is calculated on the basis of the concentrations of the products and reactants in the chemical equation. This factor is critically dependant on

the pH of the system, i.e. the number of H^+ ions, as pH is a logarithmic scale, variance between pH 6 and pH 7 (both within the tolerance of bacteria) has a large effect on the Q value and therefore the overall potential of the cell. An example of this is shown below where the pH of the anode in an acetate system as described in the equations above at 25 ^oC is varied between pH 5 and 8, the cathode potential is kept constant under standard conditions. The potential difference ranges from 0.97 to 1.24 V.

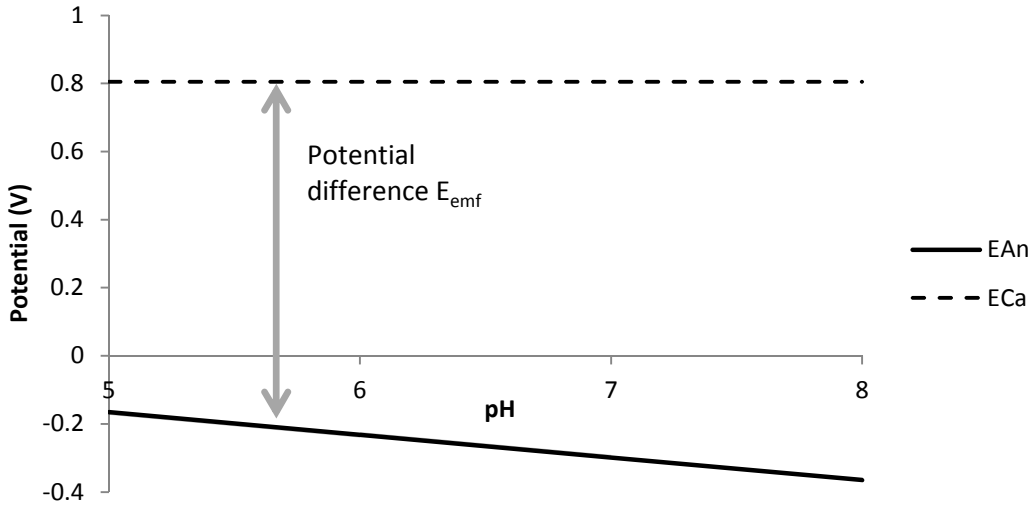


Figure 11-2 Calculated theoretical anode and cathode potential through a range of pHs using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; temperature 25 ^oC; and partial pressure of O_2 as 0.2

11.3. Appendix III – Table of calculated kJ/gCOD of various organic compounds

Compound	Formula	ΔH/gCOD
Benzene	C_6H_6	10.2
Linoleic acid	$C_{18}H_{32}O_2$	13.4
Benzoic acid	C_6H_5COOH	13.4
Myristic acid	$CH_3(CH_2)_{12}CO_2H$	13.6
Acetic acid (Acetate)	CH_3COOH	13.6
Phenol	C_6H_5OH	13.6
Palmitic Acid	$CH_3(CH_2)_{14}CO_2H$	13.6
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$	13.7
Methane	CH_4	13.9
Ethane	C_2H_6	13.9
Lactic acid	$CH_3CH(OH)COOH$	14.0
Ethanol	C_2H_5OH	14.3
Glucose	$C_6H_{12}O_6$	14.3
Propene	C_3H_6	14.3
Cyclopropane	C_3H_3	14.5
Ethanal	CH_3CHO	14.6
Ethene	C_2H_4	14.7
Sucrose	$C_{12}H_{22}O_{11}$	14.7
Methanol	CH_3OH	15.1
Chloroethylene	C_2H_3Cl	15.7
Oxalic acid	$(COOH)_2$	15.9
Formic acid	$HCOOH$	15.9
Ethyne	C_2H_2	16.3
Hexachlorobenzene	C_6Cl_6	16.5
Dichloroethylene (1,1)	$C_2H_2Cl_2$	17.1
Dichloroethylene (1,2)	$C_2H_2Cl_2$	17.2
Methanal	$HCHO$	17.8
Trichloroethylene	C_2HCl_3	20.0
Teterachloroethylene	C_2Cl_4	26.0
Chloroform	$CHCl_3$	29.1
Trichloroacetic acid	CCl_3COOH	30.4

11.4. Appendix IV - Description of the calculation algorithm used in the Shizas and Bagley

Shizas and Bagley (Shizas and Bagley, 2004) use a sample of municipal wastewater which prior to drying contains 431 mg/L COD. This sample is then oven dried to give a total solids measurement of 1980 mg/L. The dried sample is used in a bomb calorimeter giving 3.2 kJ/g dried weight.

Calculations derived from this data cited in various papers (Logan, 2008, Liao et al., 2006, Schroder, 2008, Logan, 2009):

$$3.2\ \mathrm{kJ/g}\ \times\ 1.98\ \mathrm{g/L} = 6.3\ \mathrm{kJ/L\ wastewater}$$

$$6.3\ \mathrm{kJ/L}\ \times \frac{1}{0.431\ \mathrm{gCOD/L}}\ =\ 14.7\ \mathrm{kJ/gCOD}$$

If the exercise is repeated on the data from the present paper using the oven dried samples and the measurement taken for COD prior to drying the results would have been:

Cramlington

$$8.3\ \mathrm{kJ/L}\ \times \frac{1}{0.718\ \mathrm{gCOD/L}}\ =\ 11.6\ \mathrm{kJ/gCOD}$$

Hendon

$$5.6\ \mathrm{kJ/L}\ \times \frac{1}{0.576\ \mathrm{gCOD/L}}\ =\ 9.9\ \mathrm{kJ/gCOD}$$

This is an underestimation of 60% and 45% respectively.

11.5. Appendix V - Wastewater sterilisation

Several of the experiments conducted in this thesis relied on using real wastewater, but needed this to be sterile. The following method was developed:

Method

The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into Ringers sterile dilutent (APHA, 1998). Effective sterilisation was defined as colony free plates in triplicate at zero dilution. The circulation time was varied to determine the optimum. The change in chemical composition (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) of the wastewater itself as compared to autoclaving and filtering.

Results

UV sterilisation caused the least change in wastewater properties measured as shown in Table 11-3, and was able to fully sterilise the wastewater.

Table 11-3 Percentage change of wastewater characteristics caused by the different sterilisation methods

	COD	Soluble COD	Total Solids	Bacteria per 0.1ml
Autoclaved (121^{o}C for 15 mins)	-15.6% ± 0.9	21.6% ± 0.6	-13.3% ± 5.8	0
Membrane filtered (0.2um PES)	-61.5% ± 0.5	22.8% ± 1.7	-36.1% ± 11.7	40 ±19
UV sterilised (5 mins)	-1.6% ± 0.4	7.2% ± 4.6	-3.3% ± 6.7	0

Bacteria is the average number counted on triplicate plates at zero dilution. All values show mean ± standard deviation (n=3)

Conclusion

Circulation of wastewater for 5 minutes through a UV filter was effective for bacterial kill off and least detrimental treatment to the composition of the wastewater.

11.6. Appendix VI - COD removal and coulombic efficiency

In the acetate fed cells the COD removal was high for both the cells which did (85%) and did not (80%) produce current ($p = 0.051$). For the other reactors there was an average removal of 64% COD for the wastewater and 87% for the starch solution. No significant difference in the COD removal in the reactors which generated current and those that did not was found wastewater ($p = 0.188$) and starch ($p= 0.688$).

The effluent of all reactors contained no detectable VFA's. The measured anions in each cell showed that there was almost complete removal of sulphate, from a starting value of 70 ppm in the wastewater and 38 and 41 ppm in the acetate and starch solutions respectively.

The coulombic efficiency of all reactors was low, such values are reasonably typical for complex substrates, but far lower than would be expected in a functioning acetate fed cell (Logan, 2008, Liu et al., 2011).

Table 11-4 COD removal and Coulombic efficiencies of all reactors fed on the different substrates. The values in grey are the reactors where acclimatisation did not occur

Inocula (ml)	**0.01**	**0.01**	**0.1**	**0.1**	**0.1**	**1**	**1**	**1**	**1**	**10**	**10**	**25**	**25**	**25**	**25**	**50**	**50**
COD removal (%)																	
Acetate	85.6	77.1	85.4	80.3	80.5	86.7	95.6	92.4	82.1	87.3	79.9	84.7	86.6	80.2	77.6	79.0	77.3
Wastewater						60.8	41.9			59.1	69.8	68.1	70.5			80.3	62.5
Starch						88.2	84.5			88.2	86.4	89.4	81.7			80.8	90.5
Coulombic efficiency (%)																	
Acetate	0.1	0.1	1.8	0.5	0.8	4.6	5.3	1.8	0.1	6.7	7.5	10.5	8.9	10.1	9.2	9.1	0.8
Wastewater						3.6	0.1			0.3	0.2	9.4	12.5			10.4	7.4
Starch						1.3	0.78			0.82	13.4	12.5	16.9			17.4	1.6*

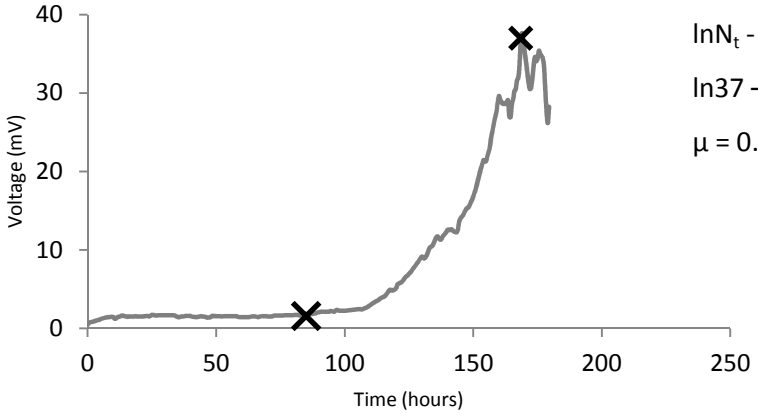
Values in grey are the reactors which did not acclimatise

*Unrepresentative value, data logging equipment failed after the point of acclimation.

11.7. Appendix VII - Yield and Specific activity calculations

Growth rate

Example calculation using 25 ml inocula

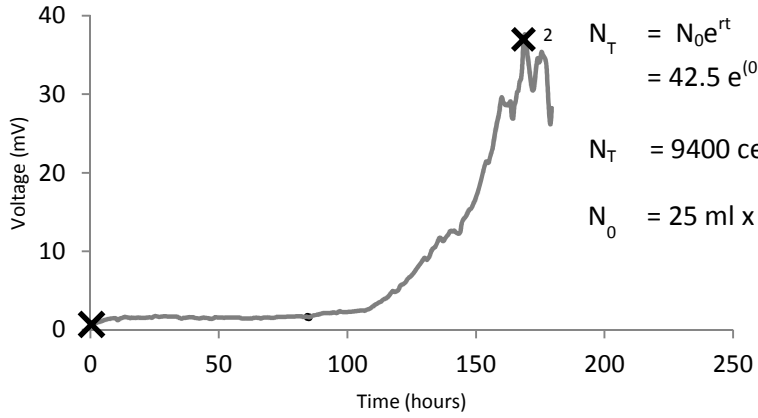


$\ln N_t - \ln N_0 = \mu(t - t_0)$

$\ln 37 - \ln 2 = \mu(170 - 85)$

$\mu = 0.034$

Specific activity



$N_T = N_0 e^{rt}$

$= 42.5\ e^{(0.034 \times 180)}$

N_T = 9400 cells

N_0 = 25 ml x 1.7 cell/ml = 42.5 cells

Each data logged voltage represents the time of 30 minutes, therefore the moles of electrons passed to the circuit per second at the data points measured is:

Moles of electrons = coulombs / Faradays constant

=((Voltage / resistance) x seconds)/Faradays constant

E.g. $\mathbf{X}_2$ =((0.037V / 470Ω)x 30mins x 60 seconds)/96485

$= 1.5 \times 10^{-6}$

Moles of electrons/cell = $1.5 \times 10^{-6} / 9400$

$= 10^{-10}$ mol e^-/cell

This value can be plotted throughout the time course of the experiment and is seen to be relatively constant.

Growth yield

The total number of cells produced up to the end of the exponential growth phase in the example above is 9400 cells.

$$\text{gCOD-cells} = (N_T - N_0) \times W \times COD_{cell}$$

where $N_T - N_0$ is the total new cell produced, W is the weight of each cell as estimated as 5.3×10^{-13} (Logan, 2008) and COD_{cell} is the estimation of 1.25 g-COD/g-cell (Rittmann, 2001).

$$\text{gCOD-cells} = (9400\text{-}43) \times 5.3 \times 10^{-13} \times 1.25$$

$$= 6.1 \times 10^{-9}$$

$$\text{gCODsubstrate} = \sum_{t0}^{t} mol\, e^{-}/8 \times 64$$

Where the sum over the growth period $t\text{-}t_0$ of the moles of electrons as calculated above is divided by 8 to give moles of acetate used, and multiplied by 64 giving the gCOD per mole of acetate.

$$\text{gCOD substrate} = 0.00011 / 8 \times 64 = 8.8 \times 10^{-4}$$

$$\text{gCOD-cell/gCOD-substrate} = 6.1 \times 10^{-9}/8.8 \times 10^{-4} = 6.9 \times 10^{-6}$$

The estimated yield of the acetate fed cells is extremely low ranging between 10^{-4} to 10^{-5} g-COD cell/g-COD substrate for the cells with between 10-50 mLs of inocula.

If exponential growth is assumed throughout the whole time period for the lower inocula cells these values are much higher up to 8 g-COD cell/g-COD for the 0.1 ml inocula. If no growth during lag is assumed these values are lower (10^{-7} g-COD cell/g-COD) and more in line with those observed for higher inocula. These yields are inconsistent with the literature on yields in microbial fuel cells (Freguia et al., 2007, Rabaey et al., 2003) although both of these studies used different methodology. They are also inconsistent with yields of other bacterial systems (Rittmann, 2001).

11.8. Appendix VIII – Calculations of performance in MFCs and MECs

Power Calculation for both MFCs and MECs

Performance can be evaluated through the amount of power produced which can be expressed as:

$$P = IE$$

Where P is the power in watts, E is the voltage as measured by the data logger in volts and I is the current in amps, calculated from the measured voltage E, at a known resistance R:

$$I = E/R$$

Power can therefore be alternatively expressed as:

$$P = E^2/R$$

This power is often also evaluated as power density (*Pd*), this is the amount of power produced per area of electrode surface (typically the size of the anode) expressed as Wm^2. Normalising the power output in this way allows different systems to be compared. This is calculated as:

$$Pd = \frac{E^2}{A_{An}R}$$

Where A_{An} is the area of the anode. The current density (A/m^2) can also be expressed in the same way normalising current to electrode size. Both power and current density can also be expressed per reactor size by substituting A_{An} above for the reactor volume in m^3, resulting in a power density measured as Wm^3. or current density as A/m^3.

Efficiency calculations for MFCs

The efficiency of an MFC is expressed as the Coulombic Efficiency (CE) and is a measure of the amount of coulombs of charge recovered from the cell from the total coulombs available in the substrate that has been removed in the reactor. It is expressed as a percentage:

$$CE = \frac{Coulombs\ recovered}{Coulombs\ in\ substrate}$$

An Amp is the transfer of 1 coulomb of charge per second, therefore by integrating the current over the course of the experiment or batch time (t) the total coulombs transferred is given. Usually the amount of coulombs in the substrate is evaluated using the amount of organic matter removed as determined by the chemical oxygen demand (COD). CE is therefore calculated as:

$$CE = \frac{8 \int_0^t I \ dt}{F \ V_{An} \Delta COD}$$

Where 8 is used as a constant derived from the molecular weight of oxygen divided by 4 the amount of electrons exchanged per mole of oxygen. Faradays constant (F) of 96485 Coulombs/mol, is the magnitude of electrical change per mole of electrons, ΔCOD is the measured change in COD in g/L and V_{An} (L) is the volume of the anode compartment containing the liquid feed at the given COD concentration. .

Efficiency calculation for MECs

The efficiency of an MEC is a more complex matter, as the output of energy is of hydrogen gas (not electricity or charge directly) and the inputs of energy are from the substrate and the additional electrical energy added to the system.

11.9. Appendix IX – Dissimilarity matrix

Unweighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.79	0.00																	
Wastewater inocula 2	0.88	**0.67**	0.00																
Acetate cold ww 1	0.88	0.67	0.70	0.00															
Acetate cold ww 2	0.88	0.65	0.69	**0.45**	0.00														
Acetate cold soil 1	0.82	0.80	0.80	0.72	0.73	0.00													
Acetate cold soil 2	0.76	0.78	0.82	0.75	0.75	**0.59**	0.00												
Acetate hot ww 1	0.89	0.71	0.69	0.54	0.60	0.73	0.78	0.00											
Acetate hot ww 2	0.89	0.73	0.71	0.56	0.58	0.74	0.78	**0.22**	0.00										
Acetate hot soil 1	0.81	0.79	0.80	0.72	0.75	0.62	0.63	0.25	0.70	0.00									
Acetate hot soil 2	0.79	0.79	0.82	0.75	0.76	0.64	0.65	0.18	0.72	**0.51**	0.00								
Wastewater cold ww 1	0.87	0.69	0.70	0.59	0.61	0.73	0.76	0.27	0.64	0.73	0.76	0.00							
Wastewater cold ww 2	0.82	0.72	0.76	0.72	0.73	0.74	0.74	0.44	0.67	0.66	0.67	**0.68**	0.00						
Wastewater cold soil 1	0.85	0.69	0.75	0.61	0.64	0.65	0.69	0.28	0.64	0.64	0.69	0.53	0.66	0.00					
Wastewater cold soil 2	0.88	0.74	0.75	0.69	0.69	0.77	0.78	0.24	0.60	0.69	0.72	0.62	0.51	**0.64**	0.00				
Wastewater hot ww 1	0.86	0.71	0.73	0.62	0.64	0.66	0.71	0.28	0.65	0.67	0.71	0.49	0.68	0.42	0.65	0.00			
Wastewater hot ww 2	0.88	0.74	0.74	0.68	0.68	0.76	0.77	0.24	0.60	0.70	0.72	0.63	0.51	0.65	0.23	**0.66**	0.00		
Wastewater hot soil 1	0.86	0.67	0.71	0.62	0.65	0.74	0.77	0.28	0.64	0.73	0.76	0.35	0.67	0.53	0.64	0.53	0.63	0.00	
Wastewater hot soil 2	0.89	0.71	0.72	0.59	0.58	0.75	0.78	0.28	0.59	0.74	0.78	0.44	0.71	0.51	0.64	0.49	0.64	**0.45**	0.00

Weighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.41	0.00																	
Wastewater inocula 2	0.52	**0.59**	0.00																
Acetate cold ww 1	0.44	0.48	0.45	0.00															
Acetate cold ww 2	0.43	0.45	0.48	**0.08**	0.00														
Acetate cold soil 1	0.50	0.56	0.53	0.17	0.16	0.00													
Acetate cold soil 2	0.44	0.48	0.47	0.09	0.10	**0.10**	0.00												
Acetate hot ww 1	0.49	0.57	0.41	0.28	0.30	0.36	0.30	0.00											
Acetate hot ww 2	0.56	0.63	0.44	0.37	0.41	0.45	0.40	**0.17**	0.00										
Acetate hot soil 1	0.36	0.43	0.50	0.23	0.22	0.30	0.23	0.32	0.43	0.00									
Acetate hot soil 2	0.41	0.51	0.43	0.14	0.16	0.22	0.15	0.23	0.34	**0.16**	0.00								
Wastewater cold ww 1	0.34	0.39	0.46	0.25	0.23	0.35	0.28	0.31	0.43	0.22	0.25	0.00							
Wastewater cold ww 2	0.37	0.42	0.55	0.43	0.40	0.45	0.42	0.46	0.52	0.31	0.39	**0.33**	0.00						
Wastewater cold soil 1	0.36	0.40	0.45	0.26	0.22	0.34	0.27	0.30	0.42	0.22	0.26	0.13	0.36	0.00					
Wastewater cold soil 2	0.41	0.50	0.41	0.31	0.33	0.39	0.33	0.30	0.36	0.37	0.31	0.32	0.33	**0.34**	0.00				
Wastewater hot ww 1	0.35	0.39	0.46	0.27	0.24	0.36	0.29	0.31	0.43	0.24	0.29	0.14	0.37	0.05	0.35	0.00			
Wastewater hot ww 2	0.38	0.49	0.40	0.31	0.32	0.40	0.33	0.26	0.35	0.35	0.29	0.29	0.33	0.31	0.08	**0.32**	0.00		
Wastewater hot soil 1	0.34	0.40	0.48	0.24	0.22	0.34	0.28	0.33	0.45	0.22	0.26	0.05	0.33	0.14	0.31	0.16	0.29	0.00	
Wastewater hot soil 2	0.38	0.38	0.52	0.28	0.24	0.35	0.30	0.36	0.49	0.25	0.30	0.12	0.32	0.18	0.34	0.19	0.34	**0.11**	0.00

Dissimilarity values calculated using UniFrac, lower values indicate greater similarity

11.10. Appendix X - Estimates of sample total diversity

Table 11-5 Estimates of total diversity using the MCMC model (Quince et al., 2008), values given are the lower 95% confidence interval : median : upper 95% confidence interval. The best fit values according to the DIC values are highlighted in bold, the model fits that had DIC scores within 6 of the best fitting model are in italics and should not be considered as plausible options for fitting the data

	Total diversity		
Sample	**Log-normal**	**Inverse Gaussian**	**Sichel**
Arctic soil inocula	**5831:7207:10593**	5151:6227:7439	3632:4403:5821
Wastewater inocula 1	**3431:4238:5572**	2217:2405:2655	2648:3275:5533
Wastewater inocula 2	2924:4260:8970	1679:2066:2752	**1716:2286:3640**
Acetate cold ww 1	**3060:5449:11740**	1273:1700:2406	1402:2197:3379
Acetate cold ww 2	13901:29226:42363	**984:1549:3049**	993:1697:3298
Acetate cold soil 1	**1380146:1393974:1407428**	*3430:5004:7687*	2960:4628:9094
Acetate cold soil 2	**1849625:1865409:1877419**	*3428:4923:7910*	3191:5018:8179
Acetate hot ww 1	1934:3511:12608	808:987:1300	**948:1310:2224**
Acetate hot ww 2	**1217:2159:6024**	643:785:1037	665:843:1264
Acetate hot soil 1	4386:8968:19150	1508:1968:2813	**1456:1984:3086**
Acetate hot soil 2	**171417:184911:197766**	2445:3773:5440	2350:3579:5577
Wastewater cold ww 1	**614:749:1014**	493:535:594	491:534:599
Wastewater cold ww 2	*859:1102:1596*	*640:708:805*	**730:906:1455**
Wastewater cold soil 1	**1079:2249:8263**	543:733:1197	651:1032:2324
Wastewater cold soil 2	556:640:789	*467:494:531*	**510:575:793**
Wastewater hot ww 1	1430:2911:9800	*637:845:1300*	**5682:16751:18608**
Wastewater hot ww 2	**483:548:660**	419:443:476	430:467:525
Wastewater hot soil 1	**820:1148:1985**	581:661:787	596:697:893
Wastewater hot soil 2	694:1135:2283	438:504:614	**468:572:954**

Table 11-6 DIC scores as defined by the sum of the deviance averaged over the posterior distribution and estimate of the sampling effort required to capture 90% of the diversity of taxa within the sample as determined by the fits of abundance distribution

	DIC			Sampling effort		
Sample	Log-normal	Inverse Gaussian	Sichel	Log-normal	Inverse Gaussian	Sichel
Arctic soil inocula	165.53	171.01	166.67	2.02E+06	4.06E+05	1.32E+05
Wastewater inocula 1	450.33	455.14	444.42	1.32E+07	2.56E+05	8.92E+05
Wastewater inocula 2	264.17	262.28	261.93	3.56E+07	2.98E+05	4.16E+05
Acetate cold ww 1	275.13	275.3	275.85	3.32E+09	1.59E+06	3.06E+06
Acetate cold ww 2	197.07	196.74	196.98	1.11E+13	1.47E+06	1.70E+06
Acetate cold soil 1	266.22	273.65	267.61	2.56E+18	1.42E+07	8.37E+06
Acetate cold soil 2	274.28	283.68	274.4	2.42E+18	7.28E+06	5.19E+06
Acetate hot ww 1	309.59	311.17	309.21	2.99E+09	5.88E+05	1.59E+06
Acetate hot ww 2	242.64	244.43	244.76	2.84E+08	3.61E+05	4.73E+05
Acetate hot soil 1	290.25	288.7	288.57	1.17E+10	1.44E+06	1.34E+06
Acetate hot soil 2	265.04	269.84	265.05	6.98E+14	4.73E+06	3.16E+06
Wastewater cold ww 1	254.73	255.02	255.23	5.22E+05	4.23E+04	4.25E+04
Wastewater cold ww 2	268.11	269.7	261.78	1.23E+06	4.91E+04	1.63E+05
Wastewater cold soil 1	201	201.99	197.99	2.68E+08	1.53E+05	5.35E+05
Wastewater cold soil 2	333.27	349.36	332.04	3.47E+05	3.70E+04	9.96E+04
Wastewater hot ww 1	252.09	254.67	246.76	1.37E+09	2.57E+05	1.05E+09
Wastewater hot ww 2	274.09	279.19	275.06	1.51E+05	2.52E+04	3.56E+04
Wastewater hot soil 1	248.04	250.28	248.96	3.54E+06	7.21E+04	9.24E+04
Wastewater hot soil 2	243.6	244.69	242.65	1.93E+07	7.44E+04	1.32E+05

11.11. Appendix XI - Details of the bacteria phyla and families found within the samples tested

It is seen in Figure 11-3 (a) that the inoculated and acclimatised reactors have become enriched Proteobacteria, this phylum dominates with about 80% abundance in the acetate fed cells, and around 60% in the wastewater fed cells. Proteobacteria are a diverse phylum of bacteria, yet most of this high abundance in the reactors is caused by the enrichment of *Geobacter* an exoelectrogenic organism, as is seen in Figure 11-4. *Rhodocyclaceae*, *Psuedomonas* and *Desulfovibrio* also added to the proportion of Proteobacteria that became enriched. The relative abundance of the other main phyla generally drops within the reactor samples, a proportion (around 10-20%) of Bacteriodietes remains, and there is some enrichment of Acidobacteria in the wastewater fed reactors. The wastewater reactors have a greater spread of abundance over the phyla groups shown, with less domination by Proteobacter.

The OTU richness shown in Figure 11-3 (b) again shows the greater diversity of the acetate reactors over the wastewater fed ones, both by the larger bar size and the Chao estimate above. It is seen many of the OTUs present in the inoculum have survived in the acetate reactor conditions, despite the metabolic narrowing of the conditions. Surprisingly this greater diversity or spread of OTUs appears to be slightly higher in the cold reactors, than the warm ones. In the case of the wastewater fed reactors the OTU richness in reduced, temperature does not appear to have an impact.

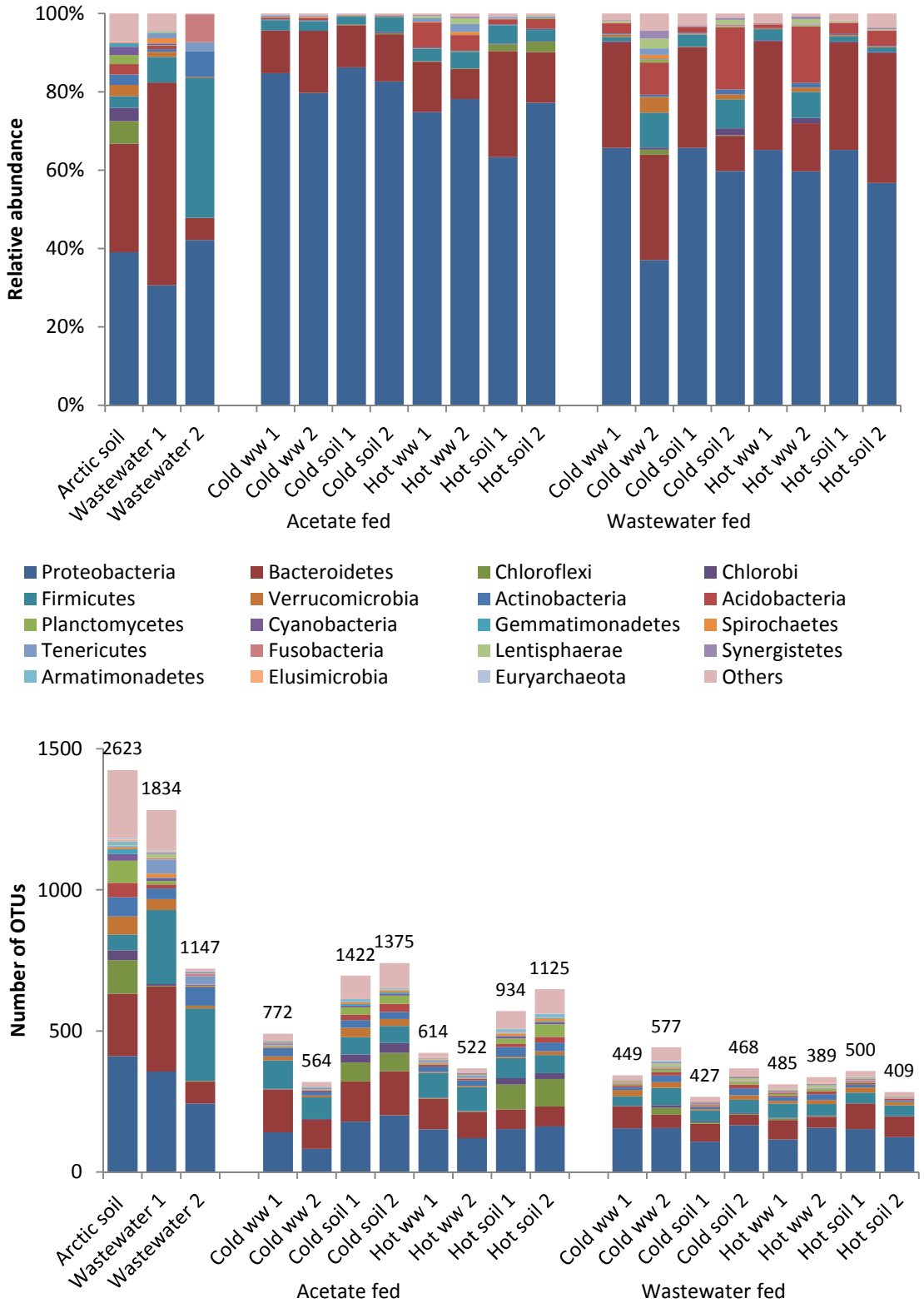


Figure 11-3 Relative abundance (a) and OTU richness (b) for all the data sets given at the phylum rank. Relative abundance is shown as the number of reads within each taxa divided by the total number of reads. The OTU richness is the number of taxa within each phylum is given by the size of the bar, the Chao 1 estimate of richness is written at the top of each bar

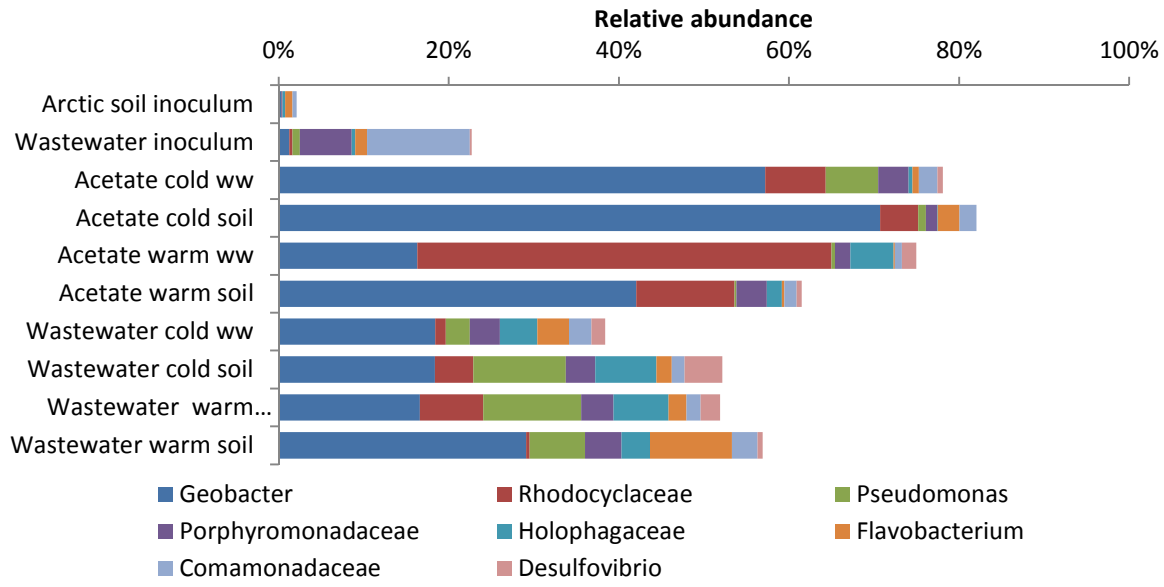


Figure 11-4 The relative abundance of the 8 most dominant genus as an average for the duplicate reactors under each condition, where the genus name was not given by the classification database family is used

It would be expected that the most dominant organisms within the reactors are the ones that are able to most competitively metabolise, grow and therefore reproduce within the conditions of the reactors. The top 8 most dominant genus are given in Figure 11-4, for Rhodocyclaceae, Porphyromonadaceae, Holophagaceae, Comamonadaceae the classification did not give the genus name, and therefore the family name is given. It is seen that for the acetate fed reactors these 8 genus make up a large proportion of the total abundance, and in the cold reactor most of this is by *Geobacter*. For the warm acetate reactors, *Geobacter* is still important, but Rhodocyclaceaea is also dominant, especially in those seeded with wastewater. The proportion of *Geobacter* is made up of 11 different species (names of which are not given by the classification), 4 of which are dominant within the reactors. Rhodocyclaceae is a diverse family of bacteria associated with wastewater treatment, further classification of this group is not made.

Within the wastewater reactors Geobacter is less dominant, between 20-30% of abundance, and there is a greater spread of the other genus and families, most notable *Pseudomonas* which make up to 10%. Within the Pseudomonas genus, 8 species were identified, of which 2 were dominant within the reactors, Pseudomonas have previously been seen within fuel cell systems fed substrates such as glucose and butyric acid and are believed to be capable of fermentation (Kiely et al., 2011c), some species such as *Pseudomonas aerunginosa* produce soluble redox shuttles and have been investigated

for their use within fuel cell systems (Marsili, 2010). The family of Holophagaceae is also quite enriched, this family includes the species of *Geothrix fermetans* which has been found in wastewater fed MFCs and is believed to be important in the hydrolysis or fermentation steps, (Kiely et al., 2011a), and has also been linked to shuttle formation (Bond and Lovley, 2005). *Flavobacteium* are also enriched, although this genus is more typically associated with freshwater environments. There is also likely to be sulphate reduction occurring in the cells due to the presence of *Desulfovibro*.

11.12. Appendix XII – Acknowledged contributions

Section	Other contributors	Contribution of E. Heidrich
Chapter 2	T.P. Curtis and J. Dolfing – editing and guidance with content	90%
Chapter 3	T.P. Curtis and J. Dolfing – editing and guidance with content	95%
Chapter 4	T.P. Curtis and J. Dolfing - editing and guidance with content, M. Wade – bioinformatics analysis, W.T.Sloan – sequencing funding	95%
Chapter 5	T.P. Curtis, K. Scott, I Head and J. Dolfing – discussion and experiment planning	95%
Chapter 6	T.P. Curtis K. Scott and J. Dolfing - editing and guidance with content, S. Edwards – site installation and running MEC	90%

Evaluation of Microbial Electrolysis Cells in the treatment of domestic wastewater

Thesis submitted to Newcastle University for the degree of Doctor of Philosophy

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Supervisor: Professor Tom Curtis

Co-Supervisor: Dr Jan Dolfing

Co-Supervisor: Professor Keith Scott

Date: May 2012

Abstract

Wastewater can be an energy source and not a problem. This study investigates whether rapidly emerging bioelectrochemical technologies can go beyond working in a laboratory under controlled temperatures with simple substrates and actually become a realistic option for a new generation of sustainable wastewater treatment plants.

The actual amount of energy available in the wastewater is established using a new methodology. The energy is found to be considerably higher than the previous measurement, or estimates based on the chemical oxygen demand with a domestic wastewater sample containing 17.8 kJ/gCOD and a mixed wastewater containing 28.7 kJ/gCOD.

With the energy content established the use of bioelectrochemical systems is examined comparing real wastewater to the 'model' substrate of acetate. The abundance of exoelectrogenic bacteria within the sample, and the acclimation of these systems is examined through the use of most probable number experiments. It is found that there may be as few as 10-20 exoelectrogens per 100 mL. The impact of temperature, substrate and inoculum source on performance and community structure is analysed using pyrosequencing. Substrate is found to have a critical role, with greater diversity in acetate fed systems than the wastewater fed ones, indicating that something other than complexity is driving diversity.

Laboratory scale microbial electrolysis cells are operated in batch mode fail when fed wastewater, whilst acetate fed reactors continue working, the reasons for this are examined. However a pilot scale, continuous flow microbial electrolysis cell is built and tested at a domestic wastewater treatment facility. Contrary to the laboratory reactors, this continues to operate after 3 months, and has achieved 70% electrical energy recovery, and an average 30% COD removal.

This study concludes that wastewater is a very complex but valuable resource, and that the biological systems required to extract this resource are equally complex. Through the work conducted here a greater understanding and confidence in the ability of these systems to treat wastewater sustainably has been gained.

Acknowledgements

It has been a great privilege to be able to study for a PhD, and I am grateful to all those who have made it possible.

I would like to thank my supervisor Tom Curtis for giving me the support and freedom to develop and explore my own ideas, always meeting these with enthusiasm and the necessary intellectual guidance. I would also like to thank Jan Dolfing who has skilfully guided me into, and through many intellectual challenges. I would like to thank Keith Scott and Ian Head for their support and guidance when needed. I would also like to thank Bill Sloan and Chris Quince for their support and guidance with the sequencing work, the contributions of individuals are detailed in Appendix XII.

I would like to thank EPSRC for giving me my doctoral training grant, and the School of Chemical Engineering and Advanced Materials for additional funding which enabled me to take up the offer of a PhD. I would like to thank Northumbrian Water Ltd. for the additional funding which has allowed for more experimental work to be carried out. I would like to thanks in particular from Northumbrian Water Ltd Chris Jones and Andrew Moore, who have been enthusiastic and supportive throughout this project and have provided me with all the assistance needed, and also Laura Stephenson, Steve Robson and all the workers at Howdon wastewater treatment plant, who have enthusiastically accommodated the pilot scale reactor for far longer than expected.

I would like to thank all my colleagues and co-workers who have provided advice, support and humour when needed. In particular to: Beate Christgen who helped with setting up MFCs and electrochemistry tests; Matt Wade who patiently nursed me through the bioinformatics process; and Stephen Edwards who has always been willing to lend a helping hand or ear.

As well as intellectual and financial support, I have also received, and needed, much emotional and moral support during the course of this PhD. This has come from many of those listed above, but also from my family who have not only made my completing a PhD possible, but are also the reason why it has been worthwhile. I would like to thank in particular: my mum Linda who has always taught me to learn for the sake of enjoyment above all else; my niece Katie whose determination and fighting spirit is a

continual inspiration; my husband Oliver, whose moral support, guidance, motivation and good humour has been unfaltering throughout; and lastly I thank my son Ben and his beautiful smile.

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Chapter 1. Introduction

There is growing consensus that wastewater is a resource not a problem (Verstraete and Vlaeminck, 2011, Sutton et al., 2011, McCarty et al., 2011). The conventional treatment of wastewater removes its organic content via aerobic processes, termed activated sludge, this is energy expensive typically 3% of the electrical energy usage of many developed countries (Curtis, 2010). Not only is the energy in wastewater removed not recovered, we expend considerable energy in performing this removal.

In the UK the water sector energy use has increased 10% in the last 10 years (Water UK, 2012, Water UK, 2011), industrial electricity prices have increased by 69% since 2000 (National Statistics, 2011). If these trends continue the energy bill for the water sector will be vastly higher than for the current 9016 GWh (Water UK, 2012). With infrastructure requiring long term planning and capital investment, it is hard to see without drastic action how the necessary changes can be made. Technologies that require relatively simple modifications to the current infrastructure to become operational are more likely to be given a chance rather than those which require wholesale change. New technology should ideally fit reasonably well into the existing infrastructure, and as a minimum achieve similar loading rates per unit area to activated sludge of 0.4-1.2 kg BOD $m^{-3}d^{-1}$ (Grady, 1999). The high capital costs of change and the uncertainty of using a different technology, coupled with the regulation of both effluent quality and pricing structures, are an obstacle to change.

There are alternatives to this approach. Replacing the aerobic activated sludge process with an anaerobic process means the energy stored in the organic content of the wastewater is converted to methane (80% efficiency) which can be combusted to produce electricity (35% efficiency) (McCarty et al., 2011). Only around 30% of the total energy in the wastewater can be captured as electricity in anaerobic systems, although with heat exchange in the combustion process, or the use of non-combustion methods of conversion, this could be increased (McCarty et al., 2011).

The scientific challenges of creating an energy neutral or even energy positive wastewater treatment process are also substantial and complex. The process needed to replace activated sludge must:

- Extract and convert energy to a useable form at an efficiency that justifies the costs.
- Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
- Treat low strength domestic wastewater, which is problematic for anaerobic digestion technologies (Rittmann, 2001).
- Work at ambient, often low temperatures, again problematic for anaerobic digestion (Lettinga et al., 1999).
- Work continuously and reliably.

An innovative and relatively new approach to wastewater treatment is through the use of bioelectrochemical systems (BES), though the fuel cell technology lying behind this process is over 100 years old (Potter, 1911) (see appendix I for a history of development). Here wastewater is consumed in a battery like cell, redox reaction catalysed by bacteria pushing electrons around in an electrical circuit, thus creating electricity (Rabaey et al., 2007). In a microbial fuel cell (MFC) the electricity is captured directly (Logan, 2005), in a microbial electrolysis cell (MEC) the electricity is supplemented by an external source to make a product such as hydrogen or methane (Rozendal et al., 2006) or to perform a process such as reductive dechlorination (Aulenta et al., 2008) or de-salination (Mehanna et al., 2010). There are substantial losses within these systems (Logan et al., 2006), it is suggested they may reach a higher conversion efficiency of 44% (McCarty et al., 2011), the performance of MFCs to date has only reached around 1 tenth of that needed to be competitive with anaerobic digestion (Pham et al., 2006). With MECs the potential higher value (energetically or commercially) of the product formed or process completed means this technology is likely to be more viable and may be the driver of development (Foley et al., 2010).

As organic matter is degraded by bacteria it releases electrons (oxidation) providing energy for the cells. These electrons then pass to an electron acceptor (or reduced species), which is normally oxygen, nitrate or sulphate depending on their availability providing further energy for the cells (Rittmann, 2001).It has been shown that there is a group of organisms that are capable of passing electrons to materials (such as metal oxides) outside the cell, which are then transferred by that material to an electron acceptor. This process is termed electrogenesis, and the group of organisms are known as exoelectrogens (Logan, 2008). MFCs exploit this, providing the bacteria with a

surface to donate electrons to, and then using the principles of all electrochemical cells to transport these electrons and create current.

MFCs, like electrochemical cells usually have two compartments, the anode chamber containing organic matter to be degraded, and the cathode chamber containing an electron acceptor. In the anode chamber organic matter is degraded by bacteria producing electrons, the absence of a preferred electron acceptor such as oxygen, means these electrons pass into the anode material then through a wire to the cathode. The H^+ ions generated in this reaction pass through the membrane from the anode to cathode chamber. At the cathode the electrons, H^+ ions and a reduced species (typically oxygen) combine to form for example H_2O. Electrical current is generated in the wire as the electrons pass from one side to the other.

An MEC reactor is an adaptation of an MFC. In an MEC both the anode and cathode chamber are anaerobic. Rather than creating H_2O in the cathode chamber, the electrons and H^+ ions are combined to generate H_2 gas rather than electricity. The process of forming H_2 is however endothermic, i.e. it requires energy. It cannot happen spontaneously. The addition of a small amount of electricity (with acetate this is in theory 0.114 V, in practice <0.25 V), is required to generate the H_2 gas (Logan et al., 2008). This is substantially less energy than is required to produce H_2 through water electrolysis, typically 1.8-2.0 V. A schematic of an MEC is shown in Figure 1-1.

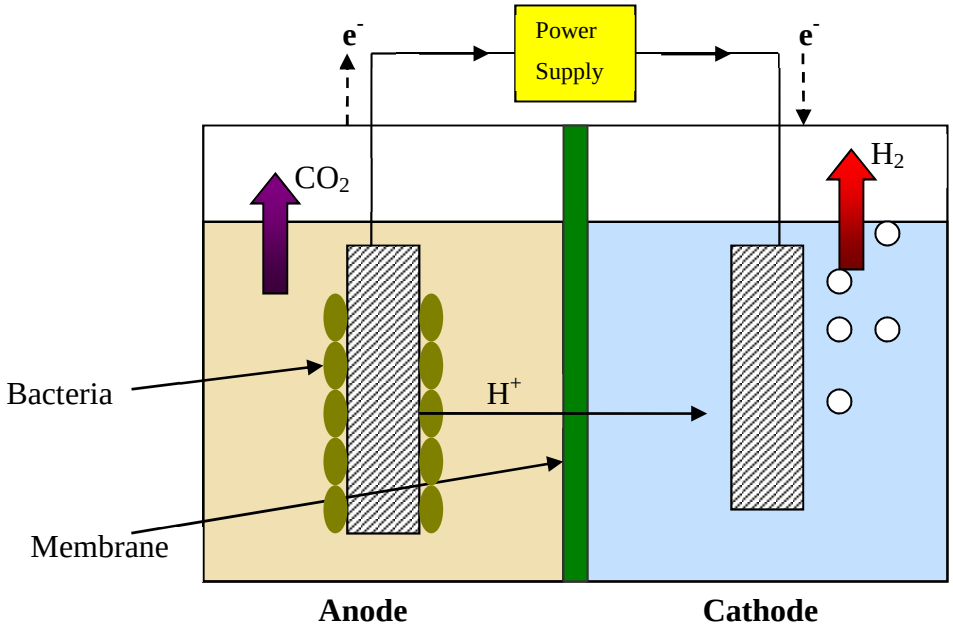

Figure 1-1 Generalised schematic of an MEC adapted from (Liu et al., 2005b) showing the flow of electrons and hydrogen ions and the function of the anode and cathode sections

The theoretical electrochemical energy gains or requirements of a MFC and MEC respectively will vary with temperatures, substrate free energy and ionic concentrations especially pH, as shown in appendix II. Even if it were possible to determine the potentials accurately in practice these theoretical values are not achieved. Energy is lost through all the transfer processes which take place to allow this reaction to happen. There are both electrochemical losses known as overpotentials caused by losses in redox reactions and transfer to the electrodes, losses in transfer of ions between the electrodes, limitations caused by transfer rates being different for different species, and on top of this there are losses caused by transfer of both electrons and ions in and out of the bacteria, losses to the bacteria themselves as they use energy, losses of electron transfer, and also losses by side or chain reactions occurring which do not advantage the fuel cell (Logan, 2008). This means that the energy gained in an MFC is less, and the energy input required in an MEC is more, than would theoretically be the case, represented in Figure 1.2.

In an MEC substantially more energy input than the theoretical is needed, in acetate fed systems these typically range from 0.4 V to 0.8 V with greater hydrogen gas production at higher voltages but less energy efficiency (Call and Logan, 2008). Glucose fed reactors have been shown to operate at applied voltages of 0.9 V (Selembo et al., 2009a), although far less work has been carried out on this substrate and its limits of applied voltage are undefined. In a larger scale system it is likely overpotentials (the difference between the theoretical potential at which the reaction occurs, and the observed potential of the electrode) will be increased and therefore the power input might be higher. In a pilot scale reactor fed on wine wastewater the input voltage of 0.9 V was used, although this performed less well than laboratory trials at a smaller laboratory scale on the same substrate, high over potentials being one of the suggested reasons (Cusick et al., 2011).

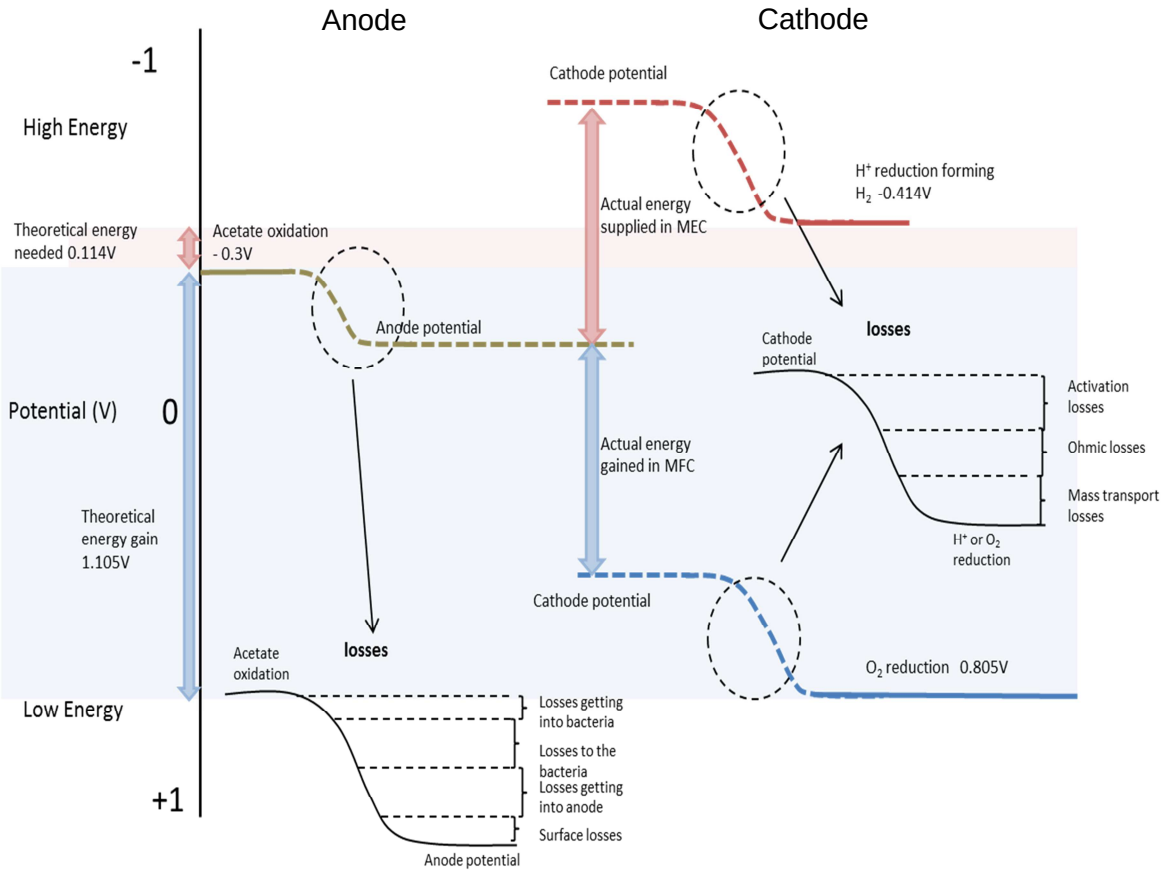

Figure 1-2 Representation of the energy losses within an MFC and MEC using acetate. Energy is shown as potential on the vertical axis, the green line shown the potential of the anode from the potential of acetate (solid line) to the actual anode potential (dotted line) which dependant on the losses. The reduction potential of the MFC and MEC cathode reactions is shown as the solid blue and red lines respectively, whereas the actual cathode potential is again shown in the dotted lines and is dependent on losses. The predicted total energy gain (MFC) and loss (MEC) is shown by the thick arrows and can be variable depending on these losses, but will always be less than that theoretically predicted as seen in the thick arrows at the vertical axis

Understanding the complexities of the electrochemistry of these systems is however only part of the challenge of understanding and ultimately manipulating BES technology. The microbiology of such systems plays a critical role in dictating their efficiency and their success or failure. The microbial community, which catalyses and enables the whole process to take place will also be affected by temperature, pH and substrates (Rittmann, 2001), it will vary with time and within the reactor, and the factors of competition, symbiosis and random assembly lead to a highly complex and unpredictable system.

BES systems run on electrochemical principles but rely on microbial communities. Therefore predicting their absolute function and output of energy, or indeed the input of energy needed, is at this stage in our understanding not possible. The empirical collection of this information is necessary in helping us identify not only if this technology is viable but also the areas that can and need to improved. Critically understanding the bacterial communities and the energy transfers within these systems lies at the heart of being able to manipulate and use this technology.

BES in general and MECs in particular have the potential to fulfil these needs of the wastewater industry (Foley et al., 2010). MECs are entirely anaerobic, eliminating the need for any aeration or complex membrane systems, meaning their engineering can be simple and 'retrofittable' within existing infrastructure. Although hydrogen production is focused on in this study, the flexibility of this process to make other high value products is an economic driver. However the key challenges to overcome are the scientific ones. An increasing body of work is amassing showing improved efficiencies and performance, however the vast majority of this is with simple substrates at warm temperatures (Rader and Logan, 2010, Call et al., 2009, Cheng et al., 2006b, Zhang et al., 2010). Evidence that BES work at low temperature is conflicting (Jadhav and Ghangrekar, 2009, Cheng et al., 2011), the only published study of a large scale 'hydrogen producing' MEC did not produce hydrogen (Cusick et al., 2011), and MECs studies using real wastewater as a substrate are limited, the longest documented study runs reactors for 7.6 days (Wagner et al., 2009).

1.1. Aim and objectives

The overall aim of this research is to understand if BES can be used as a sustainable method of wastewater treatment.

Much work has been and is being carried out fine tuning BES technologies within laboratories, testing new materials and moving towards greater output efficiencies, however large volumes of this work is conducted at warm temperatures and with simple artificial substrates (Hu et al., 2008, Logan et al., 2008, Selembo et al., 2009a, Tartakovsky et al., 2009). This research does not strive towards making such efficiencies, but answers the following fundamental questions of: can they work with

real wastewaters? and, can they work at realistic temperatures? this was addressed by completing the following objectives:

- Quantifying the amount of energy available in the wastewater
- Analysing the start-up and community development of MFC systems.
- Testing the operation and performance of MFC reactors at low temperatures
- Monitoring the performance of MEC reactors with wastewater substrate
- Building and testing a pilot scale MEC reactor run at a wastewater treatment site.

Chapter 2. Determination of the Internal Chemical Energy of Wastewater

Parts of this chapter have been published as Heidrich, E.S., Curtis T.P., and Dolfing J., Determination of the Internal Chemical Energy of Wastewater. Environmental Science & Technology, 2011. 45(2): p. 827-832.

The wastewater industry is facing a paradigm shift, learning to view domestic wastewater not as a waste stream which needs to be disposed of, but as a resource from which to generate energy. The extent of that resource is a strategically important question. However, the only previous published measurement of the internal chemical energy of wastewater measured 6.3 kJ/L, calculated to be 14.7 kJ/gCOD. It has long been assumed that the energy content in wastewater relates directly to chemical oxygen demand (COD). However there is no standard relationship between COD and energy content. In this study a new methodology of preparing samples for measuring the internal chemical energy in wastewater is developed, and an analysis made between this and the COD measurements taken. The mixed wastewater examined, using freeze drying of samples to minimise loss of volatiles, had 28.7 kJ/gCOD, whilst domestic wastewater tested had 17.8 kJ/gCOD nearly 20% higher than previously estimated. The size of the resource that wastewater presents is clearly both complex and variable, but is likely to be significantly greater than previously thought. A systematic evaluation into the energy contained in wastewaters is warranted.

2.1. Introduction

Every one of us produces at least around 40 $gBOD_5$ (biochemical oxygen demand consumed over 5 days), in waste every day, in richer countries this is likely to be nearer 80 $gBOD_5$,(Mara, 2004), equating to around 60-120 gCOD/person/day (Kiely, 1997). If there were 14.7 kJ/gCOD (Shizas and Bagley, 2004), the only previous published measurement of the energy value of wastewater, with 6.8 billion people in the world, 2.2 - 4.4 x 10^{18} joules of energy per year is available, or a continuous supply rate of 70 - 140 gigawatts of energy, the equivalent of burning 52 - 104 million tonnes of oil in a modern power station, or 12 - 24,000 of the world largest wind turbines working continuously. This estimation does not even include all the energy contained in our agricultural and industrial wastewater.

Despite the resource that wastewater represents, most developed countries spend substantial quantities of energy treating the wastewater so it can be released without harm to the environment, the US uses approximately 1.3% of its total electricity consumption doing so (Carns, 2005, Logan, 2008). The energy for wastewater treatment will be a particular burden in the urban areas of less well-off nations. Wastewater is typically viewed as a problem which we need to spend energy to solve, rather than a resource. If the energy contained in wastewater is harnessed, not only could it help the water industries become self-sufficient in energy or even net providers, but it could also be a modest source of energy in parts of the world which currently lack reliable and affordable energy supply.

Wastewater contains a largely uncharacterised and undefined mixture of compounds, including many organics, likely to range from small, simple chains through to more complex molecules. All organic compounds contain energy stored within their bonds. The energy that can be obtained from wastewater by different processes is varied, methane gas from anaerobic digestion, electricity from microbial fuel cells (MFCs), or hydrogen in the case of microbial electrolysis cells (MECs) (Logan, 2008) or a fermentation process (Davila-Vazquez et al., 2008). Large amounts of research is being undertaken in all of these areas but there has been very little work conducted in quantifying the amount of energy held in wastewater to start with.

The COD of wastewater has long been used as a relatively simple and reliable method of determining the 'strength' of waste, and by inference the energy contained within it. However there is no empirical formula for the determination of the energy content from the COD measurement. The only previous study to attempt to determine the energy content of raw municipal wastewater by experiment was conducted by Shizas and Bagley (2004) using a bomb calorimeter. Here a single grab sample of domestic wastewater from a treatment plant in Toronto was dried in an oven overnight at 103^{o}C before being analysed by bomb calorimetry. It was found that the domestic wastewater had a measured COD of 431 mg/L, and an energy value of 3.2 ± 0.1 kJ/g dry sample; with 1.98 g/L of solids this equates to 6.3 kJ/L. This interesting observation has led to the pioneering interpretation that wastewater contains 14.7 kJ/gCOD (Logan, 2008), which has been cited in the literature several times in particular with relation to microbial fuel cell work (Liao et al., 2006, Schroder, 2008, Logan, 2009). However the oven drying of samples will have driven off many volatile organic compounds, such as

methanol (boiling point 64.7 ^{o}C), ethanol (78.4 ^{o}C), and formic acid (101 ^{o}C). Moreover, the calculations were based on a single grab sample from one treatment plant, and using the COD measurement taken prior to drying, it is very likely that some of this COD will have also been lost before the energy determination was made. The work of Shizas and Bagley (Shizas and Bagley, 2004) provides a valuable starting point for the estimation of energy in wastewater, but given the volatile losses, and the measurement of the COD before these losses have occurred, this value must be an underestimation of the true internal chemical energy of wastewater.

The objectives of this study were to develop an improved methodology for measuring internal chemical energy, to better quantify the internal chemical energy of wastewaters, and to evaluate the relationship between internal chemical energy and COD.

2.2. Materials and methods

2.2.1. *Collection and storage of samples*

Two 24 hour composite samples of influent wastewater were taken, one from Cramlington Wastewater Treatment Plant, which deals with a mixed (i.e. industrial and domestic) wastewater, and the other from Hendon Treatment Plant, primarily treating domestic wastewater, both in the North East of England. Within two hours of collection, 3 L of sample was placed into the deep freeze at -80 ^{o}C, and a further 3 L was placed into an oven at 104 ^{o}C. A sample was stored in a refrigerator at 4 ^{o}C.

2.2.2. *Drying procedures*

After a period of around 48 hours in the oven at 104 ^{o}C the sample was fully dried. This was then ground into a powder using a pestle and mortar, and stored in four measured quantities of approximately 0.5 g in clean, dried sealed containers. The frozen samples were dried using a freeze dryer (Labconco Freezone, Labconco Corp. USA) which when used daily over a period of 4 weeks was capable of drying about 1.5 L of sample, each 20 hour drying period removing a few millilitres of liquid. The samples were stored at -80 ^{o}C between drying for 12 hours whilst the freeze dryer stabilised. This procedure was repeated until enough sample was dried to yield four 0.5 g samples. These were then ground and stored in the same way as the oven dried samples.

2.2.3. *Wastewater analysis*

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total organic carbon (TOC), inorganic carbon (IC), total carbon (TC) and chemical oxygen demand (COD) measurements were carried out in the two days after collection using the refrigerated samples. The methods described in Standard Methods for the Examination of Water and Wastewater (APHA, 1998) were used. TS was also measured using the freeze drying process. Further COD tests were carried out on rehydrated freeze dried and oven dried samples. All measurements were taken in triplicate.

2.2.4. *Energy content*

The energy content of the dried wastes was determined using an adiabatic bomb calorimeter, Gallenkamp Autobomb. The internal bomb was a stainless steel unit surrounded by a water jacket with a volume of 1900 mL, with a further cooling jacket outside with a flow of 300 mL/min. The system also included a mechanical stirrer, ignition unit and a digital thermometer accurate to 0.01 ^{o}C. The effective heat capacity of the system i.e. the heat required to cause a unit rise in temperature of the calorimeter was determined using triplicate samples of pure benzoic acid. This was used to calibrate the heat of combustion of the system components such as the wire and cotton, and the effective heat capacity of the bomb, its water jacket and thermometer. After this determination all of the components of the system were then kept constant throughout the tests. Four samples of benzoic acid were used on each time of operation of the bomb calorimeter to verify the technique.

The samples were dried, weighed to around 1 g, and compacted before combustion in the bomb. It was found that the samples did not fully combust, and therefore they were mixed in a 1:1 ratio with a combustion aid of benzoic acid, a method used by Shizas and Bagley (2004). The exact sample weight and the temperature rise in the surrounding water jacket was recorded and used to determine the energy content of each sample. All measurements including the benzoic acid standards were taken in a randomised order.

2.2.5. *Energy content calculations*

The bomb calorimeter measures the heat of combustion of the bomb's contents. When the bomb is ignited the contents including the fuse wire, cotton thread used to attach the sample to the fuse wire and the fuel, including any benzoic acid used is burnt, and this heat is absorbed by the bomb and its surrounding water jacket. In addition to the heat from the combustion, there is also heat created by the formation of nitric acid from the

nitrogen contained in the air inside the bomb. Moles of nitric acid formed are found by titration of the bombs contents with 0.1M NaOH. It is assumed that there is 57.8 kJ/mol of nitric acid; the oxidation state of the nitrogen is not taken into consideration as is standard practice (Rossini, 1956). The kilojoules contained in the sample are calculated in the following equation:

$$-\Delta U_{c,s} = ((V_w + B)(c_{p,w})(\Delta T) + (-\Delta U_{c,w}) + (-\Delta U_{c,c}) + (-\Delta U_{c,b})(m_b) - (Q_{f,n}\, mol_{nitric})) / m_s$$

Table 2-1 Definition of parameters in the equation above used to calculate energy of combustion

Term	Definition
$-\Delta U_{c,s}$	Energy of combustion at constant volume for sample (kJ/g)
$-\Delta U_{c,b}$	Energy of combustion at constant volume for benzoic acid = 26.42 kJ/g[a]
$-\Delta U_{c,w}$	Energy of combustion at constant volume for fuse wire = 0.013 kJ/g[b]
$-\Delta U_{c,c}$	Energy of combustion at constant volume for cotton = 0.082 kJ/g[b]
V_w	Volume of water = 1940 g[b]
B	Volume of water equivalent to the effect of the bomb container = 390 g[b]
$c_{p,w}$	Specific heat capacity of water = 0.00418/g/^oC[a]
ΔT	Temperature rise (^oC)
m_b	Mass of benzoic acid combusted (g)
m_s	Mass of sample combusted (g)
$Q_{f,n}$	Heat of formation of nitric acid = 57.8 kJ/mol[a]
mol_{nitric}	Moles of nitric acid formed (mol)

[a](Atkins, 2006)

[b]Determined in laboratory

2.2.6. *Measurement of volatile fatty acids*

The loss of known volatile fatty acids (VFA's) was measured for each drying technique using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Triplicate 20 mL samples of 50 ppm acetate solution were dried overnight in an oven at 104 ^oC, and in the freeze dryer. These were then re-hydrated with 20 mL of deionised water, and the VFAs measured.

2.2.7. *Measurement of anions*

The anion content of both wastewaters was measured in triplicate using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent.

2.2.8. *Measurement of volatile halocarbons*

Dried 20mg samples were rehydrated using 20 mL de-ionised water and, 20 mL wastewater samples were sealed within a sample jar, with the addition of 20 mg of salt (KCl). These were left for 24hrs at 30^{o}C, the headspace gas was then analysed using an Agilent 7890A GC split/split less injector linked to an Agilent 5975C MSD

2.2.9. *Statistical techniques*

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run two sample t-tests on the data. Before the tests were performed the data was checked for equal variance and normal distribution, validating the use of a two sample t-test.

2.3. Results

This paper uses an improved methodology: freeze drying the samples prior to using a bomb calorimeter. With this method only a few millilitres of liquid can be removed in a 24 hr operational period. Therefore drying enough wastewater to yield several grams of solids takes between 4 - 8 weeks. Although far more time consuming it is believed this is the best method available for drying the wastewater without raising its temperature and thus removing the volatiles.

Table 2-2 Measured wastewater parameters of the two different samples used in the energy analysis

	Cramlington	**Hendon**
COD	718.4 ± 9.7	576.2 ± 40.8
COD- oven dried	368.2 ± 12.3	324.0 ± 18.1
COD - freeze dried	587.1 ± 32.2	425.3 ± 16.5
Total solids - oven dried	1392 ± 35	1070 ± 60
Total solids - freeze dried	1597 ± 40	1130 ± 20
Total organic carbon	116.5	115.8
Total carbon	181.8 ± 2.3	196.4 ± 1.2
Inorganic carbon	65.3 ± 1.2	80.5 ± 0.1
Volatile solids (standard method)	953 ± 143	427 ± 20
Total Kjeldahl Nitrogen	92.4 ± 0.0	71.9 ± 4.3
Chloride (ppm)	391 ± 10.9	169.6 ± 17.2

Mean ± standard deviation (n=3), all values are in mg/L unless otherwise stated

Table 2-2 shows the differences between the two wastewaters, and the effects of the drying processes on the COD and solids recovery from these wastewaters. Oven drying reduces the measured COD from 718.4 mg/L in the original wet sample to 368.2 mg/L (49% loss) in the Cramlington wastewater and from 576.2 mg/L to 324.0 mg/L (44% loss) in the Hendon sample, whilst freeze drying gives losses of 18% and 26%. The freeze drying process captured 5-12% more mass than oven drying. This demonstrates that freeze drying is a more accurate method to determine the total amount of COD than oven drying. However, even freeze drying resulted in COD losses of 18-26%. This is probably due to the loss of the volatile fraction of the COD such as short chain fatty acids. This was confirmed using ion chromatography where oven dried samples contained 0.000 ppm acetate whereas freeze dried samples contained 1.8 ppm, compared to the original 54.5 ppm. Acetate is one of the smaller and therefore more volatile of the VFA's and is likely to represent some of the greatest losses.

Table 2-3 Measured internal energy content values given as both energy per litre and energy per gCOD using the post drying measurement of COD

	Cramlington		Hendon	
	Oven dried	**Freeze dried**	**Oven dried**	**Freeze dried**
kJ/L	8.3 ±1.8	16.8 ± 3.3	5.6 ±1.0	7.6 ± 0.9
kJ/gCOD	22.5 ± 4.8	28.7 ± 5.6	17.7 ± 3.2	17.8 ± 2.1

Mean of four measurements ± standard deviation
Values for kJ/gCOD are calculated from the COD measurement after drying and re-hydrating, and TS measurement for the given drying method.

The freeze drying method enabled a significantly greater proportion of the energy in the wastewater to be measured, over 50% more for Cramlington (p value 0.010), and 24% more for Hendon (p value 0.044). There are also significant differences between the two wastewaters, with the Cramlington waste being more energy rich (p value 0.019). The energy content per gram of oxidisable material measured i.e. kJ/gCOD is considerably higher for both wastewaters than previous estimates of around 14 kJ/gCOD, for the Cramlington wastewater this is even higher with the freeze dried sample.

The energy captured by the freeze drying process does not equate to all the energy available in the wastewater sample. Based on the percentage losses of measured COD from the original sample to the freeze dried sample (18% for Cramlington and 26% for

Hendon), the actual energy of the Cramlington wastewater could be as high as 20 kJ/L, and 10 kJ/L for the Hendon wastewater.

2.3.1. *Theoretical results - can internal chemical energy per gram COD be calculated from first principles?*

If we were able to evaluate the energy content of wastewater from the COD measurement, this would require an estimation of which organic compounds are present. With this, the internal chemical energy for each individual organic compound can be calculated on the basis of simple thermodynamic calculations as follows (thermodynamic values are taken from Atkins (2006)) based on the principle that 1 gram of COD equals 1/32 mol O_2, i.e. for every 1 mol O_2 there is 32 grams COD.

If we assume that the organic compound present is methane:

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \qquad (1 \text{ mol } CH_4 = 64 \text{ gCOD})$$

The overall enthalpy for the reaction can be calculated on the basis of Hess's Law, which states that the enthalpy of a reaction is equal to the sum of the enthalpy of formation ($\Delta_f H$) of all the products minus the sum of the enthalpy of formation of all the reactants. Using tabulated values for the enthalpy of formation the energy released in the above reaction with methane is as follows:

$$\begin{aligned}
\Delta_f H \text{ (kJ/mol)} &= \sum \Delta_f H \text{ products} - \sum \Delta_f H \text{ reactants} \\
&= 2(\Delta_f H\ H_2O) + \Delta_f H\ CO_2 - \Delta_f H\ CH_4 - 2(\Delta_f H\ O_2) \\
&= 2(-285.83 \text{ kJ/mol}) + -393.51 \text{ kJ/mol} - -74.81 \text{ kJ/mol} - 2(0 \text{ kJ/mol}) \\
&= -890.5 \text{ kJ/mol} \\
&= -890.5 \text{ kJ/mol} / 64 \text{ gCOD} \\
&= -13.9 \text{ kJ/gCOD}
\end{aligned}$$

Analogous calculations for a wide range of organic compounds show that the typical $\Delta_f H$ values of $C_aH_bO_c$ compounds fall within a fairly narrow range of 13-15 kJ/gCOD, with a few exceptions such as formic and oxalic acid with 15.7 kJ/gCOD, ethyne with 16.3 kJ/gCOD and methanol with 17.8 kJ/gCOD. (See Appendix III).

It could be concluded that 13.9 kJ/gCOD is the maximum amount of heat energy that can be gained from methanogenic wastewater treatment. Therefore from a relatively simple COD measurement the potential energy yield would be known. However biodegradation of organic content in wastewater does not necessarily lead to methanogenesis. Some waste streams can be used for biohydrogen production. Here 1 gCOD is equal to 1/16 mol H_2, ($2H_2 + O_2 \rightarrow 2H_2O$) therefore 1 mol H_2 equals 16 gCOD, giving an energy yield of 17.9 kJ/gCOD (286 kJ/mol H_2 / (16 gCOD / mol H_2)).

The simple $C_aH_bO_c$ compounds are not necessarily the only wastewater components, and other classes of compounds such as halocarbons can contain far more internal chemical energy per gCOD. The explanation to this can be supported by writing the equations that describe their degradation down as oxidations of the carbon moiety with reducing equivalents released as H_2, coupled to the oxidation of the H_2 to water. In highly substituted compounds such as organohalogens, less H_2 is potentially available. The oxidation reaction of H_2 to water becomes less important in the overall equation, the ratio of H:CO_2 decreases, increasing the overall value of kJ/gCOD. This is illustrated using methane and one of its halogenated equivalents trichloromethane (thermodynamic data taken from (Hanselmann, 1991)):

Methane

$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (-393.5 + 4(0)) - (-74.8 + 2(-285.8))$

$= 252.9$kJ/reaction

$$4H_2 + 2O_2 \rightarrow 4H_2O$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (4(-285.8)) - (0 + 2(0))$

$= -1143.2$ kJ/reaction

These two values are then added together to give the overall enthalpy of reaction to be -890.3 kJ/mol, this can then be divided by the COD to give -13.9 kJ/gCOD

Trichloromethane

$$CHCl_3 + 2H_2O \rightarrow CO_2 + 3HCl + H_2$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-393.5 + 3(-167.1) + 0) – (-103.1 + 2(-285.8))

= -220.1 kJ/mol

$$H_2 + \tfrac{1}{2} O_2 \rightarrow H_2O$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-285.8) - (0 + 0.5(0))

= -285kJ/mol

The total enthalpy of reaction is -505.9 kJ/mol, giving -31.6 kJ/gCOD.

It becomes clear how important the reducing equivalents of H_2 are in terms of energetic value, this is illustrated in Figure 2-1, (values given in Appendix III). As the number of substitutions of hydrogen increases, so does the value of energy per gram COD. The value of energy per gram of COD can vary far more widely than previously thought.

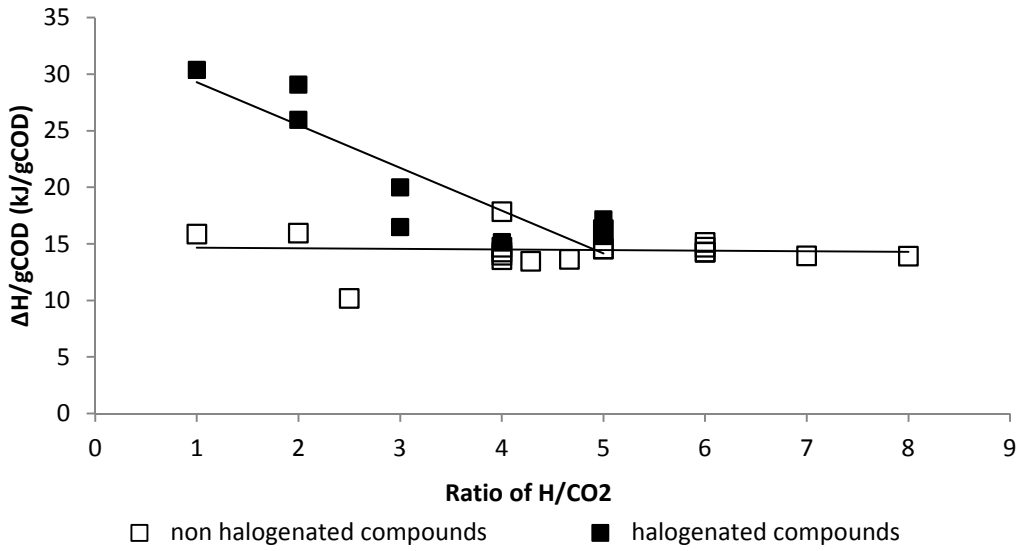

Figure 2-1 Energy content per gCOD of a variety of organic compounds plotted against their degree of oxidation

2.4. Discussion

The predicted energy gained from treatment of municipal wastewaters has been shown to be higher than the previous estimation. The domestic wastewater analysed in this paper has 20% more energy per litre than the estimation made by Shizas and Bagley (Shizas and Bagley, 2004). In addition to this, as the volatiles in their wastewater were not captured, it is likely their sample could have had an energy value around 35% higher, (based on the percentage losses between oven and freeze drying in this study) this would be 8.5 kJ/L. This has a significant impact on the development and implementation of technologies for the treatment of 'low strength' municipal wastewater which pose a greater challenge for the recovery of energy than concentrated waste. These waste streams are clearly richer in energy than previously thought.

The internal chemical energy of the wastewaters per gCOD was greater than expected by comparison to acetate (heat of combustion is 13.6 kJ/gram COD) or glucose (heat of combustion is 14.3 kJ/gram COD). From the data (Table 2-2) of the two wastewaters it can also be seen that the carbon oxidation state plays an important role in determining the energy present. Both samples have a very similar value of TOC (total organic carbon), yet very different COD values. This means that the Cramlington waste with the much higher COD has proportionally more reduction capacity and therefore chemical energy per carbon molecule than the Hendon wastewater. Another possible cause of these high values is that there are compounds within the wastewater that have an energy value, yet are not oxidised during a COD test, most notably urea, which contains 10.4 kJ/g (Atkins, 2006) when combusted, yet undergoes a hydrolysis reaction rather than an oxidation. This compound, which is certain to be present in domestic wastewater (and though it is assumed to hydrolyse in the sewer, a fraction may reach the wastewater treatment site), contributes to the overall energy of combustion of waste but not to the COD measurement, there are likely to be others compounds which do the same. Additionally there could be some compounds which have proportionally far greater energy content per gram of COD than glucose and acetate, such as organohalogens or other highly substituted compounds.

Although many simple halocarbons are no longer in use, some more complex ones are still common in many industrial processes for example as solvents and pesticides, and in the manufacture of in plastics, adhesives, sealants and paper pulp. Organic halocarbons also occur in natural systems. Chlorination treatment also introduces this halogen which

could then combine with other organics. It can be seen from the anion analysis (Table 2-2) that there is significant quantity of chloride ions in the wastewaters, with more in the Cramlington wastewater. This wastewater is likely to contain a more diverse range of organic compounds as this site takes in mixed wastes, some of which must have a high specific energy value and volatility, resulting in high energy wastewater. Volatile halocarbons, however, were not detected with the GC MS method described.

The energy values found in this study are also higher than that reported by Shizas and Bagley (2004). However the calculations in their paper were based on oven dried wastewater energy data, versus a COD measurement taken from the original wastewater sample, which in our study was found to be reduced by about 50% after oven drying. If the same calculation algorithms were used on the data in the present paper then the Cramlington and Hendon wastewaters would contain 11.6 kJ/gCOD and 9.9 kJ/gCOD respectively, while they actually contained at least 2.4 times higher (28.7 kJ/gCOD) and 1.8 times higher (17.8 kJ/gCOD), these calculations are shown in Appendix IV. Thus the energy reported per gCOD cited in the literature (Logan, 2008) based on the Shizas and Bagley paper (Shizas and Bagley, 2004) is probably a substantial underestimation. By comparison to the Hendon domestic wastewater the energy of their municipal wastewater could have had at least 26.4 kJ/gCOD, rather than the 14.7 kJ/gCOD reported.

Clearly not all the energy available in wastewater can be extracted in a useful form as no process is 100 % efficient. Ideally one would be able to measure or calculate the energy biologically available as kJ/gBOD, (although not suitable for anaerobic processes), this is not possible given the unknown and variable composition of wastewater. However knowing the potential energy available would give insight into the types of waste that might be in the waste stream which would also be of importance in the choice of treatment method. Some wastes which may be high in energy value, such as halogenated wastes may be unsuitable or unattractive to some treatment methods. For example one mole of trichloromethane at 506 kJ/mol would only yield 0.25 moles of methane equal to 222 kJ through methanogenic treatment, or one mole of H_2 equal to 286 kJ through biohydrogen production. Although these halogenated compounds are energy rich per gram of COD due to their lack of hydrogen, this actually makes them unattractive to terms of energy extraction for methane or hydrogen production, however

it may be possible to recover this energy using other treatment methods which may be able to capture electrons directly.

In microbial fuel cells (MFC's) the reaction taking place is essentially a combustion reaction, i.e. the organic compound is oxidized to carbon dioxide and water, the difference being that this reaction occurs not as combustion but as redox reactions in two half cells. Importantly, it is the free energy of the organics that determines the maximum electricity yield. This technology could theoretically capture more of the energy available in complex or halogenated compounds than for example methanogenic treatment.

The measurement of the internal or combustion energy of the wastewater and use of this as a basis for efficiency calculations will not necessarily yield all the information required to fully understand the energy flows in such systems. It can be observed using internal chemical energy data, a methanogenic process could in some cases be endothermic, the combustion energy of the methane product being higher than that of the starting substrate. This is the case with the conversion of one mole of acetate (13.6 kJ/gCOD) to one mole of methane (13.9 kJ/gCOD). In this scenario energy appears to have been created. It is actually the Gibbs free energy (the amount of energy that can be extracted from a process occurring at constant pressure) which should be examined for this and other reactions as this parameter informs us of the amount of energy available to organisms for the generation of biomass and an energy rich product. This is also the case for MFC's and MEC's where it is voltage which is measured which relates directly to Gibbs free energy. However without knowing the composition of wastewater, its Gibbs free energy content cannot be determined.

A consequential difference was found between the internal chemical energy measured on freeze dried samples as compared to oven dried samples. This difference was greater than the difference observed by measuring mass alone. This shows that there are significant losses of volatile compounds when a wastewater sample is dried at 104 ^{o}C and that in the case of the mixed wastewaters these volatiles can contain proportionally more energy per gCOD than the non-volatiles captured in both methods. It is shown that, although a clear improvement on the traditional oven drying method, the freeze drying method still results in significant loss of semi-volatiles such as acetate, so even

with the improved method we are still not capturing all the energy available in the wastewater.

Bomb Calorimetry remains the only method for measurement of internal chemical energy or calorific value, and for this method the material must be combustible i.e. dry. To give reasonably accurate results the temperature change in the bomb calorimeter must be in the region of 1 - 3 ^{o}C, usually a gram of substance will provide this. In our analyses this gram was half made up by the use of a combustion aid (benzoic acid) to ensure full combustion and the correct temperature rise. Had the proportion of wastewater to benzoic acid been decreased, making the drying process easier, it was feared that the uncertainty inherent to the introduction of the standard would overshadow the accuracy of the measurements of the samples. Although more challenging the methodology of freeze drying samples is an improvement on previous methods although it does not achieve the full capture of all volatiles. These results begin to get close to the true amount of energy in wastewater, and challenge the assumption that measured COD is equivalent to the amount of energy. Freeze drying, although far more time consuming, therefore should be the method of choice when completing such analysis in particular with complex wastes, despite its far greater time consumption rate unless or until new methods and equipment are developed to reduce the time burden using this principle. One such method could be the use of membranes, in particular through the use of reverse osmosis which would 'trap' molecules as small as salts and allow water to be removed. Such techniques may allow for more rapid, cost effective and efficient drying of samples, thus enabling more sampling to be undertaken.

It is clear from our data that the energy value of different wastewaters is variable, as would be expected; there is no standard relationship to measured COD. Values ranged from 17.7 kJ/gCOD to 28.7 kJ/gCOD, when measuring the COD remaining in the dried sample, however we cannot know how much compounds such as urea contribute to this. This means than a measurement of the amount of oxygen required to oxidise the organics within wastewater is not a simple representation of the amount of energy contained within that waste. This is particularly the case when dealing with mixed wastes, where the energy content is proportionally far greater per gCOD. It seems that 13 – 14 kJ/gCOD is the minimum energy content that could be found in wastewaters, however it may be significantly greater. Given the variability in the amount of energy

per gram COD it seems better to measure this energy directly rather than making an estimation, despite the fact that even with the better drying method there are still losses.

Given the huge amount of wastewater globally and the potential energy stored within it, it is important that this potential energy should be determined. With new technologies such as fuel cells being developed, the estimation of this resource is not as trivial as previously assumed. It has been shown that wastewaters can lie well outside the previously estimated values. A systematic review of the energy contained within different waste streams is needed. This paper examines two wastewaters from a reasonably similar geographical location and has found extremely diverse results. It is hoped that this methodology will be repeated and improved upon in terms of time taken, allowing the dissemination of multiple studies using different wastewaters building up a comprehensive and global picture of the energy available in wastewater. This would form the strategic foundation block to the establishment of new and existing technologies within the wastewater industry harnessing this valuable renewable energy source.

Chapter 3. How many exoelectrogens make a Bioelectrochemical System?

3.1. Introduction

The inoculation and subsequent acclimatisation of a bioelectrochemical system (BES) is fundamental to the operation of such systems (Logan and Regan, 2006, Rittmann, 2006). Yet the origin, abundance and physiology of these organisms is the area of greatest uncertainty in design (Oh et al., 2010).

The main goal of the inoculation and acclimatisation of a reactor is typically to 'get it going' as quickly as possible, typically the sources of seed includes: reactors already working in the lab (Jeremiasse et al., 2009, Cheng et al., 2009, Call and Logan, 2008); anaerobic sludge (Chae et al., 2010, Yang et al., 2009); return activated sludge (Torres et al., 2009); mixtures of sludges; or simply wastewater taken at various stages from the treatment plant (Kiely et al., 2011b, Wang et al., 2008). The source and volume of inoculum varies between studies. There is no consensus of how a BES reactor should be started up, or how long acclimatisation will take. This can lead to problems, highlighted by a pilot scale study where several attempts were made to acclimatise the reactor (Cusick et al., 2011).

The bacteria needed for microbial fuel cells to work are termed exoelectrogens (Logan, 2008) due to their ability to transfer electrons outside their cell. Three transfer mechanisms have been proposed.

Firstly electrons can be transferred through conduction with direct contact between the cytoplasmic membrane of the bacteria and the solid substrate being reduced, this mechanism has primarily been associated with the genera *Shewanella* and *Geobacter* (Myers and Myers, 1992, Mehta et al., 2005).

The second mechanism is an electron shuttle. Some bacteria are able to excrete compounds or shuttles into the electrolyte which are capable of transferring electron to an electrode. Rabaey *et al.,* (2005) found that *Psuedomonas aeruginosa* produced Pyocyanin, a mediator which was not only able to transfer electrons from this taxon to the anode of an MFC, but could also work for other species when introduced back into a mixed culture. Thus, a bacterium unable to transfer electrons itself, may become

exoelectrogenic due to the presence of a different shuttle producing bacteria. *Shewanella* species have been seen to do this with the production of riboflavins (von Canstein et al., 2008).

Thirdly electrons might also be transferred through conductive microscopic pili named nanowires which extend from the bacteria cell to other cells or any other electron acceptor (Reguera et al., 2005). *Geobacter* and *Shewanella* species have both been linked to this activity (Gorby et al., 2006). Putative nanowires have been observed using electron microscopy extending to a conductive surface. Conducting probe atomic force microscopy (Reguera et al., 2005) and conductive scanning tunnelling microscopy (Gorby et al., 2006) have been used to reveal that the pili which had previously been observed as attachment mechanisms for bacteria onto Fe oxides, were highly conductive.

It has been proposed that symbiotic relationships between different bacteria groups enhance the function of mixed cultures and improve process stability (Lovley, 2008), possibly by allowing inter-species electron transfer (Rabaey et al., 2005). Many of the exoelectrogens typically associated with BES's such as *Geobacter sulfurreducens* have limited metabolic diversity, and are only able to utilise the end products of fermentation (Caccavo Jr et al., 1994). A reactor fed with a waste requires bacteria which are able to digest the complex substrates, but may not necessarily be able to utilise the anode for respiration (Kiely et al., 2011c). The hydrolysis step within these food chains has been shown to be the rate limiting step with regard to the current production (Velasquez-Orta et al., 2011).

In general, growth in bacterial systems can be described through the equation $N_T = N_0 exp^{rt}$, where the number of bacteria present at a specific time period (N_T) is equal to the number of bacteria present at the start (N_0) multiplied by the exponential of the growth rate (r) over the time span (t). (Rittmann, 2001). With N_T known various other properties can be calculated such as specific activity and growth yield. However in MFCs these are not well understood (Logan, 2008), although growth rates have been defined for some of the key organisms involved in MFC reactions such as *Geobacter*, (Cord-Ruwisch et al., 1998). A cell yield of 0.07-0.22 g-COD-cell/g-COD-substrate has been calculated (Logan, 2008) from an early study by Rabaey et al. (2003) using total bacterial concentrations within the reactors determined turbidometrically and the total

COD removed during the experiments. Freguia et al. (2007) reported estimates of growth yields of -0.016 to 0.403 mol-C-biomass/mol-C-substrate, based on measurement of the substrate removal which was then used to calculate cell yield through a mass balance approach. Yield has been shown to drop with decreasing external resistance (Katuri et al., 2011).

However the value of N_T is complex and unknown. Although a body of research is growing identifying the functions of bacteria within working BES reactors, little is known of their abundance in a natural sample (N_0) and absolute number within a working system (N_T). Additionally the pattern of acclimatisation, the period is likely to be crucial in the community formation, also remains largely unexplored.

Using the acclimatisation period of reactors the aims of this study were to firstly identify the optimum level of inoculum needed to start a reactor with a view to identifying a protocol for the further experiments. Secondly to estimate the most probable number of exoelectrogens present in a sample of wastewater which can be used as a guide to the sequencing depth needed to find these organisms, and to determine N_0 for a reactor. Thirdly to define the growth rates (r) within MFC systems through examining the start-up phase. With these two factors quantified the N_T can be estimated, as can specific activity and yield. Finally by examining the pattern of acclimatisation on different substrates, key differences in community formation can be identified.

3.2. Method

3.2.1. *Reactor Set-up*

Double chamber tubular design MFC reactors (78 mL each chamber) were used, constructed in Perspex, with an internal diameter of 40mm and length of 60mm. The anode was a 2.5 cm^2 carbon felt (Olmec Advanced Materials Ltd, UK), the cathode a 2.5 cm^2 platinum coated titanium mesh with a surface area 8.13 cm^2 (Tishop.com, UK). The cation selective membrane between the reactor chambers was Nafion® 117 (DuPont, France), with an area of 12.6cm^2. The electrodes were positioned 1cm apart. The components of the reactor were cleaned before use and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK)

The cathode chamber was filled with 50 mM pH 7 phosphate buffer saturated with air for 20 minutes before being added into the reactors. Three different media were used:

1. Acetate solution with added nutrients (Call and Logan, 2008)
2. Starch solution with added nutrients (Call and Logan, 2008)
3. Primary settled wastewater (Cramlington WWTP, Northumbrian Water Ltd)

The quantities of starch and acetate in the nutrient solutions were balanced to give similar total chemical oxygen demand (TCOD) as the wastewater, and were autoclaved (121°C, 15 min) before use. The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into ¼ strength Ringers sterile dilutent (APHA, 1998). The contact time under UV was altered to give effective sterilisation as defined as colony free plates in triplicate at zero dilution. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see Appendix V).

The three medias were sparged under sterile conditions for 10 minutes using ultra high purity (UHP) nitrogen (99.998%), until the dissolved oxygen (DO) as measured on a DO probe Jenway 970 (Bibby Scientific Ltd, UK) reached zero.

3.2.2. *Inoculum*

Screened raw influent wastewater from Cramlington wastewater treatment plant (Northumbria, UK). This wastewater is a mixture of industrial and domestic origin. Samples were stored anaerobically at 4^{o}C and used within 24 hours of collection. The inoculum was also sparged with UPH nitrogen before use.

3.2.3. *Start –up and acclimatisation*

Duplicate reactors were inoculated with differing volumes of wastewater (1 mL, 10 mL, 25 mL and 50 mL). The anode compartment was then filled with the sterile substrates. Control ‘reactors’ (using no inoculum) were run during each test. An inverted 50ml syringe filled with UPH nitrogen was placed into the refilling port on top of the anode chamber to provide an anaerobic headspace. The cathode chamber once filled was left open allowing the diffusion of oxygen into the liquid. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor. A data logging

multimeter (Pico ADC-16, Pico Technologies, UK) was attached to record voltage output every 30 minutes. Reactors were allowed 800 hours at room temperature (20-25 ^{o}C) to show acclimatisation before the experiment was ended. With the acetate fed experiment a further set of reactors were run with lower dilutions of inocula, 0.01 mL, 0.1 mL and repeated 1mL with 25 mL as a positive control.

3.2.4. *Enumeration of bacteria*

The total number of aerobic culturable bacteria present in the wastewater samples used for inoculation was approximated using a spread plate method 9215C (APHA, 1998), with peptone based nutrient agar (Lab M Ltd, UK). Serial dilutions were undertaken into sterile ¼ strength ringers solution, with each dilution plated in triplicate. Plates were incubated at 37 ^{o}C for 48 hours. Anaerobic bacteria were enumerated using a basal salts media (Shelton and Tiedje, 1984) with 1 g/L of both yeast extract and glucose as a carbon source. The media was autoclaved (121 ^{o}C for 15 min) and sparged with sterile UHP nitrogen for 20 minutes. A volume of 9 mL was then added to sterilised Hungate tubes, 1 mL of wastewater was then added to five tubes, and dilutions made down to 10^{-12} with five replicates at each dilution. The headspace of the tubes was sparged with nitrogen, and the tubes incubated at 37 ^{o}C for two weeks. The number of bacteria was determined using the MPN methodology (APHA, 1998).

3.2.5. *Analytical methods*

TCOD of the medias and inocula were measured in duplicate according to standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) colorimetric reagent kit. Volatile fatty acids of the media and inocula were measured in duplicate using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. When the current of the cell had dropped to zero TCOD and VFA's of the cell were measured using the same method as inocula and media above.

3.2.6. *Most probable number (MPN) calculations*

With non-standard dilutions the pre-calculated MPN tables (APHA, 1998) cannot be used. The MNP is calculated through a series of iterations based on a Poisson and binomial distributions (Blodgett, 2005) using the following formula, solving λ for the concentration:

$$\sum_{j-1}^{k} \frac{g_j m_j}{1 - \exp\ (-\lambda m_j)} \ = \ \sum_{j-1}^{k} t_j m_j$$

K = the number of dilutions,

gj = the number of positive (or growth) tubes in the jth dilution,

mj = the amount of the original sample put in each tube in the jth dilution,

tj = denotes the number of tubes in the jth dilution

A probability is assigned to each possibility of the number of bacteria based on the outcome at each dilution, a positive outcome being voltage produced in by the reactor. The number with the highest probability is given as the MPN. Using the spreadsheet developed by Bloggett to make these iterative calculations, the most probable numbers of exoelectrogens per 100 mL of wastewater can be calculated (Garthright and Blodgett, 2003) using the inocula volumes, and the test outcome.

Thomas' simple formula which is based on the same principles as the full test, but a simpler algorithm to solve, can also be applied to the data set, this formula has been shown to have substantial agreement (Thomas, 1942). Using only the lowest dilution that doesn't have all positive tubes, the highest dilution with at least one positive tube and the dilutions in between the following calculation can be made:

$$MPN/100\ ml \ = \ \frac{no.positive\ tubes \ \times \ 100}{\sqrt{(ml\ sample\ in\ negative\ tubes) \times (ml\ sample\ in\ all\ tubes)}}$$

The confidence limits of this calculation at the 95% level can be calculated using Haldane's formula (Haldane, 1939):

m_1, m_2, m_3 ……. denotes inoculation amounts ranging from the largest to the smallest of the chosen dilutions

g_1, g_2, g_3 ……. denotes the number of positive tubes at the corresponding dilutions

$$T_1 = \exp(-MPN \times m_1)\ , T_2 = \exp(-MPN \times m_2) \ldots\ldots\ldots etc$$

$$B = [g_1 \times m_1 \times m_1 \times T_1/((T_1 - 1)^2)] + [g_2 \times m_2 \times m_2 \times T_2/((T_2 - 1)^2)] + [g_3], [\ g_4] \ldots. etc.$$

$$Standard\ Error\ of\ \log 10\ (MPN) = \ 1/\left(2.303 \times MPN \times (B^{0.5})\right)$$

95% confidence intervals are given by:

$$Log_{10}\ (MPN) \pm 1.96 \times Standard\ Error$$

3.2.1. *Growth rate, specific activity and yield calculations*

Growth rate of bacteria (μ) is classically calculated by quantifying the number of bacteria at two time intervals. In this experiment voltage is deemed to be a suitable proxy for exoelectrogenic bacteria, the rate of voltage rise being equivalent to the rate of growth. It is assumed that each bacterium is capable of donating an amount of electrons therefore an increasing number of electrons are donated to the circuit (i.e. the voltage increases at a constant resistance) as the absolute number of bacteria increases, (it does not represent an increasing ability to metabolise), i.e. voltage is deemed proportional to bacterial number. This can be from the growth rate expression:

$$N_T = N_0 e^{\mu t}$$

Where N_T is the number of bacteria at time t (in this case the voltage), N_0 is the number of bacteria (voltage) at time zero (t_0) and μ is the growth rate. Therefore growth can be defined as:

$$\mu = \frac{\ln N_t - \ln N_0}{(t - t_0)}$$

Specific activity (q), defined as moles electrons per cell per second can be calculated over the period of growth as follows:

$$q = \frac{I \times (t_1 - t_o)/F}{N_0}$$

Where I is the current in amps (coulombs/second) as calculate from the measured voltage V, and resistance R calculated through I=V/R, t_1-t_0 is representative of the time period of each measurement, (i.e. every 30 minutes, the total coulombs of charge within this period is therefore I multiplied by 30 minutes multiplied by 60 seconds) and F is Faradays constant of 96485 coulombs/mol e^-. The growth rate and starting MPN is used to calculate the number of cells at each time period N_T. This can be converted to moles of acetate per cell per second (1 mole acetate = 8 moles electrons), to give substrate utilisation (U).

Growth yield (Y) is the amount of biomass or cells produced by the bacteria per mass of degraded substrate measured in g-COD-cell/g-COD-substrate. Rather than use the total COD removed in the reactor, which would also involve COD digested via other routes only the g-COD substrate put to the circuit is used as calculated from the substrate utilisation above. The yield is calculated as follows:

$$Y = \frac{(N_T - N_0) \times W \times COD_{cell}}{\sum_{t0}^{t} U \times COD_{sub}}$$

Where the total cells produced over the growth period N_T-N_0 is multiplied by an estimation of the weight of cells W of 5.3 x 10^{-13} g-cell given in Logan (2008) and the estimation for anaerobically grown cells of the formula of $C_{4.9}H_{9.4}O_{2.9}N$ equating 1.25 g-COD/g-cell, (Rittmann, 2001). The sum of the substrate utilisation U as calculated above is multiplied by COD_{sub} the amount of COD per mole of substrate, 64 for acetate.

3.3. Results

3.3.1. *Number of bacteria in wastewater*

The spread plate counts of the wastewater, and anaerobic multiple tube count indicate there is 8.3 x 10^5 culturable aerobic bacteria, and 6.9 x 10^4 culturable anaerobic per ml of this wastewater, giving a rough estimate of the total bacteria per mL of wastewater to be 10^6. Although this method may over estimate numbers due to some bacteria being able to grow under both conditions, and underestimating numbers due to bacteria being intolerant to the media, the overall value calculated fits in with previous estimates (Tchobanoglous, 1991).

3.3.2. *Most probable number of exoelectrogens*

The number of positive outcomes of each test are shown in Table 3-1. From this the MPN can be calculated shown in Table 3-2. The MPN of exoelectrogens in an acetate fed reactor is 17 per ml of wastewater, this number drops to 1 per ml for a starch fed reactor and 0.6 per ml for a wastewater fed reactor. Superficially it appears that acetate metabolising exoelectrogens are quite rare organisms, starch metabolising exoelectrogens are even rarer and wastewater metabolising exoelectrogens are rarer still.

Table 3-1 The number of positive outcomes for each inocula size out of the total number of reactors run

Inocula size (mL)	50	25	10	1	0.1	0.01
Wastewater	2/2	2/2	0/2	1/2	-	-
Starch	2/2	2/2	1/2	0/2	-	-
Acetate	2/2	4/4	2/2	3/4	1/3	0/2

Table 3-2 The MPN in 1 ml of wastewater given by the two methods stated, numbers in brackets indicate the upper and lower bounds at 95% confidence. The probability of presence in wastewater is calculated from the total count of viable bacteria per 1 ml

Substrate	MPN calculation (Blodgett 2005)	MPN estimation (Thomas 1942)	Probability of presence in 1 ml of wastewater
Wastewater	0.6 (0.3-2.5)	0.8 (0.3-2.5)	6×10^{-7}
Starch	1.0 (0.3-3.2)	1.1 (0.3-4.0)	10^{-6}
Acetate	17.0 (5.5-52)	17.6 (6-51.5)	1.7×10^{-5}

An alternative explanation is that the lower MPNs, and therefore the probabilities of these organisms being present in 1 ml of wastewater, are the product of two or more events. In wastewater and starch there are long chain molecules present which undergo a series of steps in their breakdown. Each step is probably undertaken by different microorganisms. The electrons pass down this chain leading to the final step of donation to the electrode, represented by the acetate reactor. Thus the MPN of the wastewater and starch fed cells is the probable MPN of the acetate fed cells (the number of exoelectrogens) multiplied by the probability of each of the upstream steps. Here all of these steps are simplified into one probability step, however in reality this may be many steps the product of which is equal to 0.04 for wastewater and 0.06 for starch as shown in Figure 3.1.

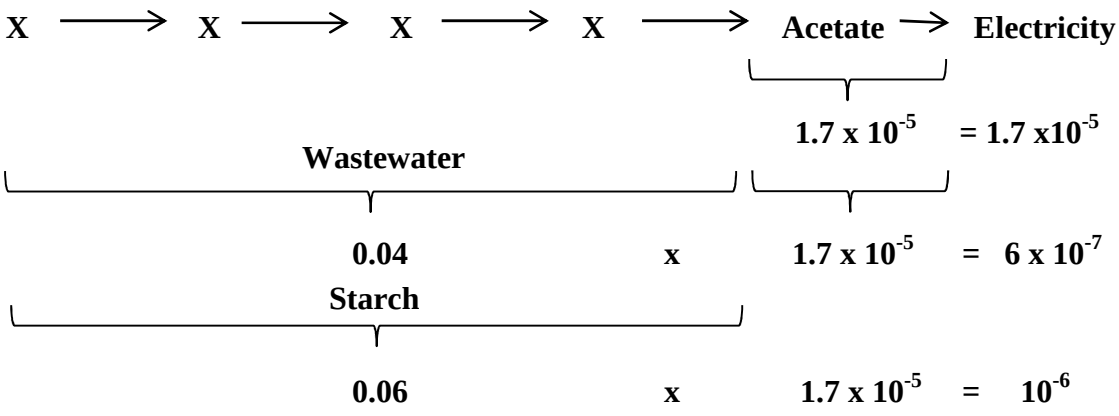

Figure 3-1 Estimated probabilities of numbers of bacteria present in the wastewater begin to produce a working MFC fed on three different substrates of acetate, starch and wastewater based on the numbers determined in the MPN method

3.3.3. *Growth rates*

The individual growth rates for the three different substrates are shown in Table 3-3. The rates were not significantly different (p=0.282 one way ANOVA), and showed agreement with other studies.

Table 3-3 Average growth rates for exoelectrogens fed on different substrates estimated using the rise in voltage measured in the acclimatising reactors

	Average growth rate
Wastewater fed community	0.028 h^{-1} ± 0.013
Starch fed community	0.023 h^{-1} ± 0.005
Acetate fed exoelectrogens	0.035 h^{-1} ± 0.020
Geobacter sulfurreducens (Cord-Ruwisch et al., 1998)	0.023 – 0.099 h^{-1}
Geobacter sulfurreducens (Esteve-Nunez et al., 2005)	0.04 – 0.09 h-1
Fermenting micro-organisms (Rittmann, 2001)	0.05 h-1

3.3.4. *Acclimatisation pattern*

Using an arbitrary value for N_0 (the starting number of bacteria per ml), the known growth rate and the time period over which the experiment was conducted, the pattern of acclimatisation can be modelled.

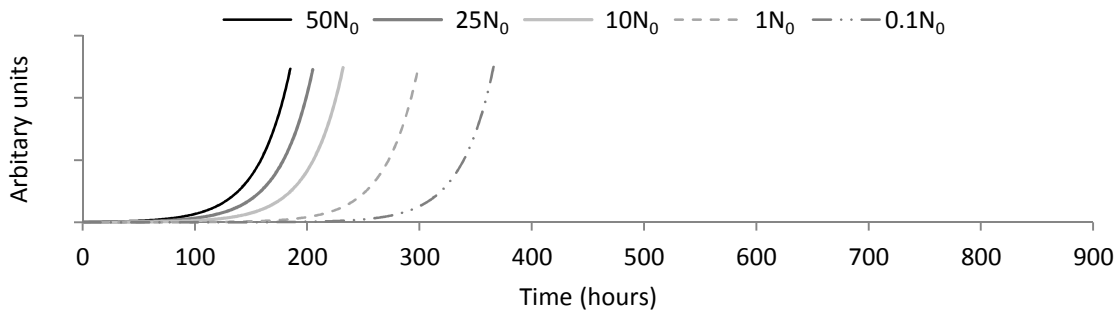

Figure 3-2 Model of the acclimatisation of reactors inoculated with varying amounts of bacteria as denoted by N_0 based on the formula $N_T = N_0 exp^{rt}$ where r the growth rate is the average growth rate determined experimentally of 0.03 hr^{-1} and t time is given on the bottom axis

The pattern of acclimatisation that occurred for the wastewater and starch fed did not follow the model. All reactors acclimatised at the approximate same time. If the growth rates and time are equal, mathematically this means that N_0 is similar for the different volumes of inocula.

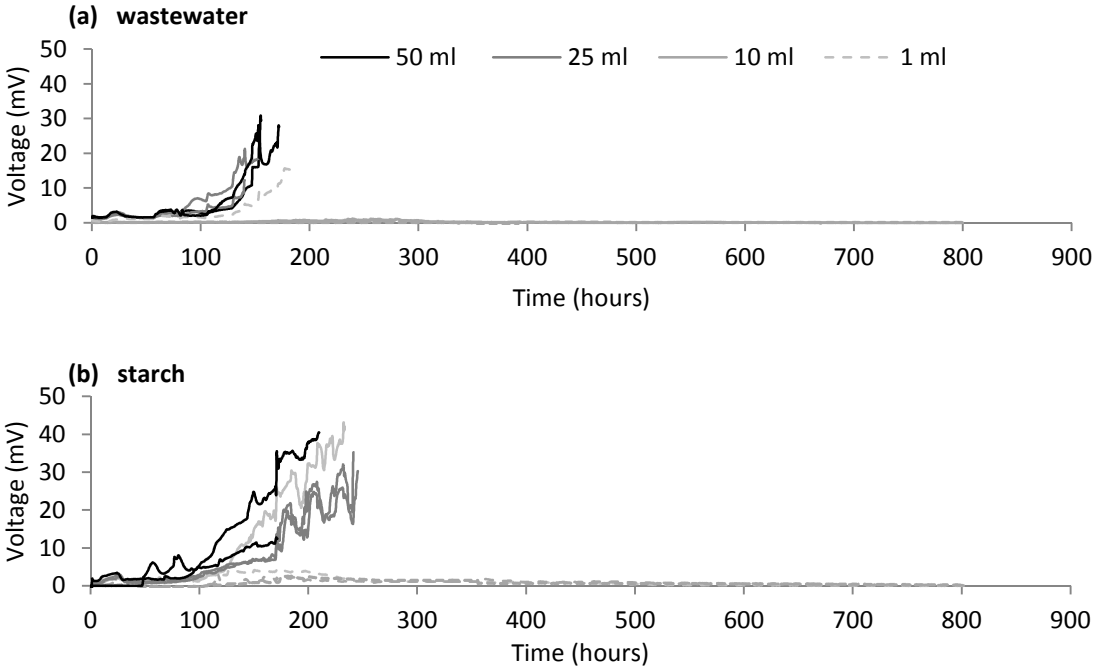

Figure 3-3 Pattern of acclimatisation of the wastewater (a) and starch (b) fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Superficially the pattern observed for the acetate fed reactors appears to follow the model pattern. However this is not the case as the lag time to acclimatisation is over extended with reducing amounts of inocula.

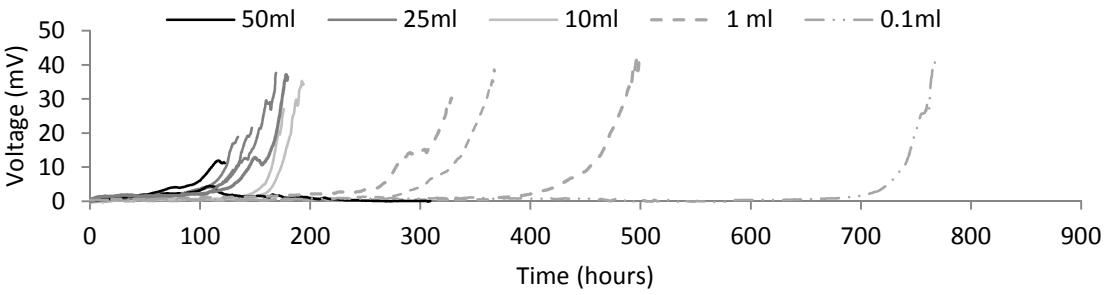

Figure 3-4 Acclimatisation of the acetate fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Using $N_T=N_0\exp^{rt}$ the calculated number of bacteria at the time the reactor inoculated with 0.1 ml (which must have contained at least one bacteria) reaches 10 mV would be 1.8×10^{11} bacteria, equivalent to the predicted number of bacteria in 1 kg of soil (Whitman et al., 1998), and 4×10^{7} times greater than the number of bacteria at 10 mV in the cell inoculated with 50 ml of wastewater (assuming an MPN of 1.7 per ml). This is clearly implausible, growth is not purely exponential, there is likely to be a lag phase with no growth. Yields calculated on the basis of these N_T and N_0 values both with (up to 8 g-COD cell/g-COD) and without (10^{-4} and 10^{-7} g-COD cell/g-COD) growth in the

lag phase give results discordant with the current literature, (these are shown in appendix VII).

3.4. Discussion

If the aim of acclimatising a reactor is to get it going, then it has been shown that a larger volume inoculum will give a quicker (in the case of acetate) and more likely (in the case of complex substrates) successful inoculation, although a proportion of the intended substrate may also be needed. As clear differences were observed between experiments, acclimatisation with the intended substrate is likely to be essential to successful operation. However, more importantly, these results also give insight into the abundance and distribution of exoelectrogenic and other crucial organisms, and to their community development within a reactor.

Discovering the number of exoelectrogenic bacteria per ml of wastewater is a strategically important question. It would inform us of the sequencing depth needed to identify these bacteria. By using the MPN methodology in a series of MFCs and aerobic and anaerobic culturing methods of the same wastewater, an estimation of this number has been gained. Acetate digesting exoelectrogens can be found at an estimated quantity of 17 per ml of wastewater, giving the probability of a bacterium in 1 ml of wastewater being an exoelectrogen as 1.7×10^{-5}, or put differently 0.0017% of the bacteria present in wastewater are exoelectrogenic. With 1000 sequencing reads there would be a reasonable chance of identifying only 1 or 2 exoelectrogens. When compared to the pyrosequencing carried out in chapter 4 a similar answer emerges. Two wastewater samples were analysed, and the total sequencing effort needed to capture 90% of all the sequences in the sample estimated using statistical algorithm as shown in Appendix X. Comparing the total number of *Geobacter* (the known exoelectrogen present in the wastewater samples) found in the sample to the estimated sampling effort, in one sample Geobacter represented an estimated 0.0012 % of the total bacteria, in the other this was lower at 0.00001 %. The two very different approached result in a similar estimation of the number of exoelectrogens present in wastewater. The use of further microbial techniques such as flow cytometry or QPCR would also help the verification of these results.

The number of acetate exoelectrogens is rare: 17 per ml. The number of starch or wastewater exoelectrogens is even lower at 1 per ml. It could be plausible that these are

even rarer organisms, however the likely explanation is that a chain of metabolism is occurring, this fits with the literature (Velasquez-Orta et al., 2011, Kiely et al., 2011c). The probability of achieving a working MFC fed on a complex substrate is therefore the probability of the exoelectrogenic step as identified above, multiplied by the probabilities of each of the upstream steps in the metabolic chain, and is therefore lower than the probability of forming with the acetate step alone. The MPN value is an approximation, yet even considering the upper and lower bounds of the calculation at 95% confidence, as shown in Table 3-2, this pattern is observed. Clearly however this is dependent on the inoculum used; with different inocula such as soil or sludge one would expect different results.

Growth rates, although intuitively demonstrated by the rise in voltage within an MFC, have not previously been calculated. It is an important value to know, especially when modelling such systems. This study calculated the average growth rate of 0.03 hr^{-1}, this value agrees with those documented in the literature from known exoelectrogenic bacteria. No statistical difference is found between reactors fed on acetate and more complex wastewaters, contrary to previous work (Velasquez-Orta et al., 2011) this study shows that the growth rate of exoelectrogens is likely to be the limiting factor.

The pattern of acclimatisation demonstrated within these reactors did not follow the expected pattern. Additionally the pattern observed in the acetate reactors is different to the pattern observed in the reactors fed with more complex substrates. Simple exponential growth does not appear to be happening in either system. The values of N_T within these systems are therefore questionable, as are the calculated yields and specific activities (see appendix VII).

The positive starch and wastewater fed reactors were fewer in number due to the reduced probabilities of the communities forming, but all acclimatised at approximately the same time regardless of the inoculum volume. The growth rates calculated were not statistically different between the different inocula, time was recorded accurately. Explaining this mathematically on the basis of $N_T = N_0 exp^{rt}$ this means either: N_0 is the same for the different inoculum sizes; the N_T of the reactors producing the same voltage is actually different; the rates as defined by voltage rise are not representative of growth rates; or the system may not be described by the equation $N_T = N_0 exp^{rt}$.

More of the acetate cells acclimatised leading to a higher MPN value, the pattern of acclimatisation here does show a clear link to inoculum size, however the size of the lag phase is far greater than would be predicted. Again the rates calculated were not statistically different between the different inoculum sizes and time was also recorded accurately. Here on the basis of $N_T = N_0 exp^{rt}$ either; N_0 is not linearly related to inoculum sizes, i.e. 50 mLs of wastewater contains more exoelectrogens than 50 times 1 ml; the N_T of the reactors producing the same voltage is actually different; there is a lag period before the growth rate starts which is also related, but not linear to, the inoculum size; or again the system is not described by $N_T = N_0 exp^{rt}$.

The MPN method and therefore N_0, is based on the following assumptions: bacteria are distributed randomly within the sample; they are separate, not clustered together; they do not repel each other; and every reactor whose inoculum contains even one viable organism will produce detectable growth or change and the reactors are independent (Blodgett, 2009). It seems likely that exoelectrogens will cluster, there function of passing electrons outside the cell may be used for passing electrons between cells when no external electron sink is available (Bretschger, 2010). In the sequencing data in chapter 1063 *Geobacter* are found in one wastewater sample and 4 in the other, also indicative of clustering. If clustering is occurring, the MPN is likely to be an underestimation as will be N_0 and N_T. This does not however explain the different patterns of acclimatisation observed between the substrates. Additionally the large upper and lower bounds given in the MPN calculations due to the relatively low sample size, could also lead to both under and over estimations of N_0 where the MPN is used.

The relationship of voltage with N_T could be more complex than assumed. Voltage generated from the electrode may be limited by properties relating to the anode itself rather than the bacteria on it, or may quickly reach saturation point of the biofilm, however then one would expect to observe the same pattern in all reactors.

Growth rates are assumed to be represented by the rising voltage measured across the reactors. This may not be the case if the bacterial population has to grow to a certain threshold level (at an unknown growth rate which may different for different inocula sizes) before any voltage is produced. Additionally an assumption is made that increasing voltage is caused by an increasing number of bacteria, not an increasing

capability of these bacteria to donate electrons, this may not be true. Again this does not account for the differences between substrates.

The period of acclimatisation is both highly complex and variable between substrates, yet does show a clear observable pattern, indicating an underlying mechanism. It seems likely that these systems are not described by $N_T = N_0 exp^{rt}$. Such deviations could be caused if the exoelectrogens present N_0 were able to induce electrogenic activity in other bacteria through the excretion of electron shuttles: $N_T > N_0 exp^{rt}$, and in addition a further growth equation of the 'induced' exoelectrogens would act to confuse the picture. In the case of the complex substrate systems something within the chain of metabolism which is unrelated to the bacteria quantity could be triggering the start of the acclimatisation, this causes the reactor to work or fail regardless of the number of exoelectrogens present at the start. In the acetate fed reactors a further factor related to the inoculum size could be causing the extended lag observed, such as the movement of the exoelectrogens to the anode surface.

The period of acclimatisation is not only complex, it is likely to be a period of high competition for resources and possible low efficiency for the exoelectrogens as seen from the low coulombic efficiencies and comparable COD removal in both the positive and negative reactors (see appendix VI).

If the aim of acclimatisation is to merely 'get the reactor going' this study has shown that using a large proportion of wastewater is best. The experiment has also demonstrated that the abundance of organisms needed to start an MFC is low within wastewater, and even lower when these systems are to be fed on complex substrates. The growth rates defined are similar to those observed for exoelectrogenic species in other environments, and are likely to be the limiting factor in MFC acclimatisation. The pattern of acclimatisation a fuel cell is complex and not explained solely by exponential growth. The clear differences between these systems demonstrate the vital importance of acclimatising a community for the eventual use of the reactor. A reactor fed on acetate is different to one fed on wastewater. By developing a greater understanding of this ecology and its development, the move towards more stable biological system can be made. Understanding the nature, abundance and location of these exoelectrogens is crucial.

Chapter 4. Can Microbial Fuel Cells operate at low temperature?

4.1. Introduction

Bioelectrochemical Systems (BES's) are being heralded as a new method of energy efficient wastewater treatment, yielding electrical energy or other products from the bacterial breakdown of organics in an electrochemical cell. For future application of this technology understanding the microbial ecology, community structure and relating this to performance is desirable (Parameswaran et al., 2010) . The majority of fuel cell research is carried out using acetate as a feed at 30^{o}C with the implicit assumption that this will translate into the treatment of real wastewaters at ambient temperatures. To use low strength high volume wastes like wastewater the bacterial communities within BES need to be able to digest complex and variable substrates and do so outside, which in the UK, Europe and many parts of the USA means at low temperatures. If the communities of bacteria able to perform this task do not occur naturally further work and investment into this area may be futile.

As noted above most BES studies are conducted in laboratories at a temperature of 30 ^{o}C (Call and Logan, 2008, Cheng and Logan, 2007a, Selembo et al., 2009b). Few ambient treatment plants will get this warm. Several studies investigating the performance of MFCs over temperatures between 20-30 ^{o}C have found that the maximum power output with acetate was reduced by 9% (Liu et al., 2005a) and 12% (Ahn and Logan, 2010) when the temperature was lowered from 30 ^{o}C to 20 ^{o}C and 23 ^{o}C respectively, using beer waste a 10% drop was seen at these temperatures (Wang et al., 2008). The reduction in performance was lower than predicted by biological process modelling, suggesting that bacterial growth at 32 ^{o}C is not optimal, or that other factors are more limiting (Liu et al., 2005a). Complex wastes were also treated by Ahn and Logan (2010), and it was found that temperature had a greater effect on these than the simple compounds.

Lower (below 20 ^{o}C) and more realistic temperatures have been even less well studied. Min et al (2008) found that at 15 ^{o}C no successful operation was achieved, after 200 hours of operation the experiment was stopped. Cheng et al. (2011) found at 15 ^{o}C start up took 210 hours but at 4 ^{o}C there was no appreciable power output after one month (720 hours) and the experiment was stopped. In the same study a reactor started at 30 ^{o}C was then dropped to 4 ^{o}C and power output was achieved, but around 60% lower than

that the higher temperature. Larrosa-Guerrero *et al*. (2010) operated reactors at 4 ^{o}C and 35 ^{o}C using a mixture of domestic and brewery wastewater, observing a decline in COD removal from 94% to 58% and power density from 174.0 mWm^{-3} to 15.1 mWm^{-3} at the lower temperature.

By contrast Jadhav and Ghangrekar (2009) operated an MFC's in a temperature range of 8-22 ^{o}C and found that the current and coulombic efficiencies were higher than that produced in the temperature range of 20-35 ^{o}C. However in this study temperatures were ambient not controlled and thus confounded by time. They inferred that a reduction in methanogenic bacterial activity at lower temperatures increased MFC performance, although the microbiology of the systems was not examined. Similar results were obtained by Catal *et al.* (2011), here the biofilm was examined using scanning electron microscopy and found to be thicker in the higher temperature reactors.

MFC systems are based on electrochemical and microbiological principles: temperature affects both. The electrochemical impacts of temperature can be calculated using the Nernst equation based on known free energies for substrates such as acetate, or estimated free energies if wastewater is used (Logan, 2008). In bacterial systems rates of reaction roughly double for every 10^{o}C rise in temperature (Rittmann, 2001). However, the actual behaviour of these complex systems at different temperatures and fed on different substrates remains an area of great uncertainty in this field of research.

An increasing number of studies into the microbial communities of BES using techniques such as restriction fragment length polymorphism (RFLP), clone libraries and denaturing gradient gel electrophoresis (DGGE) are adding to the knowledge base we have about these communities. There are advantages to these various techniques such as the high reproducibility and in the case of DGGE and RFLP the large number of samples than can be run (van Elsas and Boersma, 2011, Kirk et al., 2004). However all these techniques are limited in that only a small fraction, (in the case of DDGE estimated at 1-2 % (Macnaughton et al., 1999), of the species present are targeted in these studies, total diversity cannot be estimated from these limited results. Never the less it has been repeatedly shown that *Geobacter sulfurreducens* dominates in acetate fed reactors, although this can vary when reactors are inoculated with different media (Kiely et al., 2011c). As substrates become increasingly complex moving from VFA's

to carbohydrates to actual wastewater the dominant species become more varied (Kiely et al., 2011c). Some wastewater fed reactors were found to be dominated by *Betaproteobacteria* (Patil et al., 2009), although in other studies *Geobacter* still dominates (Cusick et al., 2010).

Most of the techniques that have been used are limited by their capacity to identify the most dominant species within the communities. Next generation sequencing (capable of sequencing to a far greater depth) has now been used in two MFC studies. Lee *et al*. (2010) used FLX Titanium pyrosequencing to sequence four samples of biofilm, triplicate samples were taken from an acetate fed reactor comparing this to a single sample taken from a glucose fed reactor. The profiles found in the samples were not significantly different. A further study by Parameswaran (2010) analysed the biofilm of two MFC reactors fed on ethanol examining the impact to the communities when methanogenesis was prevented in one, identifying the role of hydrogen scavengers.

The aim of this study was to determine if microbial fuel cells can work at low temperatures, and if the inocula affects this. By running reactors fed on both wastewater and acetate the relative importance of the final 'electrogenic' step, and the up- stream hydrolysis and fermentation steps can be evaluated. The impact of temperature, inoculum and substrate on the microbial communities and total diversity within these reactors was examined using next generation sequencing techniques.

4.2. Methods

4.2.1. *Experimental design*

The variables examined were: temperature (warm 26.5 ^{o}C and cold 7.5 ^{o}C); substrate (acetate and wastewater); and inoculum (Arctic soil and wastewater). Each set of conditions were run in parallel duplicate reactors and biofilm samples taken from each. The two series of experiments, acetate and wastewater, were conducted using the same 8 reactors under identical conditions, the two wastewater inoculum samples were used to seed the acetate (wastewater sample1) and wastewater fed (wastewater sample 2) experiments. This is represented in Figure 4-1.

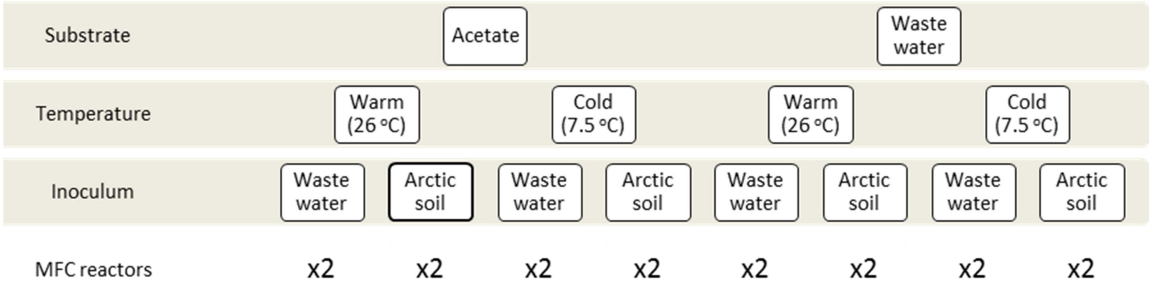

Figure 4-1 Illustration of the multi-tiered reactor conditions used

The warm temperature was chosen to represent the typical ambient laboratory temperatures of many MFC studies. The low temperature is the lowest sustained temperature of a wastewater treatment plant in the North of England (54°58'N, 01°36'W) experienced over a winter period (Northumbrian Water Ltd). The different substrates represent the most commonly used laboratory substrate acetate, and compared to wastewater. The two different inocula were the usual inoculum of wastewater, and Arctic soil (see below) which could potentially have more bacteria with low temperature, exoelectrogenic capability.

Wastewater typically contains 10^5 - 10^6 bacteria per mL (Tchobanoglous, 1991) soils can contain around 10^9 bacteria per gram (Whitman et al., 1998). Many soil environments are low in oxygen, and iron rich, favouring anaerobes and iron reducers and potentially therefore exoelectrogens. Arctic soils have been shown to have to be biologically active, accounting for around 6% of the total global methane sources (Ehhalt et al., 2001). (Hoj et al., 2005, Kotsyurbenko et al., 2004, Metje and Frenzel, 2005). Soil taken from Ny-Ålesund, in the Spitsbergen area of Norway has been shown to contain a wide range of methanogenic groups active at temperatures ranging from 1-25 °C (Hoj et al., 2005, Hoj et al., 2008).

4.2.2. *Reactor design and operation*

Eight identical double chamber tubular MFC reactors (78 mL each chamber) with an internal diameter of 40mm and length of 60mm were used. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (Tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor, and a multimeter to measure the voltage (Pico ADC-16, Pico Technology, UK). The membrane between the reactor

chambers was Nafion 117, with an area of 12.6cm^2. Reactors were sparged with 99.99% pure N2 in the anode chamber, and air in the cathode chamber for 15 minutes after every re-fill.

Four reactors were operated at a temperature of 26.5 ^{o}C in an incubator (Stuart Scientific SI 50, UK), the other four at 7.5 ^{o}C in a low temperature incubator (Sanyo MIR-254, (Sanyo Biomedical, USA). The temperature was logged continuously over the experiment using a EL-USB-1 temperature data logger (Lascar Electronics, UK). The reactors were inoculated and filled with substrate, replacing this every 5-6 days until a stable power generation was achieved. The reactors were then re-filled and three successive 3 day cycles were run logging the voltage over this time. Chemical oxygen demand (COD) removal during each batch was determined using standard methods (APHA, 1998) and Spectroquant ® test kits (Merck & Co. Inc., USA).

4.2.3. *Media and inocula*

Autoclaved acetate media (Call and Logan, 2008) containing 1 g/L sodium acetate was compared to wastewater taken from Cramlington wastewater treatment site (Northumbrian Water Ltd, UK) which was UV sterilised prior to use. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see appendix V). The cathode chamber was filled with 1M pH 7 phosphate buffer. The conductivity of the nutrient media, wastewater and the phosphate buffer was measured using an EC 300 (VWR Ltd, UK) and equalised for the temperatures of 7.5 ^{o}C and at 26.5 ^{o}C.

The wastewater inoculum was collected from Cramlington wastewater treatment plant, a Northumbrian Water site in the North of England, it was raw wastewater collected prior to any form of treatment, and is believed to be of mixed industrial and domestic, COD 0.7-0.8g/L. Once collected the sample was stored in a fridge at 4 ^{o}C within a closed container. The Arctic soil was collected from Ny-Ålesund, Spitsbergen in Svalbard. This was wrapped within three sealed bags and stored at 4 ^{o}C until used. The inocula of wastewater and soil were measured out to 5 mL or 5 g respectively before being added to the reactors. Samples of each inocula were preserved in a 50:50 in a mix of ethanol and autoclaved PBS pH7 in the freezer at -20 ^{o}C for microbial analysis.

4.2.4. *Microbiological techniques*

At the end of each experiment the anode was removed aseptically from the chamber using aseptic technique and preserved in a 50:50 mix of ethanol and autoclaved PBS pH7 and stored in a freezer at -20 ^{o}C. A 5 ml or 5 g sample of the original inocula was also taken and preserved in this way. The inocula samples were pelletized and the DNA then extracted. With the anode samples the bacteria that had dispersed into the liquid was pelletized and then added to the central section of the anode felt cut from the whole anode. The DNA was extracted by placing this sample into the beaded tube of a FastDNA Spin Kit for Soil (Qbiogene MP Biomedicals, UK). Extraction was completed according to the manufacturer's instructions. The samples were then pyrosequenced following amplification of the 16s rRNA gene fragments.

The primers used were F515 (GTGNCAGCMGCCGCGGTAA) and R926 (CCGYCAAT-TYMTTTRAGTTT). Each sample was labelled with a unique 8 base pairs (bp) barcode connected to a GA linker. Sequencing was completed from the Titanium A adaptor only forward from the F515, capturing the V4 region and most of the V5 region with a Titanium read of 400-500 bp. Triplicate PCR reactions were carried out using the Roche FastStart HiFi reaction kit (Roche Diagnostics Ltd., UK) and subject to the following optimised thermal cycles: initial denaturation at 95°C for 4 minutes; 23 cycles of denaturation at 95°C for 1 minutes; annealing at 55°C for 45 seconds; extension at 72°C for 1 minute; final extension at 78°C for 8 minutes. An automated thermal cycle Techne TC-5000 (Bibby Scientific, UK) was used.

The triplicate samples were then pooled and cleaned using QIAquick PCR Purification Kit (Qiagen, UK). The DNA concentration was quantified by UV-Vis spectrophotometry using a Nanodrop 2000 (Thermo scientific, USA). The individual samples were pooled to give equal concentrations of all reactor samples, and double concentration of the wastewater and arctic soil seed. Sequencing was carried out by the Centre for Genomic Research (University of Liverpool, UK) using the Roche 454 sequencing GS FLX Titanium Series.

4.2.5. *Data analysis*

The pyrosequencing data set was split according to the barcodes and unassigned sequences were removed[1]. The flowgram files were cleaned using a filtering algorithm Amplicon Noise (Quince et al., 2009) to give the filtered flowgram file. Filtering at a minimum flowgram length of 360 bp including the key and primer before first noisy signal, all flowgrams were then truncated to 360 bp. A pairwise distance matrix was then calculated using the Pyronoise algorithm (Quince et al., 2009). This uses an iterative Expectation-Maximization algorithm which constructs denoised sequences by clustering flowgrams using the initial hierarchical clusters generated in the previous step and the filtered flowgram file. The cut-off for initial clustering is set at 0.01 and the cluster size is 60, as recommended by Quince et al. (2009). The flowgrams can then be denoised.

PCR errors were then removed again using Seqnoise, generating a distance matrix using the Needleman-Wunsch algorithm for pairwise alignment. The optimal parameters used here were the cut-off for initial clustering of 0.08 and cluster size of 30. Chimera removal was completed using the Perseus algorithm (Quince et al., 2011) which for each sequence searches for the closest chimeric match using the other sequences as possible parents. (Quince et al., 2011). The sequences are then classified and the good classes filtered at a 50% probability of being chimeric, producing the final FASTA file which is denoised and chimera free ready for analysis in QIIME (Caporaso et al., 2010).

Using the QIIME pipeline tutorial the following analysis was completed: assigning taxonomy using Greengenes (http://greengenes.lbl.gov) at the 97% similarity level; creating an OTU table; classification using the RDP classifier; summary of taxonomic data from classification; generation of rarefaction data of the diversity in a reactor; calculation of the differences between the reactors; performing Principle Co-ordinates Analysis (PCoA); jackknifing and bootstrapping to understand uncertainty in beta diversity output; and generating Unweighted Pair Group Method with Arithmetic Mean (UPMGA) trees for hierarchical clustering of samples. The dissimilarity of the community structure between duplicates was examined using both a weighted (relative abundance) and unweighted (presence/absence) phylogenetic diversity metrics using

[1] *The analysis of the pyrosequencing data was carried out by Dr Matthew Wade, a Bioinformatics researcher within the School of Civil Engineering and Geosciences at Newcastle University.*

UniFrac, giving a distance matrix containing a dissimilarity value for each pairwise comparison. The raw OTU table generated was used to produce the species abundance pattern (with the log abundance normalised to the number of sequences in each sample) and the rank abundance curves, (where percentage abundance is used to normalise samples).

An estimate of the total diversity for each sample was calculated using the Bayesian approach as described in Quince et al. (2008), where the 'posterior distribution' of the taxa area curve is estimated, from the known distribution of the data gathered in the sequencing. Three distributions are modelled: log-normal; inverse Gaussian; and Sichel, and deviance information criterion (DIC) are used to compare the fit from each model. The lower the deviance or DIC values the better the model fit, those models within 6 of the best DIC value can be considered as a plausible fit. Using the fitted abundance distributions the sampling effort required to capture 90% of the taxa within that sample is estimated.

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run analysis of variance (ANOVA) tests on the experimental data, and t-tests on the distance matrix data for the sequences samples. Data were checked for normality prior to completing ANOVA, and if necessary the Box-Cox transformation was used.

The performance of the MFC reactors were analysed on the basis of three variables: % COD removal as measured; coulombic efficiency (CE); and power density (mW/m^2). The latter two variables were calculated using the measured COD and voltage within the cells, as described in Appendix VIII. Correlation of the community structure with these performance factors was done using BEST (Biological Environmental and Stepwise method) within Primer 6 (Primer-E Ltd. UK).

4.3. Results

4.3.1. *Cell acclimatisation*

All 16 reactors acclimatised and produced voltage. The acetate fed reactors showed a clear pattern of acclimatisation related to both temperature and inocula with the warm reactors acclimatising first, and the Arctic soil inoculated reactors starting first as shown in Figure 4-2. The cold wastewater inoculated reactors did not produce current until after around 800 hours, longer than the time allowed in previous studies (Cheng et al.

2011, Min et al (2008). The acclimatisation of the wastewater fed reactors was only affected by temperature: the warm reactors started producing current at day 1, the cold reactors at day 20. All duplicates behaved in a very similar way.

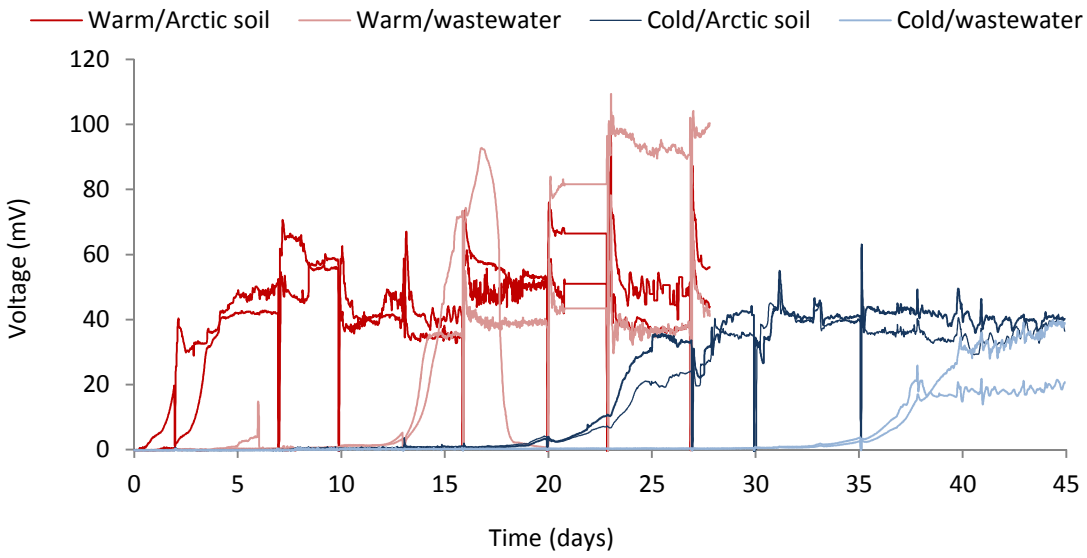

Figure 4-2 Acclimatisation of the acetate fed reactors inoculated with the two different inocula and run at warm (27.5 ^{o}C) and cold (7.5 ^{o}C) temperatures

4.3.2. *Cell performance*

Over the three batch runs, the reactor performance was variable especially within the warm reactors, as seen in Figure 4-3. The variation in performance was not a function of either the inocula or the substrate and the highest variation was seen between the duplicates.

Three measures of performance averaged for each reactor over the triplicate batches are shown in Figure 4-4. The coulombic efficiency is higher in the acetate fed reactors; and the COD removal is higher in the wastewater fed reactors. Power densities do not appear to vary with substrate, inoculum or temperature, however two individual reactors had considerably higher power densities than the others and their duplicates: acetate warm ww 2; and wastewater warm soil 1.

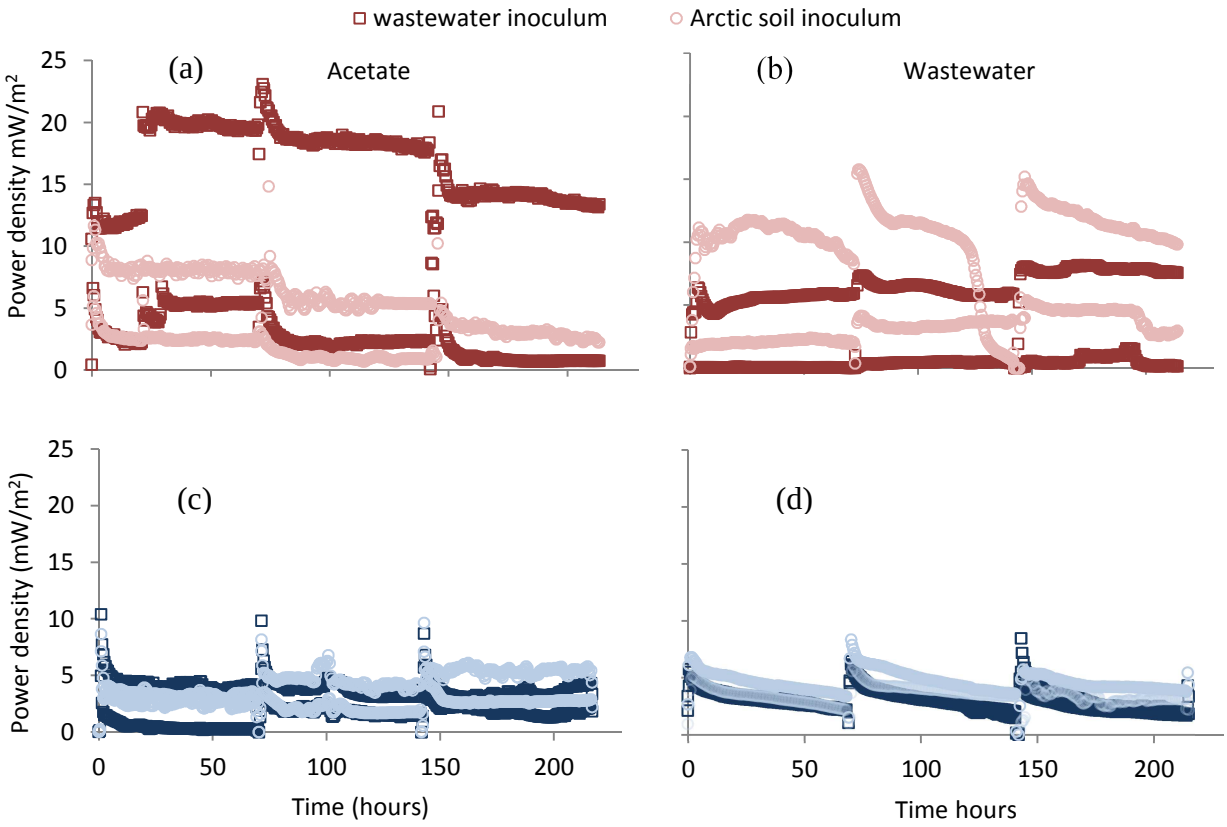

Figure 4-3 Power density plots showing the three consecutive batch runs for: (a) acetate fed reactors run at 27.5 °C, (b) wastewater fed reactor run at 27.5 °C (c) acetate fed reactor run at 7.5 °C (d) wastewater fed reactor run at 7.5 °C

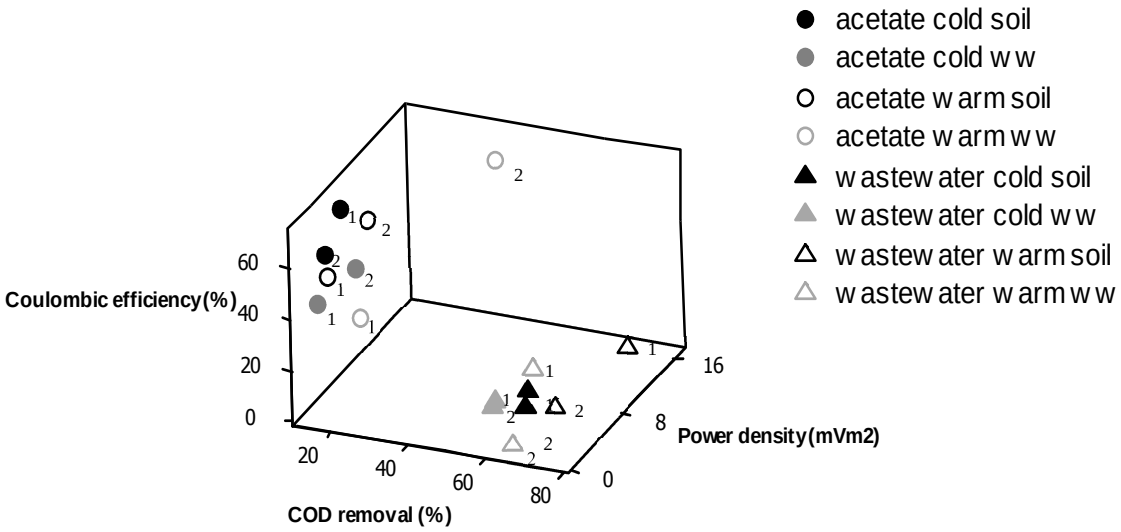

Figure 4-4 3D plot showing reactor performance in terms of Coulombic efficiency, COD removal and power density of the various reactor conditions, duplicates of each condition are labelled on the plot next to the symbols

By performing an ANOVA on the three performance indicators using the factors of feed, temperature and inocula a complex picture emerges. The power density results, i.e. the ability of the biofilm to put electrons to the circuit, were not normally distributed, when transformed, none of the performance factors analysed were significant (feed p = 0.746, inoculum p = 0.249, and temperature p = 0.147). For coulombic efficiency both inoculum (p=0.009) and feed (p=0.000) were significant yet temperature was not. The acetate fed reactors performing better (54.5%) than wastewater fed ones (12.3%), and the Arctic soil inoculated reactors performing better (37.4%) than the wastewater inoculated ones (29.4%). The reactors fed wastewater removed significantly more COD (62.1%), than the acetate reactors (19.4%) (p=0.000) the warm reactors also removed more (45.9%) than the cold ones (33.7%) (p=0.000), the type of inoculum was not significant. Two way ANOVA was performed between each interaction with each performance indicator. For CE the interaction between substrate and inoculum was significant (p = 0.057) with the inoculum having a much stronger effect with the acetate feed than the wastewater feed, and the Artic soil acetate fed reactors performing the best. The interaction between substrate and inoculum was also significant in the COD removal (p = 0.008), the Arctic soil inoculum having a higher COD removal in the wastewater fed reactors, but a lower COD removal in the acetate fed reactors than the wastewater inoculum. No other interactions were significant.

4.3.3. *Similarity of duplicate reactors*

It is seen in the data above that the duplicate reactors performance varied considerably, especially for the warm temperature reactors. Using the sequencing data a Unifrac dissimilarity matrix was plotted, using phylogenetic information the ‘distance’ between each sample is quantified and corresponds to the degree of similarity (Appendix IX). The values show that the duplicate reactors fed with acetate are indistinguishable (p=0.000). This was observed with both the weighted analysis which incorporates information on relative abundance of each OTU, and the unweighted analysis which is based on the presence or absence of each OTU. The wastewater fed duplicate reactors were typically different, with the exception of the hot Arctic soil inoculated reactors (p=0.000). The two wastewater inocula samples taken from the same treatment plant but at different plants were also indistinguishable (p=0.000). This pattern is also observed in Figure 4-5, where the acetate duplicates are paired, and appear to cluster on the basis of temperature. The wastewater fed reactor duplicates are not paired together and do not

cluster with temperature or inoculum. Further details of the bacteria groups present within these reactors can be found in Appendix XI.

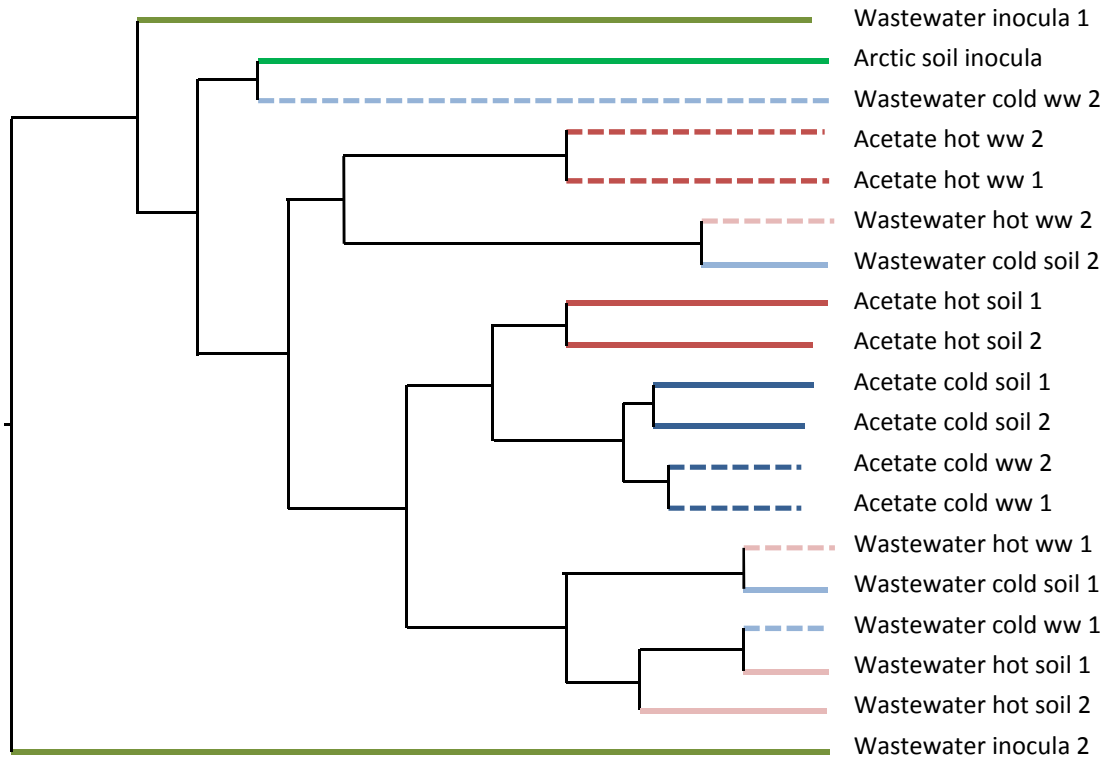

Figure 4-5 Dendrogram resulting from the UPMGA hierachical weighted clustering of samples, the length of lines is relative to the dissimilarity between samples, groupings of samples are denoted by the coloured end portion of the lines

4.3.4. *Microbial diversity*

In total 19 samples were analysed. The number of sequences per sample ranged from 8112 to 77436 with a total number of observations of 549178. The species abundance pattern plotted from the OTU table shows a large variation in the diversity of the samples shown in Figure 4-6. As expected the Arctic soil inoculum is the most diverse, followed by the wastewater inocula. The acetate fed reactors however are considerably more diverse that the wastewater fed reactors, the most diverse of these (acetate cold soil 2) has a similar diversity to the wastewater inoculum, and the least diverse (acetate warm ww 2, the reactor with the highest power density) is similar to the most diverse of the wastewater fed reactors.

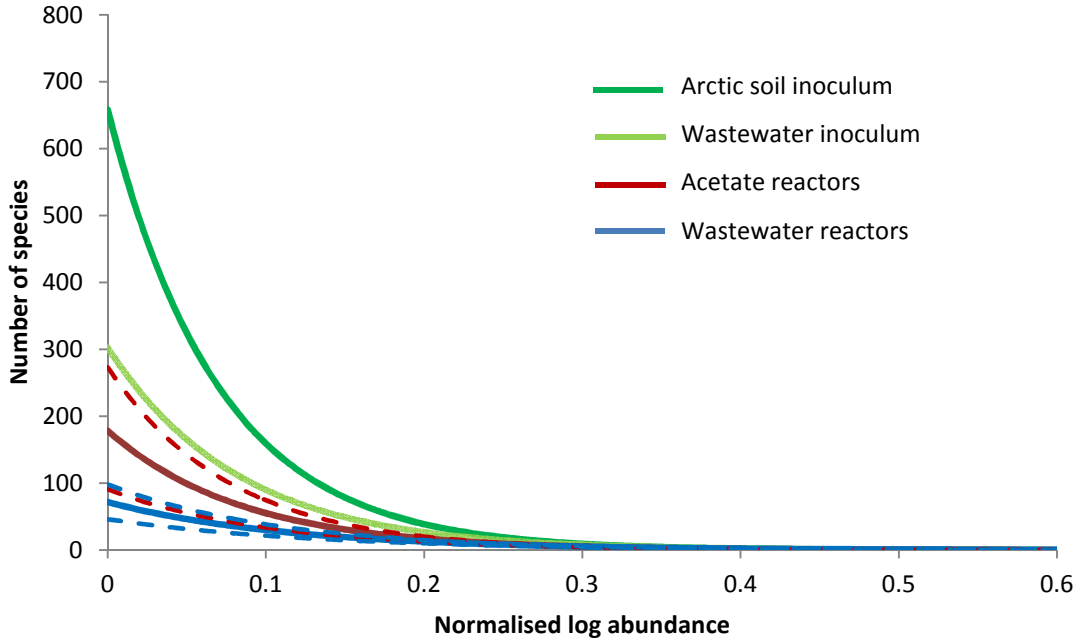

Figure 4-6 Species abundance pattern, the number of species is plotted against the log abundance normalised to the total number of observations for each sample. The plots for the acetate and wastewater fed reactors are averages of the eight reactors used, the highest and lowest within each substrate grouping are shown with the dashed lines. The wastewater inoculum line is an average of the two samples

The observation of the greater diversity in the acetate fed reactors is also seen in the total diversity estimates. A summary of these values is presented in Figure 4-7 where is clearly seen that for all the three distribution models the acetate fed cells have a higher predicted diversity, and that the acetate soil inoculated reactors have a higher total diversity than the wastewater inoculated ones. Performing a nested ANOVA on the Box Cox transformed total diversity estimates, shows that the acetate fed reactors have a statistically significantly higher diversity (log-normal $p = 0.001$; inverse Gaussian $p = 0.000$; and Sichel $p = 0.027$). Within the acetate fed reactors the Arctic soil inoculated reactors have a higher predicted diversity (log-normal $p = 0.006$; inverse Gaussian $p = 0.003$; and Sichel $p = 0.013$), the lower temperatures also give higher diversity (log-normal $p = 0.037$; inverse Gaussian $p = 0.012$; and Sichel $p = 0.029$). There is a strong interaction between the acetate feed and the inoculum type ($p = 0.024$) but not with temperature ($p = 0.156$) observed in both the log-normal and inverse Gaussian distributions. The full tables of diversity predictions, DIC values and estimate sampling requirements can be found in appendix X.

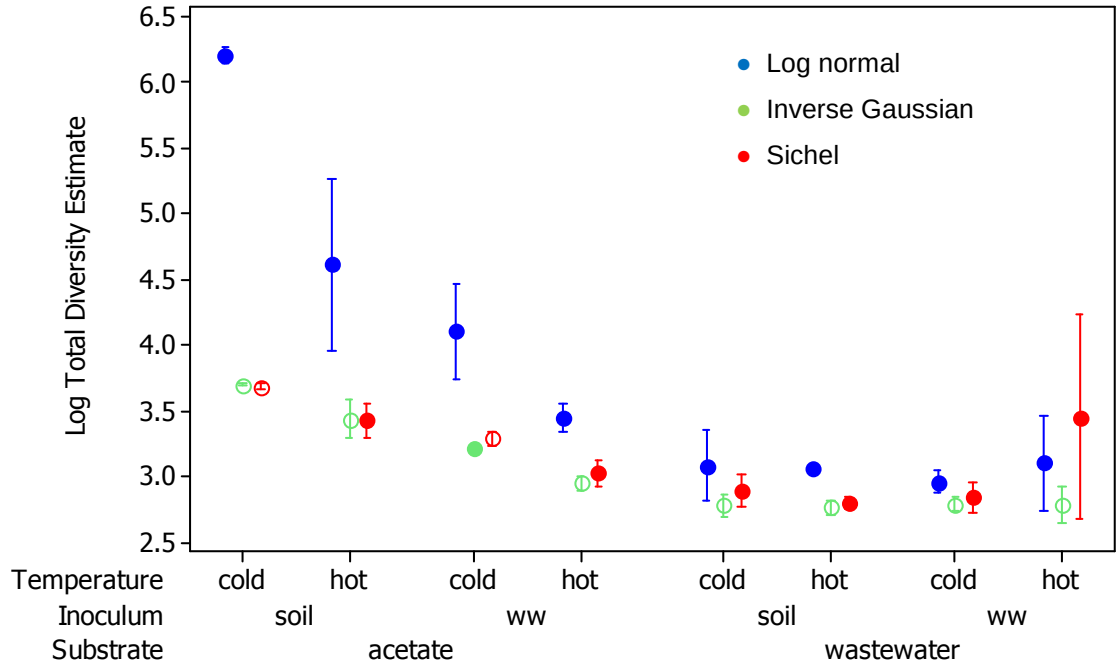

Figure 4-7 The estimates of total diversity for each set of reactor conditions, the three points within each sample are the mean of the duplicate samples modelled to log-normal, inverse Gaussian, and Sichel estimates, the best fit according to the DIC values is denoted by a closed circle, lines are one standard error of the mean

4.4. Discussion

All the reactor conditions tested produced current showing that MFCs can function at low temperatures, with real wastewaters and the bacteria required for them to do so can be found within the wastewater itself. This finding is of great significance to the industrial feasibility of MFC technology for wastewater treatment.

The power output produced by the MFCs was not significantly affected by either temperature feed or inoculum. Although some warm reactors achieved a power density much higher than the cold reactors, due to the variability between reactors this was not significant. The reasons for this variability, were not discovered, no statistical link could be made between the community structure and the power density. The higher coulombic efficiencies within the acetate fed reactors did not translate into higher power densities, only low amounts of COD was converted efficiently into power. Whereas in the wastewater fed reactors more COD was converted less efficiently producing a similar power. In terms of wastewater treatment, this high COD removal, albeit at low CE, is an advantage.

The lack of temperature effect seems at first to be unlikely. Based on the laws of thermodynamics, the free energy available in many chemical reactions decreases as temperature decreases. However in a fuel cell system the energy available is the difference in energy between two half reactions. As both the half cells are equally affected by temperature, the difference between them, or energy available does not decrease with lower temperatures (Appendix II). This is a simplification, many other factors such as dissociation constants and partial pressures of gases will affect the energy, additionally the metabolic activity of the bacteria also reduces with lower temperatures (Rittmann, 2001), however these do not appear to be having a significant impact although may be responsible for some of the variability in performance. On the basis of the results presented here, it can be asserted that low temperature systems have a similar level of energy available for both bacterial metabolism and electricity production as higher temperature systems.

The lack of temperature effect could be caused by the reactor design itself. The inherent inefficiencies and overpotentials within the reactors could be limiting the performance such that the temperature effect is not observed, i.e. all the reactors are working at the limit of their performance and warming them cannot result in improvements. If lower temperature reactors did prove to have slower microbial kinetics, as would be expected and as is indicated by the slower acclimatisation in the cold reactors this could be overcome through relatively simple engineering solutions such as increasing the size of the anode. An increase in the size of the anode would give a greater surface area for the biofilm to grow, and therefore more active bacteria to compensate for the slower metabolic rates.

A further counter intuitive result of this study it that the acetate fed cells have a higher microbial diversity than the wastewater fed cells. It would be assumed that in a wastewater fed systems that the complexity of the substrates available for metabolism, and different metabolic pathways would result in a higher diversity of bacteria, with different groups digesting different substrates at different times. With acetate fed reactors, the only metabolic pathway within a fuel cell should be the direct breakdown of acetate and donation of electrons to the electrode, the most efficient species should dominate theoretically leading to a much less diverse community. This is not seen to be

the case, with a higher diversity in the acetate fed cells being shown both by the species abundance pattern and by the analysis of all the total diversity estimates.

It is proposed that the diversity of the systems is determined not by the diversity of the metabolism within it, but by the overall energy available to the bacteria, and that the free energy available to bacteria in the acetate reactors is greater than in the wastewater reactors. This energy difference could be due to several reasons: acetate may have more free energy per g COD than wastewater; the free energy in acetate may be more accessible to the bacteria, i.e. it is easier to degrade than many of the compounds in wastewater; or that energy is lost during the metabolic chain, with acetate this chain is short, therefore the losses are low, within wastewater these chains are much longer and therefore the losses of energy are greater, this would also produce the coulombic efficiencies observed. The fact that there is no observed difference in the diversity between the warm and cold reactors is further evidence that the energy available in these is actually similar.

Results indicate that the energy flux within a microbial system is key to determining the ecology of that system. The total free energy available is likely to affect the balance of births and deaths of individual species, with greater energy resulting in more births i.e. greater abundance and therefore ultimately greater diversity. The free energy will also impact on the speciation rate (i.e. a greater number of births will ultimately lead to greater chances for speciation). This is counter to the theory that a diverse range of substrates available would provide a variety of different metabolic pathways for different organism to exploit, and therefore lead to a higher diversity.

If a quantitative link could be made between the free energy in a system and the diversity modelling of these complex biological ecologies, being able to understand such phenomena as acclimatisation, adaptation and functional redundancy, and ultimately therefore the manipulation of biological systems becomes a greater possibility (Curtis and Sloan, 2006). We are however still a long way from this in the plant and animal world ecologists have argued there is no single species/energy link (Clarke and Gaston, 2006) and even if it was the key parameter the free energy in wastewater systems cannot yet be reliably measured. Although it is evidenced here that free energy may be the key in determining diversity, a conclusive answer cannot be

given let alone a quantitative link on the basis of these results alone, further research is required.

A further effect on diversity is seen with the inoculum, which interacts with the substrate. The Arctic soil inocula has a greater diversity which seems to be carried forward into the acetate fed cells, a greater number of these species surviving within the reactors where energy may be plentiful. As the performance of the acetate and wastewater fed cells is similar despite the increased diversity of the acetate reactors, it could be concluded that this increased diversity is non-beneficial, or at least neutral to the performance of the reactor. Thus although wastewater reactors will always have lower coulombic efficiencies due to the losses within the metabolic chain, they may actually be more efficient at turning the energy available into wastewater digesting biomass and electricity.

The majority of fuel cell research is conducted at warm temperatures and with simple substrates. It has been shown in this research that reactor performance is not significantly affected by the temperature, neither is the diversity of the community developed. Inoculating reactors with cold adapted organisms does not have any benefit on the performance of the reactors. The substrate fed to the reactor again has little impact on the performance, however results in very different diversities.

It is generally assumed that an acetate fed reactor may represent the optimum conditions for an MFC, however this may not be the case. These findings suggest that wastewater feed has less available energy and therefore results in a more efficient biomass being formed. This has positive implications for the introduction of bioelectrochemical systems into wastewater treatment.

Chapter 5. Time taken until failure for MEC's fed on acetate compared to those fed on wastewater

5.1. Introduction

In 2005 a discovery was made that a microbial fuel cell could be turned into a microbial electrolysis cell adding a small supplement of electricity at the cathode to produce products such as hydrogen gas (Rozendal et al., 2006, Liu et al., 2005b). This new technology has spurned much excitement and research into increasing the performance and gas yield of such reactors (Wang et al., 2011b, Sleutels et al., 2011, Cheng and Logan, 2011). The aim of this research being to achieve a commercially viable and sustainable means of treating waste organics (Oh et al., 2010, Rittmann, 2008, Clauwaert et al., 2008).

Substantial steps have been taken towards enabling the implementation of this technology. Low cost and more robust alternatives to many of the materials used in an MEC have been discovered such as stainless steel (Call et al., 2009) and nickel (Selembo et al., 2009a) cathodes. Alternative membrane materials have been trialled successfully (Rozendal et al., 2008c), as well as not using a membrane at all (Clauwaert and Verstraete, 2009). Anodes with greater surface areas have been found (Call and Logan, 2008) as well as methods to enhance the performance of the carbon anodes (Cheng and Logan, 2007b). New cell architectures and configurations have also helped improve performance (Cheng and Logan, 2011, Wang et al., 2010). Such developments have seen the performance of these reactors increase from hydrogen production rates of 0.01-0.1 m^3H_2/m^3reactor/day (Liu et al., 2005b, Rozendal et al., 2006) to 17.8 m^3H_2/m^3reactor/day (Cheng and Logan, 2011), although the same rise in not seen in the electrical recoveries of these systems 169% (Rozendal et al., 2006) 533% (Liu et al., 2005b) in the initial studies to 115% (Cheng and Logan, 2011) due to the higher input voltages used. All of this research has used acetate as a model compound.

Research with complex substrates is more limited. The ability of MECs to digest complex substrates has been proved such as domestic wastewater (Ditzig et al., 2007), piggery wastewater (Jia et al., 2010), potato wastewater (Kiely et al., 2011a) and end products of fermentation (Wang et al., 2011a, Lalaurette et al., 2009). Limited research has been conducted into the long term performance of MFCs and MECs, deterioration in performance of an MFC after a year of operation has been attributed to the gas

diffusion cathode (Zhang et al., 2011). Marine MFCs used as batteries to power offshore monitoring devices have been monitored for up to a year (Reimers et al., 2001, Tender and Lowy, 2004) and 18 months (Lowy et al., 2006), power production was maintained over this period although in two studies it did deteriorate steadily (Lowy et al., 2006, Reimers et al., 2001), and in another there were occasional drops in the output (Tender and Lowy, 2004). Such studies may not directly translate to MFCs or MECs used for wastewater, in a marine environment the ionic concentrations, gradients and flows will be different, as will the bacteria.

By analysing all the published papers in the area of MECs up to October 2011 the limited scope of how well we understand the long term performance of these systems especially when fed on real wastewaters becomes clear, as seen in Figure 5-1.In 26% of papers the duration of the experiment was not given. In many other cases this time frame is not stated explicitly but can be inferred using the tables, graphs and other information given. In relatively few articles the durability is highlighted as a factor. Two research articles have however been published which indicate the technology might have long term applicability with experiments lasting 9 months (Lee and Rittmann, 2010) and 8 months (Jia et al., 2010) , both running on acetate. Although several other studies do state a decline in performance over time (Jeremiasse et al., 2009, Rozendal et al., 2008b, Lalaurette et al., 2009, Hu et al., 2009).

With acetate fed reactors, 73% of all MEC studies, the time scales mentioned range from 4 to 6480 hours, with 1159 as the average. However when wastewater is used, (only 10% of laboratory studies) the range is between 12 and 184 hours, with an average of 122.5 hours, this time of operation is significantly different ($p=0.000$, two sample T test). For other substrates such as VFA’s and glucose the average run time is 276 hours. This is shown in Figure 5-1, the studies with no time frame stated are not included in the graph. The explanation for this disparity is not evident in the literature, in one study acetate and piggery wastewater are compared directly with acetate reactors running for 8 months and the experiments with wastewater lasting just 12 hours, no reason for this experimental procedure is given (Jia et al., 2010). There is a clear gap in this area of research.

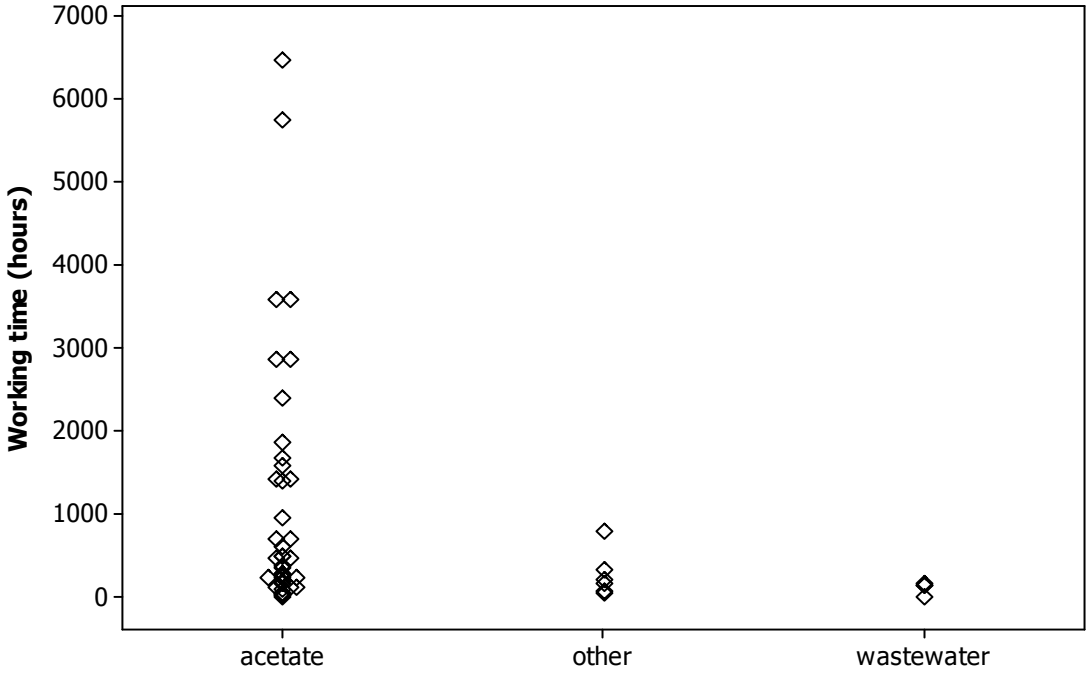

Figure 5-1 The working time of all MEC studies documented in the literature to date (Oct 2011), shown for the different substrates

If MECs are to be a viable and sustainable treatment option for the future then we need to gain an understanding of their long term performance with real wastewaters. Most of the research in MECs does not use real, or even complex artificial wastewaters, and most are run over a relatively short period of time. If this research is to translate into application, this relies on two key assumptions:

1. Real wastewaters containing mixture of simple and complex organic molecules will behave in the same way as acetate, a simple readily digestible molecule most frequently used in BES research. We know this not to be the case with anaerobic digestion (Rittmann, 2001).
2. A system that works at a particular efficiency for a short period of time will do for a long period of time. This is again unlikely as even with the clean technology of chemical fuel cells, long term durability tests have lasted around 4000 hours (166 days), although a couple of studies have extended this to 1.5 and even 3 years (Schmittinger and Vahidi, 2008). Failure is associated with blocked membranes, electrode deterioration and many other factors that may increase overpotentials. Biological systems have the added complexity of the behaviour of microorganisms.

Failure in laboratory batch fed wastewater reactors has been observed many times during preliminary laboratory testing. The aim of this research is to determine if wastewater fed MEC laboratory reactors are capable of operating over the same time periods as acetate fed reactors, and, if this is not the case, to identify the reasons why.

5.2. Method

5.2.1. *Reactor design and set up*

Double chamber MEC reactors (78 mL each chamber) were used which were of a tubular design, internal diameter of 40mm, length 60mm. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. The membrane between the reactor chambers was Nafion 117, with an area of 12.6cm^2. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 1 Ω resistor, 0.7 V supplied using a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong), and a multimeter to measure the voltage (Pico ADC-16), logged every 30 minutes onto a computer.

All reactors were cleaned and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK). The cathode media was 50 mM phosphate buffer, which was sparged with 99.99% pure N_2 for 10 minutes prior to being put into the reactors. The acetate based anode media used was that of Call and Logan (Call and Logan, 2008), during the tests where this was supplemented with protein, Aspargine was added to give an equivalent level of nitrogen to that measured in the real wastewater. The wastewater used was raw influent wastewater (post screens prior to primary sedimentation) from Cramlington wastewater treatment plant. The anode media was sparged for 10 minutes with N_2 prior to use. All reactors were initially acclimatised in MFC mode as per the method used in other studies (Call and Logan, 2008, Cheng and Logan, 2007a, Hu et al., 2008, Wagner et al., 2009), inoculated with 25 ml of raw wastewater and fed acetate media.

The gas produced by the cathode side was captured via a liquid displacement method in a 12 ml glass tube with a septa fitted to the top for sampling. The volume of this gas was measured by drawing it into a 5 ml gas tight syringe (SGE Analytical Science,

Australia). The anode gas was captured in an inverted 10 ml syringe placed into the top of the reactor and filled with the N_2 gas.

5.2.2. *Analytical procedures*

The following analysis was conducted in duplicate for both the effluent and influent of the cathode and anode liquids of each batch run. The chemical oxygen demand (COD) using standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) kit tubes. Volatile fatty acids (VFA's) were measured using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. The anion content using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The pH was measured using a pH probe (Jenway 3310, U.K.) and conductivity using an EC 300 probe (VWR Ltd, UK). The anode and cathode potential was measured using Ag/AgCl reference electrodes (BASI, U.K.) during each batch.

Hydrogen gas was measured on a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using triplicate injections of each sample, set against a three point calibration run once at the start of the measuring period and once at the end using standard calibration gases (Scientific and Technical Gases, U.K.). These gas measurements were verified using a Trace Ultra GC TCD with a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

GC-MS analysis of gaseous hydrocarbons, including halomethanes, was performed on a Agilent 7890A GC in split mode; injector at (280°C), linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software in full scan mode (10-150 amu/sec). A standard containing 100 ppm of three chloromethanes was injected (100ul headspace) followed by the reactor headspace samples (100ul) every 2 minutes. Separation was

performed on an Agilent fused silica capillary column (60m x 0.25mm i.d) coated with 0.25um dimethyl poly-siloxane (HP-5) phase. The GC remained at 30°C temperature for 90 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 20 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library if greater than 90% fit.

5.2.3. *Microbial analysis*

An assessment of the level of microbial activity occurring in the reactors was needed to give an understanding if failure was caused by a reduction or complete elimination of microbial activity, or conversely a competitive but non complementary microbial process. Methods involving the extraction and quantification of DNA from the anode biofilm were not suitable for this purpose as this would capture both the alive and active DNA and that DNA remaining on the biofilm from bacteria which were dead or inactive. Ribonucleic acid (RNA) is used within cells to convert DNA i.e. the genetic code into working proteins (Rittmann, 2001); it can therefore be used as a proxy for the amount of biological activity occurring in the cell (Milner et al., 2008, Low et al., 2000). As RNA is so susceptible to contamination and degradation, the simple and relatively quick approach of measuring the amount of nucleic acid extracted on a Nanodrop, and then comparing this directly to the amount of DNA extracted at the same time, would give the most reliable quantitative results.

Duplicate samples of anode material were taken for RNA and DNA extraction, from duplicate reactors sacrificed whilst working, and duplicate reactors after failure. The following procedure was carried out as quickly as possible inside a microbiological cabinet, to prevent the loss of RNA which readily breaks down if contaminated with RNases. All working areas and equipment was cleaned thoroughly with ethanol followed by RNase AWAY (Invitrogen Life Sciences, U.K.), including the anode cutting equipment which had also been washed with detergent and then heated to 240 ^{o}C for 4 hours in a furnace, prior to use. Each reactor at the point of sampling was taken into the microbiological cabinet maintaining the electrical circuit. The reactor was quickly dismantled and using a coring device duplicate 4mm diameter sections of the anode were cut and placed into a sterile RNase free 2 ml eppendorf, containing 1 ml of TRIzol Reagent (Invitrogen Invitrogen Life Sciences, U.K.), the sample was vortexed for 5 seconds to ensure complete submersion in the reagent, and then the samples frozen

at -80 ^{o}C. Duplicate cores were taken in the same way afterwards for DNA extraction and stored in 50:50 ethanol and phosphate buffer at -20 ^{o}C.

Extraction and clean-up of the RNA sample was then completed using a RNeasy Mini Kit (QIAGEN, Germany) as per the manufacturer's instructions. Once cleaned the samples were frozen at -20 ^{o}C. The DNA was extracted using a QBiogene FastDNA spin kit for soil (MP Biomedicals, U.K.) and also frozen in two samples at -20 ^{o}C. The quantity of nucleic acid present was then measured in duplicate on a Nanodrop Nanodrop 2000 (Thermo scientific, USA). The ratio of DNA to RNA could then be calculated for each sample.

5.2.4. *Experimental procedure*

Failure had been observed several times in these bench scale reactors used as MEC's when fed with wastewater. The purpose of these experiments was to determine if this failure was statistically significant, and if so to try and identify the particular cause. In total 12 wastewater fed reactors and 10 acetate fed reactors were used in this study, the materials and architecture of all the reactors were the same, and the same operating procedures observed throughout. The work was conducted at laboratory room temperatures of between 20-25 ^{o}C.

Initially 8 reactors were run, 4 of fed with acetate media and 4 with real wastewater. After each batch of 3-4 days the effluent was analysed for COD, VFA's, anions, pH and conductivity and the gas measured, the reactors were then refilled with N_2 sparged media to the anode and phosphate buffer to the cathode. Once having completed two batch runs producing gas, 2 reactors of each feed were sacrificed and the RNA and DNA were sampled, the remaining reactors were run and sampled as described until gas production ceased, or in the case of the acetate ones until they were stopped at 130 days.

A further experiment was conducted using 4 wastewater fed reactors to eliminate the possibility that a drop in pH in the wastewater fed reactors was causing failure. Duplicate reactors were run containing wastewater, and the same wastewater buffered to pH 7 using 50 mM phosphate buffer. All reactors were run in batch mode and samples as described above until gas production ceased. Examination as to whether the biofilm was damaged/killed during failure was gained by switching the failed MECs to MFC

mode (increased resistance and no external load), and refilling with UV sterilised wastewater (see Appendix V for details of this method).

Due to the observed drop in Cl^- ions prior to the point of failure, it was hypothesised that locally high levels of NH_4^+ at the anode, caused by the degradation of proteins present in the wastewater could be reacting with the chloride ions to form chloramines, which would then kill off the biofilm resulting in failure of the cell. This hypothesis was tested running 4 acetate fed reactors, by supplementing duplicate reactors with protein Aspargine at levels comparable to the wastewater levels as detected through the use of the TKN Standard Method 4500-Norg (APHA, 1998), comparing these to duplicate control reactors with no protein. Again sampling was carried out as above, in addition the effluent of the reactors was analysed for residual chlorine using the DPD test, Standard Methods 4500-Cl D, (APHA, 1998).

A further hypothesis to account for failure and the drop in chlorine was that the chlorination of organics, especially methane could be occurring in the reactors due to the potential of the anode. Under standard conditions, at pH 7 the required potential for chlorination of methane at a Cl concentration of 1 mM is 0.44 V, when considering that the reactors may have a pH slightly deviant from 7, and that the partial pressures of the methane and chloromethane produced would not be equivalent, it is conceivable that the anode potential needed for this reaction could be occurring in the reactors, producing chloromethanes and therefore removing the hydrogen ions from the system and eliminating H_2 production. Again 4 wastewater reactors were run in batch mode with the same analysis as described above, in addition both the anode and cathode gasses were captured and analysed for methane, hydrogen and chloromethane using the instruments and methods stated above. Duplicate reactors fed with acetate were run at the same time and subject to the same analysis. After failure reactors were again switched to MFC mode and the anode gas continued to be sampled.

5.2.5. *Calculations*

The reactor performance was evaluated in terms of the volume of hydrogen produced, and also the coulombic efficiency and electrical energy recovery. The definition of these two efficiencies can be found in section 6.2.5.

5.2.6. *Statistics*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

5.3. Results

5.3.1. *Time taken until failure*

The run time of the reactors is shown in Figure 5-2 as the amount of hydrogen produced at the end of each batch, the reactors terminated at 7 days for RNA sampling are not shown. It is seen that the Acetate fed reactors run for a longer period of time, including those supplemented with protein and produce more hydrogen than the wastewater reactors. The buffered wastewater reactors initially perform well, but then stop producing hydrogen after a short time period.

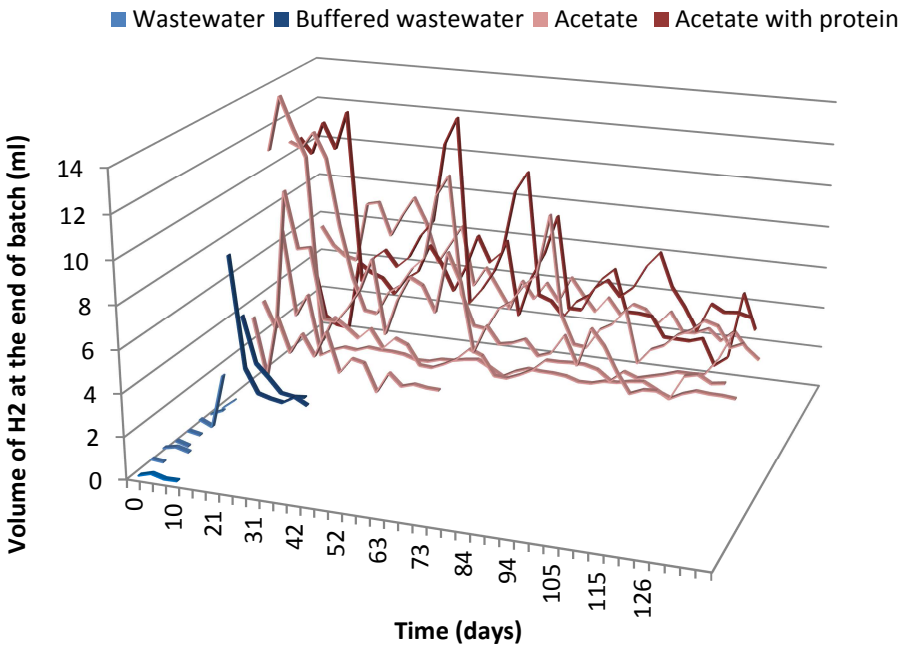

Figure 5-2 Graphic showing the working period of all reactors as indicated by the length on the line along the time axis, the volume of H_2 produced at the end of each batch is given on the y axis as an indication of reactor performance which is seen to be variable, where the line is discontinued this illustrates zero H_2 production and the reactor is deemed to have failed

All 10 of the reactors fed on wastewater failed within 7-17 days of operation, failure was determined by no measureable gas production at the cathode. Of the 8 acetate fed reactors one failed at 56 days, but the others remained functioning until the experiment was terminated after 130 days. With 130 days used as the minimum run time for the acetate fed reactors, the difference in time to failure is significant ($p=0.000$, two sample t-test) as shown graphically in Figure 5-2.

5.3.2. *Reactor performance*

The average performance data collected over the duration of different experiments is shown in Table 5-1. The acetate fed cells have a greater coulombic efficiency and electrical energy recovery. The COD removal is reasonably similar for all substrates, but higher for the buffered wastewater, although this does not translate into improved coulombic efficiency or energy recovery. In all cases there is a large degree of variation, as is seen by the standard deviations. This is also seen through the hydrogen production data in Figure 5-2, which is higher for the acetate fed reactors, but does deteriorate throughout the test period.

Table 5-1 Summary of reactor performance using three different parameters other than H_2 production for the experiments using different substrates, values are the average values of all the reactors run on the given substrate

	COD removal	Coulombic Efficiency	Electrical Energy Recovery
Wastewater	23.2% ± 12.2	7.5% ± 3.9	15.7% ± 20.1
Buffered wastewater	43.8% ± 7.8	3.7% ± 1.7	13.5% ± 16.6
Acetate	28.6% ± 11.5	10.9% ± 2.0	33.0% ± 15.1
Acetate with protein	32.3% ± 13.4	10.4% ± 3.6	35.1% ± 22.9

Values represent average of all the batch experiment run on the given substrates where hydrogen was produced, ± one standard deviation.

There is a reduced performance between the acetate fed reactors as compared to the wastewater ones of around 50 % if energy recovery is considered.

5.3.3. *Biological processes*

The average RNA: DNA ratio of the duplicate samples show that there is significant difference between the working and failed reactors at the 90% confidence interval ($p=0.068$ two-sampled t-test). This difference is more pronounced with the wastewater fed reactors, where the average ratio value for the working reactors is 11.5 compared to the failed reactors 3.9. The acetate working reactors have an average a ratio of 6.1, with the single failed cell being 4.2.

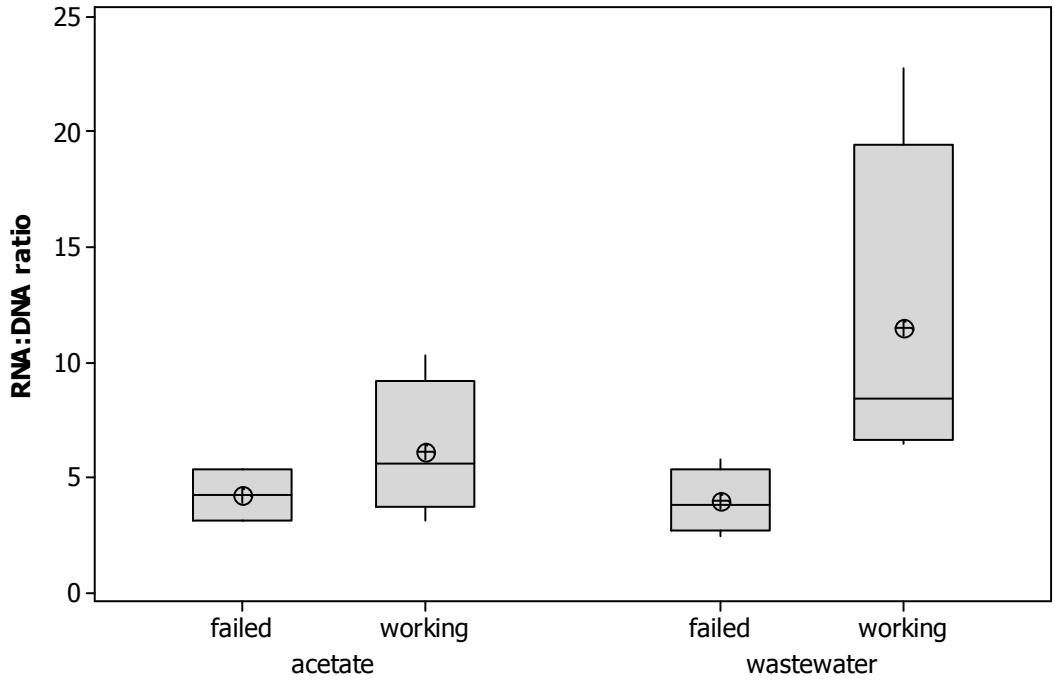

Figure 5-3 Box plot of the RNA: DNA ratios of failed and working reactors fed with both acetate and wastewater, the data represents a summary of the duplicate samples taken from duplicate reactors (i.e. four samples in total) with the central line representing the median and the mean given by the circle with cross

5.3.4. *Low pH*

In the wastewater fed reactors, which contained no additional buffering, it was observed that at around the point of failure there was a decline in the pH of the anode effluent from a starting value 6-6.5 to around 5.5. The acetate fed reactors, (the nutrient media containing 50mM pH 7 phosphate buffer) did not show any significant fall in pH during the full time period over which their function was monitored.

With the additional duplicate reactors fed on wastewater and buffered wastewater there was the same observed drop in pH with the non-buffered reactors. The buffered reactors kept a constant pH and initially performed better but then also failed within 17 days of operation. No significant difference in the run time between the buffered and non-buffered reactors ($p=0.306$, two sample t-test).

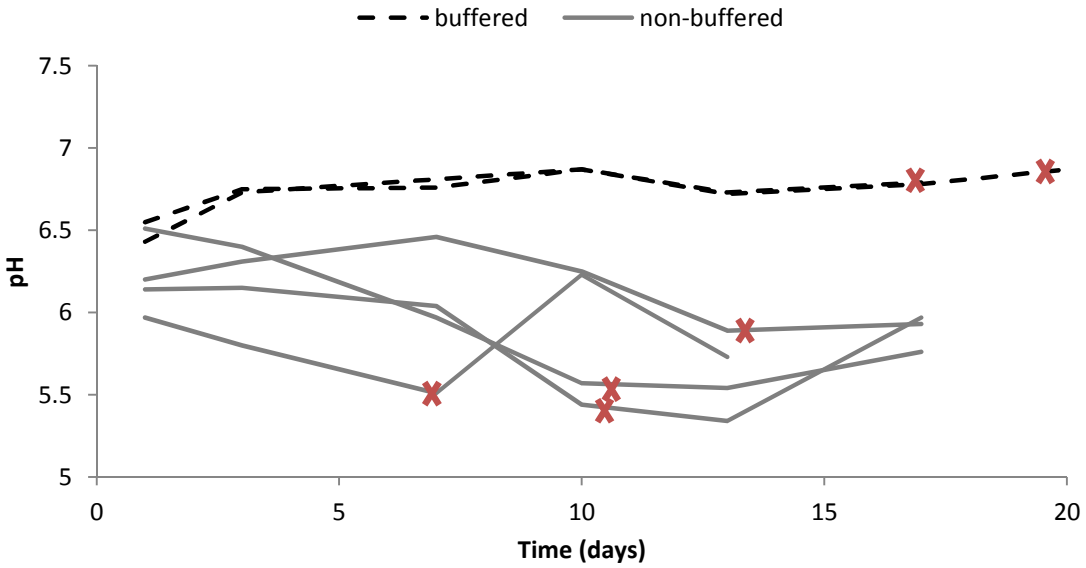

Figure 5-4 Measured pH of the wastewater reactor liquid during the course of the batch experiments, the point of failure is denoted by the red cross where gas production ceased

5.3.5. *Toxic build up within the reactors*

The full anion analysis of the cell effluent showed that there was a fall in chloride ions prior to failure of the wastewater reactors. Both the acetate media and the wastewater contained approximately 250-300 mg/L of chloride. During the course of each batch run with the acetate fed reactors, approximately 50 mg/L of the chloride would be taken up in the reactor, this remained relatively constant throughout the full time period the acetate reactors were operated for. However in the wastewater reactors, when working and producing hydrogen, the chloride removal in the cell was observed to be virtually complete prior to the reactor failure, i.e. 250-300 mg/L of chloride ions were being removed. The levels of chloride in the cathode compartment of these reactors remained the same as the original influent. After failure of the reactors when no hydrogen was produced, this chloride removal stopped. The only wastewater reactors that this drop was not observed in were the duplicate buffered wastewater reactors, here chloride removal remained constant at around 50-100 mg/L during each batch, the reactors did however also fail.

In the acetate reactors supplemented with protein the chloride removal remained roughly constant throughout the experiment at between 50-100 mg/L, and the reactors did not fail. No chloramines could be detected in the effluent of these reactors, disproving the hypothesis of chloramine formation. The performance of the protein

supplemented reactors in terms of electrical energy recovery was not significantly different to the non-supplemented ones (p=0.376, two sample t-test).

Further evidence that a toxic chlorine based product was not being formed was gained using four failed wastewater reactors, duplicate reactors were refilled with UV sterilised wastewater non sterile wastewater, put into MFC mode, i.e. increased resistance and no external load. With all four reactors biological activity started within 1 hour, and reached a level of current production as would be expected of a fully acclimatised MFC cell using the same cell materials. The electrogenic biofilm was capable of functioning. After one batch in MFC mode, the reactors were then all returned to MEC mode, where no gas was produced and the failed status continued. In MFC mode, the chloride removal was relatively constant again at around 50 mg/L.

5.3.6. *Formation of halogenated organics*

Analysis of the headspace gas for 4 wastewater fed reactors and 2 acetate fed did not show detectable levels of halogenated organics, levels were below 0.01% of the 10 ml headspace. This was the case for wastewater fed reactors before, during and after failure and for acetate fed reactors. The same observed drop in chlorides was seen in these reactors.

5.3.7. *Other factors*

The analysis of VFA's in the effluent of the reactors showed that in all cases for both acetate and wastewater there was some acetate remaining at the end of each batch. There was no acetate in the influent wastewater, but always a small amount 20-40 mg/L in the effluent of these reactors, this did not alter once the reactors had failed.

The conductivity for the wastewater was around 1.8 mS, the buffered wastewater was 6.3 mS, and the acetate media was 5.9 mS. The conductivity of the reactor effluent was on average 1.6 mS for the wastewater fed cells both before and after failure even when a drop in chloride ions was recorded, the average for the buffered wastewater cell effluent was 5.5 mS and again did not change after failure, the acetate cells also showed a slight drop in conductivity of the effluent to 5.2 mS.

The production of methane at the anode of the reactors was on average 0.002 ml for the wastewater reactors when working, after failure this increased slightly to 0.029 ml. The methane production remained relatively constant throughout the course of the

experiment and the slight rise after failure is not likely to represent a competitive biological process which is the cause of cell failure, as the average methane production in the acetate fed cells was always higher at 0.072 ml per batch, and also the converted MFC cells that functioned well, also produced on average 0.035 ml per batch.

The materials used in these reactors that could become degraded during use, i.e. the cathode and membrane, could be directly and successfully re-used in a new cell, the failure was not due to cathode degradation or membrane clogging. In addition, by increasing the applied voltage of the reactors from 0.7 V to 1.0 V immediately after failure, thus combating any increased overpotentials that could have built up during the short operation period, the reactors could not be revived and did not produce hydrogen. Failure was not therefore caused by the simple the deterioration of the cell components.

5.4. Discussion

Small laboratory scale wastewater fed reactors fail after a short period of time whereas acetate fed reactors do not. This is significant. The cause of this failure could not be identified during the course of this study. Relatively ‘simple’ explanations such as degradation of electrodes or membranes, a drop in conductivity, or lack of available VFA’s have been ruled out as possible causes of failure.

A further hypothesis that failure of the reactors is caused by a reduced or eliminated level of electrogenic activity in the reactors was also seen not to be the case. If true this hypothesis would result in the reduced DNA:RNA ratio observed and low current production. However once failure had occurred the reactors could be instantly ‘revived’ by switching them into MFC mode. The electrogenic bacteria were therefore present on the electrode and were capable of donating electrons.

The hypothesis that there is a competitive biological process occurring such as methanogenesis, as suggested in other studies (Cusick et al., 2011), has been shown not to be the case. The RNA to DNA ratio indicates a reduced biological activity in the failed wastewater cells, suggesting that the biofilm is less able to function and metabolise after failure. It is not likely that a non-complementary competing biological activity is taking over the reactor and eliminating the MEC process. It can be seen that there is greater activity in the wastewater reactors than the acetate reactors, this might be an indication of the greater and more multi-layered metabolism that has to occur in

these reactors when fed complex substrates. It is also observed that the failed acetate reactor did not differ significantly to the working ones, suggesting the reason for failure here was different to that for the wastewater reactors. Additionally the levels of methane generated in the wastewater reactors after was less than in the working acetate reactors. A competitive process such as methanogenesis is therefore unlikely to be the cause.

The hypothesis that a low pH was causing failure, either through altering the electrochemistry or affecting biological function is shown not to be correct. The simple experiment adding buffer to the wastewater also resulted in failure despite initial improvement in reactor performance, here the drop in chloride was not observed. The slightly lowered pH is likely to have a detrimental effect on the cell though. The pH measurement taken is of the whole of the liquid in the reactor, in reality the pH near the anode may be greater. Such a pH will impact on the microorganisms present and the electrochemical reactions within the cell, as pH is a logarithmic function of the concentration of H+ ions, then even a small change in this value has a large impact on the overall thermodynamic balance of the system as is calculated via the Nernst equation. Torres et al (2008) found that an increase in phosphate buffer in the anode media lead to a thicker biofilm and greater current generation in a microbial fuel cell due to the increased diffusion of H^+ out of the biofilm layer, thus making it more accessible to transport to the cathode. Although pH could be limiting the performance of non-buffered reactor it is not the cause of failure.

The formation of halomethanes such as chloromethane could potentially occur at the potentials within these reactors account for the loss of chloride and would cause failure as these compounds are toxic. This would fit the pattern of failure exhibited in the reactors as it would take some time for the levels of methane to build up which could then be converted to the halomethanes, this would ‘use up’ the H^+ ions in the anode section and H_2 would cease to be produced at the cathode. However no chloromethanes could be detected in the headspace gas of these reactors, (below 0.01%) either before or after failure, in fact no halogenated organics could be detected. Additionally the acetate fed cells did not fail when supplemented with protein, and most importantly the exoelectrogenic biofilm is able to work as an MFC after failure so has not been killed. It could be possible that the negative chlorine ions were simply temporarily attracted to the positive anode during the operation of the fuel cell, and therefore not measured in the bulk liquid of the cell. This would account for the observed ‘disappearance’ of the

chloride ions, but is not likely to affect the performance of the cell. The range of analysis carried out indicates that failure is not caused by a chlorine effect; the observed chlorine drop is simply co-incidental to the failure.

The problem of failure needs to be resolved. If MECs are to be a useable technology they need to function with real wastewater. Studying these systems when they are prone to sudden and rapid failure is difficult, therefore identifying the reasons for failure, solving them, and increasing efficiency becomes very challenging. This difficulty leads to acetate being used in most research as this does allow greater scope for experimentation. However it is clear that the processes operating in a reactor fed with real wastewater are different to those occurring in a reactor fed with acetate. The acetate research will not directly inform us of performance with wastewater.

The failure in wastewater fed, laboratory scale, batch fed reactors has been proved, but the reason not identified. Conversely, as part of this research, a larger scale MEC run in continuous mode at a wastewater treatment site fed on raw wastewater has worked producing almost pure hydrogen for a period of over 3 months, (see chapter 6). It is likely that something is occurring within the small batch reactors to prevent either the production of hydrogen ions at the anode, the transfer of these ions, or the hydrogen evolution reaction at the cathode. It may be the case that at this small scale and fed with batch mode that the system and in particular the microbial community involved is fragile and unable to adapt to change, and therefore a build-up of something at an undetectable level has catastrophic consequences. Further work is still needed to identify the cause of this failure, and therefore be able to take steps to resolve it. This can only be done by using real wastewater rather than simple artificial media. The long term performance of wastewater fed MECs is a research gap that must be filled.

Chapter 6. Production of hydrogen from domestic wastewater in a pilot scale microbial electrolysis cell

Addressing the need to recover energy from the treatment of wastewater the first working pilot scale demonstration of a wastewater fed microbial electrolysis cell is presented. A 120 litre (L) microbial electrolysis cell (MEC) was operated on a site in Northern England, using raw domestic wastewater to produce virtually pure hydrogen gas for a period of over 3 months. The volumetric loading rate was 0.14 kgCOD/m^3/day, just below the typical loading rates for activated sludge of 0.2-2 kgCOD/m^3/day, at an energetic cost of 2.3 kJ/gCOD, below the values for activated sludge 2.5-7.2 kJ/gCOD. The reactor produced an equivalent of 0.015 L H_2/L/day, and recovered around 70% of the electrical energy input, with a coulombic efficiency of 55-60%. Although the reactor did not reach the breakeven energy recovery of 100%, this value appears well within reach with improved hydrogen capture, and reactor design. Importantly for the first time a 'proof of concept' has been made, with a technology that is capable of energy capture using low strength domestic wastewaters at ambient temperatures.

6.1. Introduction

In an era of increasing energy costs and environmental awareness, wastewater treatment industries need to look at alternative treatment options to reduce their energy bills. It has been estimated that domestic wastewater alone may contain 7.6 kJ/L of energy, while stronger industrial wastewaters contain substantially more (Heidrich et al., 2011). There is an increasingly urgent need to recover some of this energy, or at the very least not expend additional energy on treatment; the activated sludge process uses 2.5-7.2 kJ/gCOD (Pant et al., 2011). Energy recovery could be achieved through anaerobic digestion to methane gas or microbial fuel cell technology directly to electricity; however life cycle assessment has shown that the production of a higher value product through the suite of bioelectrochemical systems (BES) may be the most viable solution (Foley et al., 2010). One such technology is the production of hydrogen in a microbial electrolysis cell (MEC) (Rozendal et al., 2006).

Since the MEC process was first reported (Rozendal et al., 2006, Liu et al., 2005b) MECs have emerged as a potential technology option for a new generation of wastewater treatment systems (Rozendal et al., 2008a). In an MEC bacteria use the

energy stored in the organic compounds of wastewater to metabolise and grow, donating electrons to an electrode (Rozendal et al., 2006). The electrons then travel in a circuit producing current and therefore electrical power; in an MEC these electrons are consumed at the cathode along with a supplement of electrical power. The H^+ ions also created by the breakdown of organics at the anode travel across the microbial fuel cell membrane to the cathode. Here they can combine to form H_2, however this process is endothermic requiring energy, so a supplement of electrical energy is added to the system to allow it to take place (Liu et al., 2005b).

Fuel cell technologies may offer a sustainable future for wastewater treatment, although there are still many hurdles to overcome. Progress is being made with new reactor design (Call and Logan, 2008, Rozendal et al., 2008b), improved materials (Cheng et al., 2006a, Cheng and Logan, 2008), greater understanding of the mechanisms involved (Aelterman et al., 2008, Clauwaert et al., 2008), and even improved understanding of the microbes that are at work in these systems (Holmes et al., 2004, Kim et al., 2004, Lovley, 2008, Rabaey et al., 2004). Most of this research is performed at laboratory scale, using simple substrates, often at a controlled warm temperature. Many problems have been overcome, such as validation of using multi electrode systems (Rader and Logan, 2010) and finding a low cost alternative to the platinum cathode (Zhang et al., 2010). Although of great value in improving our understanding of MEC's, these studies do not tell us about the challenges or even benefits of running such systems at a larger scale with real wastewaters in temperate climates. There is a need to demonstrate that these systems can work at a larger scale and under realistic conditions, elevating the technology from a laboratory curiosity into a practical solution to an industrial problem.

A pioneering study by Cusick et al (2011) published on the largest MEC reactor to date, a 1000 L pilot scale reactor at a winery in California. The reactor proved slow to start up with pH and temperature control being problematic. When these issues were corrected by heating to 31 ^{o}C and the addition of buffer and acetic acid, the reactor did improve in performance. The energy produced during the operation exceeded the input energy (heating not included), but this was primarily due to methane production (86%) with only trace amounts of hydrogen. Methane production was attributed to the reactor being membraneless allowing hydrogen produced at the cathode to be directly consumed by hydrogenotrophic methanogens within the reactor. The reactor performance tailed off at around 90 days, when the heating unit broke (Cusick et al., 2011). The study has

provided valuable insights into the operation of MECs: (i) the membraneless systems that work well at laboratory scale and when fed in batch mode may not be so good at larger scale and under continuous feed, and (ii) inoculation and start-up are important parameters.

Addressing the issue of a membrane is critical to reactor performance. Most laboratory scale membrane systems use Nafion 117 (Logan et al., 2006), an expensive and delicate proton exchange membrane (Logan et al., 2006); this would be both impractical and costly on a large scale. Also the high efficiencies published: 406% electrical energy recovery (the amount of electrical energy put in that is recovered, this can be higher that 100% as there is also substrate energy within the system) and 86% total energy efficiency (the amount of substrate and electrical energy recovered) (Call and Logan, 2008) are from membrane-less systems. The lack of membrane greatly reduces the resistance in the cell, improving the transmission of protons to the cathode. Membrane systems have lower efficiencies: 169% electrical energy recovery and 53% overall energy efficiency has been reported (Rozendal et al., 2006). These efficiencies are likely to decrease further with time as the membrane becomes fouled.

The issues of inoculation and start-up are poorly understood (Oh et al., 2010) Although the use of acetate is likely to reduce the acclimatisation period (Cusick et al., 2011). However the biological community needed for the degradation of complex substrates is thought to be different to that needed for acetate (Kiely et al., 2011c). A community of acetate degraders able to work at 30 ^{o}C is not likely to be the community needed to degrade wastewater at ambient UK temperatures. There is evidence in the literature that microbes exist that are able to digest wastewater (Ditzig et al., 2007) and operate at low temperatures (Lu et al., 2011). Like anaerobic digestion, however, it may well be that a long period of acclimatisation is needed and unavoidable to achieve a stable community (Rittmann, 2001).

If these start-up issues can be resolved, then the reactor in theory will function, however it would also need to reach a neutral or positive energy balance, i.e. recovering all the electrical energy input plus a substantial fraction of the substrate energy input.

To test whether these systems have a chance of achieving these goals under realistic conditions, a pilot scale 120 L reactor was placed on a wastewater treatment site in

North East England. This site takes in primarily domestic wastewater with an average Total COD of 450 mg/L. The reactor was built using low cost alternatives to the standard lab materials used for the cathode and membrane. The reactor was not heated, held inside a large unheated building, and run throughout a UK spring and summer (5-20 ^{o}C minimum and maximum temperatures) and is still in operation at the time of writing this paper. These operating conditions are likely therefore to represent close to a worst case scenario i.e. low concentration feed; non optimal components; no heating; and no additional supplement of acetate or buffering capacity after the initial acclimatisation period.

Working closely with partners at Northumbrian Water Ltd. the aim of this study was to establish reactor operation and to determine if a neutral or positive energy recovery is achievable. From that data we can evaluate if MEC technology is likely to be a viable treatment option for the future.

6.2. Methods

6.2.1. *Field Site*

The pilot scale reactor was set up and run at Howdon wastewater treatment site, situated near the city of Newcastle Upon-Tyne in the North East of England (54^{o}58'N, 01^{o}36'W). An average of 246500 m^{3} of domestic wastewater is treated daily, using 96 MWh; the activated sludge process uses around 60% of this. The wastewater used in the MEC was taken from the grit channels after primary screening, but before settling.

6.2.2. *MEC reactor*

The reactor was based on a cassette style design, with six identical cassettes being placed into a rectangular reactor with a total working volume of 120 L. The tank has a Perspex plate fitted over the liquid layer giving a small head room to the anode compartment of 2.2 L. Each of the cathode gas tubes from the cassettes projected above this Perspex sheet. The cassettes were set along alternate sides of the reactor to allow s-shaped flow, and once in place gave a final anode volume of 88 L.

Each cassette was constructed using 10 mm thick plastic sheeting and consisted of an internal cathode section 0.280 m by 0.200 m by 0.048 m deep, of a volume 2.6 L. The cathode material was stainless steel wire wool grade 1 (Merlin, UK), 20g was used in each cathode, giving a projected cathode surface area for each electrode of 0.056 m^{2}. A

0.8 m length of stainless steel wire was wound several times into the wire wool to make a firm electrical connection, and then to the outside of the cell. Each cathode electrical assembly had an internal resistance from the extremities of the wire wool to the end of the exposed wire of less than 2.75 Ω. The cathode was separated using a membrane wrapped around a plastic frame inserted into the electrode assembly on both sides. The membrane used was RhinoHide® (Entek Ltd, UK), a durable low cost microporous membrane traditionally used as a battery separator. The anode material was a sheet of carbon felt (Olmec Advanced Materials Ltd, UK), 0.2 m wide by 0.3m high and 10 mm thick. This was sandwiched between two sheets of stainless steel mesh acting a current collector. The anode assemblies were also connected by a 0.8 m length of stainless steel wire fed through the centre of the felt material, each electrode having an internal resistance less than 3.4 Ω.

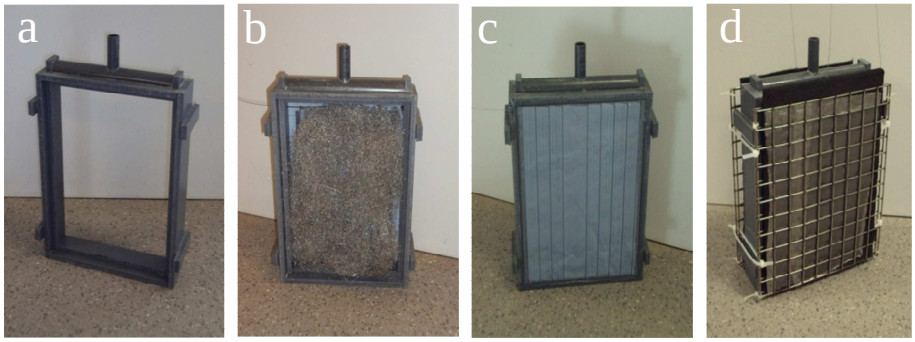

Figure 6-1 Photographs of the electrode assembly unit – a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane, d) anode with wire mesh current collector

The gas production from the anode compartment was captured from the ports in the Perspex lid, using 3mm ID PVC tubing (VWR Jencons, UK). The cathode gas was initially captured using 4mm annealed copper GC tubing connected to each cathode compartment using copper compression fittings, (Hamilton Gas Products Ltd, Northern Ireland), due to rapid corrosion this was later replaced with 3mm ID PVC tubing (VWR, UK). Both pipelines contained a gas sampling port.

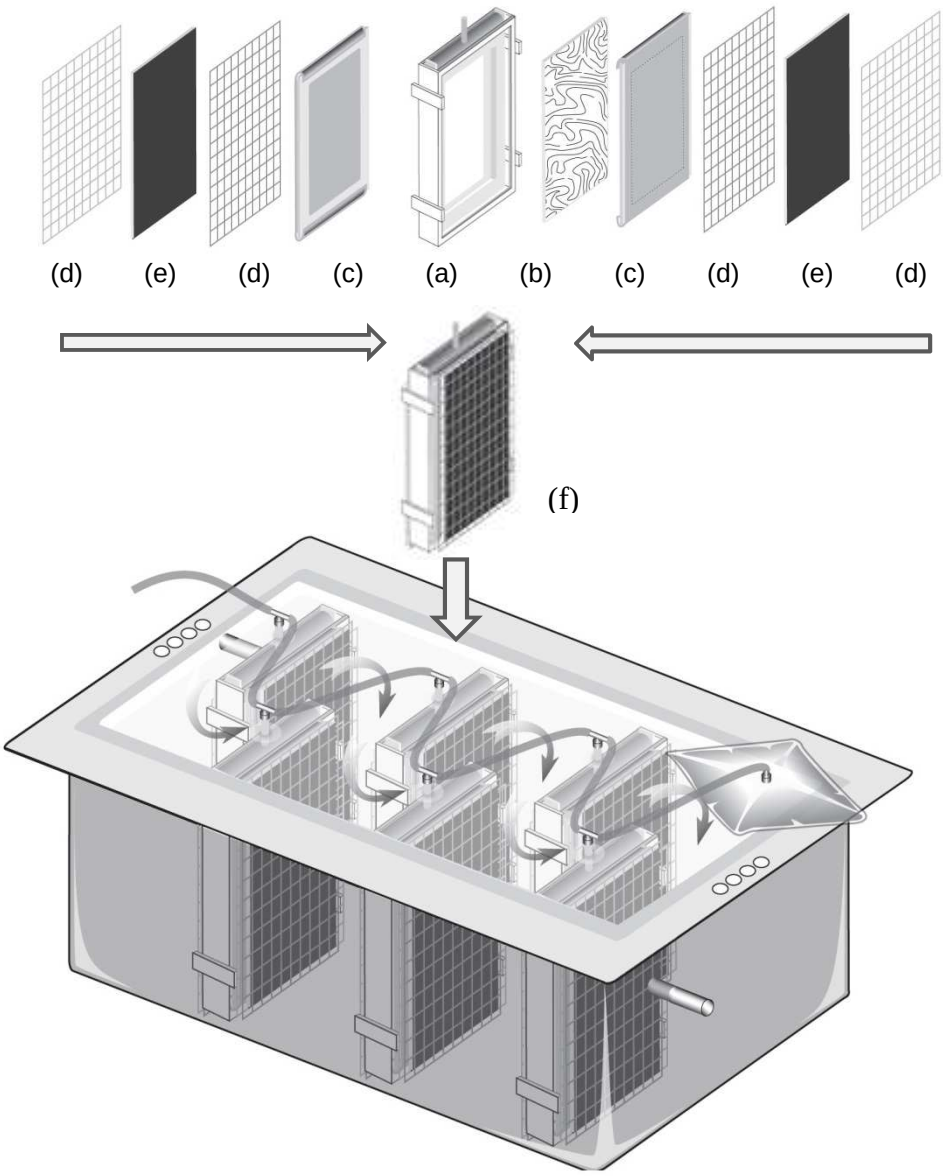

Figure 6-2 Schematic diagram of the reactor module components, a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane fixed around a PVC frame, d) stainless steel wire mesh, e) anode with wire mesh current collector. These component fit together to form a single module (f), six of these go into the reactor vessel where wastewater flows around them. Gas is collected through tubing into a gas bag

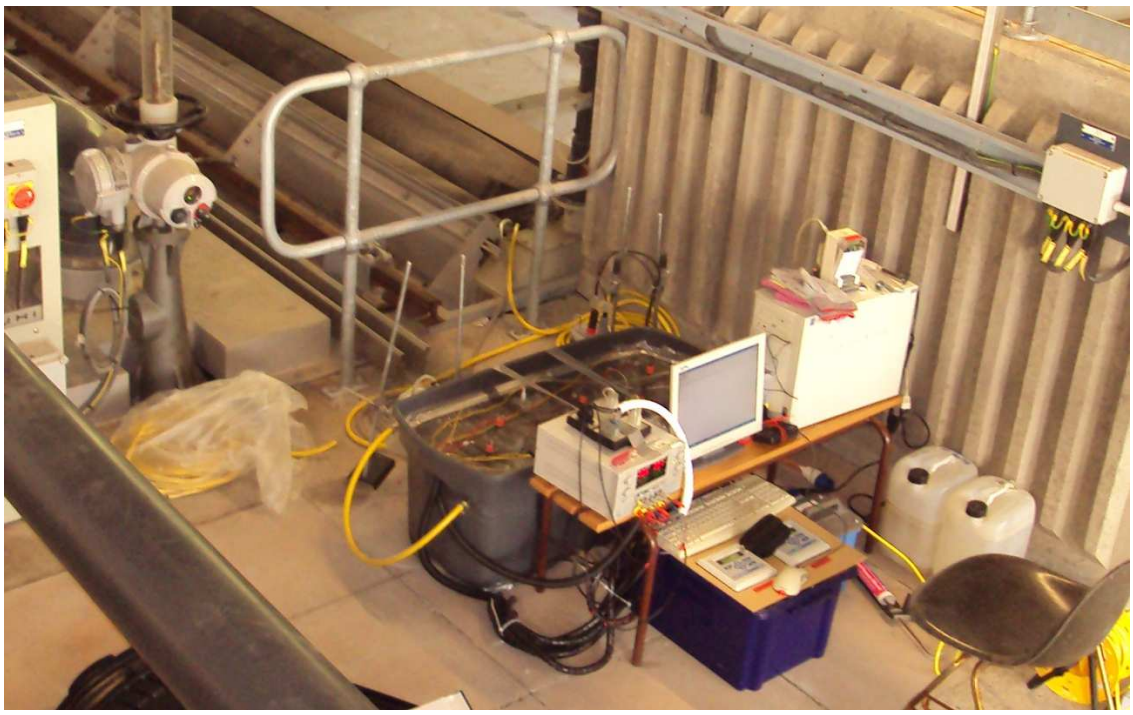

Figure 6-3 Photograph of the reactor in situ at Howden wastewater treatment site the grit lane where the influent was drawn from is seen in the top left hand corner of the picture

The reactor was situated on site in a large unheated building housing the grit channels, wastewater was pumped from the grit channels into a preliminary storage tank, providing some primary settling. During operation a peristaltic pump (Watson Marlow 520S, UK) was used to pump water into the storage tank, where it could then flow into and through the reactor, and back out to the grit channels via a smaller sampling tank at the end. These tanks were used for sampling and monitoring of the influent and effluent.

6.2.3. *Analytical procedures*

Power was provided to the electrodes using a PSM 2/2A power supply (Caltek Industrial Ltd, Hong Kong), the voltage of each cassette was monitored across a 0.1 Ω Multicomp Resistor (Farnell Ltd, UK) using a Pico AC-16 Data Logger (Pico Technology, UK), and recorded on a computer every 30 minutes.

In both the influent settling tank and the effluent tank the dissolved oxygen (DO) and pH were measured using pH and DO submersion probes (Broadley James Corporation, USA) connected to a pH DO transmitter (Model 30, Broadley James Corporation, USA), feeding an electrical output to a Pico EL 037 Converter and Pico EL 005 Enviromon Data Logger (Pico Technology, UK); these data were recorded onto the

computer every 30 minutes. Temperature was logged using 3 EL-USB-TC Thermocouple data logger (Lascar Electronics, UK) placed in the settling and effluent tanks and one placed in the reactor itself.

The gas pipelines were connected to optical gas bubble counters (made 'in-house' at Newcastle University), giving a measurement of gas volume. The operation of these counters failed after several weeks of operation. They were replaced with 1 L and then 5 L Tedlar gas bags (Sigma Aldrich, U.K.); the volume of gas was then measured by removal from the bags initially using a 100ml borosilicate gas tight syringe, and then using a larger 1 L glass tight syringe (both SGE Analytical Science, Australia). The sampling ports on each pipeline were initially used to take a sample of cathode gas 3 times a week, into a Labco Evacuated Exetainer (Labco Ltd, UK). Once gas production had risen to a higher volume, 2 L of the cathode gas was dispensed from the collecting gas bag into another 5L gas bag which was taken away for analysis. Anode gas was not measured volumetrically due to leakage but was sampled directly from the anode compartment into a 3 ml exetainers for compositional analysis.

Hydrogen gas was measured using a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using duplicate injections, set against a three point calibration. These gas measurements were verified using a Trace Ultra gas chromatograph (GC) with a thermal conduction detector (TCD) and a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements for anode and cathode gas were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

To ensure accuracy calibration standards used for the gas measurements were injected into a Labco evacuated exetainers in the laboratory at the same time (+/- 10 minutes) as the samples taken in the field. Tests carried out previously had indicated that these containers were not completely gas tight especially for hydrogen. This procedure did not have to be carried out for the cathode gas once operation had been switched to gas bags.

Liquid samples of the influent and effluent were taken 3 times a week. The total chemical oxygen demand (COD), and soluble chemical oxygen demand (SCOD) were measured in duplicate using standard methods (APHA, 1998) (Spectroquant ® test kits, Merck & Co. Inc., USA). Volatile Fatty Acids (VFA's) were determined using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The conductivity of the solution was measured using a conductivity meter, EC 300 (VWR Ltd, UK).

6.2.4. *Start up and operation*

The reactor was initially started up in batch mode, allowing all the oxygen, nitrates and sulphates within the wastewater to be consumed. Based on the lessons learnt from the previous pilot study, (Cusick et al., 2011), (Logan, B.E. personal communication),the wastewater was supplemented with acetate at a concentration of 0.5g/L. The applied voltage of 0.6 V was provided by a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong). The dosing was repeated and the reactor refilled after a 2 week period, during which time no gas production was observed.

6.2.5. *Efficiency calculations*

Four efficiency calculations are made in this study on the basis of the electrical and substrate energy used (Logan, 2008).

(i) Electrical energy recovery (η_E)- Energy recovery is the amount of electrical energy put into the reactor that is recovered as hydrogen.

The electrical energy input W_E is calculated as:

$$W_E = \sum_{1}^{n} (I\ E_{ps} \Delta t - I^2 R_{ex} \Delta t)$$

Where I is the current calculated for the circuit based on the measured voltage E and external resistor R_{ex} ($I=E/R_{ex}$), E_{ps} is the applied voltage of the power supply, this value is adjusted for the losses caused by the external resistor (I^2R_{ex}), which in reality are negligible. The time increment denoted by Δt represents the conversion of samples taken every 30 minutes into seconds. The data is summed for all 6 cells over the each batch cycle. The output of energy (W_{out}) is calculated from the measured moles of

hydrogen produced $N_{H2,}$ and the standard higher heating value of hydrogen of 285.83 kJ/mol ΔH_{H2}.

$$W_{out} = \Delta H_{H2}\, N_{H2}$$

The higher heating value is chosen over the lower heating value which takes into account the heat lost through the production of water vapour during burning. It is expected that this H_2 product would be used either as a commercial product for industry, or in a clean H_2 consuming fuel cell to create electricity, not for combustion. Methane could also be added to this value to further increase the quantity of output energy, but was not included for these same reasons.

Total Energy recovery (excluding pump requirements) can then be calculated as follows:

$$\eta_E = \frac{W_{out}}{W_E}$$

(ii) Total energy efficiency (η_{E+S}) the amount of input energy both electrical and substrate that is recovered as hydrogen.

The substrate energy (*Ws*) is calculate as

$$W_s = \Delta COD\, \Delta H_{ww/COD}$$

Where ΔCOD is the change in COD in grams, estimated as the difference in COD of the influent and effluent at the end of each batch. $\Delta H_{ww/COD}$ is the energy content per gCOD as measured on similar domestic wastewater of of 17.8 kJ/gCOD (Heidrich et al., 2011). Total energy efficiency is then calculated as:

$$\eta_{E+S} = \frac{W_{out}}{W_E + W_S}$$

(iii) Coulombic efficiency (CE) - the amount of hydrogen produced compared to the amount theoretically possible based on the current, or total charge passing through the cell.

Theoretical hydrogen production based on current (N_{CE}) is calculated as:

$$N_{CE} = \frac{\sum_1^n I\Delta t}{2F}$$

Where *I* is the current calculated from the measure voltage, Δt is the conversion of the time interval 30 minutes to 1 second to give coulombs per data sample, this is then summed over the 6 cells for the whole batch. Faradays constant (*F*) is 96485 coulombs/mol e^-, and is the moles of electrons per mole of hydrogen. Coulombic efficiency *CE* is then calculated as:

$$CE = \frac{N_{CE}}{N_{H2}}$$

(iv) Substrate efficiency - the amount of hydrogen produced compared to the amount theoretically possible based on substrate removed in the reactor.

Theoretical hydrogen production based on substrate removal *(N_S)* is calculated as:

$$N_S = 0.0625\, \Delta COD \Delta t$$

As 64 gCOD can be converted to 4 moles H_2, each g COD is equivalent to 0.0625 moles H_2. The change in COD is measured at the end of each batch, and used to calculate the total COD removed from the 88 L reactor over the duration of the sampling period based on a HRT of 1 day. Substrate efficiency is then calculated as:

$$S_E = \frac{N_S}{N_{H2}}$$

The (η_E) correlates directly to the coulombic efficiency (CE) by re-arrangement of their respective equations. It is assumed that the phrase $I^2 R_{ex} \Delta t$ in calculating W_E is negligible by comparison to the first term (this is observed to be the case in practice):

$$\eta_E = \frac{\Delta H_{H2} \times 1000}{2F \times E_{ps}}\, CE$$

This means halving the E_{ps} doubles the η_E if the CE can be maintained. An increase in CE at the same E_{ps} causes a linear increase in η_E.

6.2.6. *Statistical analysis*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

6.3. Results

6.3.1. *Reactor design and resistance limitations*

The internal resistance of a BES design is critical to its performance. Resistance is mainly caused by electrode overpotential and ohmic losses in the liquid, although there may also be losses in the bacterial transfer etc. as shown in Figure 1.2. These losses impact on the amount of energy that can be gained in and MFC and the amount for energy needed in an MEC, these effects are even greater in a scaled up system where losses become proportionally more significant (Rozendal et al., 2008a). Within the cell

designed the anode and cathode, although separated by a membrane, were relatively close together, with around 1cm distance between them, this will have minimised the ohmic losses within the liquid phase (i.e. the resistance in the movement of ions from the anode to cathode) which is especially important when using real wastewaters with no artificial increase in liquid conductivity.

However the electrode resistance with this design is high, with the cathode having a resistance of 2.8Ω and each anode sheet being 3.4Ω from the extremities of the electrode to the end of the connecting wire. With a total anode surface area for the whole reactor of 0.76 m^2 and a further 0.3 m^2 of cathode, these resistances will have a large impact in reducing the efficiency of the reactor performance. With a 0.6V load, as would be desirable based on laboratory studies (Call and Logan, 2008) this anode resistance would result in an approximate maximum current of 0.2A, increasing the load to 0.9 as needed with other wastewater studies (Kiely et al., 2011a, Cusick et al., 2011) would produce a maximum of 0.3A, and the 1.1V load used would result in around 0.4 A maximum current, assuming no other losses. This would give anode current densities of 0.3, 0.4 and 0.5 A/m^2 respectively, well below the target for BES of 10 A/m^2 which would enable similar treatment rates to activated sludge (Rozendal et al., 2008a), although current densities within MECs do tend to be lower than those of MFCs (Kiely et al., 2011a).

In reality there was greater resistance within the reactor than the electrode overpotentials alone. The current densities measured were 0.04, 0.1 and 0.3 A/m^2 at 0.7, 0.9 and 1.1V load added respectively. This means that the current density only increases by around 0.6 A/m^2/volt, far lower than two early MEC laboratory studies (1.3 A/m2/volt in (Liu et al., 2005b) and 1.78 A/m^2/volt in (Rozendal et al., 2006)). Additionally this shows that there is an inherent overpotential in the system also of over 0.6 volts as seen in Figure 6.4, over this voltage needs to be added to generate any current.

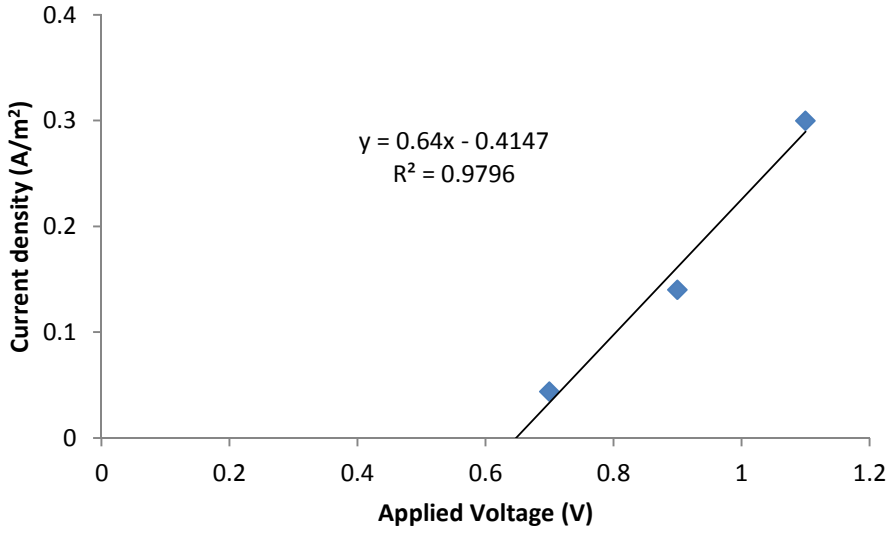

Figure 6-4 Current density as a function of applied voltage as measured in the pilot scale reactor after the initial two week acclimatisation period, showing the linear regression equation and R2 value. The intersect of the x-axis indicates the overpotential of the system

6.3.2. *Start-up and acclimatisation*

During the first 30 days of operation the reactor was run in batch mode with a supplement of 0.5 g/L of sodium acetate and an input voltage of 0.6 V. During this time there was no observed gas production and the current density was very low reaching 0.04 A/m^2 after the first two weeks. After this period wastewater was pumped through the reactor with a HRT of one day with no further addition of acetate. For the subsequent 10 days very little gas was produced and the current density remained at this very low level. At day 40 the input voltage was raised from 0.6 V to 0.9 V. The reactor was run with this input of voltage for the next 24 days; the average power density during this time reached 0.1 A/m^2. Gas production was low with an average of 9 mL/day, however once the gas lines had been flushed the purity of this gas (H_2) began to reach 100%. The electrical energy efficiency η_E was only 1 %. The voltage was then further increased to 1.1 V, and power densities rose and stabilised at 0.3 A/m^2. This led to a dramatic improvement in gas production, and the reactor entered its "working phase", the results of which are shown below. The start-up period took 64 days.

6.3.3. *Working performance of MEC reactor*

After the long start-up, and subsequent increase in the voltage to 1.1 volts, the MEC worked for the following 85 days, and continues to do so. The results presented here are for this period.

The volume of gas produced per day was highly variable. However the gas composition was consistent, hydrogen 100% ± 6.4, methane 1.8% ± 0.9. No trace of CO_2, N_2 or O_2 could be detected using the GC's or MIMS. H_2S could not be measured accurately however the MIMS did not detect any gas at this atomic weight and there was no detectable odour present. The daily H_2 production is shown in Figure 6-5. Production gradually increased during the first 30 days; after this the average production was around 1.2 L per day for the reactor, equivalent to 0.015 L-H_2/L/day.

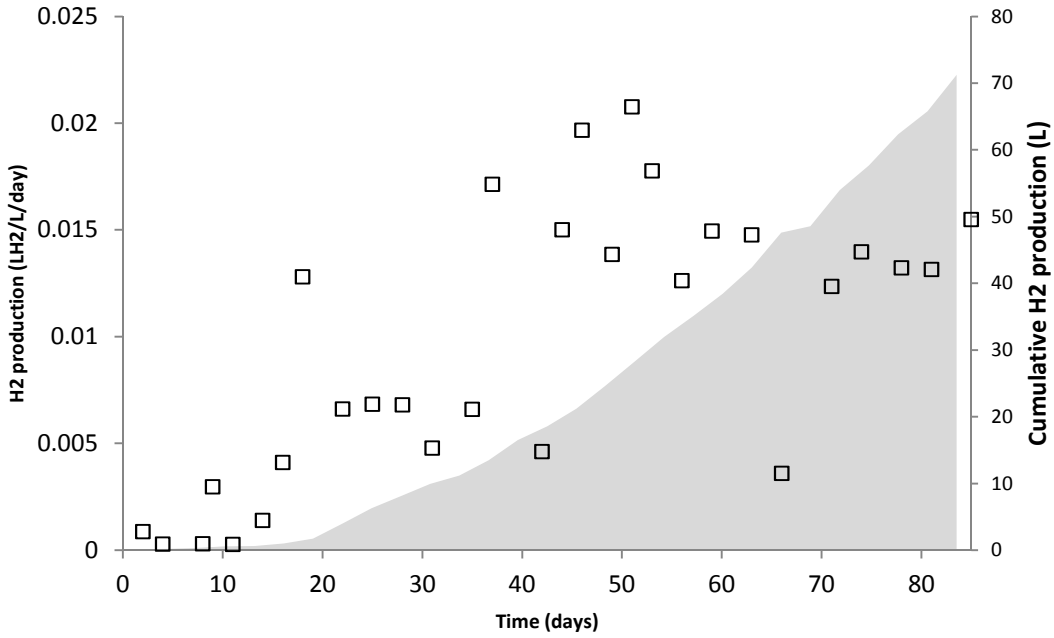

Figure 6-5 Hydrogen production during the working phase of reactor after the 64 day acclimatisation period, points showing the production rate at each time of sampling, and the area showing the cumulative production of the course of this period

The electrical energy recovery of the cell was quite variable as seen in Figure 6-6 (a), but did show an increasing trend and on occasion approached 100% (complete energy recovery) . The total energy efficiency (b) which gives the true performance of the cell was also variable, and considerably lower as both the electrical and substrate energy are considered as inputs. The energy efficiency shows an increasing trend reaching the 30 % level at the end of the study. The peak values are associated with very low COD removal measurements (making substrate energy input very low), and are not therefore likely to be representative of the true performance of the reactor. Coulombic efficiency (Fig. 5c) shows a similar trend to energy recovery (Fig. 5a), stabilising at around 55-60 % in the last 30 days.

The coulombic efficiency (CE) correlates with energy recovery (η_E) (R^2 = 0.998, Pearsons correlation). This correlation factor is calculated as N_E = 1.29 CE using the average input power voltage, this value is also seen in the data and is consistent over the course of the study. If the CE could remain at the 60% and the power input dropped to 0.9 volts 100% η_E would be achieved. Alternatively with this power input CE needs to reach 75% to achieve 100% η_E. The substrate efficiency (d), due to the highly variable influent and effluent COD values (as shown in Figure 6-7 can exceed 100%, and was often very low and even negative. The average substrate efficiency for whole the operational period is 10%.

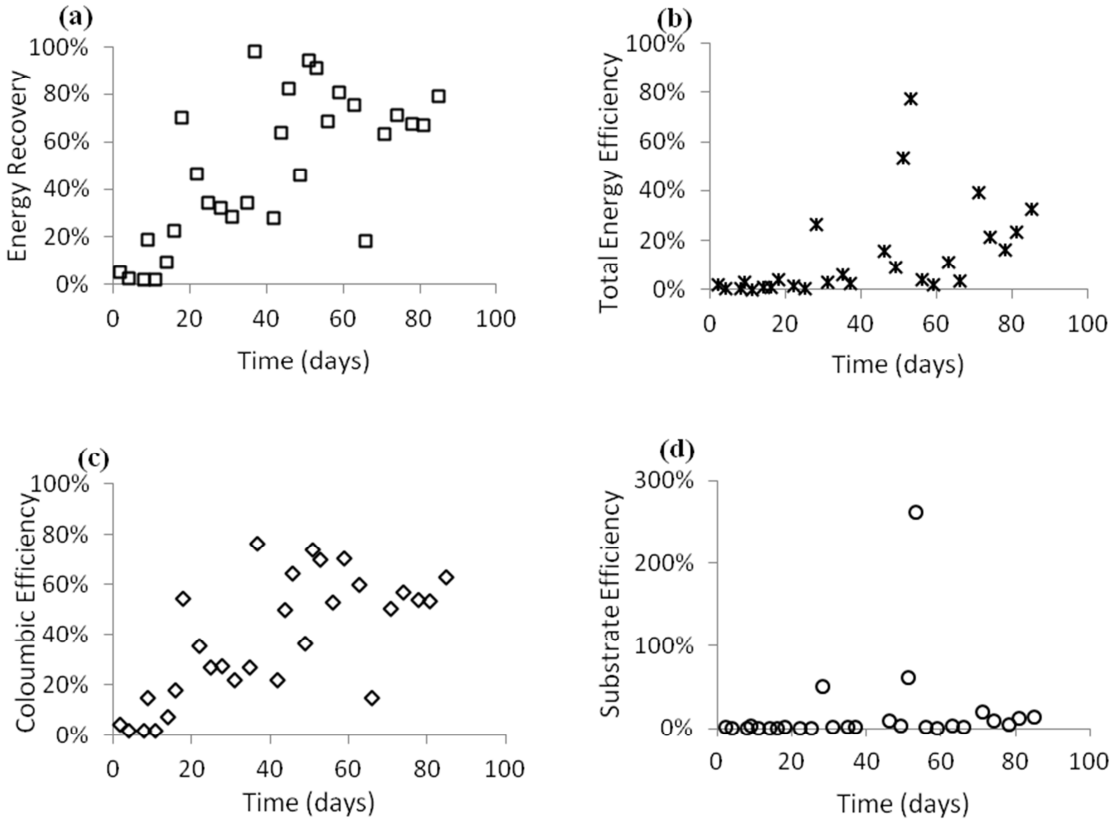

Figure 6-6 MEC reactor efficiencies over the 85 day working period a) electrical energy recovery b) total energy efficiency c) coulombic efficiency d) substrate efficiency

The levels of influent COD was highly variable which is likely to be one of the factors underlying the variation in performance. This factor was particularly the case at day 30 when the settling tank became full with sludge and influent COD was extremely high. This variability led to occasional negative values for % COD removal. The average removal of 33.7%, equates to 0.14 kgCOD/m^3/day, just below the range for activated sludge of 0.2-2 kgCOD/m^3/day (Grady, 1999). The COD effluent levels occasionally approached and dropped below the UK standard of 125 mg/l (EEC, 1991).

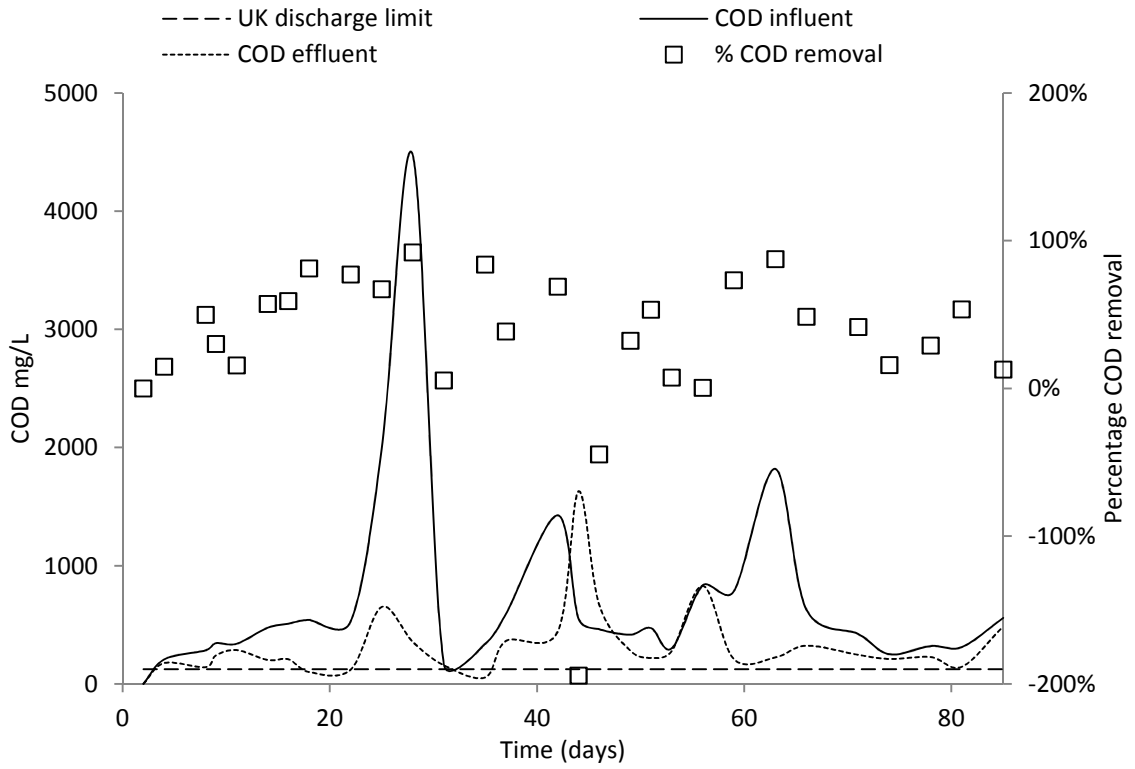

Figure 6-7 COD influent and effluent shown by the lines along with the UK discharge standard of 125 mg/l, percentage COD removal is also shown using the squares

Despite the variable influent COD and therefore variable performance, many of the other measured factors remained relatively constant throughout the operational period. The headspace of the anode compartment (2.2 L volume) contained elevated levels of CO_2 (1.9%) and low levels of CH_4 (0.4%), equivalent to 8.8 ml of CH_4, or 0.006 mg COD and 0.3 kJ. The gas production at the anode could not be measured quantitatively due to leakage. The daily production of methane at the cathode was 22 mL/day, equivalent to 0.014 mg COD, and 0.8 kJ of energy, approximately 5-6% of the amount of energy recovered as hydrogen.

The pH of the influent and effluent were continuously monitored, the influent was on average pH 7, the effluent pH 6.7, never dropping below pH 6. The DO of the influent was on average 4.2 mg/L and the effluent was 0 mg/L. The amount of VFA's dropped between the influent and the effluent, but there was frequently some acetic acid left in the effluent up to 45 mg/L, i.e. the available food source was not used up. This was confirmed by the average SCOD of the effluent of 115 mg/L. There was an average removal of 1.8 g/day of sulphate in the reactor, but never full depletion with the effluent containing 89.6 mg/L on average. The reactor removed an average of 0.2 g/day of chloride, although this value was highly variable. Fluoride and phosphate remained

relatively constant between the influent and effluent, nitrates were not present in either. There was no measured drop in conductivity between the influent and effluent.

The temperature of the influent wastewater varied considerably throughout the working period between June and September. The range of temperature was more stable within the reactor, and was on average 0.9 ^{o}C higher than the temperature of the influent. With a 88 L capacity and HRT of 1 day, this means 0.37 kJ/day of energy was lost to heat, equivalent to 20 mg COD, or 31 ml H_2. Temperature did not significantly influence energy recovery (p=0.678 influent, p=0.664 reactor, p=0.778 effluent, Pearson Correlation). Most of the fluctuation observed was diurnal and periods of the more extreme temperatures were short lived.

Table 6-1 Maximum, minimum and average temperature (^{o}C) of the influent, effluent and reactor ± 1 standard deviation which were continually logged over the experimental period

	Influent	Reactor	Effluent
Maximum	27.0 ± 2.3	21.0 ± 1.2	22.5 ± 1.6
Minimum	8.5 ± 2.3	13.5 ± 1.2	12.0 ± 1.6
Average	15.8 ± 2.3	16.6 ± 1.2	16.6 ± 1.6

The total material costs of the reactor, not including pumps, power supply and computing/recording instruments, was equivalent to £2344/m^3, of which the cathode and membrane combined represented less than 2%.

6.4. Discussion

This pilot scale reactor worked, producing almost pure hydrogen gas from raw influent domestic wastewater at U.K. ambient temperatures for a 3 month period and continues to do so. It is believed to be the first successful study of its kind, which brings the prospect of sustainable wastewater treatment and hydrogen production through the use of bioelectrochemical systems onto a new and exciting phase.

The reactor has removed on average 34% of COD, and occasionally reaching the UK discharge standard of 125 mgCOD/L, equating to a treatment rate of 0.14 kgCOD/m^3/day, just below the range for activated sludge. The reactor has performed this task using less energy than would be needed for aeration in a traditional activated sludge process. The electrical energy recovery on occasion nearly reached values of 100%, and was consistently around 70% during the later stages of the study. At this

level of performance (i.e. 70%) the energetic treatment costs were 2.3 kJ/gCOD, below the values for activated sludge of 2.5-7.2 kJ/gCOD (Pant et al., 2011). By implementing improvements to the reactor such as: increasing electrode surface areas; reducing the distance between electrodes; having a more efficient flow paths; consistent pumping; and improved materials, the η_E could be greater than 100%, making it a net energy producer. On the basis of this fairly large proof of concept study, energy neutral or even energy positive wastewater treatment is clearly a realistic goal.

The total energy recovery showed an increasing trend during the course of the study, levelling out at around 30%, with around a third of all energy both from the wastewater and from the power supply being recovered as hydrogen gas. Coulombic efficiencies of the reactor were high, levelling out at around 55-60 %, methane production accounts for an additional 3.5%. Other losses might be caused by some short circuiting in the reactor. It is likely therefore that a large proportion of the missing 40% of CE can be attributed to a loss of hydrogen gas from the system. Hydrogen is an extremely small molecule and is able to permeate most plastics, and is therefore likely to be leaking out of the reactor. In a tightly engineered system theoretically the coulombic efficiency could approach its maximum of 100%, resulting in an electrical energy recovery of 129%.

The substrate efficiency of the cell was considerably lower than the other efficiencies measured. This efficiency represents how much of the substrate is actually recovered as hydrogen, and gives an indication of how much substrate is used in the MEC process. Even if the 40% loss of hydrogen through leakage (as suggested by the CE of 60%) is accounted for in this calculation then the substrate efficiency would only increase from 10% to around 23%. Losses may be taken to suggest that substrate is being used in competitive oxidation processes, but only low levels of oxygen entered the cell with the influent. Sulphate reduction equated to about 3.6% of the total COD removal. Limited nitrates were available. Further losses can be accounted for by the probable build-up of sludge within the reactor as evidenced by the constant COD removal value throughout the study despite the increasing efficiency of the reactor, and that on three occasions a very high COD peak entered the reactor, on two of these occasions the peak of COD is not seen to leave the reactor see Figure 6-7.

Clearly the high resistance of the reactor means the overall efficiencies of the reactor will be low. The resistance observed is more problematic in this larger scale system than at the laboratory scale, and would also become increasingly challenging with further scale up. Improved reactor design is needed to overcome these problems. In a large scale system a considerable wire length is likely to be inevitable, resistance could be reduced through the use of a thicker wire, additionally resistance could be reduced in the electrode by improving the connection between the electrode, current collectors and wire. Further research into different materials and different configurations of materials would hopefully lead to improvements at a larger scale.

Further efficiency losses as identified above could be minimised by improving the engineering of the system. The two 'new' materials used in this study for the membrane and cathode have not been truly evaluated. More expensive alternatives such as Nafion membrane and a Pt coated cathode may prove to be worthwhile investments if performance increases greatly with their use. The biological MEC process works, and works relatively consistently for a period of at least three months. Although tested in realistic conditions, this was over a spring/summer period, survival over periods of sustained low temperature has yet to be confirmed.

The relationship between electrical energy recovery, electrical power input and coulombic efficiency has been defined however the prediction energy requirements for a larger scale MEC system may be difficult to make. Theoretical input voltages lie far from those needed in reality even for acetate fed cells, typically between 0.4-1.0 V compared to the 0.114 V theoretically needed (pH 7, 298 K) (Logan, 2008). A relatively small change in the electrical power input can have a large effect of the overall electrical energy recovery, yet if this value is not high enough to overcome the losses in the cell no hydrogen will be produced.

Undoubtedly there are many factors that require further investigation. Many of the inefficiencies could be overcome by improved engineering, but also a greater understanding of the biological processes (both working with and against the cell performance), community structure and ecology would allow for more confident design and manipulation.

The aim of this research was to determine if MEC technology could be a viable and alternative to the activated sludge process. The pilot scale reactor has worked producing hydrogen, with real wastewaters at ambient temperatures for over 3 months at a volumetric treatment rate just below that for activated sludge. A breakeven energy was not consistently achieved during the course of the study, yet is believed to be within reach with improved hydrogen capture and improved design to increase efficiencies. With this proof of concept now made we are a large step closer to using MEC technology for sustainable wastewater treatment.

Chapter 7. Conclusions

The overall aim of this research is to reach an understanding of whether microbial electrolysis cells could be a domestic wastewater treatment option.

I conclude that energy neutral or energy positive wastewater treatment should be possible. This research started by looking into how much energy is held intrinsically within the wastewater, and concluded that the amount of energy in the wastewater is substantial, more than previously thought, and more that the energy costs currently incurred in its treatment (18-29 kJ/gCOD vs. 2.5-7.2 kJ/gCOD in activated sludge treatment). Although this energy measured is internal chemical energy which is higher than the Gibbs Free Energy that would be available to microorganisms, with a biological system engineered for energy extraction from wastewater rather than an energy input, i.e. utilising other redox pathways rather than simple aerobic oxidation.

With the conclusion made that there is enough energy inherently contained in wastewater to treat it, the next question was to determine if Microbial Electrolysis Cells could meet this demand, replacing the high energy demanding activated sludge process with an energy yielding process. Parts of the thesis, in particular the low temperature work, suggested this might be possible yet other parts of the research did not such as the failure in MEC wastewater fed reactors. However by building and testing a pilot scale reactor on site at a wastewater treatment the most positive and conclusive evidence that this technology could work for real wastewater applications was gained. The reactor, even though it was a ‘first design’ using low cost alternatives to the optimum materials, and with many other problems such as non-optimised flow and hydrogen leakage and high resistance, it came reasonably close to its breakeven energy point. Even without breaking even it was more effective in terms of energy used per gCOD removed, and came close to the volumetric loading rates of the activated sludge process.

There is still much work to be done at this scale and larger to: understand the issues of scaling; economic feasibility; hydrogen capture and storage; design and materials; and optimisation. This work could then lead to retrofitting old activated sludge lanes with microbial electrolysis cells, radically changing the wastewater industry.

All the research conducted in this PhD has shown that the substrate acetate is not an adequate model of wastewater. This has been shown simply in terms of the energy available per gCOD, the acclimatisation and number of exoelectrogens able to digest these substrates, the diversity of the community fed with these substrates and their function within microbial electrolysis cells. The higher diversity estimates and complex acclimatisation pattern of acetate fed reactors suggest acetate may not be the optimum compound to use in BES's. Wastewater fed systems may have less free energy available, and therefore result in a more efficient biomass being formed. The lower coulombic efficiencies observed in wastewater fed reactors might be an inevitable result of electrons being lost within the longer chains of digestion, and not necessarily an indication of inefficient biomass.

The conclusion that temperature does not affect the performance of MFCs is surprising, although does correspond to some of the literature in this area (Catal et al., 2011, Jadhav and Ghangrekar, 2009). This suggests that there is a similar level of free energy available in systems run at different temperatures, and that low temperatures do not represent a disadvantage for BES. This is also observed in the pilot reactor, here low temperatures may be an advantage reducing methanogenic activity which proved fatal in the only other pilot scale MEC study to be published (run at 30 ^{o}C) (Cusick et al., 2011).

A further surprising conclusion was that inoculum did not have an effect on reactor performance, although the inoculum did interact with substrate to produce higher diversities within acetate fed reactors inoculated with high diversity soil. Exoelectrogenic bacteria were present naturally in all the wastewater inocula, and the Arctic soil inocula used throughout this research, albeit at low levels. The number or proportion of exoelectrogens was estimated to be 0.0017% using the very old methodology of MPNs, using the most recent next generation sequencing techniques and mathematical modelling algorithms, the estimates were 0.0012% and 0.00001% for two different wastewater samples. This therefore appears to be a reasonable good estimate of the rarity of such species.

BES reactors have been shown to work in challenging, real life, environments, and many observations have been made about the abundance and diversity of the organisms needed for the operation of these systems. This research has moved a substantial step

forward in proving that these technologies could be an energy efficient replacement of the activated sludge process. However we are still a long way from a deep and holistic understanding of the bacterial world operating within these systems, the energy requirements of these communities, their metabolic limits, their response to stress and ultimately their stability and function. Without this deep understanding we are reliant upon empirical data gathering, testing reactors in various environments until these limits are found. If we could model the free energy needs of the bacterial community, estimate the free energy available in the substrate, and calculate the efficiencies of the electrochemical cell, such systems could be modelled accurately and ultimately engineered to produce positive energy recovery.

Chapter 8. Perspectives on the use of MECs in the treatment of wastewater

This work has demonstrated a proof of concept of the use of MECs with domestic wastewater to produce hydrogen at the 100L scale over a 3 month time period. However this does not mean that they will be a viable wastewater treatment option. The work conducted in this research goes some way to confirming to technical feasibility of this technology in the treatment of domestic wastewaters, it does not however prove or suggest that this will be an economic viability, such an assertion is beyond the scope of this study.

There are many considerations which would need to be focused on in order to determine this economic viability for any technology to replace activated sludge treatment (AS), including those criteria stated in the introduction:

1. Extract and convert energy to a useable form at an efficiency that justifies the costs.
2. Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
3. Treat low strength domestic wastewater.
4. Work at ambient, often low temperatures.
5. Work continuously and reliably.

The detailed costing of this technology is beyond the scope of this thesis. It has been suggested that MEC technology may be an economically viable alternative to AS over other treatments such as anaerobic digestion (AD) or MFCs (Foley et al., 2010, Curtis, 2010) based on the reduction in aeration costs and the potential value of products produced. However to change the UK wastewater infrastructure would require exchanging the current AS process components for a system with higher capital costs (estimated at 0.4 €/kgCOD for an MEC compared to 0.1 €/kgCOD for AS, (Rozendal et al., 2008a)) aiming to recover the costs through the product generated. It is clear that even with low cost materials used in this research, and the idea of retrofitting the cells into existing infrastructure (Cha et al., 2010), the capital costs of filling tanks with complex electrode assemblies would be far higher than installing the aeration pipework. It would need to be ascertained whether the ‘payback’ in terms of reduction of the

energy costs and the products generated would equal the higher capital costs over the lifetime of the cells (which is again unknown at this stage).

The design life of typical wastewater treatment infrastructure is at least 25 years. MECs have not been tested over such time periods in even in the relatively clean conditions of laboratories. It is highly likely the many of the components of a typical MEC would not survive for long periods when handling real wastes, membranes for example are particularly problematical clogging over time (Zhang et al., 2011), yet membraneless are also problematic at large scale (Cusick et al., 2011). Even the estimates for a 5 year life span of electrodes and membranes used in the estimates above (Rozendal et al., 2008a) are untested under real conditions and may be unrealistic. The life span and maintenance requirements of BES will be a critical factor in determining if this technology can be used economically within the wastewater industry.

A further cost consideration is the labour costs associated with this new technology. The level of maintenance required in the MEC process is again unknown, but is likely to be higher than the AS, though may be compensated for by the reduction in sludge treatment which is a considerable fraction of the operational costs (Verstraete and Vlaeminck, 2011). The hydrogen or product produced may also require purification again the costs of this would need to be accounted for in identifying if the economic benefits of the product outweigh the costs.

The full economic costing of the MEC process versus other processes is complex, with many unknowns. It is likely to vary with: the scale and wastewater type of different treatment plants; water usage and availability; energy and material prices; and therefore inherently through time (McCarty et al., 2011). The ‘upgrading’ of AS plants with improved energy recovery from sludge AD, improved process control and greater levels of primary settling such as the Strass plant in Austria which generates 108% of its electricity use (Nowak et al., 2011) may prove to be more economically viable. The addition of AD onto the AS process is the route many UK water companies are taking including Northumbria Water Ltd who have one large sludge AD plant in operation and one under construction. However such a high degree energy recovery is exceptional, and many experts in the field question the concept of using the energy intensive process of AS to insolubalise waste organics to sludge which then can undergo energy recovery (Verstraete and Vlaeminck, 2011).

The treatment levels of the pilot MEC run were both low and variable, averaging only at 34%, the AS process can remove up to 95% of the COD (Tchobanoglous, 1991) although this is rarely the case as they are usually part of a treatment flow with pre-settling and post clarification removing a proportion of the COD (Grady, 1999). The MEC reactor demonstrated did on occasions remove the COD down to the discharge limit of 125 mgCOD/L (EEC, 1991) so operation at this level is possible. The ability to use domestic wastewaters is a clear advantage over AD which tends to be restricted to high strength industrial or farm wastes, or sludge generated by AD. Further work would be needed to demonstrate that this treatment could consistently reach discharge standards, and the electrical conductivity of the wastewater at these low strengths is sufficient for the cells to function.

Even if part of a treatment flow with pre-settling and post clarification it is likely that the MEC would need to improve treatment rates to encourage investment, additionally the more organics removed the higher the energy yield can be. Treatment rates could be improved by reducing electrode spacing; however this would have the knock on effect of reducing the volumetric loading rate. The MEC could therefore end up requiring the same unit space as trickling filters, and therefore not be a viable option either due to land restrictions or poor economic comparability to this low energy treatment option.
There is an increasing body of research demonstrating that BES technologies will work at ambient temperatures (Jadhav and Ghangrekar, 2009, Catal et al., 2011, Larrosa-Guerrero et al., 2010), added to by the work in this thesis. Further work may be required in demonstrating this with real wastewaters at a larger scale, and also in quantifying and overcoming the kinetic effect of the lower temperatures on bacterial metabolism.

Many challenges lie ahead with BES research both from a technological and economic perspective. Only through completing and importantly combining these research areas will we be able to reach an understanding as to whether the technology can be used in the wastewater treatment plants of the future.

Chapter 9. Recommendations for future research

This research set out to answer the question as to whether microbial electrolysis cells could be used for wastewater treatment. Most of this research has strengthened the case that they are, however many more research and application questions remain unanswered. Each piece of research described in this thesis could be developed further to give more conclusive answers:

Chapter 2: A comprehensive survey into the amount of energy contained within wastewater is warranted. In the research conducted two samples were tested from different wastewater treatment plants and the results showed a large difference in the energy content between the samples and with that which would be predicted. Discovering the energy in wastewater is fundamental to the study of bioelectrochemical systems, and other technologies which aim to yield energy from wastewater. If we are to evaluate the true potential of these technologies we need to know how much energy is actually encapsulated in domestic wastewater, enabling efficiencies to be calculated and therefore better solutions engineered.

Measuring internal energy by calorimetry is a standard method in the solid waste industry (Garg et al., 2007, Lupa et al., 2011), yet when applied to wastewater the problem arises that samples have to be dry, and even with the improved and extremely laborious freeze drying method used in this research 20-30% of the volatiles in wastewater were lost. With an improved and quicker method, such as the use of distillation or reverse osmosis, a comprehensive survey of wastewaters in the UK could be made. This would: facilitate decisions on where best to invest in new technologies; give an indication of which technologies might be more suitable for different wastewaters; inform of the efficiency of processes; and most importantly – make decision makers believe energy extraction from wastewaters is economically viable and worthwhile.

Chapter 3: With a more definitive answer to the number of bacteria present and their growth pattern, accurate assessments of specific activity and growth yields could be made. Accurate estimations of these values are needed for parameterising models of these systems. By redesigning these experiments, and the reactors used to minimise or at least quantify all losses, a mass balance could be made and these values determined.

However the most intriguing question arising from this work was the difference in the pattern of acclimatisation observed in the acetate fed cells and those with complex substrates. Although possible reasons for this difference were suggested, a conclusive answer was not found. By conducting further research scaling between acetate and starch in terms of substrate complexity, the step causing the change in response of acclimatisation could be found, which may give valuable insight into the development and ultimately the function of these communities. The use of other microbiological techniques such as flow cytometry and QPCR may also help in the accurate determination of these values.

Chapter 4: The finding that temperature and inoculum had little effect on reactor performance is significant to the eventual implementation of this technology. The high variability within the warmer reactors would however be worth investigating further, if all the warm reactors were able to work at the maximum level shown by some, temperature would be a significant factor. The reactor configuration used in these experiments may have been limiting factor, thus if repeated with a higher performing reactor design, the temperature effect may be observed.

The counterintuitive observation that acetate fed cells produced a higher diversity was of great interest in this work. Further research is needed to determine if it is energy that controls the diversity, not the complexity of the substrate. This could be examined by scaling through simple compounds with known and increasing free energies (e.g. from the ΔG of the reaction under standard conditions at pH 7: acetate 27.40 kJ/e^- eq, pyruvate 35.09 kJ/ e^- eq and glucose 41.35 kJ/e^- eq) and observing how diversity changes.

Chapter 5: The conclusion that laboratory wastewater fed reactors fail after a short period of time is contradicted by chapter 6 where the pilot MEC worked. Determining the reason for failure at the small scale is a priority for any further lab scale research studies. Other than scale, the two different factors in the lab based experiments compared to the pilot, are that feed is continuous not batch, and that the laboratory reactors are acclimatised as a MFCs. Research into these factors, and a solution to the failure is needed to achieve the working laboratory wastewater fed systems required for investigations into the use of this technology for wastewater treatment.

Chapter 6: The final part of this research gave the most conclusive answer as to whether MECs can work for wastewater treatment and will, when published, put the research of MECs onto a new platform. Much research is still needed into improving efficiencies and critically achieving the breakeven energy recovery, further scaling, different materials and design, and the economic feasibility of implementing this technology at scale. If the use of this technology is validated, research is needed into the strategic implications this will have on the wastewater treatment industry.

Further recommendations: The research described has increased our understanding of how BES can function in wastewater treatment. A more fundamental direction of research would be the use of BES in understanding the energetic laws and rules which underpin biological systems. Such rules would have huge impact on design in both the near and distant future (Curtis et al., 2003). BES offer the unique opportunity, effectively opening a window on the energy involved in biological reaction, as this energy is routed through an external circuit and can therefore be measured allowing energetic interactions to be unravelled.

By designing a biocalorimeter type BES reactor, where all energetic inputs and outputs are measured (with no leakage) this could be tested using simple substrates and monocultures, and simple laws developed. For example if a substrate chemically yields 'x' kilojoules of Gibbs free energy (ΔG), exactly how much of this can be accessed by bacteria at a set pH and temperature, what proportions go to growth and maintenance for the BES to be stable and what the energy transfer efficiency is. By then scaling to more complex substrates and mixed cultures insight could be gained on: the fermentation processes and on how and why some reaction routes may be favored over others; if the overall ΔG of a complex substrate adequate to model outcome or is more complexity required; and if the energy needs are similar amongst trophic layers.

Through manipulating the systems thermodynamic constraints (temperature, pressure, and ionic strength) to give predictable outcomes, the rules identified above could be verified. Knowledge would also be gained on which thresholds of energy can change community behavior, and how easily these can be manipulated, how much the bacteria can compensate for these changes. Additionally by taking the system to the energetic edge the real limits can be defined and compered to theoretical limits. Ultimately an understanding of how energy requirements of a community link to abundance and

diversity could be gained, and allow for these to be manipulated to increase system stability.

By using a BES in this novel way, the thermodynamic laws which underpin the microbial world may be discovered. The rules generated could be used to create a model allowing biotechnologies to be reliably engineered. The feasibility and efficiency of a bioprocess being modeled at the investment stage without relying on estimates from empirical data. This would have huge scope to promote change and development across the scientific and engineering community.

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Chapter 11. Appendices

11.1. Appendix I - History of microbial fuel cell technology

The concept of fuel cells, a device that can convert electrochemical energy into electricity is not new. The first working chemical fuel cell is attributed to Sir William Grove in 1839 (Lewis, 1966). Progress since then has been slow and sporadic. Although it was understood that the direct conversion of chemical energy to electrical energy was more efficient than combustion in a heat engine (where up to 80% of the energy in the fuel is lost through heat in the exhaust, friction, air turbulence and the heating up and movement of engine parts), historically the abundance of fuel meant that the simpler combustion engine took precedence. The main surge of work in fuel cells has been in the last 10-15 years as fossil fuel prices, and the need for cleaner and more efficient energy production has increased (Logan, 2008).

The first biologically catalysed fuel cell was made in 1911 by a Professor of Botany M.C. Potter at Newcastle University. He discovered that an electrical current could be produced using bacteria as the catalyst on the anode, with a glucose and yeast mixture under various conditions of temperature and concentration he produced a maximum of 0.3 to 0.5 volts (Potter, 1911). This work was added to by Barnet Cohen who built a small bacterial battery using a series of half cells. This work drew more attention to the area, however the major drawback of the system was highlighted, only a very low current is able to be produced and it is rapidly discharged. The use of mediators such as potassium ferrycyanide and benzoquinone did enable greater voltage to be produced however the current remained low (Cohen, 1930).

Del Duca et al. (1963) re-visited the idea and set up a working laboratory model built using urea as a fuel. Urea was broken down enzymically by urease to produce ammonia at the anode, which then reacted with an air cathode producing current. A conceptual design was put forward for a 20-Watt portable urea battery, containing 64 individual cells, however the battery life was only 2 weeks.

Karube et al.(1976), described how carbohydrates were broken down to hydrogen using a fixed matrix of fermentative bacteria, the hydrogen reacted in the electrochemical cell. These studies were the first to use a design very similar to those MFCs used today, but with a salt bridge rather than an artificial membrane. It was believed that the bacteria's

role was to break down the carbohydrate to make electrochemically active products, which were entirely responsible for the current generation. It was not seen that the bacteria themselves were creating the electrochemical current, through the donation of electrons, though this was almost certainly the case.

R. M. Allen and then H. P. Bennetto worked on microbial fuel cells throughout the 1980's at Kings College, London. They had the vision that fuels cells could be a solution to the poor sanitation and lack of electricity supply in the then termed 'third world'. A paper which was the culmination of this work was published in 1993, simply titled Microbial Fuel-Cells – Electricity Production from Carbohydrates, was the first to show an understanding of the mechanism at work (Allen and Bennetto, 1993), although electron transfer was still not understood. It was thought that electrons were extracted from the oxidation of carbohydrates; these would then become trapped within the bacteria, but would become available for transfer to the anode through the use of a chemical redox mediator. Chemical mediators such as ferricyanide were expensive, non-sustainable and toxic to the environment.

The breakthrough discovery was made in 1999 that chemical mediators where not needed in the cells (Kim et al., 1999). This critical discovery that MFCs do not require these mediators, and the ever increasing pressures to reduce pollution, has led to an explosion of research in this area.

In 2005 it was discovered that microbes could be used in an electrolysis cell (Rozendal et al., 2006, Liu et al., 2005b). Electrical energy input can be combined with the energy derived from the fuel by bacteria to drive electrolysis reactions making products which would otherwise require much larger inputs of energy, most notably hydrogen. Thus hydrogen can be produced at greater efficiencies than is the limit with fermentation, and in theory at around one tenth of the electrical energy input of water electrolysis.

11.2. Appendix II - Theoretical cell energetics

The basic reaction occurring in an MFC or MEC can be split into two half reactions, the anode reaction which is the catabolic breakdown of the organic substrate to produce electrons, and the cathode reaction which is the donation of these electrons. The quantity of energy released per electron transferred is dependent on the chemical properties of those compounds involved, and is given by the Gibbs free energy of the reaction or ΔG_r:

$$\Delta G_r = \Delta G_r^0 + RT \ln Q$$

Equation 1

Where ΔG_r is the Gibbs free energy of the reaction, ΔG_r^0 is the Gibbs free energy for the reaction under standard conditions (temperature of 298 K and chemical concentrations of 1M for liquids and 1 bar for gases) as tabulated (Atkins, 2006), R is the gas constant 8.31 J/mol-K, T is temperature, and Q is the reaction quotient i.e. the ratio of the activities of the products and the reactants.

The cell potential (E_{emf}) can be calculated from Gibbs free energy of each half reaction:

$$E_{emf}^0 = -\Delta G_r^0 / nF$$

Equation 2

Where n is the number of moles of electrons transferred and F is Faradays constant 96485 J/mol e^-.

Alternatively the potential can be calculated directly when the potential under standard conditions is known:

$$E_{emf} = {E_{emf}}^0 - \frac{RT}{nF} \ln Q$$

Equation 3

Using acetate as an example electron donor, the half-cell, and full reaction values are given for ΔG_r and E_{emf} in Table 11-1 under standard environmental conditions pH 7, 298 K:

Table 11-1 Calculated theoretical energies (as Gibbs free energy and Potential) of half-cell reactions occurring within BES fed with acetate

	Reaction	ΔG_r/ kJ/ e- eq	Potential E (V)
Anode/ donor	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$	27.40	-0.300 (-0.284)
Cathode /acceptor MFC	$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O$	-78.72	0.805 (0.816)
Overall MFC	$\frac{1}{8}CH_3COO^- + \frac{1}{4}O_2 \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}H_2O + \frac{1}{8}HCO_3^-$	-106.12	1.105 (1.100)
Cathode /acceptor MEC	$H^+ + e^- \rightarrow \frac{1}{2}H_2$	39.94	-0.414
Overall MEC	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{2}H_2 + \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^-$	12.54	-0.114 (-0.130)

Values for E_{emf} written in bracket are those calculated from the tabulated ΔG_r and E_{emf} values which vary slightly (Rittmann, 2001, Atkins, 2006).

From the equations above it can be seen that anode and cathode potentials vary with temperatures (T), substrates (ΔG_r^0 or E_{emf}^0) and ionic concentrations (Q), especially pH. These can be calculated as shown below (except in the case of wastewater). However in a real system they may vary from time to time, place to place, and even within the same reactor as substrates are utilised and H^+ ions produced:

Substrate

In an acetate fed MEC the theoretical anode potential (E_{An}) under standard biological conditions (i.e. pH 7, temperature 25 ^oC) would be -0.284 V and the for the hydrogen evolution reaction (again at pH 7) it is -0.414 V, giving a cell potential E_{emf} of -0.13V an additional 0.13V would need to be added, with glucose this difference is positive 0.015V, theoretically no energy would need to be added. With wastewater and its unknown composition and variability the theoretical anode potential cannot calculated,

the potential of a variety of compounds which may be found within wastewater are shown in Table 11-2.

Table 11-2 Known Gibbs free energy and potential values for a variety of compounds which may be present in wastewater

Substrate	ΔGr (kJ/mol e-)	E_{An} (V)	E_{emf} (V)
Methane	23.53	-0.244	-0.170
Acetate	27.40	-0.284	-0.130
Propionate	27.63	-0.286	-0.128
Ethanol	31.18	-0.323	-0.091
Protein	32.22	-0.334	-0.080
Lactate	32.29	-0.335	-0.079
Citrate	33.08	-0.343	-0.071
Methanol	36.84	-0.382	-0.032
Glycerol	38.88	-0.403	-0.011
Formate	39.19	-0.406	-0.008
Glucose	41.35	-0.429	0.015

ΔGr values from (Rittmann, 2001)

Temperature

Using acetate in an MFC as an example, with an acetate concentration of 0.12M (1 g/L of Na-acetate), bicarbonate concentration of 0.005M, at pH 7, and partial pressure of O_2 as 0.2, the potential, E_{emf} of the anode and cathode can be calculated through a range of temperatures from 0 to 30 ^{o}C:

Anode reaction

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O$$

Cathode reaction

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O$$

The potential under standard environmental conditions (E^0) for these reactions are 0.187V and 1.229V respectively. Using Equation 3 above:

Anode

$$E_{An} = {E_{An}}^0 - \frac{RT}{nF}\ln\frac{[CH_3COO^-]}{[HCO_3^-]^2[H^+]^9}$$

$$E_{An} = 0.187 - \frac{(8.31\,J/mol\,K)\,(T)}{(8)(\,96485\,C/mol)}\ln\frac{[0.012]}{[0.005]^2[10^{-7}]^9}$$

Cathode

$$E_{Ca} = E_{Ca}{}^{0} - \frac{RT}{nF}\ln\frac{[H_2O]}{[O_2]^{1/2}[H^+]^2}$$

$$E_{Ca} = 1.229 - \frac{(8.31\ J/mol\ K)\ (T)}{(2)(\ 96485\ C/mol)}\ \ln\frac{[1]}{[0.02]^{1/2}[10^{-7}]^2}$$

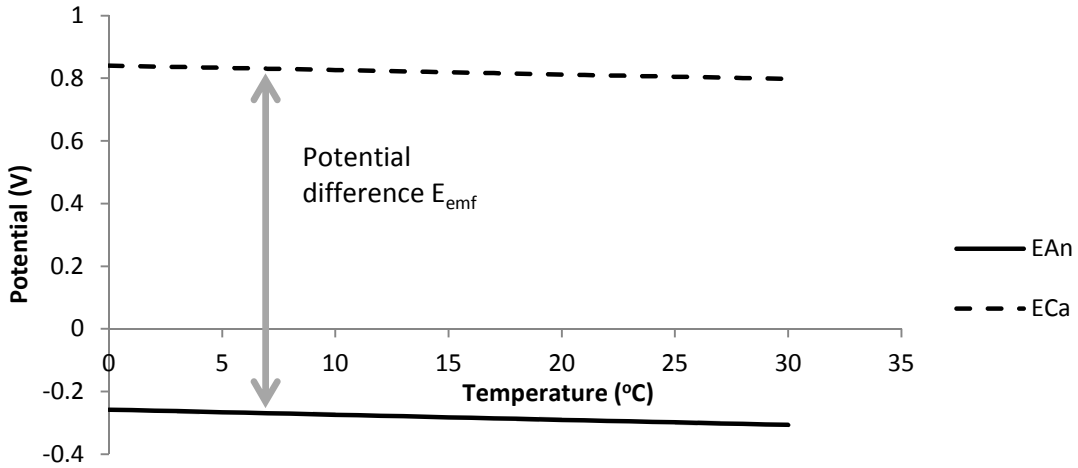


Figure 11-1 Calculated anode and cathode potential though a range of temperatures using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; pH 7; and partial pressure of O_2 as 0.2

The difference between the anode and cathode potential seen in Figure 11-1 varies only slightly from -1.098 V at 0 ^{o}C to -1.104 V at 30 ^{o}C. Theoretically therefore the energy available to be produced via a fuel cell is not greatly affected by temperature within the ranges given. This is however a simplistic approach to a system which, as stated previously is highly complex. As temperatures vary, so will many other factors including dissociation constants, partial pressures of gases and metabolic activity of the bacteria. It is therefore unlikely that the fuel cell will be able to generate as much current at lower temperatures as higher ones, yet it may not be as detrimentally affected by temperature as straight anaerobic digestion.

pH

The reaction co-efficient (Q) is calculated on the basis of the concentrations of the products and reactants in the chemical equation. This factor is critically dependant on

the pH of the system, i.e. the number of H^+ ions, as pH is a logarithmic scale, variance between pH 6 and pH 7 (both within the tolerance of bacteria) has a large effect on the Q value and therefore the overall potential of the cell. An example of this is shown below where the pH of the anode in an acetate system as described in the equations above at 25 ^oC is varied between pH 5 and 8, the cathode potential is kept constant under standard conditions. The potential difference ranges from 0.97 to 1.24 V.

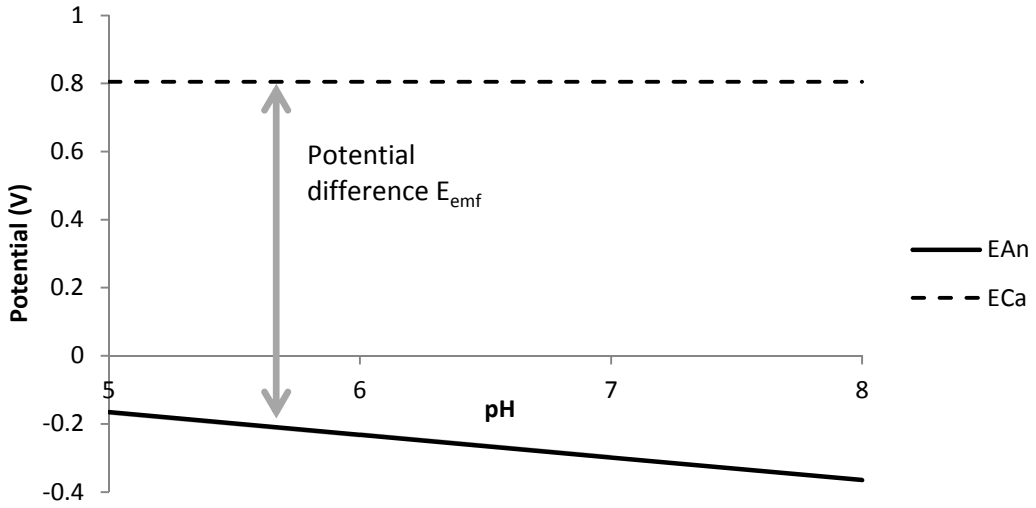


Figure 11-2 Calculated theoretical anode and cathode potential through a range of pHs using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; temperature 25 ^oC; and partial pressure of O_2 as 0.2

11.3. Appendix III – Table of calculated kJ/gCOD of various organic compounds

Compound	Formula	ΔH/gCOD
Benzene	C_6H_6	10.2
Linoleic acid	$C_{18}H_{32}O_2$	13.4
Benzoic acid	C_6H_5COOH	13.4
Myristic acid	$CH_3(CH_2)_{12}CO_2H$	13.6
Acetic acid (Acetate)	CH_3COOH	13.6
Phenol	C_6H_5OH	13.6
Palmitic Acid	$CH_3(CH_2)_{14}CO_2H$	13.6
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$	13.7
Methane	CH_4	13.9
Ethane	C_2H_6	13.9
Lactic acid	$CH_3CH(OH)COOH$	14.0
Ethanol	C_2H_5OH	14.3
Glucose	$C_6H_{12}O_6$	14.3
Propene	C_3H_6	14.3
Cyclopropane	C_3H_3	14.5
Ethanal	CH_3CHO	14.6
Ethene	C_2H_4	14.7
Sucrose	$C_{12}H_{22}O_{11}$	14.7
Methanol	CH_3OH	15.1
Chloroethylene	C_2H_3Cl	15.7
Oxalic acid	$(COOH)_2$	15.9
Formic acid	$HCOOH$	15.9
Ethyne	C_2H_2	16.3
Hexachlorobenzene	C_6Cl_6	16.5
Dichloroethylene (1,1)	$C_2H_2Cl_2$	17.1
Dichloroethylene (1,2)	$C_2H_2Cl_2$	17.2
Methanal	$HCHO$	17.8
Trichloroethylene	C_2HCl_3	20.0
Teterachloroethylene	C_2Cl_4	26.0
Chloroform	$CHCl_3$	29.1
Trichloroacetic acid	CCl_3COOH	30.4

11.4. Appendix IV - Description of the calculation algorithm used in the Shizas and Bagley

Shizas and Bagley (Shizas and Bagley, 2004) use a sample of municipal wastewater which prior to drying contains 431 mg/L COD. This sample is then oven dried to give a total solids measurement of 1980 mg/L. The dried sample is used in a bomb calorimeter giving 3.2 kJ/g dried weight.

Calculations derived from this data cited in various papers (Logan, 2008, Liao et al., 2006, Schroder, 2008, Logan, 2009):

$$3.2\ \mathrm{kJ/g}\ \times\ 1.98\ \mathrm{g/L} = 6.3\ \mathrm{kJ/L\ wastewater}$$

$$6.3\ \mathrm{kJ/L}\ \times \frac{1}{0.431\ \mathrm{gCOD/L}}\ =\ 14.7\ \mathrm{kJ/gCOD}$$

If the exercise is repeated on the data from the present paper using the oven dried samples and the measurement taken for COD prior to drying the results would have been:

Cramlington

$$8.3\ \mathrm{kJ/L}\ \times \frac{1}{0.718\ \mathrm{gCOD/L}}\ =\ 11.6\ \mathrm{kJ/gCOD}$$

Hendon

$$5.6\ \mathrm{kJ/L}\ \times \frac{1}{0.576\ \mathrm{gCOD/L}}\ =\ 9.9\ \mathrm{kJ/gCOD}$$

This is an underestimation of 60% and 45% respectively.

11.5. Appendix V - Wastewater sterilisation

Several of the experiments conducted in this thesis relied on using real wastewater, but needed this to be sterile. The following method was developed:

Method

The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into Ringers sterile dilutent (APHA, 1998). Effective sterilisation was defined as colony free plates in triplicate at zero dilution. The circulation time was varied to determine the optimum. The change in chemical composition (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) of the wastewater itself as compared to autoclaving and filtering.

Results

UV sterilisation caused the least change in wastewater properties measured as shown in Table 11-3, and was able to fully sterilise the wastewater.

Table 11-3 Percentage change of wastewater characteristics caused by the different sterilisation methods

	COD	Soluble COD	Total Solids	Bacteria per 0.1ml
Autoclaved (121^{o}C for 15 mins)	-15.6% ± 0.9	21.6% ± 0.6	-13.3% ± 5.8	0
Membrane filtered (0.2um PES)	-61.5% ± 0.5	22.8% ± 1.7	-36.1% ± 11.7	40 ±19
UV sterilised (5 mins)	-1.6% ± 0.4	7.2% ± 4.6	-3.3% ± 6.7	0

Bacteria is the average number counted on triplicate plates at zero dilution. All values show mean ± standard deviation (n=3)

Conclusion

Circulation of wastewater for 5 minutes through a UV filter was effective for bacterial kill off and least detrimental treatment to the composition of the wastewater.

11.6. Appendix VI - COD removal and coulombic efficiency

In the acetate fed cells the COD removal was high for both the cells which did (85%) and did not (80%) produce current ($p = 0.051$). For the other reactors there was an average removal of 64% COD for the wastewater and 87% for the starch solution. No significant difference in the COD removal in the reactors which generated current and those that did not was found wastewater ($p = 0.188$) and starch ($p= 0.688$).

The effluent of all reactors contained no detectable VFA's. The measured anions in each cell showed that there was almost complete removal of sulphate, from a starting value of 70 ppm in the wastewater and 38 and 41 ppm in the acetate and starch solutions respectively.

The coulombic efficiency of all reactors was low, such values are reasonably typical for complex substrates, but far lower than would be expected in a functioning acetate fed cell (Logan, 2008, Liu et al., 2011).

Table 11-4 COD removal and Coulombic efficiencies of all reactors fed on the different substrates. The values in grey are the reactors where acclimatisation did not occur

Inocula (ml)	**0.01**	**0.01**	**0.1**	**0.1**	**0.1**	**1**	**1**	**1**	**1**	**10**	**10**	**25**	**25**	**25**	**25**	**50**	**50**
COD removal (%)																	
Acetate	85.6	77.1	85.4	80.3	80.5	86.7	95.6	92.4	82.1	87.3	79.9	84.7	86.6	80.2	77.6	79.0	77.3
Wastewater						60.8	41.9			59.1	69.8	68.1	70.5			80.3	62.5
Starch						88.2	84.5			88.2	86.4	89.4	81.7			80.8	90.5
Coulombic efficiency (%)																	
Acetate	0.1	0.1	1.8	0.5	0.8	4.6	5.3	1.8	0.1	6.7	7.5	10.5	8.9	10.1	9.2	9.1	0.8
Wastewater						3.6	0.1			0.3	0.2	9.4	12.5			10.4	7.4
Starch						1.3	0.78			0.82	13.4	12.5	16.9			17.4	1.6*

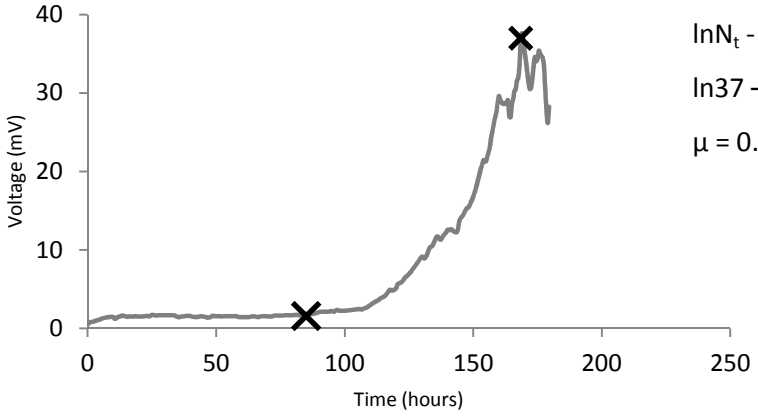
Values in grey are the reactors which did not acclimatise

*Unrepresentative value, data logging equipment failed after the point of acclimation.

11.7. Appendix VII - Yield and Specific activity calculations

Growth rate

Example calculation using 25 ml inocula

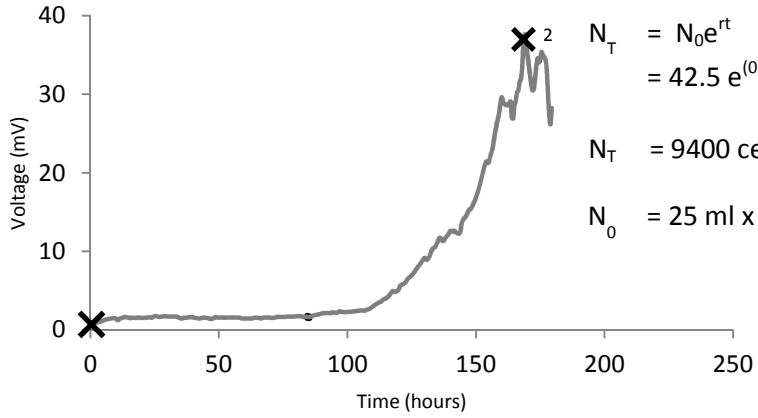


$\ln N_t - \ln N_0 = \mu(t - t_0)$

$\ln 37 - \ln 2 = \mu(170 - 85)$

$\mu = 0.034$

Specific activity



$N_T = N_0 e^{rt}$

$= 42.5\ e^{(0.034 \times 180)}$

N_T = 9400 cells

N_0 = 25 ml x 1.7 cell/ml = 42.5 cells

Each data logged voltage represents the time of 30 minutes, therefore the moles of electrons passed to the circuit per second at the data points measured is:

Moles of electrons = coulombs / Faradays constant

=((Voltage / resistance) x seconds)/Faradays constant

E.g. $\mathbf{X}_2$ =((0.037V / 470Ω)x 30mins x 60 seconds)/96485

$= 1.5 \times 10^{-6}$

Moles of electrons/cell = $1.5 \times 10^{-6} / 9400$

$= 10^{-10}$ mol e^-/cell

This value can be plotted throughout the time course of the experiment and is seen to be relatively constant.

Growth yield

The total number of cells produced up to the end of the exponential growth phase in the example above is 9400 cells.

$$\text{gCOD-cells} = (N_T - N_0) \times W \times COD_{cell}$$

where $N_T - N_0$ is the total new cell produced, W is the weight of each cell as estimated as 5.3×10^{-13} (Logan, 2008) and COD_{cell} is the estimation of 1.25 g-COD/g-cell (Rittmann, 2001).

$$\text{gCOD-cells} = (9400\text{-}43) \times 5.3 \times 10^{-13} \times 1.25$$

$$= 6.1 \times 10^{-9}$$

$$\text{gCODsubstrate} = \sum_{t0}^{t} mol\, e^{-}/8 \times 64$$

Where the sum over the growth period $t\text{-}t_0$ of the moles of electrons as calculated above is divided by 8 to give moles of acetate used, and multiplied by 64 giving the gCOD per mole of acetate.

$$\text{gCOD substrate} = 0.00011 / 8 \times 64 = 8.8 \times 10^{-4}$$

$$\text{gCOD-cell/gCOD-substrate} = 6.1 \times 10^{-9}/8.8 \times 10^{-4} = 6.9 \times 10^{-6}$$

The estimated yield of the acetate fed cells is extremely low ranging between 10^{-4} to 10^{-5} g-COD cell/g-COD substrate for the cells with between 10-50 mLs of inocula.

If exponential growth is assumed throughout the whole time period for the lower inocula cells these values are much higher up to 8 g-COD cell/g-COD for the 0.1 ml inocula. If no growth during lag is assumed these values are lower (10^{-7} g-COD cell/g-COD) and more in line with those observed for higher inocula. These yields are inconsistent with the literature on yields in microbial fuel cells (Freguia et al., 2007, Rabaey et al., 2003) although both of these studies used different methodology. They are also inconsistent with yields of other bacterial systems (Rittmann, 2001).

11.8. Appendix VIII – Calculations of performance in MFCs and MECs

Power Calculation for both MFCs and MECs

Performance can be evaluated through the amount of power produced which can be expressed as:

$$P = IE$$

Where P is the power in watts, E is the voltage as measured by the data logger in volts and I is the current in amps, calculated from the measured voltage E, at a known resistance R:

$$I = E/R$$

Power can therefore be alternatively expressed as:

$$P = E^2/R$$

This power is often also evaluated as power density (*Pd*), this is the amount of power produced per area of electrode surface (typically the size of the anode) expressed as Wm^2. Normalising the power output in this way allows different systems to be compared. This is calculated as:

$$Pd = \frac{E^2}{A_{An}R}$$

Where A_{An} is the area of the anode. The current density (A/m^2) can also be expressed in the same way normalising current to electrode size. Both power and current density can also be expressed per reactor size by substituting A_{An} above for the reactor volume in m^3, resulting in a power density measured as Wm^3. or current density as A/m^3.

Efficiency calculations for MFCs

The efficiency of an MFC is expressed as the Coulombic Efficiency (CE) and is a measure of the amount of coulombs of charge recovered from the cell from the total coulombs available in the substrate that has been removed in the reactor. It is expressed as a percentage:

$$CE = \frac{Coulombs\ recovered}{Coulombs\ in\ substrate}$$

An Amp is the transfer of 1 coulomb of charge per second, therefore by integrating the current over the course of the experiment or batch time (t) the total coulombs transferred is given. Usually the amount of coulombs in the substrate is evaluated using the amount of organic matter removed as determined by the chemical oxygen demand (COD). CE is therefore calculated as:

$$CE = \frac{8 \int_0^t I \ dt}{F \ V_{An} \Delta COD}$$

Where 8 is used as a constant derived from the molecular weight of oxygen divided by 4 the amount of electrons exchanged per mole of oxygen. Faradays constant (F) of 96485 Coulombs/mol, is the magnitude of electrical change per mole of electrons, ΔCOD is the measured change in COD in g/L and V_{An} (L) is the volume of the anode compartment containing the liquid feed at the given COD concentration. .

Efficiency calculation for MECs

The efficiency of an MEC is a more complex matter, as the output of energy is of hydrogen gas (not electricity or charge directly) and the inputs of energy are from the substrate and the additional electrical energy added to the system.

11.9. Appendix IX – Dissimilarity matrix

Unweighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.79	0.00																	
Wastewater inocula 2	0.88	**0.67**	0.00																
Acetate cold ww 1	0.88	0.67	0.70	0.00															
Acetate cold ww 2	0.88	0.65	0.69	**0.45**	0.00														
Acetate cold soil 1	0.82	0.80	0.80	0.72	0.73	0.00													
Acetate cold soil 2	0.76	0.78	0.82	0.75	0.75	**0.59**	0.00												
Acetate hot ww 1	0.89	0.71	0.69	0.54	0.60	0.73	0.78	0.00											
Acetate hot ww 2	0.89	0.73	0.71	0.56	0.58	0.74	0.78	**0.22**	0.00										
Acetate hot soil 1	0.81	0.79	0.80	0.72	0.75	0.62	0.63	0.25	0.70	0.00									
Acetate hot soil 2	0.79	0.79	0.82	0.75	0.76	0.64	0.65	0.18	0.72	**0.51**	0.00								
Wastewater cold ww 1	0.87	0.69	0.70	0.59	0.61	0.73	0.76	0.27	0.64	0.73	0.76	0.00							
Wastewater cold ww 2	0.82	0.72	0.76	0.72	0.73	0.74	0.74	0.44	0.67	0.66	0.67	**0.68**	0.00						
Wastewater cold soil 1	0.85	0.69	0.75	0.61	0.64	0.65	0.69	0.28	0.64	0.64	0.69	0.53	0.66	0.00					
Wastewater cold soil 2	0.88	0.74	0.75	0.69	0.69	0.77	0.78	0.24	0.60	0.69	0.72	0.62	0.51	**0.64**	0.00				
Wastewater hot ww 1	0.86	0.71	0.73	0.62	0.64	0.66	0.71	0.28	0.65	0.67	0.71	0.49	0.68	0.42	0.65	0.00			
Wastewater hot ww 2	0.88	0.74	0.74	0.68	0.68	0.76	0.77	0.24	0.60	0.70	0.72	0.63	0.51	0.65	0.23	**0.66**	0.00		
Wastewater hot soil 1	0.86	0.67	0.71	0.62	0.65	0.74	0.77	0.28	0.64	0.73	0.76	0.35	0.67	0.53	0.64	0.53	0.63	0.00	
Wastewater hot soil 2	0.89	0.71	0.72	0.59	0.58	0.75	0.78	0.28	0.59	0.74	0.78	0.44	0.71	0.51	0.64	0.49	0.64	**0.45**	0.00

Weighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.41	0.00																	
Wastewater inocula 2	0.52	**0.59**	0.00																
Acetate cold ww 1	0.44	0.48	0.45	0.00															
Acetate cold ww 2	0.43	0.45	0.48	**0.08**	0.00														
Acetate cold soil 1	0.50	0.56	0.53	0.17	0.16	0.00													
Acetate cold soil 2	0.44	0.48	0.47	0.09	0.10	**0.10**	0.00												
Acetate hot ww 1	0.49	0.57	0.41	0.28	0.30	0.36	0.30	0.00											
Acetate hot ww 2	0.56	0.63	0.44	0.37	0.41	0.45	0.40	**0.17**	0.00										
Acetate hot soil 1	0.36	0.43	0.50	0.23	0.22	0.30	0.23	0.32	0.43	0.00									
Acetate hot soil 2	0.41	0.51	0.43	0.14	0.16	0.22	0.15	0.23	0.34	**0.16**	0.00								
Wastewater cold ww 1	0.34	0.39	0.46	0.25	0.23	0.35	0.28	0.31	0.43	0.22	0.25	0.00							
Wastewater cold ww 2	0.37	0.42	0.55	0.43	0.40	0.45	0.42	0.46	0.52	0.31	0.39	**0.33**	0.00						
Wastewater cold soil 1	0.36	0.40	0.45	0.26	0.22	0.34	0.27	0.30	0.42	0.22	0.26	0.13	0.36	0.00					
Wastewater cold soil 2	0.41	0.50	0.41	0.31	0.33	0.39	0.33	0.30	0.36	0.37	0.31	0.32	0.33	**0.34**	0.00				
Wastewater hot ww 1	0.35	0.39	0.46	0.27	0.24	0.36	0.29	0.31	0.43	0.24	0.29	0.14	0.37	0.05	0.35	0.00			
Wastewater hot ww 2	0.38	0.49	0.40	0.31	0.32	0.40	0.33	0.26	0.35	0.35	0.29	0.29	0.33	0.31	0.08	**0.32**	0.00		
Wastewater hot soil 1	0.34	0.40	0.48	0.24	0.22	0.34	0.28	0.33	0.45	0.22	0.26	0.05	0.33	0.14	0.31	0.16	0.29	0.00	
Wastewater hot soil 2	0.38	0.38	0.52	0.28	0.24	0.35	0.30	0.36	0.49	0.25	0.30	0.12	0.32	0.18	0.34	0.19	0.34	**0.11**	0.00

Dissimilarity values calculated using UniFrac, lower values indicate greater similarity

11.10. Appendix X - Estimates of sample total diversity

Table 11-5 Estimates of total diversity using the MCMC model (Quince et al., 2008), values given are the lower 95% confidence interval : median : upper 95% confidence interval. The best fit values according to the DIC values are highlighted in bold, the model fits that had DIC scores within 6 of the best fitting model are in italics and should not be considered as plausible options for fitting the data

	Total diversity		
Sample	**Log-normal**	**Inverse Gaussian**	**Sichel**
Arctic soil inocula	**5831:7207:10593**	5151:6227:7439	3632:4403:5821
Wastewater inocula 1	**3431:4238:5572**	2217:2405:2655	2648:3275:5533
Wastewater inocula 2	2924:4260:8970	1679:2066:2752	**1716:2286:3640**
Acetate cold ww 1	**3060:5449:11740**	1273:1700:2406	1402:2197:3379
Acetate cold ww 2	13901:29226:42363	**984:1549:3049**	993:1697:3298
Acetate cold soil 1	**1380146:1393974:1407428**	*3430:5004:7687*	2960:4628:9094
Acetate cold soil 2	**1849625:1865409:1877419**	*3428:4923:7910*	3191:5018:8179
Acetate hot ww 1	1934:3511:12608	808:987:1300	**948:1310:2224**
Acetate hot ww 2	**1217:2159:6024**	643:785:1037	665:843:1264
Acetate hot soil 1	4386:8968:19150	1508:1968:2813	**1456:1984:3086**
Acetate hot soil 2	**171417:184911:197766**	2445:3773:5440	2350:3579:5577
Wastewater cold ww 1	**614:749:1014**	493:535:594	491:534:599
Wastewater cold ww 2	*859:1102:1596*	*640:708:805*	**730:906:1455**
Wastewater cold soil 1	**1079:2249:8263**	543:733:1197	651:1032:2324
Wastewater cold soil 2	556:640:789	*467:494:531*	**510:575:793**
Wastewater hot ww 1	1430:2911:9800	*637:845:1300*	**5682:16751:18608**
Wastewater hot ww 2	**483:548:660**	419:443:476	430:467:525
Wastewater hot soil 1	**820:1148:1985**	581:661:787	596:697:893
Wastewater hot soil 2	694:1135:2283	438:504:614	**468:572:954**

Table 11-6 DIC scores as defined by the sum of the deviance averaged over the posterior distribution and estimate of the sampling effort required to capture 90% of the diversity of taxa within the sample as determined by the fits of abundance distribution

	DIC			Sampling effort		
Sample	Log-normal	Inverse Gaussian	Sichel	Log-normal	Inverse Gaussian	Sichel
Arctic soil inocula	165.53	171.01	166.67	2.02E+06	4.06E+05	1.32E+05
Wastewater inocula 1	450.33	455.14	444.42	1.32E+07	2.56E+05	8.92E+05
Wastewater inocula 2	264.17	262.28	261.93	3.56E+07	2.98E+05	4.16E+05
Acetate cold ww 1	275.13	275.3	275.85	3.32E+09	1.59E+06	3.06E+06
Acetate cold ww 2	197.07	196.74	196.98	1.11E+13	1.47E+06	1.70E+06
Acetate cold soil 1	266.22	273.65	267.61	2.56E+18	1.42E+07	8.37E+06
Acetate cold soil 2	274.28	283.68	274.4	2.42E+18	7.28E+06	5.19E+06
Acetate hot ww 1	309.59	311.17	309.21	2.99E+09	5.88E+05	1.59E+06
Acetate hot ww 2	242.64	244.43	244.76	2.84E+08	3.61E+05	4.73E+05
Acetate hot soil 1	290.25	288.7	288.57	1.17E+10	1.44E+06	1.34E+06
Acetate hot soil 2	265.04	269.84	265.05	6.98E+14	4.73E+06	3.16E+06
Wastewater cold ww 1	254.73	255.02	255.23	5.22E+05	4.23E+04	4.25E+04
Wastewater cold ww 2	268.11	269.7	261.78	1.23E+06	4.91E+04	1.63E+05
Wastewater cold soil 1	201	201.99	197.99	2.68E+08	1.53E+05	5.35E+05
Wastewater cold soil 2	333.27	349.36	332.04	3.47E+05	3.70E+04	9.96E+04
Wastewater hot ww 1	252.09	254.67	246.76	1.37E+09	2.57E+05	1.05E+09
Wastewater hot ww 2	274.09	279.19	275.06	1.51E+05	2.52E+04	3.56E+04
Wastewater hot soil 1	248.04	250.28	248.96	3.54E+06	7.21E+04	9.24E+04
Wastewater hot soil 2	243.6	244.69	242.65	1.93E+07	7.44E+04	1.32E+05

11.11. Appendix XI - Details of the bacteria phyla and families found within the samples tested

It is seen in Figure 11-3 (a) that the inoculated and acclimatised reactors have become enriched Proteobacteria, this phylum dominates with about 80% abundance in the acetate fed cells, and around 60% in the wastewater fed cells. Proteobacteria are a diverse phylum of bacteria, yet most of this high abundance in the reactors is caused by the enrichment of *Geobacter* an exoelectrogenic organism, as is seen in Figure 11-4. *Rhodocyclaceae*, *Psuedomonas* and *Desulfovibrio* also added to the proportion of Proteobacteria that became enriched. The relative abundance of the other main phyla generally drops within the reactor samples, a proportion (around 10-20%) of Bacteriodietes remains, and there is some enrichment of Acidobacteria in the wastewater fed reactors. The wastewater reactors have a greater spread of abundance over the phyla groups shown, with less domination by Proteobacter.

The OTU richness shown in Figure 11-3 (b) again shows the greater diversity of the acetate reactors over the wastewater fed ones, both by the larger bar size and the Chao estimate above. It is seen many of the OTUs present in the inoculum have survived in the acetate reactor conditions, despite the metabolic narrowing of the conditions. Surprisingly this greater diversity or spread of OTUs appears to be slightly higher in the cold reactors, than the warm ones. In the case of the wastewater fed reactors the OTU richness in reduced, temperature does not appear to have an impact.

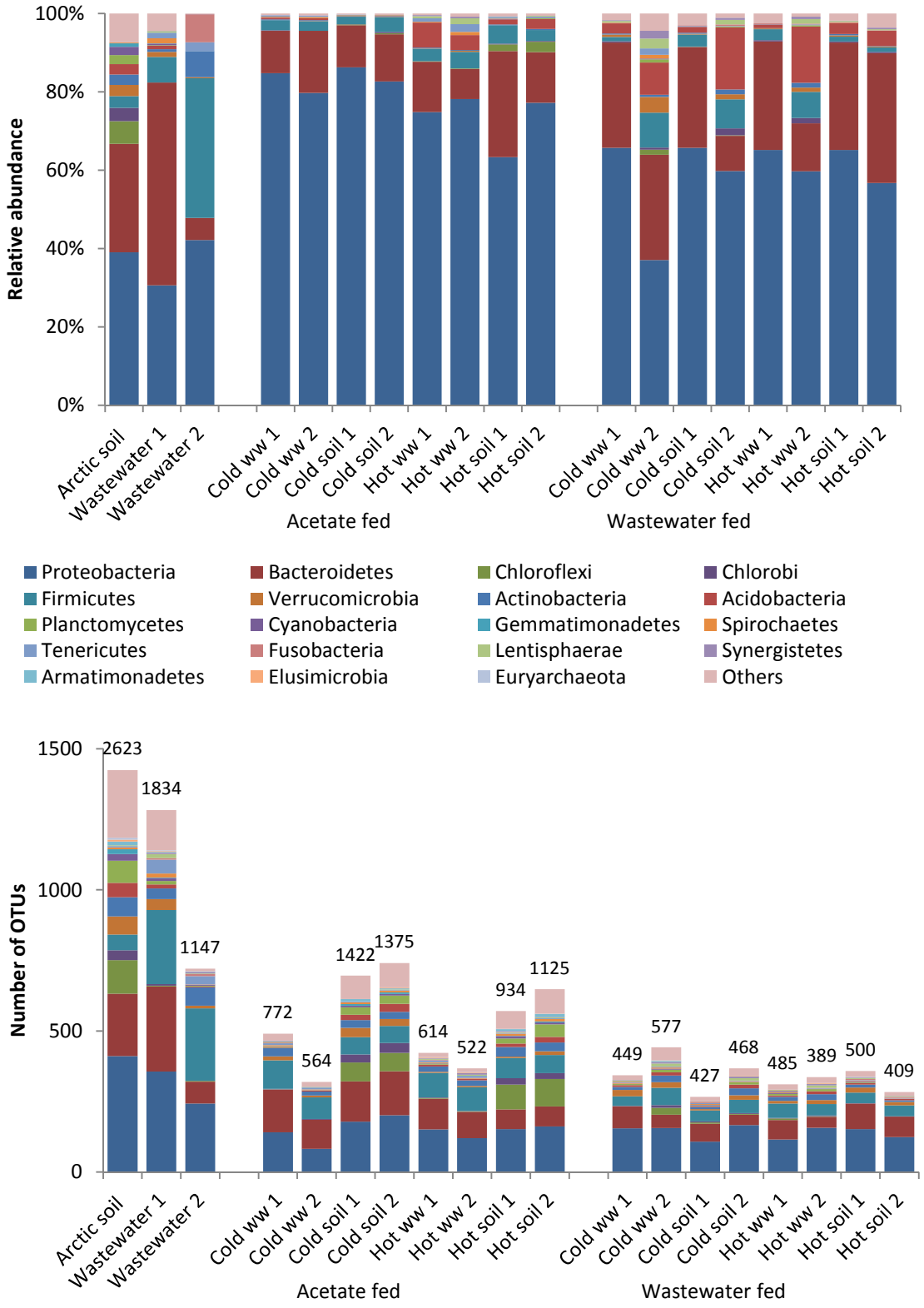


Figure 11-3 Relative abundance (a) and OTU richness (b) for all the data sets given at the phylum rank. Relative abundance is shown as the number of reads within each taxa divided by the total number of reads. The OTU richness is the number of taxa within each phylum is given by the size of the bar, the Chao 1 estimate of richness is written at the top of each bar

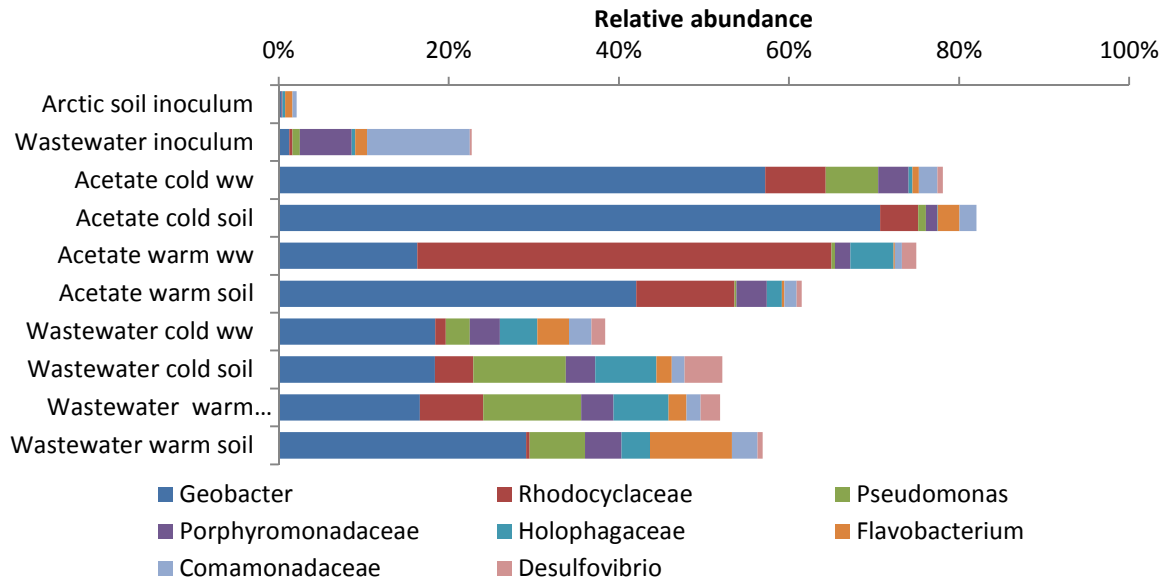


Figure 11-4 The relative abundance of the 8 most dominant genus as an average for the duplicate reactors under each condition, where the genus name was not given by the classification database family is used

It would be expected that the most dominant organisms within the reactors are the ones that are able to most competitively metabolise, grow and therefore reproduce within the conditions of the reactors. The top 8 most dominant genus are given in Figure 11-4, for Rhodocyclaceae, Porphyromonadaceae, Holophagaceae, Comamonadaceae the classification did not give the genus name, and therefore the family name is given. It is seen that for the acetate fed reactors these 8 genus make up a large proportion of the total abundance, and in the cold reactor most of this is by *Geobacter*. For the warm acetate reactors, *Geobacter* is still important, but Rhodocyclaceaea is also dominant, especially in those seeded with wastewater. The proportion of *Geobacter* is made up of 11 different species (names of which are not given by the classification), 4 of which are dominant within the reactors. Rhodocyclaceae is a diverse family of bacteria associated with wastewater treatment, further classification of this group is not made.

Within the wastewater reactors Geobacter is less dominant, between 20-30% of abundance, and there is a greater spread of the other genus and families, most notable *Pseudomonas* which make up to 10%. Within the Pseudomonas genus, 8 species were identified, of which 2 were dominant within the reactors, Pseudomonas have previously been seen within fuel cell systems fed substrates such as glucose and butyric acid and are believed to be capable of fermentation (Kiely et al., 2011c), some species such as *Pseudomonas aerunginosa* produce soluble redox shuttles and have been investigated

for their use within fuel cell systems (Marsili, 2010). The family of Holophagaceae is also quite enriched, this family includes the species of *Geothrix fermetans* which has been found in wastewater fed MFCs and is believed to be important in the hydrolysis or fermentation steps, (Kiely et al., 2011a), and has also been linked to shuttle formation (Bond and Lovley, 2005). *Flavobacteium* are also enriched, although this genus is more typically associated with freshwater environments. There is also likely to be sulphate reduction occurring in the cells due to the presence of *Desulfovibro*.

11.12. Appendix XII – Acknowledged contributions

Section	Other contributors	Contribution of E. Heidrich
Chapter 2	T.P. Curtis and J. Dolfing – editing and guidance with content	90%
Chapter 3	T.P. Curtis and J. Dolfing – editing and guidance with content	95%
Chapter 4	T.P. Curtis and J. Dolfing - editing and guidance with content, M. Wade – bioinformatics analysis, W.T.Sloan – sequencing funding	95%
Chapter 5	T.P. Curtis, K. Scott, I Head and J. Dolfing – discussion and experiment planning	95%
Chapter 6	T.P. Curtis K. Scott and J. Dolfing - editing and guidance with content, S. Edwards – site installation and running MEC	90%

Evaluation of Microbial Electrolysis Cells in the treatment of domestic wastewater

Thesis submitted to Newcastle University for the degree of Doctor of Philosophy

Author: Elizabeth Susan Heidrich

Supervisor: Professor Tom Curtis

Co-Supervisor: Dr Jan Dolfing

Co-Supervisor: Professor Keith Scott

Date: May 2012

Abstract

Wastewater can be an energy source and not a problem. This study investigates whether rapidly emerging bioelectrochemical technologies can go beyond working in a laboratory under controlled temperatures with simple substrates and actually become a realistic option for a new generation of sustainable wastewater treatment plants.

The actual amount of energy available in the wastewater is established using a new methodology. The energy is found to be considerably higher than the previous measurement, or estimates based on the chemical oxygen demand with a domestic wastewater sample containing 17.8 kJ/gCOD and a mixed wastewater containing 28.7 kJ/gCOD.

With the energy content established the use of bioelectrochemical systems is examined comparing real wastewater to the 'model' substrate of acetate. The abundance of exoelectrogenic bacteria within the sample, and the acclimation of these systems is examined through the use of most probable number experiments. It is found that there may be as few as 10-20 exoelectrogens per 100 mL. The impact of temperature, substrate and inoculum source on performance and community structure is analysed using pyrosequencing. Substrate is found to have a critical role, with greater diversity in acetate fed systems than the wastewater fed ones, indicating that something other than complexity is driving diversity.

Laboratory scale microbial electrolysis cells are operated in batch mode fail when fed wastewater, whilst acetate fed reactors continue working, the reasons for this are examined. However a pilot scale, continuous flow microbial electrolysis cell is built and tested at a domestic wastewater treatment facility. Contrary to the laboratory reactors, this continues to operate after 3 months, and has achieved 70% electrical energy recovery, and an average 30% COD removal.

This study concludes that wastewater is a very complex but valuable resource, and that the biological systems required to extract this resource are equally complex. Through the work conducted here a greater understanding and confidence in the ability of these systems to treat wastewater sustainably has been gained.

Acknowledgements

It has been a great privilege to be able to study for a PhD, and I am grateful to all those who have made it possible.

I would like to thank my supervisor Tom Curtis for giving me the support and freedom to develop and explore my own ideas, always meeting these with enthusiasm and the necessary intellectual guidance. I would also like to thank Jan Dolfing who has skilfully guided me into, and through many intellectual challenges. I would like to thank Keith Scott and Ian Head for their support and guidance when needed. I would also like to thank Bill Sloan and Chris Quince for their support and guidance with the sequencing work, the contributions of individuals are detailed in Appendix XII.

I would like to thank EPSRC for giving me my doctoral training grant, and the School of Chemical Engineering and Advanced Materials for additional funding which enabled me to take up the offer of a PhD. I would like to thank Northumbrian Water Ltd. for the additional funding which has allowed for more experimental work to be carried out. I would like to thanks in particular from Northumbrian Water Ltd Chris Jones and Andrew Moore, who have been enthusiastic and supportive throughout this project and have provided me with all the assistance needed, and also Laura Stephenson, Steve Robson and all the workers at Howdon wastewater treatment plant, who have enthusiastically accommodated the pilot scale reactor for far longer than expected.

I would like to thank all my colleagues and co-workers who have provided advice, support and humour when needed. In particular to: Beate Christgen who helped with setting up MFCs and electrochemistry tests; Matt Wade who patiently nursed me through the bioinformatics process; and Stephen Edwards who has always been willing to lend a helping hand or ear.

As well as intellectual and financial support, I have also received, and needed, much emotional and moral support during the course of this PhD. This has come from many of those listed above, but also from my family who have not only made my completing a PhD possible, but are also the reason why it has been worthwhile. I would like to thank in particular: my mum Linda who has always taught me to learn for the sake of enjoyment above all else; my niece Katie whose determination and fighting spirit is a

continual inspiration; my husband Oliver, whose moral support, guidance, motivation and good humour has been unfaltering throughout; and lastly I thank my son Ben and his beautiful smile.

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Chapter 1. Introduction

There is growing consensus that wastewater is a resource not a problem (Verstraete and Vlaeminck, 2011, Sutton et al., 2011, McCarty et al., 2011). The conventional treatment of wastewater removes its organic content via aerobic processes, termed activated sludge, this is energy expensive typically 3% of the electrical energy usage of many developed countries (Curtis, 2010). Not only is the energy in wastewater removed not recovered, we expend considerable energy in performing this removal.

In the UK the water sector energy use has increased 10% in the last 10 years (Water UK, 2012, Water UK, 2011), industrial electricity prices have increased by 69% since 2000 (National Statistics, 2011). If these trends continue the energy bill for the water sector will be vastly higher than for the current 9016 GWh (Water UK, 2012). With infrastructure requiring long term planning and capital investment, it is hard to see without drastic action how the necessary changes can be made. Technologies that require relatively simple modifications to the current infrastructure to become operational are more likely to be given a chance rather than those which require wholesale change. New technology should ideally fit reasonably well into the existing infrastructure, and as a minimum achieve similar loading rates per unit area to activated sludge of 0.4-1.2 kg BOD $m^{-3}d^{-1}$ (Grady, 1999). The high capital costs of change and the uncertainty of using a different technology, coupled with the regulation of both effluent quality and pricing structures, are an obstacle to change.

There are alternatives to this approach. Replacing the aerobic activated sludge process with an anaerobic process means the energy stored in the organic content of the wastewater is converted to methane (80% efficiency) which can be combusted to produce electricity (35% efficiency) (McCarty et al., 2011). Only around 30% of the total energy in the wastewater can be captured as electricity in anaerobic systems, although with heat exchange in the combustion process, or the use of non-combustion methods of conversion, this could be increased (McCarty et al., 2011).

The scientific challenges of creating an energy neutral or even energy positive wastewater treatment process are also substantial and complex. The process needed to replace activated sludge must:

- Extract and convert energy to a useable form at an efficiency that justifies the costs.
- Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
- Treat low strength domestic wastewater, which is problematic for anaerobic digestion technologies (Rittmann, 2001).
- Work at ambient, often low temperatures, again problematic for anaerobic digestion (Lettinga et al., 1999).
- Work continuously and reliably.

An innovative and relatively new approach to wastewater treatment is through the use of bioelectrochemical systems (BES), though the fuel cell technology lying behind this process is over 100 years old (Potter, 1911) (see appendix I for a history of development). Here wastewater is consumed in a battery like cell, redox reaction catalysed by bacteria pushing electrons around in an electrical circuit, thus creating electricity (Rabaey et al., 2007). In a microbial fuel cell (MFC) the electricity is captured directly (Logan, 2005), in a microbial electrolysis cell (MEC) the electricity is supplemented by an external source to make a product such as hydrogen or methane (Rozendal et al., 2006) or to perform a process such as reductive dechlorination (Aulenta et al., 2008) or de-salination (Mehanna et al., 2010). There are substantial losses within these systems (Logan et al., 2006), it is suggested they may reach a higher conversion efficiency of 44% (McCarty et al., 2011), the performance of MFCs to date has only reached around 1 tenth of that needed to be competitive with anaerobic digestion (Pham et al., 2006). With MECs the potential higher value (energetically or commercially) of the product formed or process completed means this technology is likely to be more viable and may be the driver of development (Foley et al., 2010).

As organic matter is degraded by bacteria it releases electrons (oxidation) providing energy for the cells. These electrons then pass to an electron acceptor (or reduced species), which is normally oxygen, nitrate or sulphate depending on their availability providing further energy for the cells (Rittmann, 2001).It has been shown that there is a group of organisms that are capable of passing electrons to materials (such as metal oxides) outside the cell, which are then transferred by that material to an electron acceptor. This process is termed electrogenesis, and the group of organisms are known as exoelectrogens (Logan, 2008). MFCs exploit this, providing the bacteria with a

surface to donate electrons to, and then using the principles of all electrochemical cells to transport these electrons and create current.

MFCs, like electrochemical cells usually have two compartments, the anode chamber containing organic matter to be degraded, and the cathode chamber containing an electron acceptor. In the anode chamber organic matter is degraded by bacteria producing electrons, the absence of a preferred electron acceptor such as oxygen, means these electrons pass into the anode material then through a wire to the cathode. The H^+ ions generated in this reaction pass through the membrane from the anode to cathode chamber. At the cathode the electrons, H^+ ions and a reduced species (typically oxygen) combine to form for example H_2O. Electrical current is generated in the wire as the electrons pass from one side to the other.

An MEC reactor is an adaptation of an MFC. In an MEC both the anode and cathode chamber are anaerobic. Rather than creating H_2O in the cathode chamber, the electrons and H^+ ions are combined to generate H_2 gas rather than electricity. The process of forming H_2 is however endothermic, i.e. it requires energy. It cannot happen spontaneously. The addition of a small amount of electricity (with acetate this is in theory 0.114 V, in practice <0.25 V), is required to generate the H_2 gas (Logan et al., 2008). This is substantially less energy than is required to produce H_2 through water electrolysis, typically 1.8-2.0 V. A schematic of an MEC is shown in Figure 1-1.

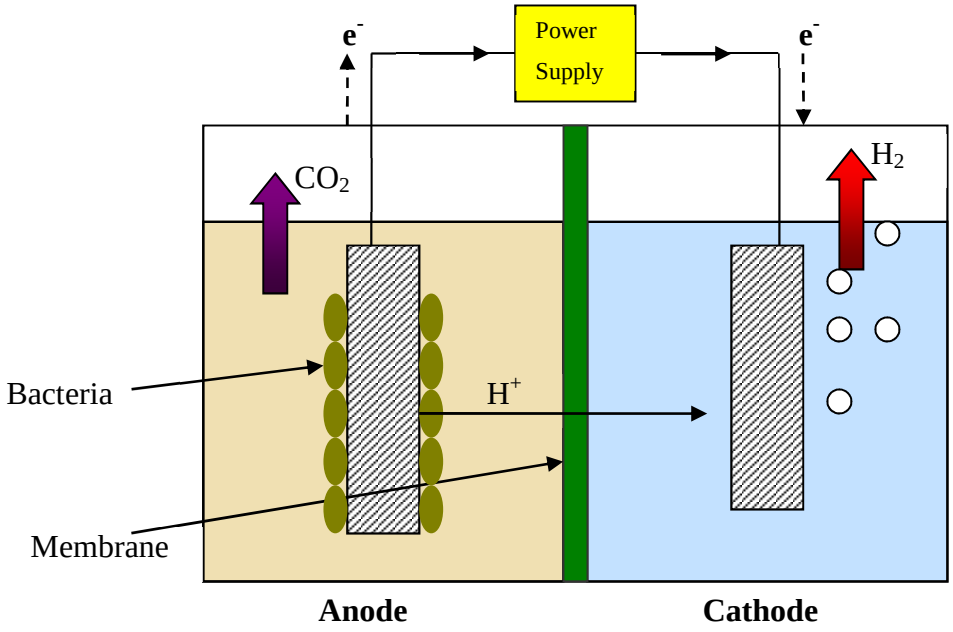

Figure 1-1 Generalised schematic of an MEC adapted from (Liu et al., 2005b) showing the flow of electrons and hydrogen ions and the function of the anode and cathode sections

The theoretical electrochemical energy gains or requirements of a MFC and MEC respectively will vary with temperatures, substrate free energy and ionic concentrations especially pH, as shown in appendix II. Even if it were possible to determine the potentials accurately in practice these theoretical values are not achieved. Energy is lost through all the transfer processes which take place to allow this reaction to happen. There are both electrochemical losses known as overpotentials caused by losses in redox reactions and transfer to the electrodes, losses in transfer of ions between the electrodes, limitations caused by transfer rates being different for different species, and on top of this there are losses caused by transfer of both electrons and ions in and out of the bacteria, losses to the bacteria themselves as they use energy, losses of electron transfer, and also losses by side or chain reactions occurring which do not advantage the fuel cell (Logan, 2008). This means that the energy gained in an MFC is less, and the energy input required in an MEC is more, than would theoretically be the case, represented in Figure 1.2.

In an MEC substantially more energy input than the theoretical is needed, in acetate fed systems these typically range from 0.4 V to 0.8 V with greater hydrogen gas production at higher voltages but less energy efficiency (Call and Logan, 2008). Glucose fed reactors have been shown to operate at applied voltages of 0.9 V (Selembo et al., 2009a), although far less work has been carried out on this substrate and its limits of applied voltage are undefined. In a larger scale system it is likely overpotentials (the difference between the theoretical potential at which the reaction occurs, and the observed potential of the electrode) will be increased and therefore the power input might be higher. In a pilot scale reactor fed on wine wastewater the input voltage of 0.9 V was used, although this performed less well than laboratory trials at a smaller laboratory scale on the same substrate, high over potentials being one of the suggested reasons (Cusick et al., 2011).

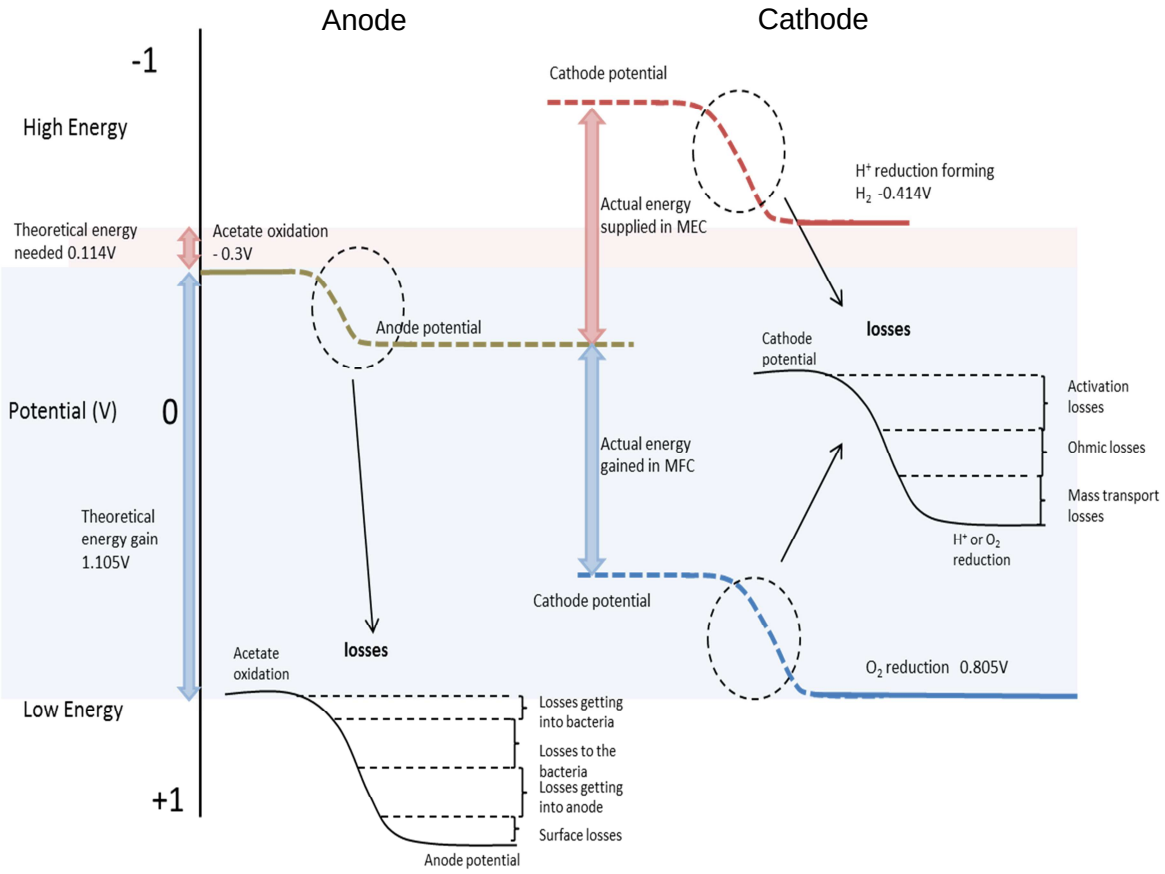

Figure 1-2 Representation of the energy losses within an MFC and MEC using acetate. Energy is shown as potential on the vertical axis, the green line shown the potential of the anode from the potential of acetate (solid line) to the actual anode potential (dotted line) which dependant on the losses. The reduction potential of the MFC and MEC cathode reactions is shown as the solid blue and red lines respectively, whereas the actual cathode potential is again shown in the dotted lines and is dependent on losses. The predicted total energy gain (MFC) and loss (MEC) is shown by the thick arrows and can be variable depending on these losses, but will always be less than that theoretically predicted as seen in the thick arrows at the vertical axis

Understanding the complexities of the electrochemistry of these systems is however only part of the challenge of understanding and ultimately manipulating BES technology. The microbiology of such systems plays a critical role in dictating their efficiency and their success or failure. The microbial community, which catalyses and enables the whole process to take place will also be affected by temperature, pH and substrates (Rittmann, 2001), it will vary with time and within the reactor, and the factors of competition, symbiosis and random assembly lead to a highly complex and unpredictable system.

BES systems run on electrochemical principles but rely on microbial communities. Therefore predicting their absolute function and output of energy, or indeed the input of energy needed, is at this stage in our understanding not possible. The empirical collection of this information is necessary in helping us identify not only if this technology is viable but also the areas that can and need to improved. Critically understanding the bacterial communities and the energy transfers within these systems lies at the heart of being able to manipulate and use this technology.

BES in general and MECs in particular have the potential to fulfil these needs of the wastewater industry (Foley et al., 2010). MECs are entirely anaerobic, eliminating the need for any aeration or complex membrane systems, meaning their engineering can be simple and 'retrofittable' within existing infrastructure. Although hydrogen production is focused on in this study, the flexibility of this process to make other high value products is an economic driver. However the key challenges to overcome are the scientific ones. An increasing body of work is amassing showing improved efficiencies and performance, however the vast majority of this is with simple substrates at warm temperatures (Rader and Logan, 2010, Call et al., 2009, Cheng et al., 2006b, Zhang et al., 2010). Evidence that BES work at low temperature is conflicting (Jadhav and Ghangrekar, 2009, Cheng et al., 2011), the only published study of a large scale 'hydrogen producing' MEC did not produce hydrogen (Cusick et al., 2011), and MECs studies using real wastewater as a substrate are limited, the longest documented study runs reactors for 7.6 days (Wagner et al., 2009).

1.1. Aim and objectives

The overall aim of this research is to understand if BES can be used as a sustainable method of wastewater treatment.

Much work has been and is being carried out fine tuning BES technologies within laboratories, testing new materials and moving towards greater output efficiencies, however large volumes of this work is conducted at warm temperatures and with simple artificial substrates (Hu et al., 2008, Logan et al., 2008, Selembo et al., 2009a, Tartakovsky et al., 2009). This research does not strive towards making such efficiencies, but answers the following fundamental questions of: can they work with

real wastewaters? and, can they work at realistic temperatures? this was addressed by completing the following objectives:

- Quantifying the amount of energy available in the wastewater
- Analysing the start-up and community development of MFC systems.
- Testing the operation and performance of MFC reactors at low temperatures
- Monitoring the performance of MEC reactors with wastewater substrate
- Building and testing a pilot scale MEC reactor run at a wastewater treatment site.

Chapter 2. Determination of the Internal Chemical Energy of Wastewater

Parts of this chapter have been published as Heidrich, E.S., Curtis T.P., and Dolfing J., Determination of the Internal Chemical Energy of Wastewater. Environmental Science & Technology, 2011. 45(2): p. 827-832.

The wastewater industry is facing a paradigm shift, learning to view domestic wastewater not as a waste stream which needs to be disposed of, but as a resource from which to generate energy. The extent of that resource is a strategically important question. However, the only previous published measurement of the internal chemical energy of wastewater measured 6.3 kJ/L, calculated to be 14.7 kJ/gCOD. It has long been assumed that the energy content in wastewater relates directly to chemical oxygen demand (COD). However there is no standard relationship between COD and energy content. In this study a new methodology of preparing samples for measuring the internal chemical energy in wastewater is developed, and an analysis made between this and the COD measurements taken. The mixed wastewater examined, using freeze drying of samples to minimise loss of volatiles, had 28.7 kJ/gCOD, whilst domestic wastewater tested had 17.8 kJ/gCOD nearly 20% higher than previously estimated. The size of the resource that wastewater presents is clearly both complex and variable, but is likely to be significantly greater than previously thought. A systematic evaluation into the energy contained in wastewaters is warranted.

2.1. Introduction

Every one of us produces at least around 40 $gBOD_5$ (biochemical oxygen demand consumed over 5 days), in waste every day, in richer countries this is likely to be nearer 80 $gBOD_5$,(Mara, 2004), equating to around 60-120 gCOD/person/day (Kiely, 1997). If there were 14.7 kJ/gCOD (Shizas and Bagley, 2004), the only previous published measurement of the energy value of wastewater, with 6.8 billion people in the world, 2.2 - 4.4 x 10^{18} joules of energy per year is available, or a continuous supply rate of 70 - 140 gigawatts of energy, the equivalent of burning 52 - 104 million tonnes of oil in a modern power station, or 12 - 24,000 of the world largest wind turbines working continuously. This estimation does not even include all the energy contained in our agricultural and industrial wastewater.

Despite the resource that wastewater represents, most developed countries spend substantial quantities of energy treating the wastewater so it can be released without harm to the environment, the US uses approximately 1.3% of its total electricity consumption doing so (Carns, 2005, Logan, 2008). The energy for wastewater treatment will be a particular burden in the urban areas of less well-off nations. Wastewater is typically viewed as a problem which we need to spend energy to solve, rather than a resource. If the energy contained in wastewater is harnessed, not only could it help the water industries become self-sufficient in energy or even net providers, but it could also be a modest source of energy in parts of the world which currently lack reliable and affordable energy supply.

Wastewater contains a largely uncharacterised and undefined mixture of compounds, including many organics, likely to range from small, simple chains through to more complex molecules. All organic compounds contain energy stored within their bonds. The energy that can be obtained from wastewater by different processes is varied, methane gas from anaerobic digestion, electricity from microbial fuel cells (MFCs), or hydrogen in the case of microbial electrolysis cells (MECs) (Logan, 2008) or a fermentation process (Davila-Vazquez et al., 2008). Large amounts of research is being undertaken in all of these areas but there has been very little work conducted in quantifying the amount of energy held in wastewater to start with.

The COD of wastewater has long been used as a relatively simple and reliable method of determining the 'strength' of waste, and by inference the energy contained within it. However there is no empirical formula for the determination of the energy content from the COD measurement. The only previous study to attempt to determine the energy content of raw municipal wastewater by experiment was conducted by Shizas and Bagley (2004) using a bomb calorimeter. Here a single grab sample of domestic wastewater from a treatment plant in Toronto was dried in an oven overnight at 103^{o}C before being analysed by bomb calorimetry. It was found that the domestic wastewater had a measured COD of 431 mg/L, and an energy value of 3.2 ± 0.1 kJ/g dry sample; with 1.98 g/L of solids this equates to 6.3 kJ/L. This interesting observation has led to the pioneering interpretation that wastewater contains 14.7 kJ/gCOD (Logan, 2008), which has been cited in the literature several times in particular with relation to microbial fuel cell work (Liao et al., 2006, Schroder, 2008, Logan, 2009). However the oven drying of samples will have driven off many volatile organic compounds, such as

methanol (boiling point 64.7 ^{o}C), ethanol (78.4 ^{o}C), and formic acid (101 ^{o}C). Moreover, the calculations were based on a single grab sample from one treatment plant, and using the COD measurement taken prior to drying, it is very likely that some of this COD will have also been lost before the energy determination was made. The work of Shizas and Bagley (Shizas and Bagley, 2004) provides a valuable starting point for the estimation of energy in wastewater, but given the volatile losses, and the measurement of the COD before these losses have occurred, this value must be an underestimation of the true internal chemical energy of wastewater.

The objectives of this study were to develop an improved methodology for measuring internal chemical energy, to better quantify the internal chemical energy of wastewaters, and to evaluate the relationship between internal chemical energy and COD.

2.2. Materials and methods

2.2.1. *Collection and storage of samples*

Two 24 hour composite samples of influent wastewater were taken, one from Cramlington Wastewater Treatment Plant, which deals with a mixed (i.e. industrial and domestic) wastewater, and the other from Hendon Treatment Plant, primarily treating domestic wastewater, both in the North East of England. Within two hours of collection, 3 L of sample was placed into the deep freeze at -80 ^{o}C, and a further 3 L was placed into an oven at 104 ^{o}C. A sample was stored in a refrigerator at 4 ^{o}C.

2.2.2. *Drying procedures*

After a period of around 48 hours in the oven at 104 ^{o}C the sample was fully dried. This was then ground into a powder using a pestle and mortar, and stored in four measured quantities of approximately 0.5 g in clean, dried sealed containers. The frozen samples were dried using a freeze dryer (Labconco Freezone, Labconco Corp. USA) which when used daily over a period of 4 weeks was capable of drying about 1.5 L of sample, each 20 hour drying period removing a few millilitres of liquid. The samples were stored at -80 ^{o}C between drying for 12 hours whilst the freeze dryer stabilised. This procedure was repeated until enough sample was dried to yield four 0.5 g samples. These were then ground and stored in the same way as the oven dried samples.

2.2.3. *Wastewater analysis*

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total organic carbon (TOC), inorganic carbon (IC), total carbon (TC) and chemical oxygen demand (COD) measurements were carried out in the two days after collection using the refrigerated samples. The methods described in Standard Methods for the Examination of Water and Wastewater (APHA, 1998) were used. TS was also measured using the freeze drying process. Further COD tests were carried out on rehydrated freeze dried and oven dried samples. All measurements were taken in triplicate.

2.2.4. *Energy content*

The energy content of the dried wastes was determined using an adiabatic bomb calorimeter, Gallenkamp Autobomb. The internal bomb was a stainless steel unit surrounded by a water jacket with a volume of 1900 mL, with a further cooling jacket outside with a flow of 300 mL/min. The system also included a mechanical stirrer, ignition unit and a digital thermometer accurate to 0.01 ^{o}C. The effective heat capacity of the system i.e. the heat required to cause a unit rise in temperature of the calorimeter was determined using triplicate samples of pure benzoic acid. This was used to calibrate the heat of combustion of the system components such as the wire and cotton, and the effective heat capacity of the bomb, its water jacket and thermometer. After this determination all of the components of the system were then kept constant throughout the tests. Four samples of benzoic acid were used on each time of operation of the bomb calorimeter to verify the technique.

The samples were dried, weighed to around 1 g, and compacted before combustion in the bomb. It was found that the samples did not fully combust, and therefore they were mixed in a 1:1 ratio with a combustion aid of benzoic acid, a method used by Shizas and Bagley (2004). The exact sample weight and the temperature rise in the surrounding water jacket was recorded and used to determine the energy content of each sample. All measurements including the benzoic acid standards were taken in a randomised order.

2.2.5. *Energy content calculations*

The bomb calorimeter measures the heat of combustion of the bomb's contents. When the bomb is ignited the contents including the fuse wire, cotton thread used to attach the sample to the fuse wire and the fuel, including any benzoic acid used is burnt, and this heat is absorbed by the bomb and its surrounding water jacket. In addition to the heat from the combustion, there is also heat created by the formation of nitric acid from the

nitrogen contained in the air inside the bomb. Moles of nitric acid formed are found by titration of the bombs contents with 0.1M NaOH. It is assumed that there is 57.8 kJ/mol of nitric acid; the oxidation state of the nitrogen is not taken into consideration as is standard practice (Rossini, 1956). The kilojoules contained in the sample are calculated in the following equation:

$$-\Delta U_{c,s} = ((V_w + B)(c_{p,w})(\Delta T) + (-\Delta U_{c,w}) + (-\Delta U_{c,c}) + (-\Delta U_{c,b})(m_b) - (Q_{f,n}\, mol_{nitric})) / m_s$$

Table 2-1 Definition of parameters in the equation above used to calculate energy of combustion

Term	Definition
$-\Delta U_{c,s}$	Energy of combustion at constant volume for sample (kJ/g)
$-\Delta U_{c,b}$	Energy of combustion at constant volume for benzoic acid = 26.42 kJ/g[a]
$-\Delta U_{c,w}$	Energy of combustion at constant volume for fuse wire = 0.013 kJ/g[b]
$-\Delta U_{c,c}$	Energy of combustion at constant volume for cotton = 0.082 kJ/g[b]
V_w	Volume of water = 1940 g[b]
B	Volume of water equivalent to the effect of the bomb container = 390 g[b]
$c_{p,w}$	Specific heat capacity of water = 0.00418/g/^oC[a]
ΔT	Temperature rise (^oC)
m_b	Mass of benzoic acid combusted (g)
m_s	Mass of sample combusted (g)
$Q_{f,n}$	Heat of formation of nitric acid = 57.8 kJ/mol[a]
mol_{nitric}	Moles of nitric acid formed (mol)

[a](Atkins, 2006)

[b]Determined in laboratory

2.2.6. *Measurement of volatile fatty acids*

The loss of known volatile fatty acids (VFA's) was measured for each drying technique using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Triplicate 20 mL samples of 50 ppm acetate solution were dried overnight in an oven at 104 ^oC, and in the freeze dryer. These were then re-hydrated with 20 mL of deionised water, and the VFAs measured.

2.2.7. *Measurement of anions*

The anion content of both wastewaters was measured in triplicate using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent.

2.2.8. *Measurement of volatile halocarbons*

Dried 20mg samples were rehydrated using 20 mL de-ionised water and, 20 mL wastewater samples were sealed within a sample jar, with the addition of 20 mg of salt (KCl). These were left for 24hrs at 30^{o}C, the headspace gas was then analysed using an Agilent 7890A GC split/split less injector linked to an Agilent 5975C MSD

2.2.9. *Statistical techniques*

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run two sample t-tests on the data. Before the tests were performed the data was checked for equal variance and normal distribution, validating the use of a two sample t-test.

2.3. Results

This paper uses an improved methodology: freeze drying the samples prior to using a bomb calorimeter. With this method only a few millilitres of liquid can be removed in a 24 hr operational period. Therefore drying enough wastewater to yield several grams of solids takes between 4 - 8 weeks. Although far more time consuming it is believed this is the best method available for drying the wastewater without raising its temperature and thus removing the volatiles.

Table 2-2 Measured wastewater parameters of the two different samples used in the energy analysis

	Cramlington	**Hendon**
COD	718.4 ± 9.7	576.2 ± 40.8
COD- oven dried	368.2 ± 12.3	324.0 ± 18.1
COD - freeze dried	587.1 ± 32.2	425.3 ± 16.5
Total solids - oven dried	1392 ± 35	1070 ± 60
Total solids - freeze dried	1597 ± 40	1130 ± 20
Total organic carbon	116.5	115.8
Total carbon	181.8 ± 2.3	196.4 ± 1.2
Inorganic carbon	65.3 ± 1.2	80.5 ± 0.1
Volatile solids (standard method)	953 ± 143	427 ± 20
Total Kjeldahl Nitrogen	92.4 ± 0.0	71.9 ± 4.3
Chloride (ppm)	391 ± 10.9	169.6 ± 17.2

Mean ± standard deviation (n=3), all values are in mg/L unless otherwise stated

Table 2-2 shows the differences between the two wastewaters, and the effects of the drying processes on the COD and solids recovery from these wastewaters. Oven drying reduces the measured COD from 718.4 mg/L in the original wet sample to 368.2 mg/L (49% loss) in the Cramlington wastewater and from 576.2 mg/L to 324.0 mg/L (44% loss) in the Hendon sample, whilst freeze drying gives losses of 18% and 26%. The freeze drying process captured 5-12% more mass than oven drying. This demonstrates that freeze drying is a more accurate method to determine the total amount of COD than oven drying. However, even freeze drying resulted in COD losses of 18-26%. This is probably due to the loss of the volatile fraction of the COD such as short chain fatty acids. This was confirmed using ion chromatography where oven dried samples contained 0.000 ppm acetate whereas freeze dried samples contained 1.8 ppm, compared to the original 54.5 ppm. Acetate is one of the smaller and therefore more volatile of the VFA's and is likely to represent some of the greatest losses.

Table 2-3 Measured internal energy content values given as both energy per litre and energy per gCOD using the post drying measurement of COD

	Cramlington		Hendon	
	Oven dried	**Freeze dried**	**Oven dried**	**Freeze dried**
kJ/L	8.3 ±1.8	16.8 ± 3.3	5.6 ±1.0	7.6 ± 0.9
kJ/gCOD	22.5 ± 4.8	28.7 ± 5.6	17.7 ± 3.2	17.8 ± 2.1

Mean of four measurements ± standard deviation
Values for kJ/gCOD are calculated from the COD measurement after drying and re-hydrating, and TS measurement for the given drying method.

The freeze drying method enabled a significantly greater proportion of the energy in the wastewater to be measured, over 50% more for Cramlington (p value 0.010), and 24% more for Hendon (p value 0.044). There are also significant differences between the two wastewaters, with the Cramlington waste being more energy rich (p value 0.019). The energy content per gram of oxidisable material measured i.e. kJ/gCOD is considerably higher for both wastewaters than previous estimates of around 14 kJ/gCOD, for the Cramlington wastewater this is even higher with the freeze dried sample.

The energy captured by the freeze drying process does not equate to all the energy available in the wastewater sample. Based on the percentage losses of measured COD from the original sample to the freeze dried sample (18% for Cramlington and 26% for

Hendon), the actual energy of the Cramlington wastewater could be as high as 20 kJ/L, and 10 kJ/L for the Hendon wastewater.

2.3.1. *Theoretical results - can internal chemical energy per gram COD be calculated from first principles?*

If we were able to evaluate the energy content of wastewater from the COD measurement, this would require an estimation of which organic compounds are present. With this, the internal chemical energy for each individual organic compound can be calculated on the basis of simple thermodynamic calculations as follows (thermodynamic values are taken from Atkins (2006)) based on the principle that 1 gram of COD equals 1/32 mol O_2, i.e. for every 1 mol O_2 there is 32 grams COD.

If we assume that the organic compound present is methane:

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \qquad (1 \text{ mol } CH_4 = 64 \text{ gCOD})$$

The overall enthalpy for the reaction can be calculated on the basis of Hess's Law, which states that the enthalpy of a reaction is equal to the sum of the enthalpy of formation ($\Delta_f H$) of all the products minus the sum of the enthalpy of formation of all the reactants. Using tabulated values for the enthalpy of formation the energy released in the above reaction with methane is as follows:

$$\begin{aligned}
\Delta_f H \text{ (kJ/mol)} &= \sum \Delta_f H \text{ products} - \sum \Delta_f H \text{ reactants} \\
&= 2(\Delta_f H\ H_2O) + \Delta_f H\ CO_2 - \Delta_f H\ CH_4 - 2(\Delta_f H\ O_2) \\
&= 2(-285.83 \text{ kJ/mol}) + -393.51 \text{ kJ/mol} - -74.81 \text{ kJ/mol} - 2(0 \text{ kJ/mol}) \\
&= -890.5 \text{ kJ/mol} \\
&= -890.5 \text{ kJ/mol} / 64 \text{ gCOD} \\
&= -13.9 \text{ kJ/gCOD}
\end{aligned}$$

Analogous calculations for a wide range of organic compounds show that the typical $\Delta_f H$ values of $C_aH_bO_c$ compounds fall within a fairly narrow range of 13-15 kJ/gCOD, with a few exceptions such as formic and oxalic acid with 15.7 kJ/gCOD, ethyne with 16.3 kJ/gCOD and methanol with 17.8 kJ/gCOD. (See Appendix III).

It could be concluded that 13.9 kJ/gCOD is the maximum amount of heat energy that can be gained from methanogenic wastewater treatment. Therefore from a relatively simple COD measurement the potential energy yield would be known. However biodegradation of organic content in wastewater does not necessarily lead to methanogenesis. Some waste streams can be used for biohydrogen production. Here 1 gCOD is equal to 1/16 mol H_2, ($2H_2 + O_2 \rightarrow 2H_2O$) therefore 1 mol H_2 equals 16 gCOD, giving an energy yield of 17.9 kJ/gCOD (286 kJ/mol H_2 / (16 gCOD / mol H_2)).

The simple $C_aH_bO_c$ compounds are not necessarily the only wastewater components, and other classes of compounds such as halocarbons can contain far more internal chemical energy per gCOD. The explanation to this can be supported by writing the equations that describe their degradation down as oxidations of the carbon moiety with reducing equivalents released as H_2, coupled to the oxidation of the H_2 to water. In highly substituted compounds such as organohalogens, less H_2 is potentially available. The oxidation reaction of H_2 to water becomes less important in the overall equation, the ratio of H:CO_2 decreases, increasing the overall value of kJ/gCOD. This is illustrated using methane and one of its halogenated equivalents trichloromethane (thermodynamic data taken from (Hanselmann, 1991)):

Methane

$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (-393.5 + 4(0)) - (-74.8 + 2(-285.8))$

$= 252.9$kJ/reaction

$$4H_2 + 2O_2 \rightarrow 4H_2O$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (4(-285.8)) - (0 + 2(0))$

$= -1143.2$ kJ/reaction

These two values are then added together to give the overall enthalpy of reaction to be -890.3 kJ/mol, this can then be divided by the COD to give -13.9 kJ/gCOD

Trichloromethane

$$CHCl_3 + 2H_2O \rightarrow CO_2 + 3HCl + H_2$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-393.5 + 3(-167.1) + 0) – (-103.1 + 2(-285.8))

= -220.1 kJ/mol

$$H_2 + \tfrac{1}{2} O_2 \rightarrow H_2O$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-285.8) - (0 + 0.5(0))

= -285kJ/mol

The total enthalpy of reaction is -505.9 kJ/mol, giving -31.6 kJ/gCOD.

It becomes clear how important the reducing equivalents of H_2 are in terms of energetic value, this is illustrated in Figure 2-1, (values given in Appendix III). As the number of substitutions of hydrogen increases, so does the value of energy per gram COD. The value of energy per gram of COD can vary far more widely than previously thought.

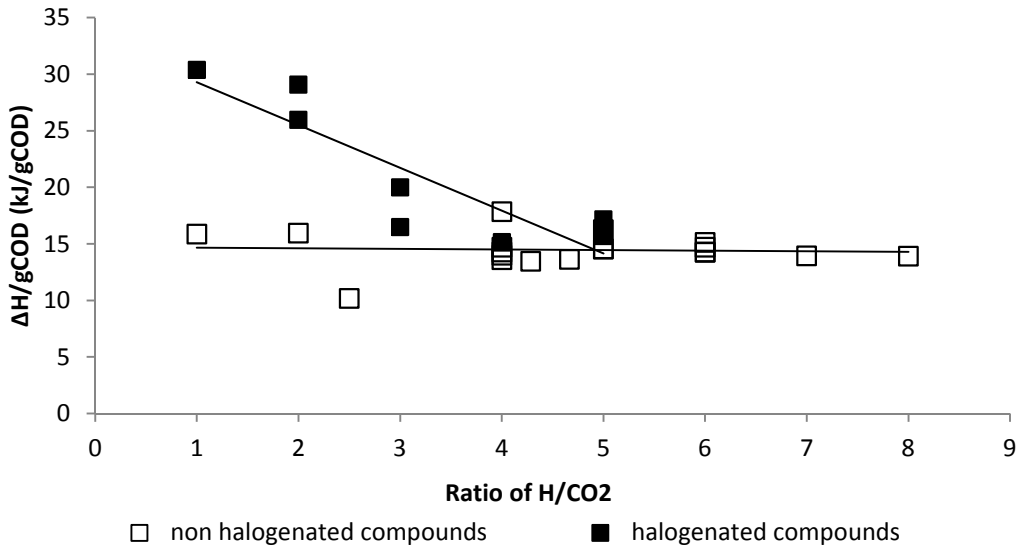

Figure 2-1 Energy content per gCOD of a variety of organic compounds plotted against their degree of oxidation

2.4. Discussion

The predicted energy gained from treatment of municipal wastewaters has been shown to be higher than the previous estimation. The domestic wastewater analysed in this paper has 20% more energy per litre than the estimation made by Shizas and Bagley (Shizas and Bagley, 2004). In addition to this, as the volatiles in their wastewater were not captured, it is likely their sample could have had an energy value around 35% higher, (based on the percentage losses between oven and freeze drying in this study) this would be 8.5 kJ/L. This has a significant impact on the development and implementation of technologies for the treatment of 'low strength' municipal wastewater which pose a greater challenge for the recovery of energy than concentrated waste. These waste streams are clearly richer in energy than previously thought.

The internal chemical energy of the wastewaters per gCOD was greater than expected by comparison to acetate (heat of combustion is 13.6 kJ/gram COD) or glucose (heat of combustion is 14.3 kJ/gram COD). From the data (Table 2-2) of the two wastewaters it can also be seen that the carbon oxidation state plays an important role in determining the energy present. Both samples have a very similar value of TOC (total organic carbon), yet very different COD values. This means that the Cramlington waste with the much higher COD has proportionally more reduction capacity and therefore chemical energy per carbon molecule than the Hendon wastewater. Another possible cause of these high values is that there are compounds within the wastewater that have an energy value, yet are not oxidised during a COD test, most notably urea, which contains 10.4 kJ/g (Atkins, 2006) when combusted, yet undergoes a hydrolysis reaction rather than an oxidation. This compound, which is certain to be present in domestic wastewater (and though it is assumed to hydrolyse in the sewer, a fraction may reach the wastewater treatment site), contributes to the overall energy of combustion of waste but not to the COD measurement, there are likely to be others compounds which do the same. Additionally there could be some compounds which have proportionally far greater energy content per gram of COD than glucose and acetate, such as organohalogens or other highly substituted compounds.

Although many simple halocarbons are no longer in use, some more complex ones are still common in many industrial processes for example as solvents and pesticides, and in the manufacture of in plastics, adhesives, sealants and paper pulp. Organic halocarbons also occur in natural systems. Chlorination treatment also introduces this halogen which

could then combine with other organics. It can be seen from the anion analysis (Table 2-2) that there is significant quantity of chloride ions in the wastewaters, with more in the Cramlington wastewater. This wastewater is likely to contain a more diverse range of organic compounds as this site takes in mixed wastes, some of which must have a high specific energy value and volatility, resulting in high energy wastewater. Volatile halocarbons, however, were not detected with the GC MS method described.

The energy values found in this study are also higher than that reported by Shizas and Bagley (2004). However the calculations in their paper were based on oven dried wastewater energy data, versus a COD measurement taken from the original wastewater sample, which in our study was found to be reduced by about 50% after oven drying. If the same calculation algorithms were used on the data in the present paper then the Cramlington and Hendon wastewaters would contain 11.6 kJ/gCOD and 9.9 kJ/gCOD respectively, while they actually contained at least 2.4 times higher (28.7 kJ/gCOD) and 1.8 times higher (17.8 kJ/gCOD), these calculations are shown in Appendix IV. Thus the energy reported per gCOD cited in the literature (Logan, 2008) based on the Shizas and Bagley paper (Shizas and Bagley, 2004) is probably a substantial underestimation. By comparison to the Hendon domestic wastewater the energy of their municipal wastewater could have had at least 26.4 kJ/gCOD, rather than the 14.7 kJ/gCOD reported.

Clearly not all the energy available in wastewater can be extracted in a useful form as no process is 100 % efficient. Ideally one would be able to measure or calculate the energy biologically available as kJ/gBOD, (although not suitable for anaerobic processes), this is not possible given the unknown and variable composition of wastewater. However knowing the potential energy available would give insight into the types of waste that might be in the waste stream which would also be of importance in the choice of treatment method. Some wastes which may be high in energy value, such as halogenated wastes may be unsuitable or unattractive to some treatment methods. For example one mole of trichloromethane at 506 kJ/mol would only yield 0.25 moles of methane equal to 222 kJ through methanogenic treatment, or one mole of H_2 equal to 286 kJ through biohydrogen production. Although these halogenated compounds are energy rich per gram of COD due to their lack of hydrogen, this actually makes them unattractive to terms of energy extraction for methane or hydrogen production, however

it may be possible to recover this energy using other treatment methods which may be able to capture electrons directly.

In microbial fuel cells (MFC's) the reaction taking place is essentially a combustion reaction, i.e. the organic compound is oxidized to carbon dioxide and water, the difference being that this reaction occurs not as combustion but as redox reactions in two half cells. Importantly, it is the free energy of the organics that determines the maximum electricity yield. This technology could theoretically capture more of the energy available in complex or halogenated compounds than for example methanogenic treatment.

The measurement of the internal or combustion energy of the wastewater and use of this as a basis for efficiency calculations will not necessarily yield all the information required to fully understand the energy flows in such systems. It can be observed using internal chemical energy data, a methanogenic process could in some cases be endothermic, the combustion energy of the methane product being higher than that of the starting substrate. This is the case with the conversion of one mole of acetate (13.6 kJ/gCOD) to one mole of methane (13.9 kJ/gCOD). In this scenario energy appears to have been created. It is actually the Gibbs free energy (the amount of energy that can be extracted from a process occurring at constant pressure) which should be examined for this and other reactions as this parameter informs us of the amount of energy available to organisms for the generation of biomass and an energy rich product. This is also the case for MFC's and MEC's where it is voltage which is measured which relates directly to Gibbs free energy. However without knowing the composition of wastewater, its Gibbs free energy content cannot be determined.

A consequential difference was found between the internal chemical energy measured on freeze dried samples as compared to oven dried samples. This difference was greater than the difference observed by measuring mass alone. This shows that there are significant losses of volatile compounds when a wastewater sample is dried at 104 ^{o}C and that in the case of the mixed wastewaters these volatiles can contain proportionally more energy per gCOD than the non-volatiles captured in both methods. It is shown that, although a clear improvement on the traditional oven drying method, the freeze drying method still results in significant loss of semi-volatiles such as acetate, so even

with the improved method we are still not capturing all the energy available in the wastewater.

Bomb Calorimetry remains the only method for measurement of internal chemical energy or calorific value, and for this method the material must be combustible i.e. dry. To give reasonably accurate results the temperature change in the bomb calorimeter must be in the region of 1 - 3 ^{o}C, usually a gram of substance will provide this. In our analyses this gram was half made up by the use of a combustion aid (benzoic acid) to ensure full combustion and the correct temperature rise. Had the proportion of wastewater to benzoic acid been decreased, making the drying process easier, it was feared that the uncertainty inherent to the introduction of the standard would overshadow the accuracy of the measurements of the samples. Although more challenging the methodology of freeze drying samples is an improvement on previous methods although it does not achieve the full capture of all volatiles. These results begin to get close to the true amount of energy in wastewater, and challenge the assumption that measured COD is equivalent to the amount of energy. Freeze drying, although far more time consuming, therefore should be the method of choice when completing such analysis in particular with complex wastes, despite its far greater time consumption rate unless or until new methods and equipment are developed to reduce the time burden using this principle. One such method could be the use of membranes, in particular through the use of reverse osmosis which would 'trap' molecules as small as salts and allow water to be removed. Such techniques may allow for more rapid, cost effective and efficient drying of samples, thus enabling more sampling to be undertaken.

It is clear from our data that the energy value of different wastewaters is variable, as would be expected; there is no standard relationship to measured COD. Values ranged from 17.7 kJ/gCOD to 28.7 kJ/gCOD, when measuring the COD remaining in the dried sample, however we cannot know how much compounds such as urea contribute to this. This means than a measurement of the amount of oxygen required to oxidise the organics within wastewater is not a simple representation of the amount of energy contained within that waste. This is particularly the case when dealing with mixed wastes, where the energy content is proportionally far greater per gCOD. It seems that 13 – 14 kJ/gCOD is the minimum energy content that could be found in wastewaters, however it may be significantly greater. Given the variability in the amount of energy

per gram COD it seems better to measure this energy directly rather than making an estimation, despite the fact that even with the better drying method there are still losses.

Given the huge amount of wastewater globally and the potential energy stored within it, it is important that this potential energy should be determined. With new technologies such as fuel cells being developed, the estimation of this resource is not as trivial as previously assumed. It has been shown that wastewaters can lie well outside the previously estimated values. A systematic review of the energy contained within different waste streams is needed. This paper examines two wastewaters from a reasonably similar geographical location and has found extremely diverse results. It is hoped that this methodology will be repeated and improved upon in terms of time taken, allowing the dissemination of multiple studies using different wastewaters building up a comprehensive and global picture of the energy available in wastewater. This would form the strategic foundation block to the establishment of new and existing technologies within the wastewater industry harnessing this valuable renewable energy source.

Chapter 3. How many exoelectrogens make a Bioelectrochemical System?

3.1. Introduction

The inoculation and subsequent acclimatisation of a bioelectrochemical system (BES) is fundamental to the operation of such systems (Logan and Regan, 2006, Rittmann, 2006). Yet the origin, abundance and physiology of these organisms is the area of greatest uncertainty in design (Oh et al., 2010).

The main goal of the inoculation and acclimatisation of a reactor is typically to 'get it going' as quickly as possible, typically the sources of seed includes: reactors already working in the lab (Jeremiasse et al., 2009, Cheng et al., 2009, Call and Logan, 2008); anaerobic sludge (Chae et al., 2010, Yang et al., 2009); return activated sludge (Torres et al., 2009); mixtures of sludges; or simply wastewater taken at various stages from the treatment plant (Kiely et al., 2011b, Wang et al., 2008). The source and volume of inoculum varies between studies. There is no consensus of how a BES reactor should be started up, or how long acclimatisation will take. This can lead to problems, highlighted by a pilot scale study where several attempts were made to acclimatise the reactor (Cusick et al., 2011).

The bacteria needed for microbial fuel cells to work are termed exoelectrogens (Logan, 2008) due to their ability to transfer electrons outside their cell. Three transfer mechanisms have been proposed.

Firstly electrons can be transferred through conduction with direct contact between the cytoplasmic membrane of the bacteria and the solid substrate being reduced, this mechanism has primarily been associated with the genera *Shewanella* and *Geobacter* (Myers and Myers, 1992, Mehta et al., 2005).

The second mechanism is an electron shuttle. Some bacteria are able to excrete compounds or shuttles into the electrolyte which are capable of transferring electron to an electrode. Rabaey *et al.,* (2005) found that *Psuedomonas aeruginosa* produced Pyocyanin, a mediator which was not only able to transfer electrons from this taxon to the anode of an MFC, but could also work for other species when introduced back into a mixed culture. Thus, a bacterium unable to transfer electrons itself, may become

exoelectrogenic due to the presence of a different shuttle producing bacteria. *Shewanella* species have been seen to do this with the production of riboflavins (von Canstein et al., 2008).

Thirdly electrons might also be transferred through conductive microscopic pili named nanowires which extend from the bacteria cell to other cells or any other electron acceptor (Reguera et al., 2005). *Geobacter* and *Shewanella* species have both been linked to this activity (Gorby et al., 2006). Putative nanowires have been observed using electron microscopy extending to a conductive surface. Conducting probe atomic force microscopy (Reguera et al., 2005) and conductive scanning tunnelling microscopy (Gorby et al., 2006) have been used to reveal that the pili which had previously been observed as attachment mechanisms for bacteria onto Fe oxides, were highly conductive.

It has been proposed that symbiotic relationships between different bacteria groups enhance the function of mixed cultures and improve process stability (Lovley, 2008), possibly by allowing inter-species electron transfer (Rabaey et al., 2005). Many of the exoelectrogens typically associated with BES's such as *Geobacter sulfurreducens* have limited metabolic diversity, and are only able to utilise the end products of fermentation (Caccavo Jr et al., 1994). A reactor fed with a waste requires bacteria which are able to digest the complex substrates, but may not necessarily be able to utilise the anode for respiration (Kiely et al., 2011c). The hydrolysis step within these food chains has been shown to be the rate limiting step with regard to the current production (Velasquez-Orta et al., 2011).

In general, growth in bacterial systems can be described through the equation $N_T = N_0 exp^{rt}$, where the number of bacteria present at a specific time period (N_T) is equal to the number of bacteria present at the start (N_0) multiplied by the exponential of the growth rate (r) over the time span (t). (Rittmann, 2001). With N_T known various other properties can be calculated such as specific activity and growth yield. However in MFCs these are not well understood (Logan, 2008), although growth rates have been defined for some of the key organisms involved in MFC reactions such as *Geobacter*, (Cord-Ruwisch et al., 1998). A cell yield of 0.07-0.22 g-COD-cell/g-COD-substrate has been calculated (Logan, 2008) from an early study by Rabaey et al. (2003) using total bacterial concentrations within the reactors determined turbidometrically and the total

COD removed during the experiments. Freguia et al. (2007) reported estimates of growth yields of -0.016 to 0.403 mol-C-biomass/mol-C-substrate, based on measurement of the substrate removal which was then used to calculate cell yield through a mass balance approach. Yield has been shown to drop with decreasing external resistance (Katuri et al., 2011).

However the value of N_T is complex and unknown. Although a body of research is growing identifying the functions of bacteria within working BES reactors, little is known of their abundance in a natural sample (N_0) and absolute number within a working system (N_T). Additionally the pattern of acclimatisation, the period is likely to be crucial in the community formation, also remains largely unexplored.

Using the acclimatisation period of reactors the aims of this study were to firstly identify the optimum level of inoculum needed to start a reactor with a view to identifying a protocol for the further experiments. Secondly to estimate the most probable number of exoelectrogens present in a sample of wastewater which can be used as a guide to the sequencing depth needed to find these organisms, and to determine N_0 for a reactor. Thirdly to define the growth rates (r) within MFC systems through examining the start-up phase. With these two factors quantified the N_T can be estimated, as can specific activity and yield. Finally by examining the pattern of acclimatisation on different substrates, key differences in community formation can be identified.

3.2. Method

3.2.1. *Reactor Set-up*

Double chamber tubular design MFC reactors (78 mL each chamber) were used, constructed in Perspex, with an internal diameter of 40mm and length of 60mm. The anode was a 2.5 cm^2 carbon felt (Olmec Advanced Materials Ltd, UK), the cathode a 2.5 cm^2 platinum coated titanium mesh with a surface area 8.13 cm^2 (Tishop.com, UK). The cation selective membrane between the reactor chambers was Nafion® 117 (DuPont, France), with an area of 12.6cm^2. The electrodes were positioned 1cm apart. The components of the reactor were cleaned before use and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK)

The cathode chamber was filled with 50 mM pH 7 phosphate buffer saturated with air for 20 minutes before being added into the reactors. Three different media were used:

1. Acetate solution with added nutrients (Call and Logan, 2008)
2. Starch solution with added nutrients (Call and Logan, 2008)
3. Primary settled wastewater (Cramlington WWTP, Northumbrian Water Ltd)

The quantities of starch and acetate in the nutrient solutions were balanced to give similar total chemical oxygen demand (TCOD) as the wastewater, and were autoclaved (121°C, 15 min) before use. The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into ¼ strength Ringers sterile dilutent (APHA, 1998). The contact time under UV was altered to give effective sterilisation as defined as colony free plates in triplicate at zero dilution. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see Appendix V).

The three medias were sparged under sterile conditions for 10 minutes using ultra high purity (UHP) nitrogen (99.998%), until the dissolved oxygen (DO) as measured on a DO probe Jenway 970 (Bibby Scientific Ltd, UK) reached zero.

3.2.2. *Inoculum*

Screened raw influent wastewater from Cramlington wastewater treatment plant (Northumbria, UK). This wastewater is a mixture of industrial and domestic origin. Samples were stored anaerobically at 4^{o}C and used within 24 hours of collection. The inoculum was also sparged with UPH nitrogen before use.

3.2.3. *Start –up and acclimatisation*

Duplicate reactors were inoculated with differing volumes of wastewater (1 mL, 10 mL, 25 mL and 50 mL). The anode compartment was then filled with the sterile substrates. Control ‘reactors’ (using no inoculum) were run during each test. An inverted 50ml syringe filled with UPH nitrogen was placed into the refilling port on top of the anode chamber to provide an anaerobic headspace. The cathode chamber once filled was left open allowing the diffusion of oxygen into the liquid. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor. A data logging

multimeter (Pico ADC-16, Pico Technologies, UK) was attached to record voltage output every 30 minutes. Reactors were allowed 800 hours at room temperature (20-25 ^{o}C) to show acclimatisation before the experiment was ended. With the acetate fed experiment a further set of reactors were run with lower dilutions of inocula, 0.01 mL, 0.1 mL and repeated 1mL with 25 mL as a positive control.

3.2.4. *Enumeration of bacteria*

The total number of aerobic culturable bacteria present in the wastewater samples used for inoculation was approximated using a spread plate method 9215C (APHA, 1998), with peptone based nutrient agar (Lab M Ltd, UK). Serial dilutions were undertaken into sterile ¼ strength ringers solution, with each dilution plated in triplicate. Plates were incubated at 37 ^{o}C for 48 hours. Anaerobic bacteria were enumerated using a basal salts media (Shelton and Tiedje, 1984) with 1 g/L of both yeast extract and glucose as a carbon source. The media was autoclaved (121 ^{o}C for 15 min) and sparged with sterile UHP nitrogen for 20 minutes. A volume of 9 mL was then added to sterilised Hungate tubes, 1 mL of wastewater was then added to five tubes, and dilutions made down to 10^{-12} with five replicates at each dilution. The headspace of the tubes was sparged with nitrogen, and the tubes incubated at 37 ^{o}C for two weeks. The number of bacteria was determined using the MPN methodology (APHA, 1998).

3.2.5. *Analytical methods*

TCOD of the medias and inocula were measured in duplicate according to standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) colorimetric reagent kit. Volatile fatty acids of the media and inocula were measured in duplicate using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. When the current of the cell had dropped to zero TCOD and VFA's of the cell were measured using the same method as inocula and media above.

3.2.6. *Most probable number (MPN) calculations*

With non-standard dilutions the pre-calculated MPN tables (APHA, 1998) cannot be used. The MNP is calculated through a series of iterations based on a Poisson and binomial distributions (Blodgett, 2005) using the following formula, solving λ for the concentration:

$$\sum_{j-1}^{k} \frac{g_j m_j}{1 - \exp\ (-\lambda m_j)} \ = \ \sum_{j-1}^{k} t_j m_j$$

K = the number of dilutions,

gj = the number of positive (or growth) tubes in the jth dilution,

mj = the amount of the original sample put in each tube in the jth dilution,

tj = denotes the number of tubes in the jth dilution

A probability is assigned to each possibility of the number of bacteria based on the outcome at each dilution, a positive outcome being voltage produced in by the reactor. The number with the highest probability is given as the MPN. Using the spreadsheet developed by Bloggett to make these iterative calculations, the most probable numbers of exoelectrogens per 100 mL of wastewater can be calculated (Garthright and Blodgett, 2003) using the inocula volumes, and the test outcome.

Thomas' simple formula which is based on the same principles as the full test, but a simpler algorithm to solve, can also be applied to the data set, this formula has been shown to have substantial agreement (Thomas, 1942). Using only the lowest dilution that doesn't have all positive tubes, the highest dilution with at least one positive tube and the dilutions in between the following calculation can be made:

$$MPN/100\ ml \ = \ \frac{no.positive\ tubes \ \times \ 100}{\sqrt{(ml\ sample\ in\ negative\ tubes) \times (ml\ sample\ in\ all\ tubes)}}$$

The confidence limits of this calculation at the 95% level can be calculated using Haldane's formula (Haldane, 1939):

m_1, m_2, m_3 ……. denotes inoculation amounts ranging from the largest to the smallest of the chosen dilutions

g_1, g_2, g_3 ……. denotes the number of positive tubes at the corresponding dilutions

$$T_1 = \exp(-MPN \times m_1)\ , T_2 = \exp(-MPN \times m_2) \ldots\ldots\ldots etc$$

$$B = [g_1 \times m_1 \times m_1 \times T_1/((T_1 - 1)^2)] + [g_2 \times m_2 \times m_2 \times T_2/((T_2 - 1)^2)] + [g_3], [\ g_4] \ldots. etc.$$

$$Standard\ Error\ of\ \log 10\ (MPN) = \ 1/\left(2.303 \times MPN \times (B^{0.5})\right)$$

95% confidence intervals are given by:

$$Log_{10}\ (MPN) \pm 1.96 \times Standard\ Error$$

3.2.1. *Growth rate, specific activity and yield calculations*

Growth rate of bacteria (μ) is classically calculated by quantifying the number of bacteria at two time intervals. In this experiment voltage is deemed to be a suitable proxy for exoelectrogenic bacteria, the rate of voltage rise being equivalent to the rate of growth. It is assumed that each bacterium is capable of donating an amount of electrons therefore an increasing number of electrons are donated to the circuit (i.e. the voltage increases at a constant resistance) as the absolute number of bacteria increases, (it does not represent an increasing ability to metabolise), i.e. voltage is deemed proportional to bacterial number. This can be from the growth rate expression:

$$N_T = N_0 e^{\mu t}$$

Where N_T is the number of bacteria at time t (in this case the voltage), N_0 is the number of bacteria (voltage) at time zero (t_0) and μ is the growth rate. Therefore growth can be defined as:

$$\mu = \frac{\ln N_t - \ln N_0}{(t - t_0)}$$

Specific activity (q), defined as moles electrons per cell per second can be calculated over the period of growth as follows:

$$q = \frac{I \times (t_1 - t_o)/F}{N_0}$$

Where I is the current in amps (coulombs/second) as calculate from the measured voltage V, and resistance R calculated through I=V/R, t_1-t_0 is representative of the time period of each measurement, (i.e. every 30 minutes, the total coulombs of charge within this period is therefore I multiplied by 30 minutes multiplied by 60 seconds) and F is Faradays constant of 96485 coulombs/mol e^-. The growth rate and starting MPN is used to calculate the number of cells at each time period N_T. This can be converted to moles of acetate per cell per second (1 mole acetate = 8 moles electrons), to give substrate utilisation (U).

Growth yield (Y) is the amount of biomass or cells produced by the bacteria per mass of degraded substrate measured in g-COD-cell/g-COD-substrate. Rather than use the total COD removed in the reactor, which would also involve COD digested via other routes only the g-COD substrate put to the circuit is used as calculated from the substrate utilisation above. The yield is calculated as follows:

$$Y = \frac{(N_T - N_0) \times W \times COD_{cell}}{\sum_{t0}^{t} U \times COD_{sub}}$$

Where the total cells produced over the growth period N_T-N_0 is multiplied by an estimation of the weight of cells W of 5.3 x 10^{-13} g-cell given in Logan (2008) and the estimation for anaerobically grown cells of the formula of $C_{4.9}H_{9.4}O_{2.9}N$ equating 1.25 g-COD/g-cell, (Rittmann, 2001). The sum of the substrate utilisation U as calculated above is multiplied by COD_{sub} the amount of COD per mole of substrate, 64 for acetate.

3.3. Results

3.3.1. *Number of bacteria in wastewater*

The spread plate counts of the wastewater, and anaerobic multiple tube count indicate there is 8.3 x 10^5 culturable aerobic bacteria, and 6.9 x 10^4 culturable anaerobic per ml of this wastewater, giving a rough estimate of the total bacteria per mL of wastewater to be 10^6. Although this method may over estimate numbers due to some bacteria being able to grow under both conditions, and underestimating numbers due to bacteria being intolerant to the media, the overall value calculated fits in with previous estimates (Tchobanoglous, 1991).

3.3.2. *Most probable number of exoelectrogens*

The number of positive outcomes of each test are shown in Table 3-1. From this the MPN can be calculated shown in Table 3-2. The MPN of exoelectrogens in an acetate fed reactor is 17 per ml of wastewater, this number drops to 1 per ml for a starch fed reactor and 0.6 per ml for a wastewater fed reactor. Superficially it appears that acetate metabolising exoelectrogens are quite rare organisms, starch metabolising exoelectrogens are even rarer and wastewater metabolising exoelectrogens are rarer still.

Table 3-1 The number of positive outcomes for each inocula size out of the total number of reactors run

Inocula size (mL)	50	25	10	1	0.1	0.01
Wastewater	2/2	2/2	0/2	1/2	-	-
Starch	2/2	2/2	1/2	0/2	-	-
Acetate	2/2	4/4	2/2	3/4	1/3	0/2

Table 3-2 The MPN in 1 ml of wastewater given by the two methods stated, numbers in brackets indicate the upper and lower bounds at 95% confidence. The probability of presence in wastewater is calculated from the total count of viable bacteria per 1 ml

Substrate	MPN calculation (Blodgett 2005)	MPN estimation (Thomas 1942)	Probability of presence in 1 ml of wastewater
Wastewater	0.6 (0.3-2.5)	0.8 (0.3-2.5)	6×10^{-7}
Starch	1.0 (0.3-3.2)	1.1 (0.3-4.0)	10^{-6}
Acetate	17.0 (5.5-52)	17.6 (6-51.5)	1.7×10^{-5}

An alternative explanation is that the lower MPNs, and therefore the probabilities of these organisms being present in 1 ml of wastewater, are the product of two or more events. In wastewater and starch there are long chain molecules present which undergo a series of steps in their breakdown. Each step is probably undertaken by different microorganisms. The electrons pass down this chain leading to the final step of donation to the electrode, represented by the acetate reactor. Thus the MPN of the wastewater and starch fed cells is the probable MPN of the acetate fed cells (the number of exoelectrogens) multiplied by the probability of each of the upstream steps. Here all of these steps are simplified into one probability step, however in reality this may be many steps the product of which is equal to 0.04 for wastewater and 0.06 for starch as shown in Figure 3.1.

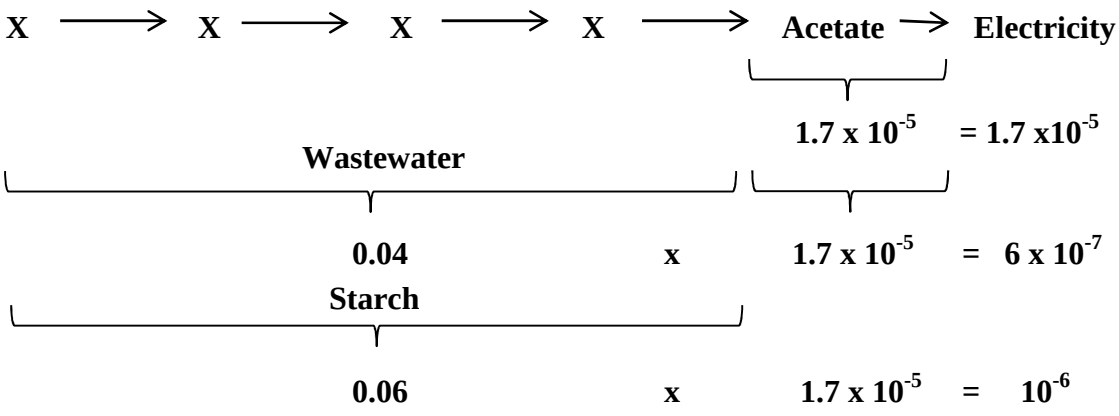

Figure 3-1 Estimated probabilities of numbers of bacteria present in the wastewater begin to produce a working MFC fed on three different substrates of acetate, starch and wastewater based on the numbers determined in the MPN method

3.3.3. *Growth rates*

The individual growth rates for the three different substrates are shown in Table 3-3. The rates were not significantly different (p=0.282 one way ANOVA), and showed agreement with other studies.

Table 3-3 Average growth rates for exoelectrogens fed on different substrates estimated using the rise in voltage measured in the acclimatising reactors

	Average growth rate
Wastewater fed community	0.028 h^{-1} ± 0.013
Starch fed community	0.023 h^{-1} ± 0.005
Acetate fed exoelectrogens	0.035 h^{-1} ± 0.020
Geobacter sulfurreducens (Cord-Ruwisch et al., 1998)	0.023 – 0.099 h^{-1}
Geobacter sulfurreducens (Esteve-Nunez et al., 2005)	0.04 – 0.09 h-1
Fermenting micro-organisms (Rittmann, 2001)	0.05 h-1

3.3.4. *Acclimatisation pattern*

Using an arbitrary value for N_0 (the starting number of bacteria per ml), the known growth rate and the time period over which the experiment was conducted, the pattern of acclimatisation can be modelled.

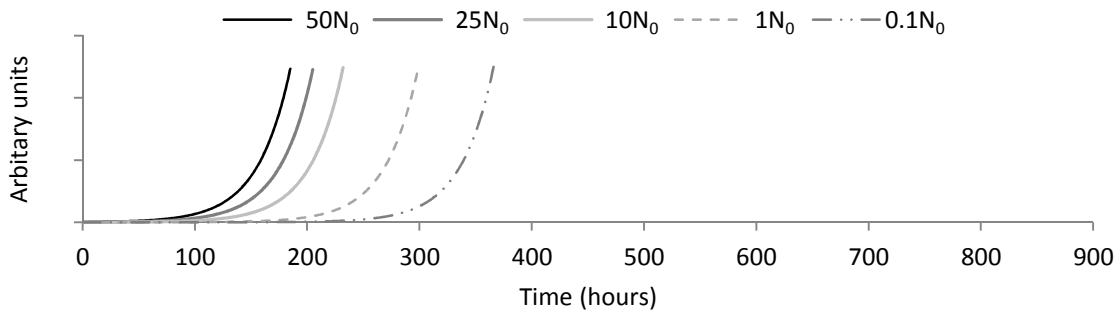

Figure 3-2 Model of the acclimatisation of reactors inoculated with varying amounts of bacteria as denoted by N_0 based on the formula $N_T = N_0 exp^{rt}$ where r the growth rate is the average growth rate determined experimentally of 0.03 hr^{-1} and t time is given on the bottom axis

The pattern of acclimatisation that occurred for the wastewater and starch fed did not follow the model. All reactors acclimatised at the approximate same time. If the growth rates and time are equal, mathematically this means that N_0 is similar for the different volumes of inocula.

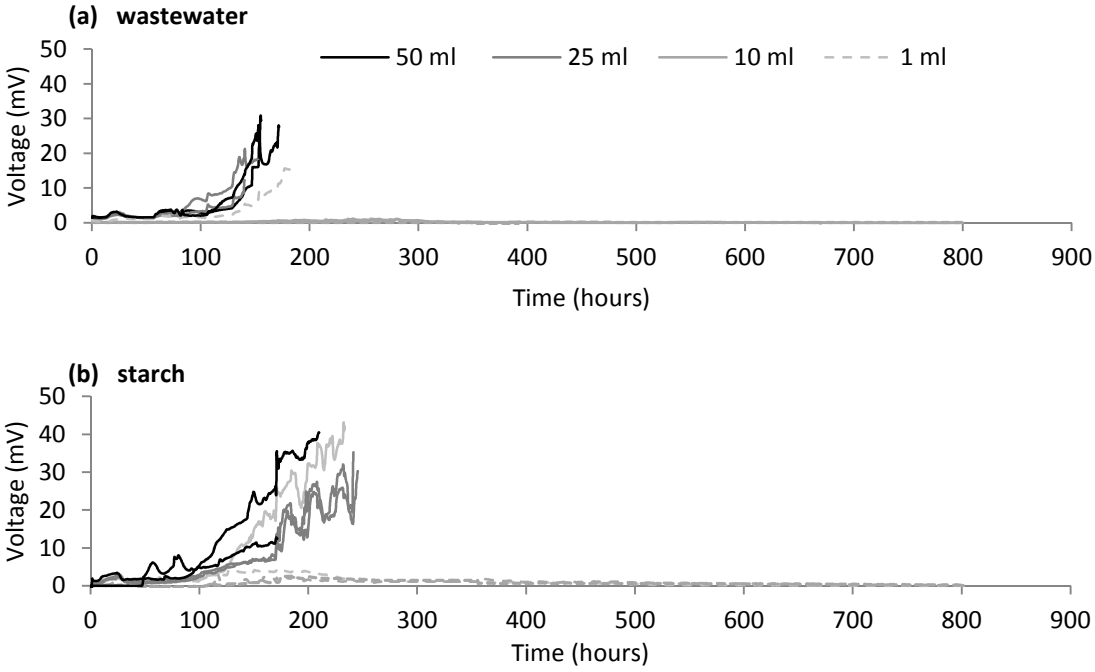

Figure 3-3 Pattern of acclimatisation of the wastewater (a) and starch (b) fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Superficially the pattern observed for the acetate fed reactors appears to follow the model pattern. However this is not the case as the lag time to acclimatisation is over extended with reducing amounts of inocula.

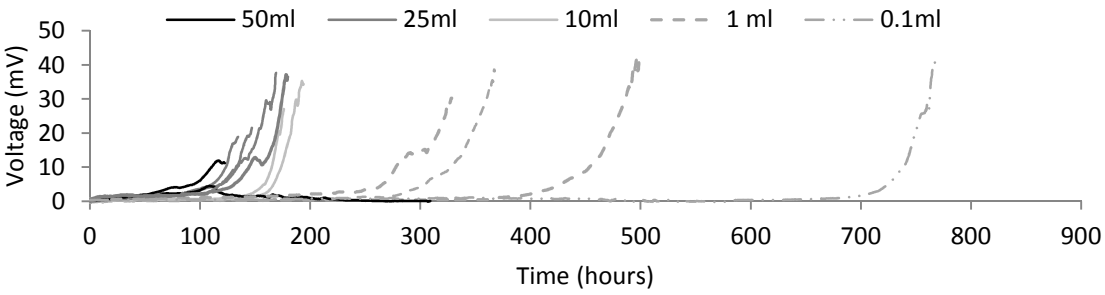

Figure 3-4 Acclimatisation of the acetate fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Using $N_T=N_0\exp^{rt}$ the calculated number of bacteria at the time the reactor inoculated with 0.1 ml (which must have contained at least one bacteria) reaches 10 mV would be 1.8×10^{11} bacteria, equivalent to the predicted number of bacteria in 1 kg of soil (Whitman et al., 1998), and 4×10^{7} times greater than the number of bacteria at 10 mV in the cell inoculated with 50 ml of wastewater (assuming an MPN of 1.7 per ml). This is clearly implausible, growth is not purely exponential, there is likely to be a lag phase with no growth. Yields calculated on the basis of these N_T and N_0 values both with (up to 8 g-COD cell/g-COD) and without (10^{-4} and 10^{-7} g-COD cell/g-COD) growth in the

lag phase give results discordant with the current literature, (these are shown in appendix VII).

3.4. Discussion

If the aim of acclimatising a reactor is to get it going, then it has been shown that a larger volume inoculum will give a quicker (in the case of acetate) and more likely (in the case of complex substrates) successful inoculation, although a proportion of the intended substrate may also be needed. As clear differences were observed between experiments, acclimatisation with the intended substrate is likely to be essential to successful operation. However, more importantly, these results also give insight into the abundance and distribution of exoelectrogenic and other crucial organisms, and to their community development within a reactor.

Discovering the number of exoelectrogenic bacteria per ml of wastewater is a strategically important question. It would inform us of the sequencing depth needed to identify these bacteria. By using the MPN methodology in a series of MFCs and aerobic and anaerobic culturing methods of the same wastewater, an estimation of this number has been gained. Acetate digesting exoelectrogens can be found at an estimated quantity of 17 per ml of wastewater, giving the probability of a bacterium in 1 ml of wastewater being an exoelectrogen as 1.7×10^{-5}, or put differently 0.0017% of the bacteria present in wastewater are exoelectrogenic. With 1000 sequencing reads there would be a reasonable chance of identifying only 1 or 2 exoelectrogens. When compared to the pyrosequencing carried out in chapter 4 a similar answer emerges. Two wastewater samples were analysed, and the total sequencing effort needed to capture 90% of all the sequences in the sample estimated using statistical algorithm as shown in Appendix X. Comparing the total number of *Geobacter* (the known exoelectrogen present in the wastewater samples) found in the sample to the estimated sampling effort, in one sample Geobacter represented an estimated 0.0012 % of the total bacteria, in the other this was lower at 0.00001 %. The two very different approached result in a similar estimation of the number of exoelectrogens present in wastewater. The use of further microbial techniques such as flow cytometry or QPCR would also help the verification of these results.

The number of acetate exoelectrogens is rare: 17 per ml. The number of starch or wastewater exoelectrogens is even lower at 1 per ml. It could be plausible that these are

even rarer organisms, however the likely explanation is that a chain of metabolism is occurring, this fits with the literature (Velasquez-Orta et al., 2011, Kiely et al., 2011c). The probability of achieving a working MFC fed on a complex substrate is therefore the probability of the exoelectrogenic step as identified above, multiplied by the probabilities of each of the upstream steps in the metabolic chain, and is therefore lower than the probability of forming with the acetate step alone. The MPN value is an approximation, yet even considering the upper and lower bounds of the calculation at 95% confidence, as shown in Table 3-2, this pattern is observed. Clearly however this is dependent on the inoculum used; with different inocula such as soil or sludge one would expect different results.

Growth rates, although intuitively demonstrated by the rise in voltage within an MFC, have not previously been calculated. It is an important value to know, especially when modelling such systems. This study calculated the average growth rate of 0.03 hr^{-1}, this value agrees with those documented in the literature from known exoelectrogenic bacteria. No statistical difference is found between reactors fed on acetate and more complex wastewaters, contrary to previous work (Velasquez-Orta et al., 2011) this study shows that the growth rate of exoelectrogens is likely to be the limiting factor.

The pattern of acclimatisation demonstrated within these reactors did not follow the expected pattern. Additionally the pattern observed in the acetate reactors is different to the pattern observed in the reactors fed with more complex substrates. Simple exponential growth does not appear to be happening in either system. The values of N_T within these systems are therefore questionable, as are the calculated yields and specific activities (see appendix VII).

The positive starch and wastewater fed reactors were fewer in number due to the reduced probabilities of the communities forming, but all acclimatised at approximately the same time regardless of the inoculum volume. The growth rates calculated were not statistically different between the different inocula, time was recorded accurately. Explaining this mathematically on the basis of $N_T = N_0 exp^{rt}$ this means either: N_0 is the same for the different inoculum sizes; the N_T of the reactors producing the same voltage is actually different; the rates as defined by voltage rise are not representative of growth rates; or the system may not be described by the equation $N_T = N_0 exp^{rt}$.

More of the acetate cells acclimatised leading to a higher MPN value, the pattern of acclimatisation here does show a clear link to inoculum size, however the size of the lag phase is far greater than would be predicted. Again the rates calculated were not statistically different between the different inoculum sizes and time was also recorded accurately. Here on the basis of $N_T = N_0 exp^{rt}$ either; N_0 is not linearly related to inoculum sizes, i.e. 50 mLs of wastewater contains more exoelectrogens than 50 times 1 ml; the N_T of the reactors producing the same voltage is actually different; there is a lag period before the growth rate starts which is also related, but not linear to, the inoculum size; or again the system is not described by $N_T = N_0 exp^{rt}$.

The MPN method and therefore N_0, is based on the following assumptions: bacteria are distributed randomly within the sample; they are separate, not clustered together; they do not repel each other; and every reactor whose inoculum contains even one viable organism will produce detectable growth or change and the reactors are independent (Blodgett, 2009). It seems likely that exoelectrogens will cluster, there function of passing electrons outside the cell may be used for passing electrons between cells when no external electron sink is available (Bretschger, 2010). In the sequencing data in chapter 1063 *Geobacter* are found in one wastewater sample and 4 in the other, also indicative of clustering. If clustering is occurring, the MPN is likely to be an underestimation as will be N_0 and N_T. This does not however explain the different patterns of acclimatisation observed between the substrates. Additionally the large upper and lower bounds given in the MPN calculations due to the relatively low sample size, could also lead to both under and over estimations of N_0 where the MPN is used.

The relationship of voltage with N_T could be more complex than assumed. Voltage generated from the electrode may be limited by properties relating to the anode itself rather than the bacteria on it, or may quickly reach saturation point of the biofilm, however then one would expect to observe the same pattern in all reactors.

Growth rates are assumed to be represented by the rising voltage measured across the reactors. This may not be the case if the bacterial population has to grow to a certain threshold level (at an unknown growth rate which may different for different inocula sizes) before any voltage is produced. Additionally an assumption is made that increasing voltage is caused by an increasing number of bacteria, not an increasing

capability of these bacteria to donate electrons, this may not be true. Again this does not account for the differences between substrates.

The period of acclimatisation is both highly complex and variable between substrates, yet does show a clear observable pattern, indicating an underlying mechanism. It seems likely that these systems are not described by $N_T = N_0 exp^{rt}$. Such deviations could be caused if the exoelectrogens present N_0 were able to induce electrogenic activity in other bacteria through the excretion of electron shuttles: $N_T > N_0 exp^{rt}$, and in addition a further growth equation of the 'induced' exoelectrogens would act to confuse the picture. In the case of the complex substrate systems something within the chain of metabolism which is unrelated to the bacteria quantity could be triggering the start of the acclimatisation, this causes the reactor to work or fail regardless of the number of exoelectrogens present at the start. In the acetate fed reactors a further factor related to the inoculum size could be causing the extended lag observed, such as the movement of the exoelectrogens to the anode surface.

The period of acclimatisation is not only complex, it is likely to be a period of high competition for resources and possible low efficiency for the exoelectrogens as seen from the low coulombic efficiencies and comparable COD removal in both the positive and negative reactors (see appendix VI).

If the aim of acclimatisation is to merely 'get the reactor going' this study has shown that using a large proportion of wastewater is best. The experiment has also demonstrated that the abundance of organisms needed to start an MFC is low within wastewater, and even lower when these systems are to be fed on complex substrates. The growth rates defined are similar to those observed for exoelectrogenic species in other environments, and are likely to be the limiting factor in MFC acclimatisation. The pattern of acclimatisation a fuel cell is complex and not explained solely by exponential growth. The clear differences between these systems demonstrate the vital importance of acclimatising a community for the eventual use of the reactor. A reactor fed on acetate is different to one fed on wastewater. By developing a greater understanding of this ecology and its development, the move towards more stable biological system can be made. Understanding the nature, abundance and location of these exoelectrogens is crucial.

Chapter 4. Can Microbial Fuel Cells operate at low temperature?

4.1. Introduction

Bioelectrochemical Systems (BES's) are being heralded as a new method of energy efficient wastewater treatment, yielding electrical energy or other products from the bacterial breakdown of organics in an electrochemical cell. For future application of this technology understanding the microbial ecology, community structure and relating this to performance is desirable (Parameswaran et al., 2010) . The majority of fuel cell research is carried out using acetate as a feed at 30^{o}C with the implicit assumption that this will translate into the treatment of real wastewaters at ambient temperatures. To use low strength high volume wastes like wastewater the bacterial communities within BES need to be able to digest complex and variable substrates and do so outside, which in the UK, Europe and many parts of the USA means at low temperatures. If the communities of bacteria able to perform this task do not occur naturally further work and investment into this area may be futile.

As noted above most BES studies are conducted in laboratories at a temperature of 30 ^{o}C (Call and Logan, 2008, Cheng and Logan, 2007a, Selembo et al., 2009b). Few ambient treatment plants will get this warm. Several studies investigating the performance of MFCs over temperatures between 20-30 ^{o}C have found that the maximum power output with acetate was reduced by 9% (Liu et al., 2005a) and 12% (Ahn and Logan, 2010) when the temperature was lowered from 30 ^{o}C to 20 ^{o}C and 23 ^{o}C respectively, using beer waste a 10% drop was seen at these temperatures (Wang et al., 2008). The reduction in performance was lower than predicted by biological process modelling, suggesting that bacterial growth at 32 ^{o}C is not optimal, or that other factors are more limiting (Liu et al., 2005a). Complex wastes were also treated by Ahn and Logan (2010), and it was found that temperature had a greater effect on these than the simple compounds.

Lower (below 20 ^{o}C) and more realistic temperatures have been even less well studied. Min et al (2008) found that at 15 ^{o}C no successful operation was achieved, after 200 hours of operation the experiment was stopped. Cheng et al. (2011) found at 15 ^{o}C start up took 210 hours but at 4 ^{o}C there was no appreciable power output after one month (720 hours) and the experiment was stopped. In the same study a reactor started at 30 ^{o}C was then dropped to 4 ^{o}C and power output was achieved, but around 60% lower than

that the higher temperature. Larrosa-Guerrero *et al*. (2010) operated reactors at 4 ^{o}C and 35 ^{o}C using a mixture of domestic and brewery wastewater, observing a decline in COD removal from 94% to 58% and power density from 174.0 mWm^{-3} to 15.1 mWm^{-3} at the lower temperature.

By contrast Jadhav and Ghangrekar (2009) operated an MFC's in a temperature range of 8-22 ^{o}C and found that the current and coulombic efficiencies were higher than that produced in the temperature range of 20-35 ^{o}C. However in this study temperatures were ambient not controlled and thus confounded by time. They inferred that a reduction in methanogenic bacterial activity at lower temperatures increased MFC performance, although the microbiology of the systems was not examined. Similar results were obtained by Catal *et al.* (2011), here the biofilm was examined using scanning electron microscopy and found to be thicker in the higher temperature reactors.

MFC systems are based on electrochemical and microbiological principles: temperature affects both. The electrochemical impacts of temperature can be calculated using the Nernst equation based on known free energies for substrates such as acetate, or estimated free energies if wastewater is used (Logan, 2008). In bacterial systems rates of reaction roughly double for every 10^{o}C rise in temperature (Rittmann, 2001). However, the actual behaviour of these complex systems at different temperatures and fed on different substrates remains an area of great uncertainty in this field of research.

An increasing number of studies into the microbial communities of BES using techniques such as restriction fragment length polymorphism (RFLP), clone libraries and denaturing gradient gel electrophoresis (DGGE) are adding to the knowledge base we have about these communities. There are advantages to these various techniques such as the high reproducibility and in the case of DGGE and RFLP the large number of samples than can be run (van Elsas and Boersma, 2011, Kirk et al., 2004). However all these techniques are limited in that only a small fraction, (in the case of DDGE estimated at 1-2 % (Macnaughton et al., 1999), of the species present are targeted in these studies, total diversity cannot be estimated from these limited results. Never the less it has been repeatedly shown that *Geobacter sulfurreducens* dominates in acetate fed reactors, although this can vary when reactors are inoculated with different media (Kiely et al., 2011c). As substrates become increasingly complex moving from VFA's

to carbohydrates to actual wastewater the dominant species become more varied (Kiely et al., 2011c). Some wastewater fed reactors were found to be dominated by *Betaproteobacteria* (Patil et al., 2009), although in other studies *Geobacter* still dominates (Cusick et al., 2010).

Most of the techniques that have been used are limited by their capacity to identify the most dominant species within the communities. Next generation sequencing (capable of sequencing to a far greater depth) has now been used in two MFC studies. Lee *et al*. (2010) used FLX Titanium pyrosequencing to sequence four samples of biofilm, triplicate samples were taken from an acetate fed reactor comparing this to a single sample taken from a glucose fed reactor. The profiles found in the samples were not significantly different. A further study by Parameswaran (2010) analysed the biofilm of two MFC reactors fed on ethanol examining the impact to the communities when methanogenesis was prevented in one, identifying the role of hydrogen scavengers.

The aim of this study was to determine if microbial fuel cells can work at low temperatures, and if the inocula affects this. By running reactors fed on both wastewater and acetate the relative importance of the final 'electrogenic' step, and the up- stream hydrolysis and fermentation steps can be evaluated. The impact of temperature, inoculum and substrate on the microbial communities and total diversity within these reactors was examined using next generation sequencing techniques.

4.2. Methods

4.2.1. *Experimental design*

The variables examined were: temperature (warm 26.5 ^{o}C and cold 7.5 ^{o}C); substrate (acetate and wastewater); and inoculum (Arctic soil and wastewater). Each set of conditions were run in parallel duplicate reactors and biofilm samples taken from each. The two series of experiments, acetate and wastewater, were conducted using the same 8 reactors under identical conditions, the two wastewater inoculum samples were used to seed the acetate (wastewater sample1) and wastewater fed (wastewater sample 2) experiments. This is represented in Figure 4-1.

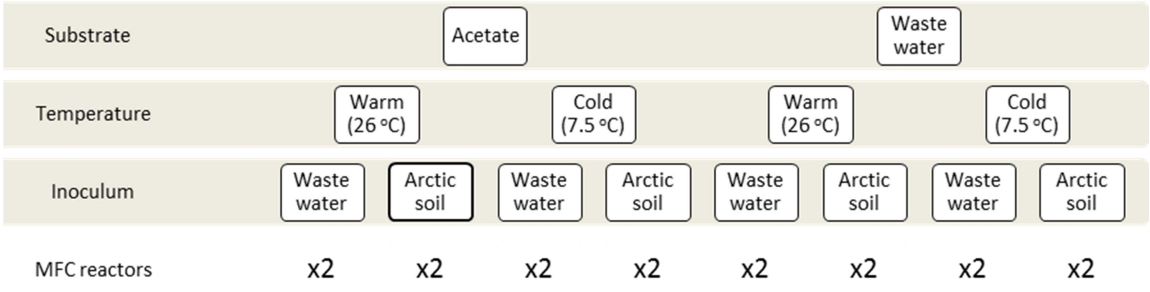

Figure 4-1 Illustration of the multi-tiered reactor conditions used

The warm temperature was chosen to represent the typical ambient laboratory temperatures of many MFC studies. The low temperature is the lowest sustained temperature of a wastewater treatment plant in the North of England (54°58'N, 01°36'W) experienced over a winter period (Northumbrian Water Ltd). The different substrates represent the most commonly used laboratory substrate acetate, and compared to wastewater. The two different inocula were the usual inoculum of wastewater, and Arctic soil (see below) which could potentially have more bacteria with low temperature, exoelectrogenic capability.

Wastewater typically contains 10^5 - 10^6 bacteria per mL (Tchobanoglous, 1991) soils can contain around 10^9 bacteria per gram (Whitman et al., 1998). Many soil environments are low in oxygen, and iron rich, favouring anaerobes and iron reducers and potentially therefore exoelectrogens. Arctic soils have been shown to have to be biologically active, accounting for around 6% of the total global methane sources (Ehhalt et al., 2001). (Hoj et al., 2005, Kotsyurbenko et al., 2004, Metje and Frenzel, 2005). Soil taken from Ny-Ålesund, in the Spitsbergen area of Norway has been shown to contain a wide range of methanogenic groups active at temperatures ranging from 1-25 °C (Hoj et al., 2005, Hoj et al., 2008).

4.2.2. *Reactor design and operation*

Eight identical double chamber tubular MFC reactors (78 mL each chamber) with an internal diameter of 40mm and length of 60mm were used. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (Tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor, and a multimeter to measure the voltage (Pico ADC-16, Pico Technology, UK). The membrane between the reactor

chambers was Nafion 117, with an area of 12.6cm^2. Reactors were sparged with 99.99% pure N2 in the anode chamber, and air in the cathode chamber for 15 minutes after every re-fill.

Four reactors were operated at a temperature of 26.5 ^{o}C in an incubator (Stuart Scientific SI 50, UK), the other four at 7.5 ^{o}C in a low temperature incubator (Sanyo MIR-254, (Sanyo Biomedical, USA). The temperature was logged continuously over the experiment using a EL-USB-1 temperature data logger (Lascar Electronics, UK). The reactors were inoculated and filled with substrate, replacing this every 5-6 days until a stable power generation was achieved. The reactors were then re-filled and three successive 3 day cycles were run logging the voltage over this time. Chemical oxygen demand (COD) removal during each batch was determined using standard methods (APHA, 1998) and Spectroquant ® test kits (Merck & Co. Inc., USA).

4.2.3. *Media and inocula*

Autoclaved acetate media (Call and Logan, 2008) containing 1 g/L sodium acetate was compared to wastewater taken from Cramlington wastewater treatment site (Northumbrian Water Ltd, UK) which was UV sterilised prior to use. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see appendix V). The cathode chamber was filled with 1M pH 7 phosphate buffer. The conductivity of the nutrient media, wastewater and the phosphate buffer was measured using an EC 300 (VWR Ltd, UK) and equalised for the temperatures of 7.5 ^{o}C and at 26.5 ^{o}C.

The wastewater inoculum was collected from Cramlington wastewater treatment plant, a Northumbrian Water site in the North of England, it was raw wastewater collected prior to any form of treatment, and is believed to be of mixed industrial and domestic, COD 0.7-0.8g/L. Once collected the sample was stored in a fridge at 4 ^{o}C within a closed container. The Arctic soil was collected from Ny-Ålesund, Spitsbergen in Svalbard. This was wrapped within three sealed bags and stored at 4 ^{o}C until used. The inocula of wastewater and soil were measured out to 5 mL or 5 g respectively before being added to the reactors. Samples of each inocula were preserved in a 50:50 in a mix of ethanol and autoclaved PBS pH7 in the freezer at -20 ^{o}C for microbial analysis.

4.2.4. *Microbiological techniques*

At the end of each experiment the anode was removed aseptically from the chamber using aseptic technique and preserved in a 50:50 mix of ethanol and autoclaved PBS pH7 and stored in a freezer at -20 ^{o}C. A 5 ml or 5 g sample of the original inocula was also taken and preserved in this way. The inocula samples were pelletized and the DNA then extracted. With the anode samples the bacteria that had dispersed into the liquid was pelletized and then added to the central section of the anode felt cut from the whole anode. The DNA was extracted by placing this sample into the beaded tube of a FastDNA Spin Kit for Soil (Qbiogene MP Biomedicals, UK). Extraction was completed according to the manufacturer's instructions. The samples were then pyrosequenced following amplification of the 16s rRNA gene fragments.

The primers used were F515 (GTGNCAGCMGCCGCGGTAA) and R926 (CCGYCAAT-TYMTTTRAGTTT). Each sample was labelled with a unique 8 base pairs (bp) barcode connected to a GA linker. Sequencing was completed from the Titanium A adaptor only forward from the F515, capturing the V4 region and most of the V5 region with a Titanium read of 400-500 bp. Triplicate PCR reactions were carried out using the Roche FastStart HiFi reaction kit (Roche Diagnostics Ltd., UK) and subject to the following optimised thermal cycles: initial denaturation at 95°C for 4 minutes; 23 cycles of denaturation at 95°C for 1 minutes; annealing at 55°C for 45 seconds; extension at 72°C for 1 minute; final extension at 78°C for 8 minutes. An automated thermal cycle Techne TC-5000 (Bibby Scientific, UK) was used.

The triplicate samples were then pooled and cleaned using QIAquick PCR Purification Kit (Qiagen, UK). The DNA concentration was quantified by UV-Vis spectrophotometry using a Nanodrop 2000 (Thermo scientific, USA). The individual samples were pooled to give equal concentrations of all reactor samples, and double concentration of the wastewater and arctic soil seed. Sequencing was carried out by the Centre for Genomic Research (University of Liverpool, UK) using the Roche 454 sequencing GS FLX Titanium Series.

4.2.5. *Data analysis*

The pyrosequencing data set was split according to the barcodes and unassigned sequences were removed[1]. The flowgram files were cleaned using a filtering algorithm Amplicon Noise (Quince et al., 2009) to give the filtered flowgram file. Filtering at a minimum flowgram length of 360 bp including the key and primer before first noisy signal, all flowgrams were then truncated to 360 bp. A pairwise distance matrix was then calculated using the Pyronoise algorithm (Quince et al., 2009). This uses an iterative Expectation-Maximization algorithm which constructs denoised sequences by clustering flowgrams using the initial hierarchical clusters generated in the previous step and the filtered flowgram file. The cut-off for initial clustering is set at 0.01 and the cluster size is 60, as recommended by Quince et al. (2009). The flowgrams can then be denoised.

PCR errors were then removed again using Seqnoise, generating a distance matrix using the Needleman-Wunsch algorithm for pairwise alignment. The optimal parameters used here were the cut-off for initial clustering of 0.08 and cluster size of 30. Chimera removal was completed using the Perseus algorithm (Quince et al., 2011) which for each sequence searches for the closest chimeric match using the other sequences as possible parents. (Quince et al., 2011). The sequences are then classified and the good classes filtered at a 50% probability of being chimeric, producing the final FASTA file which is denoised and chimera free ready for analysis in QIIME (Caporaso et al., 2010).

Using the QIIME pipeline tutorial the following analysis was completed: assigning taxonomy using Greengenes (http://greengenes.lbl.gov) at the 97% similarity level; creating an OTU table; classification using the RDP classifier; summary of taxonomic data from classification; generation of rarefaction data of the diversity in a reactor; calculation of the differences between the reactors; performing Principle Co-ordinates Analysis (PCoA); jackknifing and bootstrapping to understand uncertainty in beta diversity output; and generating Unweighted Pair Group Method with Arithmetic Mean (UPMGA) trees for hierarchical clustering of samples. The dissimilarity of the community structure between duplicates was examined using both a weighted (relative abundance) and unweighted (presence/absence) phylogenetic diversity metrics using

[1] *The analysis of the pyrosequencing data was carried out by Dr Matthew Wade, a Bioinformatics researcher within the School of Civil Engineering and Geosciences at Newcastle University.*

UniFrac, giving a distance matrix containing a dissimilarity value for each pairwise comparison. The raw OTU table generated was used to produce the species abundance pattern (with the log abundance normalised to the number of sequences in each sample) and the rank abundance curves, (where percentage abundance is used to normalise samples).

An estimate of the total diversity for each sample was calculated using the Bayesian approach as described in Quince et al. (2008), where the 'posterior distribution' of the taxa area curve is estimated, from the known distribution of the data gathered in the sequencing. Three distributions are modelled: log-normal; inverse Gaussian; and Sichel, and deviance information criterion (DIC) are used to compare the fit from each model. The lower the deviance or DIC values the better the model fit, those models within 6 of the best DIC value can be considered as a plausible fit. Using the fitted abundance distributions the sampling effort required to capture 90% of the taxa within that sample is estimated.

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run analysis of variance (ANOVA) tests on the experimental data, and t-tests on the distance matrix data for the sequences samples. Data were checked for normality prior to completing ANOVA, and if necessary the Box-Cox transformation was used.

The performance of the MFC reactors were analysed on the basis of three variables: % COD removal as measured; coulombic efficiency (CE); and power density (mW/m^2). The latter two variables were calculated using the measured COD and voltage within the cells, as described in Appendix VIII. Correlation of the community structure with these performance factors was done using BEST (Biological Environmental and Stepwise method) within Primer 6 (Primer-E Ltd. UK).

4.3. Results

4.3.1. *Cell acclimatisation*

All 16 reactors acclimatised and produced voltage. The acetate fed reactors showed a clear pattern of acclimatisation related to both temperature and inocula with the warm reactors acclimatising first, and the Arctic soil inoculated reactors starting first as shown in Figure 4-2. The cold wastewater inoculated reactors did not produce current until after around 800 hours, longer than the time allowed in previous studies (Cheng et al.

2011, Min et al (2008). The acclimatisation of the wastewater fed reactors was only affected by temperature: the warm reactors started producing current at day 1, the cold reactors at day 20. All duplicates behaved in a very similar way.

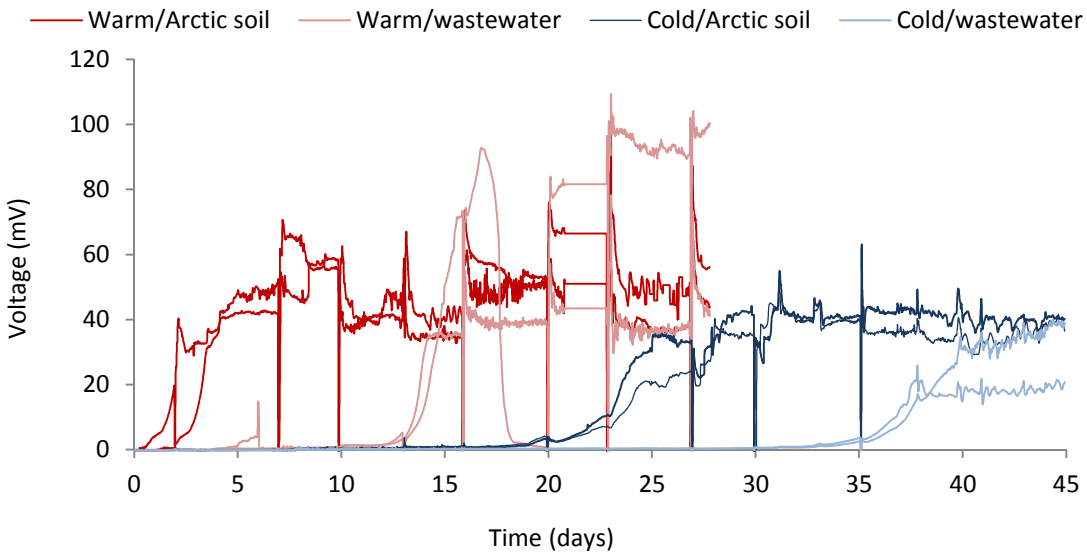

Figure 4-2 Acclimatisation of the acetate fed reactors inoculated with the two different inocula and run at warm (27.5 ^{o}C) and cold (7.5 ^{o}C) temperatures

4.3.2. *Cell performance*

Over the three batch runs, the reactor performance was variable especially within the warm reactors, as seen in Figure 4-3. The variation in performance was not a function of either the inocula or the substrate and the highest variation was seen between the duplicates.

Three measures of performance averaged for each reactor over the triplicate batches are shown in Figure 4-4. The coulombic efficiency is higher in the acetate fed reactors; and the COD removal is higher in the wastewater fed reactors. Power densities do not appear to vary with substrate, inoculum or temperature, however two individual reactors had considerably higher power densities than the others and their duplicates: acetate warm ww 2; and wastewater warm soil 1.

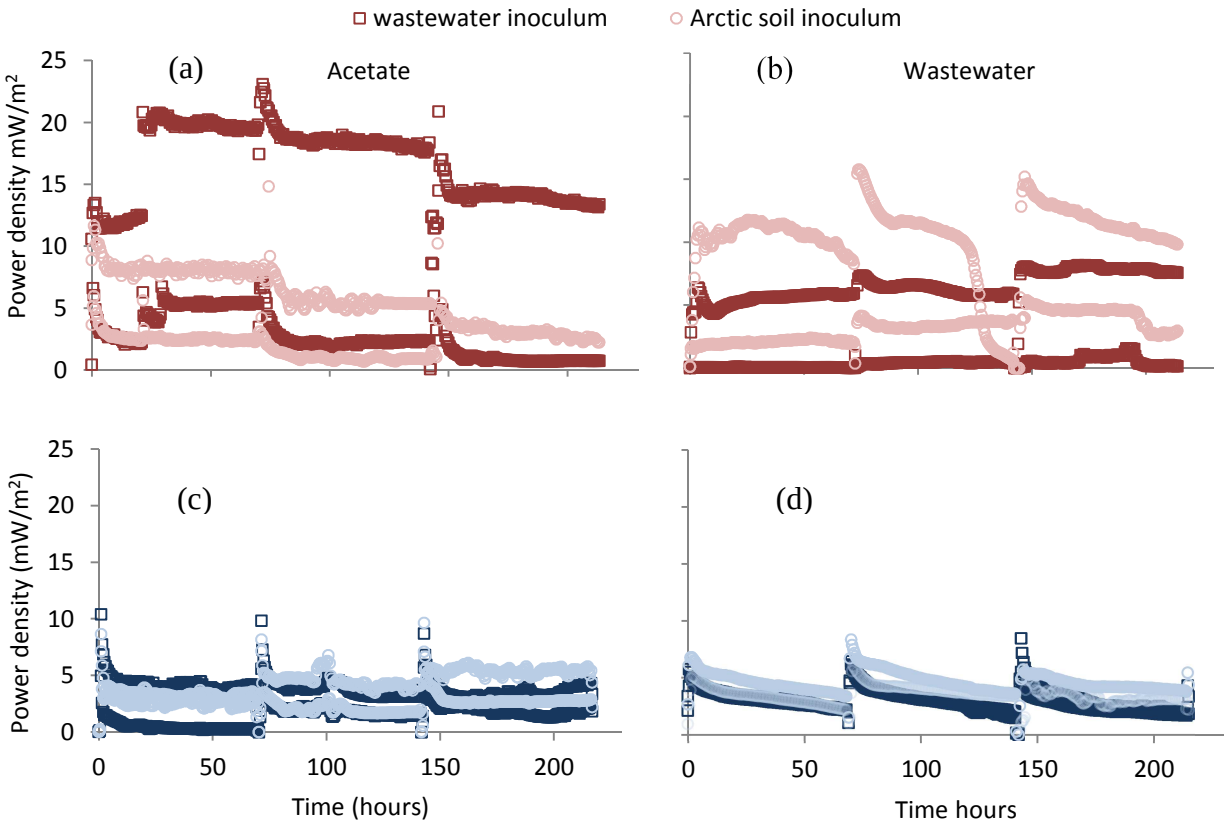

Figure 4-3 Power density plots showing the three consecutive batch runs for: (a) acetate fed reactors run at 27.5 °C, (b) wastewater fed reactor run at 27.5 °C (c) acetate fed reactor run at 7.5 °C (d) wastewater fed reactor run at 7.5 °C

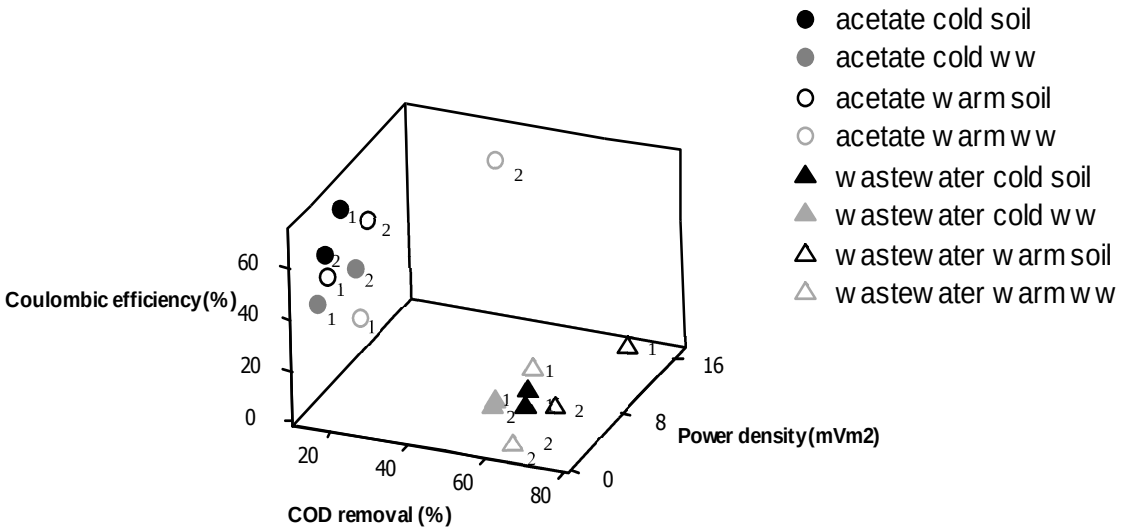

Figure 4-4 3D plot showing reactor performance in terms of Coulombic efficiency, COD removal and power density of the various reactor conditions, duplicates of each condition are labelled on the plot next to the symbols

By performing an ANOVA on the three performance indicators using the factors of feed, temperature and inocula a complex picture emerges. The power density results, i.e. the ability of the biofilm to put electrons to the circuit, were not normally distributed, when transformed, none of the performance factors analysed were significant (feed p = 0.746, inoculum p = 0.249, and temperature p = 0.147). For coulombic efficiency both inoculum (p=0.009) and feed (p=0.000) were significant yet temperature was not. The acetate fed reactors performing better (54.5%) than wastewater fed ones (12.3%), and the Arctic soil inoculated reactors performing better (37.4%) than the wastewater inoculated ones (29.4%). The reactors fed wastewater removed significantly more COD (62.1%), than the acetate reactors (19.4%) (p=0.000) the warm reactors also removed more (45.9%) than the cold ones (33.7%) (p=0.000), the type of inoculum was not significant. Two way ANOVA was performed between each interaction with each performance indicator. For CE the interaction between substrate and inoculum was significant (p = 0.057) with the inoculum having a much stronger effect with the acetate feed than the wastewater feed, and the Artic soil acetate fed reactors performing the best. The interaction between substrate and inoculum was also significant in the COD removal (p = 0.008), the Arctic soil inoculum having a higher COD removal in the wastewater fed reactors, but a lower COD removal in the acetate fed reactors than the wastewater inoculum. No other interactions were significant.

4.3.3. *Similarity of duplicate reactors*

It is seen in the data above that the duplicate reactors performance varied considerably, especially for the warm temperature reactors. Using the sequencing data a Unifrac dissimilarity matrix was plotted, using phylogenetic information the ‘distance’ between each sample is quantified and corresponds to the degree of similarity (Appendix IX). The values show that the duplicate reactors fed with acetate are indistinguishable (p=0.000). This was observed with both the weighted analysis which incorporates information on relative abundance of each OTU, and the unweighted analysis which is based on the presence or absence of each OTU. The wastewater fed duplicate reactors were typically different, with the exception of the hot Arctic soil inoculated reactors (p=0.000). The two wastewater inocula samples taken from the same treatment plant but at different plants were also indistinguishable (p=0.000). This pattern is also observed in Figure 4-5, where the acetate duplicates are paired, and appear to cluster on the basis of temperature. The wastewater fed reactor duplicates are not paired together and do not

cluster with temperature or inoculum. Further details of the bacteria groups present within these reactors can be found in Appendix XI.

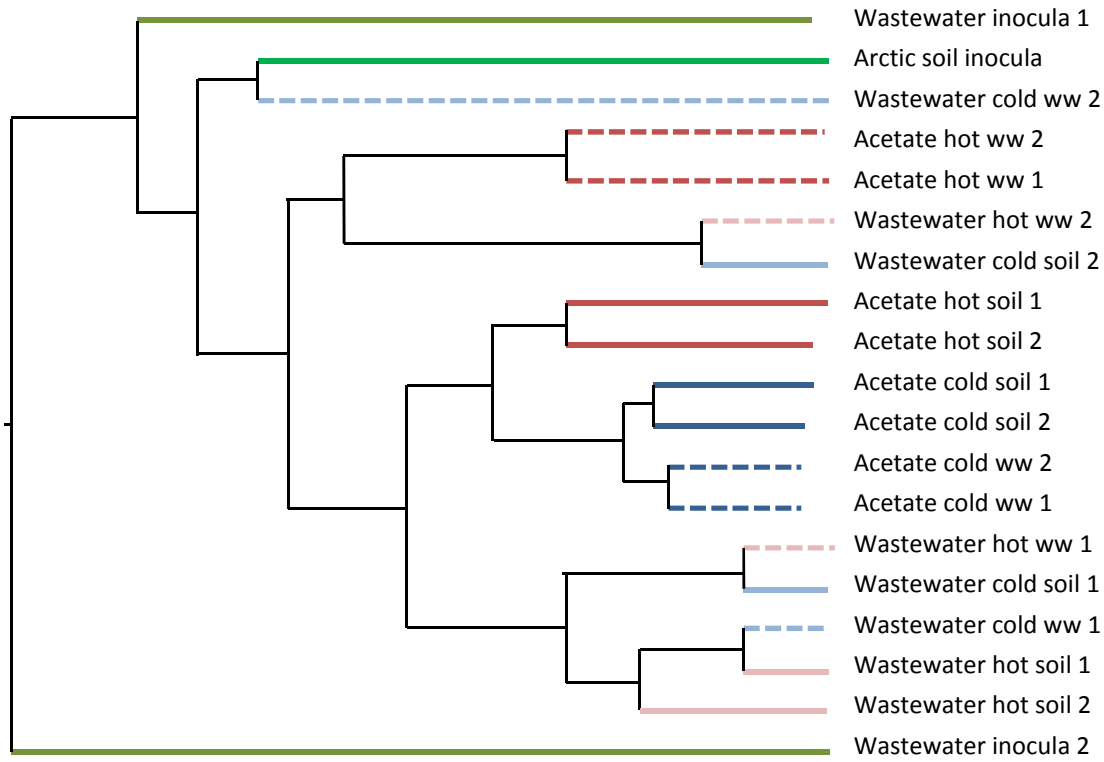

Figure 4-5 Dendrogram resulting from the UPMGA hierachical weighted clustering of samples, the length of lines is relative to the dissimilarity between samples, groupings of samples are denoted by the coloured end portion of the lines

4.3.4. *Microbial diversity*

In total 19 samples were analysed. The number of sequences per sample ranged from 8112 to 77436 with a total number of observations of 549178. The species abundance pattern plotted from the OTU table shows a large variation in the diversity of the samples shown in Figure 4-6. As expected the Arctic soil inoculum is the most diverse, followed by the wastewater inocula. The acetate fed reactors however are considerably more diverse that the wastewater fed reactors, the most diverse of these (acetate cold soil 2) has a similar diversity to the wastewater inoculum, and the least diverse (acetate warm ww 2, the reactor with the highest power density) is similar to the most diverse of the wastewater fed reactors.

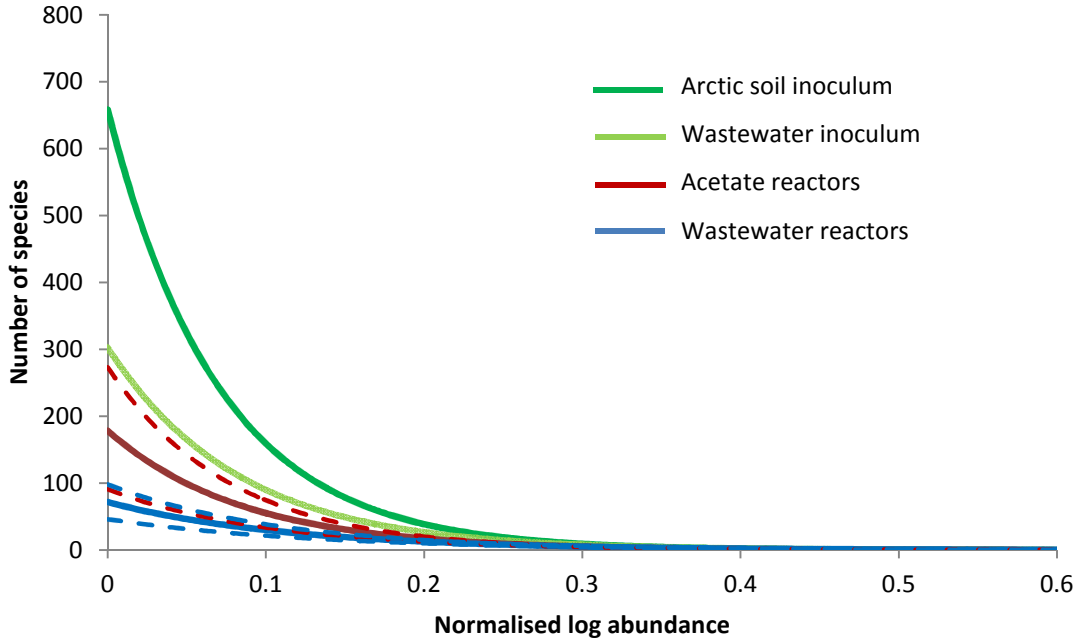

Figure 4-6 Species abundance pattern, the number of species is plotted against the log abundance normalised to the total number of observations for each sample. The plots for the acetate and wastewater fed reactors are averages of the eight reactors used, the highest and lowest within each substrate grouping are shown with the dashed lines. The wastewater inoculum line is an average of the two samples

The observation of the greater diversity in the acetate fed reactors is also seen in the total diversity estimates. A summary of these values is presented in Figure 4-7 where is clearly seen that for all the three distribution models the acetate fed cells have a higher predicted diversity, and that the acetate soil inoculated reactors have a higher total diversity than the wastewater inoculated ones. Performing a nested ANOVA on the Box Cox transformed total diversity estimates, shows that the acetate fed reactors have a statistically significantly higher diversity (log-normal $p = 0.001$; inverse Gaussian $p = 0.000$; and Sichel $p = 0.027$). Within the acetate fed reactors the Arctic soil inoculated reactors have a higher predicted diversity (log-normal $p = 0.006$; inverse Gaussian $p = 0.003$; and Sichel $p = 0.013$), the lower temperatures also give higher diversity (log-normal $p = 0.037$; inverse Gaussian $p = 0.012$; and Sichel $p = 0.029$). There is a strong interaction between the acetate feed and the inoculum type ($p = 0.024$) but not with temperature ($p = 0.156$) observed in both the log-normal and inverse Gaussian distributions. The full tables of diversity predictions, DIC values and estimate sampling requirements can be found in appendix X.

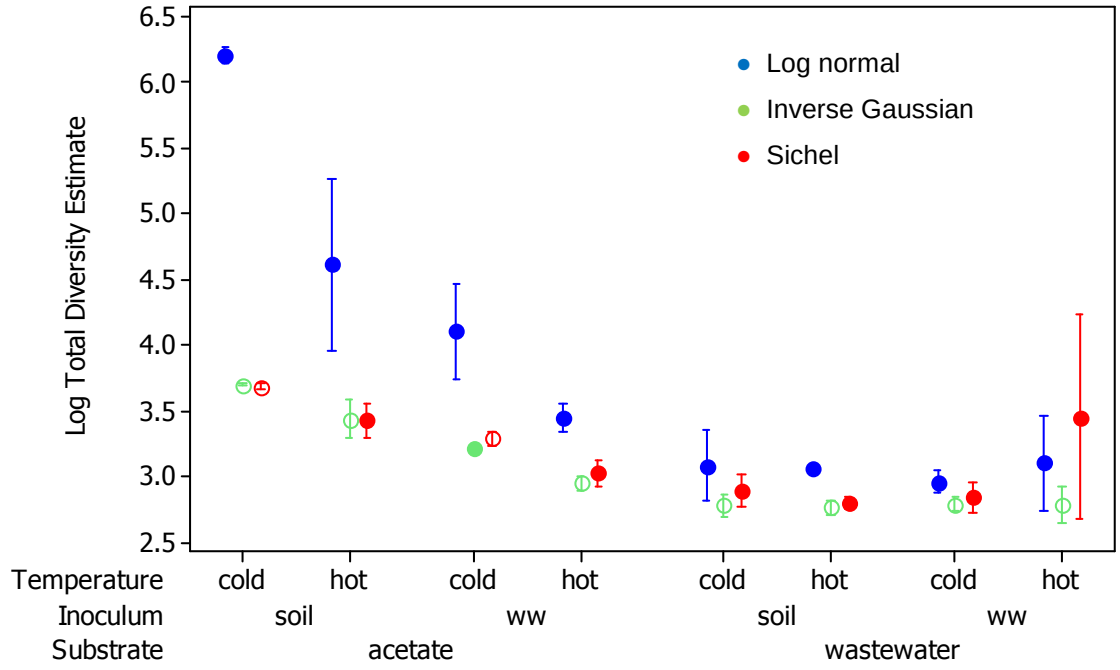

Figure 4-7 The estimates of total diversity for each set of reactor conditions, the three points within each sample are the mean of the duplicate samples modelled to log-normal, inverse Gaussian, and Sichel estimates, the best fit according to the DIC values is denoted by a closed circle, lines are one standard error of the mean

4.4. Discussion

All the reactor conditions tested produced current showing that MFCs can function at low temperatures, with real wastewaters and the bacteria required for them to do so can be found within the wastewater itself. This finding is of great significance to the industrial feasibility of MFC technology for wastewater treatment.

The power output produced by the MFCs was not significantly affected by either temperature feed or inoculum. Although some warm reactors achieved a power density much higher than the cold reactors, due to the variability between reactors this was not significant. The reasons for this variability, were not discovered, no statistical link could be made between the community structure and the power density. The higher coulombic efficiencies within the acetate fed reactors did not translate into higher power densities, only low amounts of COD was converted efficiently into power. Whereas in the wastewater fed reactors more COD was converted less efficiently producing a similar power. In terms of wastewater treatment, this high COD removal, albeit at low CE, is an advantage.

The lack of temperature effect seems at first to be unlikely. Based on the laws of thermodynamics, the free energy available in many chemical reactions decreases as temperature decreases. However in a fuel cell system the energy available is the difference in energy between two half reactions. As both the half cells are equally affected by temperature, the difference between them, or energy available does not decrease with lower temperatures (Appendix II). This is a simplification, many other factors such as dissociation constants and partial pressures of gases will affect the energy, additionally the metabolic activity of the bacteria also reduces with lower temperatures (Rittmann, 2001), however these do not appear to be having a significant impact although may be responsible for some of the variability in performance. On the basis of the results presented here, it can be asserted that low temperature systems have a similar level of energy available for both bacterial metabolism and electricity production as higher temperature systems.

The lack of temperature effect could be caused by the reactor design itself. The inherent inefficiencies and overpotentials within the reactors could be limiting the performance such that the temperature effect is not observed, i.e. all the reactors are working at the limit of their performance and warming them cannot result in improvements. If lower temperature reactors did prove to have slower microbial kinetics, as would be expected and as is indicated by the slower acclimatisation in the cold reactors this could be overcome through relatively simple engineering solutions such as increasing the size of the anode. An increase in the size of the anode would give a greater surface area for the biofilm to grow, and therefore more active bacteria to compensate for the slower metabolic rates.

A further counter intuitive result of this study it that the acetate fed cells have a higher microbial diversity than the wastewater fed cells. It would be assumed that in a wastewater fed systems that the complexity of the substrates available for metabolism, and different metabolic pathways would result in a higher diversity of bacteria, with different groups digesting different substrates at different times. With acetate fed reactors, the only metabolic pathway within a fuel cell should be the direct breakdown of acetate and donation of electrons to the electrode, the most efficient species should dominate theoretically leading to a much less diverse community. This is not seen to be

the case, with a higher diversity in the acetate fed cells being shown both by the species abundance pattern and by the analysis of all the total diversity estimates.

It is proposed that the diversity of the systems is determined not by the diversity of the metabolism within it, but by the overall energy available to the bacteria, and that the free energy available to bacteria in the acetate reactors is greater than in the wastewater reactors. This energy difference could be due to several reasons: acetate may have more free energy per g COD than wastewater; the free energy in acetate may be more accessible to the bacteria, i.e. it is easier to degrade than many of the compounds in wastewater; or that energy is lost during the metabolic chain, with acetate this chain is short, therefore the losses are low, within wastewater these chains are much longer and therefore the losses of energy are greater, this would also produce the coulombic efficiencies observed. The fact that there is no observed difference in the diversity between the warm and cold reactors is further evidence that the energy available in these is actually similar.

Results indicate that the energy flux within a microbial system is key to determining the ecology of that system. The total free energy available is likely to affect the balance of births and deaths of individual species, with greater energy resulting in more births i.e. greater abundance and therefore ultimately greater diversity. The free energy will also impact on the speciation rate (i.e. a greater number of births will ultimately lead to greater chances for speciation). This is counter to the theory that a diverse range of substrates available would provide a variety of different metabolic pathways for different organism to exploit, and therefore lead to a higher diversity.

If a quantitative link could be made between the free energy in a system and the diversity modelling of these complex biological ecologies, being able to understand such phenomena as acclimatisation, adaptation and functional redundancy, and ultimately therefore the manipulation of biological systems becomes a greater possibility (Curtis and Sloan, 2006). We are however still a long way from this in the plant and animal world ecologists have argued there is no single species/energy link (Clarke and Gaston, 2006) and even if it was the key parameter the free energy in wastewater systems cannot yet be reliably measured. Although it is evidenced here that free energy may be the key in determining diversity, a conclusive answer cannot be

given let alone a quantitative link on the basis of these results alone, further research is required.

A further effect on diversity is seen with the inoculum, which interacts with the substrate. The Arctic soil inocula has a greater diversity which seems to be carried forward into the acetate fed cells, a greater number of these species surviving within the reactors where energy may be plentiful. As the performance of the acetate and wastewater fed cells is similar despite the increased diversity of the acetate reactors, it could be concluded that this increased diversity is non-beneficial, or at least neutral to the performance of the reactor. Thus although wastewater reactors will always have lower coulombic efficiencies due to the losses within the metabolic chain, they may actually be more efficient at turning the energy available into wastewater digesting biomass and electricity.

The majority of fuel cell research is conducted at warm temperatures and with simple substrates. It has been shown in this research that reactor performance is not significantly affected by the temperature, neither is the diversity of the community developed. Inoculating reactors with cold adapted organisms does not have any benefit on the performance of the reactors. The substrate fed to the reactor again has little impact on the performance, however results in very different diversities.

It is generally assumed that an acetate fed reactor may represent the optimum conditions for an MFC, however this may not be the case. These findings suggest that wastewater feed has less available energy and therefore results in a more efficient biomass being formed. This has positive implications for the introduction of bioelectrochemical systems into wastewater treatment.

Chapter 5. Time taken until failure for MEC's fed on acetate compared to those fed on wastewater

5.1. Introduction

In 2005 a discovery was made that a microbial fuel cell could be turned into a microbial electrolysis cell adding a small supplement of electricity at the cathode to produce products such as hydrogen gas (Rozendal et al., 2006, Liu et al., 2005b). This new technology has spurned much excitement and research into increasing the performance and gas yield of such reactors (Wang et al., 2011b, Sleutels et al., 2011, Cheng and Logan, 2011). The aim of this research being to achieve a commercially viable and sustainable means of treating waste organics (Oh et al., 2010, Rittmann, 2008, Clauwaert et al., 2008).

Substantial steps have been taken towards enabling the implementation of this technology. Low cost and more robust alternatives to many of the materials used in an MEC have been discovered such as stainless steel (Call et al., 2009) and nickel (Selembo et al., 2009a) cathodes. Alternative membrane materials have been trialled successfully (Rozendal et al., 2008c), as well as not using a membrane at all (Clauwaert and Verstraete, 2009). Anodes with greater surface areas have been found (Call and Logan, 2008) as well as methods to enhance the performance of the carbon anodes (Cheng and Logan, 2007b). New cell architectures and configurations have also helped improve performance (Cheng and Logan, 2011, Wang et al., 2010). Such developments have seen the performance of these reactors increase from hydrogen production rates of 0.01-0.1 m^3H_2/m^3reactor/day (Liu et al., 2005b, Rozendal et al., 2006) to 17.8 m^3H_2/m^3reactor/day (Cheng and Logan, 2011), although the same rise in not seen in the electrical recoveries of these systems 169% (Rozendal et al., 2006) 533% (Liu et al., 2005b) in the initial studies to 115% (Cheng and Logan, 2011) due to the higher input voltages used. All of this research has used acetate as a model compound.

Research with complex substrates is more limited. The ability of MECs to digest complex substrates has been proved such as domestic wastewater (Ditzig et al., 2007), piggery wastewater (Jia et al., 2010), potato wastewater (Kiely et al., 2011a) and end products of fermentation (Wang et al., 2011a, Lalaurette et al., 2009). Limited research has been conducted into the long term performance of MFCs and MECs, deterioration in performance of an MFC after a year of operation has been attributed to the gas

diffusion cathode (Zhang et al., 2011). Marine MFCs used as batteries to power offshore monitoring devices have been monitored for up to a year (Reimers et al., 2001, Tender and Lowy, 2004) and 18 months (Lowy et al., 2006), power production was maintained over this period although in two studies it did deteriorate steadily (Lowy et al., 2006, Reimers et al., 2001), and in another there were occasional drops in the output (Tender and Lowy, 2004). Such studies may not directly translate to MFCs or MECs used for wastewater, in a marine environment the ionic concentrations, gradients and flows will be different, as will the bacteria.

By analysing all the published papers in the area of MECs up to October 2011 the limited scope of how well we understand the long term performance of these systems especially when fed on real wastewaters becomes clear, as seen in Figure 5-1.In 26% of papers the duration of the experiment was not given. In many other cases this time frame is not stated explicitly but can be inferred using the tables, graphs and other information given. In relatively few articles the durability is highlighted as a factor. Two research articles have however been published which indicate the technology might have long term applicability with experiments lasting 9 months (Lee and Rittmann, 2010) and 8 months (Jia et al., 2010) , both running on acetate. Although several other studies do state a decline in performance over time (Jeremiasse et al., 2009, Rozendal et al., 2008b, Lalaurette et al., 2009, Hu et al., 2009).

With acetate fed reactors, 73% of all MEC studies, the time scales mentioned range from 4 to 6480 hours, with 1159 as the average. However when wastewater is used, (only 10% of laboratory studies) the range is between 12 and 184 hours, with an average of 122.5 hours, this time of operation is significantly different ($p=0.000$, two sample T test). For other substrates such as VFA’s and glucose the average run time is 276 hours. This is shown in Figure 5-1, the studies with no time frame stated are not included in the graph. The explanation for this disparity is not evident in the literature, in one study acetate and piggery wastewater are compared directly with acetate reactors running for 8 months and the experiments with wastewater lasting just 12 hours, no reason for this experimental procedure is given (Jia et al., 2010). There is a clear gap in this area of research.

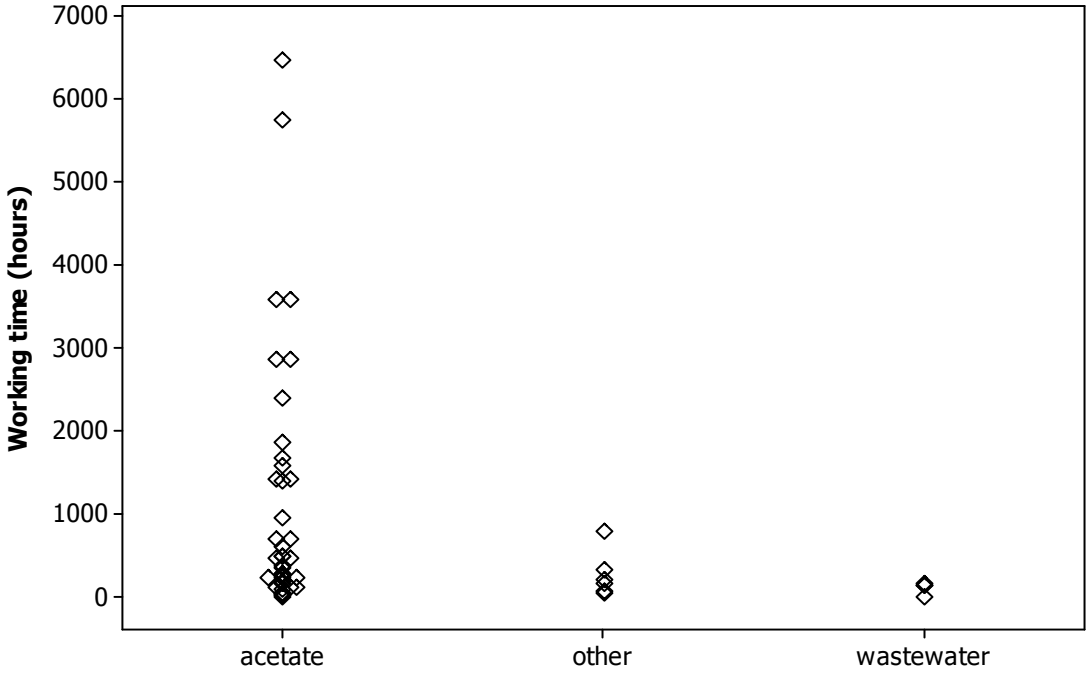

Figure 5-1 The working time of all MEC studies documented in the literature to date (Oct 2011), shown for the different substrates

If MECs are to be a viable and sustainable treatment option for the future then we need to gain an understanding of their long term performance with real wastewaters. Most of the research in MECs does not use real, or even complex artificial wastewaters, and most are run over a relatively short period of time. If this research is to translate into application, this relies on two key assumptions:

1. Real wastewaters containing mixture of simple and complex organic molecules will behave in the same way as acetate, a simple readily digestible molecule most frequently used in BES research. We know this not to be the case with anaerobic digestion (Rittmann, 2001).
2. A system that works at a particular efficiency for a short period of time will do for a long period of time. This is again unlikely as even with the clean technology of chemical fuel cells, long term durability tests have lasted around 4000 hours (166 days), although a couple of studies have extended this to 1.5 and even 3 years (Schmittinger and Vahidi, 2008). Failure is associated with blocked membranes, electrode deterioration and many other factors that may increase overpotentials. Biological systems have the added complexity of the behaviour of microorganisms.

Failure in laboratory batch fed wastewater reactors has been observed many times during preliminary laboratory testing. The aim of this research is to determine if wastewater fed MEC laboratory reactors are capable of operating over the same time periods as acetate fed reactors, and, if this is not the case, to identify the reasons why.

5.2. Method

5.2.1. *Reactor design and set up*

Double chamber MEC reactors (78 mL each chamber) were used which were of a tubular design, internal diameter of 40mm, length 60mm. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. The membrane between the reactor chambers was Nafion 117, with an area of 12.6cm^2. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 1 Ω resistor, 0.7 V supplied using a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong), and a multimeter to measure the voltage (Pico ADC-16), logged every 30 minutes onto a computer.

All reactors were cleaned and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK). The cathode media was 50 mM phosphate buffer, which was sparged with 99.99% pure N_2 for 10 minutes prior to being put into the reactors. The acetate based anode media used was that of Call and Logan (Call and Logan, 2008), during the tests where this was supplemented with protein, Aspargine was added to give an equivalent level of nitrogen to that measured in the real wastewater. The wastewater used was raw influent wastewater (post screens prior to primary sedimentation) from Cramlington wastewater treatment plant. The anode media was sparged for 10 minutes with N_2 prior to use. All reactors were initially acclimatised in MFC mode as per the method used in other studies (Call and Logan, 2008, Cheng and Logan, 2007a, Hu et al., 2008, Wagner et al., 2009), inoculated with 25 ml of raw wastewater and fed acetate media.

The gas produced by the cathode side was captured via a liquid displacement method in a 12 ml glass tube with a septa fitted to the top for sampling. The volume of this gas was measured by drawing it into a 5 ml gas tight syringe (SGE Analytical Science,

Australia). The anode gas was captured in an inverted 10 ml syringe placed into the top of the reactor and filled with the N_2 gas.

5.2.2. *Analytical procedures*

The following analysis was conducted in duplicate for both the effluent and influent of the cathode and anode liquids of each batch run. The chemical oxygen demand (COD) using standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) kit tubes. Volatile fatty acids (VFA's) were measured using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. The anion content using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The pH was measured using a pH probe (Jenway 3310, U.K.) and conductivity using an EC 300 probe (VWR Ltd, UK). The anode and cathode potential was measured using Ag/AgCl reference electrodes (BASI, U.K.) during each batch.

Hydrogen gas was measured on a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using triplicate injections of each sample, set against a three point calibration run once at the start of the measuring period and once at the end using standard calibration gases (Scientific and Technical Gases, U.K.). These gas measurements were verified using a Trace Ultra GC TCD with a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

GC-MS analysis of gaseous hydrocarbons, including halomethanes, was performed on a Agilent 7890A GC in split mode; injector at (280°C), linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software in full scan mode (10-150 amu/sec). A standard containing 100 ppm of three chloromethanes was injected (100ul headspace) followed by the reactor headspace samples (100ul) every 2 minutes. Separation was

performed on an Agilent fused silica capillary column (60m x 0.25mm i.d) coated with 0.25um dimethyl poly-siloxane (HP-5) phase. The GC remained at 30°C temperature for 90 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 20 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library if greater than 90% fit.

5.2.3. *Microbial analysis*

An assessment of the level of microbial activity occurring in the reactors was needed to give an understanding if failure was caused by a reduction or complete elimination of microbial activity, or conversely a competitive but non complementary microbial process. Methods involving the extraction and quantification of DNA from the anode biofilm were not suitable for this purpose as this would capture both the alive and active DNA and that DNA remaining on the biofilm from bacteria which were dead or inactive. Ribonucleic acid (RNA) is used within cells to convert DNA i.e. the genetic code into working proteins (Rittmann, 2001); it can therefore be used as a proxy for the amount of biological activity occurring in the cell (Milner et al., 2008, Low et al., 2000). As RNA is so susceptible to contamination and degradation, the simple and relatively quick approach of measuring the amount of nucleic acid extracted on a Nanodrop, and then comparing this directly to the amount of DNA extracted at the same time, would give the most reliable quantitative results.

Duplicate samples of anode material were taken for RNA and DNA extraction, from duplicate reactors sacrificed whilst working, and duplicate reactors after failure. The following procedure was carried out as quickly as possible inside a microbiological cabinet, to prevent the loss of RNA which readily breaks down if contaminated with RNases. All working areas and equipment was cleaned thoroughly with ethanol followed by RNase AWAY (Invitrogen Life Sciences, U.K.), including the anode cutting equipment which had also been washed with detergent and then heated to 240 ^{o}C for 4 hours in a furnace, prior to use. Each reactor at the point of sampling was taken into the microbiological cabinet maintaining the electrical circuit. The reactor was quickly dismantled and using a coring device duplicate 4mm diameter sections of the anode were cut and placed into a sterile RNase free 2 ml eppendorf, containing 1 ml of TRIzol Reagent (Invitrogen Invitrogen Life Sciences, U.K.), the sample was vortexed for 5 seconds to ensure complete submersion in the reagent, and then the samples frozen

at -80 ^{o}C. Duplicate cores were taken in the same way afterwards for DNA extraction and stored in 50:50 ethanol and phosphate buffer at -20 ^{o}C.

Extraction and clean-up of the RNA sample was then completed using a RNeasy Mini Kit (QIAGEN, Germany) as per the manufacturer's instructions. Once cleaned the samples were frozen at -20 ^{o}C. The DNA was extracted using a QBiogene FastDNA spin kit for soil (MP Biomedicals, U.K.) and also frozen in two samples at -20 ^{o}C. The quantity of nucleic acid present was then measured in duplicate on a Nanodrop Nanodrop 2000 (Thermo scientific, USA). The ratio of DNA to RNA could then be calculated for each sample.

5.2.4. *Experimental procedure*

Failure had been observed several times in these bench scale reactors used as MEC's when fed with wastewater. The purpose of these experiments was to determine if this failure was statistically significant, and if so to try and identify the particular cause. In total 12 wastewater fed reactors and 10 acetate fed reactors were used in this study, the materials and architecture of all the reactors were the same, and the same operating procedures observed throughout. The work was conducted at laboratory room temperatures of between 20-25 ^{o}C.

Initially 8 reactors were run, 4 of fed with acetate media and 4 with real wastewater. After each batch of 3-4 days the effluent was analysed for COD, VFA's, anions, pH and conductivity and the gas measured, the reactors were then refilled with N_2 sparged media to the anode and phosphate buffer to the cathode. Once having completed two batch runs producing gas, 2 reactors of each feed were sacrificed and the RNA and DNA were sampled, the remaining reactors were run and sampled as described until gas production ceased, or in the case of the acetate ones until they were stopped at 130 days.

A further experiment was conducted using 4 wastewater fed reactors to eliminate the possibility that a drop in pH in the wastewater fed reactors was causing failure. Duplicate reactors were run containing wastewater, and the same wastewater buffered to pH 7 using 50 mM phosphate buffer. All reactors were run in batch mode and samples as described above until gas production ceased. Examination as to whether the biofilm was damaged/killed during failure was gained by switching the failed MECs to MFC

mode (increased resistance and no external load), and refilling with UV sterilised wastewater (see Appendix V for details of this method).

Due to the observed drop in Cl^- ions prior to the point of failure, it was hypothesised that locally high levels of NH_4^+ at the anode, caused by the degradation of proteins present in the wastewater could be reacting with the chloride ions to form chloramines, which would then kill off the biofilm resulting in failure of the cell. This hypothesis was tested running 4 acetate fed reactors, by supplementing duplicate reactors with protein Aspargine at levels comparable to the wastewater levels as detected through the use of the TKN Standard Method 4500-Norg (APHA, 1998), comparing these to duplicate control reactors with no protein. Again sampling was carried out as above, in addition the effluent of the reactors was analysed for residual chlorine using the DPD test, Standard Methods 4500-Cl D, (APHA, 1998).

A further hypothesis to account for failure and the drop in chlorine was that the chlorination of organics, especially methane could be occurring in the reactors due to the potential of the anode. Under standard conditions, at pH 7 the required potential for chlorination of methane at a Cl concentration of 1 mM is 0.44 V, when considering that the reactors may have a pH slightly deviant from 7, and that the partial pressures of the methane and chloromethane produced would not be equivalent, it is conceivable that the anode potential needed for this reaction could be occurring in the reactors, producing chloromethanes and therefore removing the hydrogen ions from the system and eliminating H_2 production. Again 4 wastewater reactors were run in batch mode with the same analysis as described above, in addition both the anode and cathode gasses were captured and analysed for methane, hydrogen and chloromethane using the instruments and methods stated above. Duplicate reactors fed with acetate were run at the same time and subject to the same analysis. After failure reactors were again switched to MFC mode and the anode gas continued to be sampled.

5.2.5. *Calculations*

The reactor performance was evaluated in terms of the volume of hydrogen produced, and also the coulombic efficiency and electrical energy recovery. The definition of these two efficiencies can be found in section 6.2.5.

5.2.6. *Statistics*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

5.3. Results

5.3.1. *Time taken until failure*

The run time of the reactors is shown in Figure 5-2 as the amount of hydrogen produced at the end of each batch, the reactors terminated at 7 days for RNA sampling are not shown. It is seen that the Acetate fed reactors run for a longer period of time, including those supplemented with protein and produce more hydrogen than the wastewater reactors. The buffered wastewater reactors initially perform well, but then stop producing hydrogen after a short time period.

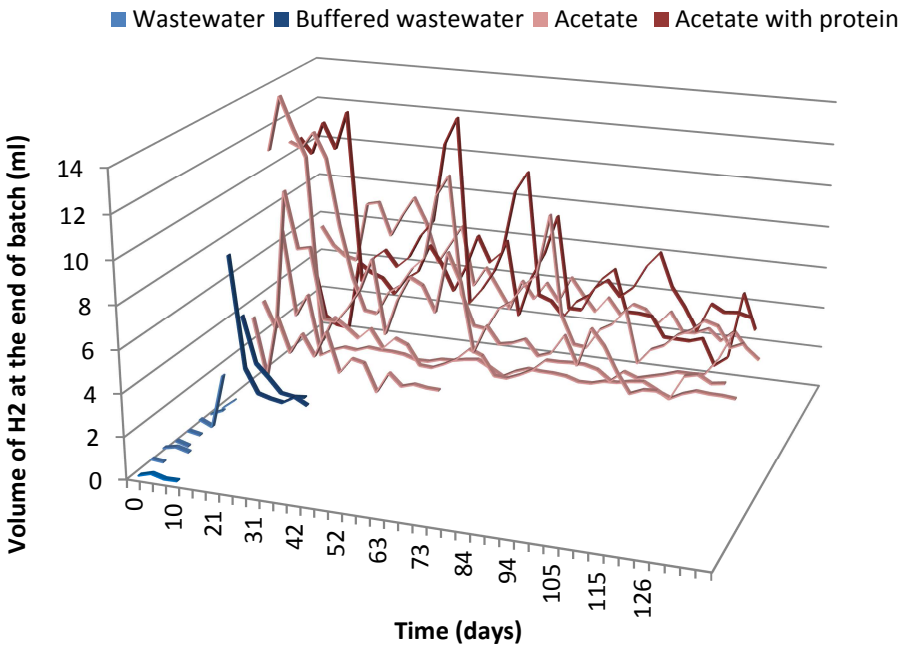

Figure 5-2 Graphic showing the working period of all reactors as indicated by the length on the line along the time axis, the volume of H_2 produced at the end of each batch is given on the y axis as an indication of reactor performance which is seen to be variable, where the line is discontinued this illustrates zero H_2 production and the reactor is deemed to have failed

All 10 of the reactors fed on wastewater failed within 7-17 days of operation, failure was determined by no measureable gas production at the cathode. Of the 8 acetate fed reactors one failed at 56 days, but the others remained functioning until the experiment was terminated after 130 days. With 130 days used as the minimum run time for the acetate fed reactors, the difference in time to failure is significant ($p=0.000$, two sample t-test) as shown graphically in Figure 5-2.

5.3.2. *Reactor performance*

The average performance data collected over the duration of different experiments is shown in Table 5-1. The acetate fed cells have a greater coulombic efficiency and electrical energy recovery. The COD removal is reasonably similar for all substrates, but higher for the buffered wastewater, although this does not translate into improved coulombic efficiency or energy recovery. In all cases there is a large degree of variation, as is seen by the standard deviations. This is also seen through the hydrogen production data in Figure 5-2, which is higher for the acetate fed reactors, but does deteriorate throughout the test period.

Table 5-1 Summary of reactor performance using three different parameters other than H_2 production for the experiments using different substrates, values are the average values of all the reactors run on the given substrate

	COD removal	Coulombic Efficiency	Electrical Energy Recovery
Wastewater	23.2% ± 12.2	7.5% ± 3.9	15.7% ± 20.1
Buffered wastewater	43.8% ± 7.8	3.7% ± 1.7	13.5% ± 16.6
Acetate	28.6% ± 11.5	10.9% ± 2.0	33.0% ± 15.1
Acetate with protein	32.3% ± 13.4	10.4% ± 3.6	35.1% ± 22.9

Values represent average of all the batch experiment run on the given substrates where hydrogen was produced, ± one standard deviation.

There is a reduced performance between the acetate fed reactors as compared to the wastewater ones of around 50 % if energy recovery is considered.

5.3.3. *Biological processes*

The average RNA: DNA ratio of the duplicate samples show that there is significant difference between the working and failed reactors at the 90% confidence interval ($p=0.068$ two-sampled t-test). This difference is more pronounced with the wastewater fed reactors, where the average ratio value for the working reactors is 11.5 compared to the failed reactors 3.9. The acetate working reactors have an average a ratio of 6.1, with the single failed cell being 4.2.

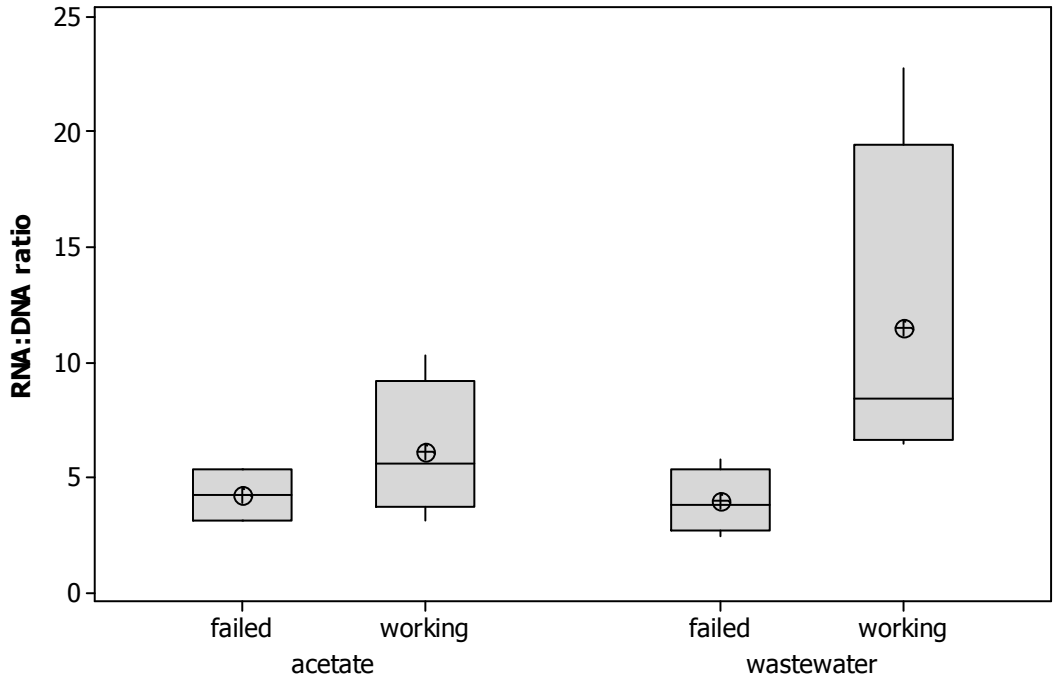

Figure 5-3 Box plot of the RNA: DNA ratios of failed and working reactors fed with both acetate and wastewater, the data represents a summary of the duplicate samples taken from duplicate reactors (i.e. four samples in total) with the central line representing the median and the mean given by the circle with cross

5.3.4. *Low pH*

In the wastewater fed reactors, which contained no additional buffering, it was observed that at around the point of failure there was a decline in the pH of the anode effluent from a starting value 6-6.5 to around 5.5. The acetate fed reactors, (the nutrient media containing 50mM pH 7 phosphate buffer) did not show any significant fall in pH during the full time period over which their function was monitored.

With the additional duplicate reactors fed on wastewater and buffered wastewater there was the same observed drop in pH with the non-buffered reactors. The buffered reactors kept a constant pH and initially performed better but then also failed within 17 days of operation. No significant difference in the run time between the buffered and non-buffered reactors ($p=0.306$, two sample t-test).

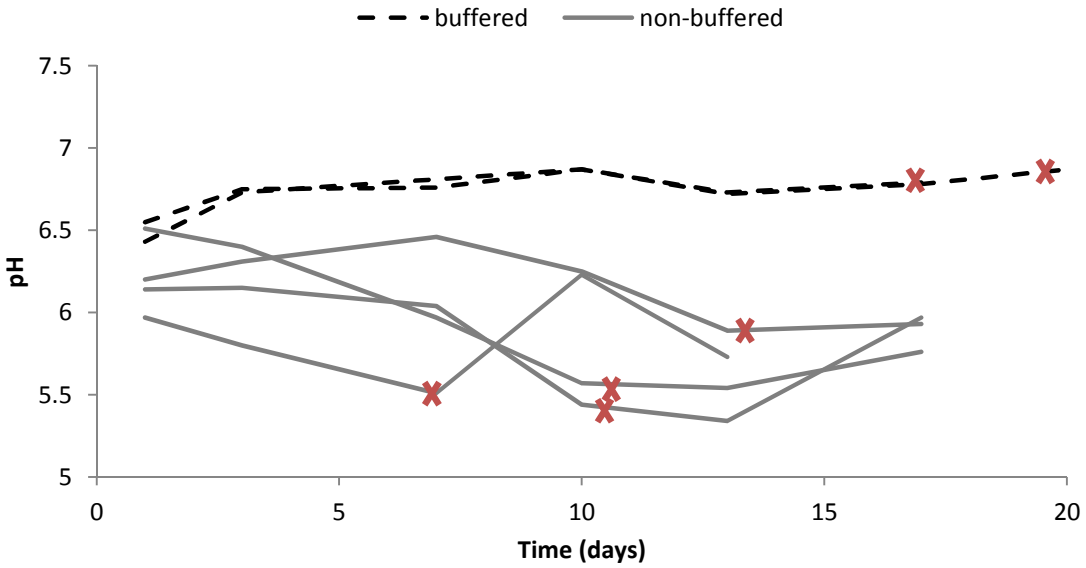

Figure 5-4 Measured pH of the wastewater reactor liquid during the course of the batch experiments, the point of failure is denoted by the red cross where gas production ceased

5.3.5. *Toxic build up within the reactors*

The full anion analysis of the cell effluent showed that there was a fall in chloride ions prior to failure of the wastewater reactors. Both the acetate media and the wastewater contained approximately 250-300 mg/L of chloride. During the course of each batch run with the acetate fed reactors, approximately 50 mg/L of the chloride would be taken up in the reactor, this remained relatively constant throughout the full time period the acetate reactors were operated for. However in the wastewater reactors, when working and producing hydrogen, the chloride removal in the cell was observed to be virtually complete prior to the reactor failure, i.e. 250-300 mg/L of chloride ions were being removed. The levels of chloride in the cathode compartment of these reactors remained the same as the original influent. After failure of the reactors when no hydrogen was produced, this chloride removal stopped. The only wastewater reactors that this drop was not observed in were the duplicate buffered wastewater reactors, here chloride removal remained constant at around 50-100 mg/L during each batch, the reactors did however also fail.

In the acetate reactors supplemented with protein the chloride removal remained roughly constant throughout the experiment at between 50-100 mg/L, and the reactors did not fail. No chloramines could be detected in the effluent of these reactors, disproving the hypothesis of chloramine formation. The performance of the protein

supplemented reactors in terms of electrical energy recovery was not significantly different to the non-supplemented ones (p=0.376, two sample t-test).

Further evidence that a toxic chlorine based product was not being formed was gained using four failed wastewater reactors, duplicate reactors were refilled with UV sterilised wastewater non sterile wastewater, put into MFC mode, i.e. increased resistance and no external load. With all four reactors biological activity started within 1 hour, and reached a level of current production as would be expected of a fully acclimatised MFC cell using the same cell materials. The electrogenic biofilm was capable of functioning. After one batch in MFC mode, the reactors were then all returned to MEC mode, where no gas was produced and the failed status continued. In MFC mode, the chloride removal was relatively constant again at around 50 mg/L.

5.3.6. *Formation of halogenated organics*

Analysis of the headspace gas for 4 wastewater fed reactors and 2 acetate fed did not show detectable levels of halogenated organics, levels were below 0.01% of the 10 ml headspace. This was the case for wastewater fed reactors before, during and after failure and for acetate fed reactors. The same observed drop in chlorides was seen in these reactors.

5.3.7. *Other factors*

The analysis of VFA's in the effluent of the reactors showed that in all cases for both acetate and wastewater there was some acetate remaining at the end of each batch. There was no acetate in the influent wastewater, but always a small amount 20-40 mg/L in the effluent of these reactors, this did not alter once the reactors had failed.

The conductivity for the wastewater was around 1.8 mS, the buffered wastewater was 6.3 mS, and the acetate media was 5.9 mS. The conductivity of the reactor effluent was on average 1.6 mS for the wastewater fed cells both before and after failure even when a drop in chloride ions was recorded, the average for the buffered wastewater cell effluent was 5.5 mS and again did not change after failure, the acetate cells also showed a slight drop in conductivity of the effluent to 5.2 mS.

The production of methane at the anode of the reactors was on average 0.002 ml for the wastewater reactors when working, after failure this increased slightly to 0.029 ml. The methane production remained relatively constant throughout the course of the

experiment and the slight rise after failure is not likely to represent a competitive biological process which is the cause of cell failure, as the average methane production in the acetate fed cells was always higher at 0.072 ml per batch, and also the converted MFC cells that functioned well, also produced on average 0.035 ml per batch.

The materials used in these reactors that could become degraded during use, i.e. the cathode and membrane, could be directly and successfully re-used in a new cell, the failure was not due to cathode degradation or membrane clogging. In addition, by increasing the applied voltage of the reactors from 0.7 V to 1.0 V immediately after failure, thus combating any increased overpotentials that could have built up during the short operation period, the reactors could not be revived and did not produce hydrogen. Failure was not therefore caused by the simple the deterioration of the cell components.

5.4. Discussion

Small laboratory scale wastewater fed reactors fail after a short period of time whereas acetate fed reactors do not. This is significant. The cause of this failure could not be identified during the course of this study. Relatively ‘simple’ explanations such as degradation of electrodes or membranes, a drop in conductivity, or lack of available VFA’s have been ruled out as possible causes of failure.

A further hypothesis that failure of the reactors is caused by a reduced or eliminated level of electrogenic activity in the reactors was also seen not to be the case. If true this hypothesis would result in the reduced DNA:RNA ratio observed and low current production. However once failure had occurred the reactors could be instantly ‘revived’ by switching them into MFC mode. The electrogenic bacteria were therefore present on the electrode and were capable of donating electrons.

The hypothesis that there is a competitive biological process occurring such as methanogenesis, as suggested in other studies (Cusick et al., 2011), has been shown not to be the case. The RNA to DNA ratio indicates a reduced biological activity in the failed wastewater cells, suggesting that the biofilm is less able to function and metabolise after failure. It is not likely that a non-complementary competing biological activity is taking over the reactor and eliminating the MEC process. It can be seen that there is greater activity in the wastewater reactors than the acetate reactors, this might be an indication of the greater and more multi-layered metabolism that has to occur in

these reactors when fed complex substrates. It is also observed that the failed acetate reactor did not differ significantly to the working ones, suggesting the reason for failure here was different to that for the wastewater reactors. Additionally the levels of methane generated in the wastewater reactors after was less than in the working acetate reactors. A competitive process such as methanogenesis is therefore unlikely to be the cause.

The hypothesis that a low pH was causing failure, either through altering the electrochemistry or affecting biological function is shown not to be correct. The simple experiment adding buffer to the wastewater also resulted in failure despite initial improvement in reactor performance, here the drop in chloride was not observed. The slightly lowered pH is likely to have a detrimental effect on the cell though. The pH measurement taken is of the whole of the liquid in the reactor, in reality the pH near the anode may be greater. Such a pH will impact on the microorganisms present and the electrochemical reactions within the cell, as pH is a logarithmic function of the concentration of H+ ions, then even a small change in this value has a large impact on the overall thermodynamic balance of the system as is calculated via the Nernst equation. Torres et al (2008) found that an increase in phosphate buffer in the anode media lead to a thicker biofilm and greater current generation in a microbial fuel cell due to the increased diffusion of H^+ out of the biofilm layer, thus making it more accessible to transport to the cathode. Although pH could be limiting the performance of non-buffered reactor it is not the cause of failure.

The formation of halomethanes such as chloromethane could potentially occur at the potentials within these reactors account for the loss of chloride and would cause failure as these compounds are toxic. This would fit the pattern of failure exhibited in the reactors as it would take some time for the levels of methane to build up which could then be converted to the halomethanes, this would ‘use up’ the H^+ ions in the anode section and H_2 would cease to be produced at the cathode. However no chloromethanes could be detected in the headspace gas of these reactors, (below 0.01%) either before or after failure, in fact no halogenated organics could be detected. Additionally the acetate fed cells did not fail when supplemented with protein, and most importantly the exoelectrogenic biofilm is able to work as an MFC after failure so has not been killed. It could be possible that the negative chlorine ions were simply temporarily attracted to the positive anode during the operation of the fuel cell, and therefore not measured in the bulk liquid of the cell. This would account for the observed ‘disappearance’ of the

chloride ions, but is not likely to affect the performance of the cell. The range of analysis carried out indicates that failure is not caused by a chlorine effect; the observed chlorine drop is simply co-incidental to the failure.

The problem of failure needs to be resolved. If MECs are to be a useable technology they need to function with real wastewater. Studying these systems when they are prone to sudden and rapid failure is difficult, therefore identifying the reasons for failure, solving them, and increasing efficiency becomes very challenging. This difficulty leads to acetate being used in most research as this does allow greater scope for experimentation. However it is clear that the processes operating in a reactor fed with real wastewater are different to those occurring in a reactor fed with acetate. The acetate research will not directly inform us of performance with wastewater.

The failure in wastewater fed, laboratory scale, batch fed reactors has been proved, but the reason not identified. Conversely, as part of this research, a larger scale MEC run in continuous mode at a wastewater treatment site fed on raw wastewater has worked producing almost pure hydrogen for a period of over 3 months, (see chapter 6). It is likely that something is occurring within the small batch reactors to prevent either the production of hydrogen ions at the anode, the transfer of these ions, or the hydrogen evolution reaction at the cathode. It may be the case that at this small scale and fed with batch mode that the system and in particular the microbial community involved is fragile and unable to adapt to change, and therefore a build-up of something at an undetectable level has catastrophic consequences. Further work is still needed to identify the cause of this failure, and therefore be able to take steps to resolve it. This can only be done by using real wastewater rather than simple artificial media. The long term performance of wastewater fed MECs is a research gap that must be filled.

Chapter 6. Production of hydrogen from domestic wastewater in a pilot scale microbial electrolysis cell

Addressing the need to recover energy from the treatment of wastewater the first working pilot scale demonstration of a wastewater fed microbial electrolysis cell is presented. A 120 litre (L) microbial electrolysis cell (MEC) was operated on a site in Northern England, using raw domestic wastewater to produce virtually pure hydrogen gas for a period of over 3 months. The volumetric loading rate was 0.14 kgCOD/m^3/day, just below the typical loading rates for activated sludge of 0.2-2 kgCOD/m^3/day, at an energetic cost of 2.3 kJ/gCOD, below the values for activated sludge 2.5-7.2 kJ/gCOD. The reactor produced an equivalent of 0.015 L H_2/L/day, and recovered around 70% of the electrical energy input, with a coulombic efficiency of 55-60%. Although the reactor did not reach the breakeven energy recovery of 100%, this value appears well within reach with improved hydrogen capture, and reactor design. Importantly for the first time a 'proof of concept' has been made, with a technology that is capable of energy capture using low strength domestic wastewaters at ambient temperatures.

6.1. Introduction

In an era of increasing energy costs and environmental awareness, wastewater treatment industries need to look at alternative treatment options to reduce their energy bills. It has been estimated that domestic wastewater alone may contain 7.6 kJ/L of energy, while stronger industrial wastewaters contain substantially more (Heidrich et al., 2011). There is an increasingly urgent need to recover some of this energy, or at the very least not expend additional energy on treatment; the activated sludge process uses 2.5-7.2 kJ/gCOD (Pant et al., 2011). Energy recovery could be achieved through anaerobic digestion to methane gas or microbial fuel cell technology directly to electricity; however life cycle assessment has shown that the production of a higher value product through the suite of bioelectrochemical systems (BES) may be the most viable solution (Foley et al., 2010). One such technology is the production of hydrogen in a microbial electrolysis cell (MEC) (Rozendal et al., 2006).

Since the MEC process was first reported (Rozendal et al., 2006, Liu et al., 2005b) MECs have emerged as a potential technology option for a new generation of wastewater treatment systems (Rozendal et al., 2008a). In an MEC bacteria use the

energy stored in the organic compounds of wastewater to metabolise and grow, donating electrons to an electrode (Rozendal et al., 2006). The electrons then travel in a circuit producing current and therefore electrical power; in an MEC these electrons are consumed at the cathode along with a supplement of electrical power. The H^+ ions also created by the breakdown of organics at the anode travel across the microbial fuel cell membrane to the cathode. Here they can combine to form H_2, however this process is endothermic requiring energy, so a supplement of electrical energy is added to the system to allow it to take place (Liu et al., 2005b).

Fuel cell technologies may offer a sustainable future for wastewater treatment, although there are still many hurdles to overcome. Progress is being made with new reactor design (Call and Logan, 2008, Rozendal et al., 2008b), improved materials (Cheng et al., 2006a, Cheng and Logan, 2008), greater understanding of the mechanisms involved (Aelterman et al., 2008, Clauwaert et al., 2008), and even improved understanding of the microbes that are at work in these systems (Holmes et al., 2004, Kim et al., 2004, Lovley, 2008, Rabaey et al., 2004). Most of this research is performed at laboratory scale, using simple substrates, often at a controlled warm temperature. Many problems have been overcome, such as validation of using multi electrode systems (Rader and Logan, 2010) and finding a low cost alternative to the platinum cathode (Zhang et al., 2010). Although of great value in improving our understanding of MEC's, these studies do not tell us about the challenges or even benefits of running such systems at a larger scale with real wastewaters in temperate climates. There is a need to demonstrate that these systems can work at a larger scale and under realistic conditions, elevating the technology from a laboratory curiosity into a practical solution to an industrial problem.

A pioneering study by Cusick et al (2011) published on the largest MEC reactor to date, a 1000 L pilot scale reactor at a winery in California. The reactor proved slow to start up with pH and temperature control being problematic. When these issues were corrected by heating to 31 ^{o}C and the addition of buffer and acetic acid, the reactor did improve in performance. The energy produced during the operation exceeded the input energy (heating not included), but this was primarily due to methane production (86%) with only trace amounts of hydrogen. Methane production was attributed to the reactor being membraneless allowing hydrogen produced at the cathode to be directly consumed by hydrogenotrophic methanogens within the reactor. The reactor performance tailed off at around 90 days, when the heating unit broke (Cusick et al., 2011). The study has

provided valuable insights into the operation of MECs: (i) the membraneless systems that work well at laboratory scale and when fed in batch mode may not be so good at larger scale and under continuous feed, and (ii) inoculation and start-up are important parameters.

Addressing the issue of a membrane is critical to reactor performance. Most laboratory scale membrane systems use Nafion 117 (Logan et al., 2006), an expensive and delicate proton exchange membrane (Logan et al., 2006); this would be both impractical and costly on a large scale. Also the high efficiencies published: 406% electrical energy recovery (the amount of electrical energy put in that is recovered, this can be higher that 100% as there is also substrate energy within the system) and 86% total energy efficiency (the amount of substrate and electrical energy recovered) (Call and Logan, 2008) are from membrane-less systems. The lack of membrane greatly reduces the resistance in the cell, improving the transmission of protons to the cathode. Membrane systems have lower efficiencies: 169% electrical energy recovery and 53% overall energy efficiency has been reported (Rozendal et al., 2006). These efficiencies are likely to decrease further with time as the membrane becomes fouled.

The issues of inoculation and start-up are poorly understood (Oh et al., 2010) Although the use of acetate is likely to reduce the acclimatisation period (Cusick et al., 2011). However the biological community needed for the degradation of complex substrates is thought to be different to that needed for acetate (Kiely et al., 2011c). A community of acetate degraders able to work at 30 ^{o}C is not likely to be the community needed to degrade wastewater at ambient UK temperatures. There is evidence in the literature that microbes exist that are able to digest wastewater (Ditzig et al., 2007) and operate at low temperatures (Lu et al., 2011). Like anaerobic digestion, however, it may well be that a long period of acclimatisation is needed and unavoidable to achieve a stable community (Rittmann, 2001).

If these start-up issues can be resolved, then the reactor in theory will function, however it would also need to reach a neutral or positive energy balance, i.e. recovering all the electrical energy input plus a substantial fraction of the substrate energy input.

To test whether these systems have a chance of achieving these goals under realistic conditions, a pilot scale 120 L reactor was placed on a wastewater treatment site in

North East England. This site takes in primarily domestic wastewater with an average Total COD of 450 mg/L. The reactor was built using low cost alternatives to the standard lab materials used for the cathode and membrane. The reactor was not heated, held inside a large unheated building, and run throughout a UK spring and summer (5-20 ^{o}C minimum and maximum temperatures) and is still in operation at the time of writing this paper. These operating conditions are likely therefore to represent close to a worst case scenario i.e. low concentration feed; non optimal components; no heating; and no additional supplement of acetate or buffering capacity after the initial acclimatisation period.

Working closely with partners at Northumbrian Water Ltd. the aim of this study was to establish reactor operation and to determine if a neutral or positive energy recovery is achievable. From that data we can evaluate if MEC technology is likely to be a viable treatment option for the future.

6.2. Methods

6.2.1. *Field Site*

The pilot scale reactor was set up and run at Howdon wastewater treatment site, situated near the city of Newcastle Upon-Tyne in the North East of England (54^{o}58'N, 01^{o}36'W). An average of 246500 m^{3} of domestic wastewater is treated daily, using 96 MWh; the activated sludge process uses around 60% of this. The wastewater used in the MEC was taken from the grit channels after primary screening, but before settling.

6.2.2. *MEC reactor*

The reactor was based on a cassette style design, with six identical cassettes being placed into a rectangular reactor with a total working volume of 120 L. The tank has a Perspex plate fitted over the liquid layer giving a small head room to the anode compartment of 2.2 L. Each of the cathode gas tubes from the cassettes projected above this Perspex sheet. The cassettes were set along alternate sides of the reactor to allow s-shaped flow, and once in place gave a final anode volume of 88 L.

Each cassette was constructed using 10 mm thick plastic sheeting and consisted of an internal cathode section 0.280 m by 0.200 m by 0.048 m deep, of a volume 2.6 L. The cathode material was stainless steel wire wool grade 1 (Merlin, UK), 20g was used in each cathode, giving a projected cathode surface area for each electrode of 0.056 m^{2}. A

0.8 m length of stainless steel wire was wound several times into the wire wool to make a firm electrical connection, and then to the outside of the cell. Each cathode electrical assembly had an internal resistance from the extremities of the wire wool to the end of the exposed wire of less than 2.75 Ω. The cathode was separated using a membrane wrapped around a plastic frame inserted into the electrode assembly on both sides. The membrane used was RhinoHide® (Entek Ltd, UK), a durable low cost microporous membrane traditionally used as a battery separator. The anode material was a sheet of carbon felt (Olmec Advanced Materials Ltd, UK), 0.2 m wide by 0.3m high and 10 mm thick. This was sandwiched between two sheets of stainless steel mesh acting a current collector. The anode assemblies were also connected by a 0.8 m length of stainless steel wire fed through the centre of the felt material, each electrode having an internal resistance less than 3.4 Ω.

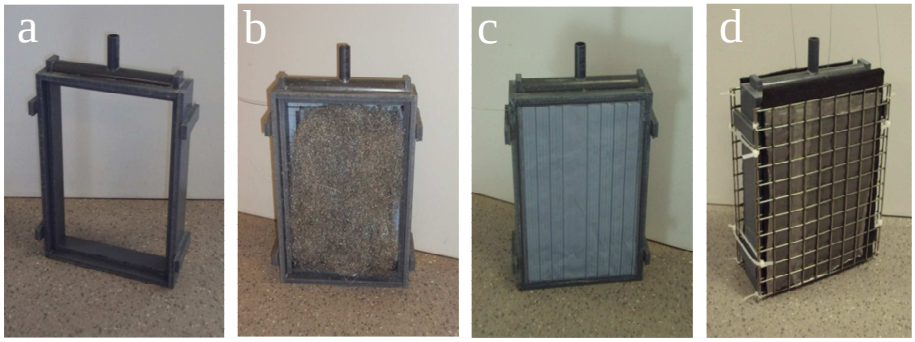

Figure 6-1 Photographs of the electrode assembly unit – a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane, d) anode with wire mesh current collector

The gas production from the anode compartment was captured from the ports in the Perspex lid, using 3mm ID PVC tubing (VWR Jencons, UK). The cathode gas was initially captured using 4mm annealed copper GC tubing connected to each cathode compartment using copper compression fittings, (Hamilton Gas Products Ltd, Northern Ireland), due to rapid corrosion this was later replaced with 3mm ID PVC tubing (VWR, UK). Both pipelines contained a gas sampling port.

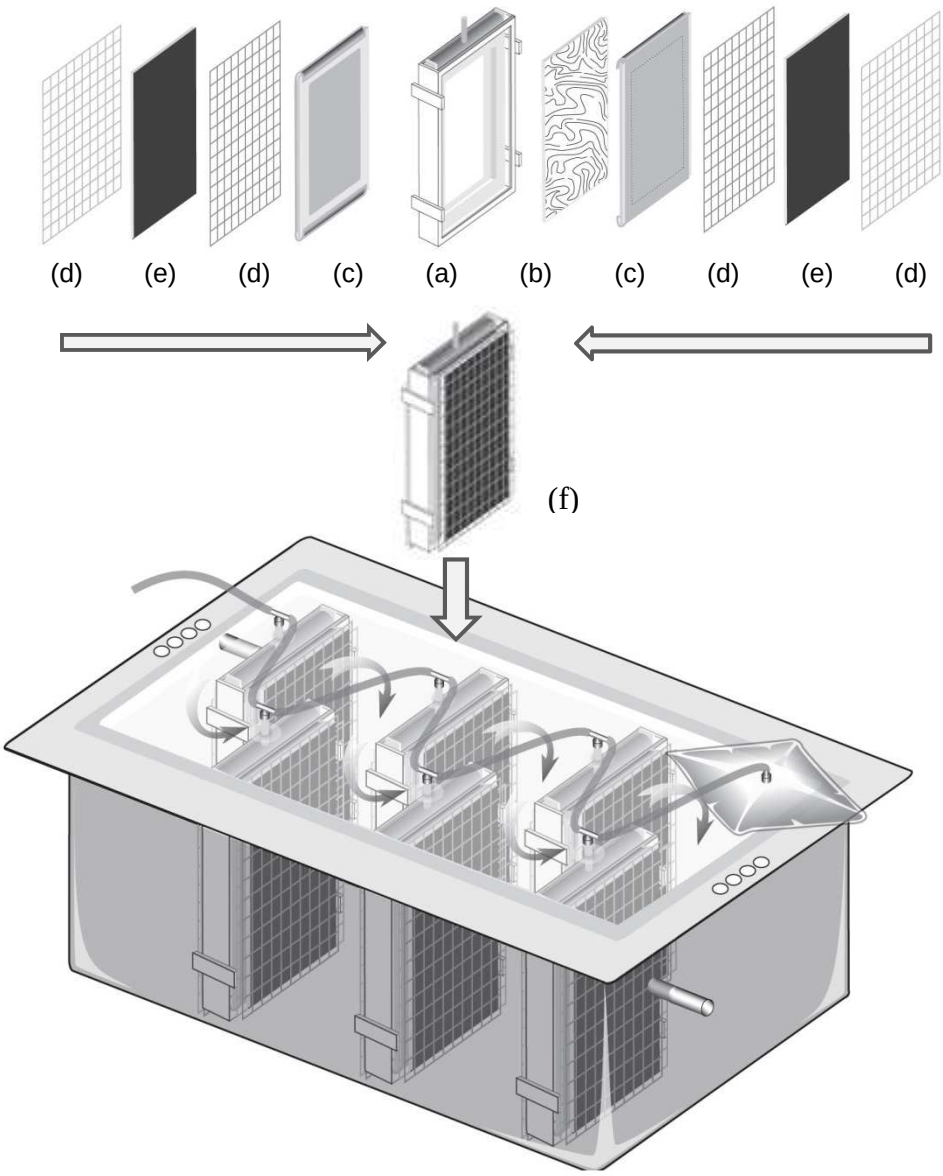

Figure 6-2 Schematic diagram of the reactor module components, a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane fixed around a PVC frame, d) stainless steel wire mesh, e) anode with wire mesh current collector. These component fit together to form a single module (f), six of these go into the reactor vessel where wastewater flows around them. Gas is collected through tubing into a gas bag

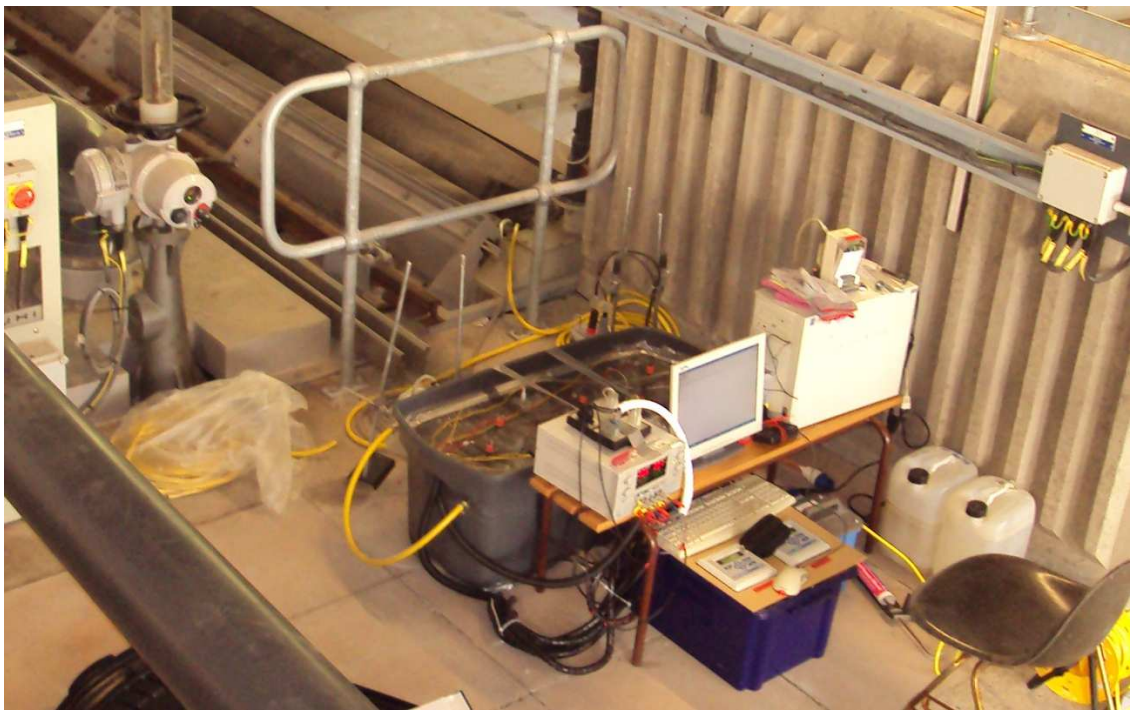

Figure 6-3 Photograph of the reactor in situ at Howden wastewater treatment site the grit lane where the influent was drawn from is seen in the top left hand corner of the picture

The reactor was situated on site in a large unheated building housing the grit channels, wastewater was pumped from the grit channels into a preliminary storage tank, providing some primary settling. During operation a peristaltic pump (Watson Marlow 520S, UK) was used to pump water into the storage tank, where it could then flow into and through the reactor, and back out to the grit channels via a smaller sampling tank at the end. These tanks were used for sampling and monitoring of the influent and effluent.

6.2.3. *Analytical procedures*

Power was provided to the electrodes using a PSM 2/2A power supply (Caltek Industrial Ltd, Hong Kong), the voltage of each cassette was monitored across a 0.1 Ω Multicomp Resistor (Farnell Ltd, UK) using a Pico AC-16 Data Logger (Pico Technology, UK), and recorded on a computer every 30 minutes.

In both the influent settling tank and the effluent tank the dissolved oxygen (DO) and pH were measured using pH and DO submersion probes (Broadley James Corporation, USA) connected to a pH DO transmitter (Model 30, Broadley James Corporation, USA), feeding an electrical output to a Pico EL 037 Converter and Pico EL 005 Enviromon Data Logger (Pico Technology, UK); these data were recorded onto the

computer every 30 minutes. Temperature was logged using 3 EL-USB-TC Thermocouple data logger (Lascar Electronics, UK) placed in the settling and effluent tanks and one placed in the reactor itself.

The gas pipelines were connected to optical gas bubble counters (made 'in-house' at Newcastle University), giving a measurement of gas volume. The operation of these counters failed after several weeks of operation. They were replaced with 1 L and then 5 L Tedlar gas bags (Sigma Aldrich, U.K.); the volume of gas was then measured by removal from the bags initially using a 100ml borosilicate gas tight syringe, and then using a larger 1 L glass tight syringe (both SGE Analytical Science, Australia). The sampling ports on each pipeline were initially used to take a sample of cathode gas 3 times a week, into a Labco Evacuated Exetainer (Labco Ltd, UK). Once gas production had risen to a higher volume, 2 L of the cathode gas was dispensed from the collecting gas bag into another 5L gas bag which was taken away for analysis. Anode gas was not measured volumetrically due to leakage but was sampled directly from the anode compartment into a 3 ml exetainers for compositional analysis.

Hydrogen gas was measured using a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using duplicate injections, set against a three point calibration. These gas measurements were verified using a Trace Ultra gas chromatograph (GC) with a thermal conduction detector (TCD) and a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements for anode and cathode gas were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

To ensure accuracy calibration standards used for the gas measurements were injected into a Labco evacuated exetainers in the laboratory at the same time (+/- 10 minutes) as the samples taken in the field. Tests carried out previously had indicated that these containers were not completely gas tight especially for hydrogen. This procedure did not have to be carried out for the cathode gas once operation had been switched to gas bags.

Liquid samples of the influent and effluent were taken 3 times a week. The total chemical oxygen demand (COD), and soluble chemical oxygen demand (SCOD) were measured in duplicate using standard methods (APHA, 1998) (Spectroquant ® test kits, Merck & Co. Inc., USA). Volatile Fatty Acids (VFA's) were determined using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The conductivity of the solution was measured using a conductivity meter, EC 300 (VWR Ltd, UK).

6.2.4. *Start up and operation*

The reactor was initially started up in batch mode, allowing all the oxygen, nitrates and sulphates within the wastewater to be consumed. Based on the lessons learnt from the previous pilot study, (Cusick et al., 2011), (Logan, B.E. personal communication),the wastewater was supplemented with acetate at a concentration of 0.5g/L. The applied voltage of 0.6 V was provided by a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong). The dosing was repeated and the reactor refilled after a 2 week period, during which time no gas production was observed.

6.2.5. *Efficiency calculations*

Four efficiency calculations are made in this study on the basis of the electrical and substrate energy used (Logan, 2008).

(i) Electrical energy recovery (η_E)- Energy recovery is the amount of electrical energy put into the reactor that is recovered as hydrogen.

The electrical energy input W_E is calculated as:

$$W_E = \sum_{1}^{n} (I\ E_{ps} \Delta t - I^2 R_{ex} \Delta t)$$

Where I is the current calculated for the circuit based on the measured voltage E and external resistor R_{ex} ($I=E/R_{ex}$), E_{ps} is the applied voltage of the power supply, this value is adjusted for the losses caused by the external resistor (I^2R_{ex}), which in reality are negligible. The time increment denoted by Δt represents the conversion of samples taken every 30 minutes into seconds. The data is summed for all 6 cells over the each batch cycle. The output of energy (W_{out}) is calculated from the measured moles of

hydrogen produced $N_{H2,}$ and the standard higher heating value of hydrogen of 285.83 kJ/mol ΔH_{H2}.

$$W_{out} = \Delta H_{H2}\, N_{H2}$$

The higher heating value is chosen over the lower heating value which takes into account the heat lost through the production of water vapour during burning. It is expected that this H_2 product would be used either as a commercial product for industry, or in a clean H_2 consuming fuel cell to create electricity, not for combustion. Methane could also be added to this value to further increase the quantity of output energy, but was not included for these same reasons.

Total Energy recovery (excluding pump requirements) can then be calculated as follows:

$$\eta_E = \frac{W_{out}}{W_E}$$

(ii) Total energy efficiency (η_{E+S}) the amount of input energy both electrical and substrate that is recovered as hydrogen.

The substrate energy (*Ws*) is calculate as

$$W_s = \Delta COD\, \Delta H_{ww/COD}$$

Where ΔCOD is the change in COD in grams, estimated as the difference in COD of the influent and effluent at the end of each batch. $\Delta H_{ww/COD}$ is the energy content per gCOD as measured on similar domestic wastewater of of 17.8 kJ/gCOD (Heidrich et al., 2011). Total energy efficiency is then calculated as:

$$\eta_{E+S} = \frac{W_{out}}{W_E + W_S}$$

(iii) Coulombic efficiency (CE) - the amount of hydrogen produced compared to the amount theoretically possible based on the current, or total charge passing through the cell.

Theoretical hydrogen production based on current (N_{CE}) is calculated as:

$$N_{CE} = \frac{\sum_1^n I\Delta t}{2F}$$

Where *I* is the current calculated from the measure voltage, Δt is the conversion of the time interval 30 minutes to 1 second to give coulombs per data sample, this is then summed over the 6 cells for the whole batch. Faradays constant (*F*) is 96485 coulombs/mol e^-, and is the moles of electrons per mole of hydrogen. Coulombic efficiency *CE* is then calculated as:

$$CE = \frac{N_{CE}}{N_{H2}}$$

(iv) Substrate efficiency - the amount of hydrogen produced compared to the amount theoretically possible based on substrate removed in the reactor.

Theoretical hydrogen production based on substrate removal *(N_S)* is calculated as:

$$N_S = 0.0625\, \Delta COD \Delta t$$

As 64 gCOD can be converted to 4 moles H_2, each g COD is equivalent to 0.0625 moles H_2. The change in COD is measured at the end of each batch, and used to calculate the total COD removed from the 88 L reactor over the duration of the sampling period based on a HRT of 1 day. Substrate efficiency is then calculated as:

$$S_E = \frac{N_S}{N_{H2}}$$

The (η_E) correlates directly to the coulombic efficiency (CE) by re-arrangement of their respective equations. It is assumed that the phrase $I^2 R_{ex} \Delta t$ in calculating W_E is negligible by comparison to the first term (this is observed to be the case in practice):

$$\eta_E = \frac{\Delta H_{H2} \times 1000}{2F \times E_{ps}}\, CE$$

This means halving the E_{ps} doubles the η_E if the CE can be maintained. An increase in CE at the same E_{ps} causes a linear increase in η_E.

6.2.6. *Statistical analysis*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

6.3. Results

6.3.1. *Reactor design and resistance limitations*

The internal resistance of a BES design is critical to its performance. Resistance is mainly caused by electrode overpotential and ohmic losses in the liquid, although there may also be losses in the bacterial transfer etc. as shown in Figure 1.2. These losses impact on the amount of energy that can be gained in and MFC and the amount for energy needed in an MEC, these effects are even greater in a scaled up system where losses become proportionally more significant (Rozendal et al., 2008a). Within the cell

designed the anode and cathode, although separated by a membrane, were relatively close together, with around 1cm distance between them, this will have minimised the ohmic losses within the liquid phase (i.e. the resistance in the movement of ions from the anode to cathode) which is especially important when using real wastewaters with no artificial increase in liquid conductivity.

However the electrode resistance with this design is high, with the cathode having a resistance of 2.8Ω and each anode sheet being 3.4Ω from the extremities of the electrode to the end of the connecting wire. With a total anode surface area for the whole reactor of 0.76 m^2 and a further 0.3 m^2 of cathode, these resistances will have a large impact in reducing the efficiency of the reactor performance. With a 0.6V load, as would be desirable based on laboratory studies (Call and Logan, 2008) this anode resistance would result in an approximate maximum current of 0.2A, increasing the load to 0.9 as needed with other wastewater studies (Kiely et al., 2011a, Cusick et al., 2011) would produce a maximum of 0.3A, and the 1.1V load used would result in around 0.4 A maximum current, assuming no other losses. This would give anode current densities of 0.3, 0.4 and 0.5 A/m^2 respectively, well below the target for BES of 10 A/m^2 which would enable similar treatment rates to activated sludge (Rozendal et al., 2008a), although current densities within MECs do tend to be lower than those of MFCs (Kiely et al., 2011a).

In reality there was greater resistance within the reactor than the electrode overpotentials alone. The current densities measured were 0.04, 0.1 and 0.3 A/m^2 at 0.7, 0.9 and 1.1V load added respectively. This means that the current density only increases by around 0.6 A/m^2/volt, far lower than two early MEC laboratory studies (1.3 A/m2/volt in (Liu et al., 2005b) and 1.78 A/m^2/volt in (Rozendal et al., 2006)). Additionally this shows that there is an inherent overpotential in the system also of over 0.6 volts as seen in Figure 6.4, over this voltage needs to be added to generate any current.

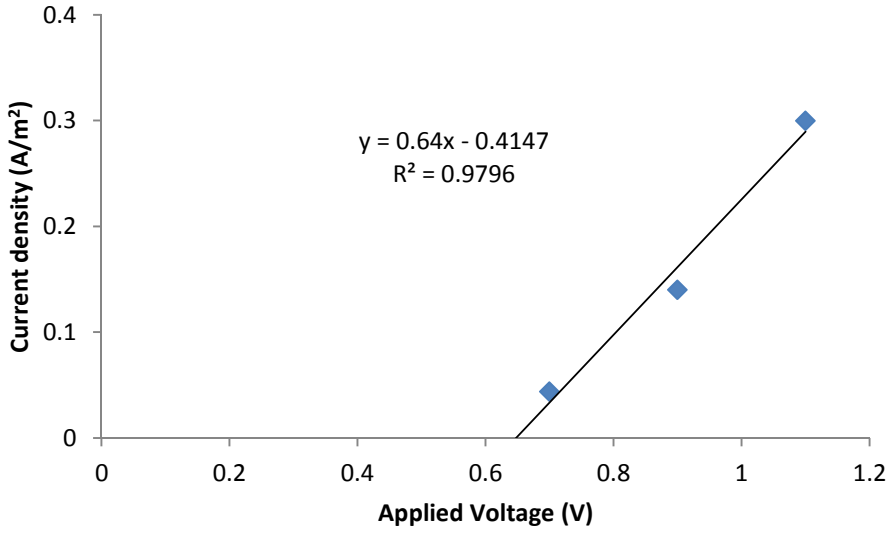

Figure 6-4 Current density as a function of applied voltage as measured in the pilot scale reactor after the initial two week acclimatisation period, showing the linear regression equation and R2 value. The intersect of the x-axis indicates the overpotential of the system

6.3.2. *Start-up and acclimatisation*

During the first 30 days of operation the reactor was run in batch mode with a supplement of 0.5 g/L of sodium acetate and an input voltage of 0.6 V. During this time there was no observed gas production and the current density was very low reaching 0.04 A/m^2 after the first two weeks. After this period wastewater was pumped through the reactor with a HRT of one day with no further addition of acetate. For the subsequent 10 days very little gas was produced and the current density remained at this very low level. At day 40 the input voltage was raised from 0.6 V to 0.9 V. The reactor was run with this input of voltage for the next 24 days; the average power density during this time reached 0.1 A/m^2. Gas production was low with an average of 9 mL/day, however once the gas lines had been flushed the purity of this gas (H_2) began to reach 100%. The electrical energy efficiency η_E was only 1 %. The voltage was then further increased to 1.1 V, and power densities rose and stabilised at 0.3 A/m^2. This led to a dramatic improvement in gas production, and the reactor entered its "working phase", the results of which are shown below. The start-up period took 64 days.

6.3.3. *Working performance of MEC reactor*

After the long start-up, and subsequent increase in the voltage to 1.1 volts, the MEC worked for the following 85 days, and continues to do so. The results presented here are for this period.

The volume of gas produced per day was highly variable. However the gas composition was consistent, hydrogen 100% ± 6.4, methane 1.8% ± 0.9. No trace of CO_2, N_2 or O_2 could be detected using the GC's or MIMS. H_2S could not be measured accurately however the MIMS did not detect any gas at this atomic weight and there was no detectable odour present. The daily H_2 production is shown in Figure 6-5. Production gradually increased during the first 30 days; after this the average production was around 1.2 L per day for the reactor, equivalent to 0.015 L-H_2/L/day.

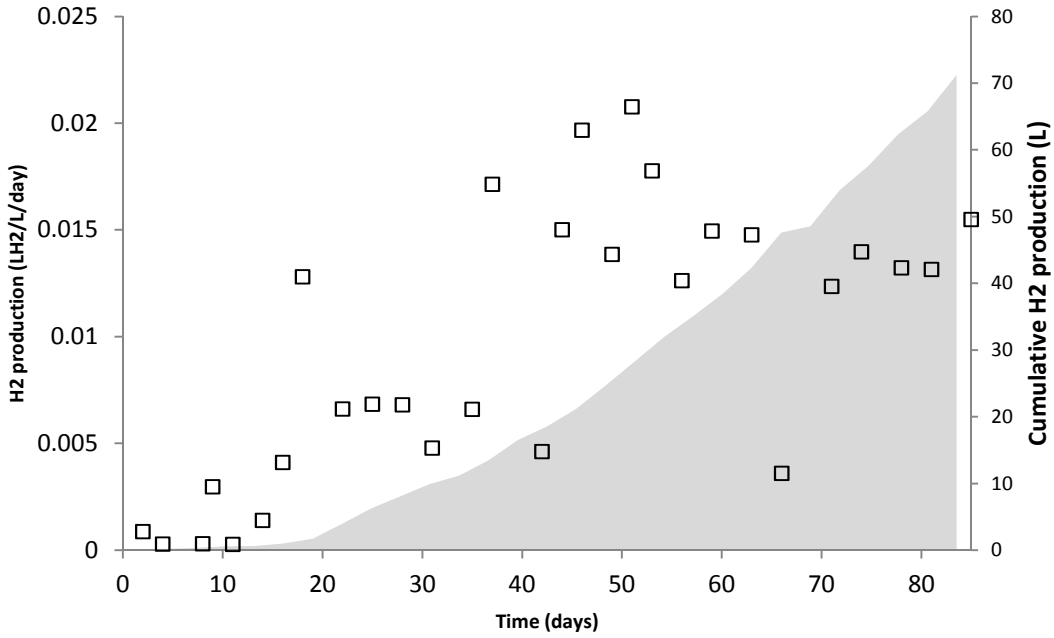

Figure 6-5 Hydrogen production during the working phase of reactor after the 64 day acclimatisation period, points showing the production rate at each time of sampling, and the area showing the cumulative production of the course of this period

The electrical energy recovery of the cell was quite variable as seen in Figure 6-6 (a), but did show an increasing trend and on occasion approached 100% (complete energy recovery) . The total energy efficiency (b) which gives the true performance of the cell was also variable, and considerably lower as both the electrical and substrate energy are considered as inputs. The energy efficiency shows an increasing trend reaching the 30 % level at the end of the study. The peak values are associated with very low COD removal measurements (making substrate energy input very low), and are not therefore likely to be representative of the true performance of the reactor. Coulombic efficiency (Fig. 5c) shows a similar trend to energy recovery (Fig. 5a), stabilising at around 55-60 % in the last 30 days.

The coulombic efficiency (CE) correlates with energy recovery (η_E) (R^2 = 0.998, Pearsons correlation). This correlation factor is calculated as N_E = 1.29 CE using the average input power voltage, this value is also seen in the data and is consistent over the course of the study. If the CE could remain at the 60% and the power input dropped to 0.9 volts 100% η_E would be achieved. Alternatively with this power input CE needs to reach 75% to achieve 100% η_E. The substrate efficiency (d), due to the highly variable influent and effluent COD values (as shown in Figure 6-7 can exceed 100%, and was often very low and even negative. The average substrate efficiency for whole the operational period is 10%.

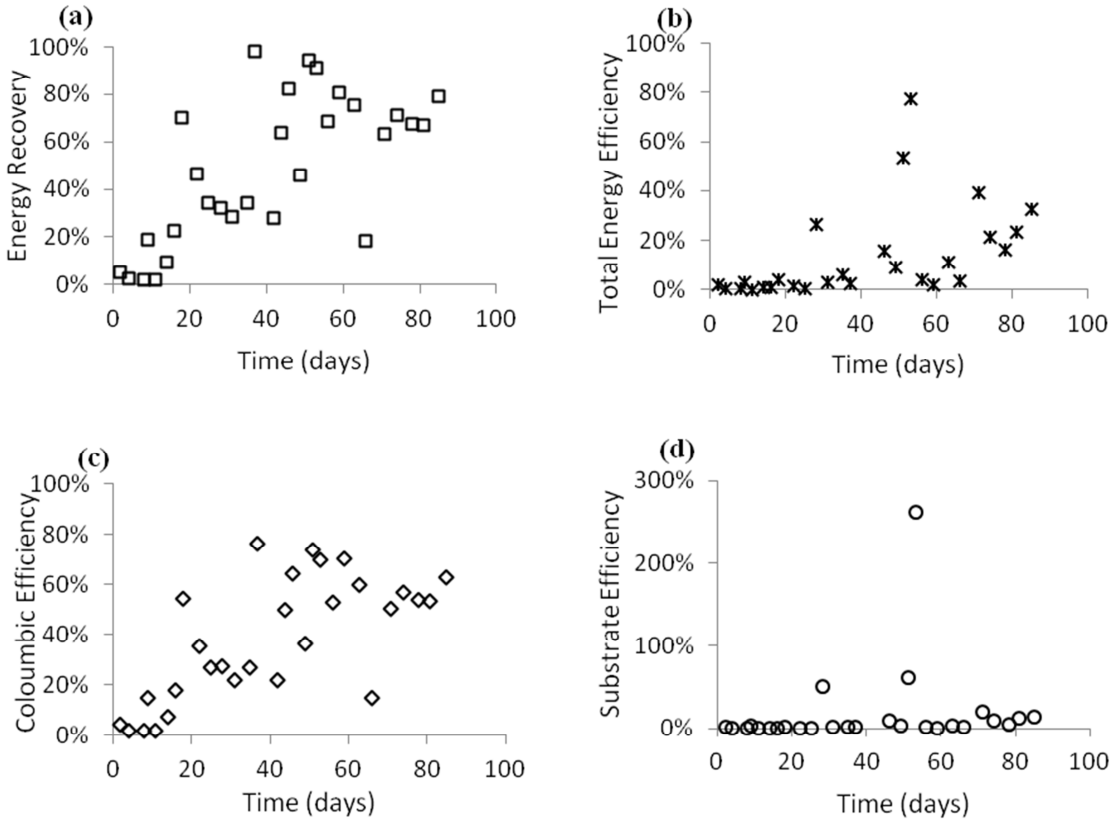

Figure 6-6 MEC reactor efficiencies over the 85 day working period a) electrical energy recovery b) total energy efficiency c) coulombic efficiency d) substrate efficiency

The levels of influent COD was highly variable which is likely to be one of the factors underlying the variation in performance. This factor was particularly the case at day 30 when the settling tank became full with sludge and influent COD was extremely high. This variability led to occasional negative values for % COD removal. The average removal of 33.7%, equates to 0.14 kgCOD/m^3/day, just below the range for activated sludge of 0.2-2 kgCOD/m^3/day (Grady, 1999). The COD effluent levels occasionally approached and dropped below the UK standard of 125 mg/l (EEC, 1991).

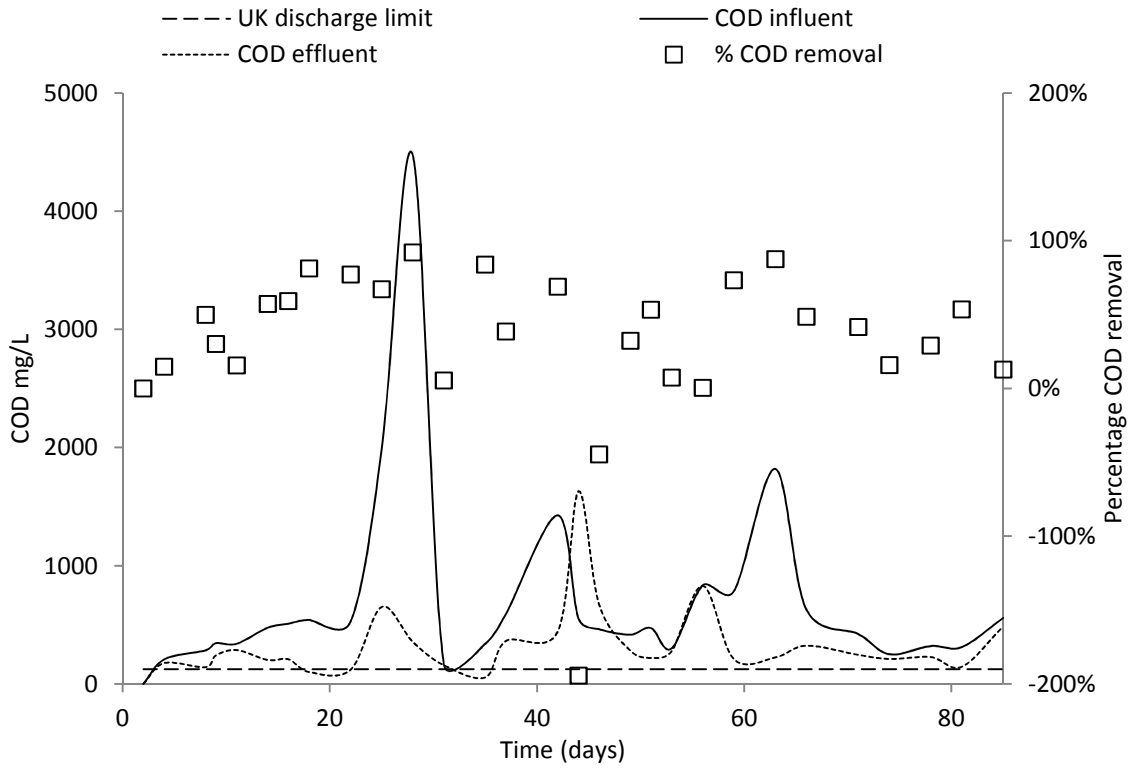

Figure 6-7 COD influent and effluent shown by the lines along with the UK discharge standard of 125 mg/l, percentage COD removal is also shown using the squares

Despite the variable influent COD and therefore variable performance, many of the other measured factors remained relatively constant throughout the operational period. The headspace of the anode compartment (2.2 L volume) contained elevated levels of CO_2 (1.9%) and low levels of CH_4 (0.4%), equivalent to 8.8 ml of CH_4, or 0.006 mg COD and 0.3 kJ. The gas production at the anode could not be measured quantitatively due to leakage. The daily production of methane at the cathode was 22 mL/day, equivalent to 0.014 mg COD, and 0.8 kJ of energy, approximately 5-6% of the amount of energy recovered as hydrogen.

The pH of the influent and effluent were continuously monitored, the influent was on average pH 7, the effluent pH 6.7, never dropping below pH 6. The DO of the influent was on average 4.2 mg/L and the effluent was 0 mg/L. The amount of VFA's dropped between the influent and the effluent, but there was frequently some acetic acid left in the effluent up to 45 mg/L, i.e. the available food source was not used up. This was confirmed by the average SCOD of the effluent of 115 mg/L. There was an average removal of 1.8 g/day of sulphate in the reactor, but never full depletion with the effluent containing 89.6 mg/L on average. The reactor removed an average of 0.2 g/day of chloride, although this value was highly variable. Fluoride and phosphate remained

relatively constant between the influent and effluent, nitrates were not present in either. There was no measured drop in conductivity between the influent and effluent.

The temperature of the influent wastewater varied considerably throughout the working period between June and September. The range of temperature was more stable within the reactor, and was on average 0.9 ^{o}C higher than the temperature of the influent. With a 88 L capacity and HRT of 1 day, this means 0.37 kJ/day of energy was lost to heat, equivalent to 20 mg COD, or 31 ml H_2. Temperature did not significantly influence energy recovery (p=0.678 influent, p=0.664 reactor, p=0.778 effluent, Pearson Correlation). Most of the fluctuation observed was diurnal and periods of the more extreme temperatures were short lived.

Table 6-1 Maximum, minimum and average temperature (^{o}C) of the influent, effluent and reactor ± 1 standard deviation which were continually logged over the experimental period

	Influent	Reactor	Effluent
Maximum	27.0 ± 2.3	21.0 ± 1.2	22.5 ± 1.6
Minimum	8.5 ± 2.3	13.5 ± 1.2	12.0 ± 1.6
Average	15.8 ± 2.3	16.6 ± 1.2	16.6 ± 1.6

The total material costs of the reactor, not including pumps, power supply and computing/recording instruments, was equivalent to £2344/m^3, of which the cathode and membrane combined represented less than 2%.

6.4. Discussion

This pilot scale reactor worked, producing almost pure hydrogen gas from raw influent domestic wastewater at U.K. ambient temperatures for a 3 month period and continues to do so. It is believed to be the first successful study of its kind, which brings the prospect of sustainable wastewater treatment and hydrogen production through the use of bioelectrochemical systems onto a new and exciting phase.

The reactor has removed on average 34% of COD, and occasionally reaching the UK discharge standard of 125 mgCOD/L, equating to a treatment rate of 0.14 kgCOD/m^3/day, just below the range for activated sludge. The reactor has performed this task using less energy than would be needed for aeration in a traditional activated sludge process. The electrical energy recovery on occasion nearly reached values of 100%, and was consistently around 70% during the later stages of the study. At this

level of performance (i.e. 70%) the energetic treatment costs were 2.3 kJ/gCOD, below the values for activated sludge of 2.5-7.2 kJ/gCOD (Pant et al., 2011). By implementing improvements to the reactor such as: increasing electrode surface areas; reducing the distance between electrodes; having a more efficient flow paths; consistent pumping; and improved materials, the η_E could be greater than 100%, making it a net energy producer. On the basis of this fairly large proof of concept study, energy neutral or even energy positive wastewater treatment is clearly a realistic goal.

The total energy recovery showed an increasing trend during the course of the study, levelling out at around 30%, with around a third of all energy both from the wastewater and from the power supply being recovered as hydrogen gas. Coulombic efficiencies of the reactor were high, levelling out at around 55-60 %, methane production accounts for an additional 3.5%. Other losses might be caused by some short circuiting in the reactor. It is likely therefore that a large proportion of the missing 40% of CE can be attributed to a loss of hydrogen gas from the system. Hydrogen is an extremely small molecule and is able to permeate most plastics, and is therefore likely to be leaking out of the reactor. In a tightly engineered system theoretically the coulombic efficiency could approach its maximum of 100%, resulting in an electrical energy recovery of 129%.

The substrate efficiency of the cell was considerably lower than the other efficiencies measured. This efficiency represents how much of the substrate is actually recovered as hydrogen, and gives an indication of how much substrate is used in the MEC process. Even if the 40% loss of hydrogen through leakage (as suggested by the CE of 60%) is accounted for in this calculation then the substrate efficiency would only increase from 10% to around 23%. Losses may be taken to suggest that substrate is being used in competitive oxidation processes, but only low levels of oxygen entered the cell with the influent. Sulphate reduction equated to about 3.6% of the total COD removal. Limited nitrates were available. Further losses can be accounted for by the probable build-up of sludge within the reactor as evidenced by the constant COD removal value throughout the study despite the increasing efficiency of the reactor, and that on three occasions a very high COD peak entered the reactor, on two of these occasions the peak of COD is not seen to leave the reactor see Figure 6-7.

Clearly the high resistance of the reactor means the overall efficiencies of the reactor will be low. The resistance observed is more problematic in this larger scale system than at the laboratory scale, and would also become increasingly challenging with further scale up. Improved reactor design is needed to overcome these problems. In a large scale system a considerable wire length is likely to be inevitable, resistance could be reduced through the use of a thicker wire, additionally resistance could be reduced in the electrode by improving the connection between the electrode, current collectors and wire. Further research into different materials and different configurations of materials would hopefully lead to improvements at a larger scale.

Further efficiency losses as identified above could be minimised by improving the engineering of the system. The two 'new' materials used in this study for the membrane and cathode have not been truly evaluated. More expensive alternatives such as Nafion membrane and a Pt coated cathode may prove to be worthwhile investments if performance increases greatly with their use. The biological MEC process works, and works relatively consistently for a period of at least three months. Although tested in realistic conditions, this was over a spring/summer period, survival over periods of sustained low temperature has yet to be confirmed.

The relationship between electrical energy recovery, electrical power input and coulombic efficiency has been defined however the prediction energy requirements for a larger scale MEC system may be difficult to make. Theoretical input voltages lie far from those needed in reality even for acetate fed cells, typically between 0.4-1.0 V compared to the 0.114 V theoretically needed (pH 7, 298 K) (Logan, 2008). A relatively small change in the electrical power input can have a large effect of the overall electrical energy recovery, yet if this value is not high enough to overcome the losses in the cell no hydrogen will be produced.

Undoubtedly there are many factors that require further investigation. Many of the inefficiencies could be overcome by improved engineering, but also a greater understanding of the biological processes (both working with and against the cell performance), community structure and ecology would allow for more confident design and manipulation.

The aim of this research was to determine if MEC technology could be a viable and alternative to the activated sludge process. The pilot scale reactor has worked producing hydrogen, with real wastewaters at ambient temperatures for over 3 months at a volumetric treatment rate just below that for activated sludge. A breakeven energy was not consistently achieved during the course of the study, yet is believed to be within reach with improved hydrogen capture and improved design to increase efficiencies. With this proof of concept now made we are a large step closer to using MEC technology for sustainable wastewater treatment.

Chapter 7. Conclusions

The overall aim of this research is to reach an understanding of whether microbial electrolysis cells could be a domestic wastewater treatment option.

I conclude that energy neutral or energy positive wastewater treatment should be possible. This research started by looking into how much energy is held intrinsically within the wastewater, and concluded that the amount of energy in the wastewater is substantial, more than previously thought, and more that the energy costs currently incurred in its treatment (18-29 kJ/gCOD vs. 2.5-7.2 kJ/gCOD in activated sludge treatment). Although this energy measured is internal chemical energy which is higher than the Gibbs Free Energy that would be available to microorganisms, with a biological system engineered for energy extraction from wastewater rather than an energy input, i.e. utilising other redox pathways rather than simple aerobic oxidation.

With the conclusion made that there is enough energy inherently contained in wastewater to treat it, the next question was to determine if Microbial Electrolysis Cells could meet this demand, replacing the high energy demanding activated sludge process with an energy yielding process. Parts of the thesis, in particular the low temperature work, suggested this might be possible yet other parts of the research did not such as the failure in MEC wastewater fed reactors. However by building and testing a pilot scale reactor on site at a wastewater treatment the most positive and conclusive evidence that this technology could work for real wastewater applications was gained. The reactor, even though it was a ‘first design’ using low cost alternatives to the optimum materials, and with many other problems such as non-optimised flow and hydrogen leakage and high resistance, it came reasonably close to its breakeven energy point. Even without breaking even it was more effective in terms of energy used per gCOD removed, and came close to the volumetric loading rates of the activated sludge process.

There is still much work to be done at this scale and larger to: understand the issues of scaling; economic feasibility; hydrogen capture and storage; design and materials; and optimisation. This work could then lead to retrofitting old activated sludge lanes with microbial electrolysis cells, radically changing the wastewater industry.

All the research conducted in this PhD has shown that the substrate acetate is not an adequate model of wastewater. This has been shown simply in terms of the energy available per gCOD, the acclimatisation and number of exoelectrogens able to digest these substrates, the diversity of the community fed with these substrates and their function within microbial electrolysis cells. The higher diversity estimates and complex acclimatisation pattern of acetate fed reactors suggest acetate may not be the optimum compound to use in BES's. Wastewater fed systems may have less free energy available, and therefore result in a more efficient biomass being formed. The lower coulombic efficiencies observed in wastewater fed reactors might be an inevitable result of electrons being lost within the longer chains of digestion, and not necessarily an indication of inefficient biomass.

The conclusion that temperature does not affect the performance of MFCs is surprising, although does correspond to some of the literature in this area (Catal et al., 2011, Jadhav and Ghangrekar, 2009). This suggests that there is a similar level of free energy available in systems run at different temperatures, and that low temperatures do not represent a disadvantage for BES. This is also observed in the pilot reactor, here low temperatures may be an advantage reducing methanogenic activity which proved fatal in the only other pilot scale MEC study to be published (run at 30 ^{o}C) (Cusick et al., 2011).

A further surprising conclusion was that inoculum did not have an effect on reactor performance, although the inoculum did interact with substrate to produce higher diversities within acetate fed reactors inoculated with high diversity soil. Exoelectrogenic bacteria were present naturally in all the wastewater inocula, and the Arctic soil inocula used throughout this research, albeit at low levels. The number or proportion of exoelectrogens was estimated to be 0.0017% using the very old methodology of MPNs, using the most recent next generation sequencing techniques and mathematical modelling algorithms, the estimates were 0.0012% and 0.00001% for two different wastewater samples. This therefore appears to be a reasonable good estimate of the rarity of such species.

BES reactors have been shown to work in challenging, real life, environments, and many observations have been made about the abundance and diversity of the organisms needed for the operation of these systems. This research has moved a substantial step

forward in proving that these technologies could be an energy efficient replacement of the activated sludge process. However we are still a long way from a deep and holistic understanding of the bacterial world operating within these systems, the energy requirements of these communities, their metabolic limits, their response to stress and ultimately their stability and function. Without this deep understanding we are reliant upon empirical data gathering, testing reactors in various environments until these limits are found. If we could model the free energy needs of the bacterial community, estimate the free energy available in the substrate, and calculate the efficiencies of the electrochemical cell, such systems could be modelled accurately and ultimately engineered to produce positive energy recovery.

Chapter 8. Perspectives on the use of MECs in the treatment of wastewater

This work has demonstrated a proof of concept of the use of MECs with domestic wastewater to produce hydrogen at the 100L scale over a 3 month time period. However this does not mean that they will be a viable wastewater treatment option. The work conducted in this research goes some way to confirming to technical feasibility of this technology in the treatment of domestic wastewaters, it does not however prove or suggest that this will be an economic viability, such an assertion is beyond the scope of this study.

There are many considerations which would need to be focused on in order to determine this economic viability for any technology to replace activated sludge treatment (AS), including those criteria stated in the introduction:

1. Extract and convert energy to a useable form at an efficiency that justifies the costs.
2. Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
3. Treat low strength domestic wastewater.
4. Work at ambient, often low temperatures.
5. Work continuously and reliably.

The detailed costing of this technology is beyond the scope of this thesis. It has been suggested that MEC technology may be an economically viable alternative to AS over other treatments such as anaerobic digestion (AD) or MFCs (Foley et al., 2010, Curtis, 2010) based on the reduction in aeration costs and the potential value of products produced. However to change the UK wastewater infrastructure would require exchanging the current AS process components for a system with higher capital costs (estimated at 0.4 €/kgCOD for an MEC compared to 0.1 €/kgCOD for AS, (Rozendal et al., 2008a)) aiming to recover the costs through the product generated. It is clear that even with low cost materials used in this research, and the idea of retrofitting the cells into existing infrastructure (Cha et al., 2010), the capital costs of filling tanks with complex electrode assemblies would be far higher than installing the aeration pipework. It would need to be ascertained whether the ‘payback’ in terms of reduction of the

energy costs and the products generated would equal the higher capital costs over the lifetime of the cells (which is again unknown at this stage).

The design life of typical wastewater treatment infrastructure is at least 25 years. MECs have not been tested over such time periods in even in the relatively clean conditions of laboratories. It is highly likely the many of the components of a typical MEC would not survive for long periods when handling real wastes, membranes for example are particularly problematical clogging over time (Zhang et al., 2011), yet membraneless are also problematic at large scale (Cusick et al., 2011). Even the estimates for a 5 year life span of electrodes and membranes used in the estimates above (Rozendal et al., 2008a) are untested under real conditions and may be unrealistic. The life span and maintenance requirements of BES will be a critical factor in determining if this technology can be used economically within the wastewater industry.

A further cost consideration is the labour costs associated with this new technology. The level of maintenance required in the MEC process is again unknown, but is likely to be higher than the AS, though may be compensated for by the reduction in sludge treatment which is a considerable fraction of the operational costs (Verstraete and Vlaeminck, 2011). The hydrogen or product produced may also require purification again the costs of this would need to be accounted for in identifying if the economic benefits of the product outweigh the costs.

The full economic costing of the MEC process versus other processes is complex, with many unknowns. It is likely to vary with: the scale and wastewater type of different treatment plants; water usage and availability; energy and material prices; and therefore inherently through time (McCarty et al., 2011). The ‘upgrading’ of AS plants with improved energy recovery from sludge AD, improved process control and greater levels of primary settling such as the Strass plant in Austria which generates 108% of its electricity use (Nowak et al., 2011) may prove to be more economically viable. The addition of AD onto the AS process is the route many UK water companies are taking including Northumbria Water Ltd who have one large sludge AD plant in operation and one under construction. However such a high degree energy recovery is exceptional, and many experts in the field question the concept of using the energy intensive process of AS to insolubalise waste organics to sludge which then can undergo energy recovery (Verstraete and Vlaeminck, 2011).

The treatment levels of the pilot MEC run were both low and variable, averaging only at 34%, the AS process can remove up to 95% of the COD (Tchobanoglous, 1991) although this is rarely the case as they are usually part of a treatment flow with pre-settling and post clarification removing a proportion of the COD (Grady, 1999). The MEC reactor demonstrated did on occasions remove the COD down to the discharge limit of 125 mgCOD/L (EEC, 1991) so operation at this level is possible. The ability to use domestic wastewaters is a clear advantage over AD which tends to be restricted to high strength industrial or farm wastes, or sludge generated by AD. Further work would be needed to demonstrate that this treatment could consistently reach discharge standards, and the electrical conductivity of the wastewater at these low strengths is sufficient for the cells to function.

Even if part of a treatment flow with pre-settling and post clarification it is likely that the MEC would need to improve treatment rates to encourage investment, additionally the more organics removed the higher the energy yield can be. Treatment rates could be improved by reducing electrode spacing; however this would have the knock on effect of reducing the volumetric loading rate. The MEC could therefore end up requiring the same unit space as trickling filters, and therefore not be a viable option either due to land restrictions or poor economic comparability to this low energy treatment option.
There is an increasing body of research demonstrating that BES technologies will work at ambient temperatures (Jadhav and Ghangrekar, 2009, Catal et al., 2011, Larrosa-Guerrero et al., 2010), added to by the work in this thesis. Further work may be required in demonstrating this with real wastewaters at a larger scale, and also in quantifying and overcoming the kinetic effect of the lower temperatures on bacterial metabolism.

Many challenges lie ahead with BES research both from a technological and economic perspective. Only through completing and importantly combining these research areas will we be able to reach an understanding as to whether the technology can be used in the wastewater treatment plants of the future.

Chapter 9. Recommendations for future research

This research set out to answer the question as to whether microbial electrolysis cells could be used for wastewater treatment. Most of this research has strengthened the case that they are, however many more research and application questions remain unanswered. Each piece of research described in this thesis could be developed further to give more conclusive answers:

Chapter 2: A comprehensive survey into the amount of energy contained within wastewater is warranted. In the research conducted two samples were tested from different wastewater treatment plants and the results showed a large difference in the energy content between the samples and with that which would be predicted. Discovering the energy in wastewater is fundamental to the study of bioelectrochemical systems, and other technologies which aim to yield energy from wastewater. If we are to evaluate the true potential of these technologies we need to know how much energy is actually encapsulated in domestic wastewater, enabling efficiencies to be calculated and therefore better solutions engineered.

Measuring internal energy by calorimetry is a standard method in the solid waste industry (Garg et al., 2007, Lupa et al., 2011), yet when applied to wastewater the problem arises that samples have to be dry, and even with the improved and extremely laborious freeze drying method used in this research 20-30% of the volatiles in wastewater were lost. With an improved and quicker method, such as the use of distillation or reverse osmosis, a comprehensive survey of wastewaters in the UK could be made. This would: facilitate decisions on where best to invest in new technologies; give an indication of which technologies might be more suitable for different wastewaters; inform of the efficiency of processes; and most importantly – make decision makers believe energy extraction from wastewaters is economically viable and worthwhile.

Chapter 3: With a more definitive answer to the number of bacteria present and their growth pattern, accurate assessments of specific activity and growth yields could be made. Accurate estimations of these values are needed for parameterising models of these systems. By redesigning these experiments, and the reactors used to minimise or at least quantify all losses, a mass balance could be made and these values determined.

However the most intriguing question arising from this work was the difference in the pattern of acclimatisation observed in the acetate fed cells and those with complex substrates. Although possible reasons for this difference were suggested, a conclusive answer was not found. By conducting further research scaling between acetate and starch in terms of substrate complexity, the step causing the change in response of acclimatisation could be found, which may give valuable insight into the development and ultimately the function of these communities. The use of other microbiological techniques such as flow cytometry and QPCR may also help in the accurate determination of these values.

Chapter 4: The finding that temperature and inoculum had little effect on reactor performance is significant to the eventual implementation of this technology. The high variability within the warmer reactors would however be worth investigating further, if all the warm reactors were able to work at the maximum level shown by some, temperature would be a significant factor. The reactor configuration used in these experiments may have been limiting factor, thus if repeated with a higher performing reactor design, the temperature effect may be observed.

The counterintuitive observation that acetate fed cells produced a higher diversity was of great interest in this work. Further research is needed to determine if it is energy that controls the diversity, not the complexity of the substrate. This could be examined by scaling through simple compounds with known and increasing free energies (e.g. from the ΔG of the reaction under standard conditions at pH 7: acetate 27.40 kJ/e^- eq, pyruvate 35.09 kJ/ e^- eq and glucose 41.35 kJ/e^- eq) and observing how diversity changes.

Chapter 5: The conclusion that laboratory wastewater fed reactors fail after a short period of time is contradicted by chapter 6 where the pilot MEC worked. Determining the reason for failure at the small scale is a priority for any further lab scale research studies. Other than scale, the two different factors in the lab based experiments compared to the pilot, are that feed is continuous not batch, and that the laboratory reactors are acclimatised as a MFCs. Research into these factors, and a solution to the failure is needed to achieve the working laboratory wastewater fed systems required for investigations into the use of this technology for wastewater treatment.

Chapter 6: The final part of this research gave the most conclusive answer as to whether MECs can work for wastewater treatment and will, when published, put the research of MECs onto a new platform. Much research is still needed into improving efficiencies and critically achieving the breakeven energy recovery, further scaling, different materials and design, and the economic feasibility of implementing this technology at scale. If the use of this technology is validated, research is needed into the strategic implications this will have on the wastewater treatment industry.

Further recommendations: The research described has increased our understanding of how BES can function in wastewater treatment. A more fundamental direction of research would be the use of BES in understanding the energetic laws and rules which underpin biological systems. Such rules would have huge impact on design in both the near and distant future (Curtis et al., 2003). BES offer the unique opportunity, effectively opening a window on the energy involved in biological reaction, as this energy is routed through an external circuit and can therefore be measured allowing energetic interactions to be unravelled.

By designing a biocalorimeter type BES reactor, where all energetic inputs and outputs are measured (with no leakage) this could be tested using simple substrates and monocultures, and simple laws developed. For example if a substrate chemically yields 'x' kilojoules of Gibbs free energy (ΔG), exactly how much of this can be accessed by bacteria at a set pH and temperature, what proportions go to growth and maintenance for the BES to be stable and what the energy transfer efficiency is. By then scaling to more complex substrates and mixed cultures insight could be gained on: the fermentation processes and on how and why some reaction routes may be favored over others; if the overall ΔG of a complex substrate adequate to model outcome or is more complexity required; and if the energy needs are similar amongst trophic layers.

Through manipulating the systems thermodynamic constraints (temperature, pressure, and ionic strength) to give predictable outcomes, the rules identified above could be verified. Knowledge would also be gained on which thresholds of energy can change community behavior, and how easily these can be manipulated, how much the bacteria can compensate for these changes. Additionally by taking the system to the energetic edge the real limits can be defined and compered to theoretical limits. Ultimately an understanding of how energy requirements of a community link to abundance and

diversity could be gained, and allow for these to be manipulated to increase system stability.

By using a BES in this novel way, the thermodynamic laws which underpin the microbial world may be discovered. The rules generated could be used to create a model allowing biotechnologies to be reliably engineered. The feasibility and efficiency of a bioprocess being modeled at the investment stage without relying on estimates from empirical data. This would have huge scope to promote change and development across the scientific and engineering community.

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Chapter 11. Appendices

11.1. Appendix I - History of microbial fuel cell technology

The concept of fuel cells, a device that can convert electrochemical energy into electricity is not new. The first working chemical fuel cell is attributed to Sir William Grove in 1839 (Lewis, 1966). Progress since then has been slow and sporadic. Although it was understood that the direct conversion of chemical energy to electrical energy was more efficient than combustion in a heat engine (where up to 80% of the energy in the fuel is lost through heat in the exhaust, friction, air turbulence and the heating up and movement of engine parts), historically the abundance of fuel meant that the simpler combustion engine took precedence. The main surge of work in fuel cells has been in the last 10-15 years as fossil fuel prices, and the need for cleaner and more efficient energy production has increased (Logan, 2008).

The first biologically catalysed fuel cell was made in 1911 by a Professor of Botany M.C. Potter at Newcastle University. He discovered that an electrical current could be produced using bacteria as the catalyst on the anode, with a glucose and yeast mixture under various conditions of temperature and concentration he produced a maximum of 0.3 to 0.5 volts (Potter, 1911). This work was added to by Barnet Cohen who built a small bacterial battery using a series of half cells. This work drew more attention to the area, however the major drawback of the system was highlighted, only a very low current is able to be produced and it is rapidly discharged. The use of mediators such as potassium ferrycyanide and benzoquinone did enable greater voltage to be produced however the current remained low (Cohen, 1930).

Del Duca et al. (1963) re-visited the idea and set up a working laboratory model built using urea as a fuel. Urea was broken down enzymically by urease to produce ammonia at the anode, which then reacted with an air cathode producing current. A conceptual design was put forward for a 20-Watt portable urea battery, containing 64 individual cells, however the battery life was only 2 weeks.

Karube et al.(1976), described how carbohydrates were broken down to hydrogen using a fixed matrix of fermentative bacteria, the hydrogen reacted in the electrochemical cell. These studies were the first to use a design very similar to those MFCs used today, but with a salt bridge rather than an artificial membrane. It was believed that the bacteria's

role was to break down the carbohydrate to make electrochemically active products, which were entirely responsible for the current generation. It was not seen that the bacteria themselves were creating the electrochemical current, through the donation of electrons, though this was almost certainly the case.

R. M. Allen and then H. P. Bennetto worked on microbial fuel cells throughout the 1980's at Kings College, London. They had the vision that fuels cells could be a solution to the poor sanitation and lack of electricity supply in the then termed 'third world'. A paper which was the culmination of this work was published in 1993, simply titled Microbial Fuel-Cells – Electricity Production from Carbohydrates, was the first to show an understanding of the mechanism at work (Allen and Bennetto, 1993), although electron transfer was still not understood. It was thought that electrons were extracted from the oxidation of carbohydrates; these would then become trapped within the bacteria, but would become available for transfer to the anode through the use of a chemical redox mediator. Chemical mediators such as ferricyanide were expensive, non-sustainable and toxic to the environment.

The breakthrough discovery was made in 1999 that chemical mediators where not needed in the cells (Kim et al., 1999). This critical discovery that MFCs do not require these mediators, and the ever increasing pressures to reduce pollution, has led to an explosion of research in this area.

In 2005 it was discovered that microbes could be used in an electrolysis cell (Rozendal et al., 2006, Liu et al., 2005b). Electrical energy input can be combined with the energy derived from the fuel by bacteria to drive electrolysis reactions making products which would otherwise require much larger inputs of energy, most notably hydrogen. Thus hydrogen can be produced at greater efficiencies than is the limit with fermentation, and in theory at around one tenth of the electrical energy input of water electrolysis.

11.2. Appendix II - Theoretical cell energetics

The basic reaction occurring in an MFC or MEC can be split into two half reactions, the anode reaction which is the catabolic breakdown of the organic substrate to produce electrons, and the cathode reaction which is the donation of these electrons. The quantity of energy released per electron transferred is dependent on the chemical properties of those compounds involved, and is given by the Gibbs free energy of the reaction or ΔG_r:

$$\Delta G_r = \Delta G_r^0 + RT \ln Q$$

Equation 1

Where ΔG_r is the Gibbs free energy of the reaction, ΔG_r^0 is the Gibbs free energy for the reaction under standard conditions (temperature of 298 K and chemical concentrations of 1M for liquids and 1 bar for gases) as tabulated (Atkins, 2006), R is the gas constant 8.31 J/mol-K, T is temperature, and Q is the reaction quotient i.e. the ratio of the activities of the products and the reactants.

The cell potential (E_{emf}) can be calculated from Gibbs free energy of each half reaction:

$$E_{emf}^0 = -\Delta G_r^0 / nF$$

Equation 2

Where n is the number of moles of electrons transferred and F is Faradays constant 96485 J/mol e^-.

Alternatively the potential can be calculated directly when the potential under standard conditions is known:

$$E_{emf} = {E_{emf}}^0 - \frac{RT}{nF} \ln Q$$

Equation 3

Using acetate as an example electron donor, the half-cell, and full reaction values are given for ΔG_r and E_{emf} in Table 11-1 under standard environmental conditions pH 7, 298 K:

Table 11-1 Calculated theoretical energies (as Gibbs free energy and Potential) of half-cell reactions occurring within BES fed with acetate

	Reaction	ΔG_r/ kJ/ e- eq	Potential E (V)
Anode/ donor	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$	27.40	-0.300 (-0.284)
Cathode /acceptor MFC	$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O$	-78.72	0.805 (0.816)
Overall MFC	$\frac{1}{8}CH_3COO^- + \frac{1}{4}O_2 \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}H_2O + \frac{1}{8}HCO_3^-$	-106.12	1.105 (1.100)
Cathode /acceptor MEC	$H^+ + e^- \rightarrow \frac{1}{2}H_2$	39.94	-0.414
Overall MEC	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{2}H_2 + \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^-$	12.54	-0.114 (-0.130)

Values for E_{emf} written in bracket are those calculated from the tabulated ΔG_r and E_{emf} values which vary slightly (Rittmann, 2001, Atkins, 2006).

From the equations above it can be seen that anode and cathode potentials vary with temperatures (T), substrates (ΔG_r^0 or E_{emf}^0) and ionic concentrations (Q), especially pH. These can be calculated as shown below (except in the case of wastewater). However in a real system they may vary from time to time, place to place, and even within the same reactor as substrates are utilised and H^+ ions produced:

Substrate

In an acetate fed MEC the theoretical anode potential (E_{An}) under standard biological conditions (i.e. pH 7, temperature 25 ^oC) would be -0.284 V and the for the hydrogen evolution reaction (again at pH 7) it is -0.414 V, giving a cell potential E_{emf} of -0.13V an additional 0.13V would need to be added, with glucose this difference is positive 0.015V, theoretically no energy would need to be added. With wastewater and its unknown composition and variability the theoretical anode potential cannot calculated,

the potential of a variety of compounds which may be found within wastewater are shown in Table 11-2.

Table 11-2 Known Gibbs free energy and potential values for a variety of compounds which may be present in wastewater

Substrate	ΔGr (kJ/mol e-)	E_{An} (V)	E_{emf} (V)
Methane	23.53	-0.244	-0.170
Acetate	27.40	-0.284	-0.130
Propionate	27.63	-0.286	-0.128
Ethanol	31.18	-0.323	-0.091
Protein	32.22	-0.334	-0.080
Lactate	32.29	-0.335	-0.079
Citrate	33.08	-0.343	-0.071
Methanol	36.84	-0.382	-0.032
Glycerol	38.88	-0.403	-0.011
Formate	39.19	-0.406	-0.008
Glucose	41.35	-0.429	0.015

ΔGr values from (Rittmann, 2001)

Temperature

Using acetate in an MFC as an example, with an acetate concentration of 0.12M (1 g/L of Na-acetate), bicarbonate concentration of 0.005M, at pH 7, and partial pressure of O_2 as 0.2, the potential, E_{emf} of the anode and cathode can be calculated through a range of temperatures from 0 to 30 ^{o}C:

Anode reaction

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O$$

Cathode reaction

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O$$

The potential under standard environmental conditions (E^0) for these reactions are 0.187V and 1.229V respectively. Using Equation 3 above:

Anode

$$E_{An} = {E_{An}}^0 - \frac{RT}{nF}\ln\frac{[CH_3COO^-]}{[HCO_3^-]^2[H^+]^9}$$

$$E_{An} = 0.187 - \frac{(8.31\,J/mol\,K)\,(T)}{(8)(\,96485\,C/mol)}\ln\frac{[0.012]}{[0.005]^2[10^{-7}]^9}$$

Cathode

$$E_{Ca} = E_{Ca}{}^{0} - \frac{RT}{nF}\ln\frac{[H_2O]}{[O_2]^{1/2}[H^+]^2}$$

$$E_{Ca} = 1.229 - \frac{(8.31\ J/mol\ K)\ (T)}{(2)(\ 96485\ C/mol)}\ \ln\frac{[1]}{[0.02]^{1/2}[10^{-7}]^2}$$

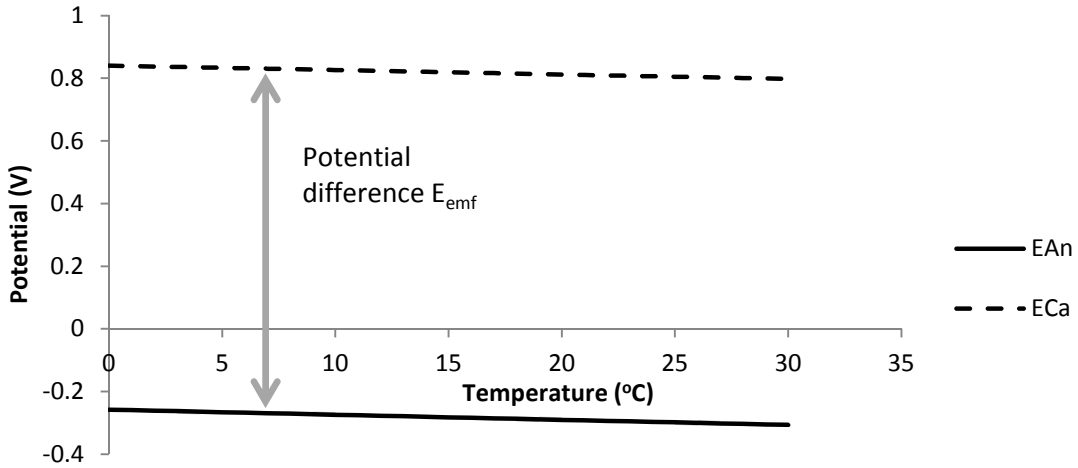


Figure 11-1 Calculated anode and cathode potential though a range of temperatures using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; pH 7; and partial pressure of O_2 as 0.2

The difference between the anode and cathode potential seen in Figure 11-1 varies only slightly from -1.098 V at 0 ^{o}C to -1.104 V at 30 ^{o}C. Theoretically therefore the energy available to be produced via a fuel cell is not greatly affected by temperature within the ranges given. This is however a simplistic approach to a system which, as stated previously is highly complex. As temperatures vary, so will many other factors including dissociation constants, partial pressures of gases and metabolic activity of the bacteria. It is therefore unlikely that the fuel cell will be able to generate as much current at lower temperatures as higher ones, yet it may not be as detrimentally affected by temperature as straight anaerobic digestion.

pH

The reaction co-efficient (Q) is calculated on the basis of the concentrations of the products and reactants in the chemical equation. This factor is critically dependant on

the pH of the system, i.e. the number of H^+ ions, as pH is a logarithmic scale, variance between pH 6 and pH 7 (both within the tolerance of bacteria) has a large effect on the Q value and therefore the overall potential of the cell. An example of this is shown below where the pH of the anode in an acetate system as described in the equations above at 25 ^oC is varied between pH 5 and 8, the cathode potential is kept constant under standard conditions. The potential difference ranges from 0.97 to 1.24 V.

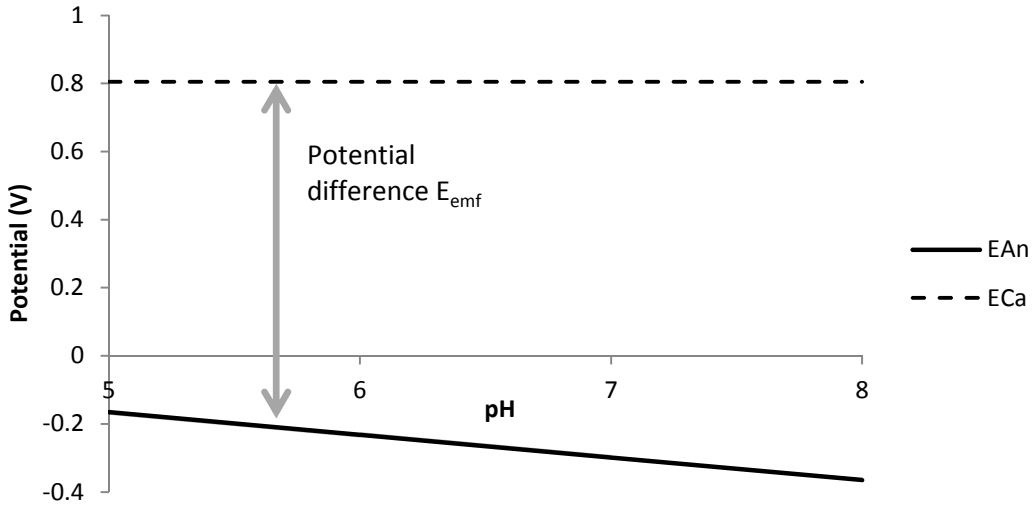


Figure 11-2 Calculated theoretical anode and cathode potential through a range of pHs using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; temperature 25 ^oC; and partial pressure of O_2 as 0.2

11.3. Appendix III – Table of calculated kJ/gCOD of various organic compounds

Compound	Formula	ΔH/gCOD
Benzene	C_6H_6	10.2
Linoleic acid	$C_{18}H_{32}O_2$	13.4
Benzoic acid	C_6H_5COOH	13.4
Myristic acid	$CH_3(CH_2)_{12}CO_2H$	13.6
Acetic acid (Acetate)	CH_3COOH	13.6
Phenol	C_6H_5OH	13.6
Palmitic Acid	$CH_3(CH_2)_{14}CO_2H$	13.6
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$	13.7
Methane	CH_4	13.9
Ethane	C_2H_6	13.9
Lactic acid	$CH_3CH(OH)COOH$	14.0
Ethanol	C_2H_5OH	14.3
Glucose	$C_6H_{12}O_6$	14.3
Propene	C_3H_6	14.3
Cyclopropane	C_3H_3	14.5
Ethanal	CH_3CHO	14.6
Ethene	C_2H_4	14.7
Sucrose	$C_{12}H_{22}O_{11}$	14.7
Methanol	CH_3OH	15.1
Chloroethylene	C_2H_3Cl	15.7
Oxalic acid	$(COOH)_2$	15.9
Formic acid	$HCOOH$	15.9
Ethyne	C_2H_2	16.3
Hexachlorobenzene	C_6Cl_6	16.5
Dichloroethylene (1,1)	$C_2H_2Cl_2$	17.1
Dichloroethylene (1,2)	$C_2H_2Cl_2$	17.2
Methanal	$HCHO$	17.8
Trichloroethylene	C_2HCl_3	20.0
Teterachloroethylene	C_2Cl_4	26.0
Chloroform	$CHCl_3$	29.1
Trichloroacetic acid	CCl_3COOH	30.4

11.4. Appendix IV - Description of the calculation algorithm used in the Shizas and Bagley

Shizas and Bagley (Shizas and Bagley, 2004) use a sample of municipal wastewater which prior to drying contains 431 mg/L COD. This sample is then oven dried to give a total solids measurement of 1980 mg/L. The dried sample is used in a bomb calorimeter giving 3.2 kJ/g dried weight.

Calculations derived from this data cited in various papers (Logan, 2008, Liao et al., 2006, Schroder, 2008, Logan, 2009):

$$3.2\ \mathrm{kJ/g}\ \times\ 1.98\ \mathrm{g/L} = 6.3\ \mathrm{kJ/L\ wastewater}$$

$$6.3\ \mathrm{kJ/L}\ \times \frac{1}{0.431\ \mathrm{gCOD/L}}\ =\ 14.7\ \mathrm{kJ/gCOD}$$

If the exercise is repeated on the data from the present paper using the oven dried samples and the measurement taken for COD prior to drying the results would have been:

Cramlington

$$8.3\ \mathrm{kJ/L}\ \times \frac{1}{0.718\ \mathrm{gCOD/L}}\ =\ 11.6\ \mathrm{kJ/gCOD}$$

Hendon

$$5.6\ \mathrm{kJ/L}\ \times \frac{1}{0.576\ \mathrm{gCOD/L}}\ =\ 9.9\ \mathrm{kJ/gCOD}$$

This is an underestimation of 60% and 45% respectively.

11.5. Appendix V - Wastewater sterilisation

Several of the experiments conducted in this thesis relied on using real wastewater, but needed this to be sterile. The following method was developed:

Method

The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into Ringers sterile dilutent (APHA, 1998). Effective sterilisation was defined as colony free plates in triplicate at zero dilution. The circulation time was varied to determine the optimum. The change in chemical composition (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) of the wastewater itself as compared to autoclaving and filtering.

Results

UV sterilisation caused the least change in wastewater properties measured as shown in Table 11-3, and was able to fully sterilise the wastewater.

Table 11-3 Percentage change of wastewater characteristics caused by the different sterilisation methods

	COD	Soluble COD	Total Solids	Bacteria per 0.1ml
Autoclaved (121^{o}C for 15 mins)	-15.6% ± 0.9	21.6% ± 0.6	-13.3% ± 5.8	0
Membrane filtered (0.2um PES)	-61.5% ± 0.5	22.8% ± 1.7	-36.1% ± 11.7	40 ±19
UV sterilised (5 mins)	-1.6% ± 0.4	7.2% ± 4.6	-3.3% ± 6.7	0

Bacteria is the average number counted on triplicate plates at zero dilution. All values show mean ± standard deviation (n=3)

Conclusion

Circulation of wastewater for 5 minutes through a UV filter was effective for bacterial kill off and least detrimental treatment to the composition of the wastewater.

11.6. Appendix VI - COD removal and coulombic efficiency

In the acetate fed cells the COD removal was high for both the cells which did (85%) and did not (80%) produce current ($p = 0.051$). For the other reactors there was an average removal of 64% COD for the wastewater and 87% for the starch solution. No significant difference in the COD removal in the reactors which generated current and those that did not was found wastewater ($p = 0.188$) and starch ($p= 0.688$).

The effluent of all reactors contained no detectable VFA's. The measured anions in each cell showed that there was almost complete removal of sulphate, from a starting value of 70 ppm in the wastewater and 38 and 41 ppm in the acetate and starch solutions respectively.

The coulombic efficiency of all reactors was low, such values are reasonably typical for complex substrates, but far lower than would be expected in a functioning acetate fed cell (Logan, 2008, Liu et al., 2011).

Table 11-4 COD removal and Coulombic efficiencies of all reactors fed on the different substrates. The values in grey are the reactors where acclimatisation did not occur

Inocula (ml)	**0.01**	**0.01**	**0.1**	**0.1**	**0.1**	**1**	**1**	**1**	**1**	**10**	**10**	**25**	**25**	**25**	**25**	**50**	**50**
COD removal (%)																	
Acetate	85.6	77.1	85.4	80.3	80.5	86.7	95.6	92.4	82.1	87.3	79.9	84.7	86.6	80.2	77.6	79.0	77.3
Wastewater						60.8	41.9			59.1	69.8	68.1	70.5			80.3	62.5
Starch						88.2	84.5			88.2	86.4	89.4	81.7			80.8	90.5
Coulombic efficiency (%)																	
Acetate	0.1	0.1	1.8	0.5	0.8	4.6	5.3	1.8	0.1	6.7	7.5	10.5	8.9	10.1	9.2	9.1	0.8
Wastewater						3.6	0.1			0.3	0.2	9.4	12.5			10.4	7.4
Starch						1.3	0.78			0.82	13.4	12.5	16.9			17.4	1.6*

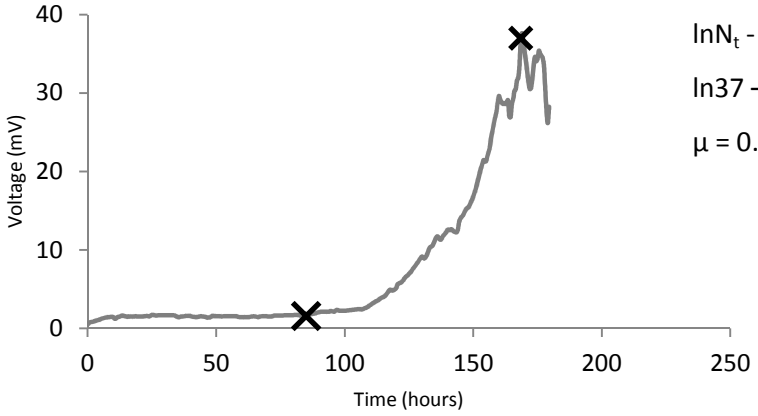
Values in grey are the reactors which did not acclimatise

*Unrepresentative value, data logging equipment failed after the point of acclimation.

11.7. Appendix VII - Yield and Specific activity calculations

Growth rate

Example calculation using 25 ml inocula

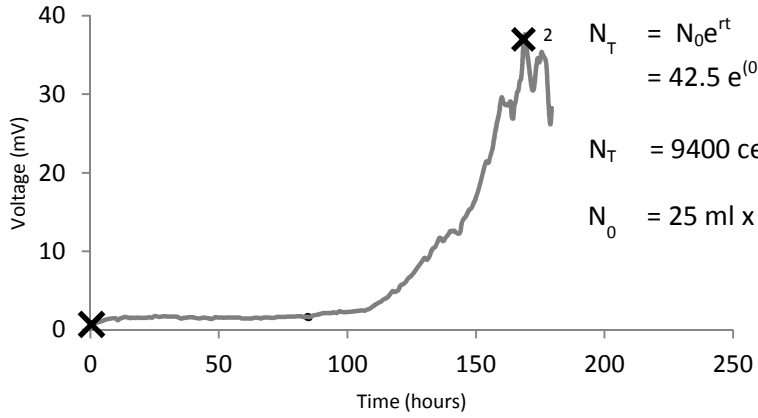


$\ln N_t - \ln N_0 = \mu(t - t_0)$

$\ln 37 - \ln 2 = \mu(170 - 85)$

$\mu = 0.034$

Specific activity



$N_T = N_0 e^{rt}$

$= 42.5\ e^{(0.034 \times 180)}$

N_T = 9400 cells

N_0 = 25 ml x 1.7 cell/ml = 42.5 cells

Each data logged voltage represents the time of 30 minutes, therefore the moles of electrons passed to the circuit per second at the data points measured is:

Moles of electrons = coulombs / Faradays constant

=((Voltage / resistance) x seconds)/Faradays constant

E.g. $\mathbf{X}_2$ =((0.037V / 470Ω)x 30mins x 60 seconds)/96485

$= 1.5 \times 10^{-6}$

Moles of electrons/cell = $1.5 \times 10^{-6} / 9400$

$= 10^{-10}$ mol e^-/cell

This value can be plotted throughout the time course of the experiment and is seen to be relatively constant.

Growth yield

The total number of cells produced up to the end of the exponential growth phase in the example above is 9400 cells.

$$\text{gCOD-cells} = (N_T - N_0) \times W \times COD_{cell}$$

where $N_T - N_0$ is the total new cell produced, W is the weight of each cell as estimated as 5.3×10^{-13} (Logan, 2008) and COD_{cell} is the estimation of 1.25 g-COD/g-cell (Rittmann, 2001).

$$\text{gCOD-cells} = (9400\text{-}43) \times 5.3 \times 10^{-13} \times 1.25$$

$$= 6.1 \times 10^{-9}$$

$$\text{gCODsubstrate} = \sum_{t0}^{t} mol\, e^{-}/8 \times 64$$

Where the sum over the growth period $t\text{-}t_0$ of the moles of electrons as calculated above is divided by 8 to give moles of acetate used, and multiplied by 64 giving the gCOD per mole of acetate.

$$\text{gCOD substrate} = 0.00011 / 8 \times 64 = 8.8 \times 10^{-4}$$

$$\text{gCOD-cell/gCOD-substrate} = 6.1 \times 10^{-9}/8.8 \times 10^{-4} = 6.9 \times 10^{-6}$$

The estimated yield of the acetate fed cells is extremely low ranging between 10^{-4} to 10^{-5} g-COD cell/g-COD substrate for the cells with between 10-50 mLs of inocula.

If exponential growth is assumed throughout the whole time period for the lower inocula cells these values are much higher up to 8 g-COD cell/g-COD for the 0.1 ml inocula. If no growth during lag is assumed these values are lower (10^{-7} g-COD cell/g-COD) and more in line with those observed for higher inocula. These yields are inconsistent with the literature on yields in microbial fuel cells (Freguia et al., 2007, Rabaey et al., 2003) although both of these studies used different methodology. They are also inconsistent with yields of other bacterial systems (Rittmann, 2001).

11.8. Appendix VIII – Calculations of performance in MFCs and MECs

Power Calculation for both MFCs and MECs

Performance can be evaluated through the amount of power produced which can be expressed as:

$$P = IE$$

Where P is the power in watts, E is the voltage as measured by the data logger in volts and I is the current in amps, calculated from the measured voltage E, at a known resistance R:

$$I = E/R$$

Power can therefore be alternatively expressed as:

$$P = E^2/R$$

This power is often also evaluated as power density (*Pd*), this is the amount of power produced per area of electrode surface (typically the size of the anode) expressed as Wm^2. Normalising the power output in this way allows different systems to be compared. This is calculated as:

$$Pd = \frac{E^2}{A_{An}R}$$

Where A_{An} is the area of the anode. The current density (A/m^2) can also be expressed in the same way normalising current to electrode size. Both power and current density can also be expressed per reactor size by substituting A_{An} above for the reactor volume in m^3, resulting in a power density measured as Wm^3. or current density as A/m^3.

Efficiency calculations for MFCs

The efficiency of an MFC is expressed as the Coulombic Efficiency (CE) and is a measure of the amount of coulombs of charge recovered from the cell from the total coulombs available in the substrate that has been removed in the reactor. It is expressed as a percentage:

$$CE = \frac{Coulombs\ recovered}{Coulombs\ in\ substrate}$$

An Amp is the transfer of 1 coulomb of charge per second, therefore by integrating the current over the course of the experiment or batch time (t) the total coulombs transferred is given. Usually the amount of coulombs in the substrate is evaluated using the amount of organic matter removed as determined by the chemical oxygen demand (COD). CE is therefore calculated as:

$$CE = \frac{8 \int_0^t I \ dt}{F \ V_{An} \Delta COD}$$

Where 8 is used as a constant derived from the molecular weight of oxygen divided by 4 the amount of electrons exchanged per mole of oxygen. Faradays constant (F) of 96485 Coulombs/mol, is the magnitude of electrical change per mole of electrons, ΔCOD is the measured change in COD in g/L and V_{An} (L) is the volume of the anode compartment containing the liquid feed at the given COD concentration. .

Efficiency calculation for MECs

The efficiency of an MEC is a more complex matter, as the output of energy is of hydrogen gas (not electricity or charge directly) and the inputs of energy are from the substrate and the additional electrical energy added to the system.

11.9. Appendix IX – Dissimilarity matrix

Unweighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.79	0.00																	
Wastewater inocula 2	0.88	**0.67**	0.00																
Acetate cold ww 1	0.88	0.67	0.70	0.00															
Acetate cold ww 2	0.88	0.65	0.69	**0.45**	0.00														
Acetate cold soil 1	0.82	0.80	0.80	0.72	0.73	0.00													
Acetate cold soil 2	0.76	0.78	0.82	0.75	0.75	**0.59**	0.00												
Acetate hot ww 1	0.89	0.71	0.69	0.54	0.60	0.73	0.78	0.00											
Acetate hot ww 2	0.89	0.73	0.71	0.56	0.58	0.74	0.78	**0.22**	0.00										
Acetate hot soil 1	0.81	0.79	0.80	0.72	0.75	0.62	0.63	0.25	0.70	0.00									
Acetate hot soil 2	0.79	0.79	0.82	0.75	0.76	0.64	0.65	0.18	0.72	**0.51**	0.00								
Wastewater cold ww 1	0.87	0.69	0.70	0.59	0.61	0.73	0.76	0.27	0.64	0.73	0.76	0.00							
Wastewater cold ww 2	0.82	0.72	0.76	0.72	0.73	0.74	0.74	0.44	0.67	0.66	0.67	**0.68**	0.00						
Wastewater cold soil 1	0.85	0.69	0.75	0.61	0.64	0.65	0.69	0.28	0.64	0.64	0.69	0.53	0.66	0.00					
Wastewater cold soil 2	0.88	0.74	0.75	0.69	0.69	0.77	0.78	0.24	0.60	0.69	0.72	0.62	0.51	**0.64**	0.00				
Wastewater hot ww 1	0.86	0.71	0.73	0.62	0.64	0.66	0.71	0.28	0.65	0.67	0.71	0.49	0.68	0.42	0.65	0.00			
Wastewater hot ww 2	0.88	0.74	0.74	0.68	0.68	0.76	0.77	0.24	0.60	0.70	0.72	0.63	0.51	0.65	0.23	**0.66**	0.00		
Wastewater hot soil 1	0.86	0.67	0.71	0.62	0.65	0.74	0.77	0.28	0.64	0.73	0.76	0.35	0.67	0.53	0.64	0.53	0.63	0.00	
Wastewater hot soil 2	0.89	0.71	0.72	0.59	0.58	0.75	0.78	0.28	0.59	0.74	0.78	0.44	0.71	0.51	0.64	0.49	0.64	**0.45**	0.00

Weighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.41	0.00																	
Wastewater inocula 2	0.52	**0.59**	0.00																
Acetate cold ww 1	0.44	0.48	0.45	0.00															
Acetate cold ww 2	0.43	0.45	0.48	**0.08**	0.00														
Acetate cold soil 1	0.50	0.56	0.53	0.17	0.16	0.00													
Acetate cold soil 2	0.44	0.48	0.47	0.09	0.10	**0.10**	0.00												
Acetate hot ww 1	0.49	0.57	0.41	0.28	0.30	0.36	0.30	0.00											
Acetate hot ww 2	0.56	0.63	0.44	0.37	0.41	0.45	0.40	**0.17**	0.00										
Acetate hot soil 1	0.36	0.43	0.50	0.23	0.22	0.30	0.23	0.32	0.43	0.00									
Acetate hot soil 2	0.41	0.51	0.43	0.14	0.16	0.22	0.15	0.23	0.34	**0.16**	0.00								
Wastewater cold ww 1	0.34	0.39	0.46	0.25	0.23	0.35	0.28	0.31	0.43	0.22	0.25	0.00							
Wastewater cold ww 2	0.37	0.42	0.55	0.43	0.40	0.45	0.42	0.46	0.52	0.31	0.39	**0.33**	0.00						
Wastewater cold soil 1	0.36	0.40	0.45	0.26	0.22	0.34	0.27	0.30	0.42	0.22	0.26	0.13	0.36	0.00					
Wastewater cold soil 2	0.41	0.50	0.41	0.31	0.33	0.39	0.33	0.30	0.36	0.37	0.31	0.32	0.33	**0.34**	0.00				
Wastewater hot ww 1	0.35	0.39	0.46	0.27	0.24	0.36	0.29	0.31	0.43	0.24	0.29	0.14	0.37	0.05	0.35	0.00			
Wastewater hot ww 2	0.38	0.49	0.40	0.31	0.32	0.40	0.33	0.26	0.35	0.35	0.29	0.29	0.33	0.31	0.08	**0.32**	0.00		
Wastewater hot soil 1	0.34	0.40	0.48	0.24	0.22	0.34	0.28	0.33	0.45	0.22	0.26	0.05	0.33	0.14	0.31	0.16	0.29	0.00	
Wastewater hot soil 2	0.38	0.38	0.52	0.28	0.24	0.35	0.30	0.36	0.49	0.25	0.30	0.12	0.32	0.18	0.34	0.19	0.34	**0.11**	0.00

Dissimilarity values calculated using UniFrac, lower values indicate greater similarity

11.10. Appendix X - Estimates of sample total diversity

Table 11-5 Estimates of total diversity using the MCMC model (Quince et al., 2008), values given are the lower 95% confidence interval : median : upper 95% confidence interval. The best fit values according to the DIC values are highlighted in bold, the model fits that had DIC scores within 6 of the best fitting model are in italics and should not be considered as plausible options for fitting the data

	Total diversity		
Sample	**Log-normal**	**Inverse Gaussian**	**Sichel**
Arctic soil inocula	**5831:7207:10593**	5151:6227:7439	3632:4403:5821
Wastewater inocula 1	**3431:4238:5572**	2217:2405:2655	2648:3275:5533
Wastewater inocula 2	2924:4260:8970	1679:2066:2752	**1716:2286:3640**
Acetate cold ww 1	**3060:5449:11740**	1273:1700:2406	1402:2197:3379
Acetate cold ww 2	13901:29226:42363	**984:1549:3049**	993:1697:3298
Acetate cold soil 1	**1380146:1393974:1407428**	*3430:5004:7687*	2960:4628:9094
Acetate cold soil 2	**1849625:1865409:1877419**	*3428:4923:7910*	3191:5018:8179
Acetate hot ww 1	1934:3511:12608	808:987:1300	**948:1310:2224**
Acetate hot ww 2	**1217:2159:6024**	643:785:1037	665:843:1264
Acetate hot soil 1	4386:8968:19150	1508:1968:2813	**1456:1984:3086**
Acetate hot soil 2	**171417:184911:197766**	2445:3773:5440	2350:3579:5577
Wastewater cold ww 1	**614:749:1014**	493:535:594	491:534:599
Wastewater cold ww 2	*859:1102:1596*	*640:708:805*	**730:906:1455**
Wastewater cold soil 1	**1079:2249:8263**	543:733:1197	651:1032:2324
Wastewater cold soil 2	556:640:789	*467:494:531*	**510:575:793**
Wastewater hot ww 1	1430:2911:9800	*637:845:1300*	**5682:16751:18608**
Wastewater hot ww 2	**483:548:660**	419:443:476	430:467:525
Wastewater hot soil 1	**820:1148:1985**	581:661:787	596:697:893
Wastewater hot soil 2	694:1135:2283	438:504:614	**468:572:954**

Table 11-6 DIC scores as defined by the sum of the deviance averaged over the posterior distribution and estimate of the sampling effort required to capture 90% of the diversity of taxa within the sample as determined by the fits of abundance distribution

	DIC			Sampling effort		
Sample	Log-normal	Inverse Gaussian	Sichel	Log-normal	Inverse Gaussian	Sichel
Arctic soil inocula	165.53	171.01	166.67	2.02E+06	4.06E+05	1.32E+05
Wastewater inocula 1	450.33	455.14	444.42	1.32E+07	2.56E+05	8.92E+05
Wastewater inocula 2	264.17	262.28	261.93	3.56E+07	2.98E+05	4.16E+05
Acetate cold ww 1	275.13	275.3	275.85	3.32E+09	1.59E+06	3.06E+06
Acetate cold ww 2	197.07	196.74	196.98	1.11E+13	1.47E+06	1.70E+06
Acetate cold soil 1	266.22	273.65	267.61	2.56E+18	1.42E+07	8.37E+06
Acetate cold soil 2	274.28	283.68	274.4	2.42E+18	7.28E+06	5.19E+06
Acetate hot ww 1	309.59	311.17	309.21	2.99E+09	5.88E+05	1.59E+06
Acetate hot ww 2	242.64	244.43	244.76	2.84E+08	3.61E+05	4.73E+05
Acetate hot soil 1	290.25	288.7	288.57	1.17E+10	1.44E+06	1.34E+06
Acetate hot soil 2	265.04	269.84	265.05	6.98E+14	4.73E+06	3.16E+06
Wastewater cold ww 1	254.73	255.02	255.23	5.22E+05	4.23E+04	4.25E+04
Wastewater cold ww 2	268.11	269.7	261.78	1.23E+06	4.91E+04	1.63E+05
Wastewater cold soil 1	201	201.99	197.99	2.68E+08	1.53E+05	5.35E+05
Wastewater cold soil 2	333.27	349.36	332.04	3.47E+05	3.70E+04	9.96E+04
Wastewater hot ww 1	252.09	254.67	246.76	1.37E+09	2.57E+05	1.05E+09
Wastewater hot ww 2	274.09	279.19	275.06	1.51E+05	2.52E+04	3.56E+04
Wastewater hot soil 1	248.04	250.28	248.96	3.54E+06	7.21E+04	9.24E+04
Wastewater hot soil 2	243.6	244.69	242.65	1.93E+07	7.44E+04	1.32E+05

11.11. Appendix XI - Details of the bacteria phyla and families found within the samples tested

It is seen in Figure 11-3 (a) that the inoculated and acclimatised reactors have become enriched Proteobacteria, this phylum dominates with about 80% abundance in the acetate fed cells, and around 60% in the wastewater fed cells. Proteobacteria are a diverse phylum of bacteria, yet most of this high abundance in the reactors is caused by the enrichment of *Geobacter* an exoelectrogenic organism, as is seen in Figure 11-4. *Rhodocyclaceae*, *Psuedomonas* and *Desulfovibrio* also added to the proportion of Proteobacteria that became enriched. The relative abundance of the other main phyla generally drops within the reactor samples, a proportion (around 10-20%) of Bacteriodietes remains, and there is some enrichment of Acidobacteria in the wastewater fed reactors. The wastewater reactors have a greater spread of abundance over the phyla groups shown, with less domination by Proteobacter.

The OTU richness shown in Figure 11-3 (b) again shows the greater diversity of the acetate reactors over the wastewater fed ones, both by the larger bar size and the Chao estimate above. It is seen many of the OTUs present in the inoculum have survived in the acetate reactor conditions, despite the metabolic narrowing of the conditions. Surprisingly this greater diversity or spread of OTUs appears to be slightly higher in the cold reactors, than the warm ones. In the case of the wastewater fed reactors the OTU richness in reduced, temperature does not appear to have an impact.

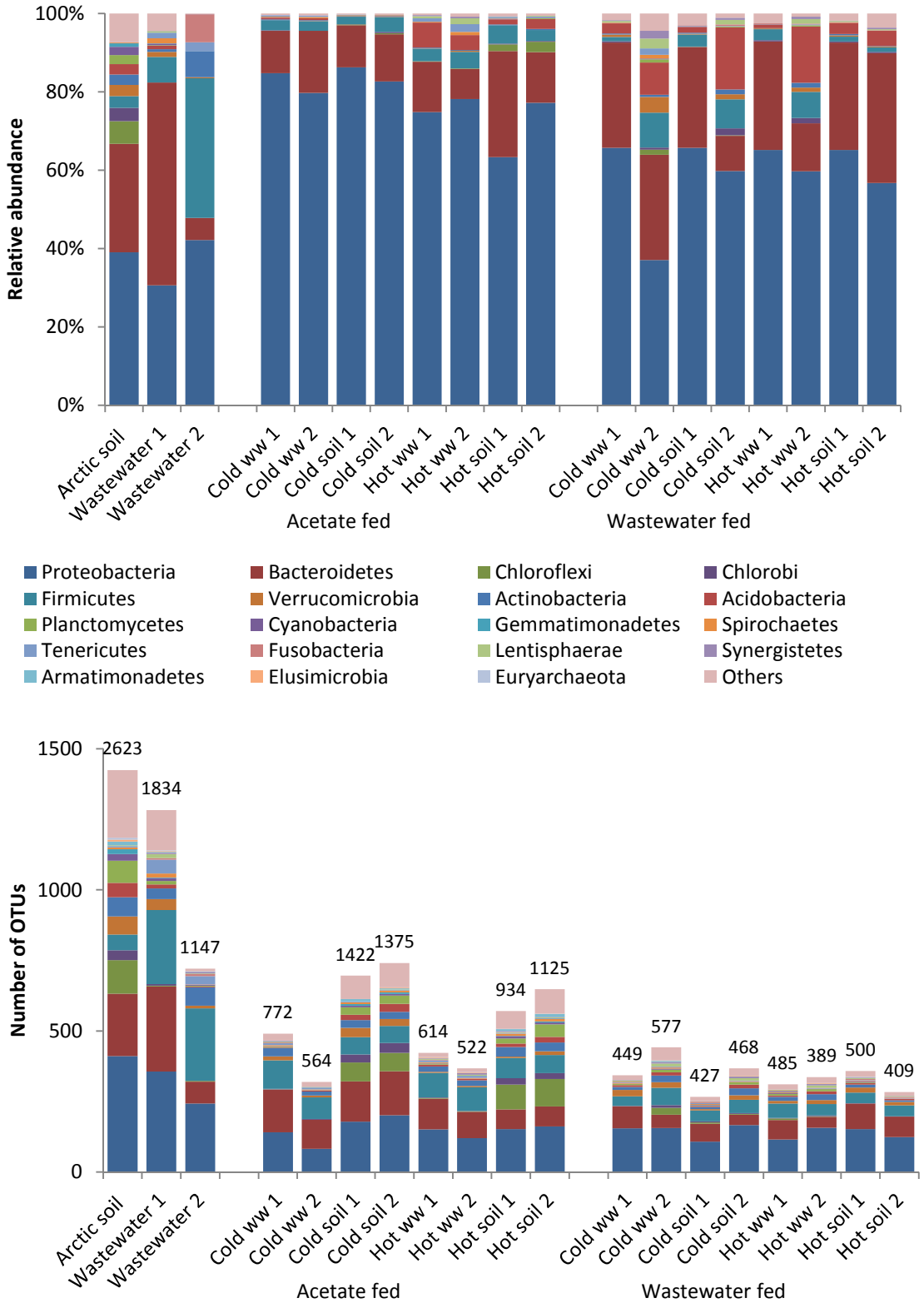


Figure 11-3 Relative abundance (a) and OTU richness (b) for all the data sets given at the phylum rank. Relative abundance is shown as the number of reads within each taxa divided by the total number of reads. The OTU richness is the number of taxa within each phylum is given by the size of the bar, the Chao 1 estimate of richness is written at the top of each bar

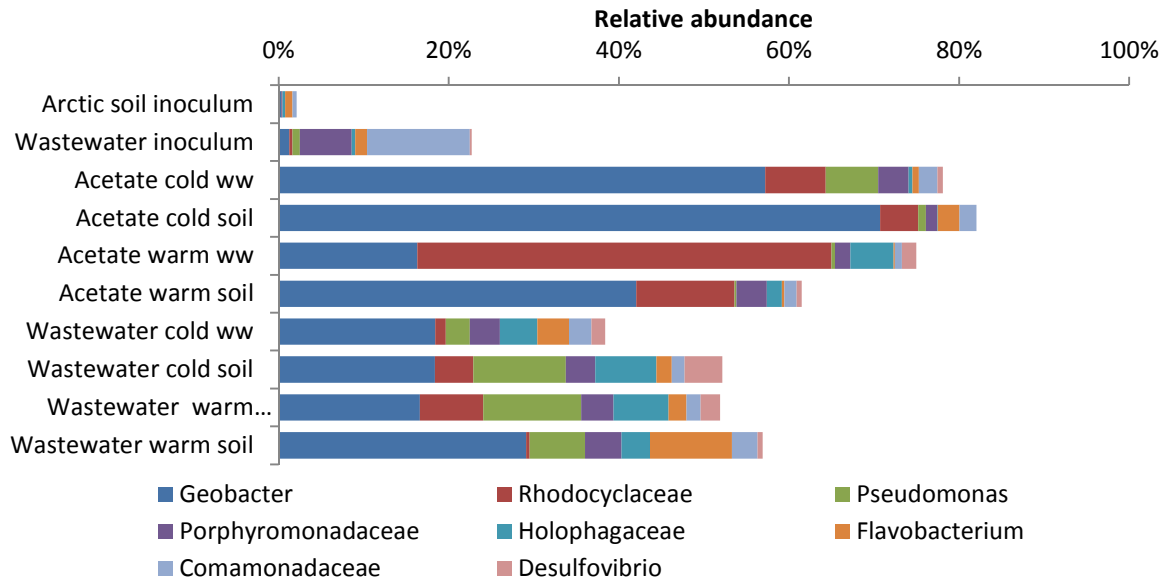


Figure 11-4 The relative abundance of the 8 most dominant genus as an average for the duplicate reactors under each condition, where the genus name was not given by the classification database family is used

It would be expected that the most dominant organisms within the reactors are the ones that are able to most competitively metabolise, grow and therefore reproduce within the conditions of the reactors. The top 8 most dominant genus are given in Figure 11-4, for Rhodocyclaceae, Porphyromonadaceae, Holophagaceae, Comamonadaceae the classification did not give the genus name, and therefore the family name is given. It is seen that for the acetate fed reactors these 8 genus make up a large proportion of the total abundance, and in the cold reactor most of this is by *Geobacter*. For the warm acetate reactors, *Geobacter* is still important, but Rhodocyclaceaea is also dominant, especially in those seeded with wastewater. The proportion of *Geobacter* is made up of 11 different species (names of which are not given by the classification), 4 of which are dominant within the reactors. Rhodocyclaceae is a diverse family of bacteria associated with wastewater treatment, further classification of this group is not made.

Within the wastewater reactors Geobacter is less dominant, between 20-30% of abundance, and there is a greater spread of the other genus and families, most notable *Pseudomonas* which make up to 10%. Within the Pseudomonas genus, 8 species were identified, of which 2 were dominant within the reactors, Pseudomonas have previously been seen within fuel cell systems fed substrates such as glucose and butyric acid and are believed to be capable of fermentation (Kiely et al., 2011c), some species such as *Pseudomonas aerunginosa* produce soluble redox shuttles and have been investigated

for their use within fuel cell systems (Marsili, 2010). The family of Holophagaceae is also quite enriched, this family includes the species of *Geothrix fermetans* which has been found in wastewater fed MFCs and is believed to be important in the hydrolysis or fermentation steps, (Kiely et al., 2011a), and has also been linked to shuttle formation (Bond and Lovley, 2005). *Flavobacteium* are also enriched, although this genus is more typically associated with freshwater environments. There is also likely to be sulphate reduction occurring in the cells due to the presence of *Desulfovibro*.

11.12. Appendix XII – Acknowledged contributions

Section	Other contributors	Contribution of E. Heidrich
Chapter 2	T.P. Curtis and J. Dolfing – editing and guidance with content	90%
Chapter 3	T.P. Curtis and J. Dolfing – editing and guidance with content	95%
Chapter 4	T.P. Curtis and J. Dolfing - editing and guidance with content, M. Wade – bioinformatics analysis, W.T.Sloan – sequencing funding	95%
Chapter 5	T.P. Curtis, K. Scott, I Head and J. Dolfing – discussion and experiment planning	95%
Chapter 6	T.P. Curtis K. Scott and J. Dolfing - editing and guidance with content, S. Edwards – site installation and running MEC	90%

Evaluation of Microbial Electrolysis Cells in the treatment of domestic wastewater

Thesis submitted to Newcastle University for the degree of Doctor of Philosophy

Author: Elizabeth Susan Heidrich

Supervisor: Professor Tom Curtis

Co-Supervisor: Dr Jan Dolfing

Co-Supervisor: Professor Keith Scott

Date: May 2012

Abstract

Wastewater can be an energy source and not a problem. This study investigates whether rapidly emerging bioelectrochemical technologies can go beyond working in a laboratory under controlled temperatures with simple substrates and actually become a realistic option for a new generation of sustainable wastewater treatment plants.

The actual amount of energy available in the wastewater is established using a new methodology. The energy is found to be considerably higher than the previous measurement, or estimates based on the chemical oxygen demand with a domestic wastewater sample containing 17.8 kJ/gCOD and a mixed wastewater containing 28.7 kJ/gCOD.

With the energy content established the use of bioelectrochemical systems is examined comparing real wastewater to the 'model' substrate of acetate. The abundance of exoelectrogenic bacteria within the sample, and the acclimation of these systems is examined through the use of most probable number experiments. It is found that there may be as few as 10-20 exoelectrogens per 100 mL. The impact of temperature, substrate and inoculum source on performance and community structure is analysed using pyrosequencing. Substrate is found to have a critical role, with greater diversity in acetate fed systems than the wastewater fed ones, indicating that something other than complexity is driving diversity.

Laboratory scale microbial electrolysis cells are operated in batch mode fail when fed wastewater, whilst acetate fed reactors continue working, the reasons for this are examined. However a pilot scale, continuous flow microbial electrolysis cell is built and tested at a domestic wastewater treatment facility. Contrary to the laboratory reactors, this continues to operate after 3 months, and has achieved 70% electrical energy recovery, and an average 30% COD removal.

This study concludes that wastewater is a very complex but valuable resource, and that the biological systems required to extract this resource are equally complex. Through the work conducted here a greater understanding and confidence in the ability of these systems to treat wastewater sustainably has been gained.

Acknowledgements

It has been a great privilege to be able to study for a PhD, and I am grateful to all those who have made it possible.

I would like to thank my supervisor Tom Curtis for giving me the support and freedom to develop and explore my own ideas, always meeting these with enthusiasm and the necessary intellectual guidance. I would also like to thank Jan Dolfing who has skilfully guided me into, and through many intellectual challenges. I would like to thank Keith Scott and Ian Head for their support and guidance when needed. I would also like to thank Bill Sloan and Chris Quince for their support and guidance with the sequencing work, the contributions of individuals are detailed in Appendix XII.

I would like to thank EPSRC for giving me my doctoral training grant, and the School of Chemical Engineering and Advanced Materials for additional funding which enabled me to take up the offer of a PhD. I would like to thank Northumbrian Water Ltd. for the additional funding which has allowed for more experimental work to be carried out. I would like to thanks in particular from Northumbrian Water Ltd Chris Jones and Andrew Moore, who have been enthusiastic and supportive throughout this project and have provided me with all the assistance needed, and also Laura Stephenson, Steve Robson and all the workers at Howdon wastewater treatment plant, who have enthusiastically accommodated the pilot scale reactor for far longer than expected.

I would like to thank all my colleagues and co-workers who have provided advice, support and humour when needed. In particular to: Beate Christgen who helped with setting up MFCs and electrochemistry tests; Matt Wade who patiently nursed me through the bioinformatics process; and Stephen Edwards who has always been willing to lend a helping hand or ear.

As well as intellectual and financial support, I have also received, and needed, much emotional and moral support during the course of this PhD. This has come from many of those listed above, but also from my family who have not only made my completing a PhD possible, but are also the reason why it has been worthwhile. I would like to thank in particular: my mum Linda who has always taught me to learn for the sake of enjoyment above all else; my niece Katie whose determination and fighting spirit is a

continual inspiration; my husband Oliver, whose moral support, guidance, motivation and good humour has been unfaltering throughout; and lastly I thank my son Ben and his beautiful smile.

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Chapter 1. Introduction

There is growing consensus that wastewater is a resource not a problem (Verstraete and Vlaeminck, 2011, Sutton et al., 2011, McCarty et al., 2011). The conventional treatment of wastewater removes its organic content via aerobic processes, termed activated sludge, this is energy expensive typically 3% of the electrical energy usage of many developed countries (Curtis, 2010). Not only is the energy in wastewater removed not recovered, we expend considerable energy in performing this removal.

In the UK the water sector energy use has increased 10% in the last 10 years (Water UK, 2012, Water UK, 2011), industrial electricity prices have increased by 69% since 2000 (National Statistics, 2011). If these trends continue the energy bill for the water sector will be vastly higher than for the current 9016 GWh (Water UK, 2012). With infrastructure requiring long term planning and capital investment, it is hard to see without drastic action how the necessary changes can be made. Technologies that require relatively simple modifications to the current infrastructure to become operational are more likely to be given a chance rather than those which require wholesale change. New technology should ideally fit reasonably well into the existing infrastructure, and as a minimum achieve similar loading rates per unit area to activated sludge of 0.4-1.2 kg BOD $m^{-3}d^{-1}$ (Grady, 1999). The high capital costs of change and the uncertainty of using a different technology, coupled with the regulation of both effluent quality and pricing structures, are an obstacle to change.

There are alternatives to this approach. Replacing the aerobic activated sludge process with an anaerobic process means the energy stored in the organic content of the wastewater is converted to methane (80% efficiency) which can be combusted to produce electricity (35% efficiency) (McCarty et al., 2011). Only around 30% of the total energy in the wastewater can be captured as electricity in anaerobic systems, although with heat exchange in the combustion process, or the use of non-combustion methods of conversion, this could be increased (McCarty et al., 2011).

The scientific challenges of creating an energy neutral or even energy positive wastewater treatment process are also substantial and complex. The process needed to replace activated sludge must:

- Extract and convert energy to a useable form at an efficiency that justifies the costs.
- Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
- Treat low strength domestic wastewater, which is problematic for anaerobic digestion technologies (Rittmann, 2001).
- Work at ambient, often low temperatures, again problematic for anaerobic digestion (Lettinga et al., 1999).
- Work continuously and reliably.

An innovative and relatively new approach to wastewater treatment is through the use of bioelectrochemical systems (BES), though the fuel cell technology lying behind this process is over 100 years old (Potter, 1911) (see appendix I for a history of development). Here wastewater is consumed in a battery like cell, redox reaction catalysed by bacteria pushing electrons around in an electrical circuit, thus creating electricity (Rabaey et al., 2007). In a microbial fuel cell (MFC) the electricity is captured directly (Logan, 2005), in a microbial electrolysis cell (MEC) the electricity is supplemented by an external source to make a product such as hydrogen or methane (Rozendal et al., 2006) or to perform a process such as reductive dechlorination (Aulenta et al., 2008) or de-salination (Mehanna et al., 2010). There are substantial losses within these systems (Logan et al., 2006), it is suggested they may reach a higher conversion efficiency of 44% (McCarty et al., 2011), the performance of MFCs to date has only reached around 1 tenth of that needed to be competitive with anaerobic digestion (Pham et al., 2006). With MECs the potential higher value (energetically or commercially) of the product formed or process completed means this technology is likely to be more viable and may be the driver of development (Foley et al., 2010).

As organic matter is degraded by bacteria it releases electrons (oxidation) providing energy for the cells. These electrons then pass to an electron acceptor (or reduced species), which is normally oxygen, nitrate or sulphate depending on their availability providing further energy for the cells (Rittmann, 2001).It has been shown that there is a group of organisms that are capable of passing electrons to materials (such as metal oxides) outside the cell, which are then transferred by that material to an electron acceptor. This process is termed electrogenesis, and the group of organisms are known as exoelectrogens (Logan, 2008). MFCs exploit this, providing the bacteria with a

surface to donate electrons to, and then using the principles of all electrochemical cells to transport these electrons and create current.

MFCs, like electrochemical cells usually have two compartments, the anode chamber containing organic matter to be degraded, and the cathode chamber containing an electron acceptor. In the anode chamber organic matter is degraded by bacteria producing electrons, the absence of a preferred electron acceptor such as oxygen, means these electrons pass into the anode material then through a wire to the cathode. The H^+ ions generated in this reaction pass through the membrane from the anode to cathode chamber. At the cathode the electrons, H^+ ions and a reduced species (typically oxygen) combine to form for example H_2O. Electrical current is generated in the wire as the electrons pass from one side to the other.

An MEC reactor is an adaptation of an MFC. In an MEC both the anode and cathode chamber are anaerobic. Rather than creating H_2O in the cathode chamber, the electrons and H^+ ions are combined to generate H_2 gas rather than electricity. The process of forming H_2 is however endothermic, i.e. it requires energy. It cannot happen spontaneously. The addition of a small amount of electricity (with acetate this is in theory 0.114 V, in practice <0.25 V), is required to generate the H_2 gas (Logan et al., 2008). This is substantially less energy than is required to produce H_2 through water electrolysis, typically 1.8-2.0 V. A schematic of an MEC is shown in Figure 1-1.

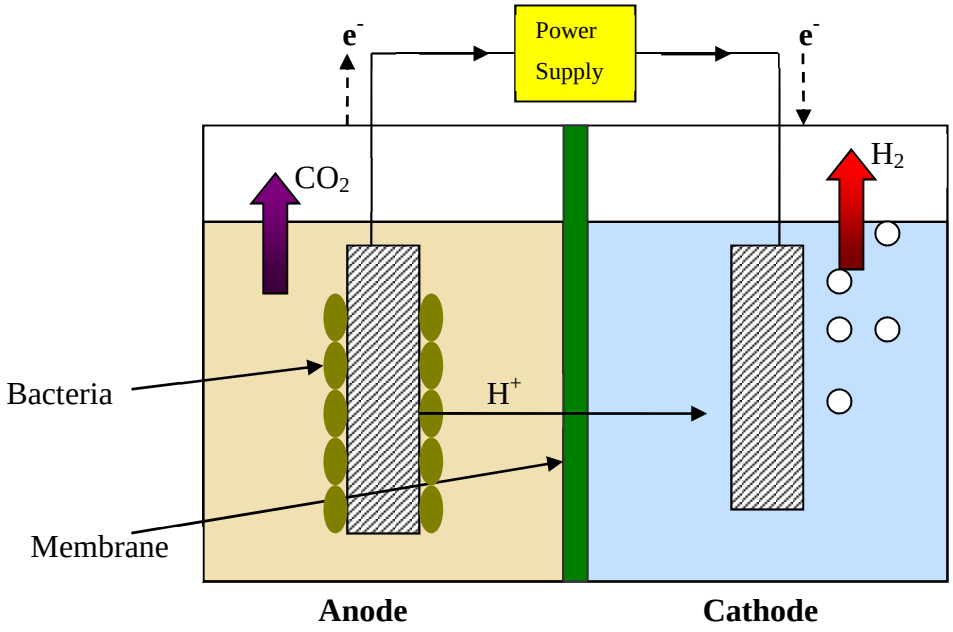

Figure 1-1 Generalised schematic of an MEC adapted from (Liu et al., 2005b) showing the flow of electrons and hydrogen ions and the function of the anode and cathode sections

The theoretical electrochemical energy gains or requirements of a MFC and MEC respectively will vary with temperatures, substrate free energy and ionic concentrations especially pH, as shown in appendix II. Even if it were possible to determine the potentials accurately in practice these theoretical values are not achieved. Energy is lost through all the transfer processes which take place to allow this reaction to happen. There are both electrochemical losses known as overpotentials caused by losses in redox reactions and transfer to the electrodes, losses in transfer of ions between the electrodes, limitations caused by transfer rates being different for different species, and on top of this there are losses caused by transfer of both electrons and ions in and out of the bacteria, losses to the bacteria themselves as they use energy, losses of electron transfer, and also losses by side or chain reactions occurring which do not advantage the fuel cell (Logan, 2008). This means that the energy gained in an MFC is less, and the energy input required in an MEC is more, than would theoretically be the case, represented in Figure 1.2.

In an MEC substantially more energy input than the theoretical is needed, in acetate fed systems these typically range from 0.4 V to 0.8 V with greater hydrogen gas production at higher voltages but less energy efficiency (Call and Logan, 2008). Glucose fed reactors have been shown to operate at applied voltages of 0.9 V (Selembo et al., 2009a), although far less work has been carried out on this substrate and its limits of applied voltage are undefined. In a larger scale system it is likely overpotentials (the difference between the theoretical potential at which the reaction occurs, and the observed potential of the electrode) will be increased and therefore the power input might be higher. In a pilot scale reactor fed on wine wastewater the input voltage of 0.9 V was used, although this performed less well than laboratory trials at a smaller laboratory scale on the same substrate, high over potentials being one of the suggested reasons (Cusick et al., 2011).

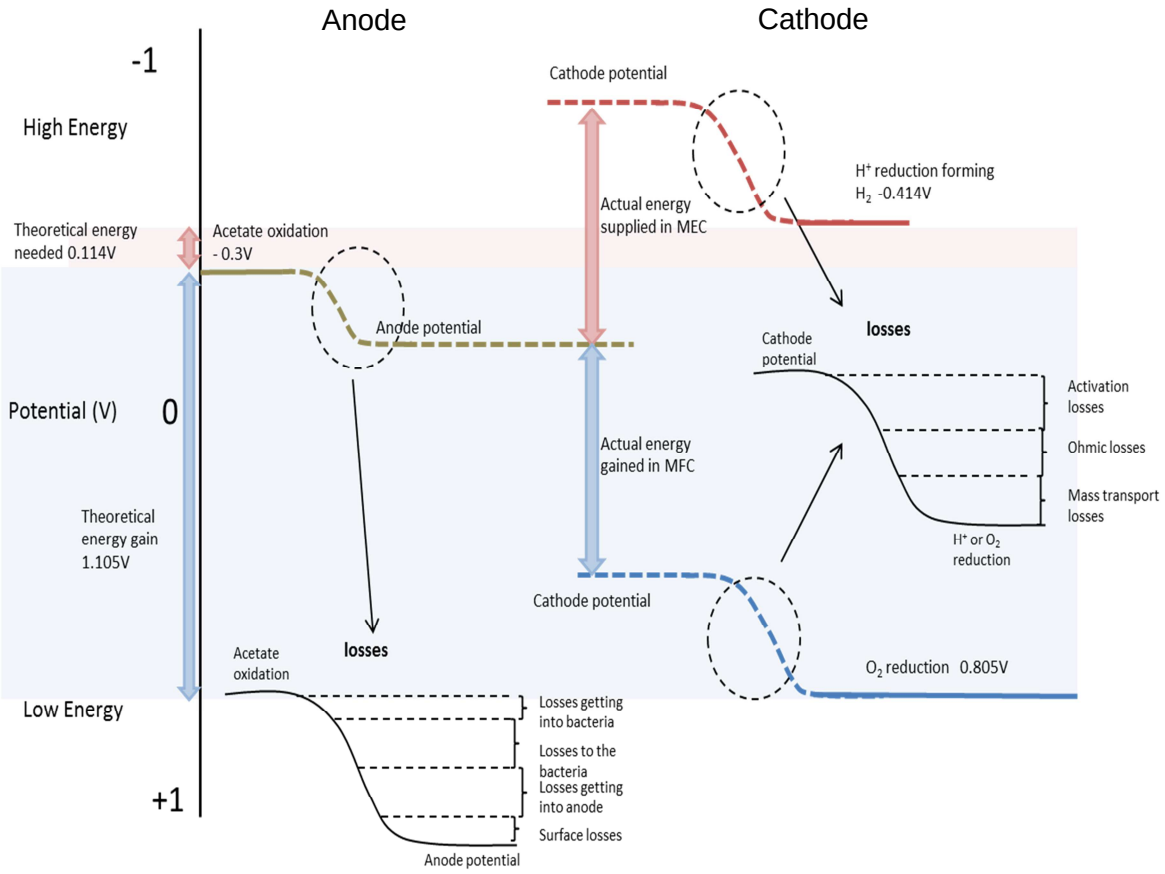

Figure 1-2 Representation of the energy losses within an MFC and MEC using acetate. Energy is shown as potential on the vertical axis, the green line shown the potential of the anode from the potential of acetate (solid line) to the actual anode potential (dotted line) which dependant on the losses. The reduction potential of the MFC and MEC cathode reactions is shown as the solid blue and red lines respectively, whereas the actual cathode potential is again shown in the dotted lines and is dependent on losses. The predicted total energy gain (MFC) and loss (MEC) is shown by the thick arrows and can be variable depending on these losses, but will always be less than that theoretically predicted as seen in the thick arrows at the vertical axis

Understanding the complexities of the electrochemistry of these systems is however only part of the challenge of understanding and ultimately manipulating BES technology. The microbiology of such systems plays a critical role in dictating their efficiency and their success or failure. The microbial community, which catalyses and enables the whole process to take place will also be affected by temperature, pH and substrates (Rittmann, 2001), it will vary with time and within the reactor, and the factors of competition, symbiosis and random assembly lead to a highly complex and unpredictable system.

BES systems run on electrochemical principles but rely on microbial communities. Therefore predicting their absolute function and output of energy, or indeed the input of energy needed, is at this stage in our understanding not possible. The empirical collection of this information is necessary in helping us identify not only if this technology is viable but also the areas that can and need to improved. Critically understanding the bacterial communities and the energy transfers within these systems lies at the heart of being able to manipulate and use this technology.

BES in general and MECs in particular have the potential to fulfil these needs of the wastewater industry (Foley et al., 2010). MECs are entirely anaerobic, eliminating the need for any aeration or complex membrane systems, meaning their engineering can be simple and 'retrofittable' within existing infrastructure. Although hydrogen production is focused on in this study, the flexibility of this process to make other high value products is an economic driver. However the key challenges to overcome are the scientific ones. An increasing body of work is amassing showing improved efficiencies and performance, however the vast majority of this is with simple substrates at warm temperatures (Rader and Logan, 2010, Call et al., 2009, Cheng et al., 2006b, Zhang et al., 2010). Evidence that BES work at low temperature is conflicting (Jadhav and Ghangrekar, 2009, Cheng et al., 2011), the only published study of a large scale 'hydrogen producing' MEC did not produce hydrogen (Cusick et al., 2011), and MECs studies using real wastewater as a substrate are limited, the longest documented study runs reactors for 7.6 days (Wagner et al., 2009).

1.1. Aim and objectives

The overall aim of this research is to understand if BES can be used as a sustainable method of wastewater treatment.

Much work has been and is being carried out fine tuning BES technologies within laboratories, testing new materials and moving towards greater output efficiencies, however large volumes of this work is conducted at warm temperatures and with simple artificial substrates (Hu et al., 2008, Logan et al., 2008, Selembo et al., 2009a, Tartakovsky et al., 2009). This research does not strive towards making such efficiencies, but answers the following fundamental questions of: can they work with

real wastewaters? and, can they work at realistic temperatures? this was addressed by completing the following objectives:

- Quantifying the amount of energy available in the wastewater
- Analysing the start-up and community development of MFC systems.
- Testing the operation and performance of MFC reactors at low temperatures
- Monitoring the performance of MEC reactors with wastewater substrate
- Building and testing a pilot scale MEC reactor run at a wastewater treatment site.

Chapter 2. Determination of the Internal Chemical Energy of Wastewater

Parts of this chapter have been published as Heidrich, E.S., Curtis T.P., and Dolfing J., Determination of the Internal Chemical Energy of Wastewater. Environmental Science & Technology, 2011. 45(2): p. 827-832.

The wastewater industry is facing a paradigm shift, learning to view domestic wastewater not as a waste stream which needs to be disposed of, but as a resource from which to generate energy. The extent of that resource is a strategically important question. However, the only previous published measurement of the internal chemical energy of wastewater measured 6.3 kJ/L, calculated to be 14.7 kJ/gCOD. It has long been assumed that the energy content in wastewater relates directly to chemical oxygen demand (COD). However there is no standard relationship between COD and energy content. In this study a new methodology of preparing samples for measuring the internal chemical energy in wastewater is developed, and an analysis made between this and the COD measurements taken. The mixed wastewater examined, using freeze drying of samples to minimise loss of volatiles, had 28.7 kJ/gCOD, whilst domestic wastewater tested had 17.8 kJ/gCOD nearly 20% higher than previously estimated. The size of the resource that wastewater presents is clearly both complex and variable, but is likely to be significantly greater than previously thought. A systematic evaluation into the energy contained in wastewaters is warranted.

2.1. Introduction

Every one of us produces at least around 40 $gBOD_5$ (biochemical oxygen demand consumed over 5 days), in waste every day, in richer countries this is likely to be nearer 80 $gBOD_5$,(Mara, 2004), equating to around 60-120 gCOD/person/day (Kiely, 1997). If there were 14.7 kJ/gCOD (Shizas and Bagley, 2004), the only previous published measurement of the energy value of wastewater, with 6.8 billion people in the world, 2.2 - 4.4 x 10^{18} joules of energy per year is available, or a continuous supply rate of 70 - 140 gigawatts of energy, the equivalent of burning 52 - 104 million tonnes of oil in a modern power station, or 12 - 24,000 of the world largest wind turbines working continuously. This estimation does not even include all the energy contained in our agricultural and industrial wastewater.

Despite the resource that wastewater represents, most developed countries spend substantial quantities of energy treating the wastewater so it can be released without harm to the environment, the US uses approximately 1.3% of its total electricity consumption doing so (Carns, 2005, Logan, 2008). The energy for wastewater treatment will be a particular burden in the urban areas of less well-off nations. Wastewater is typically viewed as a problem which we need to spend energy to solve, rather than a resource. If the energy contained in wastewater is harnessed, not only could it help the water industries become self-sufficient in energy or even net providers, but it could also be a modest source of energy in parts of the world which currently lack reliable and affordable energy supply.

Wastewater contains a largely uncharacterised and undefined mixture of compounds, including many organics, likely to range from small, simple chains through to more complex molecules. All organic compounds contain energy stored within their bonds. The energy that can be obtained from wastewater by different processes is varied, methane gas from anaerobic digestion, electricity from microbial fuel cells (MFCs), or hydrogen in the case of microbial electrolysis cells (MECs) (Logan, 2008) or a fermentation process (Davila-Vazquez et al., 2008). Large amounts of research is being undertaken in all of these areas but there has been very little work conducted in quantifying the amount of energy held in wastewater to start with.

The COD of wastewater has long been used as a relatively simple and reliable method of determining the 'strength' of waste, and by inference the energy contained within it. However there is no empirical formula for the determination of the energy content from the COD measurement. The only previous study to attempt to determine the energy content of raw municipal wastewater by experiment was conducted by Shizas and Bagley (2004) using a bomb calorimeter. Here a single grab sample of domestic wastewater from a treatment plant in Toronto was dried in an oven overnight at 103^{o}C before being analysed by bomb calorimetry. It was found that the domestic wastewater had a measured COD of 431 mg/L, and an energy value of 3.2 ± 0.1 kJ/g dry sample; with 1.98 g/L of solids this equates to 6.3 kJ/L. This interesting observation has led to the pioneering interpretation that wastewater contains 14.7 kJ/gCOD (Logan, 2008), which has been cited in the literature several times in particular with relation to microbial fuel cell work (Liao et al., 2006, Schroder, 2008, Logan, 2009). However the oven drying of samples will have driven off many volatile organic compounds, such as

methanol (boiling point 64.7 ^{o}C), ethanol (78.4 ^{o}C), and formic acid (101 ^{o}C). Moreover, the calculations were based on a single grab sample from one treatment plant, and using the COD measurement taken prior to drying, it is very likely that some of this COD will have also been lost before the energy determination was made. The work of Shizas and Bagley (Shizas and Bagley, 2004) provides a valuable starting point for the estimation of energy in wastewater, but given the volatile losses, and the measurement of the COD before these losses have occurred, this value must be an underestimation of the true internal chemical energy of wastewater.

The objectives of this study were to develop an improved methodology for measuring internal chemical energy, to better quantify the internal chemical energy of wastewaters, and to evaluate the relationship between internal chemical energy and COD.

2.2. Materials and methods

2.2.1. *Collection and storage of samples*

Two 24 hour composite samples of influent wastewater were taken, one from Cramlington Wastewater Treatment Plant, which deals with a mixed (i.e. industrial and domestic) wastewater, and the other from Hendon Treatment Plant, primarily treating domestic wastewater, both in the North East of England. Within two hours of collection, 3 L of sample was placed into the deep freeze at -80 ^{o}C, and a further 3 L was placed into an oven at 104 ^{o}C. A sample was stored in a refrigerator at 4 ^{o}C.

2.2.2. *Drying procedures*

After a period of around 48 hours in the oven at 104 ^{o}C the sample was fully dried. This was then ground into a powder using a pestle and mortar, and stored in four measured quantities of approximately 0.5 g in clean, dried sealed containers. The frozen samples were dried using a freeze dryer (Labconco Freezone, Labconco Corp. USA) which when used daily over a period of 4 weeks was capable of drying about 1.5 L of sample, each 20 hour drying period removing a few millilitres of liquid. The samples were stored at -80 ^{o}C between drying for 12 hours whilst the freeze dryer stabilised. This procedure was repeated until enough sample was dried to yield four 0.5 g samples. These were then ground and stored in the same way as the oven dried samples.

2.2.3. *Wastewater analysis*

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total organic carbon (TOC), inorganic carbon (IC), total carbon (TC) and chemical oxygen demand (COD) measurements were carried out in the two days after collection using the refrigerated samples. The methods described in Standard Methods for the Examination of Water and Wastewater (APHA, 1998) were used. TS was also measured using the freeze drying process. Further COD tests were carried out on rehydrated freeze dried and oven dried samples. All measurements were taken in triplicate.

2.2.4. *Energy content*

The energy content of the dried wastes was determined using an adiabatic bomb calorimeter, Gallenkamp Autobomb. The internal bomb was a stainless steel unit surrounded by a water jacket with a volume of 1900 mL, with a further cooling jacket outside with a flow of 300 mL/min. The system also included a mechanical stirrer, ignition unit and a digital thermometer accurate to 0.01 ^{o}C. The effective heat capacity of the system i.e. the heat required to cause a unit rise in temperature of the calorimeter was determined using triplicate samples of pure benzoic acid. This was used to calibrate the heat of combustion of the system components such as the wire and cotton, and the effective heat capacity of the bomb, its water jacket and thermometer. After this determination all of the components of the system were then kept constant throughout the tests. Four samples of benzoic acid were used on each time of operation of the bomb calorimeter to verify the technique.

The samples were dried, weighed to around 1 g, and compacted before combustion in the bomb. It was found that the samples did not fully combust, and therefore they were mixed in a 1:1 ratio with a combustion aid of benzoic acid, a method used by Shizas and Bagley (2004). The exact sample weight and the temperature rise in the surrounding water jacket was recorded and used to determine the energy content of each sample. All measurements including the benzoic acid standards were taken in a randomised order.

2.2.5. *Energy content calculations*

The bomb calorimeter measures the heat of combustion of the bomb's contents. When the bomb is ignited the contents including the fuse wire, cotton thread used to attach the sample to the fuse wire and the fuel, including any benzoic acid used is burnt, and this heat is absorbed by the bomb and its surrounding water jacket. In addition to the heat from the combustion, there is also heat created by the formation of nitric acid from the

nitrogen contained in the air inside the bomb. Moles of nitric acid formed are found by titration of the bombs contents with 0.1M NaOH. It is assumed that there is 57.8 kJ/mol of nitric acid; the oxidation state of the nitrogen is not taken into consideration as is standard practice (Rossini, 1956). The kilojoules contained in the sample are calculated in the following equation:

$$-\Delta U_{c,s} = ((V_w + B)(c_{p,w})(\Delta T) + (-\Delta U_{c,w}) + (-\Delta U_{c,c}) + (-\Delta U_{c,b})(m_b) - (Q_{f,n}\, mol_{nitric})) / m_s$$

Table 2-1 Definition of parameters in the equation above used to calculate energy of combustion

Term	Definition
$-\Delta U_{c,s}$	Energy of combustion at constant volume for sample (kJ/g)
$-\Delta U_{c,b}$	Energy of combustion at constant volume for benzoic acid = 26.42 kJ/g[a]
$-\Delta U_{c,w}$	Energy of combustion at constant volume for fuse wire = 0.013 kJ/g[b]
$-\Delta U_{c,c}$	Energy of combustion at constant volume for cotton = 0.082 kJ/g[b]
V_w	Volume of water = 1940 g[b]
B	Volume of water equivalent to the effect of the bomb container = 390 g[b]
$c_{p,w}$	Specific heat capacity of water = 0.00418/g/^oC[a]
ΔT	Temperature rise (^oC)
m_b	Mass of benzoic acid combusted (g)
m_s	Mass of sample combusted (g)
$Q_{f,n}$	Heat of formation of nitric acid = 57.8 kJ/mol[a]
mol_{nitric}	Moles of nitric acid formed (mol)

[a](Atkins, 2006)

[b]Determined in laboratory

2.2.6. *Measurement of volatile fatty acids*

The loss of known volatile fatty acids (VFA's) was measured for each drying technique using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Triplicate 20 mL samples of 50 ppm acetate solution were dried overnight in an oven at 104 ^oC, and in the freeze dryer. These were then re-hydrated with 20 mL of deionised water, and the VFAs measured.

2.2.7. *Measurement of anions*

The anion content of both wastewaters was measured in triplicate using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent.

2.2.8. *Measurement of volatile halocarbons*

Dried 20mg samples were rehydrated using 20 mL de-ionised water and, 20 mL wastewater samples were sealed within a sample jar, with the addition of 20 mg of salt (KCl). These were left for 24hrs at 30^{o}C, the headspace gas was then analysed using an Agilent 7890A GC split/split less injector linked to an Agilent 5975C MSD

2.2.9. *Statistical techniques*

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run two sample t-tests on the data. Before the tests were performed the data was checked for equal variance and normal distribution, validating the use of a two sample t-test.

2.3. Results

This paper uses an improved methodology: freeze drying the samples prior to using a bomb calorimeter. With this method only a few millilitres of liquid can be removed in a 24 hr operational period. Therefore drying enough wastewater to yield several grams of solids takes between 4 - 8 weeks. Although far more time consuming it is believed this is the best method available for drying the wastewater without raising its temperature and thus removing the volatiles.

Table 2-2 Measured wastewater parameters of the two different samples used in the energy analysis

	Cramlington	**Hendon**
COD	718.4 ± 9.7	576.2 ± 40.8
COD- oven dried	368.2 ± 12.3	324.0 ± 18.1
COD - freeze dried	587.1 ± 32.2	425.3 ± 16.5
Total solids - oven dried	1392 ± 35	1070 ± 60
Total solids - freeze dried	1597 ± 40	1130 ± 20
Total organic carbon	116.5	115.8
Total carbon	181.8 ± 2.3	196.4 ± 1.2
Inorganic carbon	65.3 ± 1.2	80.5 ± 0.1
Volatile solids (standard method)	953 ± 143	427 ± 20
Total Kjeldahl Nitrogen	92.4 ± 0.0	71.9 ± 4.3
Chloride (ppm)	391 ± 10.9	169.6 ± 17.2

Mean ± standard deviation (n=3), all values are in mg/L unless otherwise stated

Table 2-2 shows the differences between the two wastewaters, and the effects of the drying processes on the COD and solids recovery from these wastewaters. Oven drying reduces the measured COD from 718.4 mg/L in the original wet sample to 368.2 mg/L (49% loss) in the Cramlington wastewater and from 576.2 mg/L to 324.0 mg/L (44% loss) in the Hendon sample, whilst freeze drying gives losses of 18% and 26%. The freeze drying process captured 5-12% more mass than oven drying. This demonstrates that freeze drying is a more accurate method to determine the total amount of COD than oven drying. However, even freeze drying resulted in COD losses of 18-26%. This is probably due to the loss of the volatile fraction of the COD such as short chain fatty acids. This was confirmed using ion chromatography where oven dried samples contained 0.000 ppm acetate whereas freeze dried samples contained 1.8 ppm, compared to the original 54.5 ppm. Acetate is one of the smaller and therefore more volatile of the VFA's and is likely to represent some of the greatest losses.

Table 2-3 Measured internal energy content values given as both energy per litre and energy per gCOD using the post drying measurement of COD

	Cramlington		Hendon	
	Oven dried	**Freeze dried**	**Oven dried**	**Freeze dried**
kJ/L	8.3 ±1.8	16.8 ± 3.3	5.6 ±1.0	7.6 ± 0.9
kJ/gCOD	22.5 ± 4.8	28.7 ± 5.6	17.7 ± 3.2	17.8 ± 2.1

Mean of four measurements ± standard deviation
Values for kJ/gCOD are calculated from the COD measurement after drying and re-hydrating, and TS measurement for the given drying method.

The freeze drying method enabled a significantly greater proportion of the energy in the wastewater to be measured, over 50% more for Cramlington (p value 0.010), and 24% more for Hendon (p value 0.044). There are also significant differences between the two wastewaters, with the Cramlington waste being more energy rich (p value 0.019). The energy content per gram of oxidisable material measured i.e. kJ/gCOD is considerably higher for both wastewaters than previous estimates of around 14 kJ/gCOD, for the Cramlington wastewater this is even higher with the freeze dried sample.

The energy captured by the freeze drying process does not equate to all the energy available in the wastewater sample. Based on the percentage losses of measured COD from the original sample to the freeze dried sample (18% for Cramlington and 26% for

Hendon), the actual energy of the Cramlington wastewater could be as high as 20 kJ/L, and 10 kJ/L for the Hendon wastewater.

2.3.1. *Theoretical results - can internal chemical energy per gram COD be calculated from first principles?*

If we were able to evaluate the energy content of wastewater from the COD measurement, this would require an estimation of which organic compounds are present. With this, the internal chemical energy for each individual organic compound can be calculated on the basis of simple thermodynamic calculations as follows (thermodynamic values are taken from Atkins (2006)) based on the principle that 1 gram of COD equals 1/32 mol O_2, i.e. for every 1 mol O_2 there is 32 grams COD.

If we assume that the organic compound present is methane:

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \qquad (1 \text{ mol } CH_4 = 64 \text{ gCOD})$$

The overall enthalpy for the reaction can be calculated on the basis of Hess's Law, which states that the enthalpy of a reaction is equal to the sum of the enthalpy of formation ($\Delta_f H$) of all the products minus the sum of the enthalpy of formation of all the reactants. Using tabulated values for the enthalpy of formation the energy released in the above reaction with methane is as follows:

$$\begin{aligned}
\Delta_f H \text{ (kJ/mol)} &= \sum \Delta_f H \text{ products} - \sum \Delta_f H \text{ reactants} \\
&= 2(\Delta_f H\ H_2O) + \Delta_f H\ CO_2 - \Delta_f H\ CH_4 - 2(\Delta_f H\ O_2) \\
&= 2(-285.83 \text{ kJ/mol}) + -393.51 \text{ kJ/mol} - -74.81 \text{ kJ/mol} - 2(0 \text{ kJ/mol}) \\
&= -890.5 \text{ kJ/mol} \\
&= -890.5 \text{ kJ/mol} / 64 \text{ gCOD} \\
&= -13.9 \text{ kJ/gCOD}
\end{aligned}$$

Analogous calculations for a wide range of organic compounds show that the typical $\Delta_f H$ values of $C_aH_bO_c$ compounds fall within a fairly narrow range of 13-15 kJ/gCOD, with a few exceptions such as formic and oxalic acid with 15.7 kJ/gCOD, ethyne with 16.3 kJ/gCOD and methanol with 17.8 kJ/gCOD. (See Appendix III).

It could be concluded that 13.9 kJ/gCOD is the maximum amount of heat energy that can be gained from methanogenic wastewater treatment. Therefore from a relatively simple COD measurement the potential energy yield would be known. However biodegradation of organic content in wastewater does not necessarily lead to methanogenesis. Some waste streams can be used for biohydrogen production. Here 1 gCOD is equal to 1/16 mol H_2, ($2H_2 + O_2 \rightarrow 2H_2O$) therefore 1 mol H_2 equals 16 gCOD, giving an energy yield of 17.9 kJ/gCOD (286 kJ/mol H_2 / (16 gCOD / mol H_2)).

The simple $C_aH_bO_c$ compounds are not necessarily the only wastewater components, and other classes of compounds such as halocarbons can contain far more internal chemical energy per gCOD. The explanation to this can be supported by writing the equations that describe their degradation down as oxidations of the carbon moiety with reducing equivalents released as H_2, coupled to the oxidation of the H_2 to water. In highly substituted compounds such as organohalogens, less H_2 is potentially available. The oxidation reaction of H_2 to water becomes less important in the overall equation, the ratio of H:CO_2 decreases, increasing the overall value of kJ/gCOD. This is illustrated using methane and one of its halogenated equivalents trichloromethane (thermodynamic data taken from (Hanselmann, 1991)):

Methane

$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (-393.5 + 4(0)) - (-74.8 + 2(-285.8))$

$= 252.9$kJ/reaction

$$4H_2 + 2O_2 \rightarrow 4H_2O$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (4(-285.8)) - (0 + 2(0))$

$= -1143.2$ kJ/reaction

These two values are then added together to give the overall enthalpy of reaction to be -890.3 kJ/mol, this can then be divided by the COD to give -13.9 kJ/gCOD

Trichloromethane

$$CHCl_3 + 2H_2O \rightarrow CO_2 + 3HCl + H_2$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-393.5 + 3(-167.1) + 0) – (-103.1 + 2(-285.8))

= -220.1 kJ/mol

$$H_2 + \tfrac{1}{2} O_2 \rightarrow H_2O$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-285.8) - (0 + 0.5(0))

= -285kJ/mol

The total enthalpy of reaction is -505.9 kJ/mol, giving -31.6 kJ/gCOD.

It becomes clear how important the reducing equivalents of H_2 are in terms of energetic value, this is illustrated in Figure 2-1, (values given in Appendix III). As the number of substitutions of hydrogen increases, so does the value of energy per gram COD. The value of energy per gram of COD can vary far more widely than previously thought.

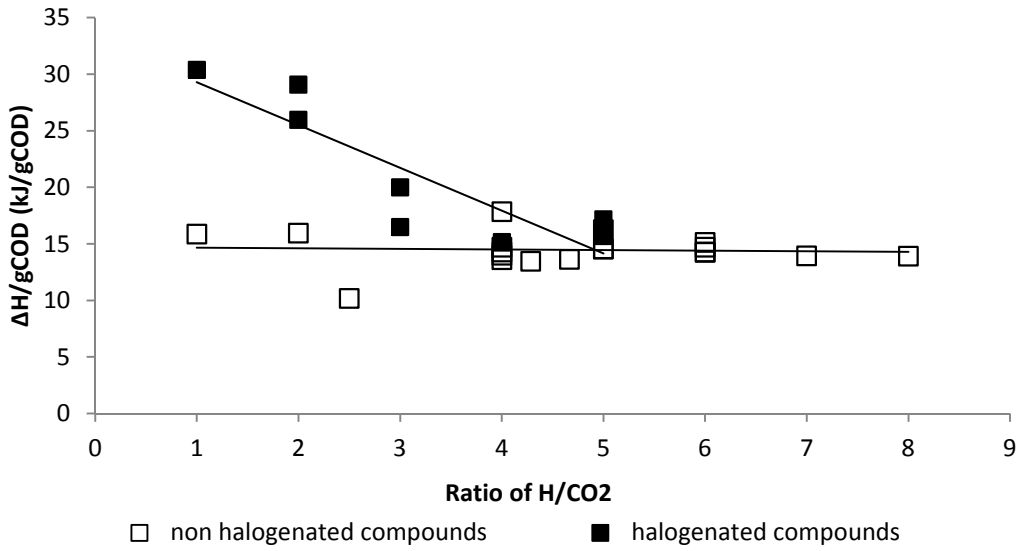

Figure 2-1 Energy content per gCOD of a variety of organic compounds plotted against their degree of oxidation

2.4. Discussion

The predicted energy gained from treatment of municipal wastewaters has been shown to be higher than the previous estimation. The domestic wastewater analysed in this paper has 20% more energy per litre than the estimation made by Shizas and Bagley (Shizas and Bagley, 2004). In addition to this, as the volatiles in their wastewater were not captured, it is likely their sample could have had an energy value around 35% higher, (based on the percentage losses between oven and freeze drying in this study) this would be 8.5 kJ/L. This has a significant impact on the development and implementation of technologies for the treatment of 'low strength' municipal wastewater which pose a greater challenge for the recovery of energy than concentrated waste. These waste streams are clearly richer in energy than previously thought.

The internal chemical energy of the wastewaters per gCOD was greater than expected by comparison to acetate (heat of combustion is 13.6 kJ/gram COD) or glucose (heat of combustion is 14.3 kJ/gram COD). From the data (Table 2-2) of the two wastewaters it can also be seen that the carbon oxidation state plays an important role in determining the energy present. Both samples have a very similar value of TOC (total organic carbon), yet very different COD values. This means that the Cramlington waste with the much higher COD has proportionally more reduction capacity and therefore chemical energy per carbon molecule than the Hendon wastewater. Another possible cause of these high values is that there are compounds within the wastewater that have an energy value, yet are not oxidised during a COD test, most notably urea, which contains 10.4 kJ/g (Atkins, 2006) when combusted, yet undergoes a hydrolysis reaction rather than an oxidation. This compound, which is certain to be present in domestic wastewater (and though it is assumed to hydrolyse in the sewer, a fraction may reach the wastewater treatment site), contributes to the overall energy of combustion of waste but not to the COD measurement, there are likely to be others compounds which do the same. Additionally there could be some compounds which have proportionally far greater energy content per gram of COD than glucose and acetate, such as organohalogens or other highly substituted compounds.

Although many simple halocarbons are no longer in use, some more complex ones are still common in many industrial processes for example as solvents and pesticides, and in the manufacture of in plastics, adhesives, sealants and paper pulp. Organic halocarbons also occur in natural systems. Chlorination treatment also introduces this halogen which

could then combine with other organics. It can be seen from the anion analysis (Table 2-2) that there is significant quantity of chloride ions in the wastewaters, with more in the Cramlington wastewater. This wastewater is likely to contain a more diverse range of organic compounds as this site takes in mixed wastes, some of which must have a high specific energy value and volatility, resulting in high energy wastewater. Volatile halocarbons, however, were not detected with the GC MS method described.

The energy values found in this study are also higher than that reported by Shizas and Bagley (2004). However the calculations in their paper were based on oven dried wastewater energy data, versus a COD measurement taken from the original wastewater sample, which in our study was found to be reduced by about 50% after oven drying. If the same calculation algorithms were used on the data in the present paper then the Cramlington and Hendon wastewaters would contain 11.6 kJ/gCOD and 9.9 kJ/gCOD respectively, while they actually contained at least 2.4 times higher (28.7 kJ/gCOD) and 1.8 times higher (17.8 kJ/gCOD), these calculations are shown in Appendix IV. Thus the energy reported per gCOD cited in the literature (Logan, 2008) based on the Shizas and Bagley paper (Shizas and Bagley, 2004) is probably a substantial underestimation. By comparison to the Hendon domestic wastewater the energy of their municipal wastewater could have had at least 26.4 kJ/gCOD, rather than the 14.7 kJ/gCOD reported.

Clearly not all the energy available in wastewater can be extracted in a useful form as no process is 100 % efficient. Ideally one would be able to measure or calculate the energy biologically available as kJ/gBOD, (although not suitable for anaerobic processes), this is not possible given the unknown and variable composition of wastewater. However knowing the potential energy available would give insight into the types of waste that might be in the waste stream which would also be of importance in the choice of treatment method. Some wastes which may be high in energy value, such as halogenated wastes may be unsuitable or unattractive to some treatment methods. For example one mole of trichloromethane at 506 kJ/mol would only yield 0.25 moles of methane equal to 222 kJ through methanogenic treatment, or one mole of H_2 equal to 286 kJ through biohydrogen production. Although these halogenated compounds are energy rich per gram of COD due to their lack of hydrogen, this actually makes them unattractive to terms of energy extraction for methane or hydrogen production, however

it may be possible to recover this energy using other treatment methods which may be able to capture electrons directly.

In microbial fuel cells (MFC's) the reaction taking place is essentially a combustion reaction, i.e. the organic compound is oxidized to carbon dioxide and water, the difference being that this reaction occurs not as combustion but as redox reactions in two half cells. Importantly, it is the free energy of the organics that determines the maximum electricity yield. This technology could theoretically capture more of the energy available in complex or halogenated compounds than for example methanogenic treatment.

The measurement of the internal or combustion energy of the wastewater and use of this as a basis for efficiency calculations will not necessarily yield all the information required to fully understand the energy flows in such systems. It can be observed using internal chemical energy data, a methanogenic process could in some cases be endothermic, the combustion energy of the methane product being higher than that of the starting substrate. This is the case with the conversion of one mole of acetate (13.6 kJ/gCOD) to one mole of methane (13.9 kJ/gCOD). In this scenario energy appears to have been created. It is actually the Gibbs free energy (the amount of energy that can be extracted from a process occurring at constant pressure) which should be examined for this and other reactions as this parameter informs us of the amount of energy available to organisms for the generation of biomass and an energy rich product. This is also the case for MFC's and MEC's where it is voltage which is measured which relates directly to Gibbs free energy. However without knowing the composition of wastewater, its Gibbs free energy content cannot be determined.

A consequential difference was found between the internal chemical energy measured on freeze dried samples as compared to oven dried samples. This difference was greater than the difference observed by measuring mass alone. This shows that there are significant losses of volatile compounds when a wastewater sample is dried at 104 ^{o}C and that in the case of the mixed wastewaters these volatiles can contain proportionally more energy per gCOD than the non-volatiles captured in both methods. It is shown that, although a clear improvement on the traditional oven drying method, the freeze drying method still results in significant loss of semi-volatiles such as acetate, so even

with the improved method we are still not capturing all the energy available in the wastewater.

Bomb Calorimetry remains the only method for measurement of internal chemical energy or calorific value, and for this method the material must be combustible i.e. dry. To give reasonably accurate results the temperature change in the bomb calorimeter must be in the region of 1 - 3 ^{o}C, usually a gram of substance will provide this. In our analyses this gram was half made up by the use of a combustion aid (benzoic acid) to ensure full combustion and the correct temperature rise. Had the proportion of wastewater to benzoic acid been decreased, making the drying process easier, it was feared that the uncertainty inherent to the introduction of the standard would overshadow the accuracy of the measurements of the samples. Although more challenging the methodology of freeze drying samples is an improvement on previous methods although it does not achieve the full capture of all volatiles. These results begin to get close to the true amount of energy in wastewater, and challenge the assumption that measured COD is equivalent to the amount of energy. Freeze drying, although far more time consuming, therefore should be the method of choice when completing such analysis in particular with complex wastes, despite its far greater time consumption rate unless or until new methods and equipment are developed to reduce the time burden using this principle. One such method could be the use of membranes, in particular through the use of reverse osmosis which would 'trap' molecules as small as salts and allow water to be removed. Such techniques may allow for more rapid, cost effective and efficient drying of samples, thus enabling more sampling to be undertaken.

It is clear from our data that the energy value of different wastewaters is variable, as would be expected; there is no standard relationship to measured COD. Values ranged from 17.7 kJ/gCOD to 28.7 kJ/gCOD, when measuring the COD remaining in the dried sample, however we cannot know how much compounds such as urea contribute to this. This means than a measurement of the amount of oxygen required to oxidise the organics within wastewater is not a simple representation of the amount of energy contained within that waste. This is particularly the case when dealing with mixed wastes, where the energy content is proportionally far greater per gCOD. It seems that 13 – 14 kJ/gCOD is the minimum energy content that could be found in wastewaters, however it may be significantly greater. Given the variability in the amount of energy

per gram COD it seems better to measure this energy directly rather than making an estimation, despite the fact that even with the better drying method there are still losses.

Given the huge amount of wastewater globally and the potential energy stored within it, it is important that this potential energy should be determined. With new technologies such as fuel cells being developed, the estimation of this resource is not as trivial as previously assumed. It has been shown that wastewaters can lie well outside the previously estimated values. A systematic review of the energy contained within different waste streams is needed. This paper examines two wastewaters from a reasonably similar geographical location and has found extremely diverse results. It is hoped that this methodology will be repeated and improved upon in terms of time taken, allowing the dissemination of multiple studies using different wastewaters building up a comprehensive and global picture of the energy available in wastewater. This would form the strategic foundation block to the establishment of new and existing technologies within the wastewater industry harnessing this valuable renewable energy source.

Chapter 3. How many exoelectrogens make a Bioelectrochemical System?

3.1. Introduction

The inoculation and subsequent acclimatisation of a bioelectrochemical system (BES) is fundamental to the operation of such systems (Logan and Regan, 2006, Rittmann, 2006). Yet the origin, abundance and physiology of these organisms is the area of greatest uncertainty in design (Oh et al., 2010).

The main goal of the inoculation and acclimatisation of a reactor is typically to 'get it going' as quickly as possible, typically the sources of seed includes: reactors already working in the lab (Jeremiasse et al., 2009, Cheng et al., 2009, Call and Logan, 2008); anaerobic sludge (Chae et al., 2010, Yang et al., 2009); return activated sludge (Torres et al., 2009); mixtures of sludges; or simply wastewater taken at various stages from the treatment plant (Kiely et al., 2011b, Wang et al., 2008). The source and volume of inoculum varies between studies. There is no consensus of how a BES reactor should be started up, or how long acclimatisation will take. This can lead to problems, highlighted by a pilot scale study where several attempts were made to acclimatise the reactor (Cusick et al., 2011).

The bacteria needed for microbial fuel cells to work are termed exoelectrogens (Logan, 2008) due to their ability to transfer electrons outside their cell. Three transfer mechanisms have been proposed.

Firstly electrons can be transferred through conduction with direct contact between the cytoplasmic membrane of the bacteria and the solid substrate being reduced, this mechanism has primarily been associated with the genera *Shewanella* and *Geobacter* (Myers and Myers, 1992, Mehta et al., 2005).

The second mechanism is an electron shuttle. Some bacteria are able to excrete compounds or shuttles into the electrolyte which are capable of transferring electron to an electrode. Rabaey *et al.,* (2005) found that *Psuedomonas aeruginosa* produced Pyocyanin, a mediator which was not only able to transfer electrons from this taxon to the anode of an MFC, but could also work for other species when introduced back into a mixed culture. Thus, a bacterium unable to transfer electrons itself, may become

exoelectrogenic due to the presence of a different shuttle producing bacteria. *Shewanella* species have been seen to do this with the production of riboflavins (von Canstein et al., 2008).

Thirdly electrons might also be transferred through conductive microscopic pili named nanowires which extend from the bacteria cell to other cells or any other electron acceptor (Reguera et al., 2005). *Geobacter* and *Shewanella* species have both been linked to this activity (Gorby et al., 2006). Putative nanowires have been observed using electron microscopy extending to a conductive surface. Conducting probe atomic force microscopy (Reguera et al., 2005) and conductive scanning tunnelling microscopy (Gorby et al., 2006) have been used to reveal that the pili which had previously been observed as attachment mechanisms for bacteria onto Fe oxides, were highly conductive.

It has been proposed that symbiotic relationships between different bacteria groups enhance the function of mixed cultures and improve process stability (Lovley, 2008), possibly by allowing inter-species electron transfer (Rabaey et al., 2005). Many of the exoelectrogens typically associated with BES's such as *Geobacter sulfurreducens* have limited metabolic diversity, and are only able to utilise the end products of fermentation (Caccavo Jr et al., 1994). A reactor fed with a waste requires bacteria which are able to digest the complex substrates, but may not necessarily be able to utilise the anode for respiration (Kiely et al., 2011c). The hydrolysis step within these food chains has been shown to be the rate limiting step with regard to the current production (Velasquez-Orta et al., 2011).

In general, growth in bacterial systems can be described through the equation $N_T = N_0 exp^{rt}$, where the number of bacteria present at a specific time period (N_T) is equal to the number of bacteria present at the start (N_0) multiplied by the exponential of the growth rate (r) over the time span (t). (Rittmann, 2001). With N_T known various other properties can be calculated such as specific activity and growth yield. However in MFCs these are not well understood (Logan, 2008), although growth rates have been defined for some of the key organisms involved in MFC reactions such as *Geobacter*, (Cord-Ruwisch et al., 1998). A cell yield of 0.07-0.22 g-COD-cell/g-COD-substrate has been calculated (Logan, 2008) from an early study by Rabaey et al. (2003) using total bacterial concentrations within the reactors determined turbidometrically and the total

COD removed during the experiments. Freguia et al. (2007) reported estimates of growth yields of -0.016 to 0.403 mol-C-biomass/mol-C-substrate, based on measurement of the substrate removal which was then used to calculate cell yield through a mass balance approach. Yield has been shown to drop with decreasing external resistance (Katuri et al., 2011).

However the value of N_T is complex and unknown. Although a body of research is growing identifying the functions of bacteria within working BES reactors, little is known of their abundance in a natural sample (N_0) and absolute number within a working system (N_T). Additionally the pattern of acclimatisation, the period is likely to be crucial in the community formation, also remains largely unexplored.

Using the acclimatisation period of reactors the aims of this study were to firstly identify the optimum level of inoculum needed to start a reactor with a view to identifying a protocol for the further experiments. Secondly to estimate the most probable number of exoelectrogens present in a sample of wastewater which can be used as a guide to the sequencing depth needed to find these organisms, and to determine N_0 for a reactor. Thirdly to define the growth rates (r) within MFC systems through examining the start-up phase. With these two factors quantified the N_T can be estimated, as can specific activity and yield. Finally by examining the pattern of acclimatisation on different substrates, key differences in community formation can be identified.

3.2. Method

3.2.1. *Reactor Set-up*

Double chamber tubular design MFC reactors (78 mL each chamber) were used, constructed in Perspex, with an internal diameter of 40mm and length of 60mm. The anode was a 2.5 cm^2 carbon felt (Olmec Advanced Materials Ltd, UK), the cathode a 2.5 cm^2 platinum coated titanium mesh with a surface area 8.13 cm^2 (Tishop.com, UK). The cation selective membrane between the reactor chambers was Nafion® 117 (DuPont, France), with an area of 12.6cm^2. The electrodes were positioned 1cm apart. The components of the reactor were cleaned before use and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK)

The cathode chamber was filled with 50 mM pH 7 phosphate buffer saturated with air for 20 minutes before being added into the reactors. Three different media were used:

1. Acetate solution with added nutrients (Call and Logan, 2008)
2. Starch solution with added nutrients (Call and Logan, 2008)
3. Primary settled wastewater (Cramlington WWTP, Northumbrian Water Ltd)

The quantities of starch and acetate in the nutrient solutions were balanced to give similar total chemical oxygen demand (TCOD) as the wastewater, and were autoclaved (121°C, 15 min) before use. The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into ¼ strength Ringers sterile dilutent (APHA, 1998). The contact time under UV was altered to give effective sterilisation as defined as colony free plates in triplicate at zero dilution. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see Appendix V).

The three medias were sparged under sterile conditions for 10 minutes using ultra high purity (UHP) nitrogen (99.998%), until the dissolved oxygen (DO) as measured on a DO probe Jenway 970 (Bibby Scientific Ltd, UK) reached zero.

3.2.2. *Inoculum*

Screened raw influent wastewater from Cramlington wastewater treatment plant (Northumbria, UK). This wastewater is a mixture of industrial and domestic origin. Samples were stored anaerobically at 4^{o}C and used within 24 hours of collection. The inoculum was also sparged with UPH nitrogen before use.

3.2.3. *Start –up and acclimatisation*

Duplicate reactors were inoculated with differing volumes of wastewater (1 mL, 10 mL, 25 mL and 50 mL). The anode compartment was then filled with the sterile substrates. Control ‘reactors’ (using no inoculum) were run during each test. An inverted 50ml syringe filled with UPH nitrogen was placed into the refilling port on top of the anode chamber to provide an anaerobic headspace. The cathode chamber once filled was left open allowing the diffusion of oxygen into the liquid. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor. A data logging

multimeter (Pico ADC-16, Pico Technologies, UK) was attached to record voltage output every 30 minutes. Reactors were allowed 800 hours at room temperature (20-25 ^{o}C) to show acclimatisation before the experiment was ended. With the acetate fed experiment a further set of reactors were run with lower dilutions of inocula, 0.01 mL, 0.1 mL and repeated 1mL with 25 mL as a positive control.

3.2.4. *Enumeration of bacteria*

The total number of aerobic culturable bacteria present in the wastewater samples used for inoculation was approximated using a spread plate method 9215C (APHA, 1998), with peptone based nutrient agar (Lab M Ltd, UK). Serial dilutions were undertaken into sterile ¼ strength ringers solution, with each dilution plated in triplicate. Plates were incubated at 37 ^{o}C for 48 hours. Anaerobic bacteria were enumerated using a basal salts media (Shelton and Tiedje, 1984) with 1 g/L of both yeast extract and glucose as a carbon source. The media was autoclaved (121 ^{o}C for 15 min) and sparged with sterile UHP nitrogen for 20 minutes. A volume of 9 mL was then added to sterilised Hungate tubes, 1 mL of wastewater was then added to five tubes, and dilutions made down to 10^{-12} with five replicates at each dilution. The headspace of the tubes was sparged with nitrogen, and the tubes incubated at 37 ^{o}C for two weeks. The number of bacteria was determined using the MPN methodology (APHA, 1998).

3.2.5. *Analytical methods*

TCOD of the medias and inocula were measured in duplicate according to standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) colorimetric reagent kit. Volatile fatty acids of the media and inocula were measured in duplicate using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. When the current of the cell had dropped to zero TCOD and VFA's of the cell were measured using the same method as inocula and media above.

3.2.6. *Most probable number (MPN) calculations*

With non-standard dilutions the pre-calculated MPN tables (APHA, 1998) cannot be used. The MNP is calculated through a series of iterations based on a Poisson and binomial distributions (Blodgett, 2005) using the following formula, solving λ for the concentration:

$$\sum_{j-1}^{k} \frac{g_j m_j}{1 - \exp\ (-\lambda m_j)} \ = \ \sum_{j-1}^{k} t_j m_j$$

K = the number of dilutions,

gj = the number of positive (or growth) tubes in the jth dilution,

mj = the amount of the original sample put in each tube in the jth dilution,

tj = denotes the number of tubes in the jth dilution

A probability is assigned to each possibility of the number of bacteria based on the outcome at each dilution, a positive outcome being voltage produced in by the reactor. The number with the highest probability is given as the MPN. Using the spreadsheet developed by Bloggett to make these iterative calculations, the most probable numbers of exoelectrogens per 100 mL of wastewater can be calculated (Garthright and Blodgett, 2003) using the inocula volumes, and the test outcome.

Thomas' simple formula which is based on the same principles as the full test, but a simpler algorithm to solve, can also be applied to the data set, this formula has been shown to have substantial agreement (Thomas, 1942). Using only the lowest dilution that doesn't have all positive tubes, the highest dilution with at least one positive tube and the dilutions in between the following calculation can be made:

$$MPN/100\ ml \ = \ \frac{no.positive\ tubes \ \times \ 100}{\sqrt{(ml\ sample\ in\ negative\ tubes) \times (ml\ sample\ in\ all\ tubes)}}$$

The confidence limits of this calculation at the 95% level can be calculated using Haldane's formula (Haldane, 1939):

m_1, m_2, m_3 ……. denotes inoculation amounts ranging from the largest to the smallest of the chosen dilutions

g_1, g_2, g_3 ……. denotes the number of positive tubes at the corresponding dilutions

$$T_1 = \exp(-MPN \times m_1)\ , T_2 = \exp(-MPN \times m_2) \ldots\ldots\ldots etc$$

$$B = [g_1 \times m_1 \times m_1 \times T_1/((T_1 - 1)^2)] + [g_2 \times m_2 \times m_2 \times T_2/((T_2 - 1)^2)] + [g_3], [\ g_4] \ldots. etc.$$

$$Standard\ Error\ of\ \log 10\ (MPN) = \ 1/\left(2.303 \times MPN \times (B^{0.5})\right)$$

95% confidence intervals are given by:

$$Log_{10}\ (MPN) \pm 1.96 \times Standard\ Error$$

3.2.1. *Growth rate, specific activity and yield calculations*

Growth rate of bacteria (μ) is classically calculated by quantifying the number of bacteria at two time intervals. In this experiment voltage is deemed to be a suitable proxy for exoelectrogenic bacteria, the rate of voltage rise being equivalent to the rate of growth. It is assumed that each bacterium is capable of donating an amount of electrons therefore an increasing number of electrons are donated to the circuit (i.e. the voltage increases at a constant resistance) as the absolute number of bacteria increases, (it does not represent an increasing ability to metabolise), i.e. voltage is deemed proportional to bacterial number. This can be from the growth rate expression:

$$N_T = N_0 e^{\mu t}$$

Where N_T is the number of bacteria at time t (in this case the voltage), N_0 is the number of bacteria (voltage) at time zero (t_0) and μ is the growth rate. Therefore growth can be defined as:

$$\mu = \frac{\ln N_t - \ln N_0}{(t - t_0)}$$

Specific activity (q), defined as moles electrons per cell per second can be calculated over the period of growth as follows:

$$q = \frac{I \times (t_1 - t_o)/F}{N_0}$$

Where I is the current in amps (coulombs/second) as calculate from the measured voltage V, and resistance R calculated through I=V/R, t_1-t_0 is representative of the time period of each measurement, (i.e. every 30 minutes, the total coulombs of charge within this period is therefore I multiplied by 30 minutes multiplied by 60 seconds) and F is Faradays constant of 96485 coulombs/mol e^-. The growth rate and starting MPN is used to calculate the number of cells at each time period N_T. This can be converted to moles of acetate per cell per second (1 mole acetate = 8 moles electrons), to give substrate utilisation (U).

Growth yield (Y) is the amount of biomass or cells produced by the bacteria per mass of degraded substrate measured in g-COD-cell/g-COD-substrate. Rather than use the total COD removed in the reactor, which would also involve COD digested via other routes only the g-COD substrate put to the circuit is used as calculated from the substrate utilisation above. The yield is calculated as follows:

$$Y = \frac{(N_T - N_0) \times W \times COD_{cell}}{\sum_{t0}^{t} U \times COD_{sub}}$$

Where the total cells produced over the growth period N_T-N_0 is multiplied by an estimation of the weight of cells W of 5.3 x 10^{-13} g-cell given in Logan (2008) and the estimation for anaerobically grown cells of the formula of $C_{4.9}H_{9.4}O_{2.9}N$ equating 1.25 g-COD/g-cell, (Rittmann, 2001). The sum of the substrate utilisation U as calculated above is multiplied by COD_{sub} the amount of COD per mole of substrate, 64 for acetate.

3.3. Results

3.3.1. *Number of bacteria in wastewater*

The spread plate counts of the wastewater, and anaerobic multiple tube count indicate there is 8.3 x 10^5 culturable aerobic bacteria, and 6.9 x 10^4 culturable anaerobic per ml of this wastewater, giving a rough estimate of the total bacteria per mL of wastewater to be 10^6. Although this method may over estimate numbers due to some bacteria being able to grow under both conditions, and underestimating numbers due to bacteria being intolerant to the media, the overall value calculated fits in with previous estimates (Tchobanoglous, 1991).

3.3.2. *Most probable number of exoelectrogens*

The number of positive outcomes of each test are shown in Table 3-1. From this the MPN can be calculated shown in Table 3-2. The MPN of exoelectrogens in an acetate fed reactor is 17 per ml of wastewater, this number drops to 1 per ml for a starch fed reactor and 0.6 per ml for a wastewater fed reactor. Superficially it appears that acetate metabolising exoelectrogens are quite rare organisms, starch metabolising exoelectrogens are even rarer and wastewater metabolising exoelectrogens are rarer still.

Table 3-1 The number of positive outcomes for each inocula size out of the total number of reactors run

Inocula size (mL)	50	25	10	1	0.1	0.01
Wastewater	2/2	2/2	0/2	1/2	-	-
Starch	2/2	2/2	1/2	0/2	-	-
Acetate	2/2	4/4	2/2	3/4	1/3	0/2

Table 3-2 The MPN in 1 ml of wastewater given by the two methods stated, numbers in brackets indicate the upper and lower bounds at 95% confidence. The probability of presence in wastewater is calculated from the total count of viable bacteria per 1 ml

Substrate	MPN calculation (Blodgett 2005)	MPN estimation (Thomas 1942)	Probability of presence in 1 ml of wastewater
Wastewater	0.6 (0.3-2.5)	0.8 (0.3-2.5)	6×10^{-7}
Starch	1.0 (0.3-3.2)	1.1 (0.3-4.0)	10^{-6}
Acetate	17.0 (5.5-52)	17.6 (6-51.5)	1.7×10^{-5}

An alternative explanation is that the lower MPNs, and therefore the probabilities of these organisms being present in 1 ml of wastewater, are the product of two or more events. In wastewater and starch there are long chain molecules present which undergo a series of steps in their breakdown. Each step is probably undertaken by different microorganisms. The electrons pass down this chain leading to the final step of donation to the electrode, represented by the acetate reactor. Thus the MPN of the wastewater and starch fed cells is the probable MPN of the acetate fed cells (the number of exoelectrogens) multiplied by the probability of each of the upstream steps. Here all of these steps are simplified into one probability step, however in reality this may be many steps the product of which is equal to 0.04 for wastewater and 0.06 for starch as shown in Figure 3.1.

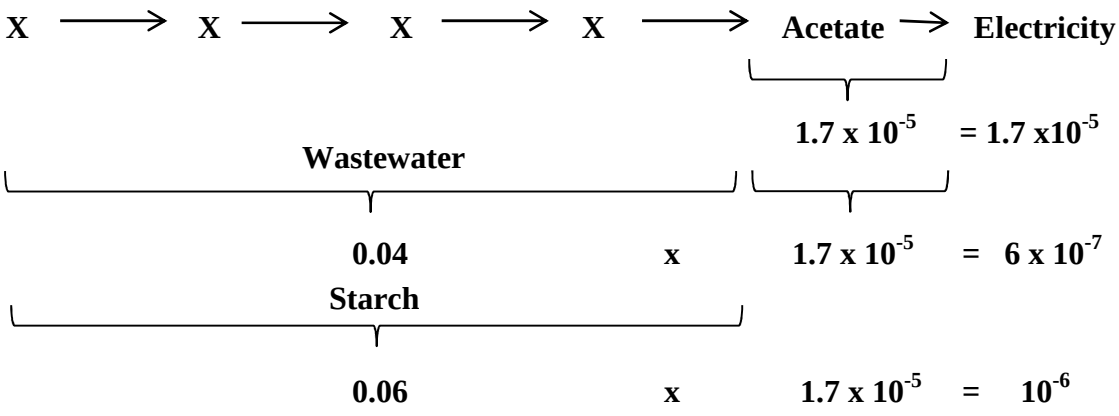

Figure 3-1 Estimated probabilities of numbers of bacteria present in the wastewater begin to produce a working MFC fed on three different substrates of acetate, starch and wastewater based on the numbers determined in the MPN method

3.3.3. *Growth rates*

The individual growth rates for the three different substrates are shown in Table 3-3. The rates were not significantly different (p=0.282 one way ANOVA), and showed agreement with other studies.

Table 3-3 Average growth rates for exoelectrogens fed on different substrates estimated using the rise in voltage measured in the acclimatising reactors

	Average growth rate
Wastewater fed community	0.028 h^{-1} ± 0.013
Starch fed community	0.023 h^{-1} ± 0.005
Acetate fed exoelectrogens	0.035 h^{-1} ± 0.020
Geobacter sulfurreducens (Cord-Ruwisch et al., 1998)	0.023 – 0.099 h^{-1}
Geobacter sulfurreducens (Esteve-Nunez et al., 2005)	0.04 – 0.09 h-1
Fermenting micro-organisms (Rittmann, 2001)	0.05 h-1

3.3.4. *Acclimatisation pattern*

Using an arbitrary value for N_0 (the starting number of bacteria per ml), the known growth rate and the time period over which the experiment was conducted, the pattern of acclimatisation can be modelled.

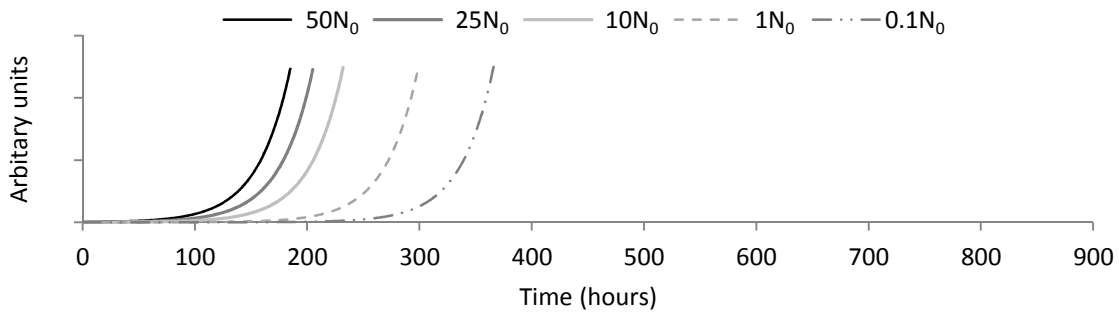

Figure 3-2 Model of the acclimatisation of reactors inoculated with varying amounts of bacteria as denoted by N_0 based on the formula $N_T = N_0 exp^{rt}$ where r the growth rate is the average growth rate determined experimentally of 0.03 hr^{-1} and t time is given on the bottom axis

The pattern of acclimatisation that occurred for the wastewater and starch fed did not follow the model. All reactors acclimatised at the approximate same time. If the growth rates and time are equal, mathematically this means that N_0 is similar for the different volumes of inocula.

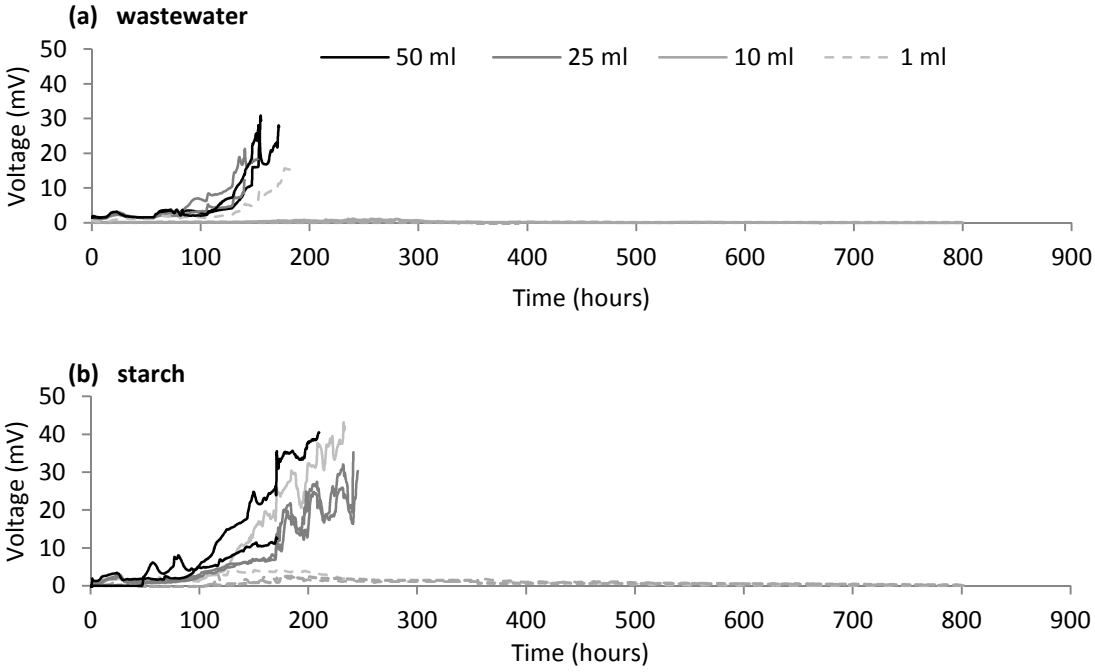

Figure 3-3 Pattern of acclimatisation of the wastewater (a) and starch (b) fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Superficially the pattern observed for the acetate fed reactors appears to follow the model pattern. However this is not the case as the lag time to acclimatisation is over extended with reducing amounts of inocula.

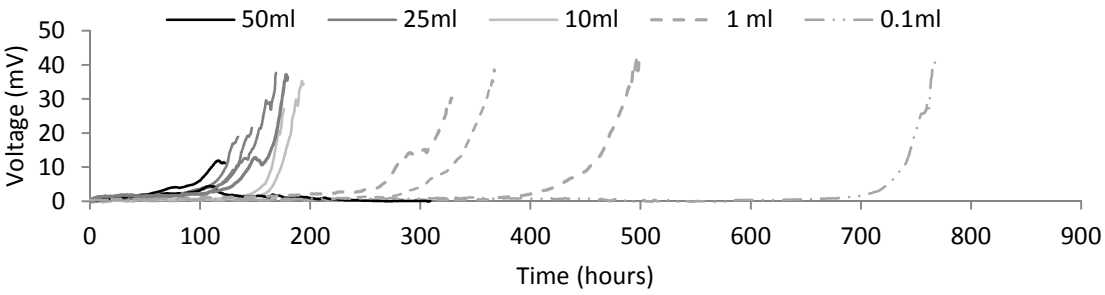

Figure 3-4 Acclimatisation of the acetate fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Using $N_T=N_0\exp^{rt}$ the calculated number of bacteria at the time the reactor inoculated with 0.1 ml (which must have contained at least one bacteria) reaches 10 mV would be 1.8×10^{11} bacteria, equivalent to the predicted number of bacteria in 1 kg of soil (Whitman et al., 1998), and 4×10^{7} times greater than the number of bacteria at 10 mV in the cell inoculated with 50 ml of wastewater (assuming an MPN of 1.7 per ml). This is clearly implausible, growth is not purely exponential, there is likely to be a lag phase with no growth. Yields calculated on the basis of these N_T and N_0 values both with (up to 8 g-COD cell/g-COD) and without (10^{-4} and 10^{-7} g-COD cell/g-COD) growth in the

lag phase give results discordant with the current literature, (these are shown in appendix VII).

3.4. Discussion

If the aim of acclimatising a reactor is to get it going, then it has been shown that a larger volume inoculum will give a quicker (in the case of acetate) and more likely (in the case of complex substrates) successful inoculation, although a proportion of the intended substrate may also be needed. As clear differences were observed between experiments, acclimatisation with the intended substrate is likely to be essential to successful operation. However, more importantly, these results also give insight into the abundance and distribution of exoelectrogenic and other crucial organisms, and to their community development within a reactor.

Discovering the number of exoelectrogenic bacteria per ml of wastewater is a strategically important question. It would inform us of the sequencing depth needed to identify these bacteria. By using the MPN methodology in a series of MFCs and aerobic and anaerobic culturing methods of the same wastewater, an estimation of this number has been gained. Acetate digesting exoelectrogens can be found at an estimated quantity of 17 per ml of wastewater, giving the probability of a bacterium in 1 ml of wastewater being an exoelectrogen as 1.7×10^{-5}, or put differently 0.0017% of the bacteria present in wastewater are exoelectrogenic. With 1000 sequencing reads there would be a reasonable chance of identifying only 1 or 2 exoelectrogens. When compared to the pyrosequencing carried out in chapter 4 a similar answer emerges. Two wastewater samples were analysed, and the total sequencing effort needed to capture 90% of all the sequences in the sample estimated using statistical algorithm as shown in Appendix X. Comparing the total number of *Geobacter* (the known exoelectrogen present in the wastewater samples) found in the sample to the estimated sampling effort, in one sample Geobacter represented an estimated 0.0012 % of the total bacteria, in the other this was lower at 0.00001 %. The two very different approached result in a similar estimation of the number of exoelectrogens present in wastewater. The use of further microbial techniques such as flow cytometry or QPCR would also help the verification of these results.

The number of acetate exoelectrogens is rare: 17 per ml. The number of starch or wastewater exoelectrogens is even lower at 1 per ml. It could be plausible that these are

even rarer organisms, however the likely explanation is that a chain of metabolism is occurring, this fits with the literature (Velasquez-Orta et al., 2011, Kiely et al., 2011c). The probability of achieving a working MFC fed on a complex substrate is therefore the probability of the exoelectrogenic step as identified above, multiplied by the probabilities of each of the upstream steps in the metabolic chain, and is therefore lower than the probability of forming with the acetate step alone. The MPN value is an approximation, yet even considering the upper and lower bounds of the calculation at 95% confidence, as shown in Table 3-2, this pattern is observed. Clearly however this is dependent on the inoculum used; with different inocula such as soil or sludge one would expect different results.

Growth rates, although intuitively demonstrated by the rise in voltage within an MFC, have not previously been calculated. It is an important value to know, especially when modelling such systems. This study calculated the average growth rate of 0.03 hr^{-1}, this value agrees with those documented in the literature from known exoelectrogenic bacteria. No statistical difference is found between reactors fed on acetate and more complex wastewaters, contrary to previous work (Velasquez-Orta et al., 2011) this study shows that the growth rate of exoelectrogens is likely to be the limiting factor.

The pattern of acclimatisation demonstrated within these reactors did not follow the expected pattern. Additionally the pattern observed in the acetate reactors is different to the pattern observed in the reactors fed with more complex substrates. Simple exponential growth does not appear to be happening in either system. The values of N_T within these systems are therefore questionable, as are the calculated yields and specific activities (see appendix VII).

The positive starch and wastewater fed reactors were fewer in number due to the reduced probabilities of the communities forming, but all acclimatised at approximately the same time regardless of the inoculum volume. The growth rates calculated were not statistically different between the different inocula, time was recorded accurately. Explaining this mathematically on the basis of $N_T = N_0 exp^{rt}$ this means either: N_0 is the same for the different inoculum sizes; the N_T of the reactors producing the same voltage is actually different; the rates as defined by voltage rise are not representative of growth rates; or the system may not be described by the equation $N_T = N_0 exp^{rt}$.

More of the acetate cells acclimatised leading to a higher MPN value, the pattern of acclimatisation here does show a clear link to inoculum size, however the size of the lag phase is far greater than would be predicted. Again the rates calculated were not statistically different between the different inoculum sizes and time was also recorded accurately. Here on the basis of $N_T = N_0 exp^{rt}$ either; N_0 is not linearly related to inoculum sizes, i.e. 50 mLs of wastewater contains more exoelectrogens than 50 times 1 ml; the N_T of the reactors producing the same voltage is actually different; there is a lag period before the growth rate starts which is also related, but not linear to, the inoculum size; or again the system is not described by $N_T = N_0 exp^{rt}$.

The MPN method and therefore N_0, is based on the following assumptions: bacteria are distributed randomly within the sample; they are separate, not clustered together; they do not repel each other; and every reactor whose inoculum contains even one viable organism will produce detectable growth or change and the reactors are independent (Blodgett, 2009). It seems likely that exoelectrogens will cluster, there function of passing electrons outside the cell may be used for passing electrons between cells when no external electron sink is available (Bretschger, 2010). In the sequencing data in chapter 1063 *Geobacter* are found in one wastewater sample and 4 in the other, also indicative of clustering. If clustering is occurring, the MPN is likely to be an underestimation as will be N_0 and N_T. This does not however explain the different patterns of acclimatisation observed between the substrates. Additionally the large upper and lower bounds given in the MPN calculations due to the relatively low sample size, could also lead to both under and over estimations of N_0 where the MPN is used.

The relationship of voltage with N_T could be more complex than assumed. Voltage generated from the electrode may be limited by properties relating to the anode itself rather than the bacteria on it, or may quickly reach saturation point of the biofilm, however then one would expect to observe the same pattern in all reactors.

Growth rates are assumed to be represented by the rising voltage measured across the reactors. This may not be the case if the bacterial population has to grow to a certain threshold level (at an unknown growth rate which may different for different inocula sizes) before any voltage is produced. Additionally an assumption is made that increasing voltage is caused by an increasing number of bacteria, not an increasing

capability of these bacteria to donate electrons, this may not be true. Again this does not account for the differences between substrates.

The period of acclimatisation is both highly complex and variable between substrates, yet does show a clear observable pattern, indicating an underlying mechanism. It seems likely that these systems are not described by $N_T = N_0 exp^{rt}$. Such deviations could be caused if the exoelectrogens present N_0 were able to induce electrogenic activity in other bacteria through the excretion of electron shuttles: $N_T > N_0 exp^{rt}$, and in addition a further growth equation of the 'induced' exoelectrogens would act to confuse the picture. In the case of the complex substrate systems something within the chain of metabolism which is unrelated to the bacteria quantity could be triggering the start of the acclimatisation, this causes the reactor to work or fail regardless of the number of exoelectrogens present at the start. In the acetate fed reactors a further factor related to the inoculum size could be causing the extended lag observed, such as the movement of the exoelectrogens to the anode surface.

The period of acclimatisation is not only complex, it is likely to be a period of high competition for resources and possible low efficiency for the exoelectrogens as seen from the low coulombic efficiencies and comparable COD removal in both the positive and negative reactors (see appendix VI).

If the aim of acclimatisation is to merely 'get the reactor going' this study has shown that using a large proportion of wastewater is best. The experiment has also demonstrated that the abundance of organisms needed to start an MFC is low within wastewater, and even lower when these systems are to be fed on complex substrates. The growth rates defined are similar to those observed for exoelectrogenic species in other environments, and are likely to be the limiting factor in MFC acclimatisation. The pattern of acclimatisation a fuel cell is complex and not explained solely by exponential growth. The clear differences between these systems demonstrate the vital importance of acclimatising a community for the eventual use of the reactor. A reactor fed on acetate is different to one fed on wastewater. By developing a greater understanding of this ecology and its development, the move towards more stable biological system can be made. Understanding the nature, abundance and location of these exoelectrogens is crucial.

Chapter 4. Can Microbial Fuel Cells operate at low temperature?

4.1. Introduction

Bioelectrochemical Systems (BES's) are being heralded as a new method of energy efficient wastewater treatment, yielding electrical energy or other products from the bacterial breakdown of organics in an electrochemical cell. For future application of this technology understanding the microbial ecology, community structure and relating this to performance is desirable (Parameswaran et al., 2010) . The majority of fuel cell research is carried out using acetate as a feed at 30^{o}C with the implicit assumption that this will translate into the treatment of real wastewaters at ambient temperatures. To use low strength high volume wastes like wastewater the bacterial communities within BES need to be able to digest complex and variable substrates and do so outside, which in the UK, Europe and many parts of the USA means at low temperatures. If the communities of bacteria able to perform this task do not occur naturally further work and investment into this area may be futile.

As noted above most BES studies are conducted in laboratories at a temperature of 30 ^{o}C (Call and Logan, 2008, Cheng and Logan, 2007a, Selembo et al., 2009b). Few ambient treatment plants will get this warm. Several studies investigating the performance of MFCs over temperatures between 20-30 ^{o}C have found that the maximum power output with acetate was reduced by 9% (Liu et al., 2005a) and 12% (Ahn and Logan, 2010) when the temperature was lowered from 30 ^{o}C to 20 ^{o}C and 23 ^{o}C respectively, using beer waste a 10% drop was seen at these temperatures (Wang et al., 2008). The reduction in performance was lower than predicted by biological process modelling, suggesting that bacterial growth at 32 ^{o}C is not optimal, or that other factors are more limiting (Liu et al., 2005a). Complex wastes were also treated by Ahn and Logan (2010), and it was found that temperature had a greater effect on these than the simple compounds.

Lower (below 20 ^{o}C) and more realistic temperatures have been even less well studied. Min et al (2008) found that at 15 ^{o}C no successful operation was achieved, after 200 hours of operation the experiment was stopped. Cheng et al. (2011) found at 15 ^{o}C start up took 210 hours but at 4 ^{o}C there was no appreciable power output after one month (720 hours) and the experiment was stopped. In the same study a reactor started at 30 ^{o}C was then dropped to 4 ^{o}C and power output was achieved, but around 60% lower than

that the higher temperature. Larrosa-Guerrero *et al*. (2010) operated reactors at 4 ^{o}C and 35 ^{o}C using a mixture of domestic and brewery wastewater, observing a decline in COD removal from 94% to 58% and power density from 174.0 mWm^{-3} to 15.1 mWm^{-3} at the lower temperature.

By contrast Jadhav and Ghangrekar (2009) operated an MFC's in a temperature range of 8-22 ^{o}C and found that the current and coulombic efficiencies were higher than that produced in the temperature range of 20-35 ^{o}C. However in this study temperatures were ambient not controlled and thus confounded by time. They inferred that a reduction in methanogenic bacterial activity at lower temperatures increased MFC performance, although the microbiology of the systems was not examined. Similar results were obtained by Catal *et al.* (2011), here the biofilm was examined using scanning electron microscopy and found to be thicker in the higher temperature reactors.

MFC systems are based on electrochemical and microbiological principles: temperature affects both. The electrochemical impacts of temperature can be calculated using the Nernst equation based on known free energies for substrates such as acetate, or estimated free energies if wastewater is used (Logan, 2008). In bacterial systems rates of reaction roughly double for every 10^{o}C rise in temperature (Rittmann, 2001). However, the actual behaviour of these complex systems at different temperatures and fed on different substrates remains an area of great uncertainty in this field of research.

An increasing number of studies into the microbial communities of BES using techniques such as restriction fragment length polymorphism (RFLP), clone libraries and denaturing gradient gel electrophoresis (DGGE) are adding to the knowledge base we have about these communities. There are advantages to these various techniques such as the high reproducibility and in the case of DGGE and RFLP the large number of samples than can be run (van Elsas and Boersma, 2011, Kirk et al., 2004). However all these techniques are limited in that only a small fraction, (in the case of DDGE estimated at 1-2 % (Macnaughton et al., 1999), of the species present are targeted in these studies, total diversity cannot be estimated from these limited results. Never the less it has been repeatedly shown that *Geobacter sulfurreducens* dominates in acetate fed reactors, although this can vary when reactors are inoculated with different media (Kiely et al., 2011c). As substrates become increasingly complex moving from VFA's

to carbohydrates to actual wastewater the dominant species become more varied (Kiely et al., 2011c). Some wastewater fed reactors were found to be dominated by *Betaproteobacteria* (Patil et al., 2009), although in other studies *Geobacter* still dominates (Cusick et al., 2010).

Most of the techniques that have been used are limited by their capacity to identify the most dominant species within the communities. Next generation sequencing (capable of sequencing to a far greater depth) has now been used in two MFC studies. Lee *et al*. (2010) used FLX Titanium pyrosequencing to sequence four samples of biofilm, triplicate samples were taken from an acetate fed reactor comparing this to a single sample taken from a glucose fed reactor. The profiles found in the samples were not significantly different. A further study by Parameswaran (2010) analysed the biofilm of two MFC reactors fed on ethanol examining the impact to the communities when methanogenesis was prevented in one, identifying the role of hydrogen scavengers.

The aim of this study was to determine if microbial fuel cells can work at low temperatures, and if the inocula affects this. By running reactors fed on both wastewater and acetate the relative importance of the final 'electrogenic' step, and the up- stream hydrolysis and fermentation steps can be evaluated. The impact of temperature, inoculum and substrate on the microbial communities and total diversity within these reactors was examined using next generation sequencing techniques.

4.2. Methods

4.2.1. *Experimental design*

The variables examined were: temperature (warm 26.5 ^{o}C and cold 7.5 ^{o}C); substrate (acetate and wastewater); and inoculum (Arctic soil and wastewater). Each set of conditions were run in parallel duplicate reactors and biofilm samples taken from each. The two series of experiments, acetate and wastewater, were conducted using the same 8 reactors under identical conditions, the two wastewater inoculum samples were used to seed the acetate (wastewater sample1) and wastewater fed (wastewater sample 2) experiments. This is represented in Figure 4-1.

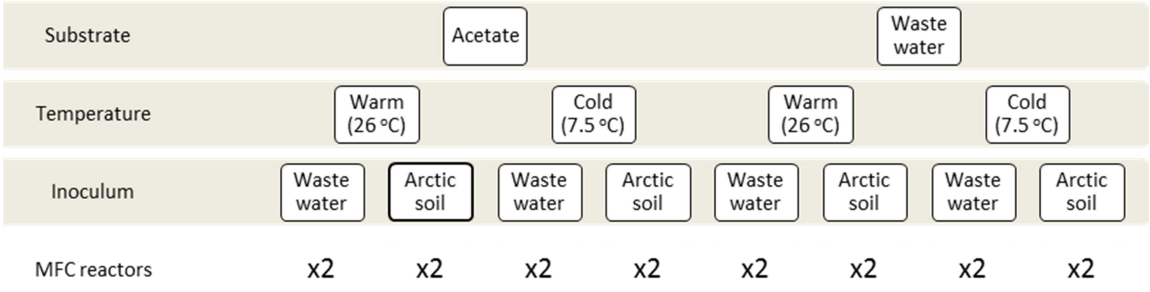

Figure 4-1 Illustration of the multi-tiered reactor conditions used

The warm temperature was chosen to represent the typical ambient laboratory temperatures of many MFC studies. The low temperature is the lowest sustained temperature of a wastewater treatment plant in the North of England (54°58'N, 01°36'W) experienced over a winter period (Northumbrian Water Ltd). The different substrates represent the most commonly used laboratory substrate acetate, and compared to wastewater. The two different inocula were the usual inoculum of wastewater, and Arctic soil (see below) which could potentially have more bacteria with low temperature, exoelectrogenic capability.

Wastewater typically contains 10^5 - 10^6 bacteria per mL (Tchobanoglous, 1991) soils can contain around 10^9 bacteria per gram (Whitman et al., 1998). Many soil environments are low in oxygen, and iron rich, favouring anaerobes and iron reducers and potentially therefore exoelectrogens. Arctic soils have been shown to have to be biologically active, accounting for around 6% of the total global methane sources (Ehhalt et al., 2001). (Hoj et al., 2005, Kotsyurbenko et al., 2004, Metje and Frenzel, 2005). Soil taken from Ny-Ålesund, in the Spitsbergen area of Norway has been shown to contain a wide range of methanogenic groups active at temperatures ranging from 1-25 °C (Hoj et al., 2005, Hoj et al., 2008).

4.2.2. *Reactor design and operation*

Eight identical double chamber tubular MFC reactors (78 mL each chamber) with an internal diameter of 40mm and length of 60mm were used. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (Tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor, and a multimeter to measure the voltage (Pico ADC-16, Pico Technology, UK). The membrane between the reactor

chambers was Nafion 117, with an area of 12.6cm^2. Reactors were sparged with 99.99% pure N2 in the anode chamber, and air in the cathode chamber for 15 minutes after every re-fill.

Four reactors were operated at a temperature of 26.5 ^{o}C in an incubator (Stuart Scientific SI 50, UK), the other four at 7.5 ^{o}C in a low temperature incubator (Sanyo MIR-254, (Sanyo Biomedical, USA). The temperature was logged continuously over the experiment using a EL-USB-1 temperature data logger (Lascar Electronics, UK). The reactors were inoculated and filled with substrate, replacing this every 5-6 days until a stable power generation was achieved. The reactors were then re-filled and three successive 3 day cycles were run logging the voltage over this time. Chemical oxygen demand (COD) removal during each batch was determined using standard methods (APHA, 1998) and Spectroquant ® test kits (Merck & Co. Inc., USA).

4.2.3. *Media and inocula*

Autoclaved acetate media (Call and Logan, 2008) containing 1 g/L sodium acetate was compared to wastewater taken from Cramlington wastewater treatment site (Northumbrian Water Ltd, UK) which was UV sterilised prior to use. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see appendix V). The cathode chamber was filled with 1M pH 7 phosphate buffer. The conductivity of the nutrient media, wastewater and the phosphate buffer was measured using an EC 300 (VWR Ltd, UK) and equalised for the temperatures of 7.5 ^{o}C and at 26.5 ^{o}C.

The wastewater inoculum was collected from Cramlington wastewater treatment plant, a Northumbrian Water site in the North of England, it was raw wastewater collected prior to any form of treatment, and is believed to be of mixed industrial and domestic, COD 0.7-0.8g/L. Once collected the sample was stored in a fridge at 4 ^{o}C within a closed container. The Arctic soil was collected from Ny-Ålesund, Spitsbergen in Svalbard. This was wrapped within three sealed bags and stored at 4 ^{o}C until used. The inocula of wastewater and soil were measured out to 5 mL or 5 g respectively before being added to the reactors. Samples of each inocula were preserved in a 50:50 in a mix of ethanol and autoclaved PBS pH7 in the freezer at -20 ^{o}C for microbial analysis.

4.2.4. *Microbiological techniques*

At the end of each experiment the anode was removed aseptically from the chamber using aseptic technique and preserved in a 50:50 mix of ethanol and autoclaved PBS pH7 and stored in a freezer at -20 ^{o}C. A 5 ml or 5 g sample of the original inocula was also taken and preserved in this way. The inocula samples were pelletized and the DNA then extracted. With the anode samples the bacteria that had dispersed into the liquid was pelletized and then added to the central section of the anode felt cut from the whole anode. The DNA was extracted by placing this sample into the beaded tube of a FastDNA Spin Kit for Soil (Qbiogene MP Biomedicals, UK). Extraction was completed according to the manufacturer's instructions. The samples were then pyrosequenced following amplification of the 16s rRNA gene fragments.

The primers used were F515 (GTGNCAGCMGCCGCGGTAA) and R926 (CCGYCAAT-TYMTTTRAGTTT). Each sample was labelled with a unique 8 base pairs (bp) barcode connected to a GA linker. Sequencing was completed from the Titanium A adaptor only forward from the F515, capturing the V4 region and most of the V5 region with a Titanium read of 400-500 bp. Triplicate PCR reactions were carried out using the Roche FastStart HiFi reaction kit (Roche Diagnostics Ltd., UK) and subject to the following optimised thermal cycles: initial denaturation at 95°C for 4 minutes; 23 cycles of denaturation at 95°C for 1 minutes; annealing at 55°C for 45 seconds; extension at 72°C for 1 minute; final extension at 78°C for 8 minutes. An automated thermal cycle Techne TC-5000 (Bibby Scientific, UK) was used.

The triplicate samples were then pooled and cleaned using QIAquick PCR Purification Kit (Qiagen, UK). The DNA concentration was quantified by UV-Vis spectrophotometry using a Nanodrop 2000 (Thermo scientific, USA). The individual samples were pooled to give equal concentrations of all reactor samples, and double concentration of the wastewater and arctic soil seed. Sequencing was carried out by the Centre for Genomic Research (University of Liverpool, UK) using the Roche 454 sequencing GS FLX Titanium Series.

4.2.5. *Data analysis*

The pyrosequencing data set was split according to the barcodes and unassigned sequences were removed[1]. The flowgram files were cleaned using a filtering algorithm Amplicon Noise (Quince et al., 2009) to give the filtered flowgram file. Filtering at a minimum flowgram length of 360 bp including the key and primer before first noisy signal, all flowgrams were then truncated to 360 bp. A pairwise distance matrix was then calculated using the Pyronoise algorithm (Quince et al., 2009). This uses an iterative Expectation-Maximization algorithm which constructs denoised sequences by clustering flowgrams using the initial hierarchical clusters generated in the previous step and the filtered flowgram file. The cut-off for initial clustering is set at 0.01 and the cluster size is 60, as recommended by Quince et al. (2009). The flowgrams can then be denoised.

PCR errors were then removed again using Seqnoise, generating a distance matrix using the Needleman-Wunsch algorithm for pairwise alignment. The optimal parameters used here were the cut-off for initial clustering of 0.08 and cluster size of 30. Chimera removal was completed using the Perseus algorithm (Quince et al., 2011) which for each sequence searches for the closest chimeric match using the other sequences as possible parents. (Quince et al., 2011). The sequences are then classified and the good classes filtered at a 50% probability of being chimeric, producing the final FASTA file which is denoised and chimera free ready for analysis in QIIME (Caporaso et al., 2010).

Using the QIIME pipeline tutorial the following analysis was completed: assigning taxonomy using Greengenes (http://greengenes.lbl.gov) at the 97% similarity level; creating an OTU table; classification using the RDP classifier; summary of taxonomic data from classification; generation of rarefaction data of the diversity in a reactor; calculation of the differences between the reactors; performing Principle Co-ordinates Analysis (PCoA); jackknifing and bootstrapping to understand uncertainty in beta diversity output; and generating Unweighted Pair Group Method with Arithmetic Mean (UPMGA) trees for hierarchical clustering of samples. The dissimilarity of the community structure between duplicates was examined using both a weighted (relative abundance) and unweighted (presence/absence) phylogenetic diversity metrics using

[1] *The analysis of the pyrosequencing data was carried out by Dr Matthew Wade, a Bioinformatics researcher within the School of Civil Engineering and Geosciences at Newcastle University.*

UniFrac, giving a distance matrix containing a dissimilarity value for each pairwise comparison. The raw OTU table generated was used to produce the species abundance pattern (with the log abundance normalised to the number of sequences in each sample) and the rank abundance curves, (where percentage abundance is used to normalise samples).

An estimate of the total diversity for each sample was calculated using the Bayesian approach as described in Quince et al. (2008), where the 'posterior distribution' of the taxa area curve is estimated, from the known distribution of the data gathered in the sequencing. Three distributions are modelled: log-normal; inverse Gaussian; and Sichel, and deviance information criterion (DIC) are used to compare the fit from each model. The lower the deviance or DIC values the better the model fit, those models within 6 of the best DIC value can be considered as a plausible fit. Using the fitted abundance distributions the sampling effort required to capture 90% of the taxa within that sample is estimated.

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run analysis of variance (ANOVA) tests on the experimental data, and t-tests on the distance matrix data for the sequences samples. Data were checked for normality prior to completing ANOVA, and if necessary the Box-Cox transformation was used.

The performance of the MFC reactors were analysed on the basis of three variables: % COD removal as measured; coulombic efficiency (CE); and power density (mW/m^2). The latter two variables were calculated using the measured COD and voltage within the cells, as described in Appendix VIII. Correlation of the community structure with these performance factors was done using BEST (Biological Environmental and Stepwise method) within Primer 6 (Primer-E Ltd. UK).

4.3. Results

4.3.1. *Cell acclimatisation*

All 16 reactors acclimatised and produced voltage. The acetate fed reactors showed a clear pattern of acclimatisation related to both temperature and inocula with the warm reactors acclimatising first, and the Arctic soil inoculated reactors starting first as shown in Figure 4-2. The cold wastewater inoculated reactors did not produce current until after around 800 hours, longer than the time allowed in previous studies (Cheng et al.

2011, Min et al (2008). The acclimatisation of the wastewater fed reactors was only affected by temperature: the warm reactors started producing current at day 1, the cold reactors at day 20. All duplicates behaved in a very similar way.

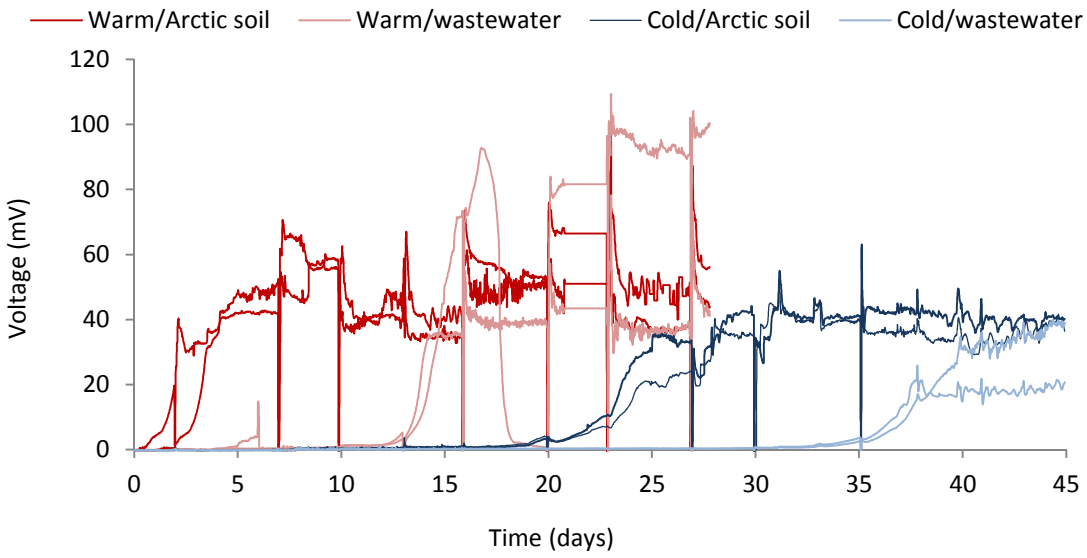

Figure 4-2 Acclimatisation of the acetate fed reactors inoculated with the two different inocula and run at warm (27.5 ^{o}C) and cold (7.5 ^{o}C) temperatures

4.3.2. *Cell performance*

Over the three batch runs, the reactor performance was variable especially within the warm reactors, as seen in Figure 4-3. The variation in performance was not a function of either the inocula or the substrate and the highest variation was seen between the duplicates.

Three measures of performance averaged for each reactor over the triplicate batches are shown in Figure 4-4. The coulombic efficiency is higher in the acetate fed reactors; and the COD removal is higher in the wastewater fed reactors. Power densities do not appear to vary with substrate, inoculum or temperature, however two individual reactors had considerably higher power densities than the others and their duplicates: acetate warm ww 2; and wastewater warm soil 1.

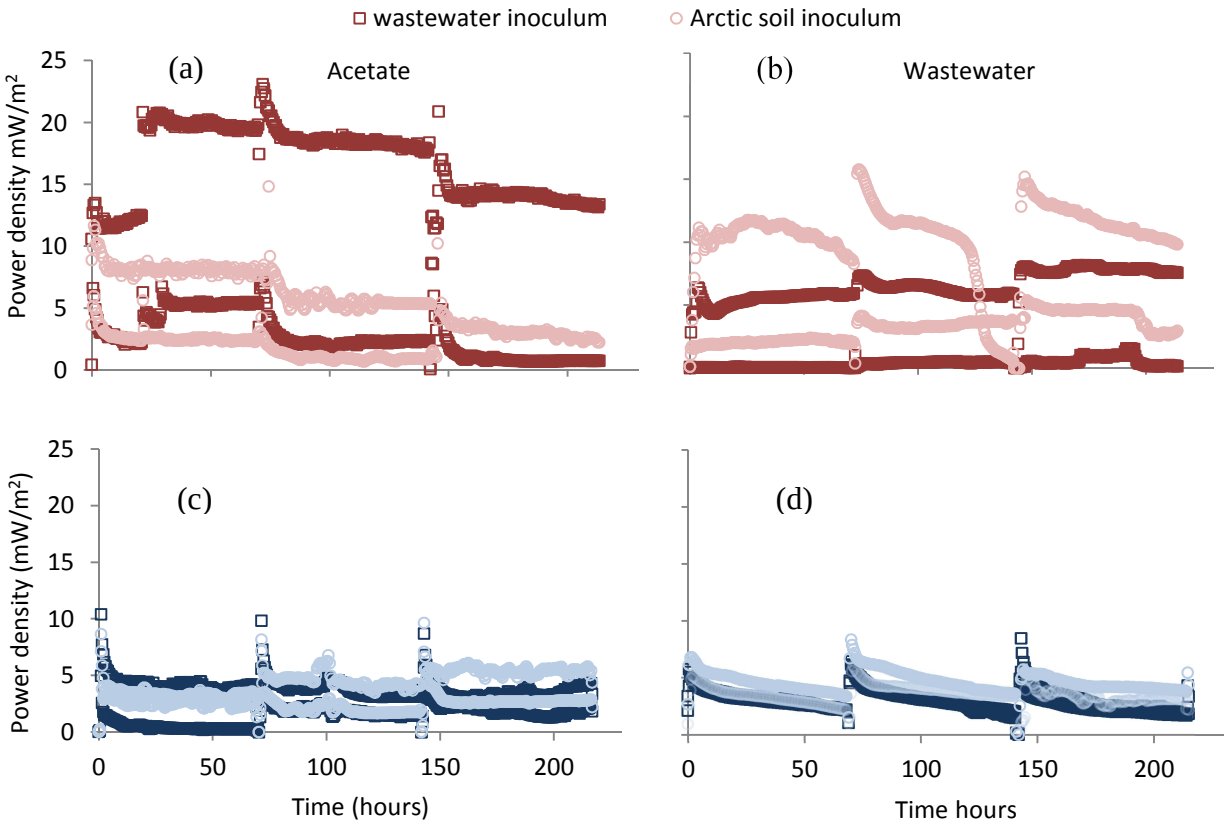

Figure 4-3 Power density plots showing the three consecutive batch runs for: (a) acetate fed reactors run at 27.5 °C, (b) wastewater fed reactor run at 27.5 °C (c) acetate fed reactor run at 7.5 °C (d) wastewater fed reactor run at 7.5 °C

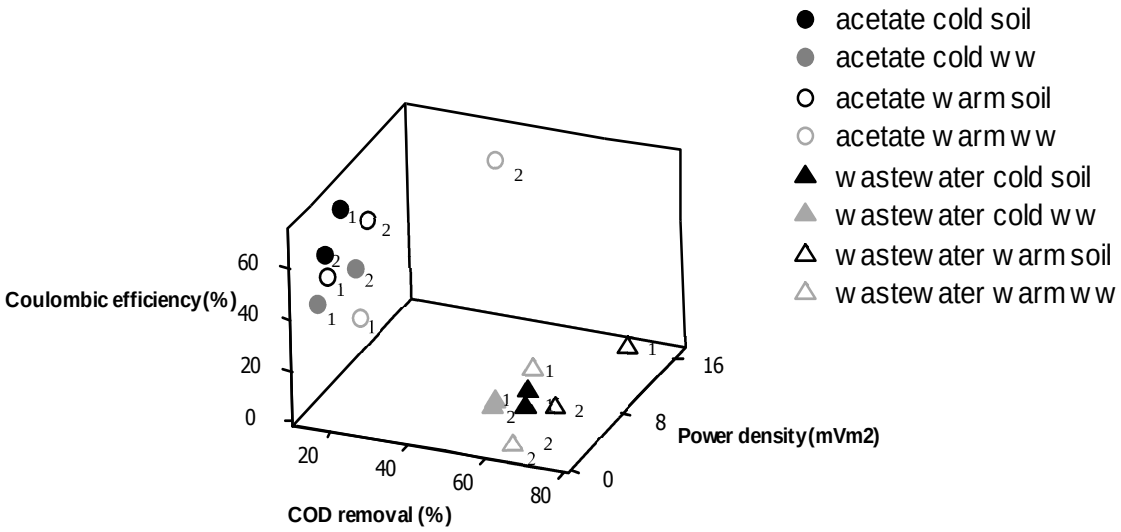

Figure 4-4 3D plot showing reactor performance in terms of Coulombic efficiency, COD removal and power density of the various reactor conditions, duplicates of each condition are labelled on the plot next to the symbols

By performing an ANOVA on the three performance indicators using the factors of feed, temperature and inocula a complex picture emerges. The power density results, i.e. the ability of the biofilm to put electrons to the circuit, were not normally distributed, when transformed, none of the performance factors analysed were significant (feed p = 0.746, inoculum p = 0.249, and temperature p = 0.147). For coulombic efficiency both inoculum (p=0.009) and feed (p=0.000) were significant yet temperature was not. The acetate fed reactors performing better (54.5%) than wastewater fed ones (12.3%), and the Arctic soil inoculated reactors performing better (37.4%) than the wastewater inoculated ones (29.4%). The reactors fed wastewater removed significantly more COD (62.1%), than the acetate reactors (19.4%) (p=0.000) the warm reactors also removed more (45.9%) than the cold ones (33.7%) (p=0.000), the type of inoculum was not significant. Two way ANOVA was performed between each interaction with each performance indicator. For CE the interaction between substrate and inoculum was significant (p = 0.057) with the inoculum having a much stronger effect with the acetate feed than the wastewater feed, and the Artic soil acetate fed reactors performing the best. The interaction between substrate and inoculum was also significant in the COD removal (p = 0.008), the Arctic soil inoculum having a higher COD removal in the wastewater fed reactors, but a lower COD removal in the acetate fed reactors than the wastewater inoculum. No other interactions were significant.

4.3.3. *Similarity of duplicate reactors*

It is seen in the data above that the duplicate reactors performance varied considerably, especially for the warm temperature reactors. Using the sequencing data a Unifrac dissimilarity matrix was plotted, using phylogenetic information the ‘distance’ between each sample is quantified and corresponds to the degree of similarity (Appendix IX). The values show that the duplicate reactors fed with acetate are indistinguishable (p=0.000). This was observed with both the weighted analysis which incorporates information on relative abundance of each OTU, and the unweighted analysis which is based on the presence or absence of each OTU. The wastewater fed duplicate reactors were typically different, with the exception of the hot Arctic soil inoculated reactors (p=0.000). The two wastewater inocula samples taken from the same treatment plant but at different plants were also indistinguishable (p=0.000). This pattern is also observed in Figure 4-5, where the acetate duplicates are paired, and appear to cluster on the basis of temperature. The wastewater fed reactor duplicates are not paired together and do not

cluster with temperature or inoculum. Further details of the bacteria groups present within these reactors can be found in Appendix XI.

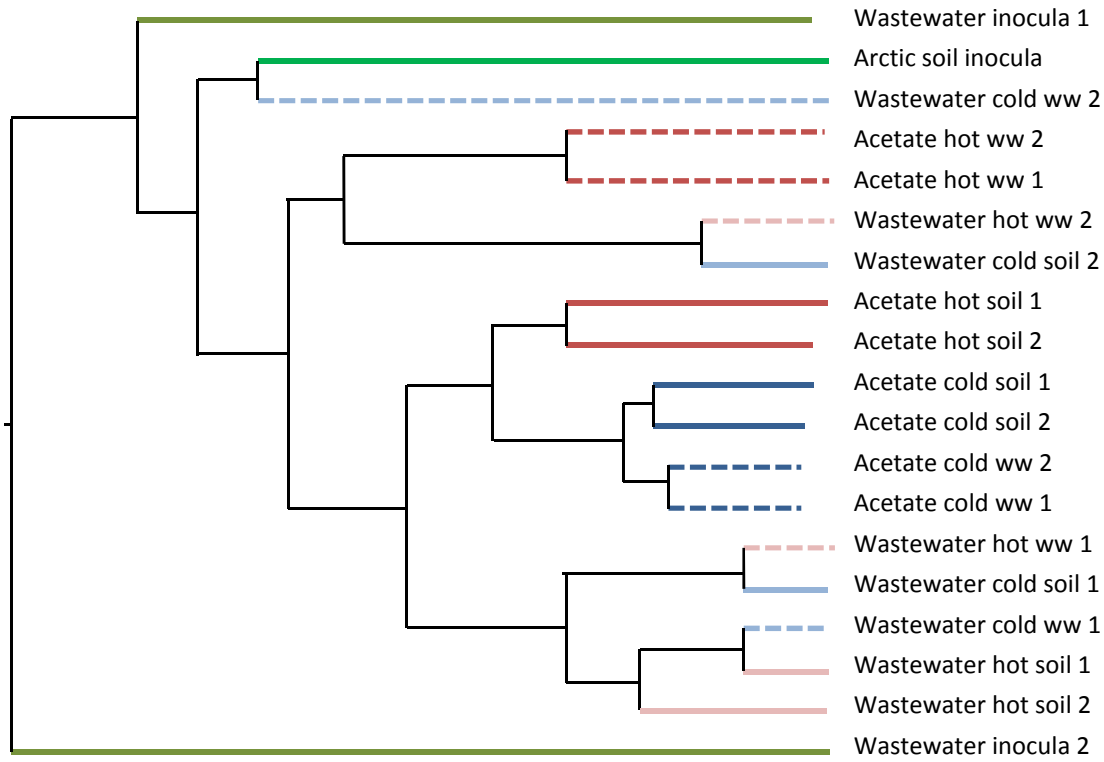

Figure 4-5 Dendrogram resulting from the UPMGA hierachical weighted clustering of samples, the length of lines is relative to the dissimilarity between samples, groupings of samples are denoted by the coloured end portion of the lines

4.3.4. *Microbial diversity*

In total 19 samples were analysed. The number of sequences per sample ranged from 8112 to 77436 with a total number of observations of 549178. The species abundance pattern plotted from the OTU table shows a large variation in the diversity of the samples shown in Figure 4-6. As expected the Arctic soil inoculum is the most diverse, followed by the wastewater inocula. The acetate fed reactors however are considerably more diverse that the wastewater fed reactors, the most diverse of these (acetate cold soil 2) has a similar diversity to the wastewater inoculum, and the least diverse (acetate warm ww 2, the reactor with the highest power density) is similar to the most diverse of the wastewater fed reactors.

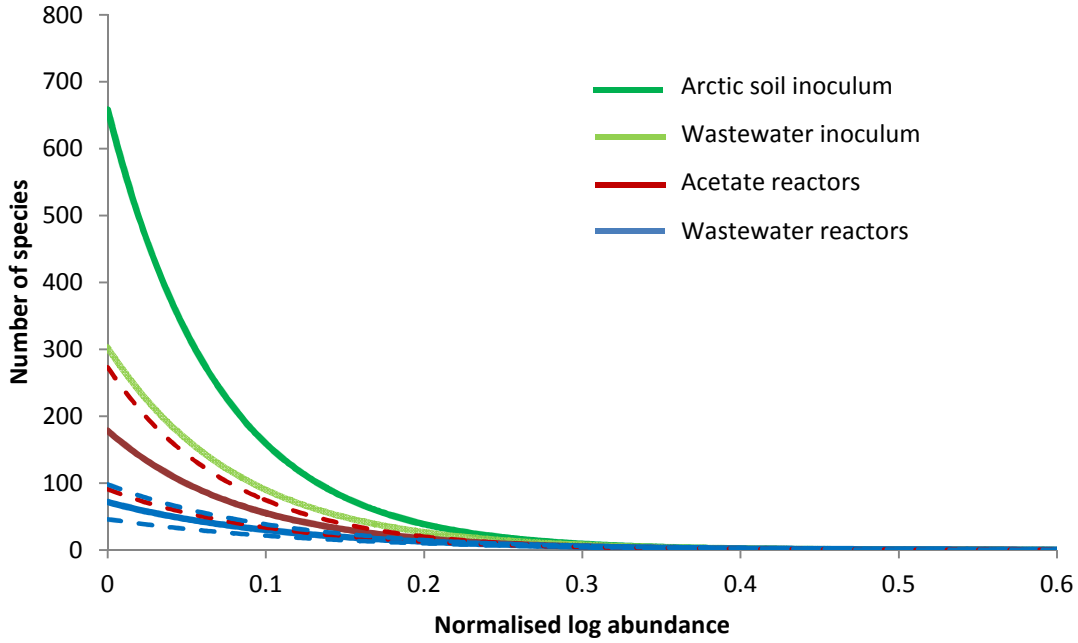

Figure 4-6 Species abundance pattern, the number of species is plotted against the log abundance normalised to the total number of observations for each sample. The plots for the acetate and wastewater fed reactors are averages of the eight reactors used, the highest and lowest within each substrate grouping are shown with the dashed lines. The wastewater inoculum line is an average of the two samples

The observation of the greater diversity in the acetate fed reactors is also seen in the total diversity estimates. A summary of these values is presented in Figure 4-7 where is clearly seen that for all the three distribution models the acetate fed cells have a higher predicted diversity, and that the acetate soil inoculated reactors have a higher total diversity than the wastewater inoculated ones. Performing a nested ANOVA on the Box Cox transformed total diversity estimates, shows that the acetate fed reactors have a statistically significantly higher diversity (log-normal $p = 0.001$; inverse Gaussian $p = 0.000$; and Sichel $p = 0.027$). Within the acetate fed reactors the Arctic soil inoculated reactors have a higher predicted diversity (log-normal $p = 0.006$; inverse Gaussian $p = 0.003$; and Sichel $p = 0.013$), the lower temperatures also give higher diversity (log-normal $p = 0.037$; inverse Gaussian $p = 0.012$; and Sichel $p = 0.029$). There is a strong interaction between the acetate feed and the inoculum type ($p = 0.024$) but not with temperature ($p = 0.156$) observed in both the log-normal and inverse Gaussian distributions. The full tables of diversity predictions, DIC values and estimate sampling requirements can be found in appendix X.

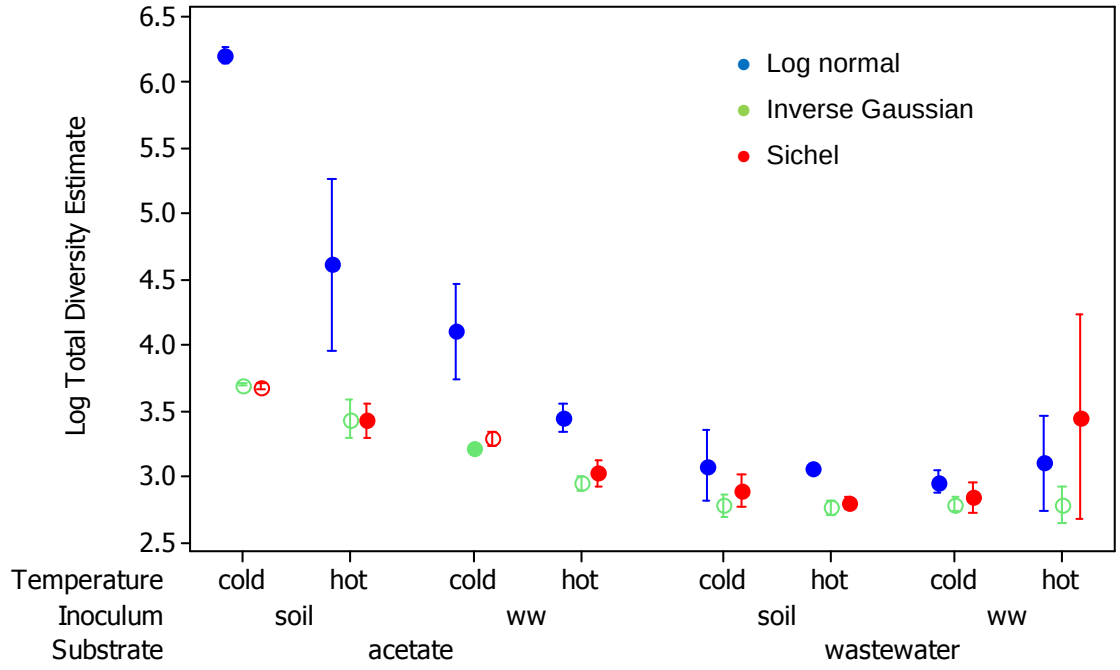

Figure 4-7 The estimates of total diversity for each set of reactor conditions, the three points within each sample are the mean of the duplicate samples modelled to log-normal, inverse Gaussian, and Sichel estimates, the best fit according to the DIC values is denoted by a closed circle, lines are one standard error of the mean

4.4. Discussion

All the reactor conditions tested produced current showing that MFCs can function at low temperatures, with real wastewaters and the bacteria required for them to do so can be found within the wastewater itself. This finding is of great significance to the industrial feasibility of MFC technology for wastewater treatment.

The power output produced by the MFCs was not significantly affected by either temperature feed or inoculum. Although some warm reactors achieved a power density much higher than the cold reactors, due to the variability between reactors this was not significant. The reasons for this variability, were not discovered, no statistical link could be made between the community structure and the power density. The higher coulombic efficiencies within the acetate fed reactors did not translate into higher power densities, only low amounts of COD was converted efficiently into power. Whereas in the wastewater fed reactors more COD was converted less efficiently producing a similar power. In terms of wastewater treatment, this high COD removal, albeit at low CE, is an advantage.

The lack of temperature effect seems at first to be unlikely. Based on the laws of thermodynamics, the free energy available in many chemical reactions decreases as temperature decreases. However in a fuel cell system the energy available is the difference in energy between two half reactions. As both the half cells are equally affected by temperature, the difference between them, or energy available does not decrease with lower temperatures (Appendix II). This is a simplification, many other factors such as dissociation constants and partial pressures of gases will affect the energy, additionally the metabolic activity of the bacteria also reduces with lower temperatures (Rittmann, 2001), however these do not appear to be having a significant impact although may be responsible for some of the variability in performance. On the basis of the results presented here, it can be asserted that low temperature systems have a similar level of energy available for both bacterial metabolism and electricity production as higher temperature systems.

The lack of temperature effect could be caused by the reactor design itself. The inherent inefficiencies and overpotentials within the reactors could be limiting the performance such that the temperature effect is not observed, i.e. all the reactors are working at the limit of their performance and warming them cannot result in improvements. If lower temperature reactors did prove to have slower microbial kinetics, as would be expected and as is indicated by the slower acclimatisation in the cold reactors this could be overcome through relatively simple engineering solutions such as increasing the size of the anode. An increase in the size of the anode would give a greater surface area for the biofilm to grow, and therefore more active bacteria to compensate for the slower metabolic rates.

A further counter intuitive result of this study it that the acetate fed cells have a higher microbial diversity than the wastewater fed cells. It would be assumed that in a wastewater fed systems that the complexity of the substrates available for metabolism, and different metabolic pathways would result in a higher diversity of bacteria, with different groups digesting different substrates at different times. With acetate fed reactors, the only metabolic pathway within a fuel cell should be the direct breakdown of acetate and donation of electrons to the electrode, the most efficient species should dominate theoretically leading to a much less diverse community. This is not seen to be

the case, with a higher diversity in the acetate fed cells being shown both by the species abundance pattern and by the analysis of all the total diversity estimates.

It is proposed that the diversity of the systems is determined not by the diversity of the metabolism within it, but by the overall energy available to the bacteria, and that the free energy available to bacteria in the acetate reactors is greater than in the wastewater reactors. This energy difference could be due to several reasons: acetate may have more free energy per g COD than wastewater; the free energy in acetate may be more accessible to the bacteria, i.e. it is easier to degrade than many of the compounds in wastewater; or that energy is lost during the metabolic chain, with acetate this chain is short, therefore the losses are low, within wastewater these chains are much longer and therefore the losses of energy are greater, this would also produce the coulombic efficiencies observed. The fact that there is no observed difference in the diversity between the warm and cold reactors is further evidence that the energy available in these is actually similar.

Results indicate that the energy flux within a microbial system is key to determining the ecology of that system. The total free energy available is likely to affect the balance of births and deaths of individual species, with greater energy resulting in more births i.e. greater abundance and therefore ultimately greater diversity. The free energy will also impact on the speciation rate (i.e. a greater number of births will ultimately lead to greater chances for speciation). This is counter to the theory that a diverse range of substrates available would provide a variety of different metabolic pathways for different organism to exploit, and therefore lead to a higher diversity.

If a quantitative link could be made between the free energy in a system and the diversity modelling of these complex biological ecologies, being able to understand such phenomena as acclimatisation, adaptation and functional redundancy, and ultimately therefore the manipulation of biological systems becomes a greater possibility (Curtis and Sloan, 2006). We are however still a long way from this in the plant and animal world ecologists have argued there is no single species/energy link (Clarke and Gaston, 2006) and even if it was the key parameter the free energy in wastewater systems cannot yet be reliably measured. Although it is evidenced here that free energy may be the key in determining diversity, a conclusive answer cannot be

given let alone a quantitative link on the basis of these results alone, further research is required.

A further effect on diversity is seen with the inoculum, which interacts with the substrate. The Arctic soil inocula has a greater diversity which seems to be carried forward into the acetate fed cells, a greater number of these species surviving within the reactors where energy may be plentiful. As the performance of the acetate and wastewater fed cells is similar despite the increased diversity of the acetate reactors, it could be concluded that this increased diversity is non-beneficial, or at least neutral to the performance of the reactor. Thus although wastewater reactors will always have lower coulombic efficiencies due to the losses within the metabolic chain, they may actually be more efficient at turning the energy available into wastewater digesting biomass and electricity.

The majority of fuel cell research is conducted at warm temperatures and with simple substrates. It has been shown in this research that reactor performance is not significantly affected by the temperature, neither is the diversity of the community developed. Inoculating reactors with cold adapted organisms does not have any benefit on the performance of the reactors. The substrate fed to the reactor again has little impact on the performance, however results in very different diversities.

It is generally assumed that an acetate fed reactor may represent the optimum conditions for an MFC, however this may not be the case. These findings suggest that wastewater feed has less available energy and therefore results in a more efficient biomass being formed. This has positive implications for the introduction of bioelectrochemical systems into wastewater treatment.

Chapter 5. Time taken until failure for MEC's fed on acetate compared to those fed on wastewater

5.1. Introduction

In 2005 a discovery was made that a microbial fuel cell could be turned into a microbial electrolysis cell adding a small supplement of electricity at the cathode to produce products such as hydrogen gas (Rozendal et al., 2006, Liu et al., 2005b). This new technology has spurned much excitement and research into increasing the performance and gas yield of such reactors (Wang et al., 2011b, Sleutels et al., 2011, Cheng and Logan, 2011). The aim of this research being to achieve a commercially viable and sustainable means of treating waste organics (Oh et al., 2010, Rittmann, 2008, Clauwaert et al., 2008).

Substantial steps have been taken towards enabling the implementation of this technology. Low cost and more robust alternatives to many of the materials used in an MEC have been discovered such as stainless steel (Call et al., 2009) and nickel (Selembo et al., 2009a) cathodes. Alternative membrane materials have been trialled successfully (Rozendal et al., 2008c), as well as not using a membrane at all (Clauwaert and Verstraete, 2009). Anodes with greater surface areas have been found (Call and Logan, 2008) as well as methods to enhance the performance of the carbon anodes (Cheng and Logan, 2007b). New cell architectures and configurations have also helped improve performance (Cheng and Logan, 2011, Wang et al., 2010). Such developments have seen the performance of these reactors increase from hydrogen production rates of 0.01-0.1 m^3H_2/m^3reactor/day (Liu et al., 2005b, Rozendal et al., 2006) to 17.8 m^3H_2/m^3reactor/day (Cheng and Logan, 2011), although the same rise in not seen in the electrical recoveries of these systems 169% (Rozendal et al., 2006) 533% (Liu et al., 2005b) in the initial studies to 115% (Cheng and Logan, 2011) due to the higher input voltages used. All of this research has used acetate as a model compound.

Research with complex substrates is more limited. The ability of MECs to digest complex substrates has been proved such as domestic wastewater (Ditzig et al., 2007), piggery wastewater (Jia et al., 2010), potato wastewater (Kiely et al., 2011a) and end products of fermentation (Wang et al., 2011a, Lalaurette et al., 2009). Limited research has been conducted into the long term performance of MFCs and MECs, deterioration in performance of an MFC after a year of operation has been attributed to the gas

diffusion cathode (Zhang et al., 2011). Marine MFCs used as batteries to power offshore monitoring devices have been monitored for up to a year (Reimers et al., 2001, Tender and Lowy, 2004) and 18 months (Lowy et al., 2006), power production was maintained over this period although in two studies it did deteriorate steadily (Lowy et al., 2006, Reimers et al., 2001), and in another there were occasional drops in the output (Tender and Lowy, 2004). Such studies may not directly translate to MFCs or MECs used for wastewater, in a marine environment the ionic concentrations, gradients and flows will be different, as will the bacteria.

By analysing all the published papers in the area of MECs up to October 2011 the limited scope of how well we understand the long term performance of these systems especially when fed on real wastewaters becomes clear, as seen in Figure 5-1.In 26% of papers the duration of the experiment was not given. In many other cases this time frame is not stated explicitly but can be inferred using the tables, graphs and other information given. In relatively few articles the durability is highlighted as a factor. Two research articles have however been published which indicate the technology might have long term applicability with experiments lasting 9 months (Lee and Rittmann, 2010) and 8 months (Jia et al., 2010) , both running on acetate. Although several other studies do state a decline in performance over time (Jeremiasse et al., 2009, Rozendal et al., 2008b, Lalaurette et al., 2009, Hu et al., 2009).

With acetate fed reactors, 73% of all MEC studies, the time scales mentioned range from 4 to 6480 hours, with 1159 as the average. However when wastewater is used, (only 10% of laboratory studies) the range is between 12 and 184 hours, with an average of 122.5 hours, this time of operation is significantly different ($p=0.000$, two sample T test). For other substrates such as VFA’s and glucose the average run time is 276 hours. This is shown in Figure 5-1, the studies with no time frame stated are not included in the graph. The explanation for this disparity is not evident in the literature, in one study acetate and piggery wastewater are compared directly with acetate reactors running for 8 months and the experiments with wastewater lasting just 12 hours, no reason for this experimental procedure is given (Jia et al., 2010). There is a clear gap in this area of research.

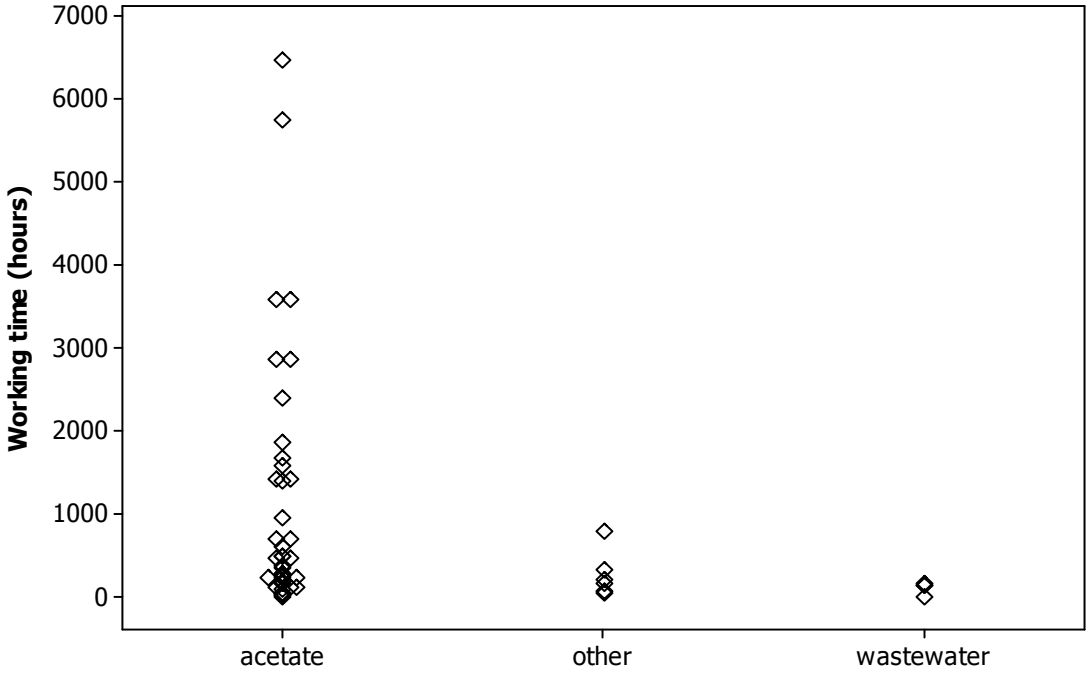

Figure 5-1 The working time of all MEC studies documented in the literature to date (Oct 2011), shown for the different substrates

If MECs are to be a viable and sustainable treatment option for the future then we need to gain an understanding of their long term performance with real wastewaters. Most of the research in MECs does not use real, or even complex artificial wastewaters, and most are run over a relatively short period of time. If this research is to translate into application, this relies on two key assumptions:

1. Real wastewaters containing mixture of simple and complex organic molecules will behave in the same way as acetate, a simple readily digestible molecule most frequently used in BES research. We know this not to be the case with anaerobic digestion (Rittmann, 2001).
2. A system that works at a particular efficiency for a short period of time will do for a long period of time. This is again unlikely as even with the clean technology of chemical fuel cells, long term durability tests have lasted around 4000 hours (166 days), although a couple of studies have extended this to 1.5 and even 3 years (Schmittinger and Vahidi, 2008). Failure is associated with blocked membranes, electrode deterioration and many other factors that may increase overpotentials. Biological systems have the added complexity of the behaviour of microorganisms.

Failure in laboratory batch fed wastewater reactors has been observed many times during preliminary laboratory testing. The aim of this research is to determine if wastewater fed MEC laboratory reactors are capable of operating over the same time periods as acetate fed reactors, and, if this is not the case, to identify the reasons why.

5.2. Method

5.2.1. *Reactor design and set up*

Double chamber MEC reactors (78 mL each chamber) were used which were of a tubular design, internal diameter of 40mm, length 60mm. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. The membrane between the reactor chambers was Nafion 117, with an area of 12.6cm^2. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 1 Ω resistor, 0.7 V supplied using a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong), and a multimeter to measure the voltage (Pico ADC-16), logged every 30 minutes onto a computer.

All reactors were cleaned and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK). The cathode media was 50 mM phosphate buffer, which was sparged with 99.99% pure N_2 for 10 minutes prior to being put into the reactors. The acetate based anode media used was that of Call and Logan (Call and Logan, 2008), during the tests where this was supplemented with protein, Aspargine was added to give an equivalent level of nitrogen to that measured in the real wastewater. The wastewater used was raw influent wastewater (post screens prior to primary sedimentation) from Cramlington wastewater treatment plant. The anode media was sparged for 10 minutes with N_2 prior to use. All reactors were initially acclimatised in MFC mode as per the method used in other studies (Call and Logan, 2008, Cheng and Logan, 2007a, Hu et al., 2008, Wagner et al., 2009), inoculated with 25 ml of raw wastewater and fed acetate media.

The gas produced by the cathode side was captured via a liquid displacement method in a 12 ml glass tube with a septa fitted to the top for sampling. The volume of this gas was measured by drawing it into a 5 ml gas tight syringe (SGE Analytical Science,

Australia). The anode gas was captured in an inverted 10 ml syringe placed into the top of the reactor and filled with the N_2 gas.

5.2.2. *Analytical procedures*

The following analysis was conducted in duplicate for both the effluent and influent of the cathode and anode liquids of each batch run. The chemical oxygen demand (COD) using standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) kit tubes. Volatile fatty acids (VFA's) were measured using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. The anion content using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The pH was measured using a pH probe (Jenway 3310, U.K.) and conductivity using an EC 300 probe (VWR Ltd, UK). The anode and cathode potential was measured using Ag/AgCl reference electrodes (BASI, U.K.) during each batch.

Hydrogen gas was measured on a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using triplicate injections of each sample, set against a three point calibration run once at the start of the measuring period and once at the end using standard calibration gases (Scientific and Technical Gases, U.K.). These gas measurements were verified using a Trace Ultra GC TCD with a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

GC-MS analysis of gaseous hydrocarbons, including halomethanes, was performed on a Agilent 7890A GC in split mode; injector at (280°C), linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software in full scan mode (10-150 amu/sec). A standard containing 100 ppm of three chloromethanes was injected (100ul headspace) followed by the reactor headspace samples (100ul) every 2 minutes. Separation was

performed on an Agilent fused silica capillary column (60m x 0.25mm i.d) coated with 0.25um dimethyl poly-siloxane (HP-5) phase. The GC remained at 30°C temperature for 90 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 20 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library if greater than 90% fit.

5.2.3. *Microbial analysis*

An assessment of the level of microbial activity occurring in the reactors was needed to give an understanding if failure was caused by a reduction or complete elimination of microbial activity, or conversely a competitive but non complementary microbial process. Methods involving the extraction and quantification of DNA from the anode biofilm were not suitable for this purpose as this would capture both the alive and active DNA and that DNA remaining on the biofilm from bacteria which were dead or inactive. Ribonucleic acid (RNA) is used within cells to convert DNA i.e. the genetic code into working proteins (Rittmann, 2001); it can therefore be used as a proxy for the amount of biological activity occurring in the cell (Milner et al., 2008, Low et al., 2000). As RNA is so susceptible to contamination and degradation, the simple and relatively quick approach of measuring the amount of nucleic acid extracted on a Nanodrop, and then comparing this directly to the amount of DNA extracted at the same time, would give the most reliable quantitative results.

Duplicate samples of anode material were taken for RNA and DNA extraction, from duplicate reactors sacrificed whilst working, and duplicate reactors after failure. The following procedure was carried out as quickly as possible inside a microbiological cabinet, to prevent the loss of RNA which readily breaks down if contaminated with RNases. All working areas and equipment was cleaned thoroughly with ethanol followed by RNase AWAY (Invitrogen Life Sciences, U.K.), including the anode cutting equipment which had also been washed with detergent and then heated to 240 ^{o}C for 4 hours in a furnace, prior to use. Each reactor at the point of sampling was taken into the microbiological cabinet maintaining the electrical circuit. The reactor was quickly dismantled and using a coring device duplicate 4mm diameter sections of the anode were cut and placed into a sterile RNase free 2 ml eppendorf, containing 1 ml of TRIzol Reagent (Invitrogen Invitrogen Life Sciences, U.K.), the sample was vortexed for 5 seconds to ensure complete submersion in the reagent, and then the samples frozen

at -80 ^{o}C. Duplicate cores were taken in the same way afterwards for DNA extraction and stored in 50:50 ethanol and phosphate buffer at -20 ^{o}C.

Extraction and clean-up of the RNA sample was then completed using a RNeasy Mini Kit (QIAGEN, Germany) as per the manufacturer's instructions. Once cleaned the samples were frozen at -20 ^{o}C. The DNA was extracted using a QBiogene FastDNA spin kit for soil (MP Biomedicals, U.K.) and also frozen in two samples at -20 ^{o}C. The quantity of nucleic acid present was then measured in duplicate on a Nanodrop Nanodrop 2000 (Thermo scientific, USA). The ratio of DNA to RNA could then be calculated for each sample.

5.2.4. *Experimental procedure*

Failure had been observed several times in these bench scale reactors used as MEC's when fed with wastewater. The purpose of these experiments was to determine if this failure was statistically significant, and if so to try and identify the particular cause. In total 12 wastewater fed reactors and 10 acetate fed reactors were used in this study, the materials and architecture of all the reactors were the same, and the same operating procedures observed throughout. The work was conducted at laboratory room temperatures of between 20-25 ^{o}C.

Initially 8 reactors were run, 4 of fed with acetate media and 4 with real wastewater. After each batch of 3-4 days the effluent was analysed for COD, VFA's, anions, pH and conductivity and the gas measured, the reactors were then refilled with N_2 sparged media to the anode and phosphate buffer to the cathode. Once having completed two batch runs producing gas, 2 reactors of each feed were sacrificed and the RNA and DNA were sampled, the remaining reactors were run and sampled as described until gas production ceased, or in the case of the acetate ones until they were stopped at 130 days.

A further experiment was conducted using 4 wastewater fed reactors to eliminate the possibility that a drop in pH in the wastewater fed reactors was causing failure. Duplicate reactors were run containing wastewater, and the same wastewater buffered to pH 7 using 50 mM phosphate buffer. All reactors were run in batch mode and samples as described above until gas production ceased. Examination as to whether the biofilm was damaged/killed during failure was gained by switching the failed MECs to MFC

mode (increased resistance and no external load), and refilling with UV sterilised wastewater (see Appendix V for details of this method).

Due to the observed drop in Cl^- ions prior to the point of failure, it was hypothesised that locally high levels of NH_4^+ at the anode, caused by the degradation of proteins present in the wastewater could be reacting with the chloride ions to form chloramines, which would then kill off the biofilm resulting in failure of the cell. This hypothesis was tested running 4 acetate fed reactors, by supplementing duplicate reactors with protein Aspargine at levels comparable to the wastewater levels as detected through the use of the TKN Standard Method 4500-Norg (APHA, 1998), comparing these to duplicate control reactors with no protein. Again sampling was carried out as above, in addition the effluent of the reactors was analysed for residual chlorine using the DPD test, Standard Methods 4500-Cl D, (APHA, 1998).

A further hypothesis to account for failure and the drop in chlorine was that the chlorination of organics, especially methane could be occurring in the reactors due to the potential of the anode. Under standard conditions, at pH 7 the required potential for chlorination of methane at a Cl concentration of 1 mM is 0.44 V, when considering that the reactors may have a pH slightly deviant from 7, and that the partial pressures of the methane and chloromethane produced would not be equivalent, it is conceivable that the anode potential needed for this reaction could be occurring in the reactors, producing chloromethanes and therefore removing the hydrogen ions from the system and eliminating H_2 production. Again 4 wastewater reactors were run in batch mode with the same analysis as described above, in addition both the anode and cathode gasses were captured and analysed for methane, hydrogen and chloromethane using the instruments and methods stated above. Duplicate reactors fed with acetate were run at the same time and subject to the same analysis. After failure reactors were again switched to MFC mode and the anode gas continued to be sampled.

5.2.5. *Calculations*

The reactor performance was evaluated in terms of the volume of hydrogen produced, and also the coulombic efficiency and electrical energy recovery. The definition of these two efficiencies can be found in section 6.2.5.

5.2.6. *Statistics*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

5.3. Results

5.3.1. *Time taken until failure*

The run time of the reactors is shown in Figure 5-2 as the amount of hydrogen produced at the end of each batch, the reactors terminated at 7 days for RNA sampling are not shown. It is seen that the Acetate fed reactors run for a longer period of time, including those supplemented with protein and produce more hydrogen than the wastewater reactors. The buffered wastewater reactors initially perform well, but then stop producing hydrogen after a short time period.

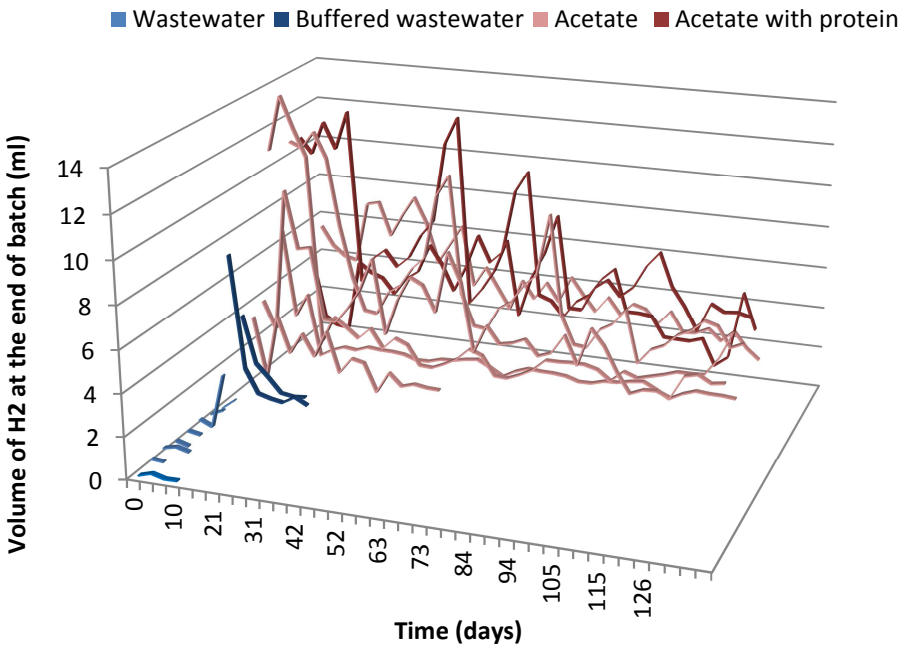

Figure 5-2 Graphic showing the working period of all reactors as indicated by the length on the line along the time axis, the volume of H_2 produced at the end of each batch is given on the y axis as an indication of reactor performance which is seen to be variable, where the line is discontinued this illustrates zero H_2 production and the reactor is deemed to have failed

All 10 of the reactors fed on wastewater failed within 7-17 days of operation, failure was determined by no measureable gas production at the cathode. Of the 8 acetate fed reactors one failed at 56 days, but the others remained functioning until the experiment was terminated after 130 days. With 130 days used as the minimum run time for the acetate fed reactors, the difference in time to failure is significant ($p=0.000$, two sample t-test) as shown graphically in Figure 5-2.

5.3.2. *Reactor performance*

The average performance data collected over the duration of different experiments is shown in Table 5-1. The acetate fed cells have a greater coulombic efficiency and electrical energy recovery. The COD removal is reasonably similar for all substrates, but higher for the buffered wastewater, although this does not translate into improved coulombic efficiency or energy recovery. In all cases there is a large degree of variation, as is seen by the standard deviations. This is also seen through the hydrogen production data in Figure 5-2, which is higher for the acetate fed reactors, but does deteriorate throughout the test period.

Table 5-1 Summary of reactor performance using three different parameters other than H_2 production for the experiments using different substrates, values are the average values of all the reactors run on the given substrate

	COD removal	Coulombic Efficiency	Electrical Energy Recovery
Wastewater	23.2% ± 12.2	7.5% ± 3.9	15.7% ± 20.1
Buffered wastewater	43.8% ± 7.8	3.7% ± 1.7	13.5% ± 16.6
Acetate	28.6% ± 11.5	10.9% ± 2.0	33.0% ± 15.1
Acetate with protein	32.3% ± 13.4	10.4% ± 3.6	35.1% ± 22.9

Values represent average of all the batch experiment run on the given substrates where hydrogen was produced, ± one standard deviation.

There is a reduced performance between the acetate fed reactors as compared to the wastewater ones of around 50 % if energy recovery is considered.

5.3.3. *Biological processes*

The average RNA: DNA ratio of the duplicate samples show that there is significant difference between the working and failed reactors at the 90% confidence interval ($p=0.068$ two-sampled t-test). This difference is more pronounced with the wastewater fed reactors, where the average ratio value for the working reactors is 11.5 compared to the failed reactors 3.9. The acetate working reactors have an average a ratio of 6.1, with the single failed cell being 4.2.

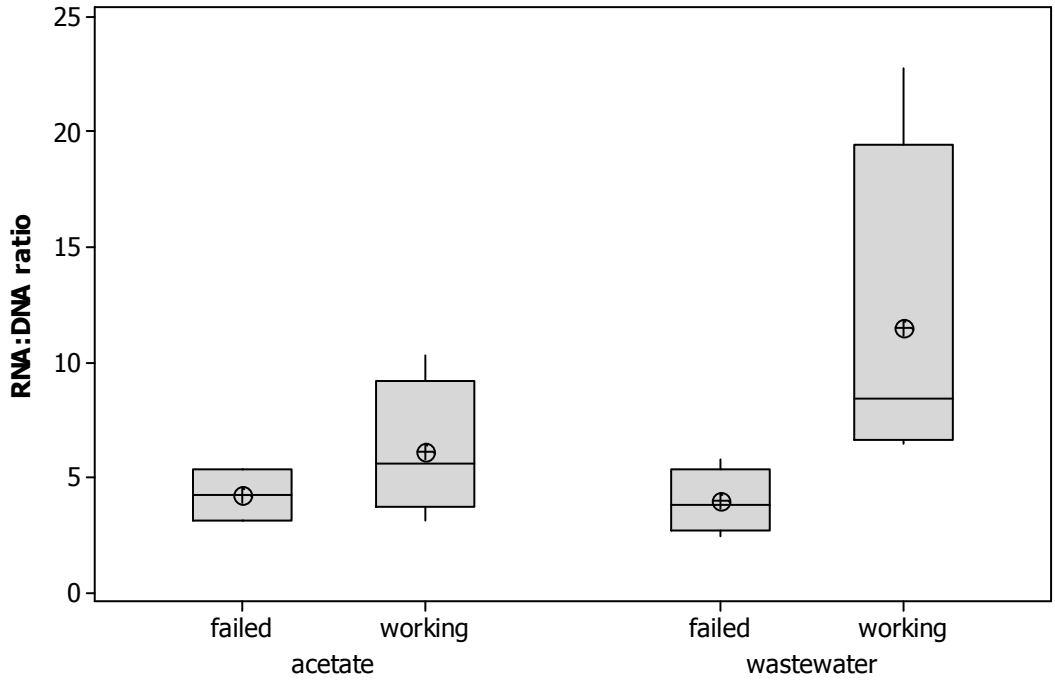

Figure 5-3 Box plot of the RNA: DNA ratios of failed and working reactors fed with both acetate and wastewater, the data represents a summary of the duplicate samples taken from duplicate reactors (i.e. four samples in total) with the central line representing the median and the mean given by the circle with cross

5.3.4. *Low pH*

In the wastewater fed reactors, which contained no additional buffering, it was observed that at around the point of failure there was a decline in the pH of the anode effluent from a starting value 6-6.5 to around 5.5. The acetate fed reactors, (the nutrient media containing 50mM pH 7 phosphate buffer) did not show any significant fall in pH during the full time period over which their function was monitored.

With the additional duplicate reactors fed on wastewater and buffered wastewater there was the same observed drop in pH with the non-buffered reactors. The buffered reactors kept a constant pH and initially performed better but then also failed within 17 days of operation. No significant difference in the run time between the buffered and non-buffered reactors ($p=0.306$, two sample t-test).

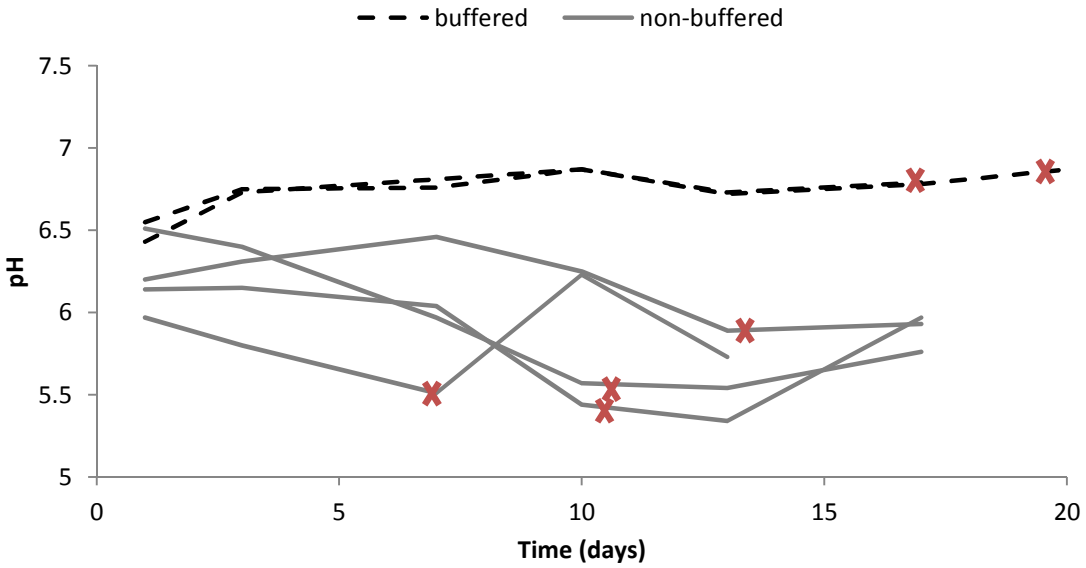

Figure 5-4 Measured pH of the wastewater reactor liquid during the course of the batch experiments, the point of failure is denoted by the red cross where gas production ceased

5.3.5. *Toxic build up within the reactors*

The full anion analysis of the cell effluent showed that there was a fall in chloride ions prior to failure of the wastewater reactors. Both the acetate media and the wastewater contained approximately 250-300 mg/L of chloride. During the course of each batch run with the acetate fed reactors, approximately 50 mg/L of the chloride would be taken up in the reactor, this remained relatively constant throughout the full time period the acetate reactors were operated for. However in the wastewater reactors, when working and producing hydrogen, the chloride removal in the cell was observed to be virtually complete prior to the reactor failure, i.e. 250-300 mg/L of chloride ions were being removed. The levels of chloride in the cathode compartment of these reactors remained the same as the original influent. After failure of the reactors when no hydrogen was produced, this chloride removal stopped. The only wastewater reactors that this drop was not observed in were the duplicate buffered wastewater reactors, here chloride removal remained constant at around 50-100 mg/L during each batch, the reactors did however also fail.

In the acetate reactors supplemented with protein the chloride removal remained roughly constant throughout the experiment at between 50-100 mg/L, and the reactors did not fail. No chloramines could be detected in the effluent of these reactors, disproving the hypothesis of chloramine formation. The performance of the protein

supplemented reactors in terms of electrical energy recovery was not significantly different to the non-supplemented ones (p=0.376, two sample t-test).

Further evidence that a toxic chlorine based product was not being formed was gained using four failed wastewater reactors, duplicate reactors were refilled with UV sterilised wastewater non sterile wastewater, put into MFC mode, i.e. increased resistance and no external load. With all four reactors biological activity started within 1 hour, and reached a level of current production as would be expected of a fully acclimatised MFC cell using the same cell materials. The electrogenic biofilm was capable of functioning. After one batch in MFC mode, the reactors were then all returned to MEC mode, where no gas was produced and the failed status continued. In MFC mode, the chloride removal was relatively constant again at around 50 mg/L.

5.3.6. *Formation of halogenated organics*

Analysis of the headspace gas for 4 wastewater fed reactors and 2 acetate fed did not show detectable levels of halogenated organics, levels were below 0.01% of the 10 ml headspace. This was the case for wastewater fed reactors before, during and after failure and for acetate fed reactors. The same observed drop in chlorides was seen in these reactors.

5.3.7. *Other factors*

The analysis of VFA's in the effluent of the reactors showed that in all cases for both acetate and wastewater there was some acetate remaining at the end of each batch. There was no acetate in the influent wastewater, but always a small amount 20-40 mg/L in the effluent of these reactors, this did not alter once the reactors had failed.

The conductivity for the wastewater was around 1.8 mS, the buffered wastewater was 6.3 mS, and the acetate media was 5.9 mS. The conductivity of the reactor effluent was on average 1.6 mS for the wastewater fed cells both before and after failure even when a drop in chloride ions was recorded, the average for the buffered wastewater cell effluent was 5.5 mS and again did not change after failure, the acetate cells also showed a slight drop in conductivity of the effluent to 5.2 mS.

The production of methane at the anode of the reactors was on average 0.002 ml for the wastewater reactors when working, after failure this increased slightly to 0.029 ml. The methane production remained relatively constant throughout the course of the

experiment and the slight rise after failure is not likely to represent a competitive biological process which is the cause of cell failure, as the average methane production in the acetate fed cells was always higher at 0.072 ml per batch, and also the converted MFC cells that functioned well, also produced on average 0.035 ml per batch.

The materials used in these reactors that could become degraded during use, i.e. the cathode and membrane, could be directly and successfully re-used in a new cell, the failure was not due to cathode degradation or membrane clogging. In addition, by increasing the applied voltage of the reactors from 0.7 V to 1.0 V immediately after failure, thus combating any increased overpotentials that could have built up during the short operation period, the reactors could not be revived and did not produce hydrogen. Failure was not therefore caused by the simple the deterioration of the cell components.

5.4. Discussion

Small laboratory scale wastewater fed reactors fail after a short period of time whereas acetate fed reactors do not. This is significant. The cause of this failure could not be identified during the course of this study. Relatively ‘simple’ explanations such as degradation of electrodes or membranes, a drop in conductivity, or lack of available VFA’s have been ruled out as possible causes of failure.

A further hypothesis that failure of the reactors is caused by a reduced or eliminated level of electrogenic activity in the reactors was also seen not to be the case. If true this hypothesis would result in the reduced DNA:RNA ratio observed and low current production. However once failure had occurred the reactors could be instantly ‘revived’ by switching them into MFC mode. The electrogenic bacteria were therefore present on the electrode and were capable of donating electrons.

The hypothesis that there is a competitive biological process occurring such as methanogenesis, as suggested in other studies (Cusick et al., 2011), has been shown not to be the case. The RNA to DNA ratio indicates a reduced biological activity in the failed wastewater cells, suggesting that the biofilm is less able to function and metabolise after failure. It is not likely that a non-complementary competing biological activity is taking over the reactor and eliminating the MEC process. It can be seen that there is greater activity in the wastewater reactors than the acetate reactors, this might be an indication of the greater and more multi-layered metabolism that has to occur in

these reactors when fed complex substrates. It is also observed that the failed acetate reactor did not differ significantly to the working ones, suggesting the reason for failure here was different to that for the wastewater reactors. Additionally the levels of methane generated in the wastewater reactors after was less than in the working acetate reactors. A competitive process such as methanogenesis is therefore unlikely to be the cause.

The hypothesis that a low pH was causing failure, either through altering the electrochemistry or affecting biological function is shown not to be correct. The simple experiment adding buffer to the wastewater also resulted in failure despite initial improvement in reactor performance, here the drop in chloride was not observed. The slightly lowered pH is likely to have a detrimental effect on the cell though. The pH measurement taken is of the whole of the liquid in the reactor, in reality the pH near the anode may be greater. Such a pH will impact on the microorganisms present and the electrochemical reactions within the cell, as pH is a logarithmic function of the concentration of H+ ions, then even a small change in this value has a large impact on the overall thermodynamic balance of the system as is calculated via the Nernst equation. Torres et al (2008) found that an increase in phosphate buffer in the anode media lead to a thicker biofilm and greater current generation in a microbial fuel cell due to the increased diffusion of H^+ out of the biofilm layer, thus making it more accessible to transport to the cathode. Although pH could be limiting the performance of non-buffered reactor it is not the cause of failure.

The formation of halomethanes such as chloromethane could potentially occur at the potentials within these reactors account for the loss of chloride and would cause failure as these compounds are toxic. This would fit the pattern of failure exhibited in the reactors as it would take some time for the levels of methane to build up which could then be converted to the halomethanes, this would ‘use up’ the H^+ ions in the anode section and H_2 would cease to be produced at the cathode. However no chloromethanes could be detected in the headspace gas of these reactors, (below 0.01%) either before or after failure, in fact no halogenated organics could be detected. Additionally the acetate fed cells did not fail when supplemented with protein, and most importantly the exoelectrogenic biofilm is able to work as an MFC after failure so has not been killed. It could be possible that the negative chlorine ions were simply temporarily attracted to the positive anode during the operation of the fuel cell, and therefore not measured in the bulk liquid of the cell. This would account for the observed ‘disappearance’ of the

chloride ions, but is not likely to affect the performance of the cell. The range of analysis carried out indicates that failure is not caused by a chlorine effect; the observed chlorine drop is simply co-incidental to the failure.

The problem of failure needs to be resolved. If MECs are to be a useable technology they need to function with real wastewater. Studying these systems when they are prone to sudden and rapid failure is difficult, therefore identifying the reasons for failure, solving them, and increasing efficiency becomes very challenging. This difficulty leads to acetate being used in most research as this does allow greater scope for experimentation. However it is clear that the processes operating in a reactor fed with real wastewater are different to those occurring in a reactor fed with acetate. The acetate research will not directly inform us of performance with wastewater.

The failure in wastewater fed, laboratory scale, batch fed reactors has been proved, but the reason not identified. Conversely, as part of this research, a larger scale MEC run in continuous mode at a wastewater treatment site fed on raw wastewater has worked producing almost pure hydrogen for a period of over 3 months, (see chapter 6). It is likely that something is occurring within the small batch reactors to prevent either the production of hydrogen ions at the anode, the transfer of these ions, or the hydrogen evolution reaction at the cathode. It may be the case that at this small scale and fed with batch mode that the system and in particular the microbial community involved is fragile and unable to adapt to change, and therefore a build-up of something at an undetectable level has catastrophic consequences. Further work is still needed to identify the cause of this failure, and therefore be able to take steps to resolve it. This can only be done by using real wastewater rather than simple artificial media. The long term performance of wastewater fed MECs is a research gap that must be filled.

Chapter 6. Production of hydrogen from domestic wastewater in a pilot scale microbial electrolysis cell

Addressing the need to recover energy from the treatment of wastewater the first working pilot scale demonstration of a wastewater fed microbial electrolysis cell is presented. A 120 litre (L) microbial electrolysis cell (MEC) was operated on a site in Northern England, using raw domestic wastewater to produce virtually pure hydrogen gas for a period of over 3 months. The volumetric loading rate was 0.14 kgCOD/m^3/day, just below the typical loading rates for activated sludge of 0.2-2 kgCOD/m^3/day, at an energetic cost of 2.3 kJ/gCOD, below the values for activated sludge 2.5-7.2 kJ/gCOD. The reactor produced an equivalent of 0.015 L H_2/L/day, and recovered around 70% of the electrical energy input, with a coulombic efficiency of 55-60%. Although the reactor did not reach the breakeven energy recovery of 100%, this value appears well within reach with improved hydrogen capture, and reactor design. Importantly for the first time a 'proof of concept' has been made, with a technology that is capable of energy capture using low strength domestic wastewaters at ambient temperatures.

6.1. Introduction

In an era of increasing energy costs and environmental awareness, wastewater treatment industries need to look at alternative treatment options to reduce their energy bills. It has been estimated that domestic wastewater alone may contain 7.6 kJ/L of energy, while stronger industrial wastewaters contain substantially more (Heidrich et al., 2011). There is an increasingly urgent need to recover some of this energy, or at the very least not expend additional energy on treatment; the activated sludge process uses 2.5-7.2 kJ/gCOD (Pant et al., 2011). Energy recovery could be achieved through anaerobic digestion to methane gas or microbial fuel cell technology directly to electricity; however life cycle assessment has shown that the production of a higher value product through the suite of bioelectrochemical systems (BES) may be the most viable solution (Foley et al., 2010). One such technology is the production of hydrogen in a microbial electrolysis cell (MEC) (Rozendal et al., 2006).

Since the MEC process was first reported (Rozendal et al., 2006, Liu et al., 2005b) MECs have emerged as a potential technology option for a new generation of wastewater treatment systems (Rozendal et al., 2008a). In an MEC bacteria use the

energy stored in the organic compounds of wastewater to metabolise and grow, donating electrons to an electrode (Rozendal et al., 2006). The electrons then travel in a circuit producing current and therefore electrical power; in an MEC these electrons are consumed at the cathode along with a supplement of electrical power. The H^+ ions also created by the breakdown of organics at the anode travel across the microbial fuel cell membrane to the cathode. Here they can combine to form H_2, however this process is endothermic requiring energy, so a supplement of electrical energy is added to the system to allow it to take place (Liu et al., 2005b).

Fuel cell technologies may offer a sustainable future for wastewater treatment, although there are still many hurdles to overcome. Progress is being made with new reactor design (Call and Logan, 2008, Rozendal et al., 2008b), improved materials (Cheng et al., 2006a, Cheng and Logan, 2008), greater understanding of the mechanisms involved (Aelterman et al., 2008, Clauwaert et al., 2008), and even improved understanding of the microbes that are at work in these systems (Holmes et al., 2004, Kim et al., 2004, Lovley, 2008, Rabaey et al., 2004). Most of this research is performed at laboratory scale, using simple substrates, often at a controlled warm temperature. Many problems have been overcome, such as validation of using multi electrode systems (Rader and Logan, 2010) and finding a low cost alternative to the platinum cathode (Zhang et al., 2010). Although of great value in improving our understanding of MEC's, these studies do not tell us about the challenges or even benefits of running such systems at a larger scale with real wastewaters in temperate climates. There is a need to demonstrate that these systems can work at a larger scale and under realistic conditions, elevating the technology from a laboratory curiosity into a practical solution to an industrial problem.

A pioneering study by Cusick et al (2011) published on the largest MEC reactor to date, a 1000 L pilot scale reactor at a winery in California. The reactor proved slow to start up with pH and temperature control being problematic. When these issues were corrected by heating to 31 ^{o}C and the addition of buffer and acetic acid, the reactor did improve in performance. The energy produced during the operation exceeded the input energy (heating not included), but this was primarily due to methane production (86%) with only trace amounts of hydrogen. Methane production was attributed to the reactor being membraneless allowing hydrogen produced at the cathode to be directly consumed by hydrogenotrophic methanogens within the reactor. The reactor performance tailed off at around 90 days, when the heating unit broke (Cusick et al., 2011). The study has

provided valuable insights into the operation of MECs: (i) the membraneless systems that work well at laboratory scale and when fed in batch mode may not be so good at larger scale and under continuous feed, and (ii) inoculation and start-up are important parameters.

Addressing the issue of a membrane is critical to reactor performance. Most laboratory scale membrane systems use Nafion 117 (Logan et al., 2006), an expensive and delicate proton exchange membrane (Logan et al., 2006); this would be both impractical and costly on a large scale. Also the high efficiencies published: 406% electrical energy recovery (the amount of electrical energy put in that is recovered, this can be higher that 100% as there is also substrate energy within the system) and 86% total energy efficiency (the amount of substrate and electrical energy recovered) (Call and Logan, 2008) are from membrane-less systems. The lack of membrane greatly reduces the resistance in the cell, improving the transmission of protons to the cathode. Membrane systems have lower efficiencies: 169% electrical energy recovery and 53% overall energy efficiency has been reported (Rozendal et al., 2006). These efficiencies are likely to decrease further with time as the membrane becomes fouled.

The issues of inoculation and start-up are poorly understood (Oh et al., 2010) Although the use of acetate is likely to reduce the acclimatisation period (Cusick et al., 2011). However the biological community needed for the degradation of complex substrates is thought to be different to that needed for acetate (Kiely et al., 2011c). A community of acetate degraders able to work at 30 ^{o}C is not likely to be the community needed to degrade wastewater at ambient UK temperatures. There is evidence in the literature that microbes exist that are able to digest wastewater (Ditzig et al., 2007) and operate at low temperatures (Lu et al., 2011). Like anaerobic digestion, however, it may well be that a long period of acclimatisation is needed and unavoidable to achieve a stable community (Rittmann, 2001).

If these start-up issues can be resolved, then the reactor in theory will function, however it would also need to reach a neutral or positive energy balance, i.e. recovering all the electrical energy input plus a substantial fraction of the substrate energy input.

To test whether these systems have a chance of achieving these goals under realistic conditions, a pilot scale 120 L reactor was placed on a wastewater treatment site in

North East England. This site takes in primarily domestic wastewater with an average Total COD of 450 mg/L. The reactor was built using low cost alternatives to the standard lab materials used for the cathode and membrane. The reactor was not heated, held inside a large unheated building, and run throughout a UK spring and summer (5-20 ^{o}C minimum and maximum temperatures) and is still in operation at the time of writing this paper. These operating conditions are likely therefore to represent close to a worst case scenario i.e. low concentration feed; non optimal components; no heating; and no additional supplement of acetate or buffering capacity after the initial acclimatisation period.

Working closely with partners at Northumbrian Water Ltd. the aim of this study was to establish reactor operation and to determine if a neutral or positive energy recovery is achievable. From that data we can evaluate if MEC technology is likely to be a viable treatment option for the future.

6.2. Methods

6.2.1. *Field Site*

The pilot scale reactor was set up and run at Howdon wastewater treatment site, situated near the city of Newcastle Upon-Tyne in the North East of England (54^{o}58'N, 01^{o}36'W). An average of 246500 m^{3} of domestic wastewater is treated daily, using 96 MWh; the activated sludge process uses around 60% of this. The wastewater used in the MEC was taken from the grit channels after primary screening, but before settling.

6.2.2. *MEC reactor*

The reactor was based on a cassette style design, with six identical cassettes being placed into a rectangular reactor with a total working volume of 120 L. The tank has a Perspex plate fitted over the liquid layer giving a small head room to the anode compartment of 2.2 L. Each of the cathode gas tubes from the cassettes projected above this Perspex sheet. The cassettes were set along alternate sides of the reactor to allow s-shaped flow, and once in place gave a final anode volume of 88 L.

Each cassette was constructed using 10 mm thick plastic sheeting and consisted of an internal cathode section 0.280 m by 0.200 m by 0.048 m deep, of a volume 2.6 L. The cathode material was stainless steel wire wool grade 1 (Merlin, UK), 20g was used in each cathode, giving a projected cathode surface area for each electrode of 0.056 m^{2}. A

0.8 m length of stainless steel wire was wound several times into the wire wool to make a firm electrical connection, and then to the outside of the cell. Each cathode electrical assembly had an internal resistance from the extremities of the wire wool to the end of the exposed wire of less than 2.75 Ω. The cathode was separated using a membrane wrapped around a plastic frame inserted into the electrode assembly on both sides. The membrane used was RhinoHide® (Entek Ltd, UK), a durable low cost microporous membrane traditionally used as a battery separator. The anode material was a sheet of carbon felt (Olmec Advanced Materials Ltd, UK), 0.2 m wide by 0.3m high and 10 mm thick. This was sandwiched between two sheets of stainless steel mesh acting a current collector. The anode assemblies were also connected by a 0.8 m length of stainless steel wire fed through the centre of the felt material, each electrode having an internal resistance less than 3.4 Ω.

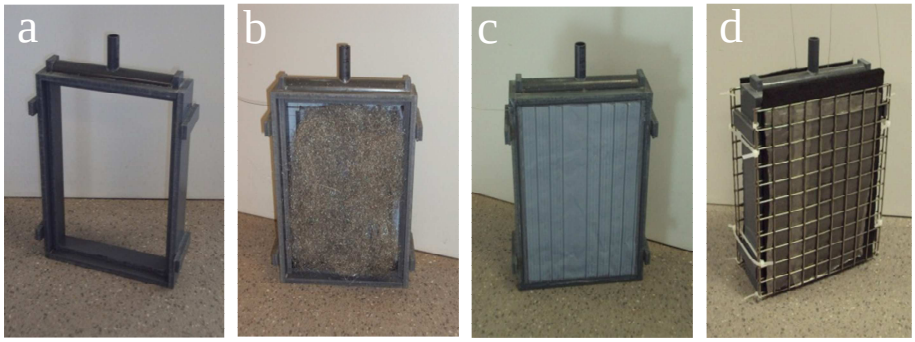

Figure 6-1 Photographs of the electrode assembly unit – a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane, d) anode with wire mesh current collector

The gas production from the anode compartment was captured from the ports in the Perspex lid, using 3mm ID PVC tubing (VWR Jencons, UK). The cathode gas was initially captured using 4mm annealed copper GC tubing connected to each cathode compartment using copper compression fittings, (Hamilton Gas Products Ltd, Northern Ireland), due to rapid corrosion this was later replaced with 3mm ID PVC tubing (VWR, UK). Both pipelines contained a gas sampling port.

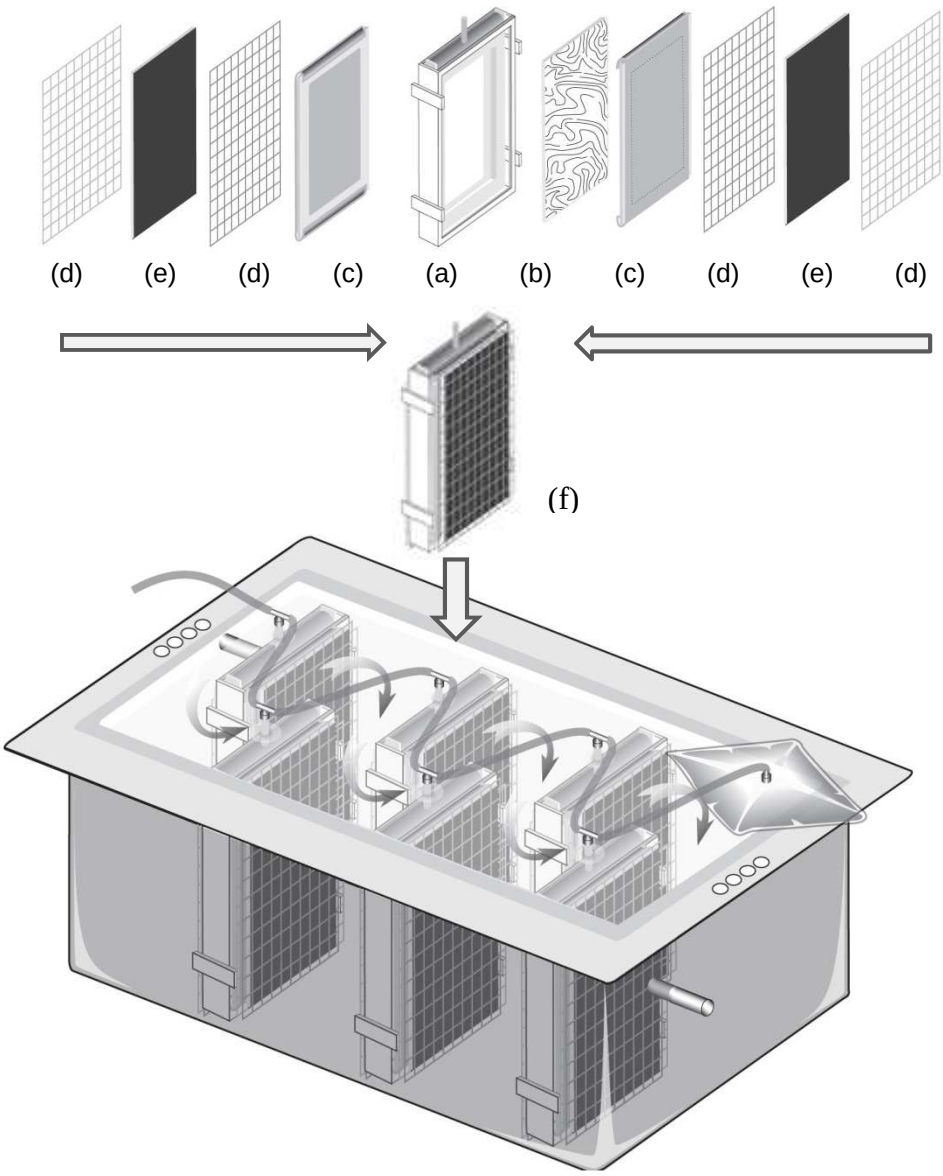

Figure 6-2 Schematic diagram of the reactor module components, a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane fixed around a PVC frame, d) stainless steel wire mesh, e) anode with wire mesh current collector. These component fit together to form a single module (f), six of these go into the reactor vessel where wastewater flows around them. Gas is collected through tubing into a gas bag

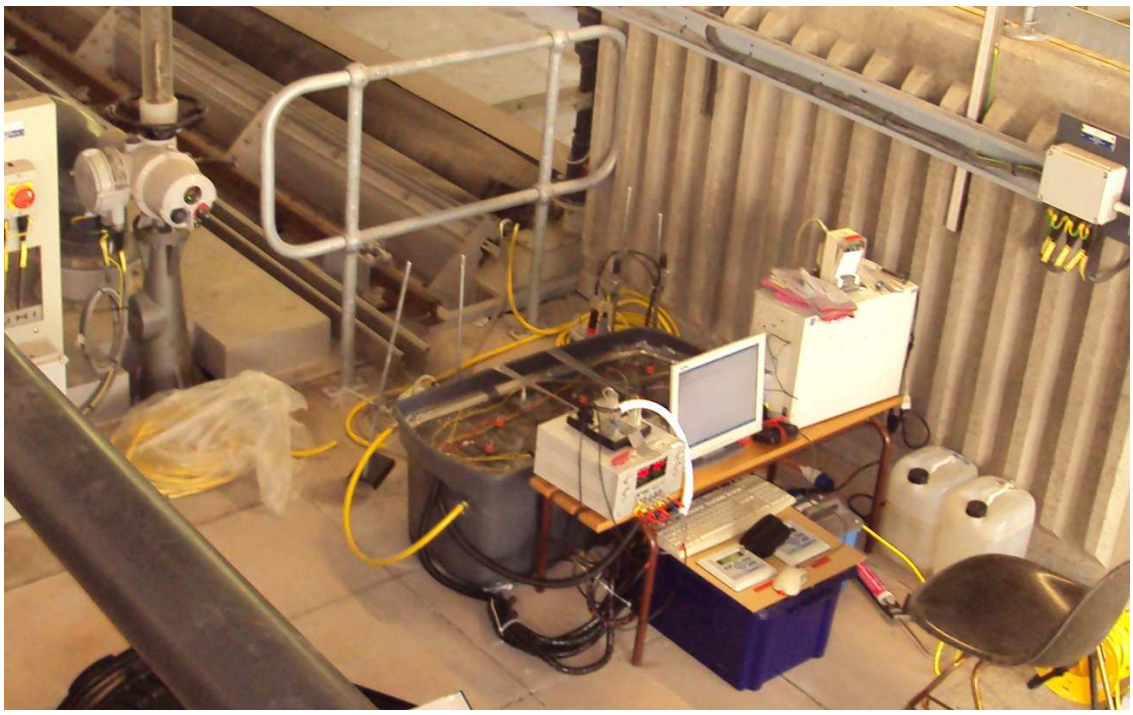

Figure 6-3 Photograph of the reactor in situ at Howden wastewater treatment site the grit lane where the influent was drawn from is seen in the top left hand corner of the picture

The reactor was situated on site in a large unheated building housing the grit channels, wastewater was pumped from the grit channels into a preliminary storage tank, providing some primary settling. During operation a peristaltic pump (Watson Marlow 520S, UK) was used to pump water into the storage tank, where it could then flow into and through the reactor, and back out to the grit channels via a smaller sampling tank at the end. These tanks were used for sampling and monitoring of the influent and effluent.

6.2.3. *Analytical procedures*

Power was provided to the electrodes using a PSM 2/2A power supply (Caltek Industrial Ltd, Hong Kong), the voltage of each cassette was monitored across a 0.1 Ω Multicomp Resistor (Farnell Ltd, UK) using a Pico AC-16 Data Logger (Pico Technology, UK), and recorded on a computer every 30 minutes.

In both the influent settling tank and the effluent tank the dissolved oxygen (DO) and pH were measured using pH and DO submersion probes (Broadley James Corporation, USA) connected to a pH DO transmitter (Model 30, Broadley James Corporation, USA), feeding an electrical output to a Pico EL 037 Converter and Pico EL 005 Enviromon Data Logger (Pico Technology, UK); these data were recorded onto the

computer every 30 minutes. Temperature was logged using 3 EL-USB-TC Thermocouple data logger (Lascar Electronics, UK) placed in the settling and effluent tanks and one placed in the reactor itself.

The gas pipelines were connected to optical gas bubble counters (made 'in-house' at Newcastle University), giving a measurement of gas volume. The operation of these counters failed after several weeks of operation. They were replaced with 1 L and then 5 L Tedlar gas bags (Sigma Aldrich, U.K.); the volume of gas was then measured by removal from the bags initially using a 100ml borosilicate gas tight syringe, and then using a larger 1 L glass tight syringe (both SGE Analytical Science, Australia). The sampling ports on each pipeline were initially used to take a sample of cathode gas 3 times a week, into a Labco Evacuated Exetainer (Labco Ltd, UK). Once gas production had risen to a higher volume, 2 L of the cathode gas was dispensed from the collecting gas bag into another 5L gas bag which was taken away for analysis. Anode gas was not measured volumetrically due to leakage but was sampled directly from the anode compartment into a 3 ml exetainers for compositional analysis.

Hydrogen gas was measured using a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using duplicate injections, set against a three point calibration. These gas measurements were verified using a Trace Ultra gas chromatograph (GC) with a thermal conduction detector (TCD) and a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements for anode and cathode gas were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

To ensure accuracy calibration standards used for the gas measurements were injected into a Labco evacuated exetainers in the laboratory at the same time (+/- 10 minutes) as the samples taken in the field. Tests carried out previously had indicated that these containers were not completely gas tight especially for hydrogen. This procedure did not have to be carried out for the cathode gas once operation had been switched to gas bags.

Liquid samples of the influent and effluent were taken 3 times a week. The total chemical oxygen demand (COD), and soluble chemical oxygen demand (SCOD) were measured in duplicate using standard methods (APHA, 1998) (Spectroquant ® test kits, Merck & Co. Inc., USA). Volatile Fatty Acids (VFA's) were determined using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The conductivity of the solution was measured using a conductivity meter, EC 300 (VWR Ltd, UK).

6.2.4. *Start up and operation*

The reactor was initially started up in batch mode, allowing all the oxygen, nitrates and sulphates within the wastewater to be consumed. Based on the lessons learnt from the previous pilot study, (Cusick et al., 2011), (Logan, B.E. personal communication),the wastewater was supplemented with acetate at a concentration of 0.5g/L. The applied voltage of 0.6 V was provided by a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong). The dosing was repeated and the reactor refilled after a 2 week period, during which time no gas production was observed.

6.2.5. *Efficiency calculations*

Four efficiency calculations are made in this study on the basis of the electrical and substrate energy used (Logan, 2008).

(i) Electrical energy recovery (η_E)- Energy recovery is the amount of electrical energy put into the reactor that is recovered as hydrogen.

The electrical energy input W_E is calculated as:

$$W_E = \sum_{1}^{n} (I\ E_{ps} \Delta t - I^2 R_{ex} \Delta t)$$

Where I is the current calculated for the circuit based on the measured voltage E and external resistor R_{ex} ($I=E/R_{ex}$), E_{ps} is the applied voltage of the power supply, this value is adjusted for the losses caused by the external resistor (I^2R_{ex}), which in reality are negligible. The time increment denoted by Δt represents the conversion of samples taken every 30 minutes into seconds. The data is summed for all 6 cells over the each batch cycle. The output of energy (W_{out}) is calculated from the measured moles of

hydrogen produced $N_{H2,}$ and the standard higher heating value of hydrogen of 285.83 kJ/mol ΔH_{H2}.

$$W_{out} = \Delta H_{H2}\, N_{H2}$$

The higher heating value is chosen over the lower heating value which takes into account the heat lost through the production of water vapour during burning. It is expected that this H_2 product would be used either as a commercial product for industry, or in a clean H_2 consuming fuel cell to create electricity, not for combustion. Methane could also be added to this value to further increase the quantity of output energy, but was not included for these same reasons.

Total Energy recovery (excluding pump requirements) can then be calculated as follows:

$$\eta_E = \frac{W_{out}}{W_E}$$

(ii) Total energy efficiency (η_{E+S}) the amount of input energy both electrical and substrate that is recovered as hydrogen.

The substrate energy (*Ws*) is calculate as

$$W_s = \Delta COD\, \Delta H_{ww/COD}$$

Where ΔCOD is the change in COD in grams, estimated as the difference in COD of the influent and effluent at the end of each batch. $\Delta H_{ww/COD}$ is the energy content per gCOD as measured on similar domestic wastewater of of 17.8 kJ/gCOD (Heidrich et al., 2011). Total energy efficiency is then calculated as:

$$\eta_{E+S} = \frac{W_{out}}{W_E + W_S}$$

(iii) Coulombic efficiency (CE) - the amount of hydrogen produced compared to the amount theoretically possible based on the current, or total charge passing through the cell.

Theoretical hydrogen production based on current (N_{CE}) is calculated as:

$$N_{CE} = \frac{\sum_1^n I\Delta t}{2F}$$

Where *I* is the current calculated from the measure voltage, Δt is the conversion of the time interval 30 minutes to 1 second to give coulombs per data sample, this is then summed over the 6 cells for the whole batch. Faradays constant (*F*) is 96485 coulombs/mol e^-, and is the moles of electrons per mole of hydrogen. Coulombic efficiency *CE* is then calculated as:

$$CE = \frac{N_{CE}}{N_{H2}}$$

(iv) Substrate efficiency - the amount of hydrogen produced compared to the amount theoretically possible based on substrate removed in the reactor.

Theoretical hydrogen production based on substrate removal *(N_S)* is calculated as:

$$N_S = 0.0625\, \Delta COD \Delta t$$

As 64 gCOD can be converted to 4 moles H_2, each g COD is equivalent to 0.0625 moles H_2. The change in COD is measured at the end of each batch, and used to calculate the total COD removed from the 88 L reactor over the duration of the sampling period based on a HRT of 1 day. Substrate efficiency is then calculated as:

$$S_E = \frac{N_S}{N_{H2}}$$

The (η_E) correlates directly to the coulombic efficiency (CE) by re-arrangement of their respective equations. It is assumed that the phrase $I^2 R_{ex} \Delta t$ in calculating W_E is negligible by comparison to the first term (this is observed to be the case in practice):

$$\eta_E = \frac{\Delta H_{H2} \times 1000}{2F \times E_{ps}}\, CE$$

This means halving the E_{ps} doubles the η_E if the CE can be maintained. An increase in CE at the same E_{ps} causes a linear increase in η_E.

6.2.6. *Statistical analysis*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

6.3. Results

6.3.1. *Reactor design and resistance limitations*

The internal resistance of a BES design is critical to its performance. Resistance is mainly caused by electrode overpotential and ohmic losses in the liquid, although there may also be losses in the bacterial transfer etc. as shown in Figure 1.2. These losses impact on the amount of energy that can be gained in and MFC and the amount for energy needed in an MEC, these effects are even greater in a scaled up system where losses become proportionally more significant (Rozendal et al., 2008a). Within the cell

designed the anode and cathode, although separated by a membrane, were relatively close together, with around 1cm distance between them, this will have minimised the ohmic losses within the liquid phase (i.e. the resistance in the movement of ions from the anode to cathode) which is especially important when using real wastewaters with no artificial increase in liquid conductivity.

However the electrode resistance with this design is high, with the cathode having a resistance of 2.8Ω and each anode sheet being 3.4Ω from the extremities of the electrode to the end of the connecting wire. With a total anode surface area for the whole reactor of 0.76 m^2 and a further 0.3 m^2 of cathode, these resistances will have a large impact in reducing the efficiency of the reactor performance. With a 0.6V load, as would be desirable based on laboratory studies (Call and Logan, 2008) this anode resistance would result in an approximate maximum current of 0.2A, increasing the load to 0.9 as needed with other wastewater studies (Kiely et al., 2011a, Cusick et al., 2011) would produce a maximum of 0.3A, and the 1.1V load used would result in around 0.4 A maximum current, assuming no other losses. This would give anode current densities of 0.3, 0.4 and 0.5 A/m^2 respectively, well below the target for BES of 10 A/m^2 which would enable similar treatment rates to activated sludge (Rozendal et al., 2008a), although current densities within MECs do tend to be lower than those of MFCs (Kiely et al., 2011a).

In reality there was greater resistance within the reactor than the electrode overpotentials alone. The current densities measured were 0.04, 0.1 and 0.3 A/m^2 at 0.7, 0.9 and 1.1V load added respectively. This means that the current density only increases by around 0.6 A/m^2/volt, far lower than two early MEC laboratory studies (1.3 A/m2/volt in (Liu et al., 2005b) and 1.78 A/m^2/volt in (Rozendal et al., 2006)). Additionally this shows that there is an inherent overpotential in the system also of over 0.6 volts as seen in Figure 6.4, over this voltage needs to be added to generate any current.

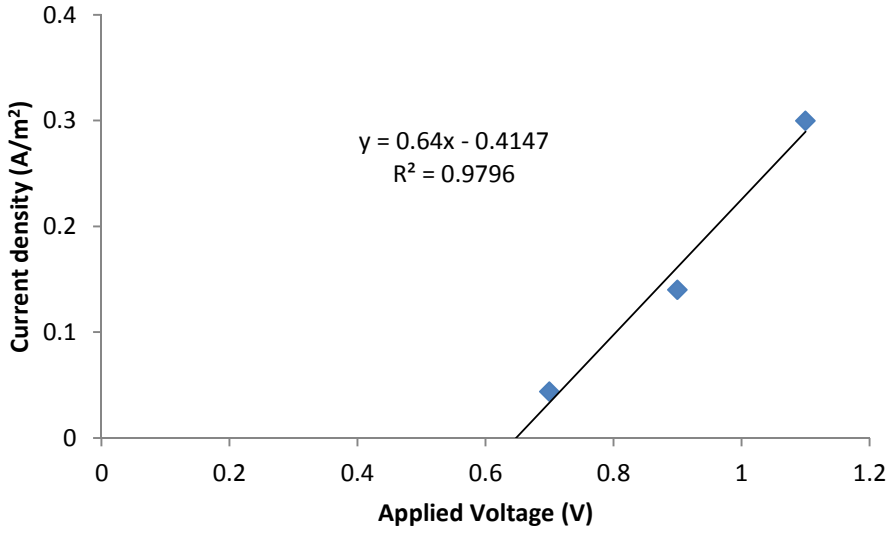

Figure 6-4 Current density as a function of applied voltage as measured in the pilot scale reactor after the initial two week acclimatisation period, showing the linear regression equation and R2 value. The intersect of the x-axis indicates the overpotential of the system

6.3.2. *Start-up and acclimatisation*

During the first 30 days of operation the reactor was run in batch mode with a supplement of 0.5 g/L of sodium acetate and an input voltage of 0.6 V. During this time there was no observed gas production and the current density was very low reaching 0.04 A/m^2 after the first two weeks. After this period wastewater was pumped through the reactor with a HRT of one day with no further addition of acetate. For the subsequent 10 days very little gas was produced and the current density remained at this very low level. At day 40 the input voltage was raised from 0.6 V to 0.9 V. The reactor was run with this input of voltage for the next 24 days; the average power density during this time reached 0.1 A/m^2. Gas production was low with an average of 9 mL/day, however once the gas lines had been flushed the purity of this gas (H_2) began to reach 100%. The electrical energy efficiency η_E was only 1 %. The voltage was then further increased to 1.1 V, and power densities rose and stabilised at 0.3 A/m^2. This led to a dramatic improvement in gas production, and the reactor entered its "working phase", the results of which are shown below. The start-up period took 64 days.

6.3.3. *Working performance of MEC reactor*

After the long start-up, and subsequent increase in the voltage to 1.1 volts, the MEC worked for the following 85 days, and continues to do so. The results presented here are for this period.

The volume of gas produced per day was highly variable. However the gas composition was consistent, hydrogen 100% ± 6.4, methane 1.8% ± 0.9. No trace of CO_2, N_2 or O_2 could be detected using the GC's or MIMS. H_2S could not be measured accurately however the MIMS did not detect any gas at this atomic weight and there was no detectable odour present. The daily H_2 production is shown in Figure 6-5. Production gradually increased during the first 30 days; after this the average production was around 1.2 L per day for the reactor, equivalent to 0.015 L-H_2/L/day.

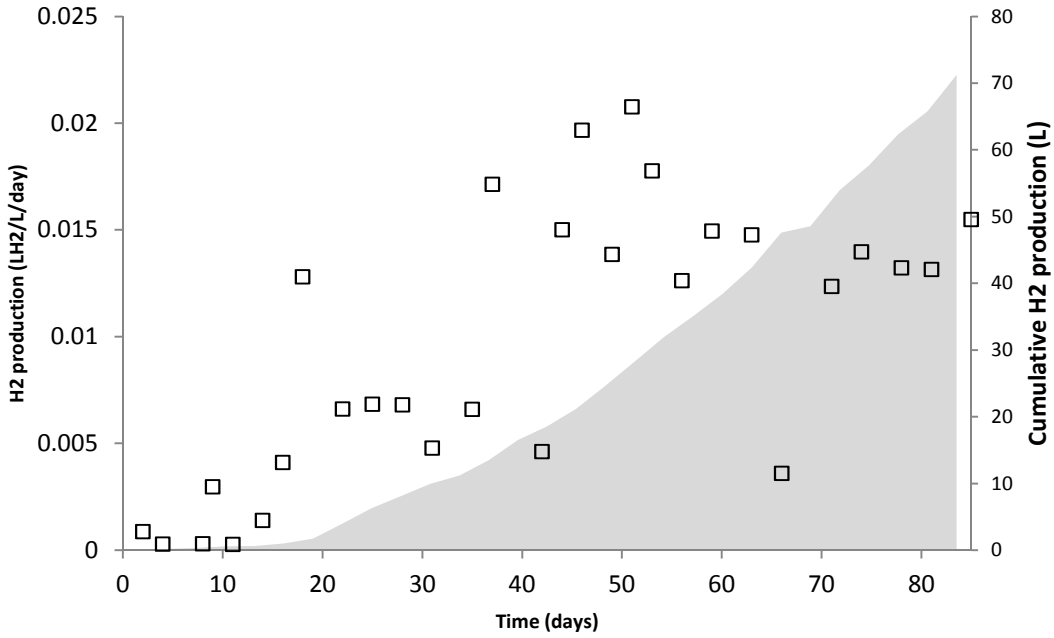

Figure 6-5 Hydrogen production during the working phase of reactor after the 64 day acclimatisation period, points showing the production rate at each time of sampling, and the area showing the cumulative production of the course of this period

The electrical energy recovery of the cell was quite variable as seen in Figure 6-6 (a), but did show an increasing trend and on occasion approached 100% (complete energy recovery) . The total energy efficiency (b) which gives the true performance of the cell was also variable, and considerably lower as both the electrical and substrate energy are considered as inputs. The energy efficiency shows an increasing trend reaching the 30 % level at the end of the study. The peak values are associated with very low COD removal measurements (making substrate energy input very low), and are not therefore likely to be representative of the true performance of the reactor. Coulombic efficiency (Fig. 5c) shows a similar trend to energy recovery (Fig. 5a), stabilising at around 55-60 % in the last 30 days.

The coulombic efficiency (CE) correlates with energy recovery (η_E) (R^2 = 0.998, Pearsons correlation). This correlation factor is calculated as N_E = 1.29 CE using the average input power voltage, this value is also seen in the data and is consistent over the course of the study. If the CE could remain at the 60% and the power input dropped to 0.9 volts 100% η_E would be achieved. Alternatively with this power input CE needs to reach 75% to achieve 100% η_E. The substrate efficiency (d), due to the highly variable influent and effluent COD values (as shown in Figure 6-7 can exceed 100%, and was often very low and even negative. The average substrate efficiency for whole the operational period is 10%.

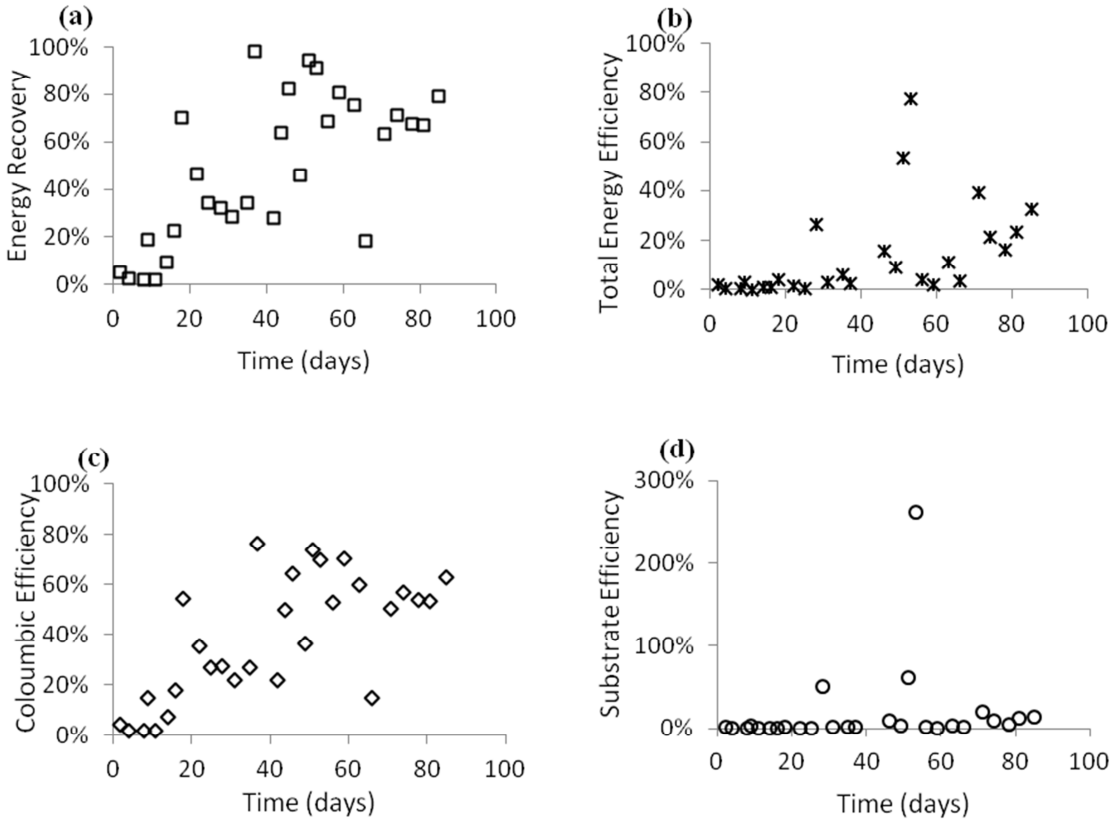

Figure 6-6 MEC reactor efficiencies over the 85 day working period a) electrical energy recovery b) total energy efficiency c) coulombic efficiency d) substrate efficiency

The levels of influent COD was highly variable which is likely to be one of the factors underlying the variation in performance. This factor was particularly the case at day 30 when the settling tank became full with sludge and influent COD was extremely high. This variability led to occasional negative values for % COD removal. The average removal of 33.7%, equates to 0.14 kgCOD/m^3/day, just below the range for activated sludge of 0.2-2 kgCOD/m^3/day (Grady, 1999). The COD effluent levels occasionally approached and dropped below the UK standard of 125 mg/l (EEC, 1991).

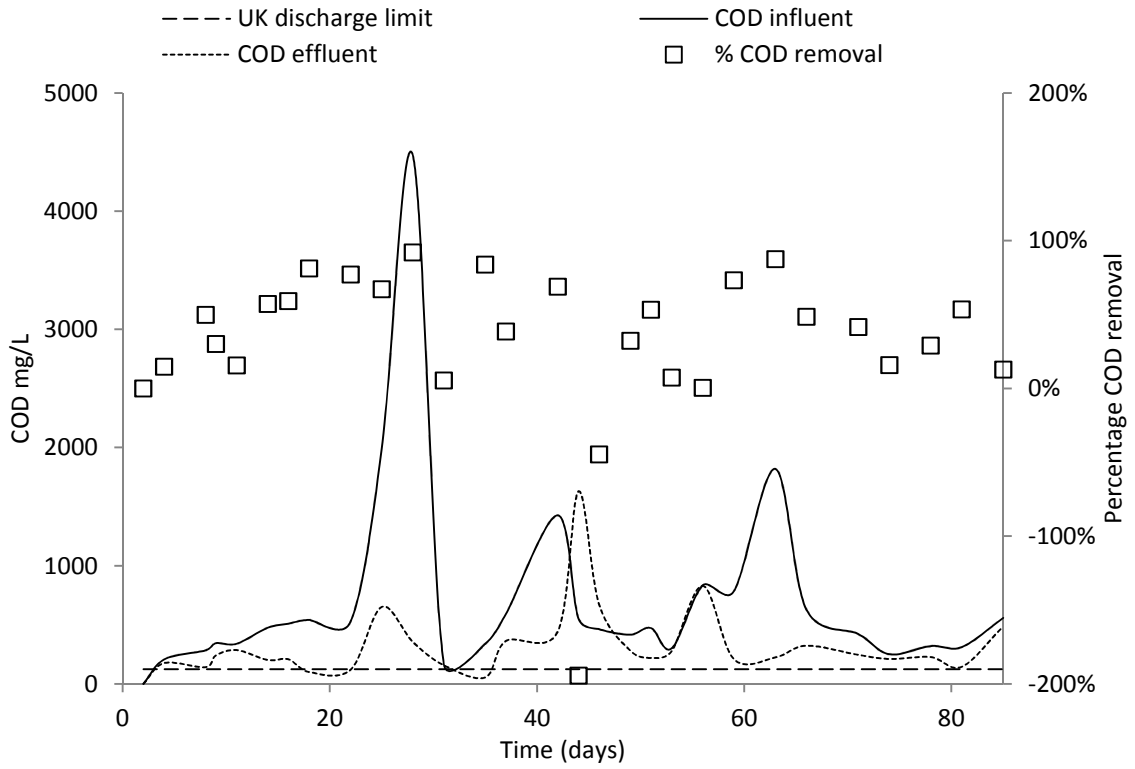

Figure 6-7 COD influent and effluent shown by the lines along with the UK discharge standard of 125 mg/l, percentage COD removal is also shown using the squares

Despite the variable influent COD and therefore variable performance, many of the other measured factors remained relatively constant throughout the operational period. The headspace of the anode compartment (2.2 L volume) contained elevated levels of CO_2 (1.9%) and low levels of CH_4 (0.4%), equivalent to 8.8 ml of CH_4, or 0.006 mg COD and 0.3 kJ. The gas production at the anode could not be measured quantitatively due to leakage. The daily production of methane at the cathode was 22 mL/day, equivalent to 0.014 mg COD, and 0.8 kJ of energy, approximately 5-6% of the amount of energy recovered as hydrogen.

The pH of the influent and effluent were continuously monitored, the influent was on average pH 7, the effluent pH 6.7, never dropping below pH 6. The DO of the influent was on average 4.2 mg/L and the effluent was 0 mg/L. The amount of VFA's dropped between the influent and the effluent, but there was frequently some acetic acid left in the effluent up to 45 mg/L, i.e. the available food source was not used up. This was confirmed by the average SCOD of the effluent of 115 mg/L. There was an average removal of 1.8 g/day of sulphate in the reactor, but never full depletion with the effluent containing 89.6 mg/L on average. The reactor removed an average of 0.2 g/day of chloride, although this value was highly variable. Fluoride and phosphate remained

relatively constant between the influent and effluent, nitrates were not present in either. There was no measured drop in conductivity between the influent and effluent.

The temperature of the influent wastewater varied considerably throughout the working period between June and September. The range of temperature was more stable within the reactor, and was on average 0.9 ^{o}C higher than the temperature of the influent. With a 88 L capacity and HRT of 1 day, this means 0.37 kJ/day of energy was lost to heat, equivalent to 20 mg COD, or 31 ml H_2. Temperature did not significantly influence energy recovery (p=0.678 influent, p=0.664 reactor, p=0.778 effluent, Pearson Correlation). Most of the fluctuation observed was diurnal and periods of the more extreme temperatures were short lived.

Table 6-1 Maximum, minimum and average temperature (^{o}C) of the influent, effluent and reactor ± 1 standard deviation which were continually logged over the experimental period

	Influent	Reactor	Effluent
Maximum	27.0 ± 2.3	21.0 ± 1.2	22.5 ± 1.6
Minimum	8.5 ± 2.3	13.5 ± 1.2	12.0 ± 1.6
Average	15.8 ± 2.3	16.6 ± 1.2	16.6 ± 1.6

The total material costs of the reactor, not including pumps, power supply and computing/recording instruments, was equivalent to £2344/m^3, of which the cathode and membrane combined represented less than 2%.

6.4. Discussion

This pilot scale reactor worked, producing almost pure hydrogen gas from raw influent domestic wastewater at U.K. ambient temperatures for a 3 month period and continues to do so. It is believed to be the first successful study of its kind, which brings the prospect of sustainable wastewater treatment and hydrogen production through the use of bioelectrochemical systems onto a new and exciting phase.

The reactor has removed on average 34% of COD, and occasionally reaching the UK discharge standard of 125 mgCOD/L, equating to a treatment rate of 0.14 kgCOD/m^3/day, just below the range for activated sludge. The reactor has performed this task using less energy than would be needed for aeration in a traditional activated sludge process. The electrical energy recovery on occasion nearly reached values of 100%, and was consistently around 70% during the later stages of the study. At this

level of performance (i.e. 70%) the energetic treatment costs were 2.3 kJ/gCOD, below the values for activated sludge of 2.5-7.2 kJ/gCOD (Pant et al., 2011). By implementing improvements to the reactor such as: increasing electrode surface areas; reducing the distance between electrodes; having a more efficient flow paths; consistent pumping; and improved materials, the η_E could be greater than 100%, making it a net energy producer. On the basis of this fairly large proof of concept study, energy neutral or even energy positive wastewater treatment is clearly a realistic goal.

The total energy recovery showed an increasing trend during the course of the study, levelling out at around 30%, with around a third of all energy both from the wastewater and from the power supply being recovered as hydrogen gas. Coulombic efficiencies of the reactor were high, levelling out at around 55-60 %, methane production accounts for an additional 3.5%. Other losses might be caused by some short circuiting in the reactor. It is likely therefore that a large proportion of the missing 40% of CE can be attributed to a loss of hydrogen gas from the system. Hydrogen is an extremely small molecule and is able to permeate most plastics, and is therefore likely to be leaking out of the reactor. In a tightly engineered system theoretically the coulombic efficiency could approach its maximum of 100%, resulting in an electrical energy recovery of 129%.

The substrate efficiency of the cell was considerably lower than the other efficiencies measured. This efficiency represents how much of the substrate is actually recovered as hydrogen, and gives an indication of how much substrate is used in the MEC process. Even if the 40% loss of hydrogen through leakage (as suggested by the CE of 60%) is accounted for in this calculation then the substrate efficiency would only increase from 10% to around 23%. Losses may be taken to suggest that substrate is being used in competitive oxidation processes, but only low levels of oxygen entered the cell with the influent. Sulphate reduction equated to about 3.6% of the total COD removal. Limited nitrates were available. Further losses can be accounted for by the probable build-up of sludge within the reactor as evidenced by the constant COD removal value throughout the study despite the increasing efficiency of the reactor, and that on three occasions a very high COD peak entered the reactor, on two of these occasions the peak of COD is not seen to leave the reactor see Figure 6-7.

Clearly the high resistance of the reactor means the overall efficiencies of the reactor will be low. The resistance observed is more problematic in this larger scale system than at the laboratory scale, and would also become increasingly challenging with further scale up. Improved reactor design is needed to overcome these problems. In a large scale system a considerable wire length is likely to be inevitable, resistance could be reduced through the use of a thicker wire, additionally resistance could be reduced in the electrode by improving the connection between the electrode, current collectors and wire. Further research into different materials and different configurations of materials would hopefully lead to improvements at a larger scale.

Further efficiency losses as identified above could be minimised by improving the engineering of the system. The two 'new' materials used in this study for the membrane and cathode have not been truly evaluated. More expensive alternatives such as Nafion membrane and a Pt coated cathode may prove to be worthwhile investments if performance increases greatly with their use. The biological MEC process works, and works relatively consistently for a period of at least three months. Although tested in realistic conditions, this was over a spring/summer period, survival over periods of sustained low temperature has yet to be confirmed.

The relationship between electrical energy recovery, electrical power input and coulombic efficiency has been defined however the prediction energy requirements for a larger scale MEC system may be difficult to make. Theoretical input voltages lie far from those needed in reality even for acetate fed cells, typically between 0.4-1.0 V compared to the 0.114 V theoretically needed (pH 7, 298 K) (Logan, 2008). A relatively small change in the electrical power input can have a large effect of the overall electrical energy recovery, yet if this value is not high enough to overcome the losses in the cell no hydrogen will be produced.

Undoubtedly there are many factors that require further investigation. Many of the inefficiencies could be overcome by improved engineering, but also a greater understanding of the biological processes (both working with and against the cell performance), community structure and ecology would allow for more confident design and manipulation.

The aim of this research was to determine if MEC technology could be a viable and alternative to the activated sludge process. The pilot scale reactor has worked producing hydrogen, with real wastewaters at ambient temperatures for over 3 months at a volumetric treatment rate just below that for activated sludge. A breakeven energy was not consistently achieved during the course of the study, yet is believed to be within reach with improved hydrogen capture and improved design to increase efficiencies. With this proof of concept now made we are a large step closer to using MEC technology for sustainable wastewater treatment.

Chapter 7. Conclusions

The overall aim of this research is to reach an understanding of whether microbial electrolysis cells could be a domestic wastewater treatment option.

I conclude that energy neutral or energy positive wastewater treatment should be possible. This research started by looking into how much energy is held intrinsically within the wastewater, and concluded that the amount of energy in the wastewater is substantial, more than previously thought, and more that the energy costs currently incurred in its treatment (18-29 kJ/gCOD vs. 2.5-7.2 kJ/gCOD in activated sludge treatment). Although this energy measured is internal chemical energy which is higher than the Gibbs Free Energy that would be available to microorganisms, with a biological system engineered for energy extraction from wastewater rather than an energy input, i.e. utilising other redox pathways rather than simple aerobic oxidation.

With the conclusion made that there is enough energy inherently contained in wastewater to treat it, the next question was to determine if Microbial Electrolysis Cells could meet this demand, replacing the high energy demanding activated sludge process with an energy yielding process. Parts of the thesis, in particular the low temperature work, suggested this might be possible yet other parts of the research did not such as the failure in MEC wastewater fed reactors. However by building and testing a pilot scale reactor on site at a wastewater treatment the most positive and conclusive evidence that this technology could work for real wastewater applications was gained. The reactor, even though it was a ‘first design’ using low cost alternatives to the optimum materials, and with many other problems such as non-optimised flow and hydrogen leakage and high resistance, it came reasonably close to its breakeven energy point. Even without breaking even it was more effective in terms of energy used per gCOD removed, and came close to the volumetric loading rates of the activated sludge process.

There is still much work to be done at this scale and larger to: understand the issues of scaling; economic feasibility; hydrogen capture and storage; design and materials; and optimisation. This work could then lead to retrofitting old activated sludge lanes with microbial electrolysis cells, radically changing the wastewater industry.

All the research conducted in this PhD has shown that the substrate acetate is not an adequate model of wastewater. This has been shown simply in terms of the energy available per gCOD, the acclimatisation and number of exoelectrogens able to digest these substrates, the diversity of the community fed with these substrates and their function within microbial electrolysis cells. The higher diversity estimates and complex acclimatisation pattern of acetate fed reactors suggest acetate may not be the optimum compound to use in BES's. Wastewater fed systems may have less free energy available, and therefore result in a more efficient biomass being formed. The lower coulombic efficiencies observed in wastewater fed reactors might be an inevitable result of electrons being lost within the longer chains of digestion, and not necessarily an indication of inefficient biomass.

The conclusion that temperature does not affect the performance of MFCs is surprising, although does correspond to some of the literature in this area (Catal et al., 2011, Jadhav and Ghangrekar, 2009). This suggests that there is a similar level of free energy available in systems run at different temperatures, and that low temperatures do not represent a disadvantage for BES. This is also observed in the pilot reactor, here low temperatures may be an advantage reducing methanogenic activity which proved fatal in the only other pilot scale MEC study to be published (run at 30 ^{o}C) (Cusick et al., 2011).

A further surprising conclusion was that inoculum did not have an effect on reactor performance, although the inoculum did interact with substrate to produce higher diversities within acetate fed reactors inoculated with high diversity soil. Exoelectrogenic bacteria were present naturally in all the wastewater inocula, and the Arctic soil inocula used throughout this research, albeit at low levels. The number or proportion of exoelectrogens was estimated to be 0.0017% using the very old methodology of MPNs, using the most recent next generation sequencing techniques and mathematical modelling algorithms, the estimates were 0.0012% and 0.00001% for two different wastewater samples. This therefore appears to be a reasonable good estimate of the rarity of such species.

BES reactors have been shown to work in challenging, real life, environments, and many observations have been made about the abundance and diversity of the organisms needed for the operation of these systems. This research has moved a substantial step

forward in proving that these technologies could be an energy efficient replacement of the activated sludge process. However we are still a long way from a deep and holistic understanding of the bacterial world operating within these systems, the energy requirements of these communities, their metabolic limits, their response to stress and ultimately their stability and function. Without this deep understanding we are reliant upon empirical data gathering, testing reactors in various environments until these limits are found. If we could model the free energy needs of the bacterial community, estimate the free energy available in the substrate, and calculate the efficiencies of the electrochemical cell, such systems could be modelled accurately and ultimately engineered to produce positive energy recovery.

Chapter 8. Perspectives on the use of MECs in the treatment of wastewater

This work has demonstrated a proof of concept of the use of MECs with domestic wastewater to produce hydrogen at the 100L scale over a 3 month time period. However this does not mean that they will be a viable wastewater treatment option. The work conducted in this research goes some way to confirming to technical feasibility of this technology in the treatment of domestic wastewaters, it does not however prove or suggest that this will be an economic viability, such an assertion is beyond the scope of this study.

There are many considerations which would need to be focused on in order to determine this economic viability for any technology to replace activated sludge treatment (AS), including those criteria stated in the introduction:

1. Extract and convert energy to a useable form at an efficiency that justifies the costs.
2. Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
3. Treat low strength domestic wastewater.
4. Work at ambient, often low temperatures.
5. Work continuously and reliably.

The detailed costing of this technology is beyond the scope of this thesis. It has been suggested that MEC technology may be an economically viable alternative to AS over other treatments such as anaerobic digestion (AD) or MFCs (Foley et al., 2010, Curtis, 2010) based on the reduction in aeration costs and the potential value of products produced. However to change the UK wastewater infrastructure would require exchanging the current AS process components for a system with higher capital costs (estimated at 0.4 €/kgCOD for an MEC compared to 0.1 €/kgCOD for AS, (Rozendal et al., 2008a)) aiming to recover the costs through the product generated. It is clear that even with low cost materials used in this research, and the idea of retrofitting the cells into existing infrastructure (Cha et al., 2010), the capital costs of filling tanks with complex electrode assemblies would be far higher than installing the aeration pipework. It would need to be ascertained whether the ‘payback’ in terms of reduction of the

energy costs and the products generated would equal the higher capital costs over the lifetime of the cells (which is again unknown at this stage).

The design life of typical wastewater treatment infrastructure is at least 25 years. MECs have not been tested over such time periods in even in the relatively clean conditions of laboratories. It is highly likely the many of the components of a typical MEC would not survive for long periods when handling real wastes, membranes for example are particularly problematical clogging over time (Zhang et al., 2011), yet membraneless are also problematic at large scale (Cusick et al., 2011). Even the estimates for a 5 year life span of electrodes and membranes used in the estimates above (Rozendal et al., 2008a) are untested under real conditions and may be unrealistic. The life span and maintenance requirements of BES will be a critical factor in determining if this technology can be used economically within the wastewater industry.

A further cost consideration is the labour costs associated with this new technology. The level of maintenance required in the MEC process is again unknown, but is likely to be higher than the AS, though may be compensated for by the reduction in sludge treatment which is a considerable fraction of the operational costs (Verstraete and Vlaeminck, 2011). The hydrogen or product produced may also require purification again the costs of this would need to be accounted for in identifying if the economic benefits of the product outweigh the costs.

The full economic costing of the MEC process versus other processes is complex, with many unknowns. It is likely to vary with: the scale and wastewater type of different treatment plants; water usage and availability; energy and material prices; and therefore inherently through time (McCarty et al., 2011). The ‘upgrading’ of AS plants with improved energy recovery from sludge AD, improved process control and greater levels of primary settling such as the Strass plant in Austria which generates 108% of its electricity use (Nowak et al., 2011) may prove to be more economically viable. The addition of AD onto the AS process is the route many UK water companies are taking including Northumbria Water Ltd who have one large sludge AD plant in operation and one under construction. However such a high degree energy recovery is exceptional, and many experts in the field question the concept of using the energy intensive process of AS to insolubalise waste organics to sludge which then can undergo energy recovery (Verstraete and Vlaeminck, 2011).

The treatment levels of the pilot MEC run were both low and variable, averaging only at 34%, the AS process can remove up to 95% of the COD (Tchobanoglous, 1991) although this is rarely the case as they are usually part of a treatment flow with pre-settling and post clarification removing a proportion of the COD (Grady, 1999). The MEC reactor demonstrated did on occasions remove the COD down to the discharge limit of 125 mgCOD/L (EEC, 1991) so operation at this level is possible. The ability to use domestic wastewaters is a clear advantage over AD which tends to be restricted to high strength industrial or farm wastes, or sludge generated by AD. Further work would be needed to demonstrate that this treatment could consistently reach discharge standards, and the electrical conductivity of the wastewater at these low strengths is sufficient for the cells to function.

Even if part of a treatment flow with pre-settling and post clarification it is likely that the MEC would need to improve treatment rates to encourage investment, additionally the more organics removed the higher the energy yield can be. Treatment rates could be improved by reducing electrode spacing; however this would have the knock on effect of reducing the volumetric loading rate. The MEC could therefore end up requiring the same unit space as trickling filters, and therefore not be a viable option either due to land restrictions or poor economic comparability to this low energy treatment option.
There is an increasing body of research demonstrating that BES technologies will work at ambient temperatures (Jadhav and Ghangrekar, 2009, Catal et al., 2011, Larrosa-Guerrero et al., 2010), added to by the work in this thesis. Further work may be required in demonstrating this with real wastewaters at a larger scale, and also in quantifying and overcoming the kinetic effect of the lower temperatures on bacterial metabolism.

Many challenges lie ahead with BES research both from a technological and economic perspective. Only through completing and importantly combining these research areas will we be able to reach an understanding as to whether the technology can be used in the wastewater treatment plants of the future.

Chapter 9. Recommendations for future research

This research set out to answer the question as to whether microbial electrolysis cells could be used for wastewater treatment. Most of this research has strengthened the case that they are, however many more research and application questions remain unanswered. Each piece of research described in this thesis could be developed further to give more conclusive answers:

Chapter 2: A comprehensive survey into the amount of energy contained within wastewater is warranted. In the research conducted two samples were tested from different wastewater treatment plants and the results showed a large difference in the energy content between the samples and with that which would be predicted. Discovering the energy in wastewater is fundamental to the study of bioelectrochemical systems, and other technologies which aim to yield energy from wastewater. If we are to evaluate the true potential of these technologies we need to know how much energy is actually encapsulated in domestic wastewater, enabling efficiencies to be calculated and therefore better solutions engineered.

Measuring internal energy by calorimetry is a standard method in the solid waste industry (Garg et al., 2007, Lupa et al., 2011), yet when applied to wastewater the problem arises that samples have to be dry, and even with the improved and extremely laborious freeze drying method used in this research 20-30% of the volatiles in wastewater were lost. With an improved and quicker method, such as the use of distillation or reverse osmosis, a comprehensive survey of wastewaters in the UK could be made. This would: facilitate decisions on where best to invest in new technologies; give an indication of which technologies might be more suitable for different wastewaters; inform of the efficiency of processes; and most importantly – make decision makers believe energy extraction from wastewaters is economically viable and worthwhile.

Chapter 3: With a more definitive answer to the number of bacteria present and their growth pattern, accurate assessments of specific activity and growth yields could be made. Accurate estimations of these values are needed for parameterising models of these systems. By redesigning these experiments, and the reactors used to minimise or at least quantify all losses, a mass balance could be made and these values determined.

However the most intriguing question arising from this work was the difference in the pattern of acclimatisation observed in the acetate fed cells and those with complex substrates. Although possible reasons for this difference were suggested, a conclusive answer was not found. By conducting further research scaling between acetate and starch in terms of substrate complexity, the step causing the change in response of acclimatisation could be found, which may give valuable insight into the development and ultimately the function of these communities. The use of other microbiological techniques such as flow cytometry and QPCR may also help in the accurate determination of these values.

Chapter 4: The finding that temperature and inoculum had little effect on reactor performance is significant to the eventual implementation of this technology. The high variability within the warmer reactors would however be worth investigating further, if all the warm reactors were able to work at the maximum level shown by some, temperature would be a significant factor. The reactor configuration used in these experiments may have been limiting factor, thus if repeated with a higher performing reactor design, the temperature effect may be observed.

The counterintuitive observation that acetate fed cells produced a higher diversity was of great interest in this work. Further research is needed to determine if it is energy that controls the diversity, not the complexity of the substrate. This could be examined by scaling through simple compounds with known and increasing free energies (e.g. from the ΔG of the reaction under standard conditions at pH 7: acetate 27.40 kJ/e^- eq, pyruvate 35.09 kJ/ e^- eq and glucose 41.35 kJ/e^- eq) and observing how diversity changes.

Chapter 5: The conclusion that laboratory wastewater fed reactors fail after a short period of time is contradicted by chapter 6 where the pilot MEC worked. Determining the reason for failure at the small scale is a priority for any further lab scale research studies. Other than scale, the two different factors in the lab based experiments compared to the pilot, are that feed is continuous not batch, and that the laboratory reactors are acclimatised as a MFCs. Research into these factors, and a solution to the failure is needed to achieve the working laboratory wastewater fed systems required for investigations into the use of this technology for wastewater treatment.

Chapter 6: The final part of this research gave the most conclusive answer as to whether MECs can work for wastewater treatment and will, when published, put the research of MECs onto a new platform. Much research is still needed into improving efficiencies and critically achieving the breakeven energy recovery, further scaling, different materials and design, and the economic feasibility of implementing this technology at scale. If the use of this technology is validated, research is needed into the strategic implications this will have on the wastewater treatment industry.

Further recommendations: The research described has increased our understanding of how BES can function in wastewater treatment. A more fundamental direction of research would be the use of BES in understanding the energetic laws and rules which underpin biological systems. Such rules would have huge impact on design in both the near and distant future (Curtis et al., 2003). BES offer the unique opportunity, effectively opening a window on the energy involved in biological reaction, as this energy is routed through an external circuit and can therefore be measured allowing energetic interactions to be unravelled.

By designing a biocalorimeter type BES reactor, where all energetic inputs and outputs are measured (with no leakage) this could be tested using simple substrates and monocultures, and simple laws developed. For example if a substrate chemically yields 'x' kilojoules of Gibbs free energy (ΔG), exactly how much of this can be accessed by bacteria at a set pH and temperature, what proportions go to growth and maintenance for the BES to be stable and what the energy transfer efficiency is. By then scaling to more complex substrates and mixed cultures insight could be gained on: the fermentation processes and on how and why some reaction routes may be favored over others; if the overall ΔG of a complex substrate adequate to model outcome or is more complexity required; and if the energy needs are similar amongst trophic layers.

Through manipulating the systems thermodynamic constraints (temperature, pressure, and ionic strength) to give predictable outcomes, the rules identified above could be verified. Knowledge would also be gained on which thresholds of energy can change community behavior, and how easily these can be manipulated, how much the bacteria can compensate for these changes. Additionally by taking the system to the energetic edge the real limits can be defined and compered to theoretical limits. Ultimately an understanding of how energy requirements of a community link to abundance and

diversity could be gained, and allow for these to be manipulated to increase system stability.

By using a BES in this novel way, the thermodynamic laws which underpin the microbial world may be discovered. The rules generated could be used to create a model allowing biotechnologies to be reliably engineered. The feasibility and efficiency of a bioprocess being modeled at the investment stage without relying on estimates from empirical data. This would have huge scope to promote change and development across the scientific and engineering community.

Chapter 10. Literature Cited

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Chapter 11. Appendices

11.1. Appendix I - History of microbial fuel cell technology

The concept of fuel cells, a device that can convert electrochemical energy into electricity is not new. The first working chemical fuel cell is attributed to Sir William Grove in 1839 (Lewis, 1966). Progress since then has been slow and sporadic. Although it was understood that the direct conversion of chemical energy to electrical energy was more efficient than combustion in a heat engine (where up to 80% of the energy in the fuel is lost through heat in the exhaust, friction, air turbulence and the heating up and movement of engine parts), historically the abundance of fuel meant that the simpler combustion engine took precedence. The main surge of work in fuel cells has been in the last 10-15 years as fossil fuel prices, and the need for cleaner and more efficient energy production has increased (Logan, 2008).

The first biologically catalysed fuel cell was made in 1911 by a Professor of Botany M.C. Potter at Newcastle University. He discovered that an electrical current could be produced using bacteria as the catalyst on the anode, with a glucose and yeast mixture under various conditions of temperature and concentration he produced a maximum of 0.3 to 0.5 volts (Potter, 1911). This work was added to by Barnet Cohen who built a small bacterial battery using a series of half cells. This work drew more attention to the area, however the major drawback of the system was highlighted, only a very low current is able to be produced and it is rapidly discharged. The use of mediators such as potassium ferrycyanide and benzoquinone did enable greater voltage to be produced however the current remained low (Cohen, 1930).

Del Duca et al. (1963) re-visited the idea and set up a working laboratory model built using urea as a fuel. Urea was broken down enzymically by urease to produce ammonia at the anode, which then reacted with an air cathode producing current. A conceptual design was put forward for a 20-Watt portable urea battery, containing 64 individual cells, however the battery life was only 2 weeks.

Karube et al.(1976), described how carbohydrates were broken down to hydrogen using a fixed matrix of fermentative bacteria, the hydrogen reacted in the electrochemical cell. These studies were the first to use a design very similar to those MFCs used today, but with a salt bridge rather than an artificial membrane. It was believed that the bacteria's

role was to break down the carbohydrate to make electrochemically active products, which were entirely responsible for the current generation. It was not seen that the bacteria themselves were creating the electrochemical current, through the donation of electrons, though this was almost certainly the case.

R. M. Allen and then H. P. Bennetto worked on microbial fuel cells throughout the 1980's at Kings College, London. They had the vision that fuels cells could be a solution to the poor sanitation and lack of electricity supply in the then termed 'third world'. A paper which was the culmination of this work was published in 1993, simply titled Microbial Fuel-Cells – Electricity Production from Carbohydrates, was the first to show an understanding of the mechanism at work (Allen and Bennetto, 1993), although electron transfer was still not understood. It was thought that electrons were extracted from the oxidation of carbohydrates; these would then become trapped within the bacteria, but would become available for transfer to the anode through the use of a chemical redox mediator. Chemical mediators such as ferricyanide were expensive, non-sustainable and toxic to the environment.

The breakthrough discovery was made in 1999 that chemical mediators where not needed in the cells (Kim et al., 1999). This critical discovery that MFCs do not require these mediators, and the ever increasing pressures to reduce pollution, has led to an explosion of research in this area.

In 2005 it was discovered that microbes could be used in an electrolysis cell (Rozendal et al., 2006, Liu et al., 2005b). Electrical energy input can be combined with the energy derived from the fuel by bacteria to drive electrolysis reactions making products which would otherwise require much larger inputs of energy, most notably hydrogen. Thus hydrogen can be produced at greater efficiencies than is the limit with fermentation, and in theory at around one tenth of the electrical energy input of water electrolysis.

11.2. Appendix II - Theoretical cell energetics

The basic reaction occurring in an MFC or MEC can be split into two half reactions, the anode reaction which is the catabolic breakdown of the organic substrate to produce electrons, and the cathode reaction which is the donation of these electrons. The quantity of energy released per electron transferred is dependent on the chemical properties of those compounds involved, and is given by the Gibbs free energy of the reaction or ΔG_r:

$$\Delta G_r = \Delta G_r^0 + RT \ln Q$$

Equation 1

Where ΔG_r is the Gibbs free energy of the reaction, ΔG_r^0 is the Gibbs free energy for the reaction under standard conditions (temperature of 298 K and chemical concentrations of 1M for liquids and 1 bar for gases) as tabulated (Atkins, 2006), R is the gas constant 8.31 J/mol-K, T is temperature, and Q is the reaction quotient i.e. the ratio of the activities of the products and the reactants.

The cell potential (E_{emf}) can be calculated from Gibbs free energy of each half reaction:

$$E_{emf}^0 = -\Delta G_r^0 / nF$$

Equation 2

Where n is the number of moles of electrons transferred and F is Faradays constant 96485 J/mol e^-.

Alternatively the potential can be calculated directly when the potential under standard conditions is known:

$$E_{emf} = {E_{emf}}^0 - \frac{RT}{nF} \ln Q$$

Equation 3

Using acetate as an example electron donor, the half-cell, and full reaction values are given for ΔG_r and E_{emf} in Table 11-1 under standard environmental conditions pH 7, 298 K:

Table 11-1 Calculated theoretical energies (as Gibbs free energy and Potential) of half-cell reactions occurring within BES fed with acetate

	Reaction	ΔG_r/ kJ/ e- eq	Potential E (V)
Anode/ donor	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$	27.40	-0.300 (-0.284)
Cathode /acceptor MFC	$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O$	-78.72	0.805 (0.816)
Overall MFC	$\frac{1}{8}CH_3COO^- + \frac{1}{4}O_2 \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}H_2O + \frac{1}{8}HCO_3^-$	-106.12	1.105 (1.100)
Cathode /acceptor MEC	$H^+ + e^- \rightarrow \frac{1}{2}H_2$	39.94	-0.414
Overall MEC	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{2}H_2 + \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^-$	12.54	-0.114 (-0.130)

Values for E_{emf} written in bracket are those calculated from the tabulated ΔG_r and E_{emf} values which vary slightly (Rittmann, 2001, Atkins, 2006).

From the equations above it can be seen that anode and cathode potentials vary with temperatures (T), substrates (ΔG_r^0 or E_{emf}^0) and ionic concentrations (Q), especially pH. These can be calculated as shown below (except in the case of wastewater). However in a real system they may vary from time to time, place to place, and even within the same reactor as substrates are utilised and H^+ ions produced:

Substrate

In an acetate fed MEC the theoretical anode potential (E_{An}) under standard biological conditions (i.e. pH 7, temperature 25 ^oC) would be -0.284 V and the for the hydrogen evolution reaction (again at pH 7) it is -0.414 V, giving a cell potential E_{emf} of -0.13V an additional 0.13V would need to be added, with glucose this difference is positive 0.015V, theoretically no energy would need to be added. With wastewater and its unknown composition and variability the theoretical anode potential cannot calculated,

the potential of a variety of compounds which may be found within wastewater are shown in Table 11-2.

Table 11-2 Known Gibbs free energy and potential values for a variety of compounds which may be present in wastewater

Substrate	ΔGr (kJ/mol e-)	E_{An} (V)	E_{emf} (V)
Methane	23.53	-0.244	-0.170
Acetate	27.40	-0.284	-0.130
Propionate	27.63	-0.286	-0.128
Ethanol	31.18	-0.323	-0.091
Protein	32.22	-0.334	-0.080
Lactate	32.29	-0.335	-0.079
Citrate	33.08	-0.343	-0.071
Methanol	36.84	-0.382	-0.032
Glycerol	38.88	-0.403	-0.011
Formate	39.19	-0.406	-0.008
Glucose	41.35	-0.429	0.015

ΔGr values from (Rittmann, 2001)

Temperature

Using acetate in an MFC as an example, with an acetate concentration of 0.12M (1 g/L of Na-acetate), bicarbonate concentration of 0.005M, at pH 7, and partial pressure of O_2 as 0.2, the potential, E_{emf} of the anode and cathode can be calculated through a range of temperatures from 0 to 30 ^{o}C:

Anode reaction

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O$$

Cathode reaction

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O$$

The potential under standard environmental conditions (E^0) for these reactions are 0.187V and 1.229V respectively. Using Equation 3 above:

Anode

$$E_{An} = {E_{An}}^0 - \frac{RT}{nF}\ln\frac{[CH_3COO^-]}{[HCO_3^-]^2[H^+]^9}$$

$$E_{An} = 0.187 - \frac{(8.31\,J/mol\,K)\,(T)}{(8)(\,96485\,C/mol)}\ln\frac{[0.012]}{[0.005]^2[10^{-7}]^9}$$

Cathode

$$E_{Ca} = E_{Ca}{}^{0} - \frac{RT}{nF}\ln\frac{[H_2O]}{[O_2]^{1/2}[H^+]^2}$$

$$E_{Ca} = 1.229 - \frac{(8.31\ J/mol\ K)\ (T)}{(2)(\ 96485\ C/mol)}\ \ln\frac{[1]}{[0.02]^{1/2}[10^{-7}]^2}$$

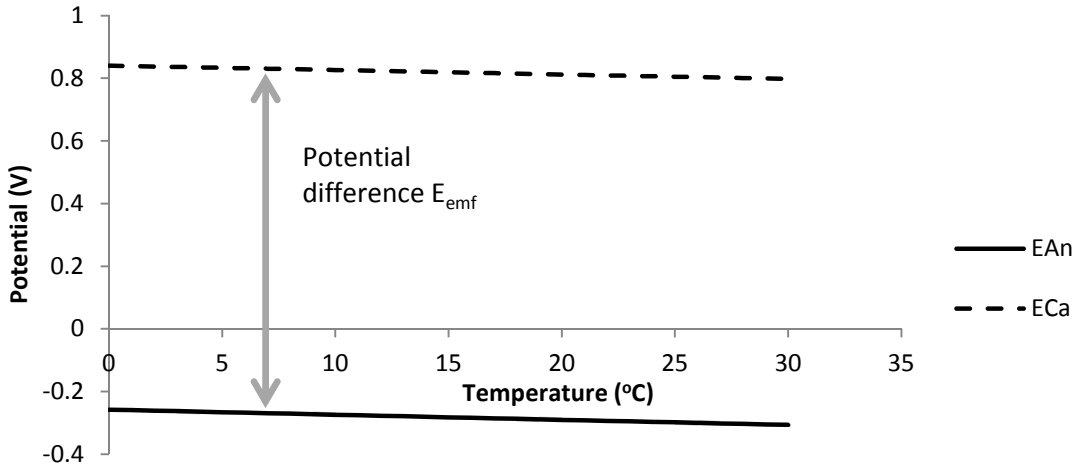


Figure 11-1 Calculated anode and cathode potential though a range of temperatures using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; pH 7; and partial pressure of O_2 as 0.2

The difference between the anode and cathode potential seen in Figure 11-1 varies only slightly from -1.098 V at 0 ^{o}C to -1.104 V at 30 ^{o}C. Theoretically therefore the energy available to be produced via a fuel cell is not greatly affected by temperature within the ranges given. This is however a simplistic approach to a system which, as stated previously is highly complex. As temperatures vary, so will many other factors including dissociation constants, partial pressures of gases and metabolic activity of the bacteria. It is therefore unlikely that the fuel cell will be able to generate as much current at lower temperatures as higher ones, yet it may not be as detrimentally affected by temperature as straight anaerobic digestion.

pH

The reaction co-efficient (Q) is calculated on the basis of the concentrations of the products and reactants in the chemical equation. This factor is critically dependant on

the pH of the system, i.e. the number of H^+ ions, as pH is a logarithmic scale, variance between pH 6 and pH 7 (both within the tolerance of bacteria) has a large effect on the Q value and therefore the overall potential of the cell. An example of this is shown below where the pH of the anode in an acetate system as described in the equations above at 25 ^oC is varied between pH 5 and 8, the cathode potential is kept constant under standard conditions. The potential difference ranges from 0.97 to 1.24 V.

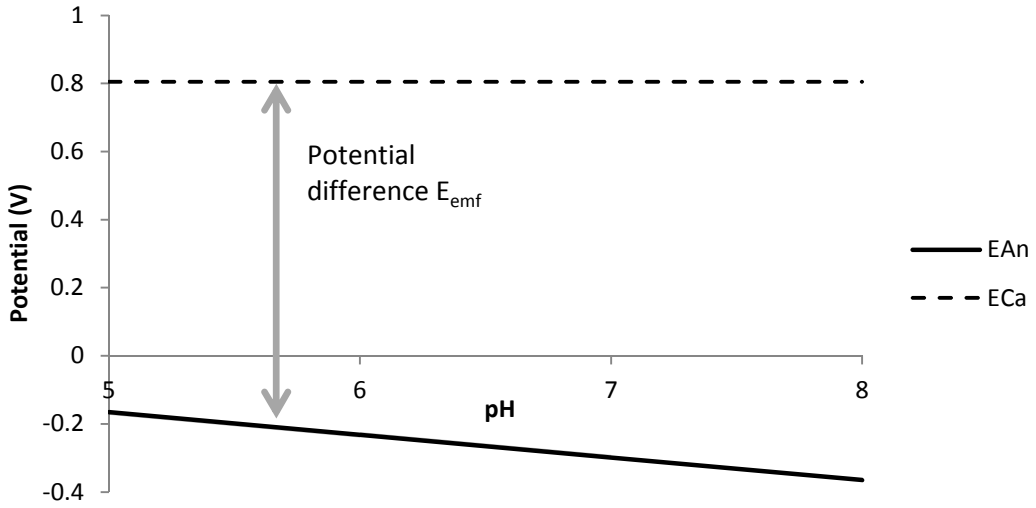


Figure 11-2 Calculated theoretical anode and cathode potential through a range of pHs using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; temperature 25 ^oC; and partial pressure of O_2 as 0.2

11.3. Appendix III – Table of calculated kJ/gCOD of various organic compounds

Compound	Formula	ΔH/gCOD
Benzene	C_6H_6	10.2
Linoleic acid	$C_{18}H_{32}O_2$	13.4
Benzoic acid	C_6H_5COOH	13.4
Myristic acid	$CH_3(CH_2)_{12}CO_2H$	13.6
Acetic acid (Acetate)	CH_3COOH	13.6
Phenol	C_6H_5OH	13.6
Palmitic Acid	$CH_3(CH_2)_{14}CO_2H$	13.6
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$	13.7
Methane	CH_4	13.9
Ethane	C_2H_6	13.9
Lactic acid	$CH_3CH(OH)COOH$	14.0
Ethanol	C_2H_5OH	14.3
Glucose	$C_6H_{12}O_6$	14.3
Propene	C_3H_6	14.3
Cyclopropane	C_3H_3	14.5
Ethanal	CH_3CHO	14.6
Ethene	C_2H_4	14.7
Sucrose	$C_{12}H_{22}O_{11}$	14.7
Methanol	CH_3OH	15.1
Chloroethylene	C_2H_3Cl	15.7
Oxalic acid	$(COOH)_2$	15.9
Formic acid	$HCOOH$	15.9
Ethyne	C_2H_2	16.3
Hexachlorobenzene	C_6Cl_6	16.5
Dichloroethylene (1,1)	$C_2H_2Cl_2$	17.1
Dichloroethylene (1,2)	$C_2H_2Cl_2$	17.2
Methanal	$HCHO$	17.8
Trichloroethylene	C_2HCl_3	20.0
Teterachloroethylene	C_2Cl_4	26.0
Chloroform	$CHCl_3$	29.1
Trichloroacetic acid	CCl_3COOH	30.4

11.4. Appendix IV - Description of the calculation algorithm used in the Shizas and Bagley

Shizas and Bagley (Shizas and Bagley, 2004) use a sample of municipal wastewater which prior to drying contains 431 mg/L COD. This sample is then oven dried to give a total solids measurement of 1980 mg/L. The dried sample is used in a bomb calorimeter giving 3.2 kJ/g dried weight.

Calculations derived from this data cited in various papers (Logan, 2008, Liao et al., 2006, Schroder, 2008, Logan, 2009):

$$3.2\ \mathrm{kJ/g}\ \times\ 1.98\ \mathrm{g/L} = 6.3\ \mathrm{kJ/L\ wastewater}$$

$$6.3\ \mathrm{kJ/L}\ \times \frac{1}{0.431\ \mathrm{gCOD/L}}\ =\ 14.7\ \mathrm{kJ/gCOD}$$

If the exercise is repeated on the data from the present paper using the oven dried samples and the measurement taken for COD prior to drying the results would have been:

Cramlington

$$8.3\ \mathrm{kJ/L}\ \times \frac{1}{0.718\ \mathrm{gCOD/L}}\ =\ 11.6\ \mathrm{kJ/gCOD}$$

Hendon

$$5.6\ \mathrm{kJ/L}\ \times \frac{1}{0.576\ \mathrm{gCOD/L}}\ =\ 9.9\ \mathrm{kJ/gCOD}$$

This is an underestimation of 60% and 45% respectively.

11.5. Appendix V - Wastewater sterilisation

Several of the experiments conducted in this thesis relied on using real wastewater, but needed this to be sterile. The following method was developed:

Method

The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into Ringers sterile dilutent (APHA, 1998). Effective sterilisation was defined as colony free plates in triplicate at zero dilution. The circulation time was varied to determine the optimum. The change in chemical composition (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) of the wastewater itself as compared to autoclaving and filtering.

Results

UV sterilisation caused the least change in wastewater properties measured as shown in Table 11-3, and was able to fully sterilise the wastewater.

Table 11-3 Percentage change of wastewater characteristics caused by the different sterilisation methods

	COD	Soluble COD	Total Solids	Bacteria per 0.1ml
Autoclaved (121^{o}C for 15 mins)	-15.6% ± 0.9	21.6% ± 0.6	-13.3% ± 5.8	0
Membrane filtered (0.2um PES)	-61.5% ± 0.5	22.8% ± 1.7	-36.1% ± 11.7	40 ±19
UV sterilised (5 mins)	-1.6% ± 0.4	7.2% ± 4.6	-3.3% ± 6.7	0

Bacteria is the average number counted on triplicate plates at zero dilution. All values show mean ± standard deviation (n=3)

Conclusion

Circulation of wastewater for 5 minutes through a UV filter was effective for bacterial kill off and least detrimental treatment to the composition of the wastewater.

11.6. Appendix VI - COD removal and coulombic efficiency

In the acetate fed cells the COD removal was high for both the cells which did (85%) and did not (80%) produce current ($p = 0.051$). For the other reactors there was an average removal of 64% COD for the wastewater and 87% for the starch solution. No significant difference in the COD removal in the reactors which generated current and those that did not was found wastewater ($p = 0.188$) and starch ($p= 0.688$).

The effluent of all reactors contained no detectable VFA's. The measured anions in each cell showed that there was almost complete removal of sulphate, from a starting value of 70 ppm in the wastewater and 38 and 41 ppm in the acetate and starch solutions respectively.

The coulombic efficiency of all reactors was low, such values are reasonably typical for complex substrates, but far lower than would be expected in a functioning acetate fed cell (Logan, 2008, Liu et al., 2011).

Table 11-4 COD removal and Coulombic efficiencies of all reactors fed on the different substrates. The values in grey are the reactors where acclimatisation did not occur

Inocula (ml)	**0.01**	**0.01**	**0.1**	**0.1**	**0.1**	**1**	**1**	**1**	**1**	**10**	**10**	**25**	**25**	**25**	**25**	**50**	**50**
COD removal (%)																	
Acetate	85.6	77.1	85.4	80.3	80.5	86.7	95.6	92.4	82.1	87.3	79.9	84.7	86.6	80.2	77.6	79.0	77.3
Wastewater						60.8	41.9			59.1	69.8	68.1	70.5			80.3	62.5
Starch						88.2	84.5			88.2	86.4	89.4	81.7			80.8	90.5
Coulombic efficiency (%)																	
Acetate	0.1	0.1	1.8	0.5	0.8	4.6	5.3	1.8	0.1	6.7	7.5	10.5	8.9	10.1	9.2	9.1	0.8
Wastewater						3.6	0.1			0.3	0.2	9.4	12.5			10.4	7.4
Starch						1.3	0.78			0.82	13.4	12.5	16.9			17.4	1.6*

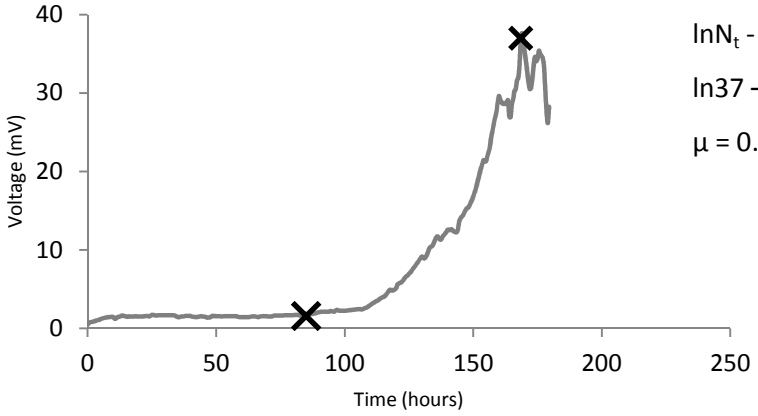
Values in grey are the reactors which did not acclimatise

*Unrepresentative value, data logging equipment failed after the point of acclimation.

11.7. Appendix VII - Yield and Specific activity calculations

Growth rate

Example calculation using 25 ml inocula

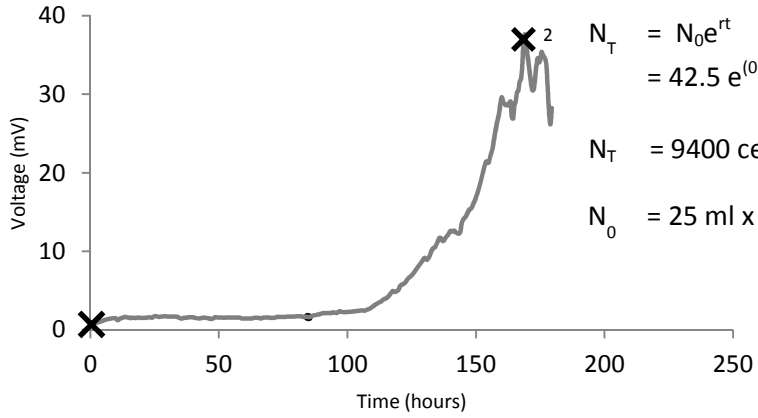


$\ln N_t - \ln N_0 = \mu(t - t_0)$

$\ln 37 - \ln 2 = \mu(170 - 85)$

$\mu = 0.034$

Specific activity



$N_T = N_0 e^{rt}$

$= 42.5\ e^{(0.034 \times 180)}$

N_T = 9400 cells

N_0 = 25 ml x 1.7 cell/ml = 42.5 cells

Each data logged voltage represents the time of 30 minutes, therefore the moles of electrons passed to the circuit per second at the data points measured is:

Moles of electrons = coulombs / Faradays constant

=((Voltage / resistance) x seconds)/Faradays constant

E.g. $\mathbf{X}_2$ =((0.037V / 470Ω)x 30mins x 60 seconds)/96485

$= 1.5 \times 10^{-6}$

Moles of electrons/cell = $1.5 \times 10^{-6} / 9400$

$= 10^{-10}$ mol e^-/cell

This value can be plotted throughout the time course of the experiment and is seen to be relatively constant.

Growth yield

The total number of cells produced up to the end of the exponential growth phase in the example above is 9400 cells.

$$\text{gCOD-cells} = (N_T - N_0) \times W \times COD_{cell}$$

where $N_T - N_0$ is the total new cell produced, W is the weight of each cell as estimated as 5.3×10^{-13} (Logan, 2008) and COD_{cell} is the estimation of 1.25 g-COD/g-cell (Rittmann, 2001).

$$\text{gCOD-cells} = (9400\text{-}43) \times 5.3 \times 10^{-13} \times 1.25$$

$$= 6.1 \times 10^{-9}$$

$$\text{gCODsubstrate} = \sum_{t0}^{t} mol\, e^{-}/8 \times 64$$

Where the sum over the growth period $t\text{-}t_0$ of the moles of electrons as calculated above is divided by 8 to give moles of acetate used, and multiplied by 64 giving the gCOD per mole of acetate.

$$\text{gCOD substrate} = 0.00011 / 8 \times 64 = 8.8 \times 10^{-4}$$

$$\text{gCOD-cell/gCOD-substrate} = 6.1 \times 10^{-9}/8.8 \times 10^{-4} = 6.9 \times 10^{-6}$$

The estimated yield of the acetate fed cells is extremely low ranging between 10^{-4} to 10^{-5} g-COD cell/g-COD substrate for the cells with between 10-50 mLs of inocula.

If exponential growth is assumed throughout the whole time period for the lower inocula cells these values are much higher up to 8 g-COD cell/g-COD for the 0.1 ml inocula. If no growth during lag is assumed these values are lower (10^{-7} g-COD cell/g-COD) and more in line with those observed for higher inocula. These yields are inconsistent with the literature on yields in microbial fuel cells (Freguia et al., 2007, Rabaey et al., 2003) although both of these studies used different methodology. They are also inconsistent with yields of other bacterial systems (Rittmann, 2001).

11.8. Appendix VIII – Calculations of performance in MFCs and MECs

Power Calculation for both MFCs and MECs

Performance can be evaluated through the amount of power produced which can be expressed as:

$$P = IE$$

Where P is the power in watts, E is the voltage as measured by the data logger in volts and I is the current in amps, calculated from the measured voltage E, at a known resistance R:

$$I = E/R$$

Power can therefore be alternatively expressed as:

$$P = E^2/R$$

This power is often also evaluated as power density (*Pd*), this is the amount of power produced per area of electrode surface (typically the size of the anode) expressed as Wm^2. Normalising the power output in this way allows different systems to be compared. This is calculated as:

$$Pd = \frac{E^2}{A_{An}R}$$

Where A_{An} is the area of the anode. The current density (A/m^2) can also be expressed in the same way normalising current to electrode size. Both power and current density can also be expressed per reactor size by substituting A_{An} above for the reactor volume in m^3, resulting in a power density measured as Wm^3. or current density as A/m^3.

Efficiency calculations for MFCs

The efficiency of an MFC is expressed as the Coulombic Efficiency (CE) and is a measure of the amount of coulombs of charge recovered from the cell from the total coulombs available in the substrate that has been removed in the reactor. It is expressed as a percentage:

$$CE = \frac{Coulombs\ recovered}{Coulombs\ in\ substrate}$$

An Amp is the transfer of 1 coulomb of charge per second, therefore by integrating the current over the course of the experiment or batch time (t) the total coulombs transferred is given. Usually the amount of coulombs in the substrate is evaluated using the amount of organic matter removed as determined by the chemical oxygen demand (COD). CE is therefore calculated as:

$$CE = \frac{8 \int_0^t I \ dt}{F \ V_{An} \Delta COD}$$

Where 8 is used as a constant derived from the molecular weight of oxygen divided by 4 the amount of electrons exchanged per mole of oxygen. Faradays constant (F) of 96485 Coulombs/mol, is the magnitude of electrical change per mole of electrons, ΔCOD is the measured change in COD in g/L and V_{An} (L) is the volume of the anode compartment containing the liquid feed at the given COD concentration. .

Efficiency calculation for MECs

The efficiency of an MEC is a more complex matter, as the output of energy is of hydrogen gas (not electricity or charge directly) and the inputs of energy are from the substrate and the additional electrical energy added to the system.

11.9. Appendix IX – Dissimilarity matrix

Unweighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.79	0.00																	
Wastewater inocula 2	0.88	**0.67**	0.00																
Acetate cold ww 1	0.88	0.67	0.70	0.00															
Acetate cold ww 2	0.88	0.65	0.69	**0.45**	0.00														
Acetate cold soil 1	0.82	0.80	0.80	0.72	0.73	0.00													
Acetate cold soil 2	0.76	0.78	0.82	0.75	0.75	**0.59**	0.00												
Acetate hot ww 1	0.89	0.71	0.69	0.54	0.60	0.73	0.78	0.00											
Acetate hot ww 2	0.89	0.73	0.71	0.56	0.58	0.74	0.78	**0.22**	0.00										
Acetate hot soil 1	0.81	0.79	0.80	0.72	0.75	0.62	0.63	0.25	0.70	0.00									
Acetate hot soil 2	0.79	0.79	0.82	0.75	0.76	0.64	0.65	0.18	0.72	**0.51**	0.00								
Wastewater cold ww 1	0.87	0.69	0.70	0.59	0.61	0.73	0.76	0.27	0.64	0.73	0.76	0.00							
Wastewater cold ww 2	0.82	0.72	0.76	0.72	0.73	0.74	0.74	0.44	0.67	0.66	0.67	**0.68**	0.00						
Wastewater cold soil 1	0.85	0.69	0.75	0.61	0.64	0.65	0.69	0.28	0.64	0.64	0.69	0.53	0.66	0.00					
Wastewater cold soil 2	0.88	0.74	0.75	0.69	0.69	0.77	0.78	0.24	0.60	0.69	0.72	0.62	0.51	**0.64**	0.00				
Wastewater hot ww 1	0.86	0.71	0.73	0.62	0.64	0.66	0.71	0.28	0.65	0.67	0.71	0.49	0.68	0.42	0.65	0.00			
Wastewater hot ww 2	0.88	0.74	0.74	0.68	0.68	0.76	0.77	0.24	0.60	0.70	0.72	0.63	0.51	0.65	0.23	**0.66**	0.00		
Wastewater hot soil 1	0.86	0.67	0.71	0.62	0.65	0.74	0.77	0.28	0.64	0.73	0.76	0.35	0.67	0.53	0.64	0.53	0.63	0.00	
Wastewater hot soil 2	0.89	0.71	0.72	0.59	0.58	0.75	0.78	0.28	0.59	0.74	0.78	0.44	0.71	0.51	0.64	0.49	0.64	**0.45**	0.00

Weighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.41	0.00																	
Wastewater inocula 2	0.52	**0.59**	0.00																
Acetate cold ww 1	0.44	0.48	0.45	0.00															
Acetate cold ww 2	0.43	0.45	0.48	**0.08**	0.00														
Acetate cold soil 1	0.50	0.56	0.53	0.17	0.16	0.00													
Acetate cold soil 2	0.44	0.48	0.47	0.09	0.10	**0.10**	0.00												
Acetate hot ww 1	0.49	0.57	0.41	0.28	0.30	0.36	0.30	0.00											
Acetate hot ww 2	0.56	0.63	0.44	0.37	0.41	0.45	0.40	**0.17**	0.00										
Acetate hot soil 1	0.36	0.43	0.50	0.23	0.22	0.30	0.23	0.32	0.43	0.00									
Acetate hot soil 2	0.41	0.51	0.43	0.14	0.16	0.22	0.15	0.23	0.34	**0.16**	0.00								
Wastewater cold ww 1	0.34	0.39	0.46	0.25	0.23	0.35	0.28	0.31	0.43	0.22	0.25	0.00							
Wastewater cold ww 2	0.37	0.42	0.55	0.43	0.40	0.45	0.42	0.46	0.52	0.31	0.39	**0.33**	0.00						
Wastewater cold soil 1	0.36	0.40	0.45	0.26	0.22	0.34	0.27	0.30	0.42	0.22	0.26	0.13	0.36	0.00					
Wastewater cold soil 2	0.41	0.50	0.41	0.31	0.33	0.39	0.33	0.30	0.36	0.37	0.31	0.32	0.33	**0.34**	0.00				
Wastewater hot ww 1	0.35	0.39	0.46	0.27	0.24	0.36	0.29	0.31	0.43	0.24	0.29	0.14	0.37	0.05	0.35	0.00			
Wastewater hot ww 2	0.38	0.49	0.40	0.31	0.32	0.40	0.33	0.26	0.35	0.35	0.29	0.29	0.33	0.31	0.08	**0.32**	0.00		
Wastewater hot soil 1	0.34	0.40	0.48	0.24	0.22	0.34	0.28	0.33	0.45	0.22	0.26	0.05	0.33	0.14	0.31	0.16	0.29	0.00	
Wastewater hot soil 2	0.38	0.38	0.52	0.28	0.24	0.35	0.30	0.36	0.49	0.25	0.30	0.12	0.32	0.18	0.34	0.19	0.34	**0.11**	0.00

Dissimilarity values calculated using UniFrac, lower values indicate greater similarity

11.10. Appendix X - Estimates of sample total diversity

Table 11-5 Estimates of total diversity using the MCMC model (Quince et al., 2008), values given are the lower 95% confidence interval : median : upper 95% confidence interval. The best fit values according to the DIC values are highlighted in bold, the model fits that had DIC scores within 6 of the best fitting model are in italics and should not be considered as plausible options for fitting the data

	Total diversity		
Sample	**Log-normal**	**Inverse Gaussian**	**Sichel**
Arctic soil inocula	**5831:7207:10593**	5151:6227:7439	3632:4403:5821
Wastewater inocula 1	**3431:4238:5572**	2217:2405:2655	2648:3275:5533
Wastewater inocula 2	2924:4260:8970	1679:2066:2752	**1716:2286:3640**
Acetate cold ww 1	**3060:5449:11740**	1273:1700:2406	1402:2197:3379
Acetate cold ww 2	13901:29226:42363	**984:1549:3049**	993:1697:3298
Acetate cold soil 1	**1380146:1393974:1407428**	*3430:5004:7687*	2960:4628:9094
Acetate cold soil 2	**1849625:1865409:1877419**	*3428:4923:7910*	3191:5018:8179
Acetate hot ww 1	1934:3511:12608	808:987:1300	**948:1310:2224**
Acetate hot ww 2	**1217:2159:6024**	643:785:1037	665:843:1264
Acetate hot soil 1	4386:8968:19150	1508:1968:2813	**1456:1984:3086**
Acetate hot soil 2	**171417:184911:197766**	2445:3773:5440	2350:3579:5577
Wastewater cold ww 1	**614:749:1014**	493:535:594	491:534:599
Wastewater cold ww 2	*859:1102:1596*	*640:708:805*	**730:906:1455**
Wastewater cold soil 1	**1079:2249:8263**	543:733:1197	651:1032:2324
Wastewater cold soil 2	556:640:789	*467:494:531*	**510:575:793**
Wastewater hot ww 1	1430:2911:9800	*637:845:1300*	**5682:16751:18608**
Wastewater hot ww 2	**483:548:660**	419:443:476	430:467:525
Wastewater hot soil 1	**820:1148:1985**	581:661:787	596:697:893
Wastewater hot soil 2	694:1135:2283	438:504:614	**468:572:954**

Table 11-6 DIC scores as defined by the sum of the deviance averaged over the posterior distribution and estimate of the sampling effort required to capture 90% of the diversity of taxa within the sample as determined by the fits of abundance distribution

	DIC			Sampling effort		
Sample	Log-normal	Inverse Gaussian	Sichel	Log-normal	Inverse Gaussian	Sichel
Arctic soil inocula	165.53	171.01	166.67	2.02E+06	4.06E+05	1.32E+05
Wastewater inocula 1	450.33	455.14	444.42	1.32E+07	2.56E+05	8.92E+05
Wastewater inocula 2	264.17	262.28	261.93	3.56E+07	2.98E+05	4.16E+05
Acetate cold ww 1	275.13	275.3	275.85	3.32E+09	1.59E+06	3.06E+06
Acetate cold ww 2	197.07	196.74	196.98	1.11E+13	1.47E+06	1.70E+06
Acetate cold soil 1	266.22	273.65	267.61	2.56E+18	1.42E+07	8.37E+06
Acetate cold soil 2	274.28	283.68	274.4	2.42E+18	7.28E+06	5.19E+06
Acetate hot ww 1	309.59	311.17	309.21	2.99E+09	5.88E+05	1.59E+06
Acetate hot ww 2	242.64	244.43	244.76	2.84E+08	3.61E+05	4.73E+05
Acetate hot soil 1	290.25	288.7	288.57	1.17E+10	1.44E+06	1.34E+06
Acetate hot soil 2	265.04	269.84	265.05	6.98E+14	4.73E+06	3.16E+06
Wastewater cold ww 1	254.73	255.02	255.23	5.22E+05	4.23E+04	4.25E+04
Wastewater cold ww 2	268.11	269.7	261.78	1.23E+06	4.91E+04	1.63E+05
Wastewater cold soil 1	201	201.99	197.99	2.68E+08	1.53E+05	5.35E+05
Wastewater cold soil 2	333.27	349.36	332.04	3.47E+05	3.70E+04	9.96E+04
Wastewater hot ww 1	252.09	254.67	246.76	1.37E+09	2.57E+05	1.05E+09
Wastewater hot ww 2	274.09	279.19	275.06	1.51E+05	2.52E+04	3.56E+04
Wastewater hot soil 1	248.04	250.28	248.96	3.54E+06	7.21E+04	9.24E+04
Wastewater hot soil 2	243.6	244.69	242.65	1.93E+07	7.44E+04	1.32E+05

11.11. Appendix XI - Details of the bacteria phyla and families found within the samples tested

It is seen in Figure 11-3 (a) that the inoculated and acclimatised reactors have become enriched Proteobacteria, this phylum dominates with about 80% abundance in the acetate fed cells, and around 60% in the wastewater fed cells. Proteobacteria are a diverse phylum of bacteria, yet most of this high abundance in the reactors is caused by the enrichment of *Geobacter* an exoelectrogenic organism, as is seen in Figure 11-4. *Rhodocyclaceae*, *Psuedomonas* and *Desulfovibrio* also added to the proportion of Proteobacteria that became enriched. The relative abundance of the other main phyla generally drops within the reactor samples, a proportion (around 10-20%) of Bacteriodietes remains, and there is some enrichment of Acidobacteria in the wastewater fed reactors. The wastewater reactors have a greater spread of abundance over the phyla groups shown, with less domination by Proteobacter.

The OTU richness shown in Figure 11-3 (b) again shows the greater diversity of the acetate reactors over the wastewater fed ones, both by the larger bar size and the Chao estimate above. It is seen many of the OTUs present in the inoculum have survived in the acetate reactor conditions, despite the metabolic narrowing of the conditions. Surprisingly this greater diversity or spread of OTUs appears to be slightly higher in the cold reactors, than the warm ones. In the case of the wastewater fed reactors the OTU richness in reduced, temperature does not appear to have an impact.

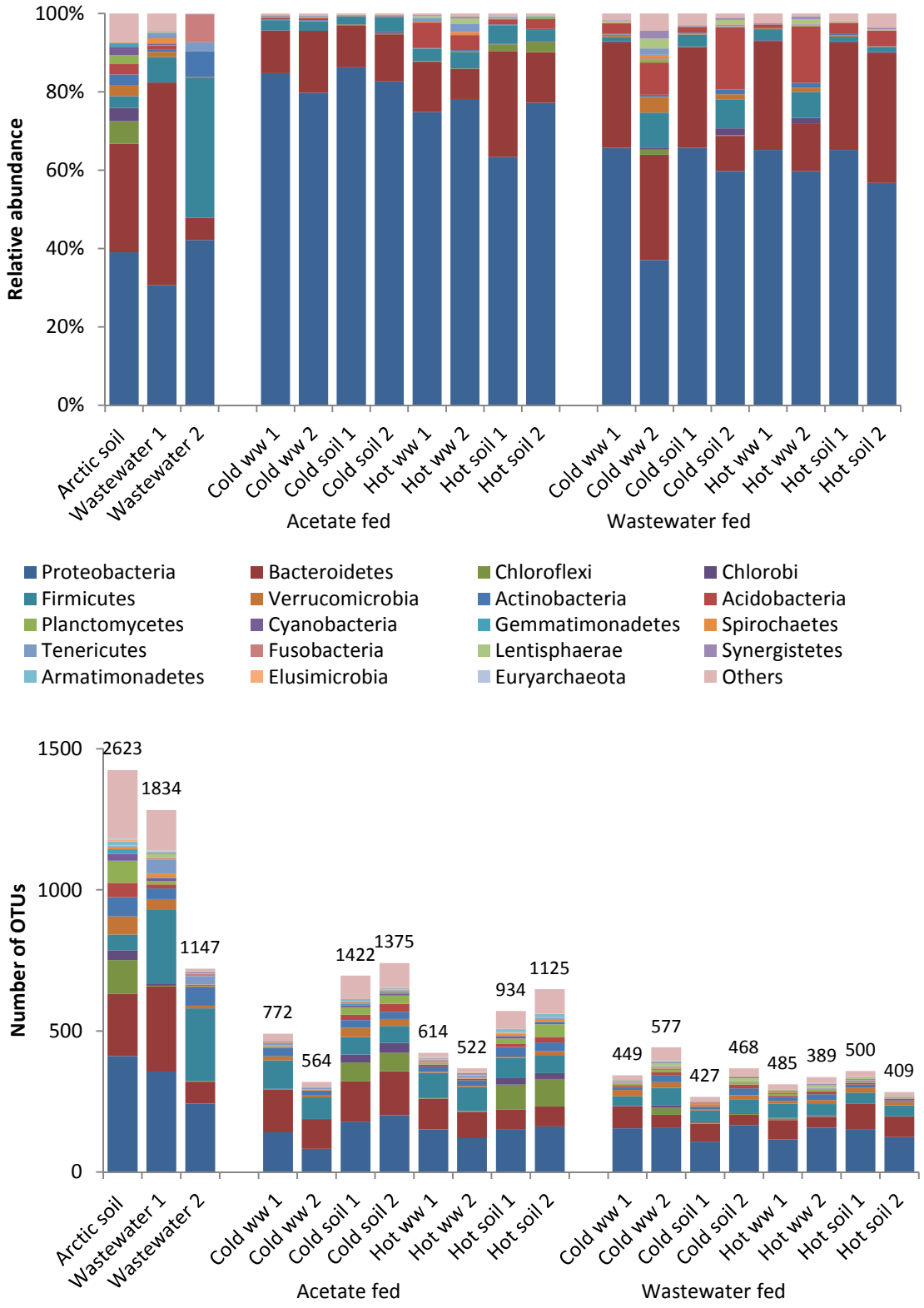


Figure 11-3 Relative abundance (a) and OTU richness (b) for all the data sets given at the phylum rank. Relative abundance is shown as the number of reads within each taxa divided by the total number of reads. The OTU richness is the number of taxa within each phylum is given by the size of the bar, the Chao 1 estimate of richness is written at the top of each bar

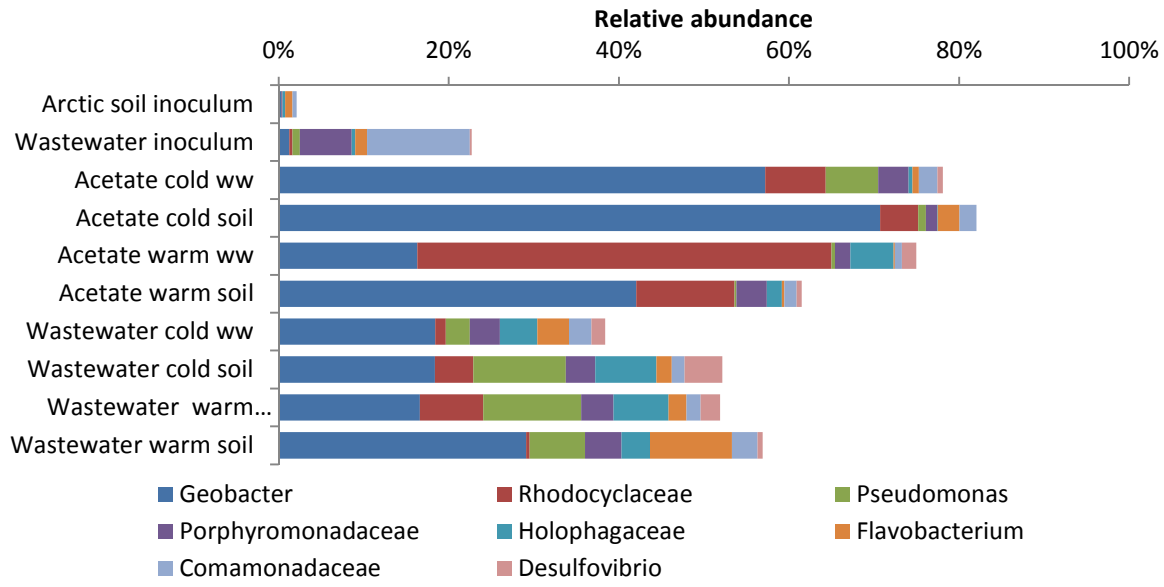


Figure 11-4 The relative abundance of the 8 most dominant genus as an average for the duplicate reactors under each condition, where the genus name was not given by the classification database family is used

It would be expected that the most dominant organisms within the reactors are the ones that are able to most competitively metabolise, grow and therefore reproduce within the conditions of the reactors. The top 8 most dominant genus are given in Figure 11-4, for Rhodocyclaceae, Porphyromonadaceae, Holophagaceae, Comamonadaceae the classification did not give the genus name, and therefore the family name is given. It is seen that for the acetate fed reactors these 8 genus make up a large proportion of the total abundance, and in the cold reactor most of this is by *Geobacter*. For the warm acetate reactors, *Geobacter* is still important, but Rhodocyclaceaea is also dominant, especially in those seeded with wastewater. The proportion of *Geobacter* is made up of 11 different species (names of which are not given by the classification), 4 of which are dominant within the reactors. Rhodocyclaceae is a diverse family of bacteria associated with wastewater treatment, further classification of this group is not made.

Within the wastewater reactors Geobacter is less dominant, between 20-30% of abundance, and there is a greater spread of the other genus and families, most notable *Pseudomonas* which make up to 10%. Within the Pseudomonas genus, 8 species were identified, of which 2 were dominant within the reactors, Pseudomonas have previously been seen within fuel cell systems fed substrates such as glucose and butyric acid and are believed to be capable of fermentation (Kiely et al., 2011c), some species such as *Pseudomonas aerunginosa* produce soluble redox shuttles and have been investigated

for their use within fuel cell systems (Marsili, 2010). The family of Holophagaceae is also quite enriched, this family includes the species of *Geothrix fermetans* which has been found in wastewater fed MFCs and is believed to be important in the hydrolysis or fermentation steps, (Kiely et al., 2011a), and has also been linked to shuttle formation (Bond and Lovley, 2005). *Flavobacteium* are also enriched, although this genus is more typically associated with freshwater environments. There is also likely to be sulphate reduction occurring in the cells due to the presence of *Desulfovibro*.

11.12. Appendix XII – Acknowledged contributions

Section	Other contributors	Contribution of E. Heidrich
Chapter 2	T.P. Curtis and J. Dolfing – editing and guidance with content	90%
Chapter 3	T.P. Curtis and J. Dolfing – editing and guidance with content	95%
Chapter 4	T.P. Curtis and J. Dolfing - editing and guidance with content, M. Wade – bioinformatics analysis, W.T.Sloan – sequencing funding	95%
Chapter 5	T.P. Curtis, K. Scott, I Head and J. Dolfing – discussion and experiment planning	95%
Chapter 6	T.P. Curtis K. Scott and J. Dolfing - editing and guidance with content, S. Edwards – site installation and running MEC	90%

Evaluation of Microbial Electrolysis Cells in the treatment of domestic wastewater

Thesis submitted to Newcastle University for the degree of Doctor of Philosophy

Author: Elizabeth Susan Heidrich

Supervisor: Professor Tom Curtis

Co-Supervisor: Dr Jan Dolfing

Co-Supervisor: Professor Keith Scott

Date: May 2012

Abstract

Wastewater can be an energy source and not a problem. This study investigates whether rapidly emerging bioelectrochemical technologies can go beyond working in a laboratory under controlled temperatures with simple substrates and actually become a realistic option for a new generation of sustainable wastewater treatment plants.

The actual amount of energy available in the wastewater is established using a new methodology. The energy is found to be considerably higher than the previous measurement, or estimates based on the chemical oxygen demand with a domestic wastewater sample containing 17.8 kJ/gCOD and a mixed wastewater containing 28.7 kJ/gCOD.

With the energy content established the use of bioelectrochemical systems is examined comparing real wastewater to the 'model' substrate of acetate. The abundance of exoelectrogenic bacteria within the sample, and the acclimation of these systems is examined through the use of most probable number experiments. It is found that there may be as few as 10-20 exoelectrogens per 100 mL. The impact of temperature, substrate and inoculum source on performance and community structure is analysed using pyrosequencing. Substrate is found to have a critical role, with greater diversity in acetate fed systems than the wastewater fed ones, indicating that something other than complexity is driving diversity.

Laboratory scale microbial electrolysis cells are operated in batch mode fail when fed wastewater, whilst acetate fed reactors continue working, the reasons for this are examined. However a pilot scale, continuous flow microbial electrolysis cell is built and tested at a domestic wastewater treatment facility. Contrary to the laboratory reactors, this continues to operate after 3 months, and has achieved 70% electrical energy recovery, and an average 30% COD removal.

This study concludes that wastewater is a very complex but valuable resource, and that the biological systems required to extract this resource are equally complex. Through the work conducted here a greater understanding and confidence in the ability of these systems to treat wastewater sustainably has been gained.

Acknowledgements

It has been a great privilege to be able to study for a PhD, and I am grateful to all those who have made it possible.

I would like to thank my supervisor Tom Curtis for giving me the support and freedom to develop and explore my own ideas, always meeting these with enthusiasm and the necessary intellectual guidance. I would also like to thank Jan Dolfing who has skilfully guided me into, and through many intellectual challenges. I would like to thank Keith Scott and Ian Head for their support and guidance when needed. I would also like to thank Bill Sloan and Chris Quince for their support and guidance with the sequencing work, the contributions of individuals are detailed in Appendix XII.

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Chapter 1. Introduction

There is growing consensus that wastewater is a resource not a problem (Verstraete and Vlaeminck, 2011, Sutton et al., 2011, McCarty et al., 2011). The conventional treatment of wastewater removes its organic content via aerobic processes, termed activated sludge, this is energy expensive typically 3% of the electrical energy usage of many developed countries (Curtis, 2010). Not only is the energy in wastewater removed not recovered, we expend considerable energy in performing this removal.

In the UK the water sector energy use has increased 10% in the last 10 years (Water UK, 2012, Water UK, 2011), industrial electricity prices have increased by 69% since 2000 (National Statistics, 2011). If these trends continue the energy bill for the water sector will be vastly higher than for the current 9016 GWh (Water UK, 2012). With infrastructure requiring long term planning and capital investment, it is hard to see without drastic action how the necessary changes can be made. Technologies that require relatively simple modifications to the current infrastructure to become operational are more likely to be given a chance rather than those which require wholesale change. New technology should ideally fit reasonably well into the existing infrastructure, and as a minimum achieve similar loading rates per unit area to activated sludge of 0.4-1.2 kg BOD $m^{-3}d^{-1}$ (Grady, 1999). The high capital costs of change and the uncertainty of using a different technology, coupled with the regulation of both effluent quality and pricing structures, are an obstacle to change.

There are alternatives to this approach. Replacing the aerobic activated sludge process with an anaerobic process means the energy stored in the organic content of the wastewater is converted to methane (80% efficiency) which can be combusted to produce electricity (35% efficiency) (McCarty et al., 2011). Only around 30% of the total energy in the wastewater can be captured as electricity in anaerobic systems, although with heat exchange in the combustion process, or the use of non-combustion methods of conversion, this could be increased (McCarty et al., 2011).

The scientific challenges of creating an energy neutral or even energy positive wastewater treatment process are also substantial and complex. The process needed to replace activated sludge must:

- Extract and convert energy to a useable form at an efficiency that justifies the costs.
- Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
- Treat low strength domestic wastewater, which is problematic for anaerobic digestion technologies (Rittmann, 2001).
- Work at ambient, often low temperatures, again problematic for anaerobic digestion (Lettinga et al., 1999).
- Work continuously and reliably.

An innovative and relatively new approach to wastewater treatment is through the use of bioelectrochemical systems (BES), though the fuel cell technology lying behind this process is over 100 years old (Potter, 1911) (see appendix I for a history of development). Here wastewater is consumed in a battery like cell, redox reaction catalysed by bacteria pushing electrons around in an electrical circuit, thus creating electricity (Rabaey et al., 2007). In a microbial fuel cell (MFC) the electricity is captured directly (Logan, 2005), in a microbial electrolysis cell (MEC) the electricity is supplemented by an external source to make a product such as hydrogen or methane (Rozendal et al., 2006) or to perform a process such as reductive dechlorination (Aulenta et al., 2008) or de-salination (Mehanna et al., 2010). There are substantial losses within these systems (Logan et al., 2006), it is suggested they may reach a higher conversion efficiency of 44% (McCarty et al., 2011), the performance of MFCs to date has only reached around 1 tenth of that needed to be competitive with anaerobic digestion (Pham et al., 2006). With MECs the potential higher value (energetically or commercially) of the product formed or process completed means this technology is likely to be more viable and may be the driver of development (Foley et al., 2010).

As organic matter is degraded by bacteria it releases electrons (oxidation) providing energy for the cells. These electrons then pass to an electron acceptor (or reduced species), which is normally oxygen, nitrate or sulphate depending on their availability providing further energy for the cells (Rittmann, 2001).It has been shown that there is a group of organisms that are capable of passing electrons to materials (such as metal oxides) outside the cell, which are then transferred by that material to an electron acceptor. This process is termed electrogenesis, and the group of organisms are known as exoelectrogens (Logan, 2008). MFCs exploit this, providing the bacteria with a

surface to donate electrons to, and then using the principles of all electrochemical cells to transport these electrons and create current.

MFCs, like electrochemical cells usually have two compartments, the anode chamber containing organic matter to be degraded, and the cathode chamber containing an electron acceptor. In the anode chamber organic matter is degraded by bacteria producing electrons, the absence of a preferred electron acceptor such as oxygen, means these electrons pass into the anode material then through a wire to the cathode. The H^+ ions generated in this reaction pass through the membrane from the anode to cathode chamber. At the cathode the electrons, H^+ ions and a reduced species (typically oxygen) combine to form for example H_2O. Electrical current is generated in the wire as the electrons pass from one side to the other.

An MEC reactor is an adaptation of an MFC. In an MEC both the anode and cathode chamber are anaerobic. Rather than creating H_2O in the cathode chamber, the electrons and H^+ ions are combined to generate H_2 gas rather than electricity. The process of forming H_2 is however endothermic, i.e. it requires energy. It cannot happen spontaneously. The addition of a small amount of electricity (with acetate this is in theory 0.114 V, in practice <0.25 V), is required to generate the H_2 gas (Logan et al., 2008). This is substantially less energy than is required to produce H_2 through water electrolysis, typically 1.8-2.0 V. A schematic of an MEC is shown in Figure 1-1.

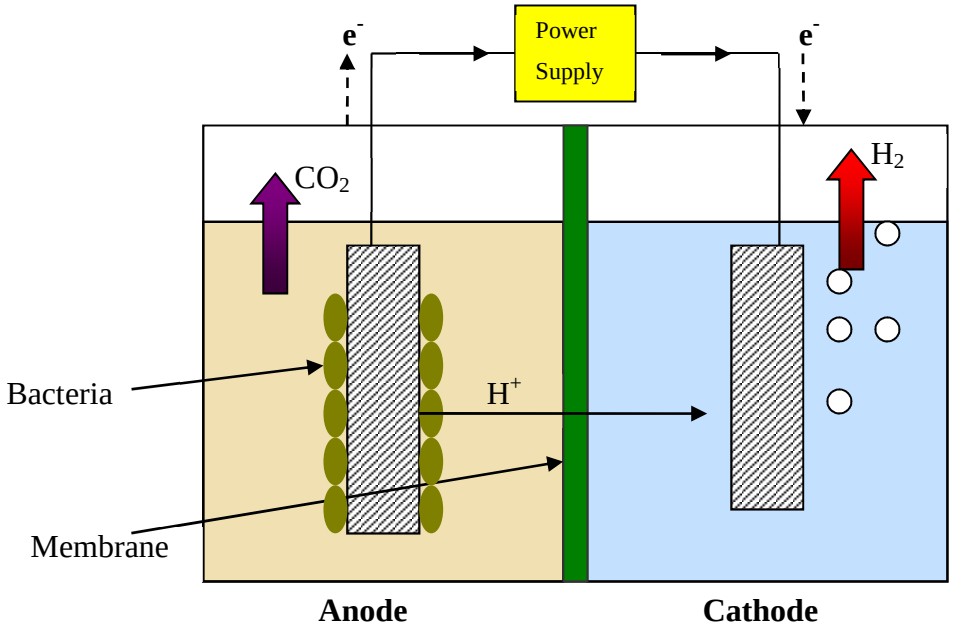

Figure 1-1 Generalised schematic of an MEC adapted from (Liu et al., 2005b) showing the flow of electrons and hydrogen ions and the function of the anode and cathode sections

The theoretical electrochemical energy gains or requirements of a MFC and MEC respectively will vary with temperatures, substrate free energy and ionic concentrations especially pH, as shown in appendix II. Even if it were possible to determine the potentials accurately in practice these theoretical values are not achieved. Energy is lost through all the transfer processes which take place to allow this reaction to happen. There are both electrochemical losses known as overpotentials caused by losses in redox reactions and transfer to the electrodes, losses in transfer of ions between the electrodes, limitations caused by transfer rates being different for different species, and on top of this there are losses caused by transfer of both electrons and ions in and out of the bacteria, losses to the bacteria themselves as they use energy, losses of electron transfer, and also losses by side or chain reactions occurring which do not advantage the fuel cell (Logan, 2008). This means that the energy gained in an MFC is less, and the energy input required in an MEC is more, than would theoretically be the case, represented in Figure 1.2.

In an MEC substantially more energy input than the theoretical is needed, in acetate fed systems these typically range from 0.4 V to 0.8 V with greater hydrogen gas production at higher voltages but less energy efficiency (Call and Logan, 2008). Glucose fed reactors have been shown to operate at applied voltages of 0.9 V (Selembo et al., 2009a), although far less work has been carried out on this substrate and its limits of applied voltage are undefined. In a larger scale system it is likely overpotentials (the difference between the theoretical potential at which the reaction occurs, and the observed potential of the electrode) will be increased and therefore the power input might be higher. In a pilot scale reactor fed on wine wastewater the input voltage of 0.9 V was used, although this performed less well than laboratory trials at a smaller laboratory scale on the same substrate, high over potentials being one of the suggested reasons (Cusick et al., 2011).

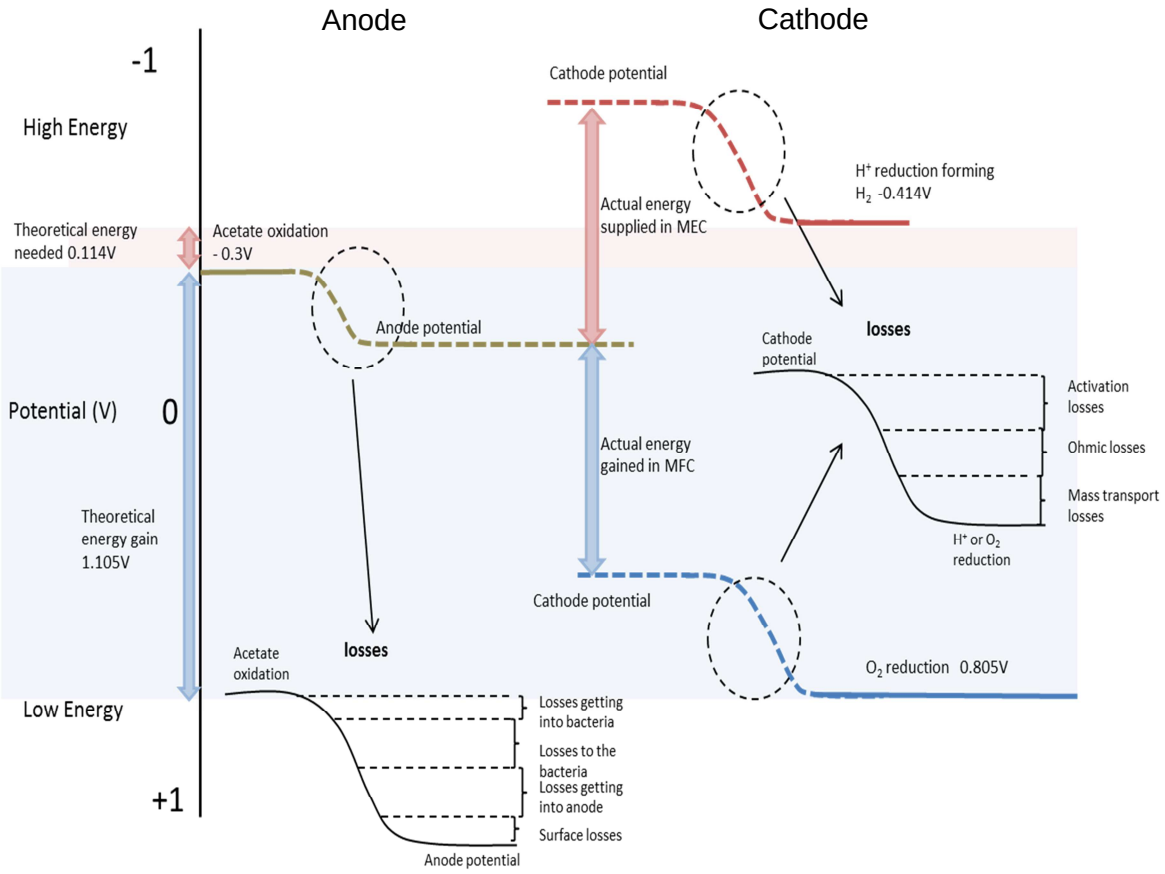

Figure 1-2 Representation of the energy losses within an MFC and MEC using acetate. Energy is shown as potential on the vertical axis, the green line shown the potential of the anode from the potential of acetate (solid line) to the actual anode potential (dotted line) which dependant on the losses. The reduction potential of the MFC and MEC cathode reactions is shown as the solid blue and red lines respectively, whereas the actual cathode potential is again shown in the dotted lines and is dependent on losses. The predicted total energy gain (MFC) and loss (MEC) is shown by the thick arrows and can be variable depending on these losses, but will always be less than that theoretically predicted as seen in the thick arrows at the vertical axis

Understanding the complexities of the electrochemistry of these systems is however only part of the challenge of understanding and ultimately manipulating BES technology. The microbiology of such systems plays a critical role in dictating their efficiency and their success or failure. The microbial community, which catalyses and enables the whole process to take place will also be affected by temperature, pH and substrates (Rittmann, 2001), it will vary with time and within the reactor, and the factors of competition, symbiosis and random assembly lead to a highly complex and unpredictable system.

BES systems run on electrochemical principles but rely on microbial communities. Therefore predicting their absolute function and output of energy, or indeed the input of energy needed, is at this stage in our understanding not possible. The empirical collection of this information is necessary in helping us identify not only if this technology is viable but also the areas that can and need to improved. Critically understanding the bacterial communities and the energy transfers within these systems lies at the heart of being able to manipulate and use this technology.

BES in general and MECs in particular have the potential to fulfil these needs of the wastewater industry (Foley et al., 2010). MECs are entirely anaerobic, eliminating the need for any aeration or complex membrane systems, meaning their engineering can be simple and 'retrofittable' within existing infrastructure. Although hydrogen production is focused on in this study, the flexibility of this process to make other high value products is an economic driver. However the key challenges to overcome are the scientific ones. An increasing body of work is amassing showing improved efficiencies and performance, however the vast majority of this is with simple substrates at warm temperatures (Rader and Logan, 2010, Call et al., 2009, Cheng et al., 2006b, Zhang et al., 2010). Evidence that BES work at low temperature is conflicting (Jadhav and Ghangrekar, 2009, Cheng et al., 2011), the only published study of a large scale 'hydrogen producing' MEC did not produce hydrogen (Cusick et al., 2011), and MECs studies using real wastewater as a substrate are limited, the longest documented study runs reactors for 7.6 days (Wagner et al., 2009).

1.1. Aim and objectives

The overall aim of this research is to understand if BES can be used as a sustainable method of wastewater treatment.

Much work has been and is being carried out fine tuning BES technologies within laboratories, testing new materials and moving towards greater output efficiencies, however large volumes of this work is conducted at warm temperatures and with simple artificial substrates (Hu et al., 2008, Logan et al., 2008, Selembo et al., 2009a, Tartakovsky et al., 2009). This research does not strive towards making such efficiencies, but answers the following fundamental questions of: can they work with

real wastewaters? and, can they work at realistic temperatures? this was addressed by completing the following objectives:

- Quantifying the amount of energy available in the wastewater
- Analysing the start-up and community development of MFC systems.
- Testing the operation and performance of MFC reactors at low temperatures
- Monitoring the performance of MEC reactors with wastewater substrate
- Building and testing a pilot scale MEC reactor run at a wastewater treatment site.

Chapter 2. Determination of the Internal Chemical Energy of Wastewater

Parts of this chapter have been published as Heidrich, E.S., Curtis T.P., and Dolfing J., Determination of the Internal Chemical Energy of Wastewater. Environmental Science & Technology, 2011. 45(2): p. 827-832.

The wastewater industry is facing a paradigm shift, learning to view domestic wastewater not as a waste stream which needs to be disposed of, but as a resource from which to generate energy. The extent of that resource is a strategically important question. However, the only previous published measurement of the internal chemical energy of wastewater measured 6.3 kJ/L, calculated to be 14.7 kJ/gCOD. It has long been assumed that the energy content in wastewater relates directly to chemical oxygen demand (COD). However there is no standard relationship between COD and energy content. In this study a new methodology of preparing samples for measuring the internal chemical energy in wastewater is developed, and an analysis made between this and the COD measurements taken. The mixed wastewater examined, using freeze drying of samples to minimise loss of volatiles, had 28.7 kJ/gCOD, whilst domestic wastewater tested had 17.8 kJ/gCOD nearly 20% higher than previously estimated. The size of the resource that wastewater presents is clearly both complex and variable, but is likely to be significantly greater than previously thought. A systematic evaluation into the energy contained in wastewaters is warranted.

2.1. Introduction

Every one of us produces at least around 40 $gBOD_5$ (biochemical oxygen demand consumed over 5 days), in waste every day, in richer countries this is likely to be nearer 80 $gBOD_5$,(Mara, 2004), equating to around 60-120 gCOD/person/day (Kiely, 1997). If there were 14.7 kJ/gCOD (Shizas and Bagley, 2004), the only previous published measurement of the energy value of wastewater, with 6.8 billion people in the world, 2.2 - 4.4 x 10^{18} joules of energy per year is available, or a continuous supply rate of 70 - 140 gigawatts of energy, the equivalent of burning 52 - 104 million tonnes of oil in a modern power station, or 12 - 24,000 of the world largest wind turbines working continuously. This estimation does not even include all the energy contained in our agricultural and industrial wastewater.

Despite the resource that wastewater represents, most developed countries spend substantial quantities of energy treating the wastewater so it can be released without harm to the environment, the US uses approximately 1.3% of its total electricity consumption doing so (Carns, 2005, Logan, 2008). The energy for wastewater treatment will be a particular burden in the urban areas of less well-off nations. Wastewater is typically viewed as a problem which we need to spend energy to solve, rather than a resource. If the energy contained in wastewater is harnessed, not only could it help the water industries become self-sufficient in energy or even net providers, but it could also be a modest source of energy in parts of the world which currently lack reliable and affordable energy supply.

Wastewater contains a largely uncharacterised and undefined mixture of compounds, including many organics, likely to range from small, simple chains through to more complex molecules. All organic compounds contain energy stored within their bonds. The energy that can be obtained from wastewater by different processes is varied, methane gas from anaerobic digestion, electricity from microbial fuel cells (MFCs), or hydrogen in the case of microbial electrolysis cells (MECs) (Logan, 2008) or a fermentation process (Davila-Vazquez et al., 2008). Large amounts of research is being undertaken in all of these areas but there has been very little work conducted in quantifying the amount of energy held in wastewater to start with.

The COD of wastewater has long been used as a relatively simple and reliable method of determining the 'strength' of waste, and by inference the energy contained within it. However there is no empirical formula for the determination of the energy content from the COD measurement. The only previous study to attempt to determine the energy content of raw municipal wastewater by experiment was conducted by Shizas and Bagley (2004) using a bomb calorimeter. Here a single grab sample of domestic wastewater from a treatment plant in Toronto was dried in an oven overnight at 103^{o}C before being analysed by bomb calorimetry. It was found that the domestic wastewater had a measured COD of 431 mg/L, and an energy value of 3.2 ± 0.1 kJ/g dry sample; with 1.98 g/L of solids this equates to 6.3 kJ/L. This interesting observation has led to the pioneering interpretation that wastewater contains 14.7 kJ/gCOD (Logan, 2008), which has been cited in the literature several times in particular with relation to microbial fuel cell work (Liao et al., 2006, Schroder, 2008, Logan, 2009). However the oven drying of samples will have driven off many volatile organic compounds, such as

methanol (boiling point 64.7 ^{o}C), ethanol (78.4 ^{o}C), and formic acid (101 ^{o}C). Moreover, the calculations were based on a single grab sample from one treatment plant, and using the COD measurement taken prior to drying, it is very likely that some of this COD will have also been lost before the energy determination was made. The work of Shizas and Bagley (Shizas and Bagley, 2004) provides a valuable starting point for the estimation of energy in wastewater, but given the volatile losses, and the measurement of the COD before these losses have occurred, this value must be an underestimation of the true internal chemical energy of wastewater.

The objectives of this study were to develop an improved methodology for measuring internal chemical energy, to better quantify the internal chemical energy of wastewaters, and to evaluate the relationship between internal chemical energy and COD.

2.2. Materials and methods

2.2.1. *Collection and storage of samples*

Two 24 hour composite samples of influent wastewater were taken, one from Cramlington Wastewater Treatment Plant, which deals with a mixed (i.e. industrial and domestic) wastewater, and the other from Hendon Treatment Plant, primarily treating domestic wastewater, both in the North East of England. Within two hours of collection, 3 L of sample was placed into the deep freeze at -80 ^{o}C, and a further 3 L was placed into an oven at 104 ^{o}C. A sample was stored in a refrigerator at 4 ^{o}C.

2.2.2. *Drying procedures*

After a period of around 48 hours in the oven at 104 ^{o}C the sample was fully dried. This was then ground into a powder using a pestle and mortar, and stored in four measured quantities of approximately 0.5 g in clean, dried sealed containers. The frozen samples were dried using a freeze dryer (Labconco Freezone, Labconco Corp. USA) which when used daily over a period of 4 weeks was capable of drying about 1.5 L of sample, each 20 hour drying period removing a few millilitres of liquid. The samples were stored at -80 ^{o}C between drying for 12 hours whilst the freeze dryer stabilised. This procedure was repeated until enough sample was dried to yield four 0.5 g samples. These were then ground and stored in the same way as the oven dried samples.

2.2.3. *Wastewater analysis*

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total organic carbon (TOC), inorganic carbon (IC), total carbon (TC) and chemical oxygen demand (COD) measurements were carried out in the two days after collection using the refrigerated samples. The methods described in Standard Methods for the Examination of Water and Wastewater (APHA, 1998) were used. TS was also measured using the freeze drying process. Further COD tests were carried out on rehydrated freeze dried and oven dried samples. All measurements were taken in triplicate.

2.2.4. *Energy content*

The energy content of the dried wastes was determined using an adiabatic bomb calorimeter, Gallenkamp Autobomb. The internal bomb was a stainless steel unit surrounded by a water jacket with a volume of 1900 mL, with a further cooling jacket outside with a flow of 300 mL/min. The system also included a mechanical stirrer, ignition unit and a digital thermometer accurate to 0.01 ^{o}C. The effective heat capacity of the system i.e. the heat required to cause a unit rise in temperature of the calorimeter was determined using triplicate samples of pure benzoic acid. This was used to calibrate the heat of combustion of the system components such as the wire and cotton, and the effective heat capacity of the bomb, its water jacket and thermometer. After this determination all of the components of the system were then kept constant throughout the tests. Four samples of benzoic acid were used on each time of operation of the bomb calorimeter to verify the technique.

The samples were dried, weighed to around 1 g, and compacted before combustion in the bomb. It was found that the samples did not fully combust, and therefore they were mixed in a 1:1 ratio with a combustion aid of benzoic acid, a method used by Shizas and Bagley (2004). The exact sample weight and the temperature rise in the surrounding water jacket was recorded and used to determine the energy content of each sample. All measurements including the benzoic acid standards were taken in a randomised order.

2.2.5. *Energy content calculations*

The bomb calorimeter measures the heat of combustion of the bomb's contents. When the bomb is ignited the contents including the fuse wire, cotton thread used to attach the sample to the fuse wire and the fuel, including any benzoic acid used is burnt, and this heat is absorbed by the bomb and its surrounding water jacket. In addition to the heat from the combustion, there is also heat created by the formation of nitric acid from the

nitrogen contained in the air inside the bomb. Moles of nitric acid formed are found by titration of the bombs contents with 0.1M NaOH. It is assumed that there is 57.8 kJ/mol of nitric acid; the oxidation state of the nitrogen is not taken into consideration as is standard practice (Rossini, 1956). The kilojoules contained in the sample are calculated in the following equation:

$$-\Delta U_{c,s} = ((V_w + B)(c_{p,w})(\Delta T) + (-\Delta U_{c,w}) + (-\Delta U_{c,c}) + (-\Delta U_{c,b})(m_b) - (Q_{f,n}\, mol_{nitric})) / m_s$$

Table 2-1 Definition of parameters in the equation above used to calculate energy of combustion

Term	Definition
$-\Delta U_{c,s}$	Energy of combustion at constant volume for sample (kJ/g)
$-\Delta U_{c,b}$	Energy of combustion at constant volume for benzoic acid = 26.42 kJ/g[a]
$-\Delta U_{c,w}$	Energy of combustion at constant volume for fuse wire = 0.013 kJ/g[b]
$-\Delta U_{c,c}$	Energy of combustion at constant volume for cotton = 0.082 kJ/g[b]
V_w	Volume of water = 1940 g[b]
B	Volume of water equivalent to the effect of the bomb container = 390 g[b]
$c_{p,w}$	Specific heat capacity of water = 0.00418/g/^oC[a]
ΔT	Temperature rise (^oC)
m_b	Mass of benzoic acid combusted (g)
m_s	Mass of sample combusted (g)
$Q_{f,n}$	Heat of formation of nitric acid = 57.8 kJ/mol[a]
mol_{nitric}	Moles of nitric acid formed (mol)

[a](Atkins, 2006)

[b]Determined in laboratory

2.2.6. *Measurement of volatile fatty acids*

The loss of known volatile fatty acids (VFA's) was measured for each drying technique using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Triplicate 20 mL samples of 50 ppm acetate solution were dried overnight in an oven at 104 ^oC, and in the freeze dryer. These were then re-hydrated with 20 mL of deionised water, and the VFAs measured.

2.2.7. *Measurement of anions*

The anion content of both wastewaters was measured in triplicate using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent.

2.2.8. *Measurement of volatile halocarbons*

Dried 20mg samples were rehydrated using 20 mL de-ionised water and, 20 mL wastewater samples were sealed within a sample jar, with the addition of 20 mg of salt (KCl). These were left for 24hrs at 30^{o}C, the headspace gas was then analysed using an Agilent 7890A GC split/split less injector linked to an Agilent 5975C MSD

2.2.9. *Statistical techniques*

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run two sample t-tests on the data. Before the tests were performed the data was checked for equal variance and normal distribution, validating the use of a two sample t-test.

2.3. Results

This paper uses an improved methodology: freeze drying the samples prior to using a bomb calorimeter. With this method only a few millilitres of liquid can be removed in a 24 hr operational period. Therefore drying enough wastewater to yield several grams of solids takes between 4 - 8 weeks. Although far more time consuming it is believed this is the best method available for drying the wastewater without raising its temperature and thus removing the volatiles.

Table 2-2 Measured wastewater parameters of the two different samples used in the energy analysis

	Cramlington	**Hendon**
COD	718.4 ± 9.7	576.2 ± 40.8
COD- oven dried	368.2 ± 12.3	324.0 ± 18.1
COD - freeze dried	587.1 ± 32.2	425.3 ± 16.5
Total solids - oven dried	1392 ± 35	1070 ± 60
Total solids - freeze dried	1597 ± 40	1130 ± 20
Total organic carbon	116.5	115.8
Total carbon	181.8 ± 2.3	196.4 ± 1.2
Inorganic carbon	65.3 ± 1.2	80.5 ± 0.1
Volatile solids (standard method)	953 ± 143	427 ± 20
Total Kjeldahl Nitrogen	92.4 ± 0.0	71.9 ± 4.3
Chloride (ppm)	391 ± 10.9	169.6 ± 17.2

Mean ± standard deviation (n=3), all values are in mg/L unless otherwise stated

Table 2-2 shows the differences between the two wastewaters, and the effects of the drying processes on the COD and solids recovery from these wastewaters. Oven drying reduces the measured COD from 718.4 mg/L in the original wet sample to 368.2 mg/L (49% loss) in the Cramlington wastewater and from 576.2 mg/L to 324.0 mg/L (44% loss) in the Hendon sample, whilst freeze drying gives losses of 18% and 26%. The freeze drying process captured 5-12% more mass than oven drying. This demonstrates that freeze drying is a more accurate method to determine the total amount of COD than oven drying. However, even freeze drying resulted in COD losses of 18-26%. This is probably due to the loss of the volatile fraction of the COD such as short chain fatty acids. This was confirmed using ion chromatography where oven dried samples contained 0.000 ppm acetate whereas freeze dried samples contained 1.8 ppm, compared to the original 54.5 ppm. Acetate is one of the smaller and therefore more volatile of the VFA's and is likely to represent some of the greatest losses.

Table 2-3 Measured internal energy content values given as both energy per litre and energy per gCOD using the post drying measurement of COD

	Cramlington		Hendon	
	Oven dried	**Freeze dried**	**Oven dried**	**Freeze dried**
kJ/L	8.3 ±1.8	16.8 ± 3.3	5.6 ±1.0	7.6 ± 0.9
kJ/gCOD	22.5 ± 4.8	28.7 ± 5.6	17.7 ± 3.2	17.8 ± 2.1

Mean of four measurements ± standard deviation
Values for kJ/gCOD are calculated from the COD measurement after drying and re-hydrating, and TS measurement for the given drying method.

The freeze drying method enabled a significantly greater proportion of the energy in the wastewater to be measured, over 50% more for Cramlington (p value 0.010), and 24% more for Hendon (p value 0.044). There are also significant differences between the two wastewaters, with the Cramlington waste being more energy rich (p value 0.019). The energy content per gram of oxidisable material measured i.e. kJ/gCOD is considerably higher for both wastewaters than previous estimates of around 14 kJ/gCOD, for the Cramlington wastewater this is even higher with the freeze dried sample.

The energy captured by the freeze drying process does not equate to all the energy available in the wastewater sample. Based on the percentage losses of measured COD from the original sample to the freeze dried sample (18% for Cramlington and 26% for

Hendon), the actual energy of the Cramlington wastewater could be as high as 20 kJ/L, and 10 kJ/L for the Hendon wastewater.

2.3.1. *Theoretical results - can internal chemical energy per gram COD be calculated from first principles?*

If we were able to evaluate the energy content of wastewater from the COD measurement, this would require an estimation of which organic compounds are present. With this, the internal chemical energy for each individual organic compound can be calculated on the basis of simple thermodynamic calculations as follows (thermodynamic values are taken from Atkins (2006)) based on the principle that 1 gram of COD equals 1/32 mol O_2, i.e. for every 1 mol O_2 there is 32 grams COD.

If we assume that the organic compound present is methane:

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \qquad (1 \text{ mol } CH_4 = 64 \text{ gCOD})$$

The overall enthalpy for the reaction can be calculated on the basis of Hess's Law, which states that the enthalpy of a reaction is equal to the sum of the enthalpy of formation ($\Delta_f H$) of all the products minus the sum of the enthalpy of formation of all the reactants. Using tabulated values for the enthalpy of formation the energy released in the above reaction with methane is as follows:

$$\begin{aligned}
\Delta_f H \text{ (kJ/mol)} &= \sum \Delta_f H \text{ products} - \sum \Delta_f H \text{ reactants} \\
&= 2(\Delta_f H\ H_2O) + \Delta_f H\ CO_2 - \Delta_f H\ CH_4 - 2(\Delta_f H\ O_2) \\
&= 2(-285.83 \text{ kJ/mol}) + -393.51 \text{ kJ/mol} - -74.81 \text{ kJ/mol} - 2(0 \text{ kJ/mol}) \\
&= -890.5 \text{ kJ/mol} \\
&= -890.5 \text{ kJ/mol} / 64 \text{ gCOD} \\
&= -13.9 \text{ kJ/gCOD}
\end{aligned}$$

Analogous calculations for a wide range of organic compounds show that the typical $\Delta_f H$ values of $C_aH_bO_c$ compounds fall within a fairly narrow range of 13-15 kJ/gCOD, with a few exceptions such as formic and oxalic acid with 15.7 kJ/gCOD, ethyne with 16.3 kJ/gCOD and methanol with 17.8 kJ/gCOD. (See Appendix III).

It could be concluded that 13.9 kJ/gCOD is the maximum amount of heat energy that can be gained from methanogenic wastewater treatment. Therefore from a relatively simple COD measurement the potential energy yield would be known. However biodegradation of organic content in wastewater does not necessarily lead to methanogenesis. Some waste streams can be used for biohydrogen production. Here 1 gCOD is equal to 1/16 mol H_2, ($2H_2 + O_2 \rightarrow 2H_2O$) therefore 1 mol H_2 equals 16 gCOD, giving an energy yield of 17.9 kJ/gCOD (286 kJ/mol H_2 / (16 gCOD / mol H_2)).

The simple $C_aH_bO_c$ compounds are not necessarily the only wastewater components, and other classes of compounds such as halocarbons can contain far more internal chemical energy per gCOD. The explanation to this can be supported by writing the equations that describe their degradation down as oxidations of the carbon moiety with reducing equivalents released as H_2, coupled to the oxidation of the H_2 to water. In highly substituted compounds such as organohalogens, less H_2 is potentially available. The oxidation reaction of H_2 to water becomes less important in the overall equation, the ratio of H:CO_2 decreases, increasing the overall value of kJ/gCOD. This is illustrated using methane and one of its halogenated equivalents trichloromethane (thermodynamic data taken from (Hanselmann, 1991)):

Methane

$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (-393.5 + 4(0)) - (-74.8 + 2(-285.8))$

$= 252.9$kJ/reaction

$$4H_2 + 2O_2 \rightarrow 4H_2O$$

Enthalpy of reaction $\Delta H_r = \sum \Delta_f H$ products $- \sum \Delta_f H$ reactants

$= (4(-285.8)) - (0 + 2(0))$

$= -1143.2$ kJ/reaction

These two values are then added together to give the overall enthalpy of reaction to be -890.3 kJ/mol, this can then be divided by the COD to give -13.9 kJ/gCOD

Trichloromethane

$$CHCl_3 + 2H_2O \rightarrow CO_2 + 3HCl + H_2$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-393.5 + 3(-167.1) + 0) – (-103.1 + 2(-285.8))

= -220.1 kJ/mol

$$H_2 + \tfrac{1}{2} O_2 \rightarrow H_2O$$

Enthalpy of reaction ΔH_r = $\sum \Delta_f H$ products - $\sum \Delta_f H$ reactants

= (-285.8) - (0 + 0.5(0))

= -285kJ/mol

The total enthalpy of reaction is -505.9 kJ/mol, giving -31.6 kJ/gCOD.

It becomes clear how important the reducing equivalents of H_2 are in terms of energetic value, this is illustrated in Figure 2-1, (values given in Appendix III). As the number of substitutions of hydrogen increases, so does the value of energy per gram COD. The value of energy per gram of COD can vary far more widely than previously thought.

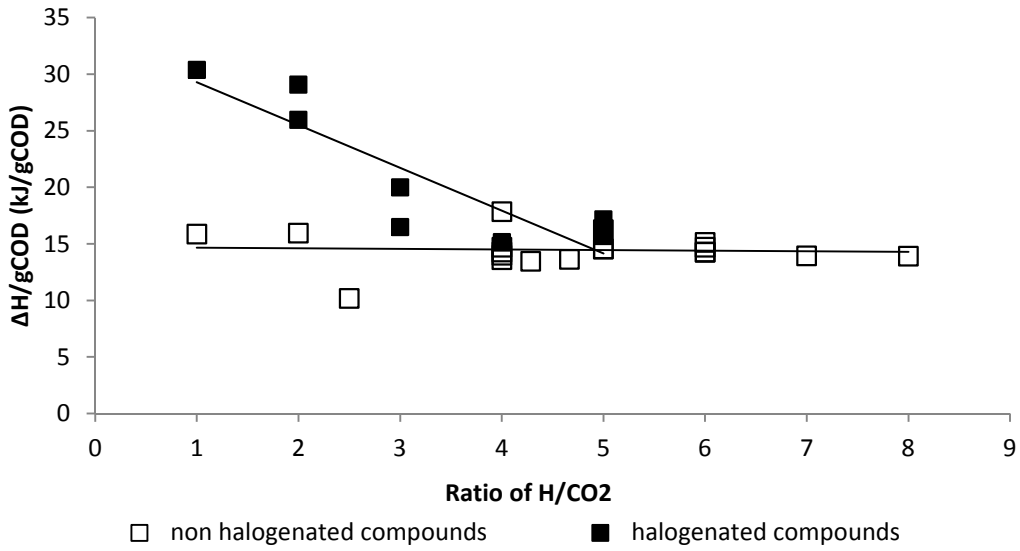

Figure 2-1 Energy content per gCOD of a variety of organic compounds plotted against their degree of oxidation

2.4. Discussion

The predicted energy gained from treatment of municipal wastewaters has been shown to be higher than the previous estimation. The domestic wastewater analysed in this paper has 20% more energy per litre than the estimation made by Shizas and Bagley (Shizas and Bagley, 2004). In addition to this, as the volatiles in their wastewater were not captured, it is likely their sample could have had an energy value around 35% higher, (based on the percentage losses between oven and freeze drying in this study) this would be 8.5 kJ/L. This has a significant impact on the development and implementation of technologies for the treatment of 'low strength' municipal wastewater which pose a greater challenge for the recovery of energy than concentrated waste. These waste streams are clearly richer in energy than previously thought.

The internal chemical energy of the wastewaters per gCOD was greater than expected by comparison to acetate (heat of combustion is 13.6 kJ/gram COD) or glucose (heat of combustion is 14.3 kJ/gram COD). From the data (Table 2-2) of the two wastewaters it can also be seen that the carbon oxidation state plays an important role in determining the energy present. Both samples have a very similar value of TOC (total organic carbon), yet very different COD values. This means that the Cramlington waste with the much higher COD has proportionally more reduction capacity and therefore chemical energy per carbon molecule than the Hendon wastewater. Another possible cause of these high values is that there are compounds within the wastewater that have an energy value, yet are not oxidised during a COD test, most notably urea, which contains 10.4 kJ/g (Atkins, 2006) when combusted, yet undergoes a hydrolysis reaction rather than an oxidation. This compound, which is certain to be present in domestic wastewater (and though it is assumed to hydrolyse in the sewer, a fraction may reach the wastewater treatment site), contributes to the overall energy of combustion of waste but not to the COD measurement, there are likely to be others compounds which do the same. Additionally there could be some compounds which have proportionally far greater energy content per gram of COD than glucose and acetate, such as organohalogens or other highly substituted compounds.

Although many simple halocarbons are no longer in use, some more complex ones are still common in many industrial processes for example as solvents and pesticides, and in the manufacture of in plastics, adhesives, sealants and paper pulp. Organic halocarbons also occur in natural systems. Chlorination treatment also introduces this halogen which

could then combine with other organics. It can be seen from the anion analysis (Table 2-2) that there is significant quantity of chloride ions in the wastewaters, with more in the Cramlington wastewater. This wastewater is likely to contain a more diverse range of organic compounds as this site takes in mixed wastes, some of which must have a high specific energy value and volatility, resulting in high energy wastewater. Volatile halocarbons, however, were not detected with the GC MS method described.

The energy values found in this study are also higher than that reported by Shizas and Bagley (2004). However the calculations in their paper were based on oven dried wastewater energy data, versus a COD measurement taken from the original wastewater sample, which in our study was found to be reduced by about 50% after oven drying. If the same calculation algorithms were used on the data in the present paper then the Cramlington and Hendon wastewaters would contain 11.6 kJ/gCOD and 9.9 kJ/gCOD respectively, while they actually contained at least 2.4 times higher (28.7 kJ/gCOD) and 1.8 times higher (17.8 kJ/gCOD), these calculations are shown in Appendix IV. Thus the energy reported per gCOD cited in the literature (Logan, 2008) based on the Shizas and Bagley paper (Shizas and Bagley, 2004) is probably a substantial underestimation. By comparison to the Hendon domestic wastewater the energy of their municipal wastewater could have had at least 26.4 kJ/gCOD, rather than the 14.7 kJ/gCOD reported.

Clearly not all the energy available in wastewater can be extracted in a useful form as no process is 100 % efficient. Ideally one would be able to measure or calculate the energy biologically available as kJ/gBOD, (although not suitable for anaerobic processes), this is not possible given the unknown and variable composition of wastewater. However knowing the potential energy available would give insight into the types of waste that might be in the waste stream which would also be of importance in the choice of treatment method. Some wastes which may be high in energy value, such as halogenated wastes may be unsuitable or unattractive to some treatment methods. For example one mole of trichloromethane at 506 kJ/mol would only yield 0.25 moles of methane equal to 222 kJ through methanogenic treatment, or one mole of H_2 equal to 286 kJ through biohydrogen production. Although these halogenated compounds are energy rich per gram of COD due to their lack of hydrogen, this actually makes them unattractive to terms of energy extraction for methane or hydrogen production, however

it may be possible to recover this energy using other treatment methods which may be able to capture electrons directly.

In microbial fuel cells (MFC's) the reaction taking place is essentially a combustion reaction, i.e. the organic compound is oxidized to carbon dioxide and water, the difference being that this reaction occurs not as combustion but as redox reactions in two half cells. Importantly, it is the free energy of the organics that determines the maximum electricity yield. This technology could theoretically capture more of the energy available in complex or halogenated compounds than for example methanogenic treatment.

The measurement of the internal or combustion energy of the wastewater and use of this as a basis for efficiency calculations will not necessarily yield all the information required to fully understand the energy flows in such systems. It can be observed using internal chemical energy data, a methanogenic process could in some cases be endothermic, the combustion energy of the methane product being higher than that of the starting substrate. This is the case with the conversion of one mole of acetate (13.6 kJ/gCOD) to one mole of methane (13.9 kJ/gCOD). In this scenario energy appears to have been created. It is actually the Gibbs free energy (the amount of energy that can be extracted from a process occurring at constant pressure) which should be examined for this and other reactions as this parameter informs us of the amount of energy available to organisms for the generation of biomass and an energy rich product. This is also the case for MFC's and MEC's where it is voltage which is measured which relates directly to Gibbs free energy. However without knowing the composition of wastewater, its Gibbs free energy content cannot be determined.

A consequential difference was found between the internal chemical energy measured on freeze dried samples as compared to oven dried samples. This difference was greater than the difference observed by measuring mass alone. This shows that there are significant losses of volatile compounds when a wastewater sample is dried at 104 ^{o}C and that in the case of the mixed wastewaters these volatiles can contain proportionally more energy per gCOD than the non-volatiles captured in both methods. It is shown that, although a clear improvement on the traditional oven drying method, the freeze drying method still results in significant loss of semi-volatiles such as acetate, so even

with the improved method we are still not capturing all the energy available in the wastewater.

Bomb Calorimetry remains the only method for measurement of internal chemical energy or calorific value, and for this method the material must be combustible i.e. dry. To give reasonably accurate results the temperature change in the bomb calorimeter must be in the region of 1 - 3 ^{o}C, usually a gram of substance will provide this. In our analyses this gram was half made up by the use of a combustion aid (benzoic acid) to ensure full combustion and the correct temperature rise. Had the proportion of wastewater to benzoic acid been decreased, making the drying process easier, it was feared that the uncertainty inherent to the introduction of the standard would overshadow the accuracy of the measurements of the samples. Although more challenging the methodology of freeze drying samples is an improvement on previous methods although it does not achieve the full capture of all volatiles. These results begin to get close to the true amount of energy in wastewater, and challenge the assumption that measured COD is equivalent to the amount of energy. Freeze drying, although far more time consuming, therefore should be the method of choice when completing such analysis in particular with complex wastes, despite its far greater time consumption rate unless or until new methods and equipment are developed to reduce the time burden using this principle. One such method could be the use of membranes, in particular through the use of reverse osmosis which would 'trap' molecules as small as salts and allow water to be removed. Such techniques may allow for more rapid, cost effective and efficient drying of samples, thus enabling more sampling to be undertaken.

It is clear from our data that the energy value of different wastewaters is variable, as would be expected; there is no standard relationship to measured COD. Values ranged from 17.7 kJ/gCOD to 28.7 kJ/gCOD, when measuring the COD remaining in the dried sample, however we cannot know how much compounds such as urea contribute to this. This means than a measurement of the amount of oxygen required to oxidise the organics within wastewater is not a simple representation of the amount of energy contained within that waste. This is particularly the case when dealing with mixed wastes, where the energy content is proportionally far greater per gCOD. It seems that 13 – 14 kJ/gCOD is the minimum energy content that could be found in wastewaters, however it may be significantly greater. Given the variability in the amount of energy

per gram COD it seems better to measure this energy directly rather than making an estimation, despite the fact that even with the better drying method there are still losses.

Given the huge amount of wastewater globally and the potential energy stored within it, it is important that this potential energy should be determined. With new technologies such as fuel cells being developed, the estimation of this resource is not as trivial as previously assumed. It has been shown that wastewaters can lie well outside the previously estimated values. A systematic review of the energy contained within different waste streams is needed. This paper examines two wastewaters from a reasonably similar geographical location and has found extremely diverse results. It is hoped that this methodology will be repeated and improved upon in terms of time taken, allowing the dissemination of multiple studies using different wastewaters building up a comprehensive and global picture of the energy available in wastewater. This would form the strategic foundation block to the establishment of new and existing technologies within the wastewater industry harnessing this valuable renewable energy source.

Chapter 3. How many exoelectrogens make a Bioelectrochemical System?

3.1. Introduction

The inoculation and subsequent acclimatisation of a bioelectrochemical system (BES) is fundamental to the operation of such systems (Logan and Regan, 2006, Rittmann, 2006). Yet the origin, abundance and physiology of these organisms is the area of greatest uncertainty in design (Oh et al., 2010).

The main goal of the inoculation and acclimatisation of a reactor is typically to 'get it going' as quickly as possible, typically the sources of seed includes: reactors already working in the lab (Jeremiasse et al., 2009, Cheng et al., 2009, Call and Logan, 2008); anaerobic sludge (Chae et al., 2010, Yang et al., 2009); return activated sludge (Torres et al., 2009); mixtures of sludges; or simply wastewater taken at various stages from the treatment plant (Kiely et al., 2011b, Wang et al., 2008). The source and volume of inoculum varies between studies. There is no consensus of how a BES reactor should be started up, or how long acclimatisation will take. This can lead to problems, highlighted by a pilot scale study where several attempts were made to acclimatise the reactor (Cusick et al., 2011).

The bacteria needed for microbial fuel cells to work are termed exoelectrogens (Logan, 2008) due to their ability to transfer electrons outside their cell. Three transfer mechanisms have been proposed.

Firstly electrons can be transferred through conduction with direct contact between the cytoplasmic membrane of the bacteria and the solid substrate being reduced, this mechanism has primarily been associated with the genera *Shewanella* and *Geobacter* (Myers and Myers, 1992, Mehta et al., 2005).

The second mechanism is an electron shuttle. Some bacteria are able to excrete compounds or shuttles into the electrolyte which are capable of transferring electron to an electrode. Rabaey *et al.,* (2005) found that *Psuedomonas aeruginosa* produced Pyocyanin, a mediator which was not only able to transfer electrons from this taxon to the anode of an MFC, but could also work for other species when introduced back into a mixed culture. Thus, a bacterium unable to transfer electrons itself, may become

exoelectrogenic due to the presence of a different shuttle producing bacteria. *Shewanella* species have been seen to do this with the production of riboflavins (von Canstein et al., 2008).

Thirdly electrons might also be transferred through conductive microscopic pili named nanowires which extend from the bacteria cell to other cells or any other electron acceptor (Reguera et al., 2005). *Geobacter* and *Shewanella* species have both been linked to this activity (Gorby et al., 2006). Putative nanowires have been observed using electron microscopy extending to a conductive surface. Conducting probe atomic force microscopy (Reguera et al., 2005) and conductive scanning tunnelling microscopy (Gorby et al., 2006) have been used to reveal that the pili which had previously been observed as attachment mechanisms for bacteria onto Fe oxides, were highly conductive.

It has been proposed that symbiotic relationships between different bacteria groups enhance the function of mixed cultures and improve process stability (Lovley, 2008), possibly by allowing inter-species electron transfer (Rabaey et al., 2005). Many of the exoelectrogens typically associated with BES's such as *Geobacter sulfurreducens* have limited metabolic diversity, and are only able to utilise the end products of fermentation (Caccavo Jr et al., 1994). A reactor fed with a waste requires bacteria which are able to digest the complex substrates, but may not necessarily be able to utilise the anode for respiration (Kiely et al., 2011c). The hydrolysis step within these food chains has been shown to be the rate limiting step with regard to the current production (Velasquez-Orta et al., 2011).

In general, growth in bacterial systems can be described through the equation $N_T = N_0 exp^{rt}$, where the number of bacteria present at a specific time period (N_T) is equal to the number of bacteria present at the start (N_0) multiplied by the exponential of the growth rate (r) over the time span (t). (Rittmann, 2001). With N_T known various other properties can be calculated such as specific activity and growth yield. However in MFCs these are not well understood (Logan, 2008), although growth rates have been defined for some of the key organisms involved in MFC reactions such as *Geobacter*, (Cord-Ruwisch et al., 1998). A cell yield of 0.07-0.22 g-COD-cell/g-COD-substrate has been calculated (Logan, 2008) from an early study by Rabaey et al. (2003) using total bacterial concentrations within the reactors determined turbidometrically and the total

COD removed during the experiments. Freguia et al. (2007) reported estimates of growth yields of -0.016 to 0.403 mol-C-biomass/mol-C-substrate, based on measurement of the substrate removal which was then used to calculate cell yield through a mass balance approach. Yield has been shown to drop with decreasing external resistance (Katuri et al., 2011).

However the value of N_T is complex and unknown. Although a body of research is growing identifying the functions of bacteria within working BES reactors, little is known of their abundance in a natural sample (N_0) and absolute number within a working system (N_T). Additionally the pattern of acclimatisation, the period is likely to be crucial in the community formation, also remains largely unexplored.

Using the acclimatisation period of reactors the aims of this study were to firstly identify the optimum level of inoculum needed to start a reactor with a view to identifying a protocol for the further experiments. Secondly to estimate the most probable number of exoelectrogens present in a sample of wastewater which can be used as a guide to the sequencing depth needed to find these organisms, and to determine N_0 for a reactor. Thirdly to define the growth rates (r) within MFC systems through examining the start-up phase. With these two factors quantified the N_T can be estimated, as can specific activity and yield. Finally by examining the pattern of acclimatisation on different substrates, key differences in community formation can be identified.

3.2. Method

3.2.1. *Reactor Set-up*

Double chamber tubular design MFC reactors (78 mL each chamber) were used, constructed in Perspex, with an internal diameter of 40mm and length of 60mm. The anode was a 2.5 cm^2 carbon felt (Olmec Advanced Materials Ltd, UK), the cathode a 2.5 cm^2 platinum coated titanium mesh with a surface area 8.13 cm^2 (Tishop.com, UK). The cation selective membrane between the reactor chambers was Nafion® 117 (DuPont, France), with an area of 12.6cm^2. The electrodes were positioned 1cm apart. The components of the reactor were cleaned before use and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK)

The cathode chamber was filled with 50 mM pH 7 phosphate buffer saturated with air for 20 minutes before being added into the reactors. Three different media were used:

1. Acetate solution with added nutrients (Call and Logan, 2008)
2. Starch solution with added nutrients (Call and Logan, 2008)
3. Primary settled wastewater (Cramlington WWTP, Northumbrian Water Ltd)

The quantities of starch and acetate in the nutrient solutions were balanced to give similar total chemical oxygen demand (TCOD) as the wastewater, and were autoclaved (121°C, 15 min) before use. The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into ¼ strength Ringers sterile dilutent (APHA, 1998). The contact time under UV was altered to give effective sterilisation as defined as colony free plates in triplicate at zero dilution. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see Appendix V).

The three medias were sparged under sterile conditions for 10 minutes using ultra high purity (UHP) nitrogen (99.998%), until the dissolved oxygen (DO) as measured on a DO probe Jenway 970 (Bibby Scientific Ltd, UK) reached zero.

3.2.2. *Inoculum*

Screened raw influent wastewater from Cramlington wastewater treatment plant (Northumbria, UK). This wastewater is a mixture of industrial and domestic origin. Samples were stored anaerobically at 4^{o}C and used within 24 hours of collection. The inoculum was also sparged with UPH nitrogen before use.

3.2.3. *Start –up and acclimatisation*

Duplicate reactors were inoculated with differing volumes of wastewater (1 mL, 10 mL, 25 mL and 50 mL). The anode compartment was then filled with the sterile substrates. Control ‘reactors’ (using no inoculum) were run during each test. An inverted 50ml syringe filled with UPH nitrogen was placed into the refilling port on top of the anode chamber to provide an anaerobic headspace. The cathode chamber once filled was left open allowing the diffusion of oxygen into the liquid. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor. A data logging

multimeter (Pico ADC-16, Pico Technologies, UK) was attached to record voltage output every 30 minutes. Reactors were allowed 800 hours at room temperature (20-25 ^{o}C) to show acclimatisation before the experiment was ended. With the acetate fed experiment a further set of reactors were run with lower dilutions of inocula, 0.01 mL, 0.1 mL and repeated 1mL with 25 mL as a positive control.

3.2.4. *Enumeration of bacteria*

The total number of aerobic culturable bacteria present in the wastewater samples used for inoculation was approximated using a spread plate method 9215C (APHA, 1998), with peptone based nutrient agar (Lab M Ltd, UK). Serial dilutions were undertaken into sterile ¼ strength ringers solution, with each dilution plated in triplicate. Plates were incubated at 37 ^{o}C for 48 hours. Anaerobic bacteria were enumerated using a basal salts media (Shelton and Tiedje, 1984) with 1 g/L of both yeast extract and glucose as a carbon source. The media was autoclaved (121 ^{o}C for 15 min) and sparged with sterile UHP nitrogen for 20 minutes. A volume of 9 mL was then added to sterilised Hungate tubes, 1 mL of wastewater was then added to five tubes, and dilutions made down to 10^{-12} with five replicates at each dilution. The headspace of the tubes was sparged with nitrogen, and the tubes incubated at 37 ^{o}C for two weeks. The number of bacteria was determined using the MPN methodology (APHA, 1998).

3.2.5. *Analytical methods*

TCOD of the medias and inocula were measured in duplicate according to standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) colorimetric reagent kit. Volatile fatty acids of the media and inocula were measured in duplicate using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. When the current of the cell had dropped to zero TCOD and VFA's of the cell were measured using the same method as inocula and media above.

3.2.6. *Most probable number (MPN) calculations*

With non-standard dilutions the pre-calculated MPN tables (APHA, 1998) cannot be used. The MNP is calculated through a series of iterations based on a Poisson and binomial distributions (Blodgett, 2005) using the following formula, solving λ for the concentration:

$$\sum_{j-1}^{k} \frac{g_j m_j}{1 - \exp\ (-\lambda m_j)} \ = \ \sum_{j-1}^{k} t_j m_j$$

K = the number of dilutions,

gj = the number of positive (or growth) tubes in the jth dilution,

mj = the amount of the original sample put in each tube in the jth dilution,

tj = denotes the number of tubes in the jth dilution

A probability is assigned to each possibility of the number of bacteria based on the outcome at each dilution, a positive outcome being voltage produced in by the reactor. The number with the highest probability is given as the MPN. Using the spreadsheet developed by Bloggett to make these iterative calculations, the most probable numbers of exoelectrogens per 100 mL of wastewater can be calculated (Garthright and Blodgett, 2003) using the inocula volumes, and the test outcome.

Thomas' simple formula which is based on the same principles as the full test, but a simpler algorithm to solve, can also be applied to the data set, this formula has been shown to have substantial agreement (Thomas, 1942). Using only the lowest dilution that doesn't have all positive tubes, the highest dilution with at least one positive tube and the dilutions in between the following calculation can be made:

$$MPN/100\ ml \ = \ \frac{no.positive\ tubes \ \times \ 100}{\sqrt{(ml\ sample\ in\ negative\ tubes) \times (ml\ sample\ in\ all\ tubes)}}$$

The confidence limits of this calculation at the 95% level can be calculated using Haldane's formula (Haldane, 1939):

m_1, m_2, m_3 ……. denotes inoculation amounts ranging from the largest to the smallest of the chosen dilutions

g_1, g_2, g_3 ……. denotes the number of positive tubes at the corresponding dilutions

$$T_1 = \exp(-MPN \times m_1)\ , T_2 = \exp(-MPN \times m_2) \ldots\ldots\ldots etc$$

$$B = [g_1 \times m_1 \times m_1 \times T_1/((T_1 - 1)^2)] + [g_2 \times m_2 \times m_2 \times T_2/((T_2 - 1)^2)] + [g_3], [\ g_4] \ldots. etc.$$

$$Standard\ Error\ of\ \log 10\ (MPN) = \ 1/\left(2.303 \times MPN \times (B^{0.5})\right)$$

95% confidence intervals are given by:

$$Log_{10}\ (MPN) \pm 1.96 \times Standard\ Error$$

3.2.1. *Growth rate, specific activity and yield calculations*

Growth rate of bacteria (μ) is classically calculated by quantifying the number of bacteria at two time intervals. In this experiment voltage is deemed to be a suitable proxy for exoelectrogenic bacteria, the rate of voltage rise being equivalent to the rate of growth. It is assumed that each bacterium is capable of donating an amount of electrons therefore an increasing number of electrons are donated to the circuit (i.e. the voltage increases at a constant resistance) as the absolute number of bacteria increases, (it does not represent an increasing ability to metabolise), i.e. voltage is deemed proportional to bacterial number. This can be from the growth rate expression:

$$N_T = N_0 e^{\mu t}$$

Where N_T is the number of bacteria at time t (in this case the voltage), N_0 is the number of bacteria (voltage) at time zero (t_0) and μ is the growth rate. Therefore growth can be defined as:

$$\mu = \frac{\ln N_t - \ln N_0}{(t - t_0)}$$

Specific activity (q), defined as moles electrons per cell per second can be calculated over the period of growth as follows:

$$q = \frac{I \times (t_1 - t_o)/F}{N_0}$$

Where I is the current in amps (coulombs/second) as calculate from the measured voltage V, and resistance R calculated through I=V/R, t_1-t_0 is representative of the time period of each measurement, (i.e. every 30 minutes, the total coulombs of charge within this period is therefore I multiplied by 30 minutes multiplied by 60 seconds) and F is Faradays constant of 96485 coulombs/mol e^-. The growth rate and starting MPN is used to calculate the number of cells at each time period N_T. This can be converted to moles of acetate per cell per second (1 mole acetate = 8 moles electrons), to give substrate utilisation (U).

Growth yield (Y) is the amount of biomass or cells produced by the bacteria per mass of degraded substrate measured in g-COD-cell/g-COD-substrate. Rather than use the total COD removed in the reactor, which would also involve COD digested via other routes only the g-COD substrate put to the circuit is used as calculated from the substrate utilisation above. The yield is calculated as follows:

$$Y = \frac{(N_T - N_0) \times W \times COD_{cell}}{\sum_{t0}^{t} U \times COD_{sub}}$$

Where the total cells produced over the growth period N_T-N_0 is multiplied by an estimation of the weight of cells W of 5.3 x 10^{-13} g-cell given in Logan (2008) and the estimation for anaerobically grown cells of the formula of $C_{4.9}H_{9.4}O_{2.9}N$ equating 1.25 g-COD/g-cell, (Rittmann, 2001). The sum of the substrate utilisation U as calculated above is multiplied by COD_{sub} the amount of COD per mole of substrate, 64 for acetate.

3.3. Results

3.3.1. *Number of bacteria in wastewater*

The spread plate counts of the wastewater, and anaerobic multiple tube count indicate there is 8.3 x 10^5 culturable aerobic bacteria, and 6.9 x 10^4 culturable anaerobic per ml of this wastewater, giving a rough estimate of the total bacteria per mL of wastewater to be 10^6. Although this method may over estimate numbers due to some bacteria being able to grow under both conditions, and underestimating numbers due to bacteria being intolerant to the media, the overall value calculated fits in with previous estimates (Tchobanoglous, 1991).

3.3.2. *Most probable number of exoelectrogens*

The number of positive outcomes of each test are shown in Table 3-1. From this the MPN can be calculated shown in Table 3-2. The MPN of exoelectrogens in an acetate fed reactor is 17 per ml of wastewater, this number drops to 1 per ml for a starch fed reactor and 0.6 per ml for a wastewater fed reactor. Superficially it appears that acetate metabolising exoelectrogens are quite rare organisms, starch metabolising exoelectrogens are even rarer and wastewater metabolising exoelectrogens are rarer still.

Table 3-1 The number of positive outcomes for each inocula size out of the total number of reactors run

Inocula size (mL)	50	25	10	1	0.1	0.01
Wastewater	2/2	2/2	0/2	1/2	-	-
Starch	2/2	2/2	1/2	0/2	-	-
Acetate	2/2	4/4	2/2	3/4	1/3	0/2

Table 3-2 The MPN in 1 ml of wastewater given by the two methods stated, numbers in brackets indicate the upper and lower bounds at 95% confidence. The probability of presence in wastewater is calculated from the total count of viable bacteria per 1 ml

Substrate	MPN calculation (Blodgett 2005)	MPN estimation (Thomas 1942)	Probability of presence in 1 ml of wastewater
Wastewater	0.6 (0.3-2.5)	0.8 (0.3-2.5)	6×10^{-7}
Starch	1.0 (0.3-3.2)	1.1 (0.3-4.0)	10^{-6}
Acetate	17.0 (5.5-52)	17.6 (6-51.5)	1.7×10^{-5}

An alternative explanation is that the lower MPNs, and therefore the probabilities of these organisms being present in 1 ml of wastewater, are the product of two or more events. In wastewater and starch there are long chain molecules present which undergo a series of steps in their breakdown. Each step is probably undertaken by different microorganisms. The electrons pass down this chain leading to the final step of donation to the electrode, represented by the acetate reactor. Thus the MPN of the wastewater and starch fed cells is the probable MPN of the acetate fed cells (the number of exoelectrogens) multiplied by the probability of each of the upstream steps. Here all of these steps are simplified into one probability step, however in reality this may be many steps the product of which is equal to 0.04 for wastewater and 0.06 for starch as shown in Figure 3.1.

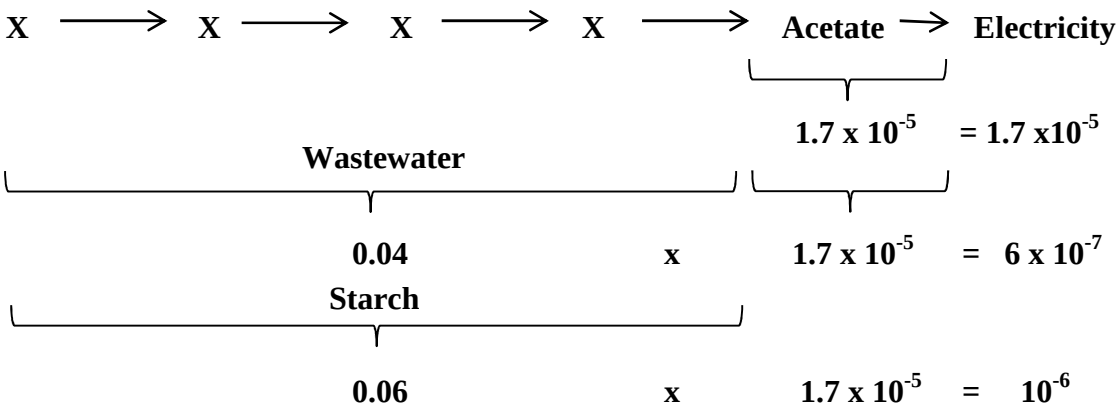

Figure 3-1 Estimated probabilities of numbers of bacteria present in the wastewater begin to produce a working MFC fed on three different substrates of acetate, starch and wastewater based on the numbers determined in the MPN method

3.3.3. *Growth rates*

The individual growth rates for the three different substrates are shown in Table 3-3. The rates were not significantly different (p=0.282 one way ANOVA), and showed agreement with other studies.

Table 3-3 Average growth rates for exoelectrogens fed on different substrates estimated using the rise in voltage measured in the acclimatising reactors

	Average growth rate
Wastewater fed community	0.028 h^{-1} ± 0.013
Starch fed community	0.023 h^{-1} ± 0.005
Acetate fed exoelectrogens	0.035 h^{-1} ± 0.020
Geobacter sulfurreducens (Cord-Ruwisch et al., 1998)	0.023 – 0.099 h^{-1}
Geobacter sulfurreducens (Esteve-Nunez et al., 2005)	0.04 – 0.09 h-1
Fermenting micro-organisms (Rittmann, 2001)	0.05 h-1

3.3.4. *Acclimatisation pattern*

Using an arbitrary value for N_0 (the starting number of bacteria per ml), the known growth rate and the time period over which the experiment was conducted, the pattern of acclimatisation can be modelled.

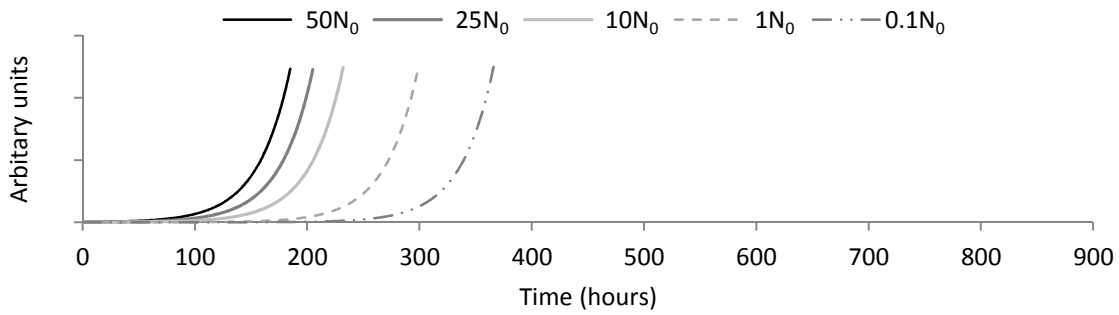

Figure 3-2 Model of the acclimatisation of reactors inoculated with varying amounts of bacteria as denoted by N_0 based on the formula $N_T = N_0 exp^{rt}$ where r the growth rate is the average growth rate determined experimentally of 0.03 hr^{-1} and t time is given on the bottom axis

The pattern of acclimatisation that occurred for the wastewater and starch fed did not follow the model. All reactors acclimatised at the approximate same time. If the growth rates and time are equal, mathematically this means that N_0 is similar for the different volumes of inocula.

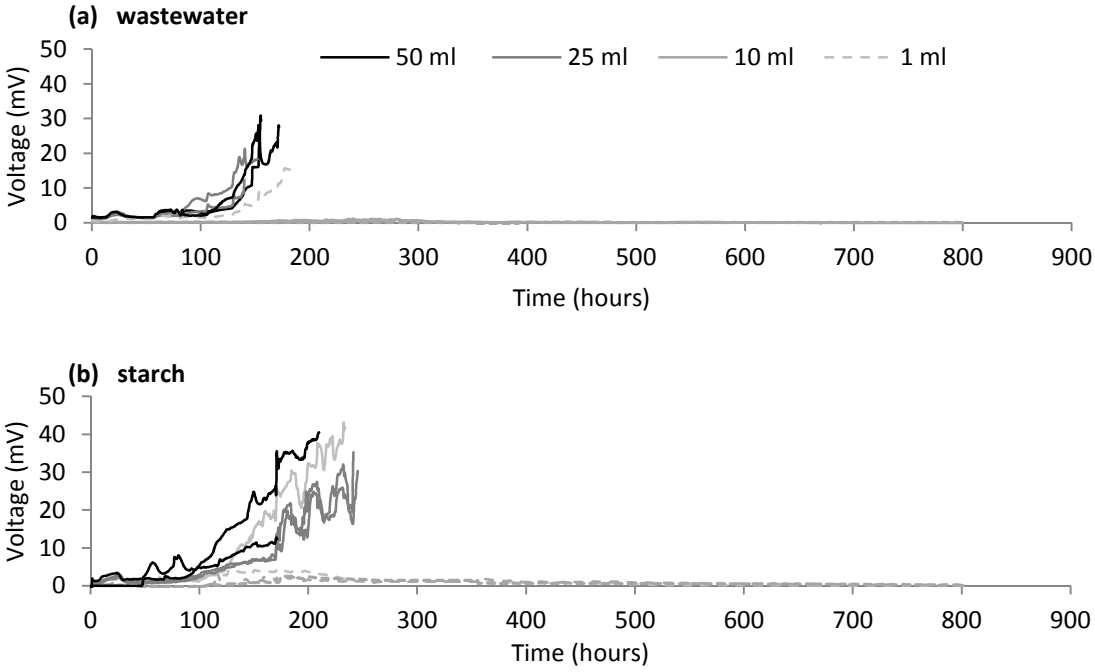

Figure 3-3 Pattern of acclimatisation of the wastewater (a) and starch (b) fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Superficially the pattern observed for the acetate fed reactors appears to follow the model pattern. However this is not the case as the lag time to acclimatisation is over extended with reducing amounts of inocula.

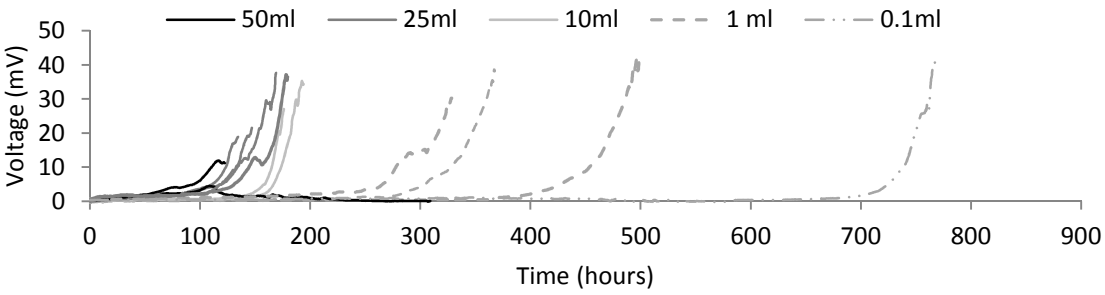

Figure 3-4 Acclimatisation of the acetate fed cells actually observed in the acclimatising cells fed on different volumes of inocula

Using $N_T=N_0\exp^{rt}$ the calculated number of bacteria at the time the reactor inoculated with 0.1 ml (which must have contained at least one bacteria) reaches 10 mV would be 1.8×10^{11} bacteria, equivalent to the predicted number of bacteria in 1 kg of soil (Whitman et al., 1998), and 4×10^{7} times greater than the number of bacteria at 10 mV in the cell inoculated with 50 ml of wastewater (assuming an MPN of 1.7 per ml). This is clearly implausible, growth is not purely exponential, there is likely to be a lag phase with no growth. Yields calculated on the basis of these N_T and N_0 values both with (up to 8 g-COD cell/g-COD) and without (10^{-4} and 10^{-7} g-COD cell/g-COD) growth in the

lag phase give results discordant with the current literature, (these are shown in appendix VII).

3.4. Discussion

If the aim of acclimatising a reactor is to get it going, then it has been shown that a larger volume inoculum will give a quicker (in the case of acetate) and more likely (in the case of complex substrates) successful inoculation, although a proportion of the intended substrate may also be needed. As clear differences were observed between experiments, acclimatisation with the intended substrate is likely to be essential to successful operation. However, more importantly, these results also give insight into the abundance and distribution of exoelectrogenic and other crucial organisms, and to their community development within a reactor.

Discovering the number of exoelectrogenic bacteria per ml of wastewater is a strategically important question. It would inform us of the sequencing depth needed to identify these bacteria. By using the MPN methodology in a series of MFCs and aerobic and anaerobic culturing methods of the same wastewater, an estimation of this number has been gained. Acetate digesting exoelectrogens can be found at an estimated quantity of 17 per ml of wastewater, giving the probability of a bacterium in 1 ml of wastewater being an exoelectrogen as 1.7×10^{-5}, or put differently 0.0017% of the bacteria present in wastewater are exoelectrogenic. With 1000 sequencing reads there would be a reasonable chance of identifying only 1 or 2 exoelectrogens. When compared to the pyrosequencing carried out in chapter 4 a similar answer emerges. Two wastewater samples were analysed, and the total sequencing effort needed to capture 90% of all the sequences in the sample estimated using statistical algorithm as shown in Appendix X. Comparing the total number of *Geobacter* (the known exoelectrogen present in the wastewater samples) found in the sample to the estimated sampling effort, in one sample Geobacter represented an estimated 0.0012 % of the total bacteria, in the other this was lower at 0.00001 %. The two very different approached result in a similar estimation of the number of exoelectrogens present in wastewater. The use of further microbial techniques such as flow cytometry or QPCR would also help the verification of these results.

The number of acetate exoelectrogens is rare: 17 per ml. The number of starch or wastewater exoelectrogens is even lower at 1 per ml. It could be plausible that these are

even rarer organisms, however the likely explanation is that a chain of metabolism is occurring, this fits with the literature (Velasquez-Orta et al., 2011, Kiely et al., 2011c). The probability of achieving a working MFC fed on a complex substrate is therefore the probability of the exoelectrogenic step as identified above, multiplied by the probabilities of each of the upstream steps in the metabolic chain, and is therefore lower than the probability of forming with the acetate step alone. The MPN value is an approximation, yet even considering the upper and lower bounds of the calculation at 95% confidence, as shown in Table 3-2, this pattern is observed. Clearly however this is dependent on the inoculum used; with different inocula such as soil or sludge one would expect different results.

Growth rates, although intuitively demonstrated by the rise in voltage within an MFC, have not previously been calculated. It is an important value to know, especially when modelling such systems. This study calculated the average growth rate of 0.03 hr^{-1}, this value agrees with those documented in the literature from known exoelectrogenic bacteria. No statistical difference is found between reactors fed on acetate and more complex wastewaters, contrary to previous work (Velasquez-Orta et al., 2011) this study shows that the growth rate of exoelectrogens is likely to be the limiting factor.

The pattern of acclimatisation demonstrated within these reactors did not follow the expected pattern. Additionally the pattern observed in the acetate reactors is different to the pattern observed in the reactors fed with more complex substrates. Simple exponential growth does not appear to be happening in either system. The values of N_T within these systems are therefore questionable, as are the calculated yields and specific activities (see appendix VII).

The positive starch and wastewater fed reactors were fewer in number due to the reduced probabilities of the communities forming, but all acclimatised at approximately the same time regardless of the inoculum volume. The growth rates calculated were not statistically different between the different inocula, time was recorded accurately. Explaining this mathematically on the basis of $N_T = N_0 exp^{rt}$ this means either: N_0 is the same for the different inoculum sizes; the N_T of the reactors producing the same voltage is actually different; the rates as defined by voltage rise are not representative of growth rates; or the system may not be described by the equation $N_T = N_0 exp^{rt}$.

More of the acetate cells acclimatised leading to a higher MPN value, the pattern of acclimatisation here does show a clear link to inoculum size, however the size of the lag phase is far greater than would be predicted. Again the rates calculated were not statistically different between the different inoculum sizes and time was also recorded accurately. Here on the basis of $N_T = N_0 exp^{rt}$ either; N_0 is not linearly related to inoculum sizes, i.e. 50 mLs of wastewater contains more exoelectrogens than 50 times 1 ml; the N_T of the reactors producing the same voltage is actually different; there is a lag period before the growth rate starts which is also related, but not linear to, the inoculum size; or again the system is not described by $N_T = N_0 exp^{rt}$.

The MPN method and therefore N_0, is based on the following assumptions: bacteria are distributed randomly within the sample; they are separate, not clustered together; they do not repel each other; and every reactor whose inoculum contains even one viable organism will produce detectable growth or change and the reactors are independent (Blodgett, 2009). It seems likely that exoelectrogens will cluster, there function of passing electrons outside the cell may be used for passing electrons between cells when no external electron sink is available (Bretschger, 2010). In the sequencing data in chapter 1063 *Geobacter* are found in one wastewater sample and 4 in the other, also indicative of clustering. If clustering is occurring, the MPN is likely to be an underestimation as will be N_0 and N_T. This does not however explain the different patterns of acclimatisation observed between the substrates. Additionally the large upper and lower bounds given in the MPN calculations due to the relatively low sample size, could also lead to both under and over estimations of N_0 where the MPN is used.

The relationship of voltage with N_T could be more complex than assumed. Voltage generated from the electrode may be limited by properties relating to the anode itself rather than the bacteria on it, or may quickly reach saturation point of the biofilm, however then one would expect to observe the same pattern in all reactors.

Growth rates are assumed to be represented by the rising voltage measured across the reactors. This may not be the case if the bacterial population has to grow to a certain threshold level (at an unknown growth rate which may different for different inocula sizes) before any voltage is produced. Additionally an assumption is made that increasing voltage is caused by an increasing number of bacteria, not an increasing

capability of these bacteria to donate electrons, this may not be true. Again this does not account for the differences between substrates.

The period of acclimatisation is both highly complex and variable between substrates, yet does show a clear observable pattern, indicating an underlying mechanism. It seems likely that these systems are not described by $N_T = N_0 exp^{rt}$. Such deviations could be caused if the exoelectrogens present N_0 were able to induce electrogenic activity in other bacteria through the excretion of electron shuttles: $N_T > N_0 exp^{rt}$, and in addition a further growth equation of the 'induced' exoelectrogens would act to confuse the picture. In the case of the complex substrate systems something within the chain of metabolism which is unrelated to the bacteria quantity could be triggering the start of the acclimatisation, this causes the reactor to work or fail regardless of the number of exoelectrogens present at the start. In the acetate fed reactors a further factor related to the inoculum size could be causing the extended lag observed, such as the movement of the exoelectrogens to the anode surface.

The period of acclimatisation is not only complex, it is likely to be a period of high competition for resources and possible low efficiency for the exoelectrogens as seen from the low coulombic efficiencies and comparable COD removal in both the positive and negative reactors (see appendix VI).

If the aim of acclimatisation is to merely 'get the reactor going' this study has shown that using a large proportion of wastewater is best. The experiment has also demonstrated that the abundance of organisms needed to start an MFC is low within wastewater, and even lower when these systems are to be fed on complex substrates. The growth rates defined are similar to those observed for exoelectrogenic species in other environments, and are likely to be the limiting factor in MFC acclimatisation. The pattern of acclimatisation a fuel cell is complex and not explained solely by exponential growth. The clear differences between these systems demonstrate the vital importance of acclimatising a community for the eventual use of the reactor. A reactor fed on acetate is different to one fed on wastewater. By developing a greater understanding of this ecology and its development, the move towards more stable biological system can be made. Understanding the nature, abundance and location of these exoelectrogens is crucial.

Chapter 4. Can Microbial Fuel Cells operate at low temperature?

4.1. Introduction

Bioelectrochemical Systems (BES's) are being heralded as a new method of energy efficient wastewater treatment, yielding electrical energy or other products from the bacterial breakdown of organics in an electrochemical cell. For future application of this technology understanding the microbial ecology, community structure and relating this to performance is desirable (Parameswaran et al., 2010) . The majority of fuel cell research is carried out using acetate as a feed at 30^{o}C with the implicit assumption that this will translate into the treatment of real wastewaters at ambient temperatures. To use low strength high volume wastes like wastewater the bacterial communities within BES need to be able to digest complex and variable substrates and do so outside, which in the UK, Europe and many parts of the USA means at low temperatures. If the communities of bacteria able to perform this task do not occur naturally further work and investment into this area may be futile.

As noted above most BES studies are conducted in laboratories at a temperature of 30 ^{o}C (Call and Logan, 2008, Cheng and Logan, 2007a, Selembo et al., 2009b). Few ambient treatment plants will get this warm. Several studies investigating the performance of MFCs over temperatures between 20-30 ^{o}C have found that the maximum power output with acetate was reduced by 9% (Liu et al., 2005a) and 12% (Ahn and Logan, 2010) when the temperature was lowered from 30 ^{o}C to 20 ^{o}C and 23 ^{o}C respectively, using beer waste a 10% drop was seen at these temperatures (Wang et al., 2008). The reduction in performance was lower than predicted by biological process modelling, suggesting that bacterial growth at 32 ^{o}C is not optimal, or that other factors are more limiting (Liu et al., 2005a). Complex wastes were also treated by Ahn and Logan (2010), and it was found that temperature had a greater effect on these than the simple compounds.

Lower (below 20 ^{o}C) and more realistic temperatures have been even less well studied. Min et al (2008) found that at 15 ^{o}C no successful operation was achieved, after 200 hours of operation the experiment was stopped. Cheng et al. (2011) found at 15 ^{o}C start up took 210 hours but at 4 ^{o}C there was no appreciable power output after one month (720 hours) and the experiment was stopped. In the same study a reactor started at 30 ^{o}C was then dropped to 4 ^{o}C and power output was achieved, but around 60% lower than

that the higher temperature. Larrosa-Guerrero *et al*. (2010) operated reactors at 4 ^{o}C and 35 ^{o}C using a mixture of domestic and brewery wastewater, observing a decline in COD removal from 94% to 58% and power density from 174.0 mWm^{-3} to 15.1 mWm^{-3} at the lower temperature.

By contrast Jadhav and Ghangrekar (2009) operated an MFC's in a temperature range of 8-22 ^{o}C and found that the current and coulombic efficiencies were higher than that produced in the temperature range of 20-35 ^{o}C. However in this study temperatures were ambient not controlled and thus confounded by time. They inferred that a reduction in methanogenic bacterial activity at lower temperatures increased MFC performance, although the microbiology of the systems was not examined. Similar results were obtained by Catal *et al.* (2011), here the biofilm was examined using scanning electron microscopy and found to be thicker in the higher temperature reactors.

MFC systems are based on electrochemical and microbiological principles: temperature affects both. The electrochemical impacts of temperature can be calculated using the Nernst equation based on known free energies for substrates such as acetate, or estimated free energies if wastewater is used (Logan, 2008). In bacterial systems rates of reaction roughly double for every 10^{o}C rise in temperature (Rittmann, 2001). However, the actual behaviour of these complex systems at different temperatures and fed on different substrates remains an area of great uncertainty in this field of research.

An increasing number of studies into the microbial communities of BES using techniques such as restriction fragment length polymorphism (RFLP), clone libraries and denaturing gradient gel electrophoresis (DGGE) are adding to the knowledge base we have about these communities. There are advantages to these various techniques such as the high reproducibility and in the case of DGGE and RFLP the large number of samples than can be run (van Elsas and Boersma, 2011, Kirk et al., 2004). However all these techniques are limited in that only a small fraction, (in the case of DDGE estimated at 1-2 % (Macnaughton et al., 1999), of the species present are targeted in these studies, total diversity cannot be estimated from these limited results. Never the less it has been repeatedly shown that *Geobacter sulfurreducens* dominates in acetate fed reactors, although this can vary when reactors are inoculated with different media (Kiely et al., 2011c). As substrates become increasingly complex moving from VFA's

to carbohydrates to actual wastewater the dominant species become more varied (Kiely et al., 2011c). Some wastewater fed reactors were found to be dominated by *Betaproteobacteria* (Patil et al., 2009), although in other studies *Geobacter* still dominates (Cusick et al., 2010).

Most of the techniques that have been used are limited by their capacity to identify the most dominant species within the communities. Next generation sequencing (capable of sequencing to a far greater depth) has now been used in two MFC studies. Lee *et al*. (2010) used FLX Titanium pyrosequencing to sequence four samples of biofilm, triplicate samples were taken from an acetate fed reactor comparing this to a single sample taken from a glucose fed reactor. The profiles found in the samples were not significantly different. A further study by Parameswaran (2010) analysed the biofilm of two MFC reactors fed on ethanol examining the impact to the communities when methanogenesis was prevented in one, identifying the role of hydrogen scavengers.

The aim of this study was to determine if microbial fuel cells can work at low temperatures, and if the inocula affects this. By running reactors fed on both wastewater and acetate the relative importance of the final 'electrogenic' step, and the up- stream hydrolysis and fermentation steps can be evaluated. The impact of temperature, inoculum and substrate on the microbial communities and total diversity within these reactors was examined using next generation sequencing techniques.

4.2. Methods

4.2.1. *Experimental design*

The variables examined were: temperature (warm 26.5 ^{o}C and cold 7.5 ^{o}C); substrate (acetate and wastewater); and inoculum (Arctic soil and wastewater). Each set of conditions were run in parallel duplicate reactors and biofilm samples taken from each. The two series of experiments, acetate and wastewater, were conducted using the same 8 reactors under identical conditions, the two wastewater inoculum samples were used to seed the acetate (wastewater sample1) and wastewater fed (wastewater sample 2) experiments. This is represented in Figure 4-1.

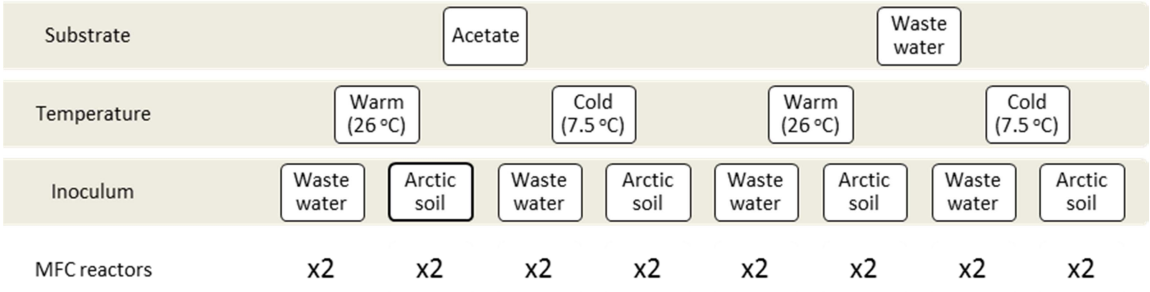

Figure 4-1 Illustration of the multi-tiered reactor conditions used

The warm temperature was chosen to represent the typical ambient laboratory temperatures of many MFC studies. The low temperature is the lowest sustained temperature of a wastewater treatment plant in the North of England (54°58'N, 01°36'W) experienced over a winter period (Northumbrian Water Ltd). The different substrates represent the most commonly used laboratory substrate acetate, and compared to wastewater. The two different inocula were the usual inoculum of wastewater, and Arctic soil (see below) which could potentially have more bacteria with low temperature, exoelectrogenic capability.

Wastewater typically contains 10^5 - 10^6 bacteria per mL (Tchobanoglous, 1991) soils can contain around 10^9 bacteria per gram (Whitman et al., 1998). Many soil environments are low in oxygen, and iron rich, favouring anaerobes and iron reducers and potentially therefore exoelectrogens. Arctic soils have been shown to have to be biologically active, accounting for around 6% of the total global methane sources (Ehhalt et al., 2001). (Hoj et al., 2005, Kotsyurbenko et al., 2004, Metje and Frenzel, 2005). Soil taken from Ny-Ålesund, in the Spitsbergen area of Norway has been shown to contain a wide range of methanogenic groups active at temperatures ranging from 1-25 °C (Hoj et al., 2005, Hoj et al., 2008).

4.2.2. *Reactor design and operation*

Eight identical double chamber tubular MFC reactors (78 mL each chamber) with an internal diameter of 40mm and length of 60mm were used. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (Tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor, and a multimeter to measure the voltage (Pico ADC-16, Pico Technology, UK). The membrane between the reactor

chambers was Nafion 117, with an area of 12.6cm^2. Reactors were sparged with 99.99% pure N2 in the anode chamber, and air in the cathode chamber for 15 minutes after every re-fill.

Four reactors were operated at a temperature of 26.5 ^{o}C in an incubator (Stuart Scientific SI 50, UK), the other four at 7.5 ^{o}C in a low temperature incubator (Sanyo MIR-254, (Sanyo Biomedical, USA). The temperature was logged continuously over the experiment using a EL-USB-1 temperature data logger (Lascar Electronics, UK). The reactors were inoculated and filled with substrate, replacing this every 5-6 days until a stable power generation was achieved. The reactors were then re-filled and three successive 3 day cycles were run logging the voltage over this time. Chemical oxygen demand (COD) removal during each batch was determined using standard methods (APHA, 1998) and Spectroquant ® test kits (Merck & Co. Inc., USA).

4.2.3. *Media and inocula*

Autoclaved acetate media (Call and Logan, 2008) containing 1 g/L sodium acetate was compared to wastewater taken from Cramlington wastewater treatment site (Northumbrian Water Ltd, UK) which was UV sterilised prior to use. This method gave the most successful sterilisation with the least change chemical composition of the wastewater (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) compared to autoclaving and filtering (see appendix V). The cathode chamber was filled with 1M pH 7 phosphate buffer. The conductivity of the nutrient media, wastewater and the phosphate buffer was measured using an EC 300 (VWR Ltd, UK) and equalised for the temperatures of 7.5 ^{o}C and at 26.5 ^{o}C.

The wastewater inoculum was collected from Cramlington wastewater treatment plant, a Northumbrian Water site in the North of England, it was raw wastewater collected prior to any form of treatment, and is believed to be of mixed industrial and domestic, COD 0.7-0.8g/L. Once collected the sample was stored in a fridge at 4 ^{o}C within a closed container. The Arctic soil was collected from Ny-Ålesund, Spitsbergen in Svalbard. This was wrapped within three sealed bags and stored at 4 ^{o}C until used. The inocula of wastewater and soil were measured out to 5 mL or 5 g respectively before being added to the reactors. Samples of each inocula were preserved in a 50:50 in a mix of ethanol and autoclaved PBS pH7 in the freezer at -20 ^{o}C for microbial analysis.

4.2.4. *Microbiological techniques*

At the end of each experiment the anode was removed aseptically from the chamber using aseptic technique and preserved in a 50:50 mix of ethanol and autoclaved PBS pH7 and stored in a freezer at -20 ^{o}C. A 5 ml or 5 g sample of the original inocula was also taken and preserved in this way. The inocula samples were pelletized and the DNA then extracted. With the anode samples the bacteria that had dispersed into the liquid was pelletized and then added to the central section of the anode felt cut from the whole anode. The DNA was extracted by placing this sample into the beaded tube of a FastDNA Spin Kit for Soil (Qbiogene MP Biomedicals, UK). Extraction was completed according to the manufacturer's instructions. The samples were then pyrosequenced following amplification of the 16s rRNA gene fragments.

The primers used were F515 (GTGNCAGCMGCCGCGGTAA) and R926 (CCGYCAAT-TYMTTTRAGTTT). Each sample was labelled with a unique 8 base pairs (bp) barcode connected to a GA linker. Sequencing was completed from the Titanium A adaptor only forward from the F515, capturing the V4 region and most of the V5 region with a Titanium read of 400-500 bp. Triplicate PCR reactions were carried out using the Roche FastStart HiFi reaction kit (Roche Diagnostics Ltd., UK) and subject to the following optimised thermal cycles: initial denaturation at 95°C for 4 minutes; 23 cycles of denaturation at 95°C for 1 minutes; annealing at 55°C for 45 seconds; extension at 72°C for 1 minute; final extension at 78°C for 8 minutes. An automated thermal cycle Techne TC-5000 (Bibby Scientific, UK) was used.

The triplicate samples were then pooled and cleaned using QIAquick PCR Purification Kit (Qiagen, UK). The DNA concentration was quantified by UV-Vis spectrophotometry using a Nanodrop 2000 (Thermo scientific, USA). The individual samples were pooled to give equal concentrations of all reactor samples, and double concentration of the wastewater and arctic soil seed. Sequencing was carried out by the Centre for Genomic Research (University of Liverpool, UK) using the Roche 454 sequencing GS FLX Titanium Series.

4.2.5. *Data analysis*

The pyrosequencing data set was split according to the barcodes and unassigned sequences were removed[1]. The flowgram files were cleaned using a filtering algorithm Amplicon Noise (Quince et al., 2009) to give the filtered flowgram file. Filtering at a minimum flowgram length of 360 bp including the key and primer before first noisy signal, all flowgrams were then truncated to 360 bp. A pairwise distance matrix was then calculated using the Pyronoise algorithm (Quince et al., 2009). This uses an iterative Expectation-Maximization algorithm which constructs denoised sequences by clustering flowgrams using the initial hierarchical clusters generated in the previous step and the filtered flowgram file. The cut-off for initial clustering is set at 0.01 and the cluster size is 60, as recommended by Quince et al. (2009). The flowgrams can then be denoised.

PCR errors were then removed again using Seqnoise, generating a distance matrix using the Needleman-Wunsch algorithm for pairwise alignment. The optimal parameters used here were the cut-off for initial clustering of 0.08 and cluster size of 30. Chimera removal was completed using the Perseus algorithm (Quince et al., 2011) which for each sequence searches for the closest chimeric match using the other sequences as possible parents. (Quince et al., 2011). The sequences are then classified and the good classes filtered at a 50% probability of being chimeric, producing the final FASTA file which is denoised and chimera free ready for analysis in QIIME (Caporaso et al., 2010).

Using the QIIME pipeline tutorial the following analysis was completed: assigning taxonomy using Greengenes (http://greengenes.lbl.gov) at the 97% similarity level; creating an OTU table; classification using the RDP classifier; summary of taxonomic data from classification; generation of rarefaction data of the diversity in a reactor; calculation of the differences between the reactors; performing Principle Co-ordinates Analysis (PCoA); jackknifing and bootstrapping to understand uncertainty in beta diversity output; and generating Unweighted Pair Group Method with Arithmetic Mean (UPMGA) trees for hierarchical clustering of samples. The dissimilarity of the community structure between duplicates was examined using both a weighted (relative abundance) and unweighted (presence/absence) phylogenetic diversity metrics using

[1] *The analysis of the pyrosequencing data was carried out by Dr Matthew Wade, a Bioinformatics researcher within the School of Civil Engineering and Geosciences at Newcastle University.*

UniFrac, giving a distance matrix containing a dissimilarity value for each pairwise comparison. The raw OTU table generated was used to produce the species abundance pattern (with the log abundance normalised to the number of sequences in each sample) and the rank abundance curves, (where percentage abundance is used to normalise samples).

An estimate of the total diversity for each sample was calculated using the Bayesian approach as described in Quince et al. (2008), where the 'posterior distribution' of the taxa area curve is estimated, from the known distribution of the data gathered in the sequencing. Three distributions are modelled: log-normal; inverse Gaussian; and Sichel, and deviance information criterion (DIC) are used to compare the fit from each model. The lower the deviance or DIC values the better the model fit, those models within 6 of the best DIC value can be considered as a plausible fit. Using the fitted abundance distributions the sampling effort required to capture 90% of the taxa within that sample is estimated.

Minitab 15 (Minitab Inc., State College, USA), statistical program was used to run analysis of variance (ANOVA) tests on the experimental data, and t-tests on the distance matrix data for the sequences samples. Data were checked for normality prior to completing ANOVA, and if necessary the Box-Cox transformation was used.

The performance of the MFC reactors were analysed on the basis of three variables: % COD removal as measured; coulombic efficiency (CE); and power density (mW/m^2). The latter two variables were calculated using the measured COD and voltage within the cells, as described in Appendix VIII. Correlation of the community structure with these performance factors was done using BEST (Biological Environmental and Stepwise method) within Primer 6 (Primer-E Ltd. UK).

4.3. Results

4.3.1. *Cell acclimatisation*

All 16 reactors acclimatised and produced voltage. The acetate fed reactors showed a clear pattern of acclimatisation related to both temperature and inocula with the warm reactors acclimatising first, and the Arctic soil inoculated reactors starting first as shown in Figure 4-2. The cold wastewater inoculated reactors did not produce current until after around 800 hours, longer than the time allowed in previous studies (Cheng et al.

2011, Min et al (2008). The acclimatisation of the wastewater fed reactors was only affected by temperature: the warm reactors started producing current at day 1, the cold reactors at day 20. All duplicates behaved in a very similar way.

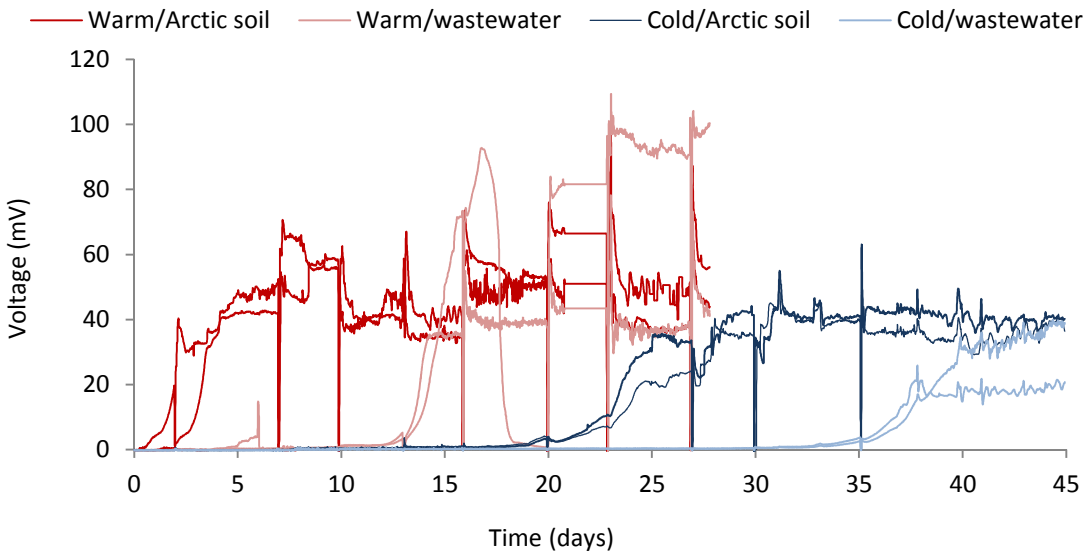

Figure 4-2 Acclimatisation of the acetate fed reactors inoculated with the two different inocula and run at warm (27.5 ^{o}C) and cold (7.5 ^{o}C) temperatures

4.3.2. *Cell performance*

Over the three batch runs, the reactor performance was variable especially within the warm reactors, as seen in Figure 4-3. The variation in performance was not a function of either the inocula or the substrate and the highest variation was seen between the duplicates.

Three measures of performance averaged for each reactor over the triplicate batches are shown in Figure 4-4. The coulombic efficiency is higher in the acetate fed reactors; and the COD removal is higher in the wastewater fed reactors. Power densities do not appear to vary with substrate, inoculum or temperature, however two individual reactors had considerably higher power densities than the others and their duplicates: acetate warm ww 2; and wastewater warm soil 1.

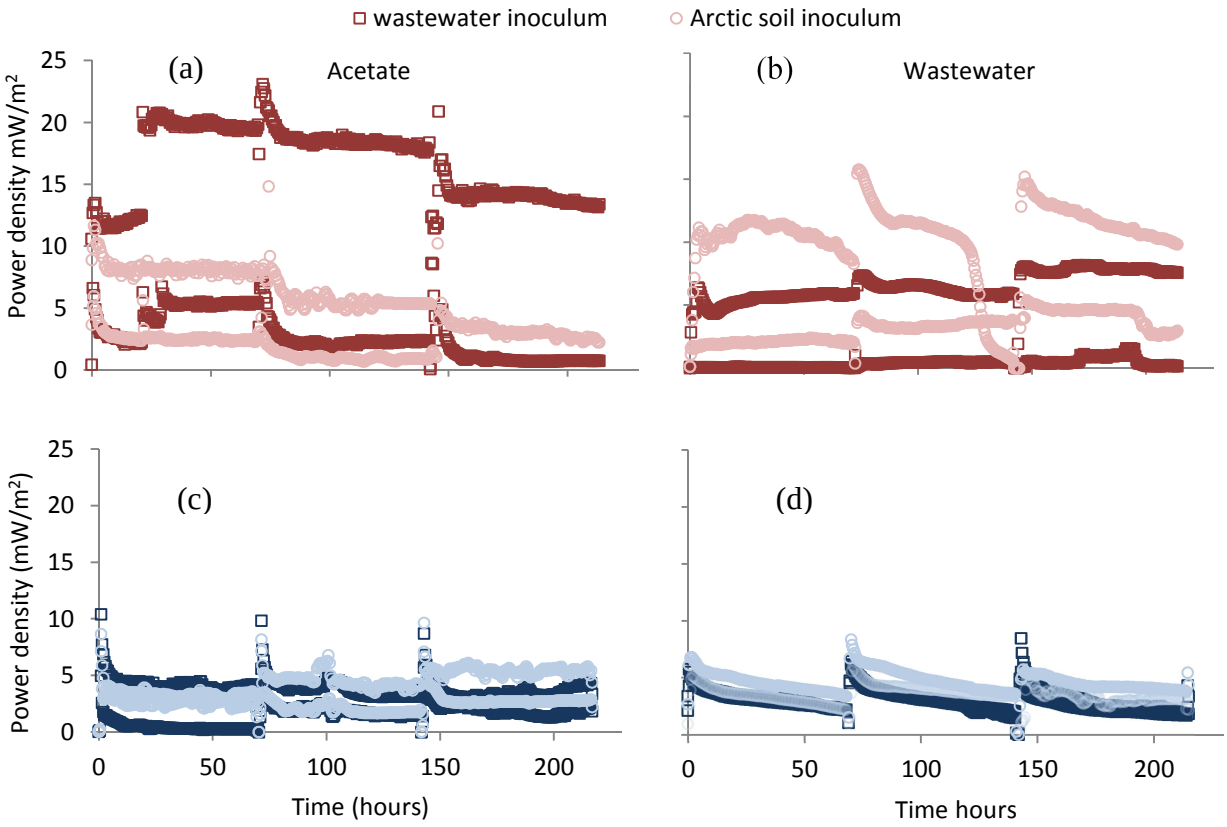

Figure 4-3 Power density plots showing the three consecutive batch runs for: (a) acetate fed reactors run at 27.5 °C, (b) wastewater fed reactor run at 27.5 °C (c) acetate fed reactor run at 7.5 °C (d) wastewater fed reactor run at 7.5 °C

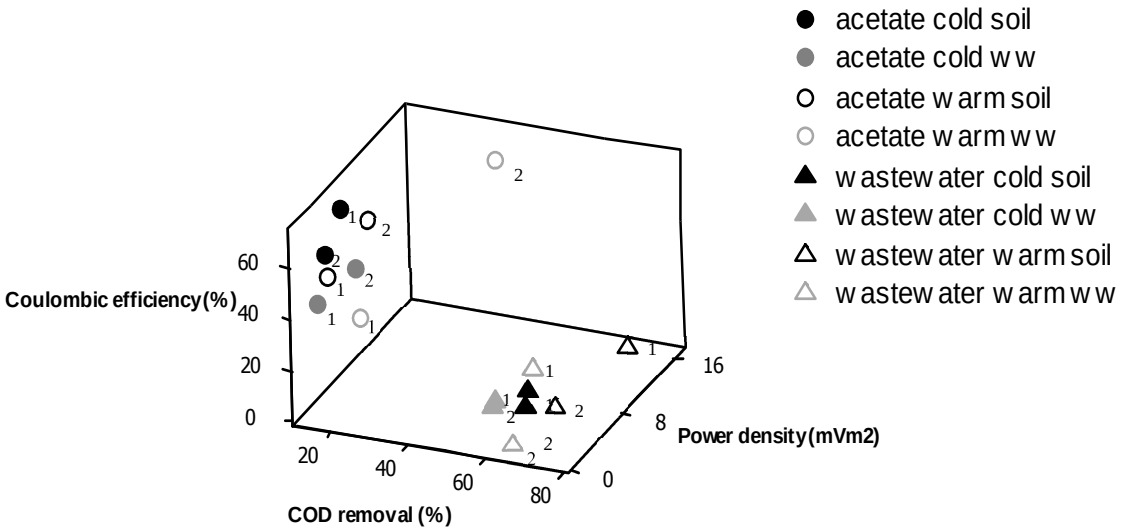

Figure 4-4 3D plot showing reactor performance in terms of Coulombic efficiency, COD removal and power density of the various reactor conditions, duplicates of each condition are labelled on the plot next to the symbols

By performing an ANOVA on the three performance indicators using the factors of feed, temperature and inocula a complex picture emerges. The power density results, i.e. the ability of the biofilm to put electrons to the circuit, were not normally distributed, when transformed, none of the performance factors analysed were significant (feed p = 0.746, inoculum p = 0.249, and temperature p = 0.147). For coulombic efficiency both inoculum (p=0.009) and feed (p=0.000) were significant yet temperature was not. The acetate fed reactors performing better (54.5%) than wastewater fed ones (12.3%), and the Arctic soil inoculated reactors performing better (37.4%) than the wastewater inoculated ones (29.4%). The reactors fed wastewater removed significantly more COD (62.1%), than the acetate reactors (19.4%) (p=0.000) the warm reactors also removed more (45.9%) than the cold ones (33.7%) (p=0.000), the type of inoculum was not significant. Two way ANOVA was performed between each interaction with each performance indicator. For CE the interaction between substrate and inoculum was significant (p = 0.057) with the inoculum having a much stronger effect with the acetate feed than the wastewater feed, and the Artic soil acetate fed reactors performing the best. The interaction between substrate and inoculum was also significant in the COD removal (p = 0.008), the Arctic soil inoculum having a higher COD removal in the wastewater fed reactors, but a lower COD removal in the acetate fed reactors than the wastewater inoculum. No other interactions were significant.

4.3.3. *Similarity of duplicate reactors*

It is seen in the data above that the duplicate reactors performance varied considerably, especially for the warm temperature reactors. Using the sequencing data a Unifrac dissimilarity matrix was plotted, using phylogenetic information the ‘distance’ between each sample is quantified and corresponds to the degree of similarity (Appendix IX). The values show that the duplicate reactors fed with acetate are indistinguishable (p=0.000). This was observed with both the weighted analysis which incorporates information on relative abundance of each OTU, and the unweighted analysis which is based on the presence or absence of each OTU. The wastewater fed duplicate reactors were typically different, with the exception of the hot Arctic soil inoculated reactors (p=0.000). The two wastewater inocula samples taken from the same treatment plant but at different plants were also indistinguishable (p=0.000). This pattern is also observed in Figure 4-5, where the acetate duplicates are paired, and appear to cluster on the basis of temperature. The wastewater fed reactor duplicates are not paired together and do not

cluster with temperature or inoculum. Further details of the bacteria groups present within these reactors can be found in Appendix XI.

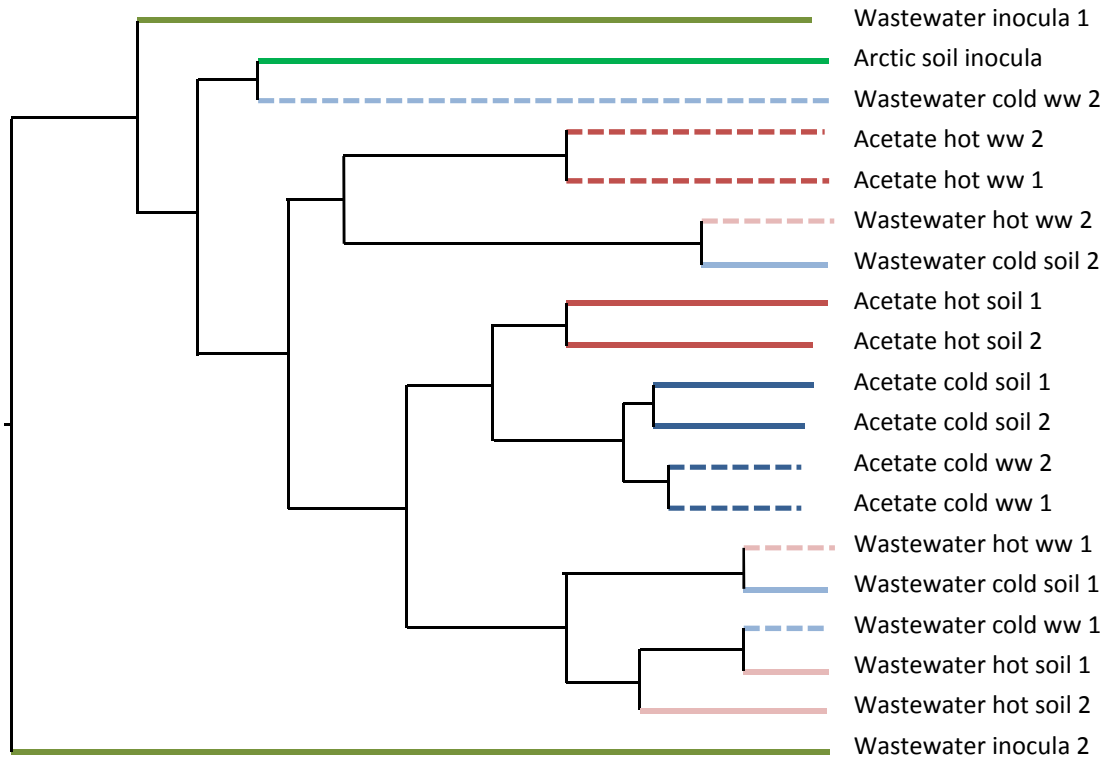

Figure 4-5 Dendrogram resulting from the UPMGA hierachical weighted clustering of samples, the length of lines is relative to the dissimilarity between samples, groupings of samples are denoted by the coloured end portion of the lines

4.3.4. *Microbial diversity*

In total 19 samples were analysed. The number of sequences per sample ranged from 8112 to 77436 with a total number of observations of 549178. The species abundance pattern plotted from the OTU table shows a large variation in the diversity of the samples shown in Figure 4-6. As expected the Arctic soil inoculum is the most diverse, followed by the wastewater inocula. The acetate fed reactors however are considerably more diverse that the wastewater fed reactors, the most diverse of these (acetate cold soil 2) has a similar diversity to the wastewater inoculum, and the least diverse (acetate warm ww 2, the reactor with the highest power density) is similar to the most diverse of the wastewater fed reactors.

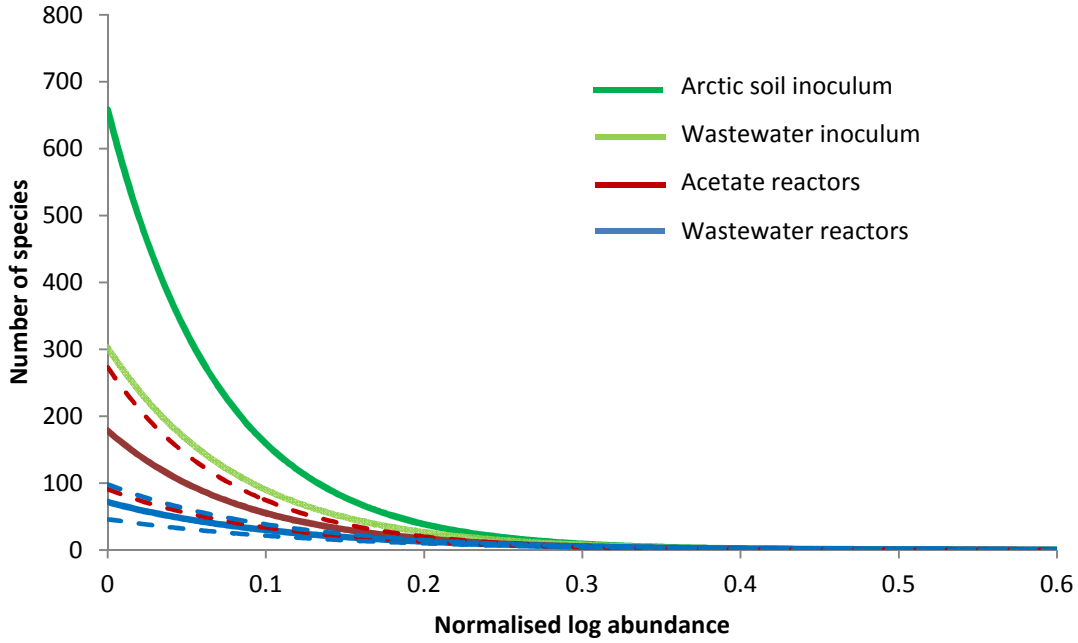

Figure 4-6 Species abundance pattern, the number of species is plotted against the log abundance normalised to the total number of observations for each sample. The plots for the acetate and wastewater fed reactors are averages of the eight reactors used, the highest and lowest within each substrate grouping are shown with the dashed lines. The wastewater inoculum line is an average of the two samples

The observation of the greater diversity in the acetate fed reactors is also seen in the total diversity estimates. A summary of these values is presented in Figure 4-7 where is clearly seen that for all the three distribution models the acetate fed cells have a higher predicted diversity, and that the acetate soil inoculated reactors have a higher total diversity than the wastewater inoculated ones. Performing a nested ANOVA on the Box Cox transformed total diversity estimates, shows that the acetate fed reactors have a statistically significantly higher diversity (log-normal $p = 0.001$; inverse Gaussian $p = 0.000$; and Sichel $p = 0.027$). Within the acetate fed reactors the Arctic soil inoculated reactors have a higher predicted diversity (log-normal $p = 0.006$; inverse Gaussian $p = 0.003$; and Sichel $p = 0.013$), the lower temperatures also give higher diversity (log-normal $p = 0.037$; inverse Gaussian $p = 0.012$; and Sichel $p = 0.029$). There is a strong interaction between the acetate feed and the inoculum type ($p = 0.024$) but not with temperature ($p = 0.156$) observed in both the log-normal and inverse Gaussian distributions. The full tables of diversity predictions, DIC values and estimate sampling requirements can be found in appendix X.

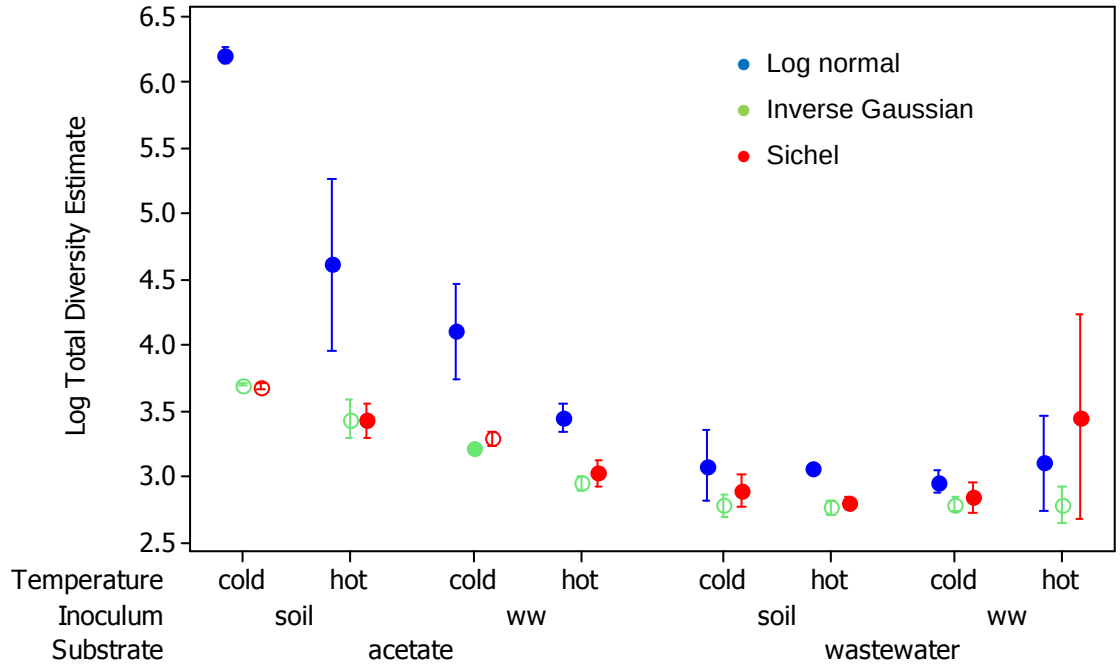

Figure 4-7 The estimates of total diversity for each set of reactor conditions, the three points within each sample are the mean of the duplicate samples modelled to log-normal, inverse Gaussian, and Sichel estimates, the best fit according to the DIC values is denoted by a closed circle, lines are one standard error of the mean

4.4. Discussion

All the reactor conditions tested produced current showing that MFCs can function at low temperatures, with real wastewaters and the bacteria required for them to do so can be found within the wastewater itself. This finding is of great significance to the industrial feasibility of MFC technology for wastewater treatment.

The power output produced by the MFCs was not significantly affected by either temperature feed or inoculum. Although some warm reactors achieved a power density much higher than the cold reactors, due to the variability between reactors this was not significant. The reasons for this variability, were not discovered, no statistical link could be made between the community structure and the power density. The higher coulombic efficiencies within the acetate fed reactors did not translate into higher power densities, only low amounts of COD was converted efficiently into power. Whereas in the wastewater fed reactors more COD was converted less efficiently producing a similar power. In terms of wastewater treatment, this high COD removal, albeit at low CE, is an advantage.

The lack of temperature effect seems at first to be unlikely. Based on the laws of thermodynamics, the free energy available in many chemical reactions decreases as temperature decreases. However in a fuel cell system the energy available is the difference in energy between two half reactions. As both the half cells are equally affected by temperature, the difference between them, or energy available does not decrease with lower temperatures (Appendix II). This is a simplification, many other factors such as dissociation constants and partial pressures of gases will affect the energy, additionally the metabolic activity of the bacteria also reduces with lower temperatures (Rittmann, 2001), however these do not appear to be having a significant impact although may be responsible for some of the variability in performance. On the basis of the results presented here, it can be asserted that low temperature systems have a similar level of energy available for both bacterial metabolism and electricity production as higher temperature systems.

The lack of temperature effect could be caused by the reactor design itself. The inherent inefficiencies and overpotentials within the reactors could be limiting the performance such that the temperature effect is not observed, i.e. all the reactors are working at the limit of their performance and warming them cannot result in improvements. If lower temperature reactors did prove to have slower microbial kinetics, as would be expected and as is indicated by the slower acclimatisation in the cold reactors this could be overcome through relatively simple engineering solutions such as increasing the size of the anode. An increase in the size of the anode would give a greater surface area for the biofilm to grow, and therefore more active bacteria to compensate for the slower metabolic rates.

A further counter intuitive result of this study it that the acetate fed cells have a higher microbial diversity than the wastewater fed cells. It would be assumed that in a wastewater fed systems that the complexity of the substrates available for metabolism, and different metabolic pathways would result in a higher diversity of bacteria, with different groups digesting different substrates at different times. With acetate fed reactors, the only metabolic pathway within a fuel cell should be the direct breakdown of acetate and donation of electrons to the electrode, the most efficient species should dominate theoretically leading to a much less diverse community. This is not seen to be

the case, with a higher diversity in the acetate fed cells being shown both by the species abundance pattern and by the analysis of all the total diversity estimates.

It is proposed that the diversity of the systems is determined not by the diversity of the metabolism within it, but by the overall energy available to the bacteria, and that the free energy available to bacteria in the acetate reactors is greater than in the wastewater reactors. This energy difference could be due to several reasons: acetate may have more free energy per g COD than wastewater; the free energy in acetate may be more accessible to the bacteria, i.e. it is easier to degrade than many of the compounds in wastewater; or that energy is lost during the metabolic chain, with acetate this chain is short, therefore the losses are low, within wastewater these chains are much longer and therefore the losses of energy are greater, this would also produce the coulombic efficiencies observed. The fact that there is no observed difference in the diversity between the warm and cold reactors is further evidence that the energy available in these is actually similar.

Results indicate that the energy flux within a microbial system is key to determining the ecology of that system. The total free energy available is likely to affect the balance of births and deaths of individual species, with greater energy resulting in more births i.e. greater abundance and therefore ultimately greater diversity. The free energy will also impact on the speciation rate (i.e. a greater number of births will ultimately lead to greater chances for speciation). This is counter to the theory that a diverse range of substrates available would provide a variety of different metabolic pathways for different organism to exploit, and therefore lead to a higher diversity.

If a quantitative link could be made between the free energy in a system and the diversity modelling of these complex biological ecologies, being able to understand such phenomena as acclimatisation, adaptation and functional redundancy, and ultimately therefore the manipulation of biological systems becomes a greater possibility (Curtis and Sloan, 2006). We are however still a long way from this in the plant and animal world ecologists have argued there is no single species/energy link (Clarke and Gaston, 2006) and even if it was the key parameter the free energy in wastewater systems cannot yet be reliably measured. Although it is evidenced here that free energy may be the key in determining diversity, a conclusive answer cannot be

given let alone a quantitative link on the basis of these results alone, further research is required.

A further effect on diversity is seen with the inoculum, which interacts with the substrate. The Arctic soil inocula has a greater diversity which seems to be carried forward into the acetate fed cells, a greater number of these species surviving within the reactors where energy may be plentiful. As the performance of the acetate and wastewater fed cells is similar despite the increased diversity of the acetate reactors, it could be concluded that this increased diversity is non-beneficial, or at least neutral to the performance of the reactor. Thus although wastewater reactors will always have lower coulombic efficiencies due to the losses within the metabolic chain, they may actually be more efficient at turning the energy available into wastewater digesting biomass and electricity.

The majority of fuel cell research is conducted at warm temperatures and with simple substrates. It has been shown in this research that reactor performance is not significantly affected by the temperature, neither is the diversity of the community developed. Inoculating reactors with cold adapted organisms does not have any benefit on the performance of the reactors. The substrate fed to the reactor again has little impact on the performance, however results in very different diversities.

It is generally assumed that an acetate fed reactor may represent the optimum conditions for an MFC, however this may not be the case. These findings suggest that wastewater feed has less available energy and therefore results in a more efficient biomass being formed. This has positive implications for the introduction of bioelectrochemical systems into wastewater treatment.

Chapter 5. Time taken until failure for MEC's fed on acetate compared to those fed on wastewater

5.1. Introduction

In 2005 a discovery was made that a microbial fuel cell could be turned into a microbial electrolysis cell adding a small supplement of electricity at the cathode to produce products such as hydrogen gas (Rozendal et al., 2006, Liu et al., 2005b). This new technology has spurned much excitement and research into increasing the performance and gas yield of such reactors (Wang et al., 2011b, Sleutels et al., 2011, Cheng and Logan, 2011). The aim of this research being to achieve a commercially viable and sustainable means of treating waste organics (Oh et al., 2010, Rittmann, 2008, Clauwaert et al., 2008).

Substantial steps have been taken towards enabling the implementation of this technology. Low cost and more robust alternatives to many of the materials used in an MEC have been discovered such as stainless steel (Call et al., 2009) and nickel (Selembo et al., 2009a) cathodes. Alternative membrane materials have been trialled successfully (Rozendal et al., 2008c), as well as not using a membrane at all (Clauwaert and Verstraete, 2009). Anodes with greater surface areas have been found (Call and Logan, 2008) as well as methods to enhance the performance of the carbon anodes (Cheng and Logan, 2007b). New cell architectures and configurations have also helped improve performance (Cheng and Logan, 2011, Wang et al., 2010). Such developments have seen the performance of these reactors increase from hydrogen production rates of 0.01-0.1 m^3H_2/m^3reactor/day (Liu et al., 2005b, Rozendal et al., 2006) to 17.8 m^3H_2/m^3reactor/day (Cheng and Logan, 2011), although the same rise in not seen in the electrical recoveries of these systems 169% (Rozendal et al., 2006) 533% (Liu et al., 2005b) in the initial studies to 115% (Cheng and Logan, 2011) due to the higher input voltages used. All of this research has used acetate as a model compound.

Research with complex substrates is more limited. The ability of MECs to digest complex substrates has been proved such as domestic wastewater (Ditzig et al., 2007), piggery wastewater (Jia et al., 2010), potato wastewater (Kiely et al., 2011a) and end products of fermentation (Wang et al., 2011a, Lalaurette et al., 2009). Limited research has been conducted into the long term performance of MFCs and MECs, deterioration in performance of an MFC after a year of operation has been attributed to the gas

diffusion cathode (Zhang et al., 2011). Marine MFCs used as batteries to power offshore monitoring devices have been monitored for up to a year (Reimers et al., 2001, Tender and Lowy, 2004) and 18 months (Lowy et al., 2006), power production was maintained over this period although in two studies it did deteriorate steadily (Lowy et al., 2006, Reimers et al., 2001), and in another there were occasional drops in the output (Tender and Lowy, 2004). Such studies may not directly translate to MFCs or MECs used for wastewater, in a marine environment the ionic concentrations, gradients and flows will be different, as will the bacteria.

By analysing all the published papers in the area of MECs up to October 2011 the limited scope of how well we understand the long term performance of these systems especially when fed on real wastewaters becomes clear, as seen in Figure 5-1.In 26% of papers the duration of the experiment was not given. In many other cases this time frame is not stated explicitly but can be inferred using the tables, graphs and other information given. In relatively few articles the durability is highlighted as a factor. Two research articles have however been published which indicate the technology might have long term applicability with experiments lasting 9 months (Lee and Rittmann, 2010) and 8 months (Jia et al., 2010) , both running on acetate. Although several other studies do state a decline in performance over time (Jeremiasse et al., 2009, Rozendal et al., 2008b, Lalaurette et al., 2009, Hu et al., 2009).

With acetate fed reactors, 73% of all MEC studies, the time scales mentioned range from 4 to 6480 hours, with 1159 as the average. However when wastewater is used, (only 10% of laboratory studies) the range is between 12 and 184 hours, with an average of 122.5 hours, this time of operation is significantly different ($p=0.000$, two sample T test). For other substrates such as VFA’s and glucose the average run time is 276 hours. This is shown in Figure 5-1, the studies with no time frame stated are not included in the graph. The explanation for this disparity is not evident in the literature, in one study acetate and piggery wastewater are compared directly with acetate reactors running for 8 months and the experiments with wastewater lasting just 12 hours, no reason for this experimental procedure is given (Jia et al., 2010). There is a clear gap in this area of research.

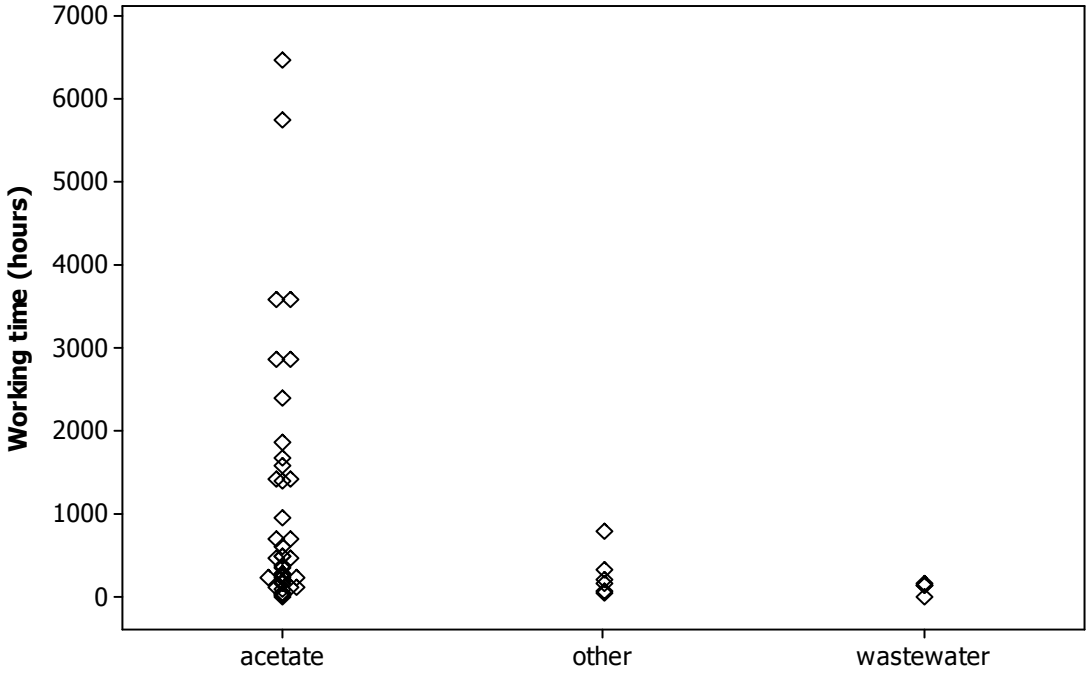

Figure 5-1 The working time of all MEC studies documented in the literature to date (Oct 2011), shown for the different substrates

If MECs are to be a viable and sustainable treatment option for the future then we need to gain an understanding of their long term performance with real wastewaters. Most of the research in MECs does not use real, or even complex artificial wastewaters, and most are run over a relatively short period of time. If this research is to translate into application, this relies on two key assumptions:

1. Real wastewaters containing mixture of simple and complex organic molecules will behave in the same way as acetate, a simple readily digestible molecule most frequently used in BES research. We know this not to be the case with anaerobic digestion (Rittmann, 2001).
2. A system that works at a particular efficiency for a short period of time will do for a long period of time. This is again unlikely as even with the clean technology of chemical fuel cells, long term durability tests have lasted around 4000 hours (166 days), although a couple of studies have extended this to 1.5 and even 3 years (Schmittinger and Vahidi, 2008). Failure is associated with blocked membranes, electrode deterioration and many other factors that may increase overpotentials. Biological systems have the added complexity of the behaviour of microorganisms.

Failure in laboratory batch fed wastewater reactors has been observed many times during preliminary laboratory testing. The aim of this research is to determine if wastewater fed MEC laboratory reactors are capable of operating over the same time periods as acetate fed reactors, and, if this is not the case, to identify the reasons why.

5.2. Method

5.2.1. *Reactor design and set up*

Double chamber MEC reactors (78 mL each chamber) were used which were of a tubular design, internal diameter of 40mm, length 60mm. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5cm^2, the cathode a 2.5cm^2 platinum coated titanium mesh cathode with a surface area 8.13cm^2 (tishop.com), in 1M pH 7 phosphate buffer within the cathode chamber. The membrane between the reactor chambers was Nafion 117, with an area of 12.6cm^2. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 1 Ω resistor, 0.7 V supplied using a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong), and a multimeter to measure the voltage (Pico ADC-16), logged every 30 minutes onto a computer.

All reactors were cleaned and sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK). The cathode media was 50 mM phosphate buffer, which was sparged with 99.99% pure N_2 for 10 minutes prior to being put into the reactors. The acetate based anode media used was that of Call and Logan (Call and Logan, 2008), during the tests where this was supplemented with protein, Aspargine was added to give an equivalent level of nitrogen to that measured in the real wastewater. The wastewater used was raw influent wastewater (post screens prior to primary sedimentation) from Cramlington wastewater treatment plant. The anode media was sparged for 10 minutes with N_2 prior to use. All reactors were initially acclimatised in MFC mode as per the method used in other studies (Call and Logan, 2008, Cheng and Logan, 2007a, Hu et al., 2008, Wagner et al., 2009), inoculated with 25 ml of raw wastewater and fed acetate media.

The gas produced by the cathode side was captured via a liquid displacement method in a 12 ml glass tube with a septa fitted to the top for sampling. The volume of this gas was measured by drawing it into a 5 ml gas tight syringe (SGE Analytical Science,

Australia). The anode gas was captured in an inverted 10 ml syringe placed into the top of the reactor and filled with the N_2 gas.

5.2.2. *Analytical procedures*

The following analysis was conducted in duplicate for both the effluent and influent of the cathode and anode liquids of each batch run. The chemical oxygen demand (COD) using standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) kit tubes. Volatile fatty acids (VFA's) were measured using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. The anion content using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The pH was measured using a pH probe (Jenway 3310, U.K.) and conductivity using an EC 300 probe (VWR Ltd, UK). The anode and cathode potential was measured using Ag/AgCl reference electrodes (BASI, U.K.) during each batch.

Hydrogen gas was measured on a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using triplicate injections of each sample, set against a three point calibration run once at the start of the measuring period and once at the end using standard calibration gases (Scientific and Technical Gases, U.K.). These gas measurements were verified using a Trace Ultra GC TCD with a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

GC-MS analysis of gaseous hydrocarbons, including halomethanes, was performed on a Agilent 7890A GC in split mode; injector at (280°C), linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software in full scan mode (10-150 amu/sec). A standard containing 100 ppm of three chloromethanes was injected (100ul headspace) followed by the reactor headspace samples (100ul) every 2 minutes. Separation was

performed on an Agilent fused silica capillary column (60m x 0.25mm i.d) coated with 0.25um dimethyl poly-siloxane (HP-5) phase. The GC remained at 30°C temperature for 90 minutes with Helium as the carrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 20 mls/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library if greater than 90% fit.

5.2.3. *Microbial analysis*

An assessment of the level of microbial activity occurring in the reactors was needed to give an understanding if failure was caused by a reduction or complete elimination of microbial activity, or conversely a competitive but non complementary microbial process. Methods involving the extraction and quantification of DNA from the anode biofilm were not suitable for this purpose as this would capture both the alive and active DNA and that DNA remaining on the biofilm from bacteria which were dead or inactive. Ribonucleic acid (RNA) is used within cells to convert DNA i.e. the genetic code into working proteins (Rittmann, 2001); it can therefore be used as a proxy for the amount of biological activity occurring in the cell (Milner et al., 2008, Low et al., 2000). As RNA is so susceptible to contamination and degradation, the simple and relatively quick approach of measuring the amount of nucleic acid extracted on a Nanodrop, and then comparing this directly to the amount of DNA extracted at the same time, would give the most reliable quantitative results.

Duplicate samples of anode material were taken for RNA and DNA extraction, from duplicate reactors sacrificed whilst working, and duplicate reactors after failure. The following procedure was carried out as quickly as possible inside a microbiological cabinet, to prevent the loss of RNA which readily breaks down if contaminated with RNases. All working areas and equipment was cleaned thoroughly with ethanol followed by RNase AWAY (Invitrogen Life Sciences, U.K.), including the anode cutting equipment which had also been washed with detergent and then heated to 240 ^{o}C for 4 hours in a furnace, prior to use. Each reactor at the point of sampling was taken into the microbiological cabinet maintaining the electrical circuit. The reactor was quickly dismantled and using a coring device duplicate 4mm diameter sections of the anode were cut and placed into a sterile RNase free 2 ml eppendorf, containing 1 ml of TRIzol Reagent (Invitrogen Invitrogen Life Sciences, U.K.), the sample was vortexed for 5 seconds to ensure complete submersion in the reagent, and then the samples frozen

at -80 ^{o}C. Duplicate cores were taken in the same way afterwards for DNA extraction and stored in 50:50 ethanol and phosphate buffer at -20 ^{o}C.

Extraction and clean-up of the RNA sample was then completed using a RNeasy Mini Kit (QIAGEN, Germany) as per the manufacturer's instructions. Once cleaned the samples were frozen at -20 ^{o}C. The DNA was extracted using a QBiogene FastDNA spin kit for soil (MP Biomedicals, U.K.) and also frozen in two samples at -20 ^{o}C. The quantity of nucleic acid present was then measured in duplicate on a Nanodrop Nanodrop 2000 (Thermo scientific, USA). The ratio of DNA to RNA could then be calculated for each sample.

5.2.4. *Experimental procedure*

Failure had been observed several times in these bench scale reactors used as MEC's when fed with wastewater. The purpose of these experiments was to determine if this failure was statistically significant, and if so to try and identify the particular cause. In total 12 wastewater fed reactors and 10 acetate fed reactors were used in this study, the materials and architecture of all the reactors were the same, and the same operating procedures observed throughout. The work was conducted at laboratory room temperatures of between 20-25 ^{o}C.

Initially 8 reactors were run, 4 of fed with acetate media and 4 with real wastewater. After each batch of 3-4 days the effluent was analysed for COD, VFA's, anions, pH and conductivity and the gas measured, the reactors were then refilled with N_2 sparged media to the anode and phosphate buffer to the cathode. Once having completed two batch runs producing gas, 2 reactors of each feed were sacrificed and the RNA and DNA were sampled, the remaining reactors were run and sampled as described until gas production ceased, or in the case of the acetate ones until they were stopped at 130 days.

A further experiment was conducted using 4 wastewater fed reactors to eliminate the possibility that a drop in pH in the wastewater fed reactors was causing failure. Duplicate reactors were run containing wastewater, and the same wastewater buffered to pH 7 using 50 mM phosphate buffer. All reactors were run in batch mode and samples as described above until gas production ceased. Examination as to whether the biofilm was damaged/killed during failure was gained by switching the failed MECs to MFC

mode (increased resistance and no external load), and refilling with UV sterilised wastewater (see Appendix V for details of this method).

Due to the observed drop in Cl^- ions prior to the point of failure, it was hypothesised that locally high levels of NH_4^+ at the anode, caused by the degradation of proteins present in the wastewater could be reacting with the chloride ions to form chloramines, which would then kill off the biofilm resulting in failure of the cell. This hypothesis was tested running 4 acetate fed reactors, by supplementing duplicate reactors with protein Aspargine at levels comparable to the wastewater levels as detected through the use of the TKN Standard Method 4500-Norg (APHA, 1998), comparing these to duplicate control reactors with no protein. Again sampling was carried out as above, in addition the effluent of the reactors was analysed for residual chlorine using the DPD test, Standard Methods 4500-Cl D, (APHA, 1998).

A further hypothesis to account for failure and the drop in chlorine was that the chlorination of organics, especially methane could be occurring in the reactors due to the potential of the anode. Under standard conditions, at pH 7 the required potential for chlorination of methane at a Cl concentration of 1 mM is 0.44 V, when considering that the reactors may have a pH slightly deviant from 7, and that the partial pressures of the methane and chloromethane produced would not be equivalent, it is conceivable that the anode potential needed for this reaction could be occurring in the reactors, producing chloromethanes and therefore removing the hydrogen ions from the system and eliminating H_2 production. Again 4 wastewater reactors were run in batch mode with the same analysis as described above, in addition both the anode and cathode gasses were captured and analysed for methane, hydrogen and chloromethane using the instruments and methods stated above. Duplicate reactors fed with acetate were run at the same time and subject to the same analysis. After failure reactors were again switched to MFC mode and the anode gas continued to be sampled.

5.2.5. *Calculations*

The reactor performance was evaluated in terms of the volume of hydrogen produced, and also the coulombic efficiency and electrical energy recovery. The definition of these two efficiencies can be found in section 6.2.5.

5.2.6. *Statistics*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

5.3. Results

5.3.1. *Time taken until failure*

The run time of the reactors is shown in Figure 5-2 as the amount of hydrogen produced at the end of each batch, the reactors terminated at 7 days for RNA sampling are not shown. It is seen that the Acetate fed reactors run for a longer period of time, including those supplemented with protein and produce more hydrogen than the wastewater reactors. The buffered wastewater reactors initially perform well, but then stop producing hydrogen after a short time period.

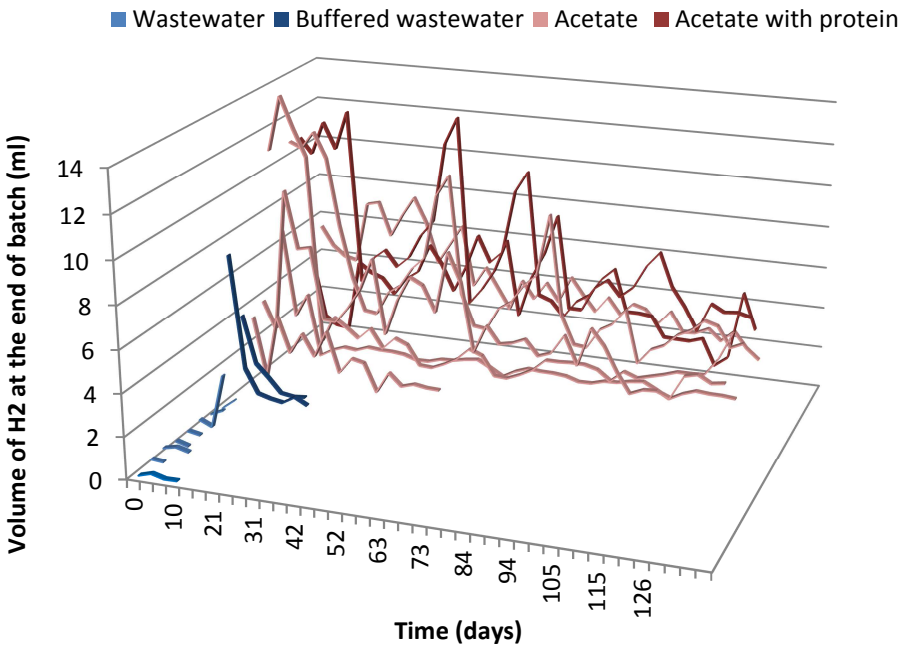

Figure 5-2 Graphic showing the working period of all reactors as indicated by the length on the line along the time axis, the volume of H_2 produced at the end of each batch is given on the y axis as an indication of reactor performance which is seen to be variable, where the line is discontinued this illustrates zero H_2 production and the reactor is deemed to have failed

All 10 of the reactors fed on wastewater failed within 7-17 days of operation, failure was determined by no measureable gas production at the cathode. Of the 8 acetate fed reactors one failed at 56 days, but the others remained functioning until the experiment was terminated after 130 days. With 130 days used as the minimum run time for the acetate fed reactors, the difference in time to failure is significant ($p=0.000$, two sample t-test) as shown graphically in Figure 5-2.

5.3.2. *Reactor performance*

The average performance data collected over the duration of different experiments is shown in Table 5-1. The acetate fed cells have a greater coulombic efficiency and electrical energy recovery. The COD removal is reasonably similar for all substrates, but higher for the buffered wastewater, although this does not translate into improved coulombic efficiency or energy recovery. In all cases there is a large degree of variation, as is seen by the standard deviations. This is also seen through the hydrogen production data in Figure 5-2, which is higher for the acetate fed reactors, but does deteriorate throughout the test period.

Table 5-1 Summary of reactor performance using three different parameters other than H_2 production for the experiments using different substrates, values are the average values of all the reactors run on the given substrate

	COD removal	Coulombic Efficiency	Electrical Energy Recovery
Wastewater	23.2% ± 12.2	7.5% ± 3.9	15.7% ± 20.1
Buffered wastewater	43.8% ± 7.8	3.7% ± 1.7	13.5% ± 16.6
Acetate	28.6% ± 11.5	10.9% ± 2.0	33.0% ± 15.1
Acetate with protein	32.3% ± 13.4	10.4% ± 3.6	35.1% ± 22.9

Values represent average of all the batch experiment run on the given substrates where hydrogen was produced, ± one standard deviation.

There is a reduced performance between the acetate fed reactors as compared to the wastewater ones of around 50 % if energy recovery is considered.

5.3.3. *Biological processes*

The average RNA: DNA ratio of the duplicate samples show that there is significant difference between the working and failed reactors at the 90% confidence interval ($p=0.068$ two-sampled t-test). This difference is more pronounced with the wastewater fed reactors, where the average ratio value for the working reactors is 11.5 compared to the failed reactors 3.9. The acetate working reactors have an average a ratio of 6.1, with the single failed cell being 4.2.

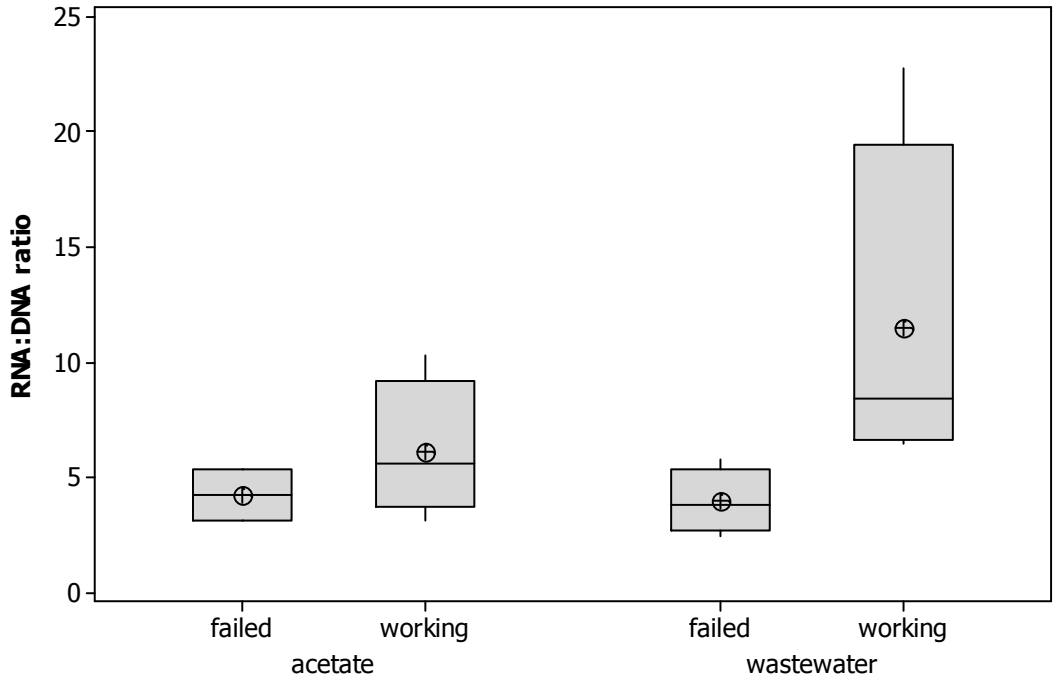

Figure 5-3 Box plot of the RNA: DNA ratios of failed and working reactors fed with both acetate and wastewater, the data represents a summary of the duplicate samples taken from duplicate reactors (i.e. four samples in total) with the central line representing the median and the mean given by the circle with cross

5.3.4. *Low pH*

In the wastewater fed reactors, which contained no additional buffering, it was observed that at around the point of failure there was a decline in the pH of the anode effluent from a starting value 6-6.5 to around 5.5. The acetate fed reactors, (the nutrient media containing 50mM pH 7 phosphate buffer) did not show any significant fall in pH during the full time period over which their function was monitored.

With the additional duplicate reactors fed on wastewater and buffered wastewater there was the same observed drop in pH with the non-buffered reactors. The buffered reactors kept a constant pH and initially performed better but then also failed within 17 days of operation. No significant difference in the run time between the buffered and non-buffered reactors ($p=0.306$, two sample t-test).

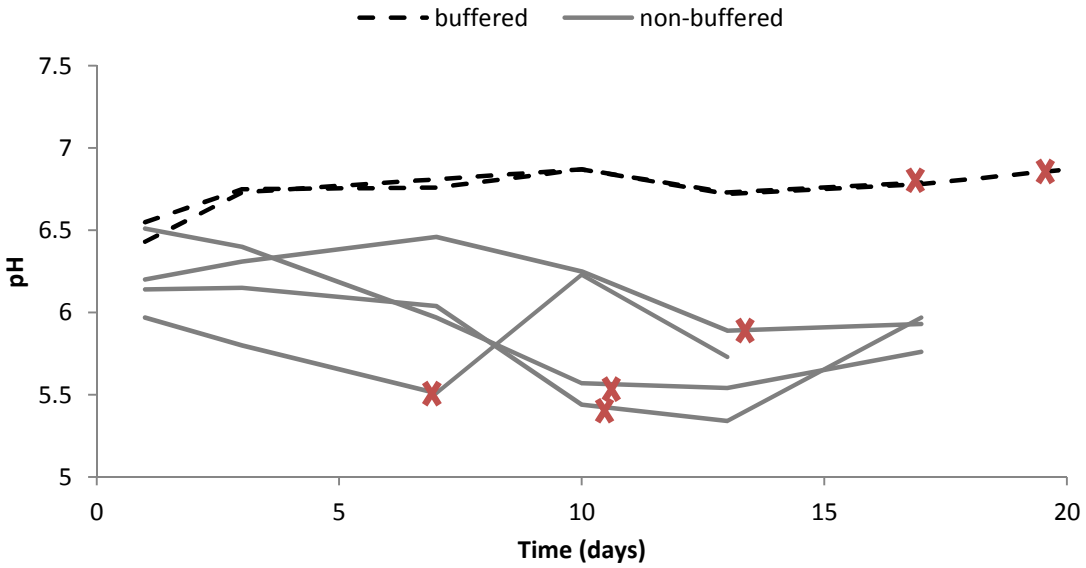

Figure 5-4 Measured pH of the wastewater reactor liquid during the course of the batch experiments, the point of failure is denoted by the red cross where gas production ceased

5.3.5. *Toxic build up within the reactors*

The full anion analysis of the cell effluent showed that there was a fall in chloride ions prior to failure of the wastewater reactors. Both the acetate media and the wastewater contained approximately 250-300 mg/L of chloride. During the course of each batch run with the acetate fed reactors, approximately 50 mg/L of the chloride would be taken up in the reactor, this remained relatively constant throughout the full time period the acetate reactors were operated for. However in the wastewater reactors, when working and producing hydrogen, the chloride removal in the cell was observed to be virtually complete prior to the reactor failure, i.e. 250-300 mg/L of chloride ions were being removed. The levels of chloride in the cathode compartment of these reactors remained the same as the original influent. After failure of the reactors when no hydrogen was produced, this chloride removal stopped. The only wastewater reactors that this drop was not observed in were the duplicate buffered wastewater reactors, here chloride removal remained constant at around 50-100 mg/L during each batch, the reactors did however also fail.

In the acetate reactors supplemented with protein the chloride removal remained roughly constant throughout the experiment at between 50-100 mg/L, and the reactors did not fail. No chloramines could be detected in the effluent of these reactors, disproving the hypothesis of chloramine formation. The performance of the protein

supplemented reactors in terms of electrical energy recovery was not significantly different to the non-supplemented ones (p=0.376, two sample t-test).

Further evidence that a toxic chlorine based product was not being formed was gained using four failed wastewater reactors, duplicate reactors were refilled with UV sterilised wastewater non sterile wastewater, put into MFC mode, i.e. increased resistance and no external load. With all four reactors biological activity started within 1 hour, and reached a level of current production as would be expected of a fully acclimatised MFC cell using the same cell materials. The electrogenic biofilm was capable of functioning. After one batch in MFC mode, the reactors were then all returned to MEC mode, where no gas was produced and the failed status continued. In MFC mode, the chloride removal was relatively constant again at around 50 mg/L.

5.3.6. *Formation of halogenated organics*

Analysis of the headspace gas for 4 wastewater fed reactors and 2 acetate fed did not show detectable levels of halogenated organics, levels were below 0.01% of the 10 ml headspace. This was the case for wastewater fed reactors before, during and after failure and for acetate fed reactors. The same observed drop in chlorides was seen in these reactors.

5.3.7. *Other factors*

The analysis of VFA's in the effluent of the reactors showed that in all cases for both acetate and wastewater there was some acetate remaining at the end of each batch. There was no acetate in the influent wastewater, but always a small amount 20-40 mg/L in the effluent of these reactors, this did not alter once the reactors had failed.

The conductivity for the wastewater was around 1.8 mS, the buffered wastewater was 6.3 mS, and the acetate media was 5.9 mS. The conductivity of the reactor effluent was on average 1.6 mS for the wastewater fed cells both before and after failure even when a drop in chloride ions was recorded, the average for the buffered wastewater cell effluent was 5.5 mS and again did not change after failure, the acetate cells also showed a slight drop in conductivity of the effluent to 5.2 mS.

The production of methane at the anode of the reactors was on average 0.002 ml for the wastewater reactors when working, after failure this increased slightly to 0.029 ml. The methane production remained relatively constant throughout the course of the

experiment and the slight rise after failure is not likely to represent a competitive biological process which is the cause of cell failure, as the average methane production in the acetate fed cells was always higher at 0.072 ml per batch, and also the converted MFC cells that functioned well, also produced on average 0.035 ml per batch.

The materials used in these reactors that could become degraded during use, i.e. the cathode and membrane, could be directly and successfully re-used in a new cell, the failure was not due to cathode degradation or membrane clogging. In addition, by increasing the applied voltage of the reactors from 0.7 V to 1.0 V immediately after failure, thus combating any increased overpotentials that could have built up during the short operation period, the reactors could not be revived and did not produce hydrogen. Failure was not therefore caused by the simple the deterioration of the cell components.

5.4. Discussion

Small laboratory scale wastewater fed reactors fail after a short period of time whereas acetate fed reactors do not. This is significant. The cause of this failure could not be identified during the course of this study. Relatively ‘simple’ explanations such as degradation of electrodes or membranes, a drop in conductivity, or lack of available VFA’s have been ruled out as possible causes of failure.

A further hypothesis that failure of the reactors is caused by a reduced or eliminated level of electrogenic activity in the reactors was also seen not to be the case. If true this hypothesis would result in the reduced DNA:RNA ratio observed and low current production. However once failure had occurred the reactors could be instantly ‘revived’ by switching them into MFC mode. The electrogenic bacteria were therefore present on the electrode and were capable of donating electrons.

The hypothesis that there is a competitive biological process occurring such as methanogenesis, as suggested in other studies (Cusick et al., 2011), has been shown not to be the case. The RNA to DNA ratio indicates a reduced biological activity in the failed wastewater cells, suggesting that the biofilm is less able to function and metabolise after failure. It is not likely that a non-complementary competing biological activity is taking over the reactor and eliminating the MEC process. It can be seen that there is greater activity in the wastewater reactors than the acetate reactors, this might be an indication of the greater and more multi-layered metabolism that has to occur in

these reactors when fed complex substrates. It is also observed that the failed acetate reactor did not differ significantly to the working ones, suggesting the reason for failure here was different to that for the wastewater reactors. Additionally the levels of methane generated in the wastewater reactors after was less than in the working acetate reactors. A competitive process such as methanogenesis is therefore unlikely to be the cause.

The hypothesis that a low pH was causing failure, either through altering the electrochemistry or affecting biological function is shown not to be correct. The simple experiment adding buffer to the wastewater also resulted in failure despite initial improvement in reactor performance, here the drop in chloride was not observed. The slightly lowered pH is likely to have a detrimental effect on the cell though. The pH measurement taken is of the whole of the liquid in the reactor, in reality the pH near the anode may be greater. Such a pH will impact on the microorganisms present and the electrochemical reactions within the cell, as pH is a logarithmic function of the concentration of H+ ions, then even a small change in this value has a large impact on the overall thermodynamic balance of the system as is calculated via the Nernst equation. Torres et al (2008) found that an increase in phosphate buffer in the anode media lead to a thicker biofilm and greater current generation in a microbial fuel cell due to the increased diffusion of H^+ out of the biofilm layer, thus making it more accessible to transport to the cathode. Although pH could be limiting the performance of non-buffered reactor it is not the cause of failure.

The formation of halomethanes such as chloromethane could potentially occur at the potentials within these reactors account for the loss of chloride and would cause failure as these compounds are toxic. This would fit the pattern of failure exhibited in the reactors as it would take some time for the levels of methane to build up which could then be converted to the halomethanes, this would ‘use up’ the H^+ ions in the anode section and H_2 would cease to be produced at the cathode. However no chloromethanes could be detected in the headspace gas of these reactors, (below 0.01%) either before or after failure, in fact no halogenated organics could be detected. Additionally the acetate fed cells did not fail when supplemented with protein, and most importantly the exoelectrogenic biofilm is able to work as an MFC after failure so has not been killed. It could be possible that the negative chlorine ions were simply temporarily attracted to the positive anode during the operation of the fuel cell, and therefore not measured in the bulk liquid of the cell. This would account for the observed ‘disappearance’ of the

chloride ions, but is not likely to affect the performance of the cell. The range of analysis carried out indicates that failure is not caused by a chlorine effect; the observed chlorine drop is simply co-incidental to the failure.

The problem of failure needs to be resolved. If MECs are to be a useable technology they need to function with real wastewater. Studying these systems when they are prone to sudden and rapid failure is difficult, therefore identifying the reasons for failure, solving them, and increasing efficiency becomes very challenging. This difficulty leads to acetate being used in most research as this does allow greater scope for experimentation. However it is clear that the processes operating in a reactor fed with real wastewater are different to those occurring in a reactor fed with acetate. The acetate research will not directly inform us of performance with wastewater.

The failure in wastewater fed, laboratory scale, batch fed reactors has been proved, but the reason not identified. Conversely, as part of this research, a larger scale MEC run in continuous mode at a wastewater treatment site fed on raw wastewater has worked producing almost pure hydrogen for a period of over 3 months, (see chapter 6). It is likely that something is occurring within the small batch reactors to prevent either the production of hydrogen ions at the anode, the transfer of these ions, or the hydrogen evolution reaction at the cathode. It may be the case that at this small scale and fed with batch mode that the system and in particular the microbial community involved is fragile and unable to adapt to change, and therefore a build-up of something at an undetectable level has catastrophic consequences. Further work is still needed to identify the cause of this failure, and therefore be able to take steps to resolve it. This can only be done by using real wastewater rather than simple artificial media. The long term performance of wastewater fed MECs is a research gap that must be filled.

Chapter 6. Production of hydrogen from domestic wastewater in a pilot scale microbial electrolysis cell

Addressing the need to recover energy from the treatment of wastewater the first working pilot scale demonstration of a wastewater fed microbial electrolysis cell is presented. A 120 litre (L) microbial electrolysis cell (MEC) was operated on a site in Northern England, using raw domestic wastewater to produce virtually pure hydrogen gas for a period of over 3 months. The volumetric loading rate was 0.14 kgCOD/m^3/day, just below the typical loading rates for activated sludge of 0.2-2 kgCOD/m^3/day, at an energetic cost of 2.3 kJ/gCOD, below the values for activated sludge 2.5-7.2 kJ/gCOD. The reactor produced an equivalent of 0.015 L H_2/L/day, and recovered around 70% of the electrical energy input, with a coulombic efficiency of 55-60%. Although the reactor did not reach the breakeven energy recovery of 100%, this value appears well within reach with improved hydrogen capture, and reactor design. Importantly for the first time a 'proof of concept' has been made, with a technology that is capable of energy capture using low strength domestic wastewaters at ambient temperatures.

6.1. Introduction

In an era of increasing energy costs and environmental awareness, wastewater treatment industries need to look at alternative treatment options to reduce their energy bills. It has been estimated that domestic wastewater alone may contain 7.6 kJ/L of energy, while stronger industrial wastewaters contain substantially more (Heidrich et al., 2011). There is an increasingly urgent need to recover some of this energy, or at the very least not expend additional energy on treatment; the activated sludge process uses 2.5-7.2 kJ/gCOD (Pant et al., 2011). Energy recovery could be achieved through anaerobic digestion to methane gas or microbial fuel cell technology directly to electricity; however life cycle assessment has shown that the production of a higher value product through the suite of bioelectrochemical systems (BES) may be the most viable solution (Foley et al., 2010). One such technology is the production of hydrogen in a microbial electrolysis cell (MEC) (Rozendal et al., 2006).

Since the MEC process was first reported (Rozendal et al., 2006, Liu et al., 2005b) MECs have emerged as a potential technology option for a new generation of wastewater treatment systems (Rozendal et al., 2008a). In an MEC bacteria use the

energy stored in the organic compounds of wastewater to metabolise and grow, donating electrons to an electrode (Rozendal et al., 2006). The electrons then travel in a circuit producing current and therefore electrical power; in an MEC these electrons are consumed at the cathode along with a supplement of electrical power. The H^+ ions also created by the breakdown of organics at the anode travel across the microbial fuel cell membrane to the cathode. Here they can combine to form H_2, however this process is endothermic requiring energy, so a supplement of electrical energy is added to the system to allow it to take place (Liu et al., 2005b).

Fuel cell technologies may offer a sustainable future for wastewater treatment, although there are still many hurdles to overcome. Progress is being made with new reactor design (Call and Logan, 2008, Rozendal et al., 2008b), improved materials (Cheng et al., 2006a, Cheng and Logan, 2008), greater understanding of the mechanisms involved (Aelterman et al., 2008, Clauwaert et al., 2008), and even improved understanding of the microbes that are at work in these systems (Holmes et al., 2004, Kim et al., 2004, Lovley, 2008, Rabaey et al., 2004). Most of this research is performed at laboratory scale, using simple substrates, often at a controlled warm temperature. Many problems have been overcome, such as validation of using multi electrode systems (Rader and Logan, 2010) and finding a low cost alternative to the platinum cathode (Zhang et al., 2010). Although of great value in improving our understanding of MEC's, these studies do not tell us about the challenges or even benefits of running such systems at a larger scale with real wastewaters in temperate climates. There is a need to demonstrate that these systems can work at a larger scale and under realistic conditions, elevating the technology from a laboratory curiosity into a practical solution to an industrial problem.

A pioneering study by Cusick et al (2011) published on the largest MEC reactor to date, a 1000 L pilot scale reactor at a winery in California. The reactor proved slow to start up with pH and temperature control being problematic. When these issues were corrected by heating to 31 ^{o}C and the addition of buffer and acetic acid, the reactor did improve in performance. The energy produced during the operation exceeded the input energy (heating not included), but this was primarily due to methane production (86%) with only trace amounts of hydrogen. Methane production was attributed to the reactor being membraneless allowing hydrogen produced at the cathode to be directly consumed by hydrogenotrophic methanogens within the reactor. The reactor performance tailed off at around 90 days, when the heating unit broke (Cusick et al., 2011). The study has

provided valuable insights into the operation of MECs: (i) the membraneless systems that work well at laboratory scale and when fed in batch mode may not be so good at larger scale and under continuous feed, and (ii) inoculation and start-up are important parameters.

Addressing the issue of a membrane is critical to reactor performance. Most laboratory scale membrane systems use Nafion 117 (Logan et al., 2006), an expensive and delicate proton exchange membrane (Logan et al., 2006); this would be both impractical and costly on a large scale. Also the high efficiencies published: 406% electrical energy recovery (the amount of electrical energy put in that is recovered, this can be higher that 100% as there is also substrate energy within the system) and 86% total energy efficiency (the amount of substrate and electrical energy recovered) (Call and Logan, 2008) are from membrane-less systems. The lack of membrane greatly reduces the resistance in the cell, improving the transmission of protons to the cathode. Membrane systems have lower efficiencies: 169% electrical energy recovery and 53% overall energy efficiency has been reported (Rozendal et al., 2006). These efficiencies are likely to decrease further with time as the membrane becomes fouled.

The issues of inoculation and start-up are poorly understood (Oh et al., 2010) Although the use of acetate is likely to reduce the acclimatisation period (Cusick et al., 2011). However the biological community needed for the degradation of complex substrates is thought to be different to that needed for acetate (Kiely et al., 2011c). A community of acetate degraders able to work at 30 ^{o}C is not likely to be the community needed to degrade wastewater at ambient UK temperatures. There is evidence in the literature that microbes exist that are able to digest wastewater (Ditzig et al., 2007) and operate at low temperatures (Lu et al., 2011). Like anaerobic digestion, however, it may well be that a long period of acclimatisation is needed and unavoidable to achieve a stable community (Rittmann, 2001).

If these start-up issues can be resolved, then the reactor in theory will function, however it would also need to reach a neutral or positive energy balance, i.e. recovering all the electrical energy input plus a substantial fraction of the substrate energy input.

To test whether these systems have a chance of achieving these goals under realistic conditions, a pilot scale 120 L reactor was placed on a wastewater treatment site in

North East England. This site takes in primarily domestic wastewater with an average Total COD of 450 mg/L. The reactor was built using low cost alternatives to the standard lab materials used for the cathode and membrane. The reactor was not heated, held inside a large unheated building, and run throughout a UK spring and summer (5-20 ^{o}C minimum and maximum temperatures) and is still in operation at the time of writing this paper. These operating conditions are likely therefore to represent close to a worst case scenario i.e. low concentration feed; non optimal components; no heating; and no additional supplement of acetate or buffering capacity after the initial acclimatisation period.

Working closely with partners at Northumbrian Water Ltd. the aim of this study was to establish reactor operation and to determine if a neutral or positive energy recovery is achievable. From that data we can evaluate if MEC technology is likely to be a viable treatment option for the future.

6.2. Methods

6.2.1. *Field Site*

The pilot scale reactor was set up and run at Howdon wastewater treatment site, situated near the city of Newcastle Upon-Tyne in the North East of England (54^{o}58'N, 01^{o}36'W). An average of 246500 m^{3} of domestic wastewater is treated daily, using 96 MWh; the activated sludge process uses around 60% of this. The wastewater used in the MEC was taken from the grit channels after primary screening, but before settling.

6.2.2. *MEC reactor*

The reactor was based on a cassette style design, with six identical cassettes being placed into a rectangular reactor with a total working volume of 120 L. The tank has a Perspex plate fitted over the liquid layer giving a small head room to the anode compartment of 2.2 L. Each of the cathode gas tubes from the cassettes projected above this Perspex sheet. The cassettes were set along alternate sides of the reactor to allow s-shaped flow, and once in place gave a final anode volume of 88 L.

Each cassette was constructed using 10 mm thick plastic sheeting and consisted of an internal cathode section 0.280 m by 0.200 m by 0.048 m deep, of a volume 2.6 L. The cathode material was stainless steel wire wool grade 1 (Merlin, UK), 20g was used in each cathode, giving a projected cathode surface area for each electrode of 0.056 m^{2}. A

0.8 m length of stainless steel wire was wound several times into the wire wool to make a firm electrical connection, and then to the outside of the cell. Each cathode electrical assembly had an internal resistance from the extremities of the wire wool to the end of the exposed wire of less than 2.75 Ω. The cathode was separated using a membrane wrapped around a plastic frame inserted into the electrode assembly on both sides. The membrane used was RhinoHide® (Entek Ltd, UK), a durable low cost microporous membrane traditionally used as a battery separator. The anode material was a sheet of carbon felt (Olmec Advanced Materials Ltd, UK), 0.2 m wide by 0.3m high and 10 mm thick. This was sandwiched between two sheets of stainless steel mesh acting a current collector. The anode assemblies were also connected by a 0.8 m length of stainless steel wire fed through the centre of the felt material, each electrode having an internal resistance less than 3.4 Ω.

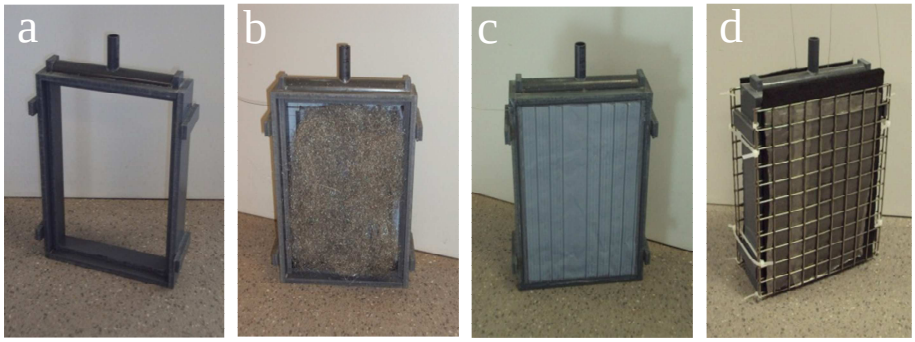

Figure 6-1 Photographs of the electrode assembly unit – a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane, d) anode with wire mesh current collector

The gas production from the anode compartment was captured from the ports in the Perspex lid, using 3mm ID PVC tubing (VWR Jencons, UK). The cathode gas was initially captured using 4mm annealed copper GC tubing connected to each cathode compartment using copper compression fittings, (Hamilton Gas Products Ltd, Northern Ireland), due to rapid corrosion this was later replaced with 3mm ID PVC tubing (VWR, UK). Both pipelines contained a gas sampling port.

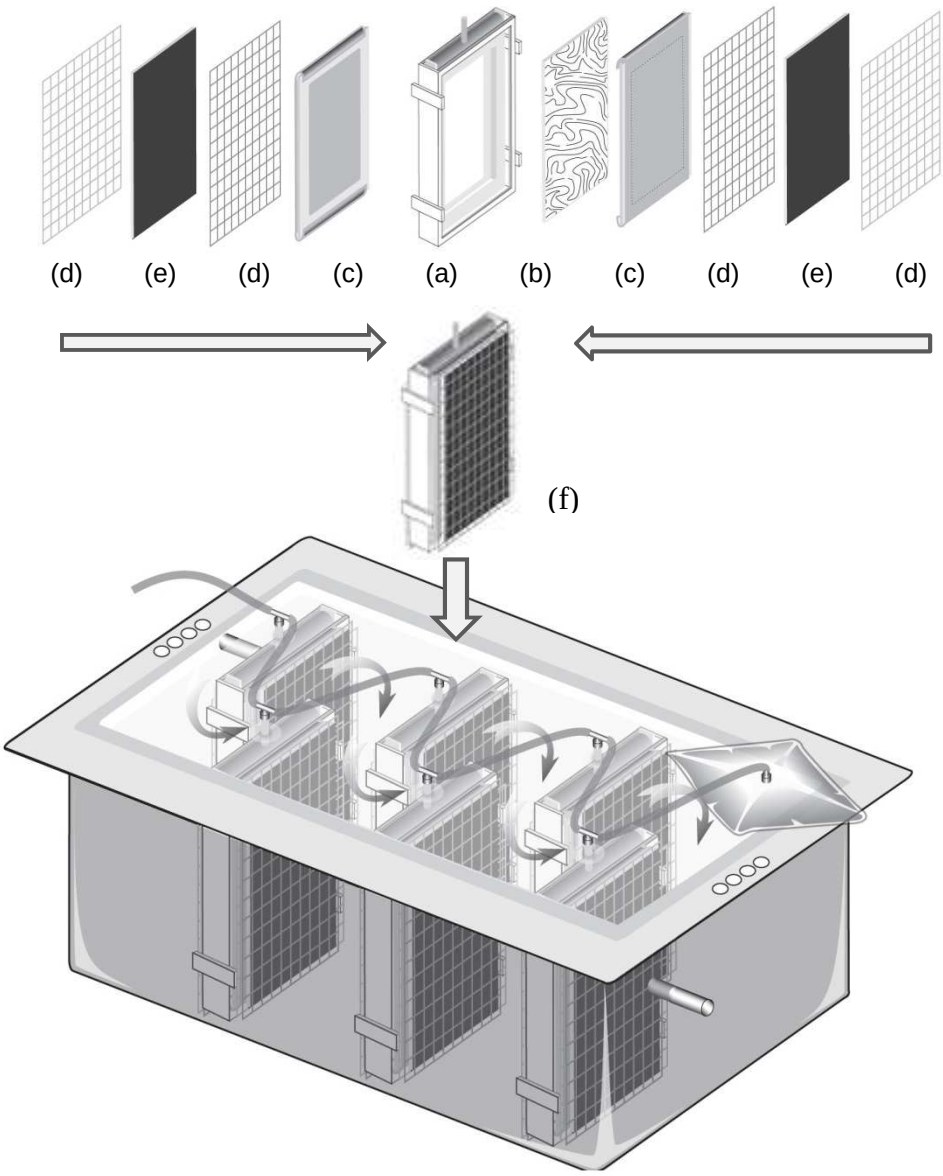

Figure 6-2 Schematic diagram of the reactor module components, a) PVC outer frame, b) wire wool cathode, c) Rhinohide membrane fixed around a PVC frame, d) stainless steel wire mesh, e) anode with wire mesh current collector. These component fit together to form a single module (f), six of these go into the reactor vessel where wastewater flows around them. Gas is collected through tubing into a gas bag

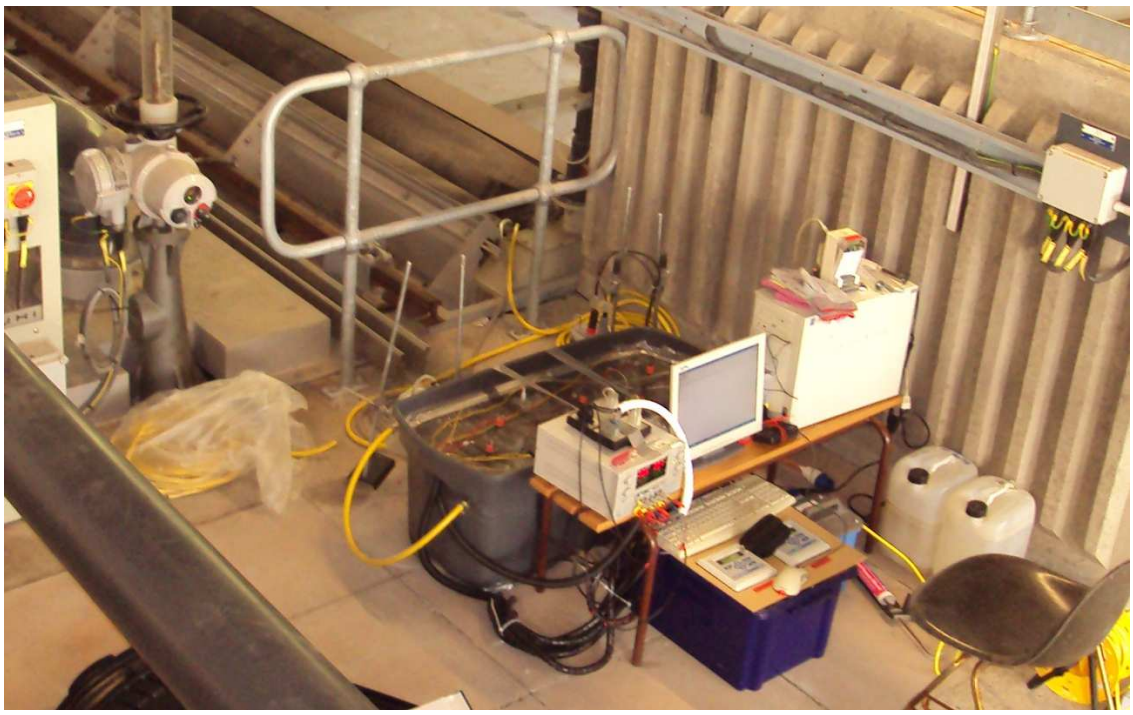

Figure 6-3 Photograph of the reactor in situ at Howden wastewater treatment site the grit lane where the influent was drawn from is seen in the top left hand corner of the picture

The reactor was situated on site in a large unheated building housing the grit channels, wastewater was pumped from the grit channels into a preliminary storage tank, providing some primary settling. During operation a peristaltic pump (Watson Marlow 520S, UK) was used to pump water into the storage tank, where it could then flow into and through the reactor, and back out to the grit channels via a smaller sampling tank at the end. These tanks were used for sampling and monitoring of the influent and effluent.

6.2.3. *Analytical procedures*

Power was provided to the electrodes using a PSM 2/2A power supply (Caltek Industrial Ltd, Hong Kong), the voltage of each cassette was monitored across a 0.1 Ω Multicomp Resistor (Farnell Ltd, UK) using a Pico AC-16 Data Logger (Pico Technology, UK), and recorded on a computer every 30 minutes.

In both the influent settling tank and the effluent tank the dissolved oxygen (DO) and pH were measured using pH and DO submersion probes (Broadley James Corporation, USA) connected to a pH DO transmitter (Model 30, Broadley James Corporation, USA), feeding an electrical output to a Pico EL 037 Converter and Pico EL 005 Enviromon Data Logger (Pico Technology, UK); these data were recorded onto the

computer every 30 minutes. Temperature was logged using 3 EL-USB-TC Thermocouple data logger (Lascar Electronics, UK) placed in the settling and effluent tanks and one placed in the reactor itself.

The gas pipelines were connected to optical gas bubble counters (made 'in-house' at Newcastle University), giving a measurement of gas volume. The operation of these counters failed after several weeks of operation. They were replaced with 1 L and then 5 L Tedlar gas bags (Sigma Aldrich, U.K.); the volume of gas was then measured by removal from the bags initially using a 100ml borosilicate gas tight syringe, and then using a larger 1 L glass tight syringe (both SGE Analytical Science, Australia). The sampling ports on each pipeline were initially used to take a sample of cathode gas 3 times a week, into a Labco Evacuated Exetainer (Labco Ltd, UK). Once gas production had risen to a higher volume, 2 L of the cathode gas was dispensed from the collecting gas bag into another 5L gas bag which was taken away for analysis. Anode gas was not measured volumetrically due to leakage but was sampled directly from the anode compartment into a 3 ml exetainers for compositional analysis.

Hydrogen gas was measured using a Membrane Inlet Mass Spectrometer (MIMS, Hiden Analytical, Warrington, U.K.) using duplicate injections, set against a three point calibration. These gas measurements were verified using a Trace Ultra gas chromatograph (GC) with a thermal conduction detector (TCD) and a Restek Micropacked 2m Shincarbon column using argon as the carrier gas (Thermo Scientific, U.S.A.) with again a three point calibration, both measurements were concordant with each other. Methane produced was measured in a GC FID Methaniser, SRI 8610C with hydrogen as the carrier gas (SRI Instruments, U.S.A.) using the same calibration approach described above. All measurements for anode and cathode gas were completed using a 100 µl gas tight syringe (SGE Analytical Science, Australia).

To ensure accuracy calibration standards used for the gas measurements were injected into a Labco evacuated exetainers in the laboratory at the same time (+/- 10 minutes) as the samples taken in the field. Tests carried out previously had indicated that these containers were not completely gas tight especially for hydrogen. This procedure did not have to be carried out for the cathode gas once operation had been switched to gas bags.

Liquid samples of the influent and effluent were taken 3 times a week. The total chemical oxygen demand (COD), and soluble chemical oxygen demand (SCOD) were measured in duplicate using standard methods (APHA, 1998) (Spectroquant ® test kits, Merck & Co. Inc., USA). Volatile Fatty Acids (VFA's) were determined using an Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using a Ion Chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. The conductivity of the solution was measured using a conductivity meter, EC 300 (VWR Ltd, UK).

6.2.4. *Start up and operation*

The reactor was initially started up in batch mode, allowing all the oxygen, nitrates and sulphates within the wastewater to be consumed. Based on the lessons learnt from the previous pilot study, (Cusick et al., 2011), (Logan, B.E. personal communication),the wastewater was supplemented with acetate at a concentration of 0.5g/L. The applied voltage of 0.6 V was provided by a regulated DC power supply PSM 2/2A, (CALTEK, Hong Kong). The dosing was repeated and the reactor refilled after a 2 week period, during which time no gas production was observed.

6.2.5. *Efficiency calculations*

Four efficiency calculations are made in this study on the basis of the electrical and substrate energy used (Logan, 2008).

(i) Electrical energy recovery (η_E)- Energy recovery is the amount of electrical energy put into the reactor that is recovered as hydrogen.

The electrical energy input W_E is calculated as:

$$W_E = \sum_{1}^{n} (I\ E_{ps} \Delta t - I^2 R_{ex} \Delta t)$$

Where I is the current calculated for the circuit based on the measured voltage E and external resistor R_{ex} ($I=E/R_{ex}$), E_{ps} is the applied voltage of the power supply, this value is adjusted for the losses caused by the external resistor (I^2R_{ex}), which in reality are negligible. The time increment denoted by Δt represents the conversion of samples taken every 30 minutes into seconds. The data is summed for all 6 cells over the each batch cycle. The output of energy (W_{out}) is calculated from the measured moles of

hydrogen produced $N_{H2,}$ and the standard higher heating value of hydrogen of 285.83 kJ/mol ΔH_{H2}.

$$W_{out} = \Delta H_{H2}\, N_{H2}$$

The higher heating value is chosen over the lower heating value which takes into account the heat lost through the production of water vapour during burning. It is expected that this H_2 product would be used either as a commercial product for industry, or in a clean H_2 consuming fuel cell to create electricity, not for combustion. Methane could also be added to this value to further increase the quantity of output energy, but was not included for these same reasons.

Total Energy recovery (excluding pump requirements) can then be calculated as follows:

$$\eta_E = \frac{W_{out}}{W_E}$$

(ii) Total energy efficiency (η_{E+S}) the amount of input energy both electrical and substrate that is recovered as hydrogen.

The substrate energy (*Ws*) is calculate as

$$W_s = \Delta COD\, \Delta H_{ww/COD}$$

Where ΔCOD is the change in COD in grams, estimated as the difference in COD of the influent and effluent at the end of each batch. $\Delta H_{ww/COD}$ is the energy content per gCOD as measured on similar domestic wastewater of of 17.8 kJ/gCOD (Heidrich et al., 2011). Total energy efficiency is then calculated as:

$$\eta_{E+S} = \frac{W_{out}}{W_E + W_S}$$

(iii) Coulombic efficiency (CE) - the amount of hydrogen produced compared to the amount theoretically possible based on the current, or total charge passing through the cell.

Theoretical hydrogen production based on current (N_{CE}) is calculated as:

$$N_{CE} = \frac{\sum_1^n I\Delta t}{2F}$$

Where *I* is the current calculated from the measure voltage, Δt is the conversion of the time interval 30 minutes to 1 second to give coulombs per data sample, this is then summed over the 6 cells for the whole batch. Faradays constant (*F*) is 96485 coulombs/mol e^-, and is the moles of electrons per mole of hydrogen. Coulombic efficiency *CE* is then calculated as:

$$CE = \frac{N_{CE}}{N_{H2}}$$

(iv) Substrate efficiency - the amount of hydrogen produced compared to the amount theoretically possible based on substrate removed in the reactor.

Theoretical hydrogen production based on substrate removal *(N_S)* is calculated as:

$$N_S = 0.0625\, \Delta COD \Delta t$$

As 64 gCOD can be converted to 4 moles H_2, each g COD is equivalent to 0.0625 moles H_2. The change in COD is measured at the end of each batch, and used to calculate the total COD removed from the 88 L reactor over the duration of the sampling period based on a HRT of 1 day. Substrate efficiency is then calculated as:

$$S_E = \frac{N_S}{N_{H2}}$$

The (η_E) correlates directly to the coulombic efficiency (CE) by re-arrangement of their respective equations. It is assumed that the phrase $I^2 R_{ex} \Delta t$ in calculating W_E is negligible by comparison to the first term (this is observed to be the case in practice):

$$\eta_E = \frac{\Delta H_{H2} \times 1000}{2F \times E_{ps}}\, CE$$

This means halving the E_{ps} doubles the η_E if the CE can be maintained. An increase in CE at the same E_{ps} causes a linear increase in η_E.

6.2.6. *Statistical analysis*

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA).

6.3. Results

6.3.1. *Reactor design and resistance limitations*

The internal resistance of a BES design is critical to its performance. Resistance is mainly caused by electrode overpotential and ohmic losses in the liquid, although there may also be losses in the bacterial transfer etc. as shown in Figure 1.2. These losses impact on the amount of energy that can be gained in and MFC and the amount for energy needed in an MEC, these effects are even greater in a scaled up system where losses become proportionally more significant (Rozendal et al., 2008a). Within the cell

designed the anode and cathode, although separated by a membrane, were relatively close together, with around 1cm distance between them, this will have minimised the ohmic losses within the liquid phase (i.e. the resistance in the movement of ions from the anode to cathode) which is especially important when using real wastewaters with no artificial increase in liquid conductivity.

However the electrode resistance with this design is high, with the cathode having a resistance of 2.8Ω and each anode sheet being 3.4Ω from the extremities of the electrode to the end of the connecting wire. With a total anode surface area for the whole reactor of 0.76 m^2 and a further 0.3 m^2 of cathode, these resistances will have a large impact in reducing the efficiency of the reactor performance. With a 0.6V load, as would be desirable based on laboratory studies (Call and Logan, 2008) this anode resistance would result in an approximate maximum current of 0.2A, increasing the load to 0.9 as needed with other wastewater studies (Kiely et al., 2011a, Cusick et al., 2011) would produce a maximum of 0.3A, and the 1.1V load used would result in around 0.4 A maximum current, assuming no other losses. This would give anode current densities of 0.3, 0.4 and 0.5 A/m^2 respectively, well below the target for BES of 10 A/m^2 which would enable similar treatment rates to activated sludge (Rozendal et al., 2008a), although current densities within MECs do tend to be lower than those of MFCs (Kiely et al., 2011a).

In reality there was greater resistance within the reactor than the electrode overpotentials alone. The current densities measured were 0.04, 0.1 and 0.3 A/m^2 at 0.7, 0.9 and 1.1V load added respectively. This means that the current density only increases by around 0.6 A/m^2/volt, far lower than two early MEC laboratory studies (1.3 A/m2/volt in (Liu et al., 2005b) and 1.78 A/m^2/volt in (Rozendal et al., 2006)). Additionally this shows that there is an inherent overpotential in the system also of over 0.6 volts as seen in Figure 6.4, over this voltage needs to be added to generate any current.

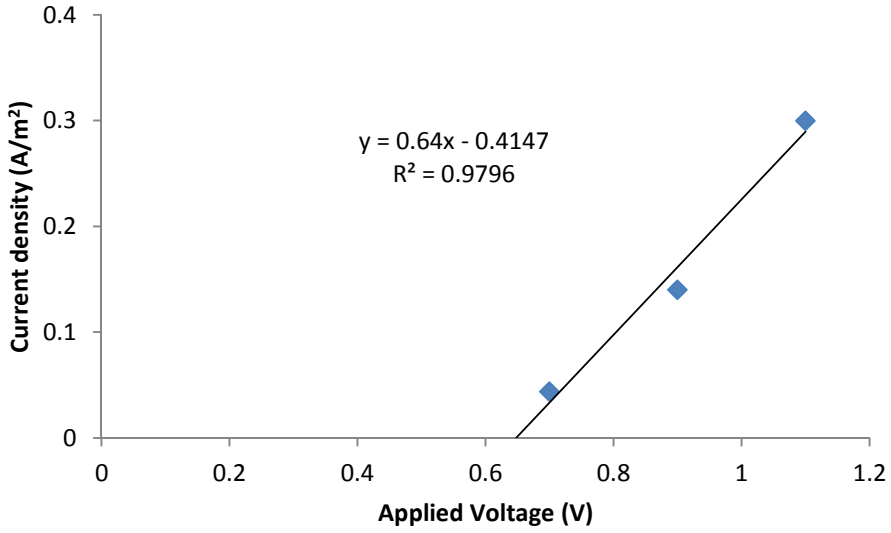

Figure 6-4 Current density as a function of applied voltage as measured in the pilot scale reactor after the initial two week acclimatisation period, showing the linear regression equation and R2 value. The intersect of the x-axis indicates the overpotential of the system

6.3.2. *Start-up and acclimatisation*

During the first 30 days of operation the reactor was run in batch mode with a supplement of 0.5 g/L of sodium acetate and an input voltage of 0.6 V. During this time there was no observed gas production and the current density was very low reaching 0.04 A/m^2 after the first two weeks. After this period wastewater was pumped through the reactor with a HRT of one day with no further addition of acetate. For the subsequent 10 days very little gas was produced and the current density remained at this very low level. At day 40 the input voltage was raised from 0.6 V to 0.9 V. The reactor was run with this input of voltage for the next 24 days; the average power density during this time reached 0.1 A/m^2. Gas production was low with an average of 9 mL/day, however once the gas lines had been flushed the purity of this gas (H_2) began to reach 100%. The electrical energy efficiency η_E was only 1 %. The voltage was then further increased to 1.1 V, and power densities rose and stabilised at 0.3 A/m^2. This led to a dramatic improvement in gas production, and the reactor entered its "working phase", the results of which are shown below. The start-up period took 64 days.

6.3.3. *Working performance of MEC reactor*

After the long start-up, and subsequent increase in the voltage to 1.1 volts, the MEC worked for the following 85 days, and continues to do so. The results presented here are for this period.

The volume of gas produced per day was highly variable. However the gas composition was consistent, hydrogen 100% ± 6.4, methane 1.8% ± 0.9. No trace of CO_2, N_2 or O_2 could be detected using the GC's or MIMS. H_2S could not be measured accurately however the MIMS did not detect any gas at this atomic weight and there was no detectable odour present. The daily H_2 production is shown in Figure 6-5. Production gradually increased during the first 30 days; after this the average production was around 1.2 L per day for the reactor, equivalent to 0.015 L-H_2/L/day.

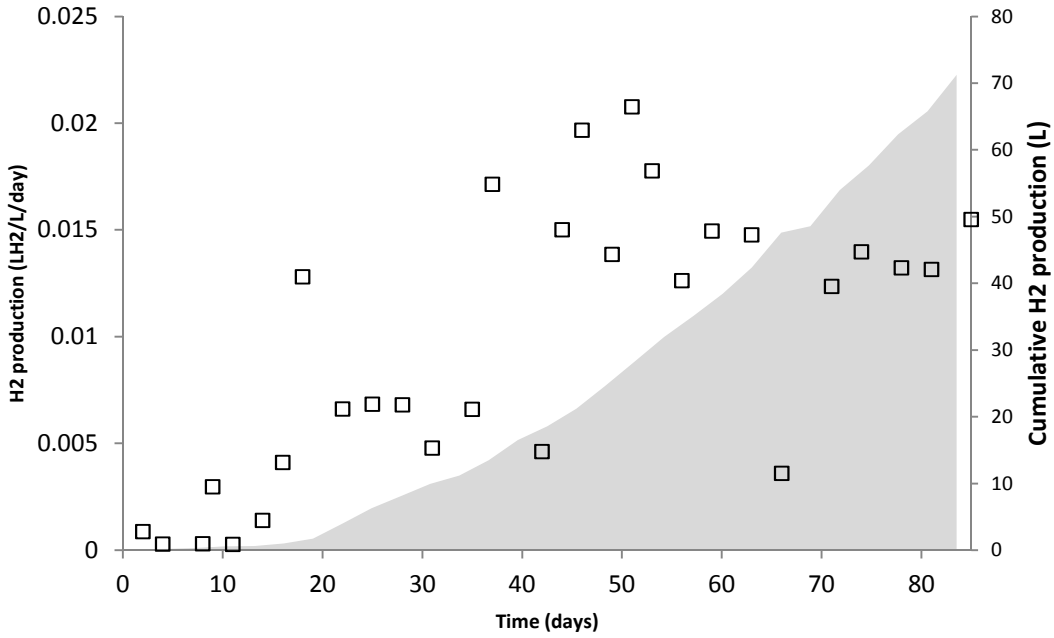

Figure 6-5 Hydrogen production during the working phase of reactor after the 64 day acclimatisation period, points showing the production rate at each time of sampling, and the area showing the cumulative production of the course of this period

The electrical energy recovery of the cell was quite variable as seen in Figure 6-6 (a), but did show an increasing trend and on occasion approached 100% (complete energy recovery) . The total energy efficiency (b) which gives the true performance of the cell was also variable, and considerably lower as both the electrical and substrate energy are considered as inputs. The energy efficiency shows an increasing trend reaching the 30 % level at the end of the study. The peak values are associated with very low COD removal measurements (making substrate energy input very low), and are not therefore likely to be representative of the true performance of the reactor. Coulombic efficiency (Fig. 5c) shows a similar trend to energy recovery (Fig. 5a), stabilising at around 55-60 % in the last 30 days.

The coulombic efficiency (CE) correlates with energy recovery (η_E) (R^2 = 0.998, Pearsons correlation). This correlation factor is calculated as N_E = 1.29 CE using the average input power voltage, this value is also seen in the data and is consistent over the course of the study. If the CE could remain at the 60% and the power input dropped to 0.9 volts 100% η_E would be achieved. Alternatively with this power input CE needs to reach 75% to achieve 100% η_E. The substrate efficiency (d), due to the highly variable influent and effluent COD values (as shown in Figure 6-7 can exceed 100%, and was often very low and even negative. The average substrate efficiency for whole the operational period is 10%.

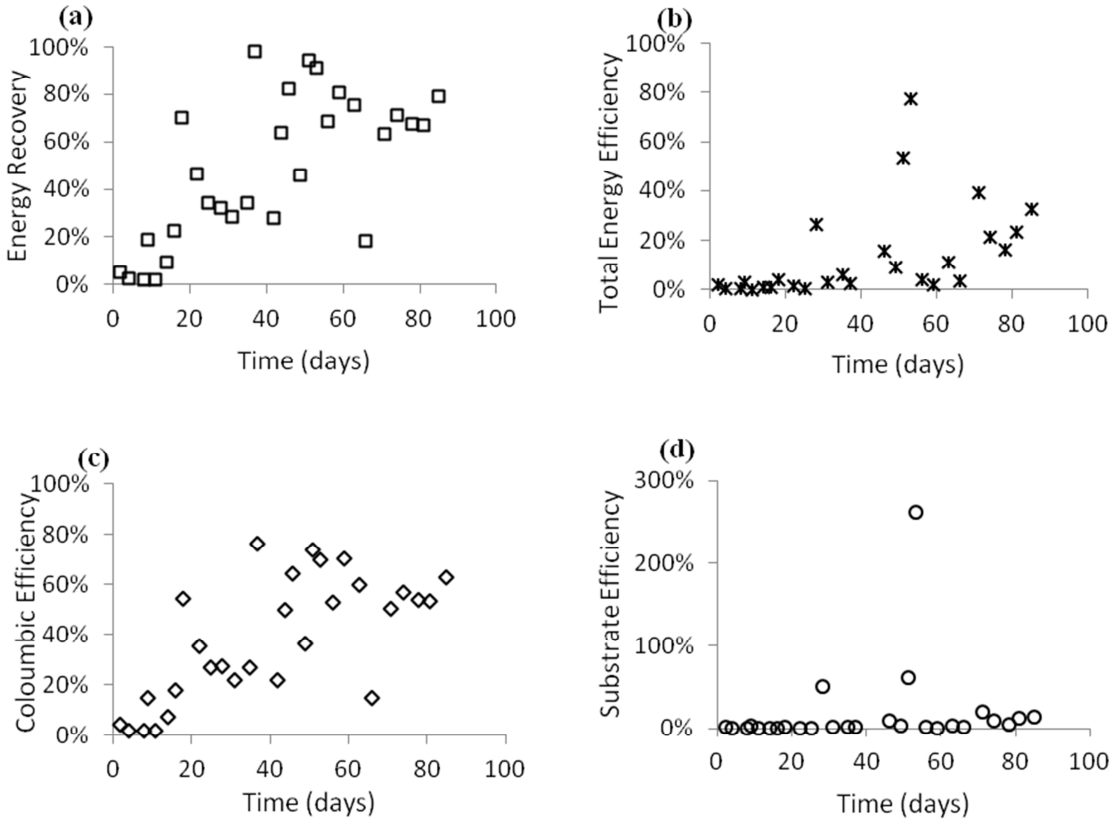

Figure 6-6 MEC reactor efficiencies over the 85 day working period a) electrical energy recovery b) total energy efficiency c) coulombic efficiency d) substrate efficiency

The levels of influent COD was highly variable which is likely to be one of the factors underlying the variation in performance. This factor was particularly the case at day 30 when the settling tank became full with sludge and influent COD was extremely high. This variability led to occasional negative values for % COD removal. The average removal of 33.7%, equates to 0.14 kgCOD/m^3/day, just below the range for activated sludge of 0.2-2 kgCOD/m^3/day (Grady, 1999). The COD effluent levels occasionally approached and dropped below the UK standard of 125 mg/l (EEC, 1991).

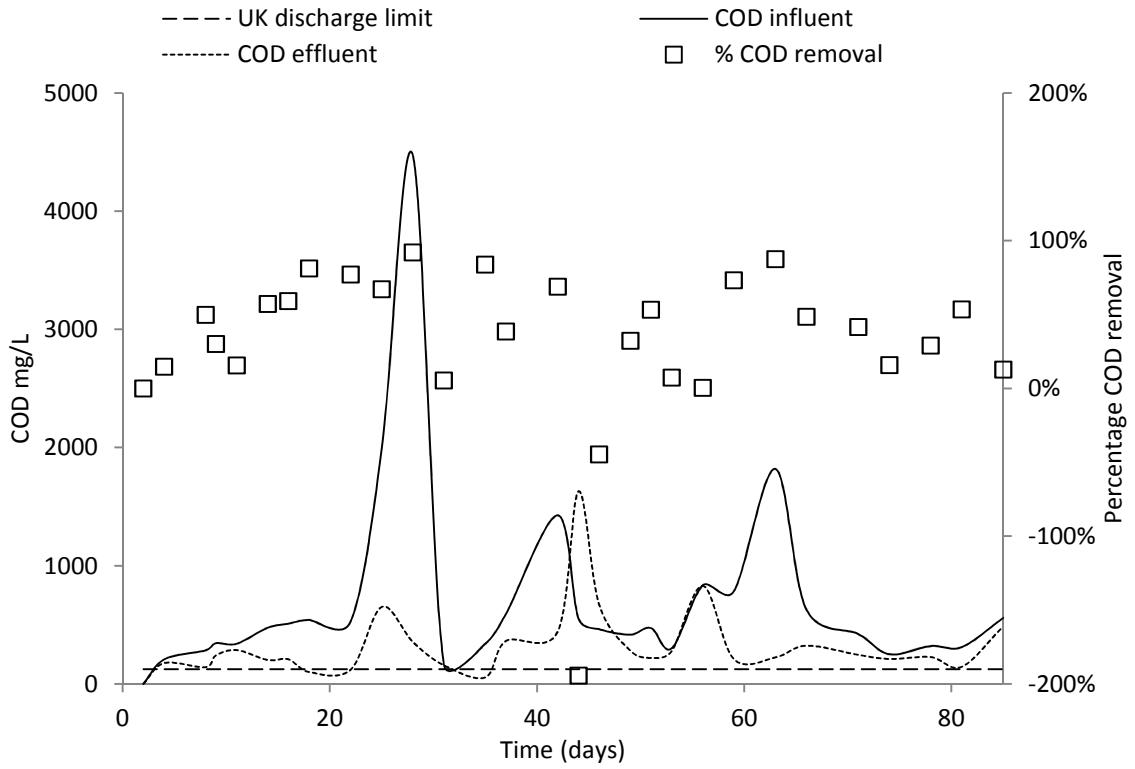

Figure 6-7 COD influent and effluent shown by the lines along with the UK discharge standard of 125 mg/l, percentage COD removal is also shown using the squares

Despite the variable influent COD and therefore variable performance, many of the other measured factors remained relatively constant throughout the operational period. The headspace of the anode compartment (2.2 L volume) contained elevated levels of CO_2 (1.9%) and low levels of CH_4 (0.4%), equivalent to 8.8 ml of CH_4, or 0.006 mg COD and 0.3 kJ. The gas production at the anode could not be measured quantitatively due to leakage. The daily production of methane at the cathode was 22 mL/day, equivalent to 0.014 mg COD, and 0.8 kJ of energy, approximately 5-6% of the amount of energy recovered as hydrogen.

The pH of the influent and effluent were continuously monitored, the influent was on average pH 7, the effluent pH 6.7, never dropping below pH 6. The DO of the influent was on average 4.2 mg/L and the effluent was 0 mg/L. The amount of VFA's dropped between the influent and the effluent, but there was frequently some acetic acid left in the effluent up to 45 mg/L, i.e. the available food source was not used up. This was confirmed by the average SCOD of the effluent of 115 mg/L. There was an average removal of 1.8 g/day of sulphate in the reactor, but never full depletion with the effluent containing 89.6 mg/L on average. The reactor removed an average of 0.2 g/day of chloride, although this value was highly variable. Fluoride and phosphate remained

relatively constant between the influent and effluent, nitrates were not present in either. There was no measured drop in conductivity between the influent and effluent.

The temperature of the influent wastewater varied considerably throughout the working period between June and September. The range of temperature was more stable within the reactor, and was on average 0.9 ^{o}C higher than the temperature of the influent. With a 88 L capacity and HRT of 1 day, this means 0.37 kJ/day of energy was lost to heat, equivalent to 20 mg COD, or 31 ml H_2. Temperature did not significantly influence energy recovery (p=0.678 influent, p=0.664 reactor, p=0.778 effluent, Pearson Correlation). Most of the fluctuation observed was diurnal and periods of the more extreme temperatures were short lived.

Table 6-1 Maximum, minimum and average temperature (^{o}C) of the influent, effluent and reactor ± 1 standard deviation which were continually logged over the experimental period

	Influent	Reactor	Effluent
Maximum	27.0 ± 2.3	21.0 ± 1.2	22.5 ± 1.6
Minimum	8.5 ± 2.3	13.5 ± 1.2	12.0 ± 1.6
Average	15.8 ± 2.3	16.6 ± 1.2	16.6 ± 1.6

The total material costs of the reactor, not including pumps, power supply and computing/recording instruments, was equivalent to £2344/m^3, of which the cathode and membrane combined represented less than 2%.

6.4. Discussion

This pilot scale reactor worked, producing almost pure hydrogen gas from raw influent domestic wastewater at U.K. ambient temperatures for a 3 month period and continues to do so. It is believed to be the first successful study of its kind, which brings the prospect of sustainable wastewater treatment and hydrogen production through the use of bioelectrochemical systems onto a new and exciting phase.

The reactor has removed on average 34% of COD, and occasionally reaching the UK discharge standard of 125 mgCOD/L, equating to a treatment rate of 0.14 kgCOD/m^3/day, just below the range for activated sludge. The reactor has performed this task using less energy than would be needed for aeration in a traditional activated sludge process. The electrical energy recovery on occasion nearly reached values of 100%, and was consistently around 70% during the later stages of the study. At this

level of performance (i.e. 70%) the energetic treatment costs were 2.3 kJ/gCOD, below the values for activated sludge of 2.5-7.2 kJ/gCOD (Pant et al., 2011). By implementing improvements to the reactor such as: increasing electrode surface areas; reducing the distance between electrodes; having a more efficient flow paths; consistent pumping; and improved materials, the η_E could be greater than 100%, making it a net energy producer. On the basis of this fairly large proof of concept study, energy neutral or even energy positive wastewater treatment is clearly a realistic goal.

The total energy recovery showed an increasing trend during the course of the study, levelling out at around 30%, with around a third of all energy both from the wastewater and from the power supply being recovered as hydrogen gas. Coulombic efficiencies of the reactor were high, levelling out at around 55-60 %, methane production accounts for an additional 3.5%. Other losses might be caused by some short circuiting in the reactor. It is likely therefore that a large proportion of the missing 40% of CE can be attributed to a loss of hydrogen gas from the system. Hydrogen is an extremely small molecule and is able to permeate most plastics, and is therefore likely to be leaking out of the reactor. In a tightly engineered system theoretically the coulombic efficiency could approach its maximum of 100%, resulting in an electrical energy recovery of 129%.

The substrate efficiency of the cell was considerably lower than the other efficiencies measured. This efficiency represents how much of the substrate is actually recovered as hydrogen, and gives an indication of how much substrate is used in the MEC process. Even if the 40% loss of hydrogen through leakage (as suggested by the CE of 60%) is accounted for in this calculation then the substrate efficiency would only increase from 10% to around 23%. Losses may be taken to suggest that substrate is being used in competitive oxidation processes, but only low levels of oxygen entered the cell with the influent. Sulphate reduction equated to about 3.6% of the total COD removal. Limited nitrates were available. Further losses can be accounted for by the probable build-up of sludge within the reactor as evidenced by the constant COD removal value throughout the study despite the increasing efficiency of the reactor, and that on three occasions a very high COD peak entered the reactor, on two of these occasions the peak of COD is not seen to leave the reactor see Figure 6-7.

Clearly the high resistance of the reactor means the overall efficiencies of the reactor will be low. The resistance observed is more problematic in this larger scale system than at the laboratory scale, and would also become increasingly challenging with further scale up. Improved reactor design is needed to overcome these problems. In a large scale system a considerable wire length is likely to be inevitable, resistance could be reduced through the use of a thicker wire, additionally resistance could be reduced in the electrode by improving the connection between the electrode, current collectors and wire. Further research into different materials and different configurations of materials would hopefully lead to improvements at a larger scale.

Further efficiency losses as identified above could be minimised by improving the engineering of the system. The two 'new' materials used in this study for the membrane and cathode have not been truly evaluated. More expensive alternatives such as Nafion membrane and a Pt coated cathode may prove to be worthwhile investments if performance increases greatly with their use. The biological MEC process works, and works relatively consistently for a period of at least three months. Although tested in realistic conditions, this was over a spring/summer period, survival over periods of sustained low temperature has yet to be confirmed.

The relationship between electrical energy recovery, electrical power input and coulombic efficiency has been defined however the prediction energy requirements for a larger scale MEC system may be difficult to make. Theoretical input voltages lie far from those needed in reality even for acetate fed cells, typically between 0.4-1.0 V compared to the 0.114 V theoretically needed (pH 7, 298 K) (Logan, 2008). A relatively small change in the electrical power input can have a large effect of the overall electrical energy recovery, yet if this value is not high enough to overcome the losses in the cell no hydrogen will be produced.

Undoubtedly there are many factors that require further investigation. Many of the inefficiencies could be overcome by improved engineering, but also a greater understanding of the biological processes (both working with and against the cell performance), community structure and ecology would allow for more confident design and manipulation.

The aim of this research was to determine if MEC technology could be a viable and alternative to the activated sludge process. The pilot scale reactor has worked producing hydrogen, with real wastewaters at ambient temperatures for over 3 months at a volumetric treatment rate just below that for activated sludge. A breakeven energy was not consistently achieved during the course of the study, yet is believed to be within reach with improved hydrogen capture and improved design to increase efficiencies. With this proof of concept now made we are a large step closer to using MEC technology for sustainable wastewater treatment.

Chapter 7. Conclusions

The overall aim of this research is to reach an understanding of whether microbial electrolysis cells could be a domestic wastewater treatment option.

I conclude that energy neutral or energy positive wastewater treatment should be possible. This research started by looking into how much energy is held intrinsically within the wastewater, and concluded that the amount of energy in the wastewater is substantial, more than previously thought, and more that the energy costs currently incurred in its treatment (18-29 kJ/gCOD vs. 2.5-7.2 kJ/gCOD in activated sludge treatment). Although this energy measured is internal chemical energy which is higher than the Gibbs Free Energy that would be available to microorganisms, with a biological system engineered for energy extraction from wastewater rather than an energy input, i.e. utilising other redox pathways rather than simple aerobic oxidation.

With the conclusion made that there is enough energy inherently contained in wastewater to treat it, the next question was to determine if Microbial Electrolysis Cells could meet this demand, replacing the high energy demanding activated sludge process with an energy yielding process. Parts of the thesis, in particular the low temperature work, suggested this might be possible yet other parts of the research did not such as the failure in MEC wastewater fed reactors. However by building and testing a pilot scale reactor on site at a wastewater treatment the most positive and conclusive evidence that this technology could work for real wastewater applications was gained. The reactor, even though it was a ‘first design’ using low cost alternatives to the optimum materials, and with many other problems such as non-optimised flow and hydrogen leakage and high resistance, it came reasonably close to its breakeven energy point. Even without breaking even it was more effective in terms of energy used per gCOD removed, and came close to the volumetric loading rates of the activated sludge process.

There is still much work to be done at this scale and larger to: understand the issues of scaling; economic feasibility; hydrogen capture and storage; design and materials; and optimisation. This work could then lead to retrofitting old activated sludge lanes with microbial electrolysis cells, radically changing the wastewater industry.

All the research conducted in this PhD has shown that the substrate acetate is not an adequate model of wastewater. This has been shown simply in terms of the energy available per gCOD, the acclimatisation and number of exoelectrogens able to digest these substrates, the diversity of the community fed with these substrates and their function within microbial electrolysis cells. The higher diversity estimates and complex acclimatisation pattern of acetate fed reactors suggest acetate may not be the optimum compound to use in BES's. Wastewater fed systems may have less free energy available, and therefore result in a more efficient biomass being formed. The lower coulombic efficiencies observed in wastewater fed reactors might be an inevitable result of electrons being lost within the longer chains of digestion, and not necessarily an indication of inefficient biomass.

The conclusion that temperature does not affect the performance of MFCs is surprising, although does correspond to some of the literature in this area (Catal et al., 2011, Jadhav and Ghangrekar, 2009). This suggests that there is a similar level of free energy available in systems run at different temperatures, and that low temperatures do not represent a disadvantage for BES. This is also observed in the pilot reactor, here low temperatures may be an advantage reducing methanogenic activity which proved fatal in the only other pilot scale MEC study to be published (run at 30 ^{o}C) (Cusick et al., 2011).

A further surprising conclusion was that inoculum did not have an effect on reactor performance, although the inoculum did interact with substrate to produce higher diversities within acetate fed reactors inoculated with high diversity soil. Exoelectrogenic bacteria were present naturally in all the wastewater inocula, and the Arctic soil inocula used throughout this research, albeit at low levels. The number or proportion of exoelectrogens was estimated to be 0.0017% using the very old methodology of MPNs, using the most recent next generation sequencing techniques and mathematical modelling algorithms, the estimates were 0.0012% and 0.00001% for two different wastewater samples. This therefore appears to be a reasonable good estimate of the rarity of such species.

BES reactors have been shown to work in challenging, real life, environments, and many observations have been made about the abundance and diversity of the organisms needed for the operation of these systems. This research has moved a substantial step

forward in proving that these technologies could be an energy efficient replacement of the activated sludge process. However we are still a long way from a deep and holistic understanding of the bacterial world operating within these systems, the energy requirements of these communities, their metabolic limits, their response to stress and ultimately their stability and function. Without this deep understanding we are reliant upon empirical data gathering, testing reactors in various environments until these limits are found. If we could model the free energy needs of the bacterial community, estimate the free energy available in the substrate, and calculate the efficiencies of the electrochemical cell, such systems could be modelled accurately and ultimately engineered to produce positive energy recovery.

Chapter 8. Perspectives on the use of MECs in the treatment of wastewater

This work has demonstrated a proof of concept of the use of MECs with domestic wastewater to produce hydrogen at the 100L scale over a 3 month time period. However this does not mean that they will be a viable wastewater treatment option. The work conducted in this research goes some way to confirming to technical feasibility of this technology in the treatment of domestic wastewaters, it does not however prove or suggest that this will be an economic viability, such an assertion is beyond the scope of this study.

There are many considerations which would need to be focused on in order to determine this economic viability for any technology to replace activated sludge treatment (AS), including those criteria stated in the introduction:

1. Extract and convert energy to a useable form at an efficiency that justifies the costs.
2. Attain the legal discharge standards of both chemical oxygen demand and nutrients, or fit with a process that would do this.
3. Treat low strength domestic wastewater.
4. Work at ambient, often low temperatures.
5. Work continuously and reliably.

The detailed costing of this technology is beyond the scope of this thesis. It has been suggested that MEC technology may be an economically viable alternative to AS over other treatments such as anaerobic digestion (AD) or MFCs (Foley et al., 2010, Curtis, 2010) based on the reduction in aeration costs and the potential value of products produced. However to change the UK wastewater infrastructure would require exchanging the current AS process components for a system with higher capital costs (estimated at 0.4 €/kgCOD for an MEC compared to 0.1 €/kgCOD for AS, (Rozendal et al., 2008a)) aiming to recover the costs through the product generated. It is clear that even with low cost materials used in this research, and the idea of retrofitting the cells into existing infrastructure (Cha et al., 2010), the capital costs of filling tanks with complex electrode assemblies would be far higher than installing the aeration pipework. It would need to be ascertained whether the ‘payback’ in terms of reduction of the

energy costs and the products generated would equal the higher capital costs over the lifetime of the cells (which is again unknown at this stage).

The design life of typical wastewater treatment infrastructure is at least 25 years. MECs have not been tested over such time periods in even in the relatively clean conditions of laboratories. It is highly likely the many of the components of a typical MEC would not survive for long periods when handling real wastes, membranes for example are particularly problematical clogging over time (Zhang et al., 2011), yet membraneless are also problematic at large scale (Cusick et al., 2011). Even the estimates for a 5 year life span of electrodes and membranes used in the estimates above (Rozendal et al., 2008a) are untested under real conditions and may be unrealistic. The life span and maintenance requirements of BES will be a critical factor in determining if this technology can be used economically within the wastewater industry.

A further cost consideration is the labour costs associated with this new technology. The level of maintenance required in the MEC process is again unknown, but is likely to be higher than the AS, though may be compensated for by the reduction in sludge treatment which is a considerable fraction of the operational costs (Verstraete and Vlaeminck, 2011). The hydrogen or product produced may also require purification again the costs of this would need to be accounted for in identifying if the economic benefits of the product outweigh the costs.

The full economic costing of the MEC process versus other processes is complex, with many unknowns. It is likely to vary with: the scale and wastewater type of different treatment plants; water usage and availability; energy and material prices; and therefore inherently through time (McCarty et al., 2011). The ‘upgrading’ of AS plants with improved energy recovery from sludge AD, improved process control and greater levels of primary settling such as the Strass plant in Austria which generates 108% of its electricity use (Nowak et al., 2011) may prove to be more economically viable. The addition of AD onto the AS process is the route many UK water companies are taking including Northumbria Water Ltd who have one large sludge AD plant in operation and one under construction. However such a high degree energy recovery is exceptional, and many experts in the field question the concept of using the energy intensive process of AS to insolubalise waste organics to sludge which then can undergo energy recovery (Verstraete and Vlaeminck, 2011).

The treatment levels of the pilot MEC run were both low and variable, averaging only at 34%, the AS process can remove up to 95% of the COD (Tchobanoglous, 1991) although this is rarely the case as they are usually part of a treatment flow with pre-settling and post clarification removing a proportion of the COD (Grady, 1999). The MEC reactor demonstrated did on occasions remove the COD down to the discharge limit of 125 mgCOD/L (EEC, 1991) so operation at this level is possible. The ability to use domestic wastewaters is a clear advantage over AD which tends to be restricted to high strength industrial or farm wastes, or sludge generated by AD. Further work would be needed to demonstrate that this treatment could consistently reach discharge standards, and the electrical conductivity of the wastewater at these low strengths is sufficient for the cells to function.

Even if part of a treatment flow with pre-settling and post clarification it is likely that the MEC would need to improve treatment rates to encourage investment, additionally the more organics removed the higher the energy yield can be. Treatment rates could be improved by reducing electrode spacing; however this would have the knock on effect of reducing the volumetric loading rate. The MEC could therefore end up requiring the same unit space as trickling filters, and therefore not be a viable option either due to land restrictions or poor economic comparability to this low energy treatment option.
There is an increasing body of research demonstrating that BES technologies will work at ambient temperatures (Jadhav and Ghangrekar, 2009, Catal et al., 2011, Larrosa-Guerrero et al., 2010), added to by the work in this thesis. Further work may be required in demonstrating this with real wastewaters at a larger scale, and also in quantifying and overcoming the kinetic effect of the lower temperatures on bacterial metabolism.

Many challenges lie ahead with BES research both from a technological and economic perspective. Only through completing and importantly combining these research areas will we be able to reach an understanding as to whether the technology can be used in the wastewater treatment plants of the future.

Chapter 9. Recommendations for future research

This research set out to answer the question as to whether microbial electrolysis cells could be used for wastewater treatment. Most of this research has strengthened the case that they are, however many more research and application questions remain unanswered. Each piece of research described in this thesis could be developed further to give more conclusive answers:

Chapter 2: A comprehensive survey into the amount of energy contained within wastewater is warranted. In the research conducted two samples were tested from different wastewater treatment plants and the results showed a large difference in the energy content between the samples and with that which would be predicted. Discovering the energy in wastewater is fundamental to the study of bioelectrochemical systems, and other technologies which aim to yield energy from wastewater. If we are to evaluate the true potential of these technologies we need to know how much energy is actually encapsulated in domestic wastewater, enabling efficiencies to be calculated and therefore better solutions engineered.

Measuring internal energy by calorimetry is a standard method in the solid waste industry (Garg et al., 2007, Lupa et al., 2011), yet when applied to wastewater the problem arises that samples have to be dry, and even with the improved and extremely laborious freeze drying method used in this research 20-30% of the volatiles in wastewater were lost. With an improved and quicker method, such as the use of distillation or reverse osmosis, a comprehensive survey of wastewaters in the UK could be made. This would: facilitate decisions on where best to invest in new technologies; give an indication of which technologies might be more suitable for different wastewaters; inform of the efficiency of processes; and most importantly – make decision makers believe energy extraction from wastewaters is economically viable and worthwhile.

Chapter 3: With a more definitive answer to the number of bacteria present and their growth pattern, accurate assessments of specific activity and growth yields could be made. Accurate estimations of these values are needed for parameterising models of these systems. By redesigning these experiments, and the reactors used to minimise or at least quantify all losses, a mass balance could be made and these values determined.

However the most intriguing question arising from this work was the difference in the pattern of acclimatisation observed in the acetate fed cells and those with complex substrates. Although possible reasons for this difference were suggested, a conclusive answer was not found. By conducting further research scaling between acetate and starch in terms of substrate complexity, the step causing the change in response of acclimatisation could be found, which may give valuable insight into the development and ultimately the function of these communities. The use of other microbiological techniques such as flow cytometry and QPCR may also help in the accurate determination of these values.

Chapter 4: The finding that temperature and inoculum had little effect on reactor performance is significant to the eventual implementation of this technology. The high variability within the warmer reactors would however be worth investigating further, if all the warm reactors were able to work at the maximum level shown by some, temperature would be a significant factor. The reactor configuration used in these experiments may have been limiting factor, thus if repeated with a higher performing reactor design, the temperature effect may be observed.

The counterintuitive observation that acetate fed cells produced a higher diversity was of great interest in this work. Further research is needed to determine if it is energy that controls the diversity, not the complexity of the substrate. This could be examined by scaling through simple compounds with known and increasing free energies (e.g. from the ΔG of the reaction under standard conditions at pH 7: acetate 27.40 kJ/e^- eq, pyruvate 35.09 kJ/ e^- eq and glucose 41.35 kJ/e^- eq) and observing how diversity changes.

Chapter 5: The conclusion that laboratory wastewater fed reactors fail after a short period of time is contradicted by chapter 6 where the pilot MEC worked. Determining the reason for failure at the small scale is a priority for any further lab scale research studies. Other than scale, the two different factors in the lab based experiments compared to the pilot, are that feed is continuous not batch, and that the laboratory reactors are acclimatised as a MFCs. Research into these factors, and a solution to the failure is needed to achieve the working laboratory wastewater fed systems required for investigations into the use of this technology for wastewater treatment.

Chapter 6: The final part of this research gave the most conclusive answer as to whether MECs can work for wastewater treatment and will, when published, put the research of MECs onto a new platform. Much research is still needed into improving efficiencies and critically achieving the breakeven energy recovery, further scaling, different materials and design, and the economic feasibility of implementing this technology at scale. If the use of this technology is validated, research is needed into the strategic implications this will have on the wastewater treatment industry.

Further recommendations: The research described has increased our understanding of how BES can function in wastewater treatment. A more fundamental direction of research would be the use of BES in understanding the energetic laws and rules which underpin biological systems. Such rules would have huge impact on design in both the near and distant future (Curtis et al., 2003). BES offer the unique opportunity, effectively opening a window on the energy involved in biological reaction, as this energy is routed through an external circuit and can therefore be measured allowing energetic interactions to be unravelled.

By designing a biocalorimeter type BES reactor, where all energetic inputs and outputs are measured (with no leakage) this could be tested using simple substrates and monocultures, and simple laws developed. For example if a substrate chemically yields 'x' kilojoules of Gibbs free energy (ΔG), exactly how much of this can be accessed by bacteria at a set pH and temperature, what proportions go to growth and maintenance for the BES to be stable and what the energy transfer efficiency is. By then scaling to more complex substrates and mixed cultures insight could be gained on: the fermentation processes and on how and why some reaction routes may be favored over others; if the overall ΔG of a complex substrate adequate to model outcome or is more complexity required; and if the energy needs are similar amongst trophic layers.

Through manipulating the systems thermodynamic constraints (temperature, pressure, and ionic strength) to give predictable outcomes, the rules identified above could be verified. Knowledge would also be gained on which thresholds of energy can change community behavior, and how easily these can be manipulated, how much the bacteria can compensate for these changes. Additionally by taking the system to the energetic edge the real limits can be defined and compered to theoretical limits. Ultimately an understanding of how energy requirements of a community link to abundance and

diversity could be gained, and allow for these to be manipulated to increase system stability.

By using a BES in this novel way, the thermodynamic laws which underpin the microbial world may be discovered. The rules generated could be used to create a model allowing biotechnologies to be reliably engineered. The feasibility and efficiency of a bioprocess being modeled at the investment stage without relying on estimates from empirical data. This would have huge scope to promote change and development across the scientific and engineering community.

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Chapter 11. Appendices

11.1. Appendix I - History of microbial fuel cell technology

The concept of fuel cells, a device that can convert electrochemical energy into electricity is not new. The first working chemical fuel cell is attributed to Sir William Grove in 1839 (Lewis, 1966). Progress since then has been slow and sporadic. Although it was understood that the direct conversion of chemical energy to electrical energy was more efficient than combustion in a heat engine (where up to 80% of the energy in the fuel is lost through heat in the exhaust, friction, air turbulence and the heating up and movement of engine parts), historically the abundance of fuel meant that the simpler combustion engine took precedence. The main surge of work in fuel cells has been in the last 10-15 years as fossil fuel prices, and the need for cleaner and more efficient energy production has increased (Logan, 2008).

The first biologically catalysed fuel cell was made in 1911 by a Professor of Botany M.C. Potter at Newcastle University. He discovered that an electrical current could be produced using bacteria as the catalyst on the anode, with a glucose and yeast mixture under various conditions of temperature and concentration he produced a maximum of 0.3 to 0.5 volts (Potter, 1911). This work was added to by Barnet Cohen who built a small bacterial battery using a series of half cells. This work drew more attention to the area, however the major drawback of the system was highlighted, only a very low current is able to be produced and it is rapidly discharged. The use of mediators such as potassium ferrycyanide and benzoquinone did enable greater voltage to be produced however the current remained low (Cohen, 1930).

Del Duca et al. (1963) re-visited the idea and set up a working laboratory model built using urea as a fuel. Urea was broken down enzymically by urease to produce ammonia at the anode, which then reacted with an air cathode producing current. A conceptual design was put forward for a 20-Watt portable urea battery, containing 64 individual cells, however the battery life was only 2 weeks.

Karube et al.(1976), described how carbohydrates were broken down to hydrogen using a fixed matrix of fermentative bacteria, the hydrogen reacted in the electrochemical cell. These studies were the first to use a design very similar to those MFCs used today, but with a salt bridge rather than an artificial membrane. It was believed that the bacteria's

role was to break down the carbohydrate to make electrochemically active products, which were entirely responsible for the current generation. It was not seen that the bacteria themselves were creating the electrochemical current, through the donation of electrons, though this was almost certainly the case.

R. M. Allen and then H. P. Bennetto worked on microbial fuel cells throughout the 1980's at Kings College, London. They had the vision that fuels cells could be a solution to the poor sanitation and lack of electricity supply in the then termed 'third world'. A paper which was the culmination of this work was published in 1993, simply titled Microbial Fuel-Cells – Electricity Production from Carbohydrates, was the first to show an understanding of the mechanism at work (Allen and Bennetto, 1993), although electron transfer was still not understood. It was thought that electrons were extracted from the oxidation of carbohydrates; these would then become trapped within the bacteria, but would become available for transfer to the anode through the use of a chemical redox mediator. Chemical mediators such as ferricyanide were expensive, non-sustainable and toxic to the environment.

The breakthrough discovery was made in 1999 that chemical mediators where not needed in the cells (Kim et al., 1999). This critical discovery that MFCs do not require these mediators, and the ever increasing pressures to reduce pollution, has led to an explosion of research in this area.

In 2005 it was discovered that microbes could be used in an electrolysis cell (Rozendal et al., 2006, Liu et al., 2005b). Electrical energy input can be combined with the energy derived from the fuel by bacteria to drive electrolysis reactions making products which would otherwise require much larger inputs of energy, most notably hydrogen. Thus hydrogen can be produced at greater efficiencies than is the limit with fermentation, and in theory at around one tenth of the electrical energy input of water electrolysis.

11.2. Appendix II - Theoretical cell energetics

The basic reaction occurring in an MFC or MEC can be split into two half reactions, the anode reaction which is the catabolic breakdown of the organic substrate to produce electrons, and the cathode reaction which is the donation of these electrons. The quantity of energy released per electron transferred is dependent on the chemical properties of those compounds involved, and is given by the Gibbs free energy of the reaction or ΔG_r:

$$\Delta G_r = \Delta G_r^0 + RT \ln Q$$

Equation 1

Where ΔG_r is the Gibbs free energy of the reaction, ΔG_r^0 is the Gibbs free energy for the reaction under standard conditions (temperature of 298 K and chemical concentrations of 1M for liquids and 1 bar for gases) as tabulated (Atkins, 2006), R is the gas constant 8.31 J/mol-K, T is temperature, and Q is the reaction quotient i.e. the ratio of the activities of the products and the reactants.

The cell potential (E_{emf}) can be calculated from Gibbs free energy of each half reaction:

$$E_{emf}^0 = -\Delta G_r^0 / nF$$

Equation 2

Where n is the number of moles of electrons transferred and F is Faradays constant 96485 J/mol e^-.

Alternatively the potential can be calculated directly when the potential under standard conditions is known:

$$E_{emf} = {E_{emf}}^0 - \frac{RT}{nF} \ln Q$$

Equation 3

Using acetate as an example electron donor, the half-cell, and full reaction values are given for ΔG_r and E_{emf} in Table 11-1 under standard environmental conditions pH 7, 298 K:

Table 11-1 Calculated theoretical energies (as Gibbs free energy and Potential) of half-cell reactions occurring within BES fed with acetate

	Reaction	ΔG_r/ kJ/ e- eq	Potential E (V)
Anode/ donor	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$	27.40	-0.300 (-0.284)
Cathode /acceptor MFC	$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O$	-78.72	0.805 (0.816)
Overall MFC	$\frac{1}{8}CH_3COO^- + \frac{1}{4}O_2 \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}H_2O + \frac{1}{8}HCO_3^-$	-106.12	1.105 (1.100)
Cathode /acceptor MEC	$H^+ + e^- \rightarrow \frac{1}{2}H_2$	39.94	-0.414
Overall MEC	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{2}H_2 + \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^-$	12.54	-0.114 (-0.130)

Values for E_{emf} written in bracket are those calculated from the tabulated ΔG_r and E_{emf} values which vary slightly (Rittmann, 2001, Atkins, 2006).

From the equations above it can be seen that anode and cathode potentials vary with temperatures (T), substrates (ΔG_r^0 or E_{emf}^0) and ionic concentrations (Q), especially pH. These can be calculated as shown below (except in the case of wastewater). However in a real system they may vary from time to time, place to place, and even within the same reactor as substrates are utilised and H^+ ions produced:

Substrate

In an acetate fed MEC the theoretical anode potential (E_{An}) under standard biological conditions (i.e. pH 7, temperature 25 ^oC) would be -0.284 V and the for the hydrogen evolution reaction (again at pH 7) it is -0.414 V, giving a cell potential E_{emf} of -0.13V an additional 0.13V would need to be added, with glucose this difference is positive 0.015V, theoretically no energy would need to be added. With wastewater and its unknown composition and variability the theoretical anode potential cannot calculated,

the potential of a variety of compounds which may be found within wastewater are shown in Table 11-2.

Table 11-2 Known Gibbs free energy and potential values for a variety of compounds which may be present in wastewater

Substrate	ΔGr (kJ/mol e-)	E_{An} (V)	E_{emf} (V)
Methane	23.53	-0.244	-0.170
Acetate	27.40	-0.284	-0.130
Propionate	27.63	-0.286	-0.128
Ethanol	31.18	-0.323	-0.091
Protein	32.22	-0.334	-0.080
Lactate	32.29	-0.335	-0.079
Citrate	33.08	-0.343	-0.071
Methanol	36.84	-0.382	-0.032
Glycerol	38.88	-0.403	-0.011
Formate	39.19	-0.406	-0.008
Glucose	41.35	-0.429	0.015

ΔGr values from (Rittmann, 2001)

Temperature

Using acetate in an MFC as an example, with an acetate concentration of 0.12M (1 g/L of Na-acetate), bicarbonate concentration of 0.005M, at pH 7, and partial pressure of O_2 as 0.2, the potential, E_{emf} of the anode and cathode can be calculated through a range of temperatures from 0 to 30 ^{o}C:

Anode reaction

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O$$

Cathode reaction

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O$$

The potential under standard environmental conditions (E^0) for these reactions are 0.187V and 1.229V respectively. Using Equation 3 above:

Anode

$$E_{An} = {E_{An}}^0 - \frac{RT}{nF}\ln\frac{[CH_3COO^-]}{[HCO_3^-]^2[H^+]^9}$$

$$E_{An} = 0.187 - \frac{(8.31\,J/mol\,K)\,(T)}{(8)(\,96485\,C/mol)}\ln\frac{[0.012]}{[0.005]^2[10^{-7}]^9}$$

Cathode

$$E_{Ca} = E_{Ca}{}^{0} - \frac{RT}{nF}\ln\frac{[H_2O]}{[O_2]^{1/2}[H^+]^2}$$

$$E_{Ca} = 1.229 - \frac{(8.31\ J/mol\ K)\ (T)}{(2)(\ 96485\ C/mol)}\ \ln\frac{[1]}{[0.02]^{1/2}[10^{-7}]^2}$$

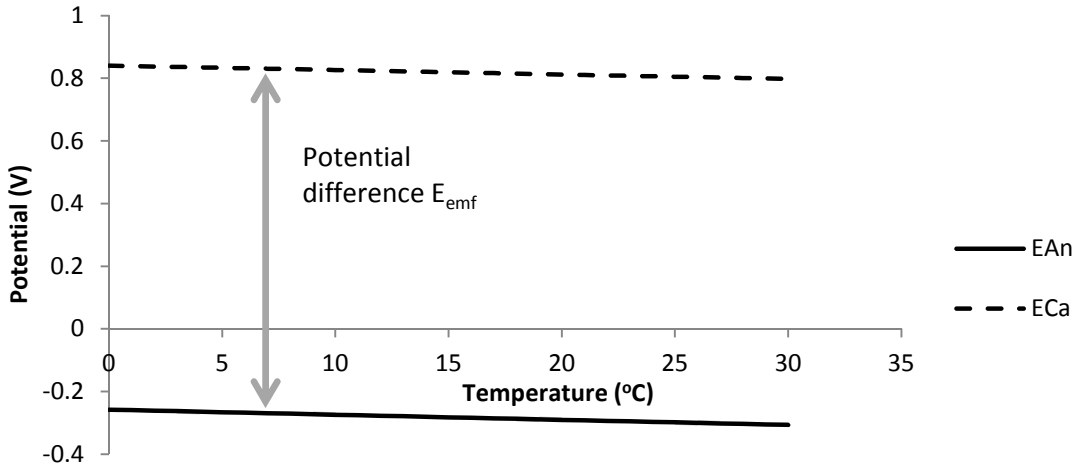


Figure 11-1 Calculated anode and cathode potential though a range of temperatures using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; pH 7; and partial pressure of O_2 as 0.2

The difference between the anode and cathode potential seen in Figure 11-1 varies only slightly from -1.098 V at 0 ^{o}C to -1.104 V at 30 ^{o}C. Theoretically therefore the energy available to be produced via a fuel cell is not greatly affected by temperature within the ranges given. This is however a simplistic approach to a system which, as stated previously is highly complex. As temperatures vary, so will many other factors including dissociation constants, partial pressures of gases and metabolic activity of the bacteria. It is therefore unlikely that the fuel cell will be able to generate as much current at lower temperatures as higher ones, yet it may not be as detrimentally affected by temperature as straight anaerobic digestion.

pH

The reaction co-efficient (Q) is calculated on the basis of the concentrations of the products and reactants in the chemical equation. This factor is critically dependant on

the pH of the system, i.e. the number of H^+ ions, as pH is a logarithmic scale, variance between pH 6 and pH 7 (both within the tolerance of bacteria) has a large effect on the Q value and therefore the overall potential of the cell. An example of this is shown below where the pH of the anode in an acetate system as described in the equations above at 25 ^oC is varied between pH 5 and 8, the cathode potential is kept constant under standard conditions. The potential difference ranges from 0.97 to 1.24 V.

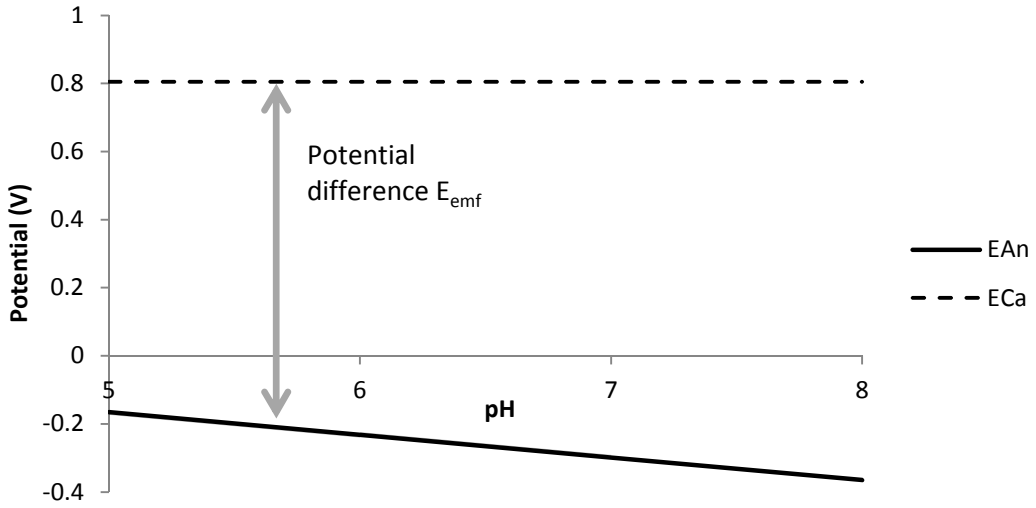


Figure 11-2 Calculated theoretical anode and cathode potential through a range of pHs using the conditions of: acetate concentration of 0.12M (1 g/L of Na-acetate); bicarbonate concentration of 0.005M; temperature 25 ^oC; and partial pressure of O_2 as 0.2

11.3. Appendix III – Table of calculated kJ/gCOD of various organic compounds

Compound	Formula	ΔH/gCOD
Benzene	C_6H_6	10.2
Linoleic acid	$C_{18}H_{32}O_2$	13.4
Benzoic acid	C_6H_5COOH	13.4
Myristic acid	$CH_3(CH_2)_{12}CO_2H$	13.6
Acetic acid (Acetate)	CH_3COOH	13.6
Phenol	C_6H_5OH	13.6
Palmitic Acid	$CH_3(CH_2)_{14}CO_2H$	13.6
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$	13.7
Methane	CH_4	13.9
Ethane	C_2H_6	13.9
Lactic acid	$CH_3CH(OH)COOH$	14.0
Ethanol	C_2H_5OH	14.3
Glucose	$C_6H_{12}O_6$	14.3
Propene	C_3H_6	14.3
Cyclopropane	C_3H_3	14.5
Ethanal	CH_3CHO	14.6
Ethene	C_2H_4	14.7
Sucrose	$C_{12}H_{22}O_{11}$	14.7
Methanol	CH_3OH	15.1
Chloroethylene	C_2H_3Cl	15.7
Oxalic acid	$(COOH)_2$	15.9
Formic acid	$HCOOH$	15.9
Ethyne	C_2H_2	16.3
Hexachlorobenzene	C_6Cl_6	16.5
Dichloroethylene (1,1)	$C_2H_2Cl_2$	17.1
Dichloroethylene (1,2)	$C_2H_2Cl_2$	17.2
Methanal	$HCHO$	17.8
Trichloroethylene	C_2HCl_3	20.0
Teterachloroethylene	C_2Cl_4	26.0
Chloroform	$CHCl_3$	29.1
Trichloroacetic acid	CCl_3COOH	30.4

11.4. Appendix IV - Description of the calculation algorithm used in the Shizas and Bagley

Shizas and Bagley (Shizas and Bagley, 2004) use a sample of municipal wastewater which prior to drying contains 431 mg/L COD. This sample is then oven dried to give a total solids measurement of 1980 mg/L. The dried sample is used in a bomb calorimeter giving 3.2 kJ/g dried weight.

Calculations derived from this data cited in various papers (Logan, 2008, Liao et al., 2006, Schroder, 2008, Logan, 2009):

$$3.2\ \mathrm{kJ/g}\ \times\ 1.98\ \mathrm{g/L} = 6.3\ \mathrm{kJ/L\ wastewater}$$

$$6.3\ \mathrm{kJ/L}\ \times \frac{1}{0.431\ \mathrm{gCOD/L}}\ =\ 14.7\ \mathrm{kJ/gCOD}$$

If the exercise is repeated on the data from the present paper using the oven dried samples and the measurement taken for COD prior to drying the results would have been:

Cramlington

$$8.3\ \mathrm{kJ/L}\ \times \frac{1}{0.718\ \mathrm{gCOD/L}}\ =\ 11.6\ \mathrm{kJ/gCOD}$$

Hendon

$$5.6\ \mathrm{kJ/L}\ \times \frac{1}{0.576\ \mathrm{gCOD/L}}\ =\ 9.9\ \mathrm{kJ/gCOD}$$

This is an underestimation of 60% and 45% respectively.

11.5. Appendix V - Wastewater sterilisation

Several of the experiments conducted in this thesis relied on using real wastewater, but needed this to be sterile. The following method was developed:

Method

The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into Ringers sterile dilutent (APHA, 1998). Effective sterilisation was defined as colony free plates in triplicate at zero dilution. The circulation time was varied to determine the optimum. The change in chemical composition (total chemical oxygen demand TCOD, soluble chemical oxygen demand SCOD and total solids TS) of the wastewater itself as compared to autoclaving and filtering.

Results

UV sterilisation caused the least change in wastewater properties measured as shown in Table 11-3, and was able to fully sterilise the wastewater.

Table 11-3 Percentage change of wastewater characteristics caused by the different sterilisation methods

	COD	Soluble COD	Total Solids	Bacteria per 0.1ml
Autoclaved (121^{o}C for 15 mins)	-15.6% ± 0.9	21.6% ± 0.6	-13.3% ± 5.8	0
Membrane filtered (0.2um PES)	-61.5% ± 0.5	22.8% ± 1.7	-36.1% ± 11.7	40 ±19
UV sterilised (5 mins)	-1.6% ± 0.4	7.2% ± 4.6	-3.3% ± 6.7	0

Bacteria is the average number counted on triplicate plates at zero dilution. All values show mean ± standard deviation (n=3)

Conclusion

Circulation of wastewater for 5 minutes through a UV filter was effective for bacterial kill off and least detrimental treatment to the composition of the wastewater.

11.6. Appendix VI - COD removal and coulombic efficiency

In the acetate fed cells the COD removal was high for both the cells which did (85%) and did not (80%) produce current ($p = 0.051$). For the other reactors there was an average removal of 64% COD for the wastewater and 87% for the starch solution. No significant difference in the COD removal in the reactors which generated current and those that did not was found wastewater ($p = 0.188$) and starch ($p= 0.688$).

The effluent of all reactors contained no detectable VFA's. The measured anions in each cell showed that there was almost complete removal of sulphate, from a starting value of 70 ppm in the wastewater and 38 and 41 ppm in the acetate and starch solutions respectively.

The coulombic efficiency of all reactors was low, such values are reasonably typical for complex substrates, but far lower than would be expected in a functioning acetate fed cell (Logan, 2008, Liu et al., 2011).

Table 11-4 COD removal and Coulombic efficiencies of all reactors fed on the different substrates. The values in grey are the reactors where acclimatisation did not occur

Inocula (ml)	**0.01**	**0.01**	**0.1**	**0.1**	**0.1**	**1**	**1**	**1**	**1**	**10**	**10**	**25**	**25**	**25**	**25**	**50**	**50**
COD removal (%)																	
Acetate	85.6	77.1	85.4	80.3	80.5	86.7	95.6	92.4	82.1	87.3	79.9	84.7	86.6	80.2	77.6	79.0	77.3
Wastewater						60.8	41.9			59.1	69.8	68.1	70.5			80.3	62.5
Starch						88.2	84.5			88.2	86.4	89.4	81.7			80.8	90.5
Coulombic efficiency (%)																	
Acetate	0.1	0.1	1.8	0.5	0.8	4.6	5.3	1.8	0.1	6.7	7.5	10.5	8.9	10.1	9.2	9.1	0.8
Wastewater						3.6	0.1			0.3	0.2	9.4	12.5			10.4	7.4
Starch						1.3	0.78			0.82	13.4	12.5	16.9			17.4	1.6*

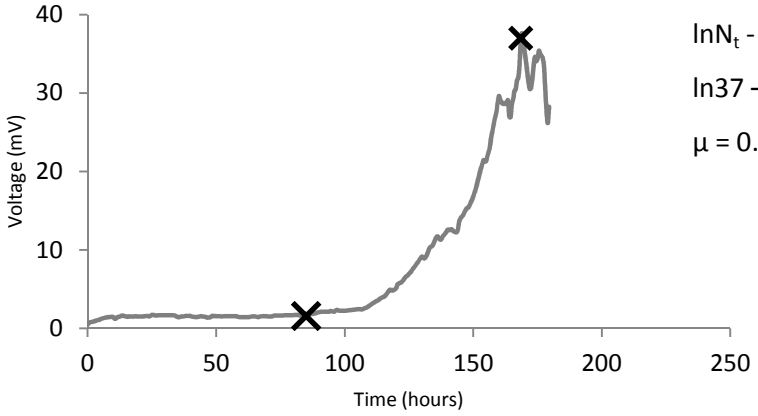
Values in grey are the reactors which did not acclimatise

*Unrepresentative value, data logging equipment failed after the point of acclimation.

11.7. Appendix VII - Yield and Specific activity calculations

Growth rate

Example calculation using 25 ml inocula

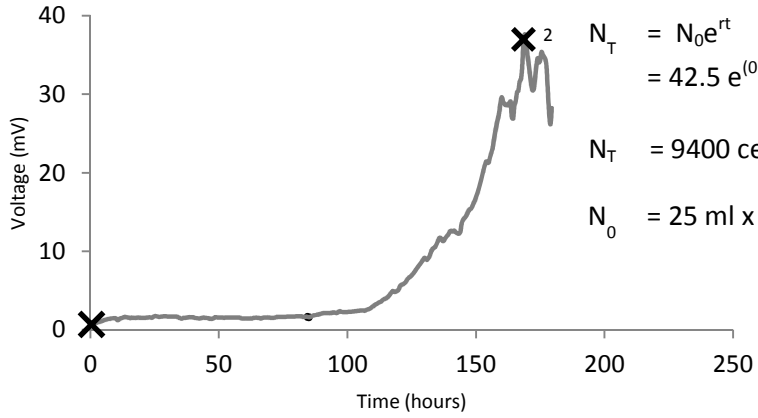


$\ln N_t - \ln N_0 = \mu(t - t_0)$

$\ln 37 - \ln 2 = \mu(170 - 85)$

$\mu = 0.034$

Specific activity



$N_T = N_0 e^{rt}$

$= 42.5\ e^{(0.034 \times 180)}$

N_T = 9400 cells

N_0 = 25 ml x 1.7 cell/ml = 42.5 cells

Each data logged voltage represents the time of 30 minutes, therefore the moles of electrons passed to the circuit per second at the data points measured is:

Moles of electrons = coulombs / Faradays constant

=((Voltage / resistance) x seconds)/Faradays constant

E.g. $\mathbf{X}_2$ =((0.037V / 470Ω)x 30mins x 60 seconds)/96485

$= 1.5 \times 10^{-6}$

Moles of electrons/cell = $1.5 \times 10^{-6} / 9400$

$= 10^{-10}$ mol e^-/cell

This value can be plotted throughout the time course of the experiment and is seen to be relatively constant.

Growth yield

The total number of cells produced up to the end of the exponential growth phase in the example above is 9400 cells.

$$\text{gCOD-cells} = (N_T - N_0) \times W \times COD_{cell}$$

where $N_T - N_0$ is the total new cell produced, W is the weight of each cell as estimated as 5.3×10^{-13} (Logan, 2008) and COD_{cell} is the estimation of 1.25 g-COD/g-cell (Rittmann, 2001).

$$\text{gCOD-cells} = (9400\text{-}43) \times 5.3 \times 10^{-13} \times 1.25$$

$$= 6.1 \times 10^{-9}$$

$$\text{gCODsubstrate} = \sum_{t0}^{t} mol\, e^{-}/8 \times 64$$

Where the sum over the growth period $t\text{-}t_0$ of the moles of electrons as calculated above is divided by 8 to give moles of acetate used, and multiplied by 64 giving the gCOD per mole of acetate.

$$\text{gCOD substrate} = 0.00011 / 8 \times 64 = 8.8 \times 10^{-4}$$

$$\text{gCOD-cell/gCOD-substrate} = 6.1 \times 10^{-9}/8.8 \times 10^{-4} = 6.9 \times 10^{-6}$$

The estimated yield of the acetate fed cells is extremely low ranging between 10^{-4} to 10^{-5} g-COD cell/g-COD substrate for the cells with between 10-50 mLs of inocula.

If exponential growth is assumed throughout the whole time period for the lower inocula cells these values are much higher up to 8 g-COD cell/g-COD for the 0.1 ml inocula. If no growth during lag is assumed these values are lower (10^{-7} g-COD cell/g-COD) and more in line with those observed for higher inocula. These yields are inconsistent with the literature on yields in microbial fuel cells (Freguia et al., 2007, Rabaey et al., 2003) although both of these studies used different methodology. They are also inconsistent with yields of other bacterial systems (Rittmann, 2001).

11.8. Appendix VIII – Calculations of performance in MFCs and MECs

Power Calculation for both MFCs and MECs

Performance can be evaluated through the amount of power produced which can be expressed as:

$$P = IE$$

Where P is the power in watts, E is the voltage as measured by the data logger in volts and I is the current in amps, calculated from the measured voltage E, at a known resistance R:

$$I = E/R$$

Power can therefore be alternatively expressed as:

$$P = E^2/R$$

This power is often also evaluated as power density (*Pd*), this is the amount of power produced per area of electrode surface (typically the size of the anode) expressed as Wm^2. Normalising the power output in this way allows different systems to be compared. This is calculated as:

$$Pd = \frac{E^2}{A_{An}R}$$

Where A_{An} is the area of the anode. The current density (A/m^2) can also be expressed in the same way normalising current to electrode size. Both power and current density can also be expressed per reactor size by substituting A_{An} above for the reactor volume in m^3, resulting in a power density measured as Wm^3. or current density as A/m^3.

Efficiency calculations for MFCs

The efficiency of an MFC is expressed as the Coulombic Efficiency (CE) and is a measure of the amount of coulombs of charge recovered from the cell from the total coulombs available in the substrate that has been removed in the reactor. It is expressed as a percentage:

$$CE = \frac{Coulombs\ recovered}{Coulombs\ in\ substrate}$$

An Amp is the transfer of 1 coulomb of charge per second, therefore by integrating the current over the course of the experiment or batch time (t) the total coulombs transferred is given. Usually the amount of coulombs in the substrate is evaluated using the amount of organic matter removed as determined by the chemical oxygen demand (COD). CE is therefore calculated as:

$$CE = \frac{8 \int_0^t I \ dt}{F \ V_{An} \Delta COD}$$

Where 8 is used as a constant derived from the molecular weight of oxygen divided by 4 the amount of electrons exchanged per mole of oxygen. Faradays constant (F) of 96485 Coulombs/mol, is the magnitude of electrical change per mole of electrons, ΔCOD is the measured change in COD in g/L and V_{An} (L) is the volume of the anode compartment containing the liquid feed at the given COD concentration. .

Efficiency calculation for MECs

The efficiency of an MEC is a more complex matter, as the output of energy is of hydrogen gas (not electricity or charge directly) and the inputs of energy are from the substrate and the additional electrical energy added to the system.

11.9. Appendix IX – Dissimilarity matrix

Unweighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.79	0.00																	
Wastewater inocula 2	0.88	**0.67**	0.00																
Acetate cold ww 1	0.88	0.67	0.70	0.00															
Acetate cold ww 2	0.88	0.65	0.69	**0.45**	0.00														
Acetate cold soil 1	0.82	0.80	0.80	0.72	0.73	0.00													
Acetate cold soil 2	0.76	0.78	0.82	0.75	0.75	**0.59**	0.00												
Acetate hot ww 1	0.89	0.71	0.69	0.54	0.60	0.73	0.78	0.00											
Acetate hot ww 2	0.89	0.73	0.71	0.56	0.58	0.74	0.78	**0.22**	0.00										
Acetate hot soil 1	0.81	0.79	0.80	0.72	0.75	0.62	0.63	0.25	0.70	0.00									
Acetate hot soil 2	0.79	0.79	0.82	0.75	0.76	0.64	0.65	0.18	0.72	**0.51**	0.00								
Wastewater cold ww 1	0.87	0.69	0.70	0.59	0.61	0.73	0.76	0.27	0.64	0.73	0.76	0.00							
Wastewater cold ww 2	0.82	0.72	0.76	0.72	0.73	0.74	0.74	0.44	0.67	0.66	0.67	**0.68**	0.00						
Wastewater cold soil 1	0.85	0.69	0.75	0.61	0.64	0.65	0.69	0.28	0.64	0.64	0.69	0.53	0.66	0.00					
Wastewater cold soil 2	0.88	0.74	0.75	0.69	0.69	0.77	0.78	0.24	0.60	0.69	0.72	0.62	0.51	**0.64**	0.00				
Wastewater hot ww 1	0.86	0.71	0.73	0.62	0.64	0.66	0.71	0.28	0.65	0.67	0.71	0.49	0.68	0.42	0.65	0.00			
Wastewater hot ww 2	0.88	0.74	0.74	0.68	0.68	0.76	0.77	0.24	0.60	0.70	0.72	0.63	0.51	0.65	0.23	**0.66**	0.00		
Wastewater hot soil 1	0.86	0.67	0.71	0.62	0.65	0.74	0.77	0.28	0.64	0.73	0.76	0.35	0.67	0.53	0.64	0.53	0.63	0.00	
Wastewater hot soil 2	0.89	0.71	0.72	0.59	0.58	0.75	0.78	0.28	0.59	0.74	0.78	0.44	0.71	0.51	0.64	0.49	0.64	**0.45**	0.00

Weighted

	Arctic soil inocula	Wastewater inocula 1	Wastewater inocula 2	Acetate cold ww 1	Acetate cold ww 2	Acetate cold soil 1	Acetate cold soil 2	Acetate hot ww 1	Acetate hot ww 2	Acetate hot soil 1	Acetate hot soil 2	Wastewater cold ww 1	Wastewater cold ww 2	Wastewater cold soil 1	Wastewater cold soil 2	Wastewater hot ww 1	Wastewater hot ww 2	Wastewater hot soil 1	Wastewater hot soil 2
Arctic soil inocula	0.00																		
Wastewater inocula 1	0.41	0.00																	
Wastewater inocula 2	0.52	**0.59**	0.00																
Acetate cold ww 1	0.44	0.48	0.45	0.00															
Acetate cold ww 2	0.43	0.45	0.48	**0.08**	0.00														
Acetate cold soil 1	0.50	0.56	0.53	0.17	0.16	0.00													
Acetate cold soil 2	0.44	0.48	0.47	0.09	0.10	**0.10**	0.00												
Acetate hot ww 1	0.49	0.57	0.41	0.28	0.30	0.36	0.30	0.00											
Acetate hot ww 2	0.56	0.63	0.44	0.37	0.41	0.45	0.40	**0.17**	0.00										
Acetate hot soil 1	0.36	0.43	0.50	0.23	0.22	0.30	0.23	0.32	0.43	0.00									
Acetate hot soil 2	0.41	0.51	0.43	0.14	0.16	0.22	0.15	0.23	0.34	**0.16**	0.00								
Wastewater cold ww 1	0.34	0.39	0.46	0.25	0.23	0.35	0.28	0.31	0.43	0.22	0.25	0.00							
Wastewater cold ww 2	0.37	0.42	0.55	0.43	0.40	0.45	0.42	0.46	0.52	0.31	0.39	**0.33**	0.00						
Wastewater cold soil 1	0.36	0.40	0.45	0.26	0.22	0.34	0.27	0.30	0.42	0.22	0.26	0.13	0.36	0.00					
Wastewater cold soil 2	0.41	0.50	0.41	0.31	0.33	0.39	0.33	0.30	0.36	0.37	0.31	0.32	0.33	**0.34**	0.00				
Wastewater hot ww 1	0.35	0.39	0.46	0.27	0.24	0.36	0.29	0.31	0.43	0.24	0.29	0.14	0.37	0.05	0.35	0.00			
Wastewater hot ww 2	0.38	0.49	0.40	0.31	0.32	0.40	0.33	0.26	0.35	0.35	0.29	0.29	0.33	0.31	0.08	**0.32**	0.00		
Wastewater hot soil 1	0.34	0.40	0.48	0.24	0.22	0.34	0.28	0.33	0.45	0.22	0.26	0.05	0.33	0.14	0.31	0.16	0.29	0.00	
Wastewater hot soil 2	0.38	0.38	0.52	0.28	0.24	0.35	0.30	0.36	0.49	0.25	0.30	0.12	0.32	0.18	0.34	0.19	0.34	**0.11**	0.00

Dissimilarity values calculated using UniFrac, lower values indicate greater similarity

11.10. Appendix X - Estimates of sample total diversity

Table 11-5 Estimates of total diversity using the MCMC model (Quince et al., 2008), values given are the lower 95% confidence interval : median : upper 95% confidence interval. The best fit values according to the DIC values are highlighted in bold, the model fits that had DIC scores within 6 of the best fitting model are in italics and should not be considered as plausible options for fitting the data

	Total diversity		
Sample	**Log-normal**	**Inverse Gaussian**	**Sichel**
Arctic soil inocula	**5831:7207:10593**	5151:6227:7439	3632:4403:5821
Wastewater inocula 1	**3431:4238:5572**	2217:2405:2655	2648:3275:5533
Wastewater inocula 2	2924:4260:8970	1679:2066:2752	**1716:2286:3640**
Acetate cold ww 1	**3060:5449:11740**	1273:1700:2406	1402:2197:3379
Acetate cold ww 2	13901:29226:42363	**984:1549:3049**	993:1697:3298
Acetate cold soil 1	**1380146:1393974:1407428**	*3430:5004:7687*	2960:4628:9094
Acetate cold soil 2	**1849625:1865409:1877419**	*3428:4923:7910*	3191:5018:8179
Acetate hot ww 1	1934:3511:12608	808:987:1300	**948:1310:2224**
Acetate hot ww 2	**1217:2159:6024**	643:785:1037	665:843:1264
Acetate hot soil 1	4386:8968:19150	1508:1968:2813	**1456:1984:3086**
Acetate hot soil 2	**171417:184911:197766**	2445:3773:5440	2350:3579:5577
Wastewater cold ww 1	**614:749:1014**	493:535:594	491:534:599
Wastewater cold ww 2	*859:1102:1596*	*640:708:805*	**730:906:1455**
Wastewater cold soil 1	**1079:2249:8263**	543:733:1197	651:1032:2324
Wastewater cold soil 2	556:640:789	*467:494:531*	**510:575:793**
Wastewater hot ww 1	1430:2911:9800	*637:845:1300*	**5682:16751:18608**
Wastewater hot ww 2	**483:548:660**	419:443:476	430:467:525
Wastewater hot soil 1	**820:1148:1985**	581:661:787	596:697:893
Wastewater hot soil 2	694:1135:2283	438:504:614	**468:572:954**

Table 11-6 DIC scores as defined by the sum of the deviance averaged over the posterior distribution and estimate of the sampling effort required to capture 90% of the diversity of taxa within the sample as determined by the fits of abundance distribution

	DIC			Sampling effort		
Sample	Log-normal	Inverse Gaussian	Sichel	Log-normal	Inverse Gaussian	Sichel
Arctic soil inocula	165.53	171.01	166.67	2.02E+06	4.06E+05	1.32E+05
Wastewater inocula 1	450.33	455.14	444.42	1.32E+07	2.56E+05	8.92E+05
Wastewater inocula 2	264.17	262.28	261.93	3.56E+07	2.98E+05	4.16E+05
Acetate cold ww 1	275.13	275.3	275.85	3.32E+09	1.59E+06	3.06E+06
Acetate cold ww 2	197.07	196.74	196.98	1.11E+13	1.47E+06	1.70E+06
Acetate cold soil 1	266.22	273.65	267.61	2.56E+18	1.42E+07	8.37E+06
Acetate cold soil 2	274.28	283.68	274.4	2.42E+18	7.28E+06	5.19E+06
Acetate hot ww 1	309.59	311.17	309.21	2.99E+09	5.88E+05	1.59E+06
Acetate hot ww 2	242.64	244.43	244.76	2.84E+08	3.61E+05	4.73E+05
Acetate hot soil 1	290.25	288.7	288.57	1.17E+10	1.44E+06	1.34E+06
Acetate hot soil 2	265.04	269.84	265.05	6.98E+14	4.73E+06	3.16E+06
Wastewater cold ww 1	254.73	255.02	255.23	5.22E+05	4.23E+04	4.25E+04
Wastewater cold ww 2	268.11	269.7	261.78	1.23E+06	4.91E+04	1.63E+05
Wastewater cold soil 1	201	201.99	197.99	2.68E+08	1.53E+05	5.35E+05
Wastewater cold soil 2	333.27	349.36	332.04	3.47E+05	3.70E+04	9.96E+04
Wastewater hot ww 1	252.09	254.67	246.76	1.37E+09	2.57E+05	1.05E+09
Wastewater hot ww 2	274.09	279.19	275.06	1.51E+05	2.52E+04	3.56E+04
Wastewater hot soil 1	248.04	250.28	248.96	3.54E+06	7.21E+04	9.24E+04
Wastewater hot soil 2	243.6	244.69	242.65	1.93E+07	7.44E+04	1.32E+05

11.11. Appendix XI - Details of the bacteria phyla and families found within the samples tested

It is seen in Figure 11-3 (a) that the inoculated and acclimatised reactors have become enriched Proteobacteria, this phylum dominates with about 80% abundance in the acetate fed cells, and around 60% in the wastewater fed cells. Proteobacteria are a diverse phylum of bacteria, yet most of this high abundance in the reactors is caused by the enrichment of *Geobacter* an exoelectrogenic organism, as is seen in Figure 11-4. *Rhodocyclaceae*, *Psuedomonas* and *Desulfovibrio* also added to the proportion of Proteobacteria that became enriched. The relative abundance of the other main phyla generally drops within the reactor samples, a proportion (around 10-20%) of Bacteriodietes remains, and there is some enrichment of Acidobacteria in the wastewater fed reactors. The wastewater reactors have a greater spread of abundance over the phyla groups shown, with less domination by Proteobacter.

The OTU richness shown in Figure 11-3 (b) again shows the greater diversity of the acetate reactors over the wastewater fed ones, both by the larger bar size and the Chao estimate above. It is seen many of the OTUs present in the inoculum have survived in the acetate reactor conditions, despite the metabolic narrowing of the conditions. Surprisingly this greater diversity or spread of OTUs appears to be slightly higher in the cold reactors, than the warm ones. In the case of the wastewater fed reactors the OTU richness in reduced, temperature does not appear to have an impact.

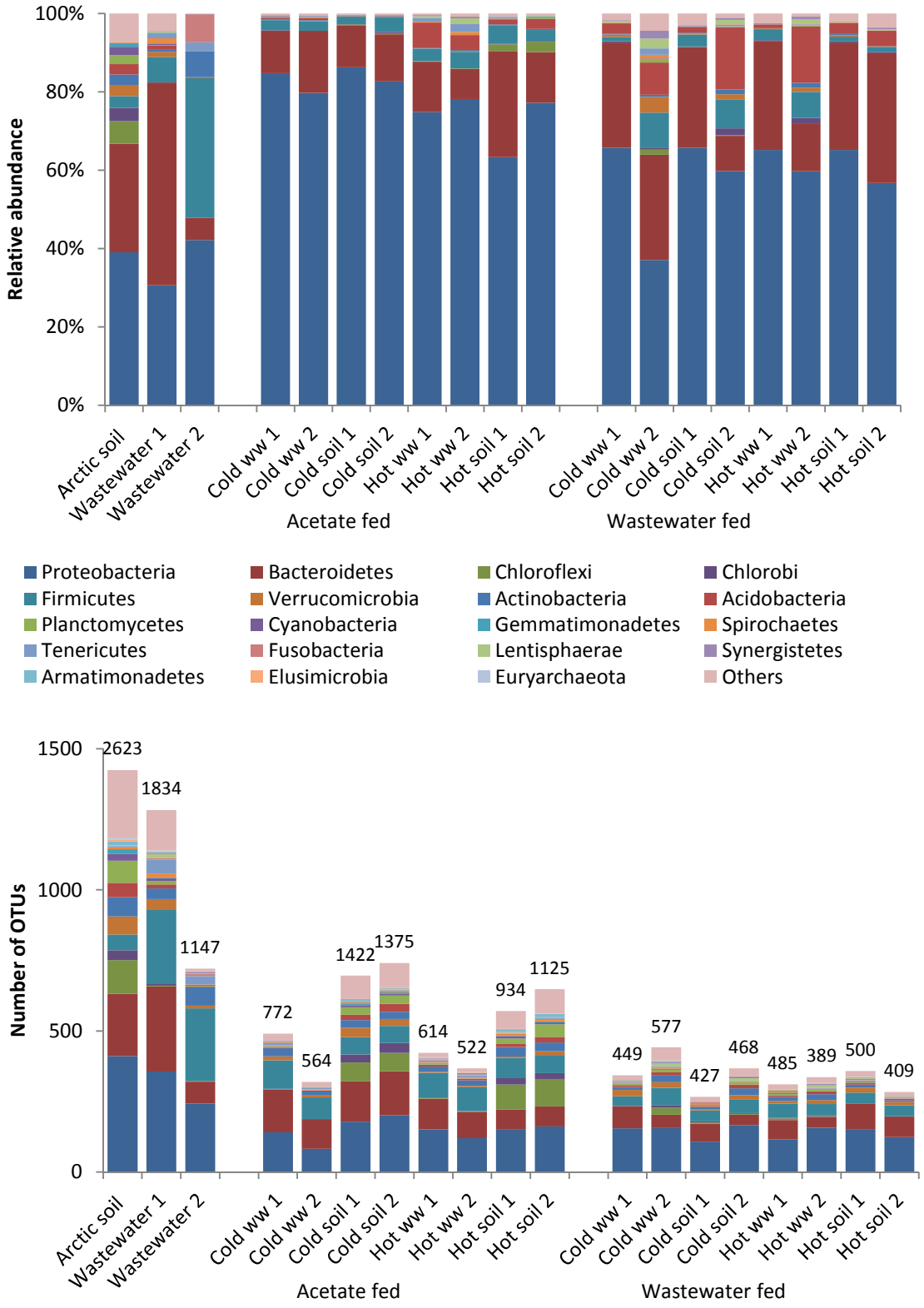


Figure 11-3 Relative abundance (a) and OTU richness (b) for all the data sets given at the phylum rank. Relative abundance is shown as the number of reads within each taxa divided by the total number of reads. The OTU richness is the number of taxa within each phylum is given by the size of the bar, the Chao 1 estimate of richness is written at the top of each bar

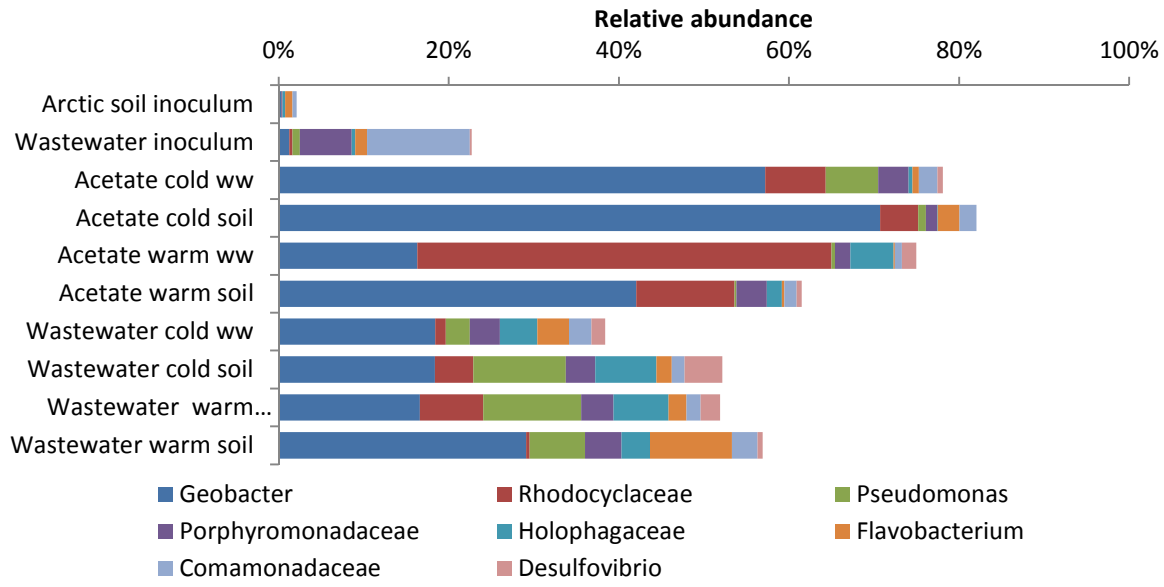


Figure 11-4 The relative abundance of the 8 most dominant genus as an average for the duplicate reactors under each condition, where the genus name was not given by the classification database family is used

It would be expected that the most dominant organisms within the reactors are the ones that are able to most competitively metabolise, grow and therefore reproduce within the conditions of the reactors. The top 8 most dominant genus are given in Figure 11-4, for Rhodocyclaceae, Porphyromonadaceae, Holophagaceae, Comamonadaceae the classification did not give the genus name, and therefore the family name is given. It is seen that for the acetate fed reactors these 8 genus make up a large proportion of the total abundance, and in the cold reactor most of this is by *Geobacter*. For the warm acetate reactors, *Geobacter* is still important, but Rhodocyclaceaea is also dominant, especially in those seeded with wastewater. The proportion of *Geobacter* is made up of 11 different species (names of which are not given by the classification), 4 of which are dominant within the reactors. Rhodocyclaceae is a diverse family of bacteria associated with wastewater treatment, further classification of this group is not made.

Within the wastewater reactors Geobacter is less dominant, between 20-30% of abundance, and there is a greater spread of the other genus and families, most notable *Pseudomonas* which make up to 10%. Within the Pseudomonas genus, 8 species were identified, of which 2 were dominant within the reactors, Pseudomonas have previously been seen within fuel cell systems fed substrates such as glucose and butyric acid and are believed to be capable of fermentation (Kiely et al., 2011c), some species such as *Pseudomonas aerunginosa* produce soluble redox shuttles and have been investigated

for their use within fuel cell systems (Marsili, 2010). The family of Holophagaceae is also quite enriched, this family includes the species of *Geothrix fermetans* which has been found in wastewater fed MFCs and is believed to be important in the hydrolysis or fermentation steps, (Kiely et al., 2011a), and has also been linked to shuttle formation (Bond and Lovley, 2005). *Flavobacteium* are also enriched, although this genus is more typically associated with freshwater environments. There is also likely to be sulphate reduction occurring in the cells due to the presence of *Desulfovibro*.

11.12. Appendix XII – Acknowledged contributions

Section	Other contributors	Contribution of E. Heidrich
Chapter 2	T.P. Curtis and J. Dolfing – editing and guidance with content	90%
Chapter 3	T.P. Curtis and J. Dolfing – editing and guidance with content	95%
Chapter 4	T.P. Curtis and J. Dolfing - editing and guidance with content, M. Wade – bioinformatics analysis, W.T.Sloan – sequencing funding	95%
Chapter 5	T.P. Curtis, K. Scott, I Head and J. Dolfing – discussion and experiment planning	95%
Chapter 6	T.P. Curtis K. Scott and J. Dolfing - editing and guidance with content, S. Edwards – site installation and running MEC	90%