Bio-electrochemical systems for treatment of petroleum hydrocarbon contaminated wastewater and sediment.

A thesis submitted for the degree of Doctor of Philosophy

by

Ekaete Okon Utuk

School of Engineering

Newcastle University, United Kingdom

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Abstract

Bio-electrochemical systems (BES), such as microbial fuel cells, can produce electric power as well as expedite anaerobic microbial biodegradation of complex contaminants such as petroleum hydrocarbons in water or sediment by using the BES anode to offset the limitation of insufficient electron acceptors. Robust bio-electrocatalytic anodic biofilms and sustained cathodic oxygen reduction reaction are imperative to achieve superior performance in BES. Petroleum hydrocarbons in sediment-water system can be removed using biological methods such as bioremediation. In this work we investigated if bio-electrocatalytic activity of anodic biofilm can be used to supplement and enhance the removal efficiency of petroleum hydrocarbon from wastewater and contaminated sediment while also producing electricity.

Bioanodes of double chamber microbial fuel cells (MFCs) were enriched with inoculum from two different sources (MFC effluent and activated sludge) and employed for removal of polyaromatic hydrocarbons (PAHs) from hydrocarbon wastewater under high and low external resistance, R_{ext} (100 and 1000 Ω). Anodic biofilms of all MFCs studied were able to tolerate low concentration of Tween 80 surfactant (100 ppm). Inoculum source influenced current density and charge output in MFCs at start-up. Remarkably, MFCs inoculated with activated sludge started up faster and achieved higher current density (59.44 ± 0.98 mA/m^2) than MFCs inoculated with MFC effluent (1.65 ± 0.03 mA/m^2). Inoculum source did not appear to have any influence on overall PAH removal efficiency. Under low R_{ext} (100Ω), similar PAH removal efficiencies were obtained (at 50 ppm PAH) in MFCs inoculated with MFC effluent (86 ± 4 %) and MFCs inoculated with activated sludge (84 ± 3 %). Higher electrochemical PAH removal and coulombic efficiency (CE) was achieved in AS under low R_{ext} (PAH removal = 84 ± 3 %; CE = 27.5 ± 2.9 %) than high R_{ext} (PAH removal = 72 ± 5 %; CE = 14.8 ± 2.6 %) at PAH concentration of 50 ppm. Cyclic voltammetry showed that stable anodic biofilm with higher bio-electrocatalytic properties were developed under low R_{ext} (100Ω).

Double and single chamber sediment microbial fuel cells (SMFCs) as well as wicking and submerged cathode configurations were investigated for enhanced removal of petroleum hydrocarbons from hydrocarbon-contaminated sediment. Wicking cathode configuration was utilized in single chamber SMFCs to evaluate trade-off between oxygen and ion transport on SMFC performance (including its PAH removal efficiency). Current density and charge output in single chamber SMFC with vertical electrode arrangement was 3 times higher using wicking cathode, CW (166 ± 78 mA/m^2, 298 ± 140 C) compared with submerged cathode, CS (54 ± 21 mA/m^2, 97 ± 38 C). Comparable PAH removal efficiencies were achieved in CW (51 ± 3 %)

and CS (46 ± 2 %). Wicking cathode was shown to enhance current/charge output in single chamber sediment microbial fuel cells using vertical electrode arrangement with marginal enhancement of PAH removal.

Microbial community analysis of bioanodes of MFCs (R_{ext} = 100Ω) and single chamber SMFC (vertical electrode arrangement) showed the presence of similar bacterial phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*) in varying proportions. In addition, *Chloroflexi*, *Planctomycetes*, and *Epsilonbacteraeota* were found in SMFC bioanodes. Individual members of these phyla found in both MFCs and single chamber SMFCs were distinct, and had potentially diverse roles within the bioreactors. Putative electroactive bacteria (EAB) *Geobacter* and *Acidovorax* were enriched in closed circuit MFCs relative to open circuit MFCs. The presence of *Pseudomonas* (some members of which are putative EAB) in both closed and open circuit MFCs was indicative of its versatile role in MFCs. This new finding showed that bacterial enrichment in each MFC was linked to their specific function in the bioreactor. SMFC anode comprised high diversity of species with more evolutionarily divergent taxa compared with MFC anode communities. Enrichment of putative fermenters *Anaerolineaceae*, *Bacteroidetes* and *Clostridiaceae* relative to other families correlated with low coulombic efficiency (< 2 %) obtained in closed circuit SMFCs. Significant enrichment (145 to 317 %) of putative fermentative anaerobe *Anaerolineaceae* in single chamber SMFCs within 35 days of operation was a novel finding in this study.

In summary, this project demonstrated that electrochemical removal of PAH from hydrocarbon wastewater could be marginally improved using activated sludge inoculum and low external resistance (R_{ext}) due to higher bio-electrocatalytic activity in anodic biofilms under low R_{ext}. Application of wicking cathode enhanced current/charge output in single chamber sediment microbial fuel cell using vertical electrode arrangement with marginal enhancement of electrochemical oxidation of PAH. Microbial community composition showed relative abundance of members with known fermentative metabolic capacities. High microbial diversity was a key factor in enhancement of MFC/SMFC performance due to synergistic interactions between various members of each community.

Keywords: microbial fuels cells, petroleum hydrocarbon, wastewater, sediment, microbial community.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AEM	Anion exchange membrane
Ag/AgCl	standard potential of the silver chloride reference electrode
ASV	amplicon sequence variants
BES	bio-electrochemical systems
BTEX	benzene, toluene, ethylbenzene, xylene
CE	coulombic efficiency
CEM	Cation exchange membranes
CMC	critical micelle concentration
COD	chemical oxygen demand
CV	cyclic voltammetry
DADA2	Divisive Amplicon Denoising Algorithm 2
DET	direct electron transfer
DGGE	denaturing gradient gel electrophoresis
DIET	direct interspecies electron transfer
DNB	denitrifying bacteria
EAB	Electrochemically active/electroactive bacteria
ECM	equivalent circuit model
EIS	electrochemical impedance spectroscopy
Emf	electromotive force
EOM	extractable organic matter
DCM	dichloromethane
DCMFC	Double chamber/dual chamber microbial fuel cell
DNA	Deoxyribonucleic acid
ΔG	Gibb's free energy
GC-MS	Gas Chromatography – Mass Spectrometry
GDL	gas diffusion layer
H_2	hydrogen
IEM	ion exchange membrane
LLE	liquid-liquid extraction
MEA	membrane electrode assembly
Med_{red}	reduced form of mediator
Med_{ox}	oxidized form of mediator
MESN	Microbial Electrochemical Snorkel
MET	mediated electron transfer

MFC	Microbial Fuel Cell
MRC	Microbial Remediation Cell
MS	mass spectrometry
m/z	mass to charge ratio
OCP	open circuit potential
ORP	oxidation reduction potential
ORR	oxygen reduction reaction
OTU	Operational taxonomic unit
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffer solution
PCB	polychlorinated biphenyls
PCR	Polymerase chain reaction
PDMS	polydimethylsiloxane
PES	Polyethersulfonate
PEM	proton exchange membrane
pH	Partial pressure of hydrogen
Pt	platinum
Pt-C	platinum-carbon
PTFE	polytetrafluoroethylene
PVA	polyvinyl alcohol
PVC	polyvinyl chloride
rRNA	ribosomal ribonucleic acid
SER	surfactant-enhanced remediation
SCMFC	Single chamber microbial fuel cell
SIM	selected ion monitoring
SMFC	sediment microbial fuel cell
SPE	solid phase extraction
SRB	sulphate -reducing
TEA	terminal electron acceptor
T80	polyoxyethylene (20) sorbitan monooleate or Tween 80
TOC	total organic carbon
TPH	total petroleum hydrocarbon
VFA	Volatile fatty acids

Chapter 1 Introduction

1.0 Chapter summary

This chapter provides an overview of petroleum hydrocarbon and their impact on the environment and public health. It highlights physical, chemical, and biological methods that have been applied to remove petroleum hydrocarbons from the environment, and the limitations of these methods. It underscores the role of bio-electrochemical systems in addressing some of the challenges identified during removal of petroleum hydrocarbons from the environment. The research problem, scope as well as aims and objectives of the research project are presented in this chapter.

1.1 Background to the Study

Petroleum hydrocarbons are complex mixture of organic compounds found in crude oil or its refined products. Origin of petroleum hydrocarbons in the environment may be biogenic (naturally occurring in the earth's crust) or anthropogenic (through human-related activities). Biogenic hydrocarbons formed through biological, physical, and geochemical processes in earth's crust, can leak into marine environment or shallow aquifers. Anthropogenic releases are derived from industrial effluents, vehicular emissions, oil and gas exploration and production activities, oil transportation and distribution, gas flaring and oil spillages. Anthropogenic hydrocarbons are more dominant in the environment and therefore, associated with water and soil/sediment contamination (Ite *et al.*, 2013; Adeniji *et al.*, 2017). Other sources of oil spills into environment include leakage from corroded or aged pipelines, operational errors as well as oil bunkering and artisanal refining activities (UNEP, 2011; Lindén and Pålsson, 2013). Petroleum hydrocarbons comprise aliphatic hydrocarbons, aromatic hydrocarbons, asphaltenes and resins. Although petroleum hydrocarbons are insoluble in water, some aliphatic hydrocarbons are easily discharged into environment due to their high volatility whereas high molecular weight aromatic hydrocarbons sink down the water column and become adsorbed onto particulate matter. Consequently, petroleum hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, xylene) and polycyclic aromatic hydrocarbons (PAHs) become persistent in soil/sediment leading to bioaccumulation and increased toxicity up the food chain when contaminated water, seafood organisms and crops are consumed (Zabbey *et al.*, 2017). For instance, physiology and growth of an edible vegetable, *Abelmoschus esculentus* (Okra), was reduced when cultivated in soil contaminated with petroleum hydrocarbons – this was

attributed to changes in soil properties caused by hydrocarbon contamination (Adenipekun *et al.*, 2009; Oyedeji *et al.*, 2012).

Petroleum hydrocarbons, found in crude oil or its refined products, constitute adverse health risks because of their linkage to neurological and respiratory problems, and cancer in humans (Mastrangelo *et al.*, 1996; Suárez *et al.*, 2005; Kponee *et al.*, 2015). In a review of detrimental effects of oil spills on human health in seven (7) cases of oil spill (ranging from 6,000 to 85,000 tonnes), Aguilera *et al.* (2010) concluded that exposure to oil spills was responsible for damage of deoxyribonucleic acid (DNA) and increased hormone levels in exposed individuals. The ecological impact of petroleum hydrocarbon contamination is evident in loss of habitat, wildlife, and biodiversity as well as any aesthetic and economical gains they might render. For example, an extensive study of the temporal changes in mangrove forest before and after the 1992 Era oil spill (South Australia) (from 1981 - 2018), using aerial imagery and predictive modelling, revealed that heavily oiled mangrove had not recovered ten (10) years after the spill incident (Connolly *et al.*, 2020). Complete recovery of oiled mangrove could take up to thirty (30) to fifty-five (55) years depending on the oil type and concentration (Duke, 2016; Connolly *et al.*, 2020).

Various physical, chemical, and biological methods have been applied for removal of petroleum hydrocarbons from the environment. Physical methods such as incineration, thermal desorption, soil vapour extraction (SVE) or soil excavation and replacement can treat large volumes of soil/sediment with rapid results but are often labour intensive and expensive, require energy inputs, and may generate harmful waste products (Zabbey *et al.*, 2017). In some instances, petroleum hydrocarbon contaminants are removed using combined methods (i.e. physicochemical methods) such as chemical washing using co-solvents or surfactants, soil flushing, solidification/stabilization, electrokinetics, and encapsulation (Trombly, 1994; Khan *et al.*, 2004; Zabbey *et al.*, 2017). Slow solvent diffusion, inadequate immobilization of contaminant, soil/sediment properties are some of the challenges of these methods that may result in prolonged remediation period. Biological methods involve biodegradation of petroleum hydrocarbons using plants (phytoremediation) or bacteria (bioremediation). Phytoremediation is a low maintenance approach but can be complicated by the interaction of multiple environmental factors such as soil parameters, plant characteristics, nutrient concentration (Lim *et al.*, 2016). Bioremediation technologies such as bioaugmentation, biostimulation and bioventilation involve addition of genetically modified microbes, alteration of environmental parameters or addition of oxygen to soil to increase growth and activity of hydrocarbon-degrading microbes (Lim *et al.*, 2016). Like phytoremediation, bioremediation technologies are cost-effective and benign to the environment but require lengthy treatment

periods ranging from a few weeks to several months or years. These lengthy treatment periods caused by insufficient electron acceptors to drive biodegradation of petroleum hydrocarbons is a major limitation in bioremediation. Oxygen can be added to promote aerobic biodegradation, however, oxygen has low solubility in water, oxygen addition requires cost and it may be used up by other chemical reactions occurring within the soil/sediment where the petroleum hydrocarbons are adsorbed (Daghio *et al.*, 2017). Limited bioavailability of petroleum hydrocarbons in water means that they accumulate in anoxic or anaerobic sediment zone where there is also a low abundance and diversity of microbes. Anaerobic biodegradation of petroleum hydrocarbons can be stimulated by addition of electron acceptors to drive the biodegradation process. Bio-electrochemical systems (BES) provide additional electron acceptors to expedite anaerobic microbial biodegradation of petroleum hydrocarbons by serving as an electron sink for electrons released during oxidation of petroleum hydrocarbons.

1.1.1 Petroleum hydrocarbon removal using Bio-electrochemical systems (BES)

Bio-electrochemical systems (BES) provide additional electrons required to accelerate anaerobic microbial biodegradation of petroleum hydrocarbons. BES use biological recognition elements as catalysts to convert chemical energy in organic substrates to electrical energy. A typical BES consists of two electrodes (anode and cathode), separated by an ion exchange membrane (IEM). The membrane could be a cation, proton, or anion exchange membrane (CEM, PEM or AEM). The electrodes are connected by an external circuit. BES may be easy to construct and install because of their simple design and configuration, most of which can be made using cost effective materials. They have low maintenance requirements and can be operated at low temperatures. They may be remotely operated and monitored with minimal disturbance to the environment. With no reported toxic by-products or damage to the environment, they are ecological-friendly options for contaminant removal (Zabihallahpoor *et al.*, 2015). BES that use enzymes or bacteria as the biological recognition element are referred to as enzyme fuel cell (EFC) or microbial fuel cell (MFC) respectively.

As can be seen in Figure 1-1, MFCs are configured as single chamber or double chamber bioreactors. Single-chamber MFCs consist of anode and cathode in the same compartment, with cathode either suspended in overlying water or exposed to atmospheric air or oxygen (air cathode). Double or dual-chamber MFCs consist of anode and cathode in separate compartments, and could be configured as cubes, rectangles, cylinders, or H-cells. H-cell MFCs are used for conducting research on new materials or membranes (Logan *et al.*, 2006). A typical double chamber MFC comprises a chamber containing anode and cathode (two black rods) separated by an IEM (patterned rectangle with dashed lines). A voltammeter and a resistor,

depicted as V and R respectively, are connected across the electrodes. Electrons released during oxidation of organic substrate (e.g. petroleum hydrocarbon) are taken up by anode via electron transfer mechanisms involving electroactive bacteria (white ovals on black rod) on anode of MFC (bioanode). The transfer of these electrons, via an external circuit, to a terminal electron acceptor (TEA) at the cathode, generates electric current. Electric current generated is measured by the voltammeter, and data is recorded on a computer using a data logging software. Current is calculated using Ohm's law (V = IR) where V is the potential difference between the two electrodes, I is current, and R is external resistance across the two electrodes.

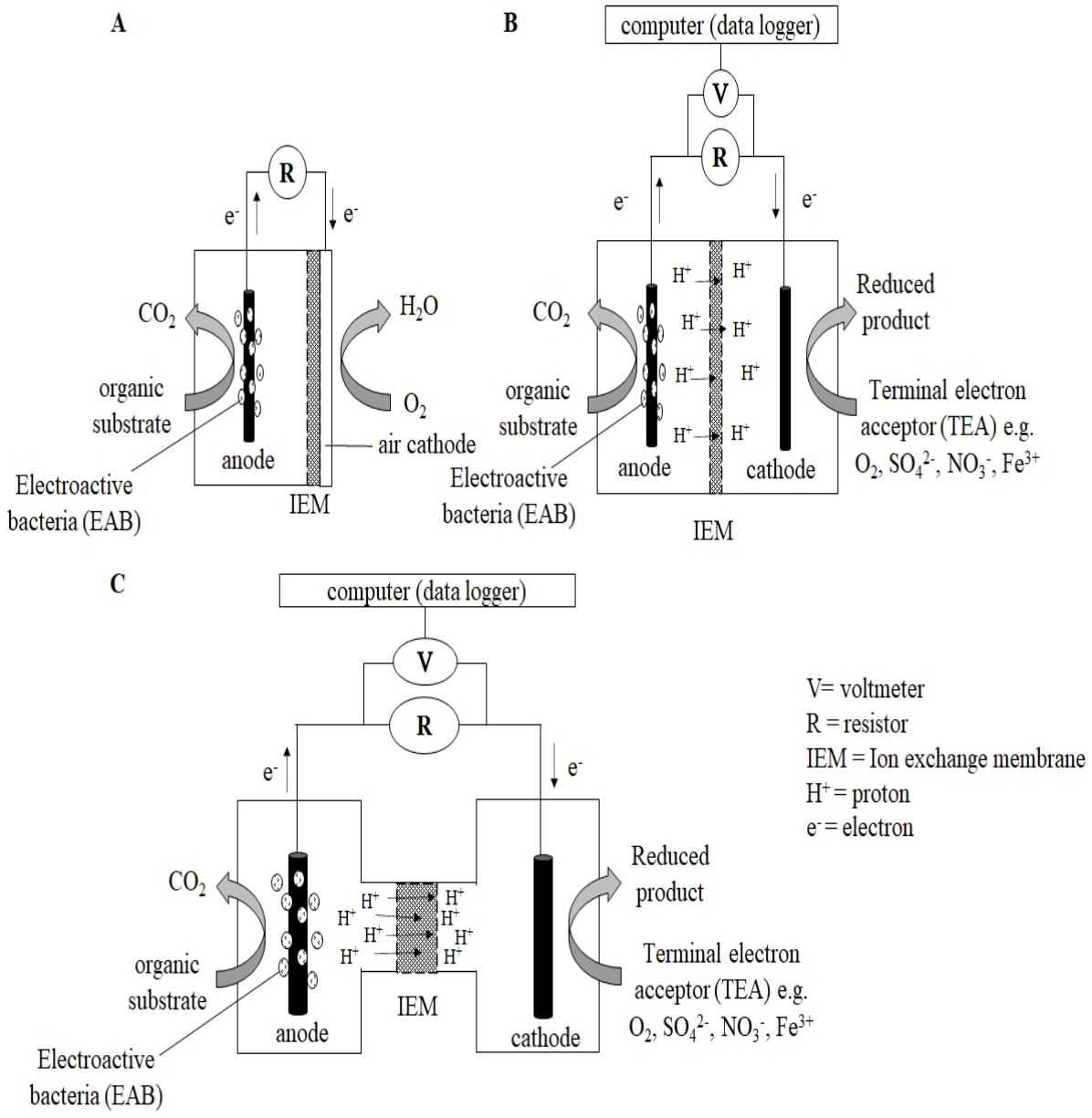


Figure 1-1. Schematic diagrams of microbial fuel cells: (A) single chamber with air-cathode, (B) double chamber (cube-cell), and (C) double chamber (H-cell).

Single chamber MFCs used for removal of contamination from soil and sediment are appropriately referred to as sediment microbial fuel cells (SMFC), marine microbial fuel cells

or benthic microbial fuel cells (Li and Yu, 2015; Zabihallahpoor et al., 2015). A sediment microbial fuel cell (SMFC) or benthic microbial fuel cell (BMFC), is a single chambered MFC with anode buried in a layer of sediment and cathode suspended in overlying water, as shown in Figure 1-2.

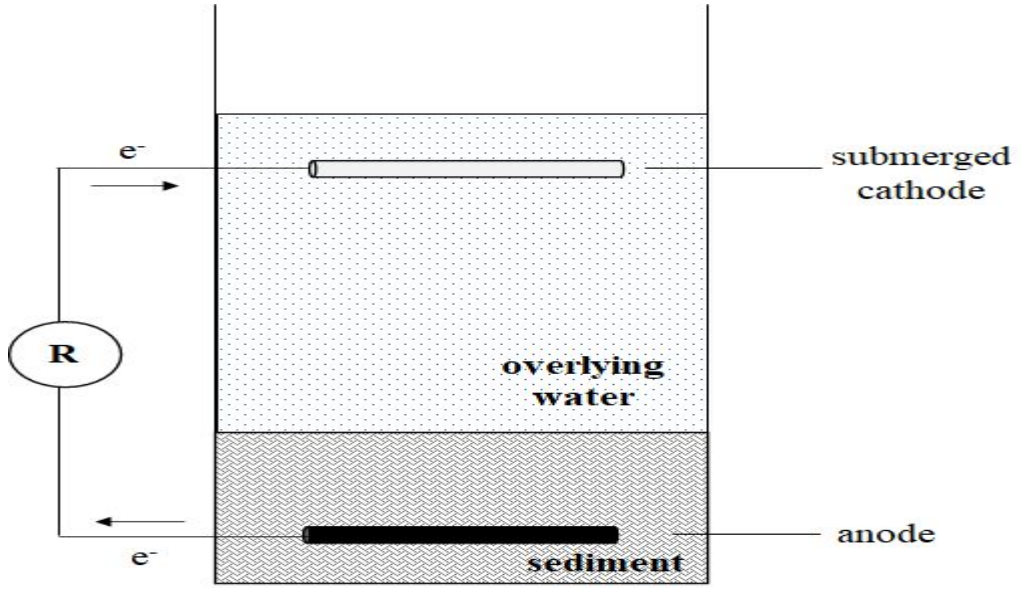


Figure 1-2. Schematic diagram of sediment or benthic sediment microbial fuel cell with anode buried in anoxic/anaerobic sediment and cathode submerged in overlying aerobic water (e^- = electron).

Biodegradation of petroleum hydrocarbons have been extensively studied using numerous MFC configurations. However, in the anoxic or anaerobic sediment zone, SMFC appear to be more suitable for *in-situ* bioremediation of these soils/sediments which serve as sinks or reservoirs for petroleum hydrocarbons (Sajana *et al.*, 2016; Viggi *et al.*, 2017). SMFC is a smart choice for *in-situ* bioremediation of soils/sediments contaminated with petroleum hydrocarbons for several reasons: (1) sediment and overlying water contaminated with petroleum hydrocarbons could be simultaneously treated (Sajana *et al.*, 2016); (2) absence of cation/proton exchange membrane reduces cost of construction of SMFC (Leong *et al.*, 2013); (3) absence of cation/proton exchange membrane could reduce charge transfer resistance leading to improved system performance (Liu and Logan, 2004; Leong *et al.*, 2013), and (4) electric current generated by SMFC can be used to power remote devices (Zhang *et al.*, 2011a; Donovan *et al.*, 2013; Thomas *et al.*, 2013; Liu *et al.*, 2015).

1.2 Research Problem and Hypothesis

As already underscored, a key advantage for use of MFC for bioremediation is removal of petroleum hydrocarbon from environment and concomitant energy recovery as electric current. However, adequate biofilm growth is required to achieve good MFC performance. The specific

type of contaminant (BTEX or PAHs) and its interaction with environmental matrix including pH, temperature, soil type, organic matter content of soil, and conductivity etc. can influence changes in microbial community in anodic biofilm (bioanode) of MFC and impact MFC performance (Atlas, 1981; Boopathy, 2000; Chae *et al.*, 2009; Pant *et al.*, 2010; Chandra *et al.*, 2013; Sajana *et al.*, 2016). Biofilm growth and development can be enhanced by using inoculum from different sources such as aerobic or anaerobic sludge, diesel-contaminated MFC, municipal waste, hydrocarbon contaminated groundwater, acetate-fed MFC, MFC treating wastewater or in situ sediment (Churchill *et al.*, 1995; Morris and Jin, 2009; Yates *et al.*, 2012; Adelaja, 2015; Venkidusamy *et al.*, 2016). The source of inoculum used for biofilm enrichment influences microbial community diversity at start-up of MFC; however, structure and function of microbial community is modified over time according to available fuel source and prevailing environmental conditions in MFC (Holmes *et al.*, 2004; Rabaey *et al.*, 2004; Chae *et al.*, 2009; Torres *et al.*, 2009; Yates *et al.*, 2012; Pasternak *et al.*, 2018; Zhang *et al.*, 2018). The mechanism and dynamics of microbial biodegradation of petroleum hydrocarbons are not yet fully understood.

Other challenges common to MFCs are low power output, non-continuous power output and scalability (Logan *et al.*, 2006). Various limitations have been identified in several MFC/SMFC designs and configurations used to study biodegradation of petroleum hydrocarbons. For instance, type of electrode material used, external resistance, the system design and configuration can influence biodegradation efficiency in SMFC. Carbon materials are quite effective but are expensive especially for scale-up purposes. Cost–effective materials such as stainless steel or modified electrodes are desirable for overall cost reduction while maximizing performance. Large distances between anode and cathode can cause ohmic losses and mass transfer limitations (Sajana *et al.*, 2016; Girguis *et al.*, 2020). High external resistance reduces active biomass and energy gain for electroactive bacteria leading to increased charge transfer resistance and reduced power output (Zhang *et al.*, 2011b; Pasternak *et al.*, 2018). Biofouling or electrode passivation can cause a decline in power output (Tender *et al.*, 2002). Increases in pH within the SMFC bioreactor may slow down or inhibit the activity of the electroactive bacteria. The presence of heavy metals could inhibit biodegradation of petroleum hydrocarbons. The area of sediment treated in SMFC is confined to a radius of influence (ROI) of 1 – 34 cm. ROI can be influenced by soil characteristics and SMFC electrode design (Lu *et al.*, 2014b; Daghio *et al.*, 2017; Wang *et al.*, 2019). The amount of organic matter content below 5% or above 10% in sediments may also diminish biodegradation of petroleum hydrocarbon (Zhao *et al.*, 2016). Low oxygen concentrations in overlying water may cause decline in cathodic oxygen reduction reaction (ORR) and impact on hydrocarbon removal efficiency (Wang *et al.*, 2012a).

To tackle the afore-mentioned challenges, this research will focus on developing appropriate configuration for performance optimization of MFC/SMFC for effective biodegradation of petroleum hydrocarbons in water and sediment. This research is expected to provide further understanding of microbial communities associated with hydrocarbon degradation; and MFC/SMFC configurations for performance evaluation for biodegradation of petroleum hydrocarbons.

The research questions to be answered are:

i. Can anodic biofilms developed from selected inocula improve hydrocarbon removal efficiency and power output of MFC/SMFC during bioremediation of petroleum hydrocarbons?

ii. Can changes in MFC/SMFC bioreactor design and configuration boost simultaneous improvement in hydrocarbon removal efficiency and MFC/SMFC current output?

iii. How are improvements in hydrocarbon removal linked with composition of microbial community on MFC/SMFC bioanode?

The research is based on the following hypotheses:

i. Anodic biofilms enriched with selected inocula can have quicker start-up time and improve current output and hydrocarbon removal efficiency of MFC during bioremediation of petroleum hydrocarbons.

ii. MFC/SMFC bioreactor design and configuration can be made to optimize simultaneous improvement in hydrocarbon removal and power output.

iii. Composition of microbial community on MFC/SMFC bioanode can be linked with improvements in hydrocarbon biodegradation.

1.3 Research Aims and Objectives

The aim of the research project is to develop microbial fuel cells (MFCs) and optimize its performance for bioremediation of water and soil/sediment contaminated with petroleum hydrocarbons. The research objectives are to:

i. Apply selected inocula to enrich and develop active anodic biofilms able to degrade petroleum hydrocarbons.

ii. Design and investigate different bioreactor configurations for biodegradation of petroleum hydrocarbons.

iii. Characterise microbial community and their changes on bioanode in MFC/SMFC for bioremediation of petroleum hydrocarbons.

Expected bias, risks and limitations of the study include, but not limited to, the following:

i. Limited or non-availability of resources (equipment, materials) could impact on the quality of data generated.

ii. The interference of other redox processes occurring within sediment and overlying water may impact on performance of microbial fuel cell/sediment microbial fuel cell.

1.4 Structure of the Thesis.

The main chapters of the thesis are as follows:

Chapter 1. Introduction.

Chapter 2. Literature Review.

Chapter 3. Methodology.

Chapter 4. Application of enriched bioanodes for enhancement of hydrocarbon removal efficiency in the presence of surfactant.

Chapter 5. Performance evaluation of sediment microbial fuel cell (SMFC) for removal of petroleum hydrocarbons from contaminated soil and sediment.

Chapter 6. Microbial community analysis of bioanodes of microbial fuel cells used for biodegradation of petroleum hydrocarbons.

Chapter 7. Conclusion and Future Work.

References.

Chapter 8. Appendices.

Chapter 2 Literature Review

2.0 Chapter summary

This chapter briefly describes principles, types, and applications of bio-electrochemical systems (BES), as well as limitations of BES, with emphasis on microbial fuel cells (MFCs) for environmental remediation. Electrode materials, membrane materials, bioreactor configurations, and operating conditions of MFC for treatment of hydrocarbon contaminated water, soil and sediments are also presented. Microbial mechanism for biodegradation of petroleum contaminants (hydrocarbons) under aerobic and anaerobic conditions, and diversity of micro-organisms involved in anaerobic biodegradation (specifically MFC) is reviewed in this chapter. Factors affecting petroleum hydrocarbon degradation, sediment MFC modifications to address these challenges, and surfactant integration in MFCs are emphasized. *Part of this chapter has been published as chapter 16 in the book: *Microbial Electrochemical Technologies*, Boca Raton: CRC Press (p. 244) edited by Sonia M. Tiquia-Arashiro and Deepak Pant in 2020 with the title 'Microbial fuel cell sensors for water and wastewater monitoring'. All authors (Sharon Velasquez-Orta, Ekaete Utuk and Martin Spurr) contributed equally to the publication.

2.1 Bio-electrochemical Systems

Bio-electrochemical systems (BES) are emerging technologies that utilize biocatalysts to produce energy from organic substrates. BES were originally explored as a source of energy. The earliest documentation of concomitant production of electrical energy by microbes during oxidation of organic compounds involved use of different substrate concentrations and temperature (Potter, 1911). Decades of research have led to improved configurations that can generate 5 to 10 times higher voltage output (Aelterman *et al.*, 2006). Conditions and parameters of BES can be adjusted to favour removal of specific organic and inorganic compounds, with the concomitant generation of energy (Jadhav and Ghangrekar, 2009; Velasquez-Orta *et al.*, 2011a). Accordingly, BES has potential for waste treatment and remediation of environmental contaminants (Chandrasekhar and Venkata Mohan, 2012; Ledezma *et al.*, 2013; Chakraborty *et al.*, 2020). Extensive research over the last 30 years has enhanced knowledge and understanding of BES, resulting in improved configurations and its potential applications as a sustainable, environmentally friendly source of energy and environmental remediation (Santoro *et al.*, 2017; Daghio *et al.*, 2018a; Chakraborty *et al.*, 2020).

2.1.1 Principles of bio-electrochemical systems

As earlier mentioned, BES generate measurable signals (such as light or electrical energy) by using biological recognition elements to catalyst electrochemical reactions on electrode surfaces (either the anode/cathode or both electrodes). BES using bacteria as catalyst can be sub-divided based on their application; these include microbial fuel cell, microbial electrosynthesis cell, microbial solar cell, and microbial desalination cell. BES with potential for environmental remediation include microbial fuel cell, microbial remediation cell and microbial electrochemical snorkel.

A typical *Microbial Fuel Cell (MFC)* comprises two electrodes (anode and cathode) in different chambers and separated by an ion or proton exchange membrane (IEM or PEM). Both MFC and sediment MFC (SMFC) have already been described in Chapter 1 of this thesis. *Microbial Remediation Cell (MRC)* are MFCs modified for removal of contaminants from the environment, using either anode as electron acceptor (Luo *et al.*, 2009; Pham *et al.*, 2009; Zhang *et al.*, 2009) or cathode as electron donor (Gregory and Lovley, 2005; Aulenta *et al.*, 2008). MRCs can be applied for in-situ bioremediation of contaminants in groundwater and sediment (Morris and Jin, 2007; Yuan *et al.*, 2010; Wang *et al.*, 2012b). Current density produced by MRCs may be applied for in situ monitoring of bioremediation (Williams *et al.*, 2010). *Microbial Electrochemical Snorkel (MESN)* consists of anaerobic anode and aerobic cathode coupled as one unit. The anode and cathode may be made of either same or different materials (Erable *et al.*, 2011). MESN cathode may be biotic or abiotic. MESNs do not generate any power, hence they are sometimes referred to as short-circuited MFCs (Hoareau *et al.*, 2019). MESN operate at maximum current, thereby increasing the rate of electrochemical reactions (Erable *et al.*, 2011). MESNs have been employed for chemical oxygen demand (COD) removal, nitrogen removal, wastewater treatment, and soil remediation (Erable *et al.*, 2011; Cruz Viggi *et al.*, 2015).

In BES, chemical energy in organic substrate is converted to electrical energy. The transfer of electrons from bacterial cell to anode of BES is an important process that connects microbiology and electrochemistry and determines the theoretical limits of the energy conversion. Thus, insight on electron transfer mechanisms and energy conversion is fundamental to understanding how BES work.

2.1.2 Electron Transfer Mechanism and Energy Conversion Efficiency

Electrochemically active bacteria (EAB) in BES obtain carbon from organic substrates and derive energy from Gibb's energy of oxidation of organic substrates via respiration or fermentation. (Schröder, 2007). A key determinant of energy conversion potential of MFC is

efficiency of anodic electron transfer. Let us assume reactions occurring at anode and cathode of MFC using acetate as substrate are as shown in equations 2-1 and 2-2:

Anode (oxidation): $CH_3COO^- + 4\,H_2O \rightarrow 2HCO_3^- + 9\,H^+ + 8\,e^-$ *2-1*

Cathode (reduction): $2O_2 + 8H^+ + 8e^- \rightarrow 4\,H_2O$ *2-2*

The electromotive force (emf) of MFC is calculated as potential difference between anode and cathode (see equation 2-3). It is the maximum potential/voltage achievable under open circuit conditions (i.e. when no current is flowing). The relationship between E_{emf} and Gibb's free energy (ΔG) as shown in equation 2-4:

$$\Delta E^{\circ}_{emf} = \Delta E^{\circ}_{cathode} - \Delta E^{\circ}_{anode} \qquad 2\text{-}3$$

$$\Delta G^{\circ} = -nF\Delta E^{\circ}_{emf} \qquad 2\text{-}4$$

where: ΔG° = Gibb's free energy (kJ mol^{-1}) under standard conditions (temperature 25^{o}C, pressure 0.1MPa, concentration 1 mol dm^{-3})

ΔE°_{emf} = cell potential (V) under standard conditions

F = Faraday's constant (96485 C mol^{-1})

n = number of electrons transferred during the reaction.

Oxidation of complex organic compounds such as hydrocarbons generate several electron donors (including ethanol, formate, acetate). Hence, theoretical value of ΔG° is very large and can be estimated from total number of electrons extracted from these electron donors as well as heat of combustion of the hydrocarbon compound (Roux *et al.*, 2008) (see Appendix A).

Total Gibb's free energy in MFC is apportioned between EAB utilization and conversion to electricity as shown in equation 2-5:

$$\Delta G^{\circ}_{TOTAL} = \Delta G^{\circ}_{EAB} + \Delta G^{\circ}_{ELEC} \qquad 2\text{-}5$$

where: ΔG°_{TOTAL}= total energy in MFC (kJ mol^{-1})

ΔG°_{EAB} = energy utilized by EAB

ΔG°_{ELEC}= energy available as electricity.

The total energy in MFC system, described in terms of the relationship between cell potential, temperature and concentration of the reactants and products, can be expressed by the Nernst equation (see equation 2-6 below)

$$E_{emf} = E^{0}_{emf} - \frac{RT}{nF} In\,[Q_r] \qquad 2\text{-}6$$

where:

R = gas constant (8.31447 J K^{-1}mol^{-1})

T = absolute temperature (298 K)

In [Q_r] = natural logarithm of reaction quotient Q_r, where $Q_r = \frac{[\text{products}]}{[\text{reactants}]}$.

Electrons transport from EAB to anode is facilitated by linking species (or mediator). Energy utilized by EAB is influenced by redox potential of linking species (Schröder, 2007; Liu *et al.*, 2020; Pirbadian *et al.*, 2020). This relationship is shown in equations 2-7 and 2-8.

$$\Delta G^{\circ}_{EAB} = nF(\Delta E^{\circ}_{anode} - \Delta E^{\circ}_{link}) \quad 2\text{-}7$$

$$\Delta G^{\circ}_{ELEC} = nF(\Delta E^{\circ}_{link} - \Delta E^{\circ}_{cathode}) \quad 2\text{-}8$$

Electrons are transported from EAB to anode either by non-mediated or direct electron transfer (DET) or by mediated electron transfer (MET).

Direct Electron Transfer. DET requires physical contact between bacterial cell and solid electron acceptor either through membrane bound proteins, electron transfer molecules or conductive nanowires. Membrane bound electron transport proteins (c-type cytochrome multi heme proteins) carry electrons from inside the bacteria to outside the bacterial membrane. As shown in Figure 2-1A, membrane-bound cytochromes on outer bacterial membrane transfer electrons to solid electron acceptors. Some bacteria develop conductive appendages or pili that facilitate electron transport (Reguera *et al.*, 2005; Gorby *et al.*, 2006). These conductive pili or nanowires are connected to outer membrane bound cytochromes, thus promoting development of thicker anodic biofilms and increased MFC performance (Schröder, 2007). Nanowires permit transfer of electrons between bacteria and electrodes that are not in direct contact.

Mediated Electron Transfer. Electron transfer can be achieved in the absence of direct contact between bacteria and solid electron acceptor by means of chemical species that exhibit reversible redox activity. These chemical species are known as mediators or electron shuttles. Electron shuttles are reduced as they harvest electrons within EAB and carry these across the bacterial membrane. These shuttles are converted back to their oxidized form when they release the electrons to MFC anode, as shown in Figure 2-1B. Electron shuttles may be exogenous (externally produced) or endogenous (produced by bacteria). Exogenous mediators include naturally-occurring humic acid or metal chelates (Hernandez and Newman, 2001; Schröder, 2007) or synthetic compounds (artificial mediators) such as neutral red, methylene blue, thionine, riboflavin, anthraquinone-2-sulphonate (AQS), and 2-hydroxy-1,4-naphthoquinone (HNQ) (Park and Zeikus, 2000; Ieropoulos *et al.*, 2005; Adelaja *et al.*, 2015). On the other hand, endogenous mediators include flavins (*Shewanella*) (Marsili *et al.*, 2008a), pyocyanin and phenazine-1-carboxamide (*Pseudomonas aeruginosa*) (Hernandez and Newman, 2001). Reduced metabolic products of oxidation of biodegradable substrate (e.g. sulfate/sulfide redox couple, hydrogen) may also be used as electron shuttles (Schröder, 2007).

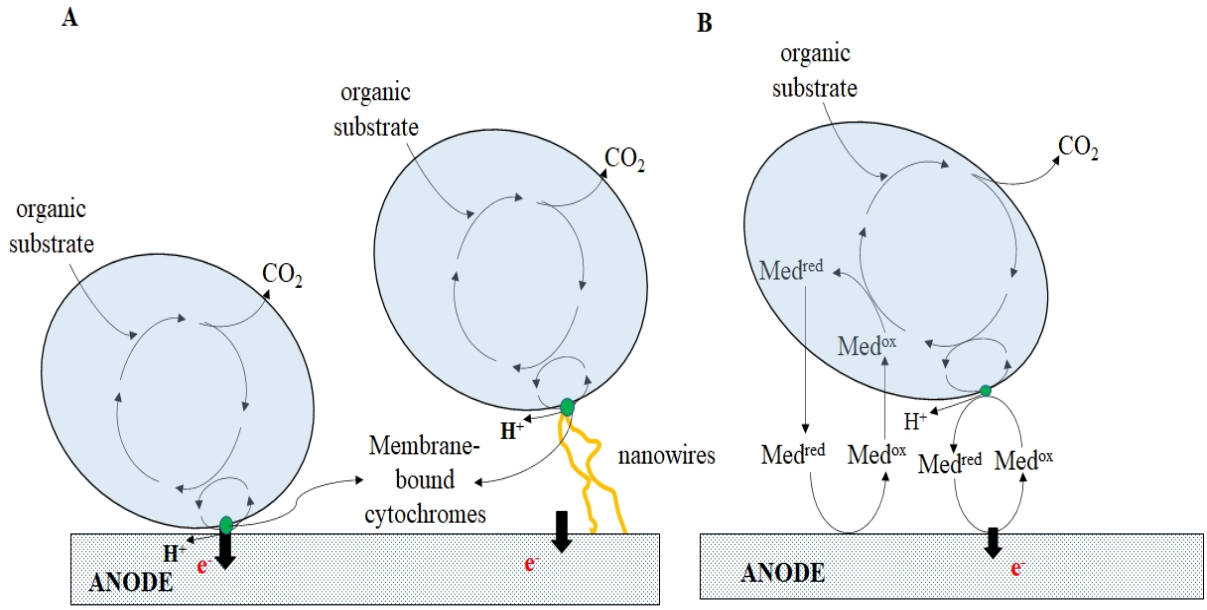


Figure 2-1. Diagram of electron transfer mechanisms: (A) Direct electron transfer (DET) using membrane-bound cytochromes and nanowire; and (B) Mediated electron transfer (MET) using electron shuttles via membrane-bound cytochromes and redox couples (Med^{red}=reduced form of mediator and Med^{ox}=oxidized form of mediator)(adapted from Schröder (2007)).

Besides energy loss from bacterial utilization for growth, energy losses occur through overpotentials or polarizations. Sources of overpotentials are: (i) energy required for reactions to proceed (activation losses), (ii) energy used to overcome internal resistance (ohmic losses) and (iii) mass transport of chemical species (concentration losses) (Logan *et al.*, 2006; Venkata Mohan *et al.*, 2014). Thus, MFC cell potential (E_{MFC}) in relation to its theoretical electromotive force (E_{emf}) is modified as shown below:

$$E_{MFC} = E_{emf} - \eta_{ohm} - \eta_{act} - \eta_{concn} \qquad 2\text{-}9$$

where η_{ohm} = ohmic losses

η_{act} = activation losses

η_{concn} = concentration losses

Coulombic efficiency (CE) is the ratio of actual electrons recovered to the total number of electrons available for recovery, and is a measure of the efficiency of conversion of electrons transferred to electric energy (Logan *et al.*, 2006). Electroanalytical techniques such as cyclic voltammetry, electrochemical impedance spectroscopy and polarization curves are used to study MFC electron transfer mechanisms (Fricke *et al.*, 2008; Marsili *et al.*, 2008b; Zhao *et al.*, 2009). Cyclic voltammetry technique is discussed in section 3.6.3.

2.1.3 Factors that influence performance of bio-electrochemical systems

The performance of BES is influenced by several physicochemical and biological factors. Physicochemical factors include electrode and membrane materials, electrode spacing,

bioreactor configuration, nature of substrate and operating conditions (i.e. temperature, pH, salinity). Biological factors include source of inoculum and nature of electroactive biofilm on anode. Electrode and membrane materials as well as BES configuration for hydrocarbon removal are discussed in section 2.2.

The nature of substrates influences power output, coulombic efficiency, and bacterial diversity in MFCs, with acetate being commonly preferred substrate for electricity generation because of its simple structure (Chae *et al.*, 2009; Pant *et al.*, 2010). As mentioned earlier in section 2.1.2, coulombic efficiency is a measure of the efficiency of conversion of electrons extracted (from organic substrate) to electric current. Simple organic substrate such as acetate has higher coulombic efficiency (65%) compared with more complex organic substrates such as starch (21%), dextran (17%), and glucose (14%) (Min and Logan, 2004). BES may be operated in batch mode where BES is fed at periodic intervals or continuous flow mode where BES is fed at a specified flow rate (Jang *et al.*, 2004; Tartakovsky and Guiot, 2006). Partial pressure of hydrogen (pH) plays a vital role in determining movement of electrons and ions and current/power output because it regulates microbial growth and diversity, and microbial metabolic pathway of available substrate (Veer Raghavulu *et al.*, 2009). Power output, COD removal and CE improves with increase in temperature (Larrosa-Guerrero *et al.*, 2010). MFCs can be operated between 20^{o}C and 55^{o}C. Maximum power density in MFCs have been reported at salinity between 1 and 1.5% (w/v) (Adelaja *et al.*, 2015). Increase in solution conductivity also enhances system performance (Cheng and Logan, 2011).

2.1.4 Applications of Bio-electrochemical systems (BES)

MFCs offer the advantage of simultaneous *wastewater treatment and energy recovery* (Gude, 2016). MFCs require ten times less energy input (0.024 kWh/m^3/ 0.076 kWh/kg COD) compared with aerobic-activated sludge or anaerobic digestion process (0.3 kWh/m^3/ 0.6 kWh/kg COD) (McCarty *et al.*, 2011; Zhang *et al.*, 2013a). These statistics highlight the potential for energy recovery from wastewater using MFCs. MFCs are used for w*ater and wastewater monitoring*, where EAB on bioanode is employed as biosensing probe. Interactions between EAB and target analyte impact on current/voltage output which is monitored and recorded in real-time as calibration signal for biosensors (Modin and Wilén, 2012; Jiang *et al.*, 2018; Velasquez-Orta *et al.*, 2020). MFC biosensors can provide real-time data within 1 to 36 minutes compared with conventional BOD and COD measurements which take up to 5 days (Moon *et al.*, 2004; Kumlanghan *et al.*, 2007; Kaur *et al.*, 2013; Di Lorenzo *et al.*, 2014; Pasternak *et al.*, 2017). By coupling anodic oxidation of organic matter with cathodic reduction, MFC has been used for *recovery of resources* such as nitrogen, phosphorus as well as

conversion of carbon dioxide to useful chemicals (e.g. formate, ethanol) (Yu, 2016). MFCs used for *environmental remediation* are based on exploitation of redox gradient between MFC electrodes and contaminants to remove contaminants by either anodic oxidation or cathodic reduction reactions (Wang *et al.*, 2015).

2.1.5 Limitations of bio-electrochemical systems

BES technology has numerous advantages such as low environmental impact because additional chemicals are not required, which could generate unwanted by-products. They generate electricity and as well as valuable chemicals. They can be integrated with other technologies e.g. greenhouses, rice paddy fields or wastewater and surface water treatment (Kaku *et al.*, 2008; Strik *et al.*, 2008; Hamelers *et al.*, 2010). BES require low energy input/consumption. For instance for hydrogen (H_2) production, energy input via MEC was 0.6 – 1.12 kWh/m^3 compared with energy input of 4.5 – 5 kWh/m^3 via water electrolysis (Liu *et al.*, 2005; Lu *et al.*, 2009). BES have low biomass production compared with conventional wastewater treatment systems such as anaerobic digestion (Gude, 2016).

Nevertheless, BES technology is limited by several factors. First, BES produce lower voltage than thermodynamically predicted because of activation, ohmic and concentration losses (Pandit *et al.*, 2017). Second, power density and contaminant removal efficiencies can be diminished because of inefficient electron transfer mechanisms (Venkata Mohan *et al.*, 2014). Third, limited understanding of electrochemically active bacteria and their role in electron transfer restrict power output and coulombic efficiency (Logan and Regan, 2006b). Fourth, system performance diminishes with increase in bioreactor size because of increase in ohmic resistance and voltage reversal (for series/parallel configuration); more data and field studies are required to improve system performance during BES scale up (Oh and Logan, 2007b; Logan, 2010). Fifth, optimization of BES system performance for commercialisation requires a combination of low-cost materials, efficient bioreactor configuration and suitable operating conditions (Venkata Mohan *et al.*, 2014). Strategies for tackling challenges mentioned above are presented in the following sections.

2.2 Microbial fuel cells (MFCs) for hydrocarbon removal from the environment

2.2.1 Electrode materials

Anode. Primary reactions in MFCs occur at the anode where the biofilm is formed, hence the name anodic biofilm or bioanode (Mohan *et al.*, 2008a; Rousseau *et al.*, 2013). MFC performance is greatly influenced by ability of anode to facilitate bacterial adhesion to electrode

surface, substrate oxidation, and electron transfer between the biofilm and the anode (Dumitru and Scott, 2016). Consequently, the anode surface must be: biocompatible; mechanically strong; highly conductive; chemically stable; highly porous; resistant to corrosion; low-cost; scalable; and have high surface area (Guo *et al.*, 2015; Sajana *et al.*, 2016). Carbon materials are commonly used as anode materials for MFCs because of their high conductivity, high specific surface area, good biocompatibility, corrosion-resistance, and low cost.

Most materials used for anodes in MFCs for hydrocarbon removal are carbon-based such as carbon cloth (Morris and Jin, 2012), carbon fiber brush (Venkidusamy *et al.*, 2016), graphite plate (Mohan and Chandrasekhar, 2011; Sherafatmand and Ng, 2015), graphite granules (Lu *et al.*, 2014b), graphite fiber (Rakoczy *et al.*, 2013; Xu *et al.*, 2017), and polyvinyl alcohol (PVA) coke (Chang *et al.*, 2017). Carbon fiber has high surface area, high porosity, and high electrical conductivity (Li *et al.*, 2016c). Metal-based or other materials used in MFCs for hydrocarbon degradation, including stainless steel (scrubber, mesh or plate) (Dumas *et al.*, 2007; Erable and Bergel, 2009; Morris *et al.*, 2009), stainless steel cylinder (Yan *et al.*, 2012), and biochar (Lu *et al.*, 2014a). Anode performance can be improved by physical or chemical modifications. Modified anodes used for hydrocarbon removal include carbon cloth modified with gas diffusion layer (GDL) and polytetrafluoroethylene (PTFE) (Li *et al.*, 2013) and carbon nanomaterial modified graphite felt (graphene, graphene oxides and carbon nanotubes) (Liang *et al.*, 2020).

Cathode. Cathodic reaction has been identified as the limiting factor of MFCs (Bajracharya *et al.*, 2016). In view of the critical importance of cathode in MFC performance, it is desirable that cathode should have high redox potential and proton capture capability (Zhou *et al.*, 2011a). Cathode materials must be chemically stable, highly conductive, mechanically strong, and inexpensive. Materials used for cathodes in MFCs for hydrocarbon removal include platinum-carbon (Pt-C) mesh (Wang *et al.*, 2012b), Pt-coated carbon paper or cloth (Morris *et al.*, 2009; Morris and Jin, 2012), Pt-coated carbon felt (Adelaja *et al.*, 2015), carbon fiber (Xu *et al.*, 2017), graphite plate (Sherafatmand and Ng, 2015) graphite rod (Zhang *et al.*, 2010), stainless steel cylinder (Yan *et al.*, 2012), stainless steel mesh (Bellagamba *et al.*, 2017), carbon cloth modified with PTFE on the air side (Li *et al.*, 2013; Venkidusamy *et al.*, 2016), activated carbon cloth with Pt/C catalyst layer (Lu *et al.*, 2014a), and stainless steel mesh modified with Pt/C catalyst layer on one side and polydimethylsiloxane (PDMS) on the other side (Lu *et al.*, 2014b). Different cathode materials have produce different hydrocarbon removal efficiencies (24.4 to 100 %) (Zhang *et al.*, 2010; Morris and Jin, 2012).

A common terminal electron acceptor (TEA) for cathodic reduction reaction is oxygen. Cathode materials are modified with catalysts to expedite oxygen reduction reaction (ORR) kinetics

(Bajracharya *et al.*, 2016). ORR cathodes may be configured as 1) aqueous or submerged cathodes using dissolved oxygen available in bulk liquid/water in which it is suspended; 2) air cathodes which use free oxygen in air; and 3) biocathodes where ORR is catalysed by micro-organisms grown on cathode surface (Mustakeem, 2015; Bajracharya *et al.*, 2016)

2.2.2 Membrane materials

A separation of anodic and cathodic chambers is important to prevent chemical imbalance, short circuiting and polarization losses triggered by movement of unwanted chemicals or substrate from one chamber to another, because these events lower coulombic efficiency of MFCs. Good separator materials exhibit properties such as chemical inertness, mechanical strength, good ionic conductivity, and zero electronic conductivity (Leong *et al.*, 2013). Examples of materials applied as separators for MFCs treating hydrocarbon contaminated matrix include glass fibre (Wang *et al.*, 2012b), polyvinyl alcohol (PVA) separator (Chang *et al.*, 2017), and proton bridge (salt bridge) (Mohan *et al.*, 2008b; Morris *et al.*, 2009; Li *et al.*, 2011).

Membranes are semipermeable selective separators that allow for diffusion of selected/relevant ions; hence pore size, porosity and ion exchange capacity of a membrane is crucial to delivery of its requisite function (Scott, 2016a). Since membrane materials contribute to the overall cost of MFC, the material used must also be cost-effective while delivering high performance. Cation exchange membranes (CEMs) are selectively permeable to cations/protons (Erable *et al.*, 2011). The most common type used in MFCs for hydrocarbon removal is perfluorosulfonic acid (Nafion®) (Mohan and Chandrasekhar, 2011; Friman *et al.*, 2012). Other CEMs used include Ultrex® CMI 7000 (Pham *et al.*, 2009; Daghio *et al.*, 2016; Adelaja *et al.*, 2017), Hyflon® (perfluoropolymer membrane) and Zirfon® (85 % ZrO_2 powder and 15% polysulfone) (Li *et al.*, 2011). Anion exchange membranes (AEMs) are selectively permeable to anions (Scott, 2016a). Membrane biofouling remains one of the main challenges in MFCs because it can reduce membrane durability, hinder proton transfer, and lower MFC performance. Sediment MFCs do not require membranes. Although the absence of membrane can increase cathode potential and reduce internal resistance, challenges of these types of systems include oxygen diffusion into anodic chamber and reduced coulombic efficiency (Jang *et al.*, 2004; Liu and Logan, 2004).

2.2.3 MFC bioreactor configurations and operating conditions

Maximum power production coupled with maximum wastewater treatment/contamination removal is achieved by establishing optimal MFC configuration and operating conditions (Premier *et al.*, 2016). Common MFC reactor configurations include single chamber, double

chamber, and membrane electrode assembly (MEA). Single chamber MFC comprise anode and cathode in one compartment, and may or may not include a membrane (Osman *et al.*, 2010). They are simple in design, cost effective and practical (Park and Zeikus, 2003). They are fabricated in different shapes, including polyvinyl chloride (PVC) tube (Adelaja *et al.*, 2017), plexi-glass cylinder (Zhou *et al.*, 2020), and serum bottle (Daghio *et al.*, 2018a). Double-chamber MFCs have been fabricated in different shapes, for example: "H cell" (consisting of anode and cathode chambers connected by a horizontal tube) (Logan *et al.*, 2006); cylinders (Wei *et al.*, 2015), cubic Perspex frames (Li *et al.*, 2013), plexiglass bottles (Adelaja *et al.*, 2015), or rectangles (Du *et al.*, 2007). Membrane electrode assemblies (MEAs) are compact MFC configurations made by combining anode, cathode, and membrane into a single unit (Miyahara *et al.*, 2013). MFCs can generate higher current when connected in series (stacked), however, voltage reversal and subsequent loss of power may occur if there is loss of bacterial activity due to fuel starvation (Oh and Logan, 2007a; Ieropoulos *et al.*, 2013). Both double chamber and single chamber MFCs may be operated in fed-batch (Morris *et al.*, 2009; Adelaja *et al.*, 2015; Venkidusamy *et al.*, 2016) or continuous flow mode (Pham *et al.*, 2009; Rakoczy *et al.*, 2013; Chang *et al.*, 2017). Most studies on microbial fuels cells for hydrocarbon removal have been studied using operating temperatures between 20 - 30˚C (Morris *et al.*, 2009; Chandrasekhar and Venkata Mohan, 2012; Morris and Jin, 2012; Lu *et al.*, 2014a; Cruz Viggi *et al.*, 2015; Sherafatmand and Ng, 2015; Hemalatha *et al.*, 2020), with few studies conducted above 30 ˚C (Adelaja *et al.*, 2015; Guo *et al.*, 2015; Sheikhyousefi *et al.*, 2017).

2.2.4 Microbial fuel cells for removal of petroleum hydrocarbons from aqueous media

Most MFCs studied for treatment of hydrocarbon contaminated wastewater are configured as double chamber cells using cation or proton exchange membrane as separator and operated using batch-fed mode. Most of these studies employed mixed culture inoculum for MFC start-up. Although, over 90% electron recovery or hydrocarbon degradation efficiency has been reported with pure culture, higher degradation rates can be obtained using mixed culture inoculum (Zhang *et al.*, 2010; Adelaja *et al.*, 2014a). In several studies, reported treatment efficiencies for hydrocarbon-contaminated wastewater/medium is mostly in the range of 50 to 100 %, and accompanied by low power output (< 1W m^{-2}). High treatment efficiencies may be due to high water content in aqueous media which support high mass transfer rates (Kronenberg *et al.*, 2017). A summary of some MFCs used for hydrocarbon removal from wastewater or aqueous media is provided in Table 2-1.

Table 2-1. Microbial Fuel Cells used for treatment of hydrocarbon-contaminated wastewater/media.

S/N	Anode	Cathode	Membrane	MFC configuration	Medium treated	Contaminant	Removal Rate (%)	Maximum current/power density	Reference
1	Carbon cloth	Carbon cloth	PEM (Nafion 212)	Double chamber	Mineral medium	Phenol	90	28.3 W m^{-3}	(Luo *et al.*, 2009)
2	Stainless steel scrubber	Pt/C paper	Proton bridge	Double chamber	Groundwater	Diesel	82	32 mW m^{-2}	(Morris *et al.*, 2009)
3	Graphite rod	Graphite rod	CEM (Ultrex CMI 7000)	Double chamber	Mineral medium	1,2-DCA	98	*15.2 W m^{-3}	(Pham *et al.*, 2009)
4	Graphite fiber brush	Carbon cloth	CEM (Ultrex CMI 7000)	Double chamber	Mineral medium	Nitrobenzene	88.2	-	(Wang *et al.*, 2011)
5	Graphite rod	Pt/carbon cloth	PEM (Nafion 115)	Double chamber	Mineral medium	Phenol	*80.24	478 mA m^{-2}	(Friman *et al.*, 2013)
6	Carbon cloth coated with GDL & PTFE	Pt carbon cloth with outer PTFE layer	Nutrient reservoir	Double chamber	Gas steam	Toluene	91.2	6.19 mW m^{-2}	(Li *et al.*, 2013)
7	Graphite fiber	Graphite fiber	CEM (Nafion 117)	Double chamber	Contaminated groundwater	Benzene	18-80	-	(Rakoczy *et al.*, 2013)

S/N	Anode	Cathode	Membrane	MFC configuration	Medium treated	Contaminant	Removal Rate (%)	Maximum current/power density	Reference
8	Carbon cloth	Carbon cloth	CEM (Nafion 117)	Double chamber	Mineral medium	Benzene	*100	3.94 mW m^{-2}	(Wu *et al.*, 2013)
9	Carbon felt	Pt-coated carbon felt	CEM: CMI-7000	Double chamber	Mineral medium	Phenanthrene Benzene	97.1	1.15 mW m^{-2}	(Adelaja *et al.*, 2015)
10	Graphite rod	Graphite rod	PEM (Nafion 117)	Double chamber	Mineral medium	1,2-DCA	68	-	(Leitão *et al.*, 2015)
11	Graphite granules	Graphite granules	CEM (Nafion-117)	Double chamber	Contaminated groundwater	Benzene	80	316 mW m^{-3}	(Wei *et al.*, 2015)
12	Activated carbon fabric	#Activated carbon fabric	CEM	Double chamber	Real produced water	TPH	69.86	-	(Jain *et al.*, 2016)
13	Activated carbon fabric	#Activated carbon fabric	CEM	Double chamber	Synthetic produced water	TPH	76.6	-	(Jain *et al.*, 2016)
14	Carbon fibre brush	Carbon cloth coated with PTFE	-	Single chamber	Mineral medium	Diesel	93.5	38.02 mW m^{-2}	(Venkidusamy *et al.*, 2016)
15	PVA coke	Carbon cloth	PVA separator	Single chamber	Contaminated groundwater	Benzene	>95	38 mW m^{-2}	(Chang *et al.*, 2017)

S/N	Anode	Cathode	Membrane	MFC configuration	Medium treated	Contaminant	Removal Rate (%)	Maximum current/power density	Reference
16	Graphite plate	Graphite plate	-	Single chamber	Refinery wastewater	Benzene Toluene Ethylbenzene Xylene	*52.9 *100 *71.4 *100	480 mA m^{-2}	(Daghio *et al.*, 2018a)
17	Carbon fiber brush	Carbon fiber brush	AEM (AMI 7001)	Double chamber	Contaminated groundwater	1,4-dioxane	52	88.9 mW m^{-3}	(Aryal *et al.*, 2019)
18	Graphite brush	Pt-coated carbon cloth	Nafion 117	Double chamber	Synthetic petroleum produced water	TPH	53.1	1089 mW m^{-2}	(Mohanakrishna *et al.*, 2019)
19	Graphite brush	Pt-coated carbon cloth	-	Single chamber	Synthetic petroleum produced water	TPH	47.6	789 mW m^{-2}	(Mohanakrishna *et al.*, 2019)
20	Carbon cloth	Pt-coated carbon cloth	-	Single chamber	Mineral medium	Phenanthrene Pyrene	89.2 51.4	-	(Zhou *et al.*, 2020)

*Calculated from data presented in the paper. Pt=platinum; GDL=gas diffusion layer; PTFE=polytetrafluoroethylene; BTEX=benzene, toluene, ethylbenzne, xylene; CEM= Cation exchange membrane. 1,2-DCA=1,2-dichloroethane. TPH=total petroleum hydrocarbon. #poised cathode potential (+400 mV vs Ag/AgCl)

As discussed above, MFC could be suitable for treatment of industrial or domestic wastewater. However, petroleum hydrocarbon can be discharged into the environment via accidental oil spill, vehicular run-off, or equipment/pipeline failure. Consequently, high molecular weight aromatic hydrocarbons may sink down the water column and become adsorbed onto soil/sediment particulate matter. Thus, treatment objectives would be targeted on the soil/sediment where the hydrocarbons have accumulated. The following section discusses how MFCs can be applied for removal of petroleum hydrocarbons from sediment.

2.2.5 Microbial fuel cells for removal of petroleum hydrocarbons from soil/sediment

Microbial degradation is the key avenue for hydrocarbon removal from the environment and normally proceeds under aerobic conditions with oxygen as terminal electron acceptor (TEA) (Kuppusamy *et al.*, 2017). However, soil and sediments have either low oxygen concentrations (anoxic) or are depleted of oxygen (anaerobic) (Venosa and Zhu, 2003), hence microbial degradation in soil and sediment tends to proceed via anaerobic pathways. In the absence of oxygen, other compounds present in soil/sediment are used as terminal electron acceptors e.g. manganese ion (Mn^{2+}), ferric ion (Fe^{3+}), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH^{4+}), and sulphate (SO_4^{2-}) (Sajana *et al.*, 2016). These terminal electron acceptors act as natural redox mediators during hydrocarbon degradation in soil/sediment (Zabihallahpoor *et al.*, 2015). However, the degradation process is a slow because of insufficient terminal electron acceptors. An anode buried in the soil/sediment can serve as terminal electron acceptor, thereby providing a reservoir for electrons generated during hydrocarbon biodegradation (Daghio *et al.*, 2017). The electrons are further transferred to cathode where they are consumed during oxygen reduction in overlying water. Hence, SMFC could be used to enhance microbial biodegradation of hydrocarbons in soil/sediment, and overlying water.

SMFC was first documented by Reimers *et al.* (2001) for power generation from marine sediments. Over the last 22 years, there had been steady increase in research on SMFC, not just as a source of bioelectricity but also as alternative remediation technology (Zabihallahpoor *et al.*, 2015). Although bioelectricity generated from SMFC is low, SMFC has also been applied as power source for a meteorological buoy (Tender *et al.*, 2008), temperature sensor (Zhang *et al.*, 2011a); wireless telecommunication system (Thomas *et al.*, 2013), submersible ultrasonic receiver (Donovan *et al.*, 2013); subsea sensors (Liu *et al.*, 2015); and magnetometer for ship detection (Arias-Thode *et al.*, 2017). SMFCs have also been used for removal of nutrients and contaminants: COD and nitrogen in aquaculture water (Sajana *et al.*, 2014), nitrate and nitrite (Zhang and Angelidaki, 2012), and uranium (Gregory and Lovley, 2005). Although there is limited documentation on SMFC for metal removal from soil and sediment, successful

applications of MFC for removal of metals and ions such as cadmium, zinc, copper, vanadium and sulphate are indicative of potentials of SMFC for metal removal from soil and sediment (Rabaey *et al.*, 2006; Abourached *et al.*, 2014; Hao *et al.*, 2015; Rikame *et al.*, 2018). SMFC could be suitable for *in-situ* bioremediation of soil and sediment because soil and sediment serve as sinks or reservoirs for contaminants including heavy metals and petroleum hydrocarbons (Zoumis *et al.*, 2001; Liang *et al.*, 2007; Feng *et al.*, 2012; Yang *et al.*, 2014). Figure 2-2 shows some configurations of SMFC used for hydrocarbon removal from sediment. Benefits of SMFC for treatment of hydrocarbon contaminated soil/sediment include: in situ conversion of chemical energy in organic contaminant to bioelectricity, simultaneous treatment of soil/water systems, reduced construction cost because of absence of additional chamber and membrane, low energy and chemical input, potential for integration with other treatment technologies such as wastewater treatment plant or constructed wetland, minimum maintenance requirements, and low environmental impact (Wang *et al.*, 2015; Sajana *et al.*, 2016; Daghio *et al.*, 2017; Cheng *et al.*, 2019).

As can be seen in Table 2-2, most SMFC studied for hydrocarbon removal are configured as single chamber membraneless bioreactors with varied anode potential. One study using single chamber MFC utilized a single rod as both anode and cathode, known as "oil spill snorkel" (Cruz Viggi *et al.*, 2015). Various treatment efficiencies (12 to 99.5%) and power densities (3.4 to 87.85 mW/m^2) have been obtained in SMFCs used for hydrocarbon removal. Duration of treatment is longer for sediment (up to 2 years in one instance) than for wastewater due to lower water content and sediment properties (e.g. soil organic content, sediment particle size or pore structure). Hydrocarbon removal in SMFC is limited when there is reduced dissolved oxygen in overlying water, hence air-cathode configuration is increasingly being used to enhance cathodic oxygen reduction reaction (Yuan *et al.*, 2010; Li *et al.*, 2015). A SMFC using 3-D floating biocathode made with graphite granules has been used to enhance removal of soil organic matter (Wang *et al.*, 2012a).

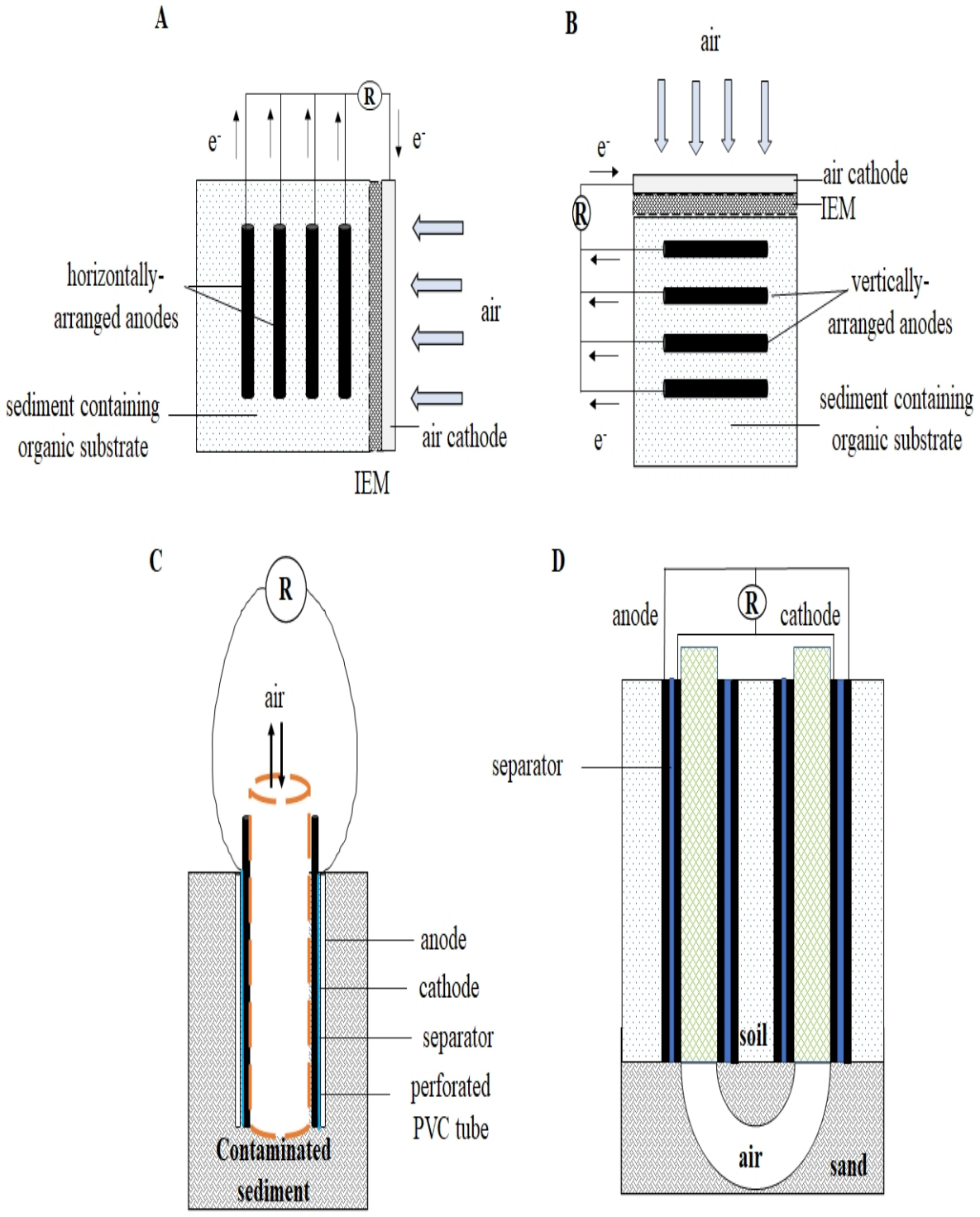


Figure 2-2. Illustration of various configurations of sediment microbial fuel cells (SMFCs) employed for treatment of hydrocarbon contaminated sediment: (A) series arrangement of anodes; (B) parallel arrangement of anodes; (C) tubular SMFC with membrane electrode assembly; and (D) U-tube SMFC with membrane electrode assembly. Adapted from Wang et al. (2012b); Lu et al. (2014a); Zhang et al. (2015).

Table 2-2. Sediment microbial fuel cells used for treatment of hydrocarbon contaminated soil/sediment.

S/N	Anode	Cathode	SMFC configuration×/ modification	Contaminant	Removal rate (%)	Duration of experiment	Maximum current density	Maximum power density	Reference
1	Graphite rod	Graphite rod	Double chamber with poised anode potential (+ 300mV vs Ag/AgCl)	Toluene Benzene	100 100	10 days 7 days	-	-	(Zhang *et al.*, 2010)
2	Graphite plate	Graphite plate	Single chamber with PEM (Nafion-117)	TPH	*68.5	17 days	198.31 mA m^{-2}	20.62 mW m^{-2}	(Chandrasekhar and Venkata Mohan, 2012)
3	Carbon cloth	Pt/C paper	Single chamber	TPH	24.4	66 days	-	2162 mW m^{-3}	(Morris and Jin, 2012)
4	SS cylinder	SS cylinder	Single chamber, FeOOH-treated sediment	Phenanthrene, Pyrene	99.5 94.8	240 days	-	-	(Yan *et al.*, 2012)
5	Carbon mesh, x3, parallel arrangement	SS mesh, Air cathode	Single chamber	TPH PAH n-alkanes	18 36 29	180 days	102 mA m^{-2}	37 mW m^{-2}	(Li *et al.*, 2014)
6	Carbon cloth Biochar	Activated carbon cloth with Pt/C	Single chamber with glass fibre membrane	TPH	73.1 78.7	64 days	73 mA m^{-2} 85.9 mA m^{-2}	39.1 mW m^{-2} 17.7 mW m^{-2}	(Lu *et al.*, 2014a)

S/N	Anode	Cathode	SMFC configuration×/ modification	Contaminant	Removal rate (%)	Duration of experiment	Maximum current density	Maximum power density	Reference
7	Biochar Graphite granule	SS mesh modified with Pt/C and PDMS layers	Single chamber with glass fibre membrane	TPH	#82.1 #89.7	120 days	35.2 mA m^{-2} 70.4 mA m^{-2}	3.4 mW m^{-2} 8.8 mW m^{-2}	(Lu *et al.*, 2014b)
8	A single conductive graphite rod served as both anode and cathode		Single chamber. Electrochemical snorkel, no electrical input or output	TPH	12 (1 snorkel) 21 (3 snorkels) (Day 200)	417 days	-	-	(Cruz Viggi *et al.*, 2015)
9	Carbon mesh, x3, parallel arrangement	Activated carbon, Air cathode	Single chamber. Sand amendment. Soil: sand (2:1)	TPH PAH n-alkanes	22 48 54	135 days	0.28 mA m^{-2} g^{-1} soil	2.76 mW m^{-2} g^{-1} soil	(Li *et al.*, 2015)
10	Graphite plate	Graphite plate (anaerobic cathode)	Single chamber. Nitrate and sulphate added	Naphthalene Acenaphthene Phenanthrene	76.9 52.5 36.8	45 days	-	3.63 mW m^{-2}	(Sherafatmand and Ng, 2015)
11	Graphite plate, x 5, parallel arrangement	Foam bonded graphite felt	Single chamber	PAH, PBDE	22.1	2 years	-	18.6 mW m^{-3}	(Yang *et al.*, 2015)
12	Graphite rod	Activated carbon, Air cathode	Single chamber. Soil amended with carbon fibre 1% (w/w)	TPH PAH n-alkanes	30 37 55	144 days	203 mA m^{-2}	17.3 mW m^{-2}	(Li *et al.*, 2016c)

S/N	Anode	Cathode	SMFC configuration×/ modification	Contaminant	Removal rate (%)	Duration of experiment	Maximum current density	Maximum power density	Reference
13	DSA (Ti mesh modified with mixed metal oxides)	SS mesh	Single chamber. Intermittent low voltage electrolysis (2 V) applied	TPH	59	202 days	-	-	(Bellagamba *et al.*, 2017)
14	Graphite fiber	Graphite fiber	Single chamber. Fe (III) oxide added	TOC	57.2	60 days	*60.78 mA m^{-2}	87.85 mW m^{-2}	(Xu *et al.*, 2017)
15	Graphite felt GO-graphite felt GR-graphite felt CNT-graphite felt	Graphite felt	Single chamber	Phenanthrene, Pyrene	45.6, 42.3 71.2, 69.6 73, 68.2 78.1, 66.7	110 days	-	-	(Liang *et al.*, 2020)

× = unless otherwise stated, single chamber bioreactors had no membrane. SS = stainless steel. FeOOH = Ferric hydroxide. PBDE = polybrominated diphenyl ethers. Pt=platinum. C=carbon. PDMS= poly(dimethylsiloxane). DSA=dimensionally stable anodes. GR=graphene. GO=graphene oxide. CNT=carbon nanotube.

within 1 – 34 cm radius of influence (ROI). *Calculated from data presented in the paper.

2.3 Microbial degradation of petroleum hydrocarbons

Petroleum hydrocarbons released into the environment undergo several transformations such as volatilisation, photo-oxidation, evaporation, dispersion, emulsification, remobilisation (Manzetti, 2013; Duran and Cravo-Laureau, 2016). Despite this, they are persistent because of their low solubility in aqueous phase. Consequently, undissolved petroleum hydrocarbons sink through the water column and accumulate in sediments where they sorb onto organic matter (Liang *et al.*, 2007). The main avenue of hydrocarbon removal from environment is through microbial biodegradation or microbial bioremediation (Kuppusamy *et al.*, 2017).

2.3.1 Microbial bioremediation

Different fractions of petroleum hydrocarbons are degraded by either individual species of micro-organisms or a consortium of micro-organisms belonging to one or more genera (Varjani, 2017). Bacteria are the key players in degradation of hydrocarbons. Hydrocarbon-degrading bacteria belong to the following classes: *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Actinomycetales*, and *Bacilli* (Joye *et al.*, 2016). Dominant hydrocarbon degrading bacterial strains in both soil and marine environment include *Micrococcus*, *Corynebacterium*, *Bacillus*, *Achromobacter*, *Arthobacter*, *Norcardia*, *Enterobacteriaceae*, *Pseudomonas*, *Alcaligenes*, *Flavobacterium*, *Acinetobacter* and *Vibrio* (Austin *et al.*, 1977; Leahy and Colwell, 1990). These strains are capable of growth in both low and high temperature environments. Fungi can degrade polycyclic aromatic hydrocarbons (PAHs) while utilizing another carbon source for energy and growth. Fungal hydrocarbon degraders include *Aspergillus*, *Penicillium*, *Candida*, *Trichoderma*, *Phanerochaete*, and *Bjerkandera* (Doble and Kumar, 2005). Marine algae *Rhodomonas baltica* is capable of degrading PAHs (Arias *et al.*, 2017). Asphaltene can be degraded by bacterium *Garciaella petrolearia* (Lavania *et al.*, 2012) and by fungus, *Neosartorya fischeri* (Hernández-López *et al.*, 2016).

Simple or low molecular weight hydrocarbons are preferentially degraded before complex or higher molecular weight hydrocarbons (Samanta *et al.*, 2001). The degradation pathway used differs with nature of hydrocarbon involved and presence or absence of oxygen. A cross-section of hydrocarbon-degrading bacteria is provided in Table 2-3. Indeed, Kostka *et al.* (2014) posited that critical understanding of relationships between microbial community structure and metabolic activities of hydrocarbon-degrading micro-organisms is required to identify metabolic potentials of oil-degrading microbial communities.

Table 2-3. Microbial diversity associated with petroleum hydrocarbon degradation.

Hydrocarbon fraction	Compound	Micro-organism(s)	References
AEROBIC METABOLIC PATHWAYS			
Aliphatics	Butane	*Arthrobacter* sp.	(Zhang *et al.*, 2013b)
	n-alkanes	*Mycobacterium vanbaalenii*	(Kim *et al.*, 2015)
	n-alkanes	*Alcanivorax borkumensis*	(Yakimov *et al.*, 1998)
	n-alkanes (C9 – C36)	Mixed bacterial consortium containing *Lysinibacillus* sp., *Bacillus flexus* sp., *Pseudomonas mendocina* sp., *Pseudomonas alcaligenes* sp., and *Bacillus thuringiensis*	(Tian *et al.*, 2019)
	n-alkanes and its oxidized derivatives (alkanoles & alkanoates)	*Oleiphilus messinensis*	(Golyshin *et al.*, 2002)
	Branched and *n*-alkanes (C11- C33)	*Planococcus alkanoclasticus*	(Engelhardt *et al.*, 2001)
	Branched and *n*-alkanes (kerosene)	*Geobacillus* sp.	(Maugeri *et al.*, 2002)
Aromatics	Benzene, Toluene, Xylene, Naphthalene	*Bacillus naphthovorans*	(Zhuang *et al.*, 2003)
	Toluene, Phenol	*Bacillus subtilis*, *Bacillus laterosporus*	(Reda and Ashraf, 2010)
	Toluene, Naphthalene, Anthracene, Phenanthrene	*Cycloclasticus pugetii*	(Dyksterhouse *et al.*, 1995)
	Naphthalene	*Pseudomonas putida*	(Samanta *et al.*, 2001)
	Naphthalene	*Staphylococcus* sp.	(Zhuang *et al.*, 2003)
	Naphthalene, Benzene	*Micrococcus* sp.	(Zhuang *et al.*, 2003)

Hydrocarbon fraction	Compound	Micro-organism(s)	References
	Phenanthrene	*Stenotrophomonas maltophilia*	(Gao *et al.*, 2013)
	Phenanthrene	*Arthrobacter sulphureus*, *Acidovorax delafieldii*, *Brevibacterium* sp., *Pseudomonas* sp.	(Samanta *et al.*, 1999)
	Phenanthrene, Fluoranthene	*Azoarcus* sp., *Chelativorans* sp., *Chelatococcus* sp.	(Patel *et al.*, 2019)
	Phenanthrene, Fluorene, Fluoranthene, Pyrene	*Mycobacterium* sp.	(Boldrin *et al.*, 1993)
	Phenanthrene, Fluoranthene, Pyrene	*Mycobacterium vanbaalenii*	(Kweon *et al.*, 2007; Kim *et al.*, 2015)
	Anthracene, Fluoranthene, Pyrene	*Kurthia* sp., *Acientobacter calcoaceticus*, *Staphylococcus aureus*, M*icrococcus lylae*, *M. kristinae*, *M. sedentarius*, *Pseudomonas stutzeri*, *Corynebacterium ammoniagen*	(Igwo-Ezikpe *et al.*, 2010)
	Benzo[*a*]pyrene (BaP), Fluoranthene, Phenanthrene	*Pseudomonas* sp. (*Pseudomonas stutzeri*)	(Liang *et al.*, 2014)
	Phenanthrene, Chrysene, Benzo[*a*]pyrene (BaP)	*Trametes hirsute* (fungus)	(Hidayat and Yanto, 2018)
	Phenanthrene, Benz[*a*]anthracene (BaA)	*Pycnoporus sanguineus* (fungus)	(Li *et al.*, 2018a)
	Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene Chrysene, Benzo[*a*]anthracene	*Irpex lacteus*, *Pleurorus ostreatus* (fungi)	(Bhatt *et al.*, 2002)
	Phenanthrene, Fluoranthene, Pyrene	*Rhodomonas baltica* (algae)	(Arias *et al.*, 2017)

Hydrocarbon fraction	Compound	Micro-organism(s)	References
ANAEROBIC METABOLIC PATHWAYS			
Aliphatics	Methane	*Methylomirabilis oxyfera*	(Shen and Hu, 2012)
	n-alkanes	*Azoarcus/Thauera* group (β-*proteobacteria*)	(Rabus *et al.*, 1999)
	n-alkanes	*Desulfatibacillum aliphaticivorans*	(Cravo-Laureau *et al.*, 2005)
	n-alkanes	*Marinobacterium*, *Clostridiales* (*Peptostreptococcaceae*), *Chloroflexi* (*Anaerolineaceae*)	(Sherry *et al.*, 2013)
	Propane, *n*-butane	strains related to *Desulfosarcina/Desulfococcus* & *Desulfotomaculum*	(Kniemeyer *et al.*, 2007)
	Cyclohexane	*Geobacter* sp., *Geobacter uraniireducens*	(Musat *et al.*, 2010)
	Ethylcyclopentane	*Syntrophobacter*, *Desulfotomaculum*, *Cytophaga-Flexibacter-Bacteroides*	(Rios-Hernandez *et al.*, 2003)
	Iso- and cycloalkanes	*Desulfotomaculum*, *Smithella*, *Methanosaeta*, *Methanoregula*, *Methanoculleus*	(Siddique *et al.*, 2020)
	1-hexadecene	*Methanospirillum hungatei*, *Methanothrix soehngenii*	(Schink, 1985)
	1-hexadecene, 1-pentadecene	*Desulfatiferula* sp., *Desulfatiferula olefinivorans*	(Grossi *et al.*, 2011)
	Acetylene	*Pelobacter acetylenicus*	(Rosner and Schink, 1995)
Aromatics	Toluene	*Desulfobacula toluolica*	(Rabus and Heider, 1998)
	Toluene	*Geobacter metallireducens*	(Kane *et al.*, 2002)
	Naphthalene	NaphS2 (affiliated with *δ-proteobacteria*)	(Musat *et al.*, 2009)
	Benzene	*Geobacter* sp.	(Rooney-Varga *et al.*, 1999)

Hydrocarbon fraction	Compound	Micro-organism(s)	References
	Benzene	*Dechloromonas* strain RCB	(Chakraborty and Coates, 2005)
	Ethylbenzene	Strain EbN1 (*β-proteobacteria*)	(Rabus and Heider, 1998)
	Ethylbenzene	*Azoarcus/Thauera* group (*β-proteobacteria*)	(Rabus *et al.*, 1999)
	Phenanthrene	*Pseudomonas stutzeri*	(Zhang *et al.*, 2020b)
	Phenanthrene	*Desulfatiglans* sp.	(Himmelberg *et al.*, 2018)
	Phenanthrene	*Pseudomonas*, *Ochrobactrum*	(Zhang *et al.*, 2021)
	Fluoranthene	*Bacillus cereus*	(Fuchedzhieva *et al.*, 2008)
	Fluoranthene	*Azoarcus*, *Alicycliphilus*, *Moheibacter*	(Zhang *et al.*, 2021)
	Phenanthrene, Fluoranthene, Benzo[*a*]pyrene (BaP)	*Pseudomonas* sp. (*Pseudomonas stutzeri*)	(Liang *et al.*, 2014)

2.3.2 Aerobic degradation pathways

Aerobic degradation begins with oxidation of the terminal methyl group (see Figure 2-3). Possible oxidation pathways are terminal oxidation or sub-terminal oxidation, ω-oxidation, and β-oxidation (Varjani, 2017). For aliphatic hydrocarbons, oxidation of the methyl group leads to formation of a primary alcohol which is further oxidized to an aldehyde. Dehydrogenation of the aldehyde results in a carboxylic acid, which then undergoes β-oxidation to form acetyl coenzyme A (acetyl-CoA) (Joye *et al.*, 2016). Sub-terminal oxidation of n-alkanes leads to formation of secondary alcohol, which is sequentially converted to ketone and ester (Singh *et al.*, 2012). Both ends of n-alkane can be oxidized (via ω-hydroxylation) to generate ω-hydroxyl fatty acid and subsequently dicarboxylic acid (Rojo, 2009). Key reactions in aerobic degradation of aromatic hydrocarbons are: 1) oxidative activation of benzene ring; and 2) oxygenolytic ring cleavage of aromatic group (Perez-Pantoja *et al.*, 2019). Oxidative activation involves introduction of hydroxyl group on the *orthro*- or *para*-position of benzene ring. These reactions transform aromatic hydrocarbons to central intermediates such as catechol and gentisate (Fuchs *et al.*, 2011) (see Figure 2-4).

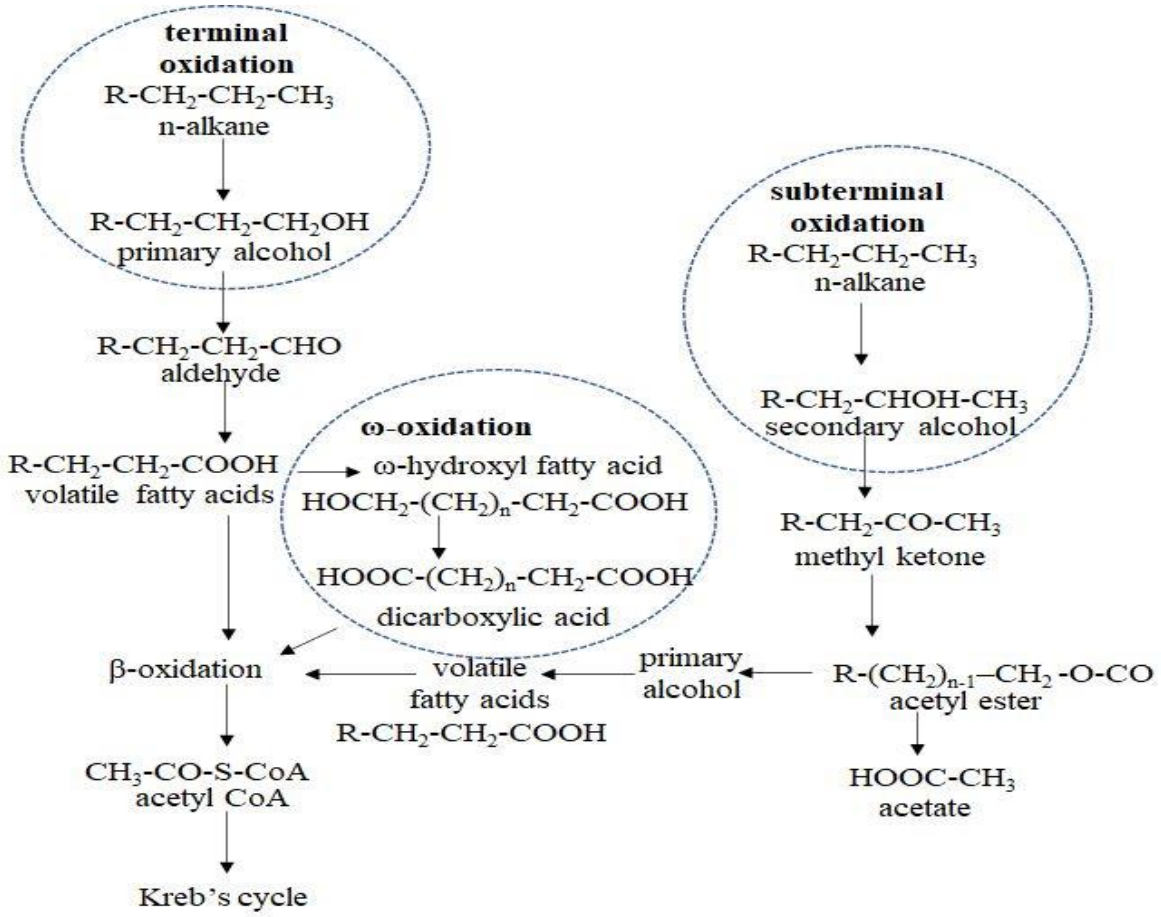


Figure 2-3. Aerobic metabolic pathways for n-alkanes (aliphatic hydrocarbons). Adapted from Zhang et al. (2006); Abbasian et al. (2015); Varjani (2017).

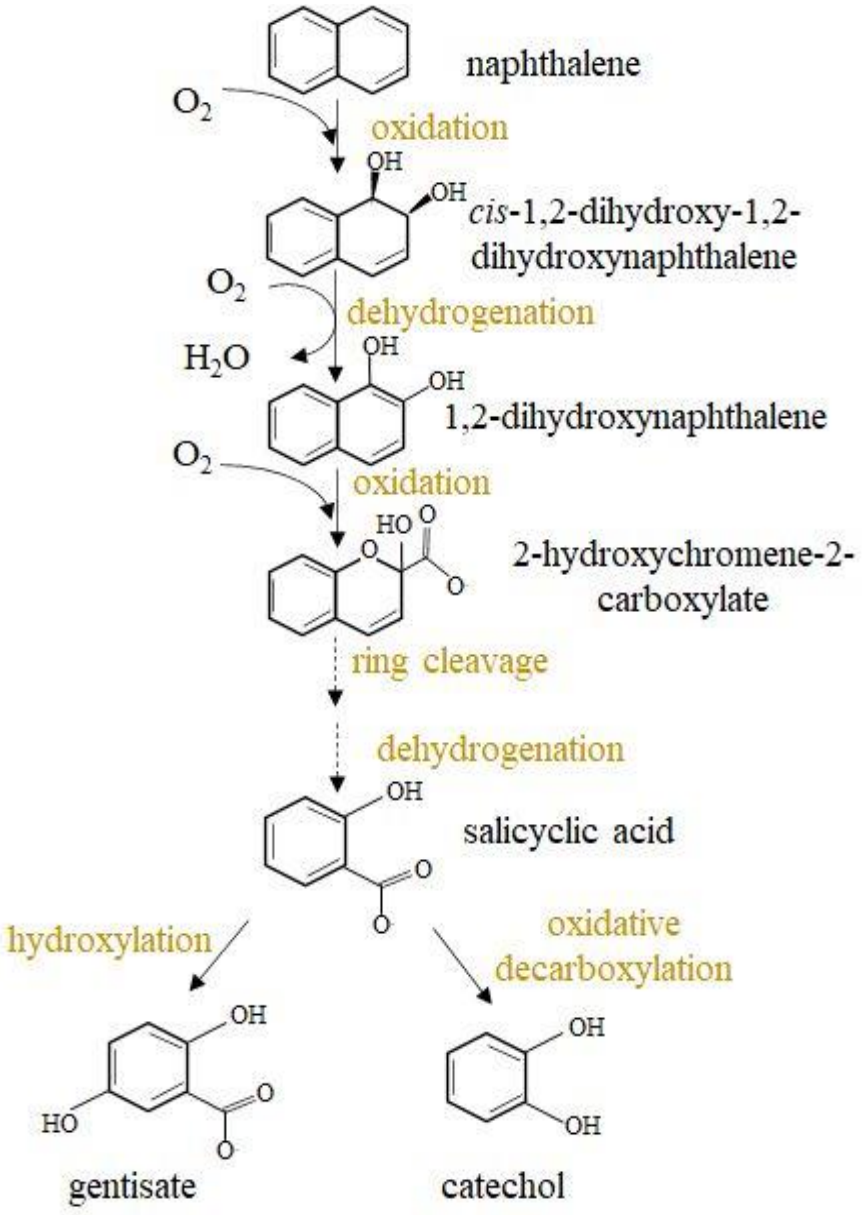


Figure 2-4. Aerobic metabolic pathways for naphthalene (aromatic hydrocarbon). Broken arrows represent multiple steps along the pathway. Adapted from Zhang et al. (2006); Abbasian et al. (2015); Varjani (2017).

2.3.3 Anaerobic degradation pathways

In low oxygen (anoxic) or zero oxygen (anaerobic) environments, metabolic pathway of hydrocarbons are more diverse and complex than aerobic degradation pathways. (Widdel and Rabus, 2001). Anaerobic degradation of hydrocarbon compounds is initiated through 5 different mechanisms – 1) addition of fumarate to methylene or methyl group of hydrocarbon; 2) oxygen-independent hydroxylation on secondary or tertiary terminal carbon; 3) hydration of double or triple bond of alkenes or alkynes; 4) carboxylation of unsubstituted carbon of aromatics; and 5) reverse methanogenesis (Abbasian *et al.*, 2015).

Some hydrocarbons undergo enzyme-catalysed co-metabolic transformation by addition of fumarate or hydroxyl group to the hydrocarbon (Wilkes *et al.*, 2016). In sulphate -reducing (SRB), n-alkanes undergo subterminal carboxylation and rearrangement of two carbon atoms to form carboxylic acid and carbon dioxide via β-oxidation pathway. With denitrifying bacteria (DNB), n-alkanes is activated by addition of fumarate, producing 4-methylalkyl-CoA and regenerating fumarate for further alkane activation (Harayama *et al.*, 2004).

A common intermediate of anaerobic degradation of aromatic hydrocarbons is benzoyl-CoA (Fuchs *et al.*, 2011). Toluene is subject to similar metabolic pathway as n-alkanes. It is activated by addition of fumarate to yield benzyl succinate which is converted via several intermediates first to succinyl-CoA, then to benzoyl-CoA (Abbasian *et al.*, 2015). Ring cleavage then occurs to transform benzoyl-CoA to aliphatic compounds. Alternatively, benzoate can be converted via CoA thioester pathway (i.e. benzoyl-CoA) and epoxidation to acetyl-CoA and succinyl-CoA (Fuchs *et al.*, 2011). In the presence of denitrifying bacteria, ethylbenzene undergoes dehydrogenation by ethylbenzene dehydrogenase to form 1-phenylethanol which is transformed via acetophenone to benzoyl-CoA and acetyl-CoA. Under sulphate-reducing conditions, however, ethylbenzene is activated, like toluene, by addition of fumarate to form 1-phenylethyl succinate (Widdel and Rabus, 2001). Although, biodegradation mechanisms of unsubstituted aromatic compounds such as benzene, naphthalene and phenanthrene is not clearly understood, it is widely agreed that their degradation is activated by carboxylation (Widdel and Musat, 2019). The central intermediate in these routes is benzyl-CoA, which is oxidized to acetyl-CoA and subsequently to carbon dioxide. These pathways are summarized in Figure 2-5 and Figure 2-6. High molecular weight PAHs are sequentially broken down or degraded resulting in generation of several low molecular weight hydrocarbons (Liang *et al.*, 2014).

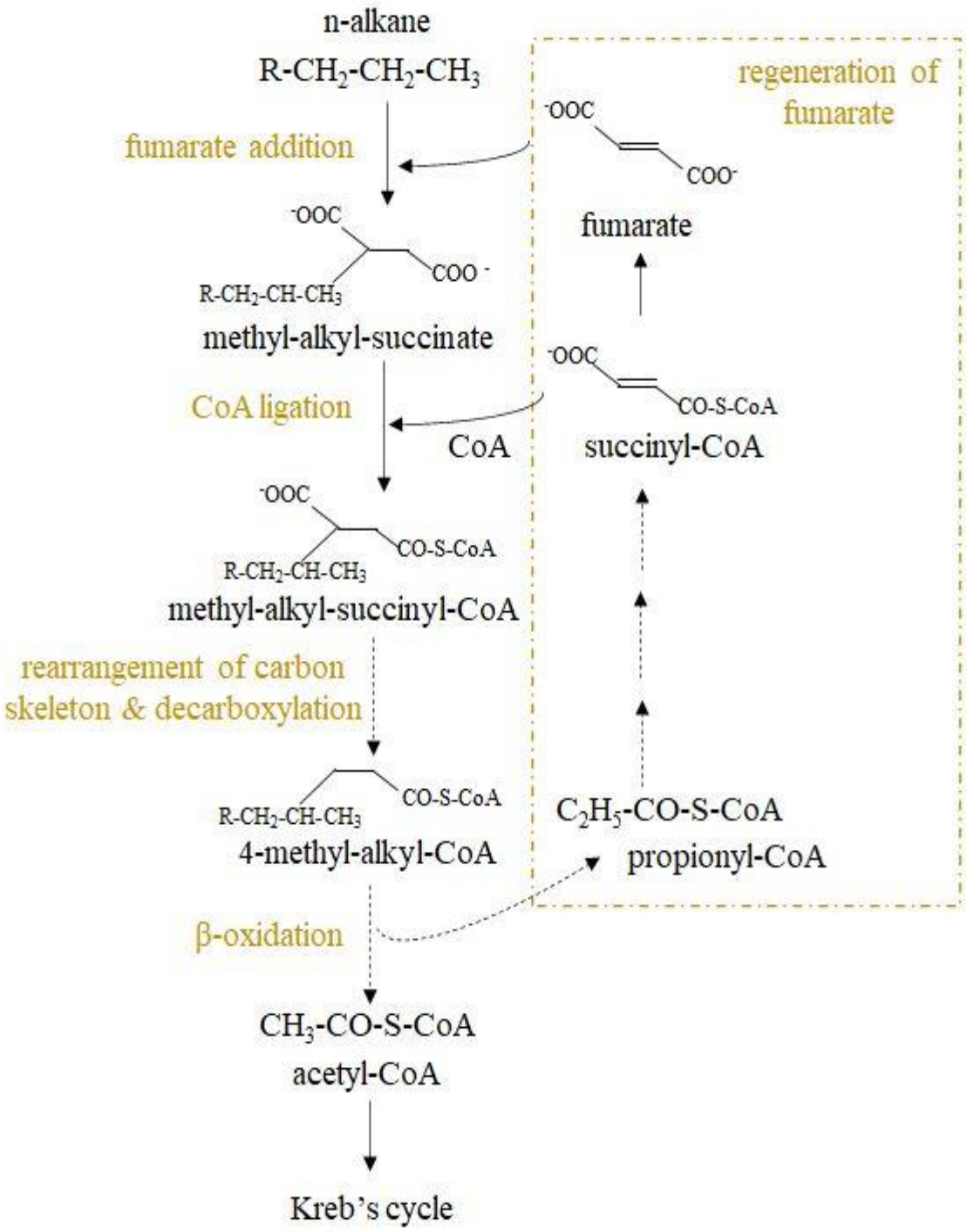


Figure 2-5. Anaerobic metabolic pathways for n-alkanes (aliphatic hydrocarbons). Broken arrows indicate multiple steps. Adapted from Heider and Fuchs (1997); Harayama et al. (2004); Zhang et al. (2006); Fuchs et al. (2011); Abbasian et al. (2015); Rabus et al. (2016).

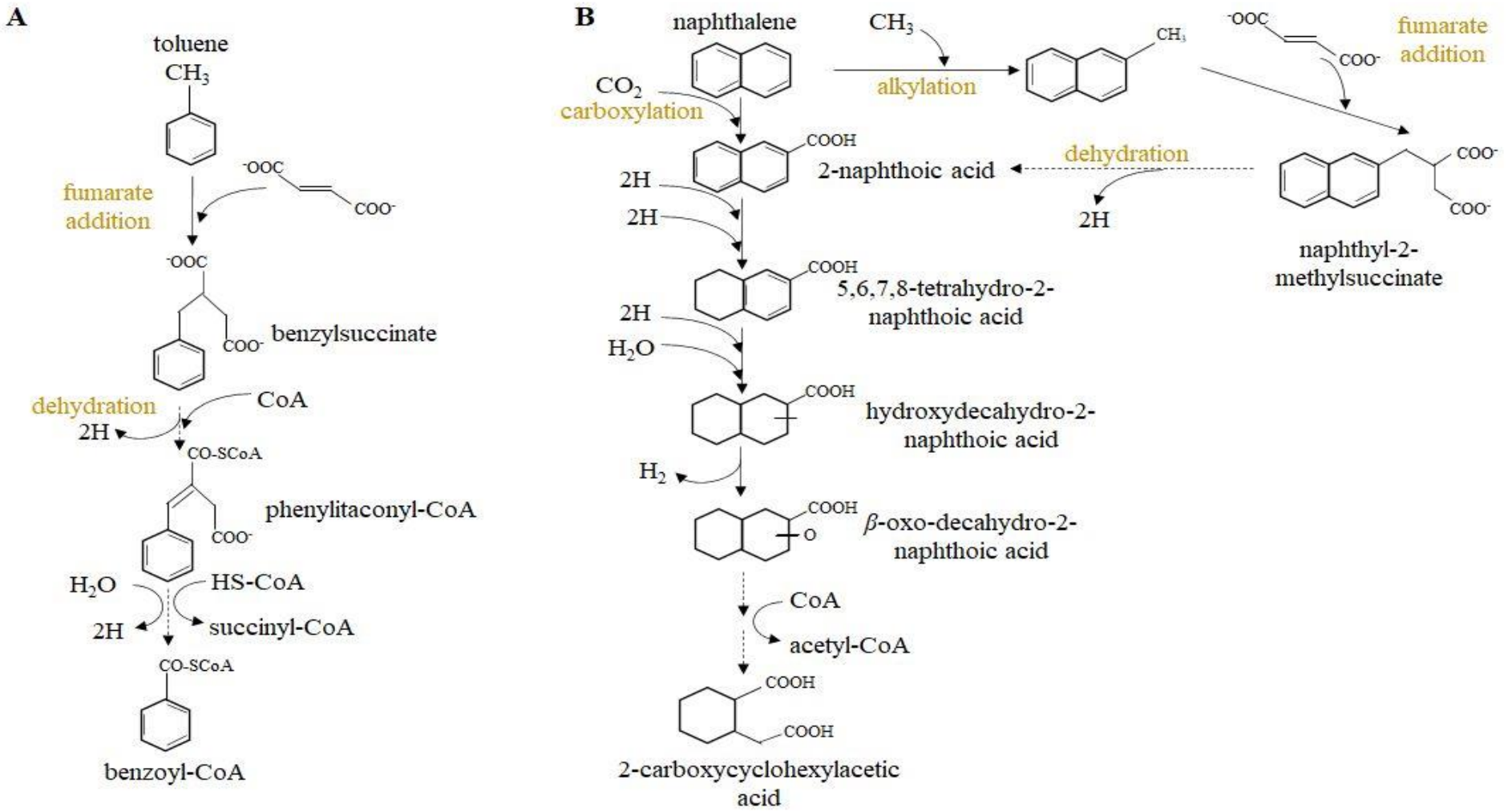


Figure 2-6. Anaerobic metabolic pathways for aromatic hydrocarbons: (A) toluene and (B) naphthalene. Broken arrows indicate multiple steps. Adapted from Heider and Fuchs (1997); Harayama et al. (2004); Zhang et al. (2006); Fuchs et al. (2011); Abbasian et al. (2015); Rabus et al. (2016).

2.4 Microbial diversity in bio-electrochemical systems

2.4.1 Microbial interactions within microbial fuel cells

Micro-organisms are ubiquitous; they interact with the environment and as well as with each other (interspecies or intraspecies). Microbial interactions are necessary for substrate degradation, electron transfer and harnessing energy. These interactions ensure that resources available are efficiently utilised to meet growth and energy needs of the microbial community (Yin *et al.,* 2021). Micro-organisms interact via syntropy and cross feeding. In syntrophic relationships, specific bacteria degrade targeted compounds, while other bacteria utilize intermediate or final products of degraded compounds (Wang *et al.,* 2015). In cross feeding, certain bacteria synthesize specific compounds that are utilized by other bacteria (Yin *et al.,* 2021). These interactions also play a vital role in removal of contaminants, including petroleum hydrocarbons, from the environment.

Micro-organisms found in microbial fuels cells (MFCs) can be found in diverse environments. Microbial diversity in MFCs is influenced by physicochemical properties of the system, including nature of substrate available (Pham *et al.*, 2009). Microbial communities differ within sections of MFC with changes in pH, conductivity, dissolved oxygen and sediment depth (Reimers *et al.*, 2006; Lu *et al.,* 2014a). Electroactive bacteria in MFCs utilise simple organic substrates such as acetate for growth and energy. However, other bacteria breakdown complex substrates into intermediate substrates that can be utilized by electroactive bacteria. For example, oxidation of petroleum hydrocarbons is coupled with sulphate reduction, where sulphide is used as electron shuttle to facilitate electron transfer between electroactive bacteria and electrode. Sulphate-reducing bacteria can be found alongside electroactive bacteria (Rakoczy *et al.,* 2013; Daghio *et al.*, 2016). Consequently, microbial community structure in MFCs consists of electroactive bacteria in syntrophic relationship with other bacteria. Although microbial community in MFC anode chamber are expected to be anaerobic bacteria, aerobic and facultative anaerobic bacteria are also found due to oxygen ingress or contamination during sampling (Daghio *et al.,* 2017). Aerobic and facultative anaerobic bacteria can thrive in low oxygen environments. For instance, *Pseudomonas sp* is a known aerobic hydrocarbon degrader but its presence in MFC suggests its ability to adapt to alternative metabolic pathways under oxygen-limited conditions (Rabaey *et al.*, 2005a; Berdugo-Clavijo and Gieg, 2014).

2.4.2 Electrochemically active bacteria

As earlier mentioned in section 2.1.2, electrochemically active or electroactive bacteria generate electricity via their interaction with electrodes (Philips *et al.,* 2016). Identified phylum in MFCs

include *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Yates *et al.*, 2012). Dominant EAB found in MFCs belong to *Gammaproteobacteria* and *Deltaproteobacteria* class (Daghio *et al.*, 2017; Kronenberg *et al.*, 2017). The dominant families associated with electricity production in freshwater and marine sediments are *Geobacteraceae* and *Delsulfobulbaceae*, respectively (Holmes *et al.*, 2004; Lovley, 2006). Iron reducing bacteria such as *Geothrix fermentans* have been found in freshwater SMFC because freshwater sediments are rich in iron oxides (Sajana *et al.*, 2016). The dominant species associated with power output in acetate fed MFC is *Geobacter sp (Yates et al., 2012)*. *Pseudomonas aeruginosa*, *Shewanella sp*, *Geobacter sulfurreducens*, and *Rhodoferax ferrireducens* have been established as EAB in MFCs (Zheng *et al.*, 2015; Santoro *et al.*, 2017).

Two phylogenetic groups *Dysgonomonas* and *Parabacteroides* as well as phylum *Firmicutes* were detected in MFC used for treating organic wastewater (Watanabe *et al.*, 2011). Families *Clostridiaceae* (*Clostridium*) *Geobacteraceae* (*Geobacter*) and *Holophagaceae* (*Geothrix*) have also been reported as dominant species in MFC used to generate electricity from organic biomass (Miyahara *et al.*, 2013). *Deltaproteobacteria* was dominant in SMFC used for removal of organic matter from sediment, especially order *Delsulfobacterales* and its family *Delsulfobulbaceae*. Sulfate reducers *Desulfobulbus propionicus* and *Thermodesulfovibrionaceae* as well as hydrogenotrophic methanogens *Methanocellaceae*, *Methanomicrobiaceae* and *Methanobacteriaceae* found in SMFC gave evidence of syntropic relationships (Zhao *et al.*, 2016).

2.4.3 Hydrocarbon degrading microbes

Micro-organisms implicated in hydrocarbon degradation have already been discussed in section 0. This section focuses on hydrocarbon-degrading micro-organisms isolated from MFCs used for treating hydrocarbon-contaminated water, soil, and sediment. The dominant bacterial community associated with removal of PAH from contaminated sediments were *Betaproteobacteria* and *Deltaproteobacteria* (including *Geobacter*) (Yan *et al.*, 2012; Cruz Viggi *et al.*, 2015). Sulfate reducing bacteria (SRB) and *Gammaproteobacteria* were dominant on anode used for diesel removal (Morris *et al.*, 2009). *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (including *Alcanivorax*, *Geobacter*, and *Escherichia*) have been linked to removal of petroleum hydrocarbons in a soil MFC (Li *et al.*, 2015; Li *et al.*, 2016b). Key hydrocarbon degrading bacteria found in MFCs for hydrocarbon removal belong to *Enterobacteriaceae* and *Pseudomonadaceae* families (Li *et al.*, 2014; Lu *et al.*, 2014a). *Desulfobulbaceae*, *Desulfuromonadaceae* and *Geobacteraceae* have been isolated in MFC used for BTEX degradation (Daghio *et al.*, 2018a). Members of genus *Geobacter* are described

as both electroactive bacteria and hydrocarbon degraders (Zhang *et al.*, 2010). Abundance of strictly anaerobic bacteria, genus *Clostridium*, has been linked with biodegradation of phenanthrene and pyrene under anaerobic conditions (Zhou *et al.*, 2020).

2.5 Factors that Influence Microbial Degradation of Petroleum Hydrocarbons

Micro-organisms are sensitive and easily respond to changes in their environment. There are several factors that influence microbial biodegradation of petroleum hydrocarbons in the environment, namely: 1) physicochemical factors such as structure and molecular weight of hydrocarbon, substrate concentration, substrate bioavailability, viscosity, aqueous phase dispersion, volatility; 2) environmental factors such as temperature, pH, soil water content, salinity, oxygen, and nutrient availability; and 3) biological factors such as microbial consortium and their adaptive response (Atlas, 1981; Boopathy, 2000; Chandra *et al.*, 2013).

Straight-chained hydrocarbons such as n-alkanes are readily biodegraded than branched or aromatic hydrocarbons (Salleh *et al.*, 2003). Though degradation rate of saturated hydrocarbons may increase in proportion to its concentration, biodegradation rates for higher molecular weight hydrocarbons are influenced by their aqueous solubilities (Leahy and Colwell, 1990). Inhibitory effects can be observed beyond specific concentration thresholds (Rahman *et al.*, 2002; Joye *et al.*, 2018). Aqueous phase dispersion breaks up hydrocarbons into water column, increasing surface area and availability to micro-organisms. Some hydrocarbon fractions are easily lost via volatilization while heavily weathered hydrocarbons are not readily accessible to micro-organisms for consumption.

Hydrocarbon degradation can proceed over a wide range of temperatures, however biodegradation rates often decrease with decreasing temperature (Venosa and Zhu, 2003). Microbial metabolic rates are reduced in hypersaline and high-pressure environment; hence biodegradation rates decrease with increased salinity (Atlas, 1981; Venosa and Zhu, 2003). Water is essential for microbial growth and metabolism. High moisture content >90% supports enhanced biodegradation rates (Chandra *et al.*, 2013). Hydrocarbon biodegradation, is frequently limited by nutrient availability, and can be enhanced by application of fertilizers containing nitrogen and phosphorus (Head and Swannell, 1999). Hydrocarbons adsorbed onto soil organic matter or clay content are not available to microbial community (Hwang and Cutright, 2004; Duan *et al.*, 2015; Kuppusamy *et al.*, 2017). Hydrocarbon desorption from soil improves mass transfer rate and thus its bioavailability (Boopathy, 2000). Hydrocarbons are easily degraded in the presence of oxygen (Chandra *et al.*, 2013). In the absence of oxygen, TEAs utilized are determined by soil/sediment redox potential (Lu *et al.*, 2011). Some micro-organisms in hydrocarbon-contaminated site may not be capable of utilizing some hydrocarbon

fractions as carbon and energy source (Atlas, 1995). Others may only be able to degrade specific hydrocarbon in the presence of another substrate. Successful biodegradation involves complex relationships between diverse micro-organisms, each with its own specificity for different hydrocarbon compounds (Srivastava *et al.*, 2014). In summary, adequate diversity and abundance of micro-organisms, coupled with favourable nutritional and environmental conditions is paramount to achieving optimal hydrocarbon biodegradation (Varjani and Upasani, 2017).

2.5.1 Challenges of petroleum hydrocarbon removal using sediment microbial fuel cells

Like conventional MFCs, the performance of SMFCs is influenced by type of electrode materials used, bioreactor configuration, temperature, salinity, pH, soil organic matter, and indigenous microbial community. These factors have already been discussed in section 2.1.3 and section 2.2. However, SMFC application for hydrocarbon removal is limited by several other factors. First, absence of membrane between cathode and anode as well as reduced distance between electrodes can lead to oxygen diffusion into anode zone and decrease SMFC performance (Hong *et al.*, 2009). Second, while increasing anode depth in sediment may enhance performance, high internal resistance can occur in SMFC due to spatial separation of anode and cathode (Sajana *et al.*, 2016; Girguis *et al.*, 2020). Third, low concentrations of dissolved oxygen in overlying water can impact reduce system performance (Wang *et al.*, 2012a; Sajana *et al.*, 2016). Fourth, electrode passivation and cathode biofouling from sediment particles may limit oxygen reduction (Song and Jiang, 2011). Fifth, mass transport limitations in soil/sediment may increase overpotentials, and reduce system efficiency (Li and Yu, 2015). Sixth, in field applications hydrocarbon removal efficiency is enhanced within a limited distance from SMFC anode (radius of influence, ROI), based on bioreactor design (Lu *et al.*, 2014b; Daghio *et al.*, 2017; Wang *et al.*, 2019).

2.5.2 Modification of sediment microbial fuel cell for enhanced hydrocarbon removal

Different methods have been investigated to improve SMFC performance. For example, contaminated soil/sediment has been either pre-treated or amended with sand (Li *et al.*, 2015), glucose (Li *et al.*, 2016a), and carbon fiber (Li *et al.*, 2016b) prior to treatment with SMFC. Petroleum hydrocarbons degradation increased by 268% after 135 days of operation when soil was amended with sand at a ratio of 2:1 (w/w) (Li *et al.*, 2015). Sand amendment increased soil porosity and facilitated mass transport of hydrocarbons. Addition of 0.5% glucose (w/v) to soil contaminated with aged petroleum hydrocarbon led to 79% increase in power density and PAH degradation of 44% (Li *et al.*, 2016a). TPH degradation rate of 60% was observed in SMFC

containing soil that was rinsed with 1:1.2 distilled water and amended with 2 % (w/w) carbon fibre as opposed to 3% TPH degradation rate in unamended control SMFC (Li *et al.*, 2016b). Addition of carbon fibre lowered charge transfer resistance (R_{CT}) and ohmic resistance (R_S) by 55% and 76% respectively; it also improved soil conductivity and enhanced mass transport of hydrocarbons. (Li *et al.*, 2016c).

Scale up of SMFC is a current area of interest with a view to real life application. A large scale SMFC (100 L) was able to remove only 22.1% of PAH and polybrominated diphenyl ethers (PBDE) over a period of 2 years. SMFC electricity was harvested using a power management system (PMS). Although overall energy efficiency was 39.1%, only 0.8% of the energy was derived from organic chemicals. The SMFC-PMS demonstrated the potential/suitability of the system for long term operations for powering small battery-operated devices (Yang et al., 2015). Improved results were obtained in a later study involving a benthic microbial electrochemical system (195L) comprising 45 L hydrocarbon contaminated sediment. Within 60 days of operation, 50% removal of benzo[k]fluoranthene (BkF) and benzo[a]pyrene (BaP) was achieved (Li et al., 2017).

2.6 Surfactant enhanced biodegradation of organic compounds

As earlier mentioned in section 2.5, degradation rate of hydrocarbon can be influenced by their low solubility in aqueous media. One approach to tackle this challenge to use surfactant to enhance hydrocarbon bioavailability to micro-organisms. Surfactants are chemical additives or surface-active compounds. Surfactants are amphiphilic or they have two segments: hydrophilic/water-soluble/polar segment and hydrophobic/water-insoluble/nonpolar segment. Surfactants can be categorized into a) petrochemical-based or synthetic surfactants and b) bio-based surfactants or biosurfactants. Biosurfactants are synthesized by micro-organisms, plants, or animals. There are generally four (4) known classes of synthetic surfactants based on charge of hydrophilic or polar segment: *anionic* (negative charge), *cationic* (positive charge), *nonionic* (no charge), and *amphoteric/zwitterionic* (positive and negative charges) (Myers, 2006). As can be seen in Figure 2-7, interactions of hydrophobic and hydrophilic segments of surfactant monomers in an aqueous system causes a linear decrease in surface and interfacial tensions in the system as surfactant concentration increases. Above a specific concentration, these monomers form stable aggregates called micelles (Rebello *et al.*, 2013). The minimum concentration of surfactant required for the formation of micelles is known as the critical micelle concentration. As the surfactant concentration increases above critical micelle concentration, formation of micelles increases solubility of hydrophobic compounds in aqueous system (Mulligan *et al.*, 2001; Makkar and Rockne, 2003).

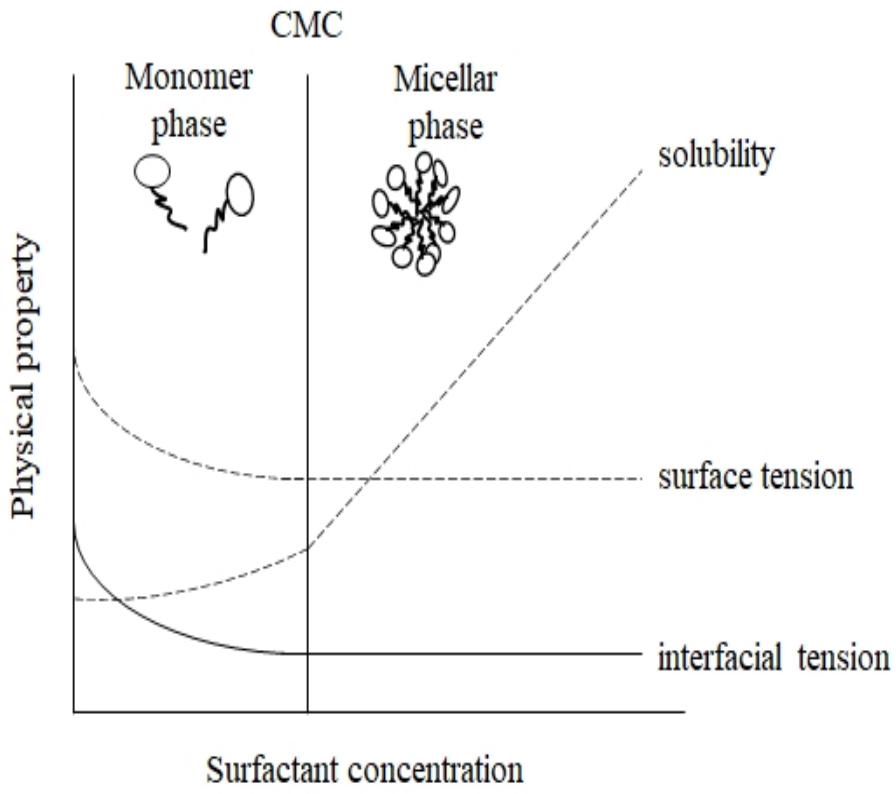


Figure 2-7. Relationship between physical properties of a system and concentration of surfactant. CMC=critical micellar concentration. (adapted from Mulligan et al. (2001)).

2.6.1 Effect of surfactants on solubilization of hydrocarbons

Bioremediation of water, soil or sediment contaminated with polycyclic aromatic hydrocarbons (PAH) is limited by low solubility of PAHs and their strong adsorption to soil and sediments. Surfactants can be used to improve solubility and biodegradability of PAH. The ability of surfactant to solubilize PAH in a soil/aqueous system depends on interactions occurring between several components of the system, namely: adsorption of PAH on soil, partitioning of PAH between soil and aqueous phase, interaction between PAH and surfactant micelles, and adsorption of surfactant on soil (Edwards *et al.*, 1991; Liu *et al.*, 1991; Edwards *et al.*, 1994). At surfactant concentrations below critical micelle concentration, amount of PAH solubilized is limited to aqueous solubility of individual compounds. PAH solubility in water or aqueous medium increases when concentration of surfactant in the system is greater than its critical micelle concentration because of reduction in surface and interfacial tensions. Surfactant facilitates mass transfer of PAH from soil to aqueous phase by desorbing PAH from soil. PAH taken up within surfactant micellar core is readily available to hydrocarbon degrading micro-organisms. However, in the presence of soil, some surfactant is adsorbed on the soil, leaving a lower amount available in aqueous phase. Surfactant concentration more than its critical micelle concentration (in a surfactant-water system) would then be required for formation of micelles.

Nevertheless, a linear relationship between surfactant concentration and PAH solubility exists only up to a maximum concentration, beyond which further surfactant addition does not result in enhanced PAH biodegradation. The surfactant may either be used up by, or become toxic to, hydrocarbon degrading micro-organisms (Tiehm *et al.*, 1997; Schreiberová *et al.*, 2012).

Extensive documentation is available on surfactant-enhanced remediation (SER) using both biosurfactants and synthetic surfactants (Mulligan *et al.*, 2001; Makkar and Rockne, 2003; Lamichhane *et al.*, 2017). Biosurfactants appear to be more effective than synthetic surfactants in solubilization of PAHs (Zhou *et al.*, 2011b; Zhou *et al.*, 2013; Wang *et al.*, 2016a). They are sourced from renewable materials, are biodegradable and have low toxicity. Nonetheless, low yields of biosurfactant by micro-organisms coupled with high recovery and production costs are common deterrents for use of biosurfactants (Mukherjee *et al.*, 2006). Synthetic surfactants are easily produced and have been successfully employed for SER (Yeom *et al.*, 1995; Zhou *et al.*, 2011b; Masrat *et al.*, 2013; Wang *et al.*, 2016b).

2.6.2 Integration of surfactants in microbial fuel cells

Surfactants have also been investigated for integration in bio-electrochemical systems during treatment of contaminated water, soil, and sediment, with mixed results. Alcántara *et al.* (2009) investigated PAH removal using surfactant and electrochemical degradation. PAH degradation (> 90%) was achieved after 3 days. Polyoxyethylene sorbitan monooleate (Tween 80) used in an air-cathode MFC decreased internal resistance resulting in improved system performance (Wen *et al.*, 2011). A binary combination of Saponin:Tween 80 has been used to treat marine sediments contaminated with phenanthrene (Iglesias *et al.*, 2014). Within 8 hours, phenanthrene removal rate was 2.3 times faster with the surfactant enhanced treatment (43 mg kg^{-1} h^{-1}) than treatment without surfactant (19 mg kg^{-1} h^{-1}). Surfactants have also been utilised in soil/sediment microbial fuel cells. Removal efficiencies of total organic carbon (TOC) and polychlorinated biphenyls (PCB) increased from 52.63 to 58.91% (TOC) and from 33.77 to 43.26% (PCB) when standard sediment microbial fuel cells (SMFC) was compared with SMFC to which mixed surfactant (Tween 80-SDS) was added (Xu *et al.*, 2015). Use of surfactant Triton X-100 in an SMFC resulted in significantly lower maximum current and power densities than obtained in the unamended SMFC (73 mA/m^{-2} and 39.1 mW/m^{-2}) (Lu *et al.*, 2014a).

Nonionic surfactants have been reported as displaying higher solubilization capacity than cationic and anionic surfactants, hence the solubility order of nonionic > cationic > anionic (Liu *et al.*, 1991; Kamil and Siddiqui, 2013; Masrat *et al.*, 2013). Tween 80 has been demonstrated to be more effective than other nonionic surfactants for PAH solubilization.

2.7 Future perspectives

Within the last two decades of research, microbial fuel cell (MFC) technology has demonstrated capability as remote power source for environmental sensors and sustainable remediation of contaminated water, soil, and sediment. To optimize MFC performance for higher power output and increased treatment efficiency, key areas requiring consideration include use of cost-effective materials, achieving high surface area biocompatible electrode, adjustment of physicochemical environmental conditions, enhanced bioreactor configuration, improved oxygen reduction reaction and better understanding of *in-situ* microbial interactions. Additional field data is required for large scale application of MFC. This research project aims to optimize MFC for: treatment of hydrocarbon contaminated water in the presence of surfactant; and treatment of hydrocarbon contaminated sediment using different bioreactor configurations. This research is expected to elucidate bacterial communities involved in hydrocarbon degradation as well as kinetics and dynamics of hydrocarbon biodegradation process.

Chapter 3 Methodology

3.0 Chapter summary

This chapter presents methods used for experiments in this research project. It describes methods and equipment used for development of electrochemically active bacteria (EAB) on anode, and its application in microbial fuel cells (MFCs) and sediment microbial fuel cells (SMFCs) for removal of petroleum hydrocarbons from contaminated water and sediment. MFC bioreactor materials, configuration, operating conditions (including composition of media used), methods for sample collection and preservation as well as parameter monitoring (electrode potentials, pH etc.) are presented. Parameter monitoring was essential to keep track of changes occurring within each MFC/SMFC bioreactor. Electrochemical tests for MFC/SMFC characterisation, and equipment used, are also described. These methods enhanced understanding of electron transfer mechanism, internal resistance component, and power output of MFC/SMFC. Materials and methods for chemical analyses are included in this chapter. Chemical analyses results were used to calculate contamination removal efficiency of MFC/SMFC bioreactors. Information gathered from parameters monitored, electrochemical tests and chemical analyses enabled interpretation of likely reactions occurring, limitations on performance, and possible deviations from expected outcome. Procedures for microbial community analyses on bioanode are also described. Microbial community analyses highlight microorganisms likely responsible for reactions in MFC/SMFC and support elucidation of probable metabolic pathways utilized.

3.1 Experimental setup

3.1.1 Microbial fuel cell bioreactors for bioanode enrichment and contamination experiments

Double chamber reactors (H-cells) were used to set up MFC bioreactors for biofilm (bioanode) growth and development. The H-cell bioreactor comprised two (2) 250 ml Pyrex glass bottles, each modified to include a lateral flange. One bottle served as cathode chamber and the second bottle as anode chamber. The final volume of each chamber with its lateral flange was 300 ml. Anode and cathode chambers were separated by a 6 cm x 6 cm perfluorinated cation exchange membrane (CEM) (Fumapem® F-950, thickness 50 µm) sandwiched between two circular silicone gaskets (4 cm inner diameter, SILEX Ltd, UK). The anode chamber was sealed airtight with a rubber stopper and cap. The cathode chamber was oxygenated by air sparging using a 4-outlet Hailea ACO-9610 600lph adjustable air pump (purchased from Amazon, UK). The anode (working electrode, WE) was Ø 2 cm x L 5 cm carbon fibre brush and the cathode (counter

electrode, CE) was 2 cm x 3 cm platinum-coated titanium plate, 1µm thick (Ti-shop.com, William Gregor Ltd, UK). To clean the electrodes, anode and cathode were soaked overnight in acetone (Sigma-Aldrich, UK), and rinsed 2 to 3 times with de-ionized water. To speed up biofilm development, closed circuit MFC bioreactors were poised for 65 days at an anode potential of -0.2 V versus silver/silver chloride (Ag/AgCl) reference electrode (RE) (RE-5B BASi MF-2052, Alvatek, UK) using a 4-channel potentiostat (Quad Potentiostat, Whistonbrook Technologies, UK) (see Figure 3-1A). When the redox potential of an electron donor is just negative to the applied anode potential, the bacteria will conserve more energy and electric energy output is maximized (Rabaey and Verstraete, 2005; Finkelstein *et al.*, 2006). Electron donors such as acetate can be oxidized at low potential of - 0.297 V vs Ag/AgCl RE (Rabaey and Verstraete, 2005; Scott, 2016c). Therefore, poised anode potentials (- 0.200 V) were used only during biofilm development to induce selective growth of acetate-utilizing microorganisms and maximize electric current output.

Double chamber reactors (H-cells) were also used to set up MFC bioreactors for hydrocarbon contamination experiments. Although acetate is a terminal product of anaerobic fermentation of complex organic compounds (including hydrocarbons), several intermediary metabolites are generated during anaerobic degradation of hydrocarbon. Therefore, during hydrocarbon contamination experiments poised anode potential was not used to allow equal opportunity for anode colonization by both acetate- and non-acetate-utilizing bacteria. Poised anode potential was exchanged for external resistance (R) connected across anode and cathode (see Figure 3-1B). Anode and cathode were connected to an external circuit using titanium wire (Strem Chemicals Ltd., UK). All MFC bioreactors were run in duplicates for 64 days.

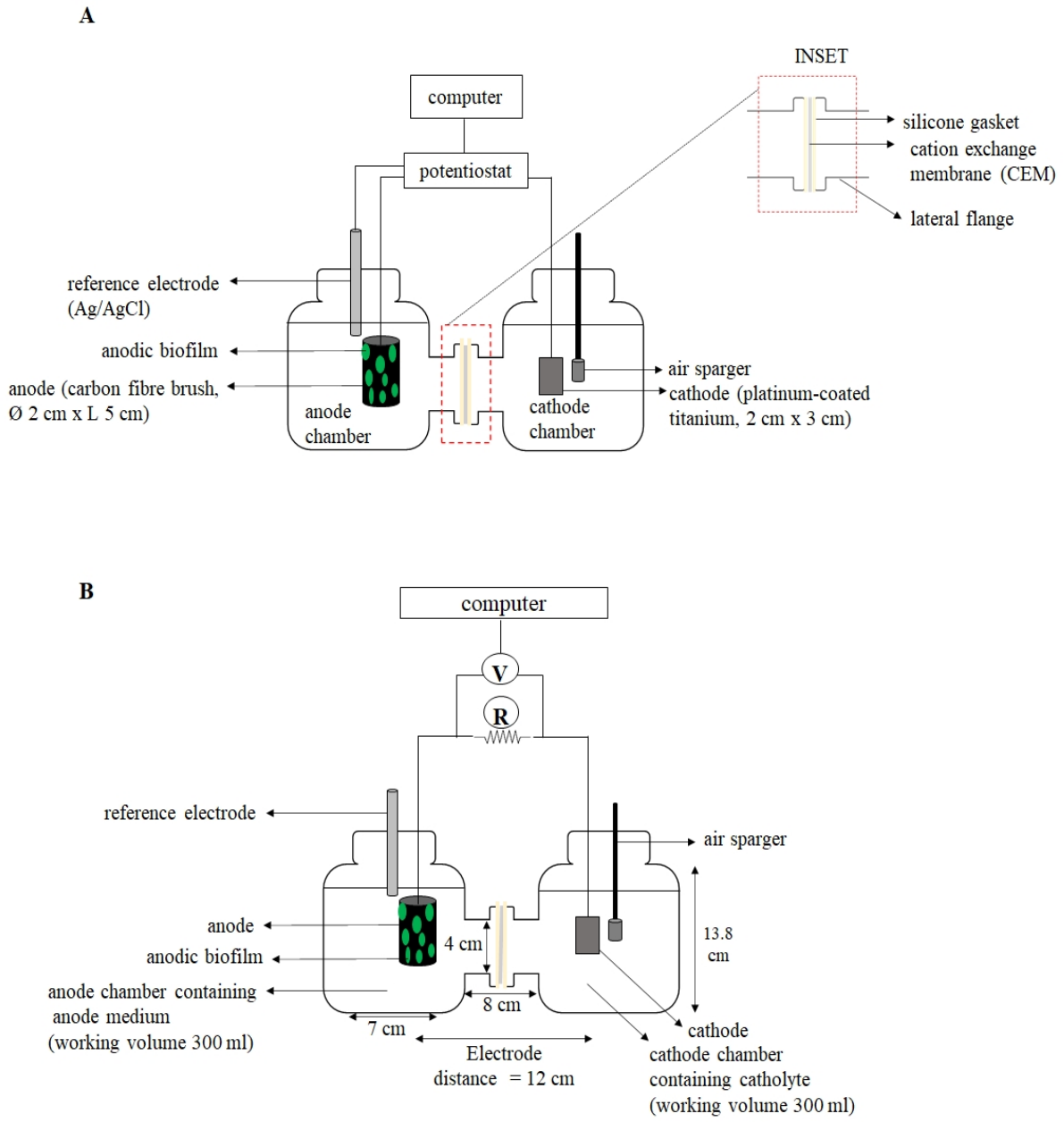


Figure 3-1. Schematic diagram of MFC bioreactor set up for (A) biofilm growth and development (B) hydrocarbon contamination experiments (V = voltameter; R = external resistance).

3.1.2 Sediment microbial fuel cell bioreactors

Grab samples of river water and hydrocarbon-contaminated sediment were collected from Tyne River at low tide using a clean 2-liter glass bottle with polytetrafluoroethylene (PTFE) liner and core samplers, respectively. Sediment samples were taken from anoxic zone. Sediment samples were stored in a cold room at 4^{o}C. Both double (H-cells) and single chamber sediment microbial fuel cells (SMFC) were set up in anaerobic chamber (Coy Laboratory) accessed via a glove box. SMFC materials were placed in air lock chamber. Gas purging cycle using 95% nitrogen:5% hydrogen was performed to remove residual oxygen from the chamber. Once

purging cycle was complete, items in air lock chamber were transferred to main chamber for SMFC set-up, once oxygen concentration in main chamber declined to 0 ppm. Once anode chamber was properly sealed, SMFC bioreactors were removed from anaerobic chamber and placed inside a polystyrene-insulated box with a heating mat at temperature 30^{o}C for the duration of the experiments.

For double chamber SMFC, anode chamber was filled with 300 g of anoxic sediment under anaerobic conditions. External resistance (R_{ext} = 1000 Ω) was connected across anode and cathode of main cells (R) (see Figure 3-2). External resistance (R_{ext} = 1000 Ω) has been shown to support quick start-up in microbial fuel cells (Ren *et al.*, 2011; Zhang *et al.*, 2017). Double chamber SMFC controls (nR) were set up at open circuit potential.

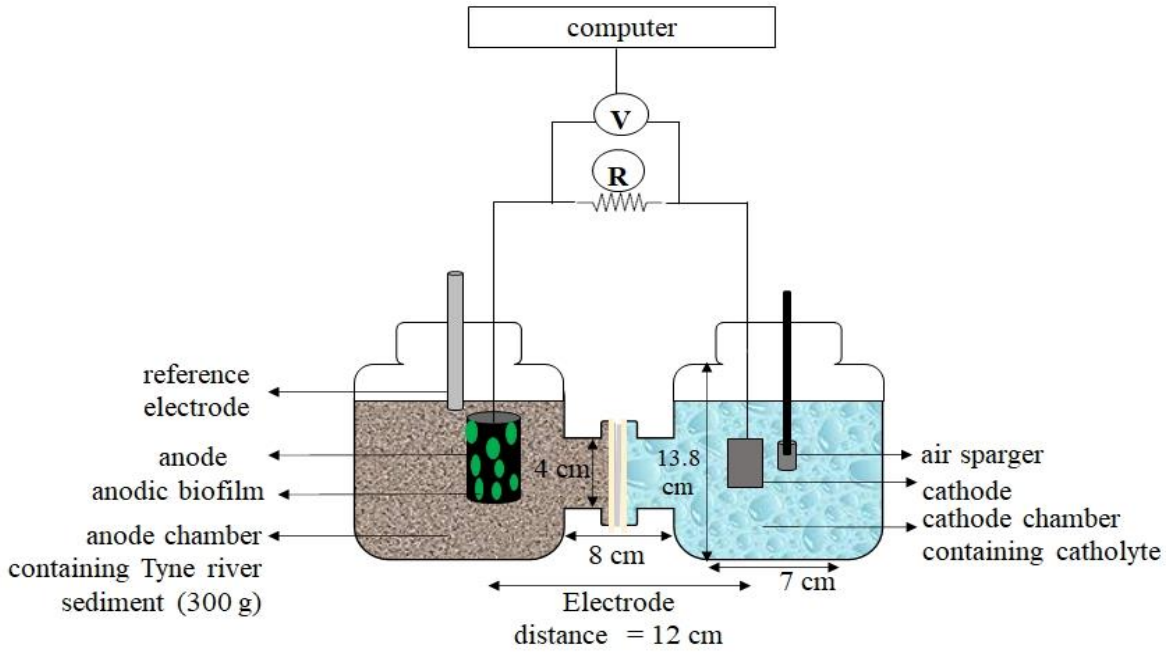


Figure 3-2. Schematic diagram showing double chamber sediment microbial fuel cells (SMFC) used for removal of hydrocarbon from contaminated sediment (V = voltameter; R = external resistance).

Single chamber sediment microbial fuel cells (SMFC) were set up using 500 ml glass bottles, comprising carbon fibre brush anode buried in 300 g of sediment and platinum-coated titanium cathode was suspended in 240ml of overlying water. Single chamber SMFCs with submerged and wicking cathode configurations were designed as shown in Figure 3-3A & B. Closed circuit cells were labelled CW (for wicking cathode configuration) and CS (for submerged cathode configuration). Although high external resistance can support faster start-up and higher microbial diversity, low external resistance could increase bacterial energy gain and support thicker and more active biomass resulting in improved performance (Katuri *et al.*, 2011; Pasternak *et al.*, 2018). However, very low external resistance can limit biofilm development and access to substrate in inner biomass layer (Aelterman *et al.*, 2008a; Zhang *et al.*, 2017). For

this reason, external resistance (R_{ext} = 200 Ω) was connected across anode and cathode to achieve the closed-circuit configuration and establish a balance between anodic biofilm development and SMFC performance. Single chamber SMFCs at open circuit configuration (OC) and biotic non-SMFC (XX) bioreactor were used as controls (see Figure 3-3C & D). All SMFC bioreactors were run in duplicates for 35 days.

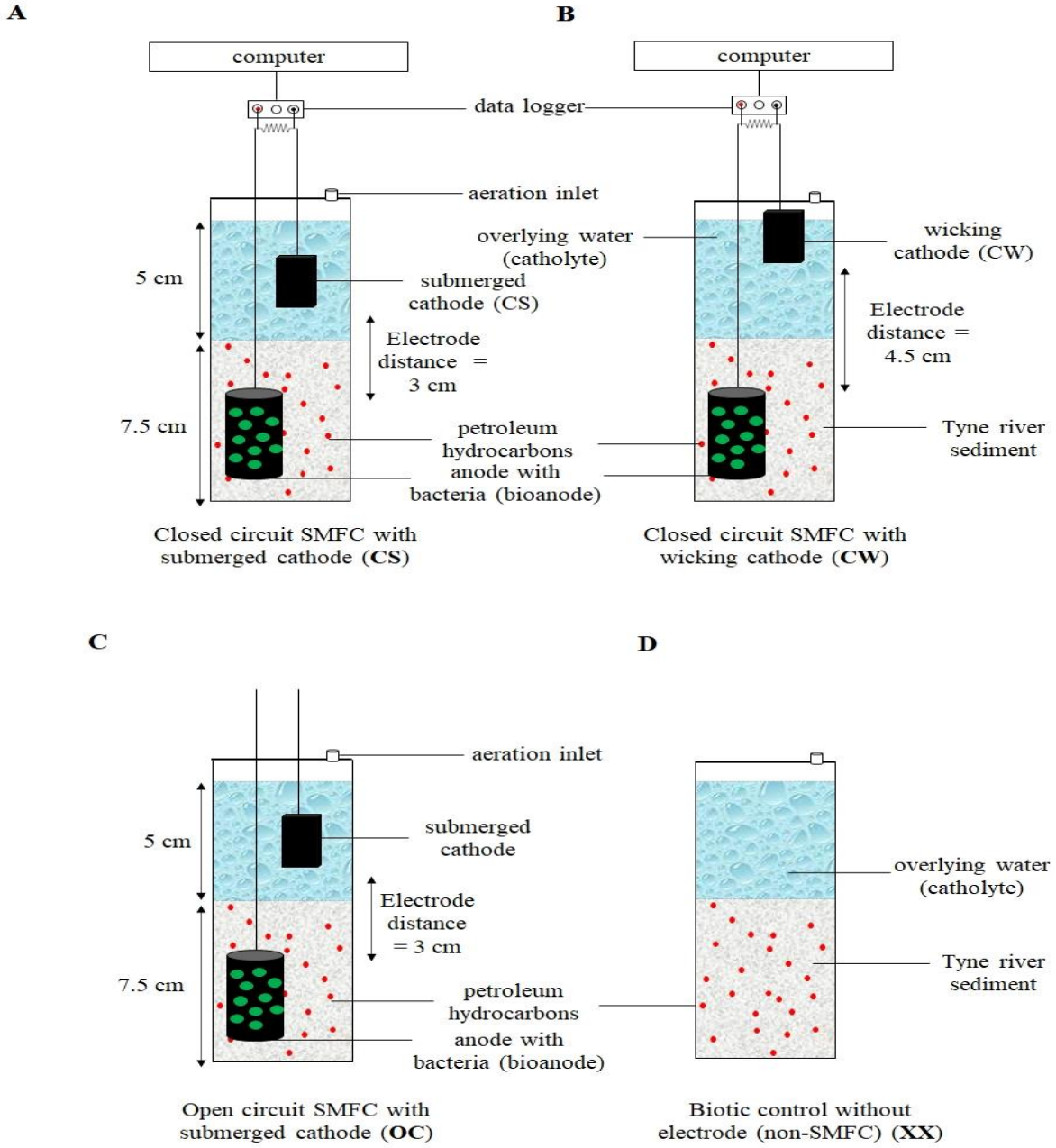


Figure 3-3. Schematic diagram showing single chamber sediment microbial fuel cells (SMFC) with used for removal of hydrocarbon from contaminated sediment: (A) closed circuit with submerged cathode configuration; (B) closed circuit with wicking cathode configuration; (C) control SMFC with submerged cathode; (D) biotic control.

Poised anode potentials were not applied to start up the SMFCs because the presence of PAHs and hydrocarbon-degrading micro-organisms in sediments from Tyne River has already been established from previous studies (Woodhead *et al.*, 1999; Hale *et al.*, 2010).

3.1.3 Media Preparation

All bioreactors were flushed with nitrogen for 15 – 20 minutes before experimental set up. The composition of anode medium (anolyte) used was modified from Milner *et al.* (2016) and Yates *et al.* (2012). 1M phosphate buffer solution (PBS) was prepared using monopotassium phosphate ($HK_2PO_4{\cdot}2H_2O$) 100.48g and dipotassium phosphate ($HK_2PO_4{\cdot}2H_2O$) 57.54g. The solution was sterilized by autoclaving at a temperature of 121˚C and pressure of 15 p.s.i for 20 minutes. One litre of Wolfe's mineral solution contained $MgSO_4{\cdot}7H_2O$ 3.00 g, Nitrilotriacetic acid 1.50 g, NaCl 1.00 g, $MnSO_4{\cdot}H_2O$ 0.50 g, $CaCl_2$ 0.10 g, $CoCl_2{\cdot}6H_2O$ 0.10 g, $FeSO_4{\cdot}6H_2O$ 0.10 g, $ZnSO_4{\cdot}7H_2O$ 0.10 g, $AlK(SO_4)_2{\cdot}12H_2O$ 0.10 g, $CuSO_4{\cdot}5H_2O$ 0.10 g, H_3BO_3 0.10 g, $Na_2MoO_4{\cdot}2H_2O$ 0.10 g, Na_2SeO_3 0.10 g, $NaWO_4{\cdot}2H_2O$ 0.10 g and $NiCl_2{\cdot}6H_2O$ 0.10 g. One litre of Wolfe's vitamin solution contained Pyridoxine HCl 10.0 mg, *p*-Aminobenzoic acid 5.0 mg, Lipoic acid 5.0 mg, Nicotinic acid 5.0 mg, Riboflavin 5.0 mg, Thiamine HCL 5.0 mg, Calcium DL-pantothenate 5.0 mg, Biotin 2.0 mg, Folic acid 2.0 mg and Vitamin B12 0.1 mg. Wolfe's mineral and vitamin solutions were prepared, filter sterilized with a 0.22μm syringe filter (Whatman, UK), and stored in refrigerator at 4˚C prior to use (see Appendix B and Appendix C for procedure for preparation of Wolfe's mineral and vitamin solutions).

For biofilm growth and development, anode medium comprised 12.5ml Wolfe's mineral solution, 5 ml Wolfe's vitamin, 1 g sodium acetate, 50 ml phosphate buffer solution (1 M), 10 ml of ammonium and potassium chloride solution (final concentration 0.31 g/L NH_4Cl & 0.13 g/L KCl), and 922.5 ml deionized water. For hydrocarbon contamination experiments, 1g sodium acetate was replaced with 1ml solution of polyoxyethylene (20) sorbitan monooleate (Tween 80, anionic surfactant, 100g/l) containing hydrocarbon compounds (preparation of the surfactant solution is described in section 3.2). Anode medium was sparged with nitrogen for 15 to 20 minutes before transfer into anode chamber. Reference electrode (Ag/AgCl RE) (in anode chamber) was placed in a Luggin capillary to minimize drift in RE potential. Salt bridge for Luggin capillary was prepared using 8.76g of NaCl and 2 g of Agar powder in 50 ml of deionized water. Luggin capillary was inserted in anode chamber. Formation of air bubbles in Luggin capillary was avoided because air bubbles could disrupt the salt bridge and result in erroneous experimental data.

For all bioreactors, cathode medium (50 mM PBS) was made up of 50 ml phosphate buffer solution (1 M), and 950 ml de-ionized water except in single chamber SMFC bioreactors, where overlying water comprised anode medium without acetate, surfactant or added hydrocarbon compound. The sediment used in single chamber SMFCs was already contaminated with petroleum hydrocarbons.

3.1.4 Operating conditions

Inoculum source may influence MFC performance. Hence, biofilm enrichment was performed to compare two sources of inoculum: defined mixture culture based on acetate (terminal product of anaerobic hydrocarbon biodegradation) (MFC effluent) and undefined mixed culture from non-MFC source (activated sludge). The experimental design for biofilm enrichment is shown in Table 3-1. Activated sludge and MFC effluent were obtained from Tudhoe Mills Wastewater Treatment Works, Durham, and a pre-existing double chamber MFC (fed continuously on 1g/L acetate for 2 years) in Newcastle University, respectively. The MFC in Newcastle University was fabricated from polyacrylic cubes with dimensions 5 cm x 5 cm x 1 cm (length x wide x depth) and operational volume of 25ml. In view of the limited size and volume of the MFC, effluent from the bioreactor was used rather than direct sample from the anode. Cathode chamber was filled with only 300 ml of 50 mM PBS, while anode chamber was filled with 240 ml anode medium and 20% volume (60 ml) inoculum. MFC bioreactors were operated using fed batch mode (50% medium change) every 2-3 days or when current generated dropped to 50 µA. The headspace in all bioreactors were purged with nitrogen for 3 – 5 minutes after each medium change. Anode chamber was inoculated during the first 2 cycles of medium change. Subsequently, medium change without further inoculation was performed until a stable biofilm was achieved. A stable biofilm was assumed to have developed when 2 – 3 repeatable cycles of current were generated. Anodic biofilms were deemed to be stable (successfully developed) if there was no further increase in current output on addition of fresh anode medium (Zhang et al., 2011b). Once biofilms were developed (with acetate as substrate), the reference electrode was removed and an external resistance connected across the anode and cathode, and the bioreactors were spiked with increasing concentrations of hydrocarbons.

For MFC bioreactors, two external resistances (R_{ext}) were studied (100 Ω versus 1000Ω). External resistance has been shown to control bacterial diversity and metabolism. Even though bacterial concentration and biomass yield could be similar, ten-fold decrease in external resistance has been shown to enhance chemical oxygen demand removal and current output (up to 100%) even with less bacterial diversity (Katuri *et al.*, 2011). As previously stated in 3.1.2, high external resistance can support fast start-up while thicker more active biofilms are developed at low external resistance (Zhang *et al.*, 2017). Above 1000 Ω, high internal resistance caused by increase in ohmic losses could reduce MFC performance (Pasternak *et al.*, 2018). For this reasons, external resistance in this study was limited to 100 Ω versus 1000Ω.

*Table 3-1. Factorial design for biofilm enrichment experiments (*poised anode potential = -0.2V vs Ag/AgCl reference electrode (RE). A=acetate. M = bioreactors inoculated with MFC effluent. AS = bioreactors inoculated with activated sludge. nM = MFC effluent bioreactors at open circuit potential, nAS = AS bioreactors at open circuit potential).*

Bioreactors	Temperature (°C)	Inoculum	Substrate	Anode potential
M1	30	M	A	Poised*
M2	30	M	A	Poised*
AS1	30	AS	A	Poised*
AS2	30	AS	A	Poised*
nM1	30	M	A	OCP
nM2	30	M	A	OCP
nAS1	30	AS	A	OCP
nAS2	30	AS	A	OCP

For hydrocarbon contamination experiments in MFCs, surfactant was used to enhance hydrocarbon bioavailability because of low aqueous solubility of hydrocarbons. Nonionic surfactant, polyoxyethylene (20) sorbitan monooleate (Tween 80 or T80), was used because: (1) nonionic exhibit higher solubilization capacity compared with cationic and anionic surfactants (Liu *et al.*, 1991; Kamil and Siddiqui, 2013; Masrat *et al.*, 2013); and (2) Tween 80 has been used successfully to enhance MFC performance (Wen *et al.*, 2011; Iglesias *et al.*, 2014; Xu *et al.*, 2015). The number '20' indicates Monolaurate as the type of major fatty acid associated with Tween 80 molecule. Bioreactors were first fed on only surfactant to observe biofilm response to surfactant. Polyaromatic hydrocarbons (PAH) were chosen for the experiments because these compounds are persistent in the environment and are carcinogenic. Only 2 PAH compounds were used: first, to reflect how they might occur in the real environment; and second, to simplify elucidation of the biodegradation pathway of the compounds. Low concentrations of PAH were chosen that may be tolerable by the anodic biofilm. Previous studies have used PAH concentrations between 5 and 100 ppm (Yan *et al.*, 2012; Adelaja *et al.*, 2015; Sherafatmand and Ng, 2015; Daghio *et al.*, 2016; Daghio *et al.*, 2018a). Two different concentrations were used to observe anodic biofilm response to change in hydrocarbon concentration.

MFC bioreactors were batch fed sequentially with increasing concentrations of polycyclic aromatic hydrocarbon (PAH) contained in 1ml surfactant solution (Tween 80 or T80, 100g/l). Preparation of the surfactant solution is described in section 3.2.1. The PAH mixture (50 & 100 ppm) contained phenanthrene ($C_{14}H_{10}$) (98%, L01921, Alfa Aesar) and fluoranthene ($C_{16}H_{10}$)

(98%, CAS No. 206-44-0, Sigma-Aldrich). T80 and/or PAH was used as carbon source instead of acetate. MFC bioreactors were batch-fed in 3 stages: stage 1: T80 100 ppm only; stage 2: T80 + 50 ppm PAH; and stage 3: T80 + 100 ppm PAH. Each stage lasted 21 days. Polycyclic aromatic hydrocarbon removal efficiency in MFCs using external resistance (R_{ext}) 100 Ω and 1000 Ω was studied. MFCs inoculated with MFC effluent were labelled M, and their open circuit counterparts labelled nM. MFCs inoculated with activated sludge were labelled AS and their open circuit counterparts labelled nAS.

No surfactant was added to SMFC bioreactors because surfactant could be adsorbed onto soil and become unavailable. It could also be used as carbon source by soil micro-organisms instead of contaminant, thereby decreasing their efficacy for contamination removal (Liu *et al.*, 1991; Cheng *et al.*, 2017; Lamichhane *et al.*, 2017). To reproduce environmental condition obtainable in a real system, Tyne River water was used as catholyte in the double chamber SMFCs for 14 days and subsequently, replaced by 0.05 M phosphate buffer solution (PBS). Overlying water in single chamber SMFCs comprised anode medium without acetate, surfactant or added hydrocarbon compound (see section 3.1.3 for description of anode medium preparation). De-ionized water was used to compensate for evaporated anode medium in single chamber SMFCs. Cathode chambers were kept oxygenated by surface aeration using a 4-outlet Hailea ACO-9610 600lph adjustable air pump (purchased from Amazon, UK).

Temperature in all bioreactors was controlled at 30^{o}C by keeping the bioreactors on a heating mat inside a box with polystyrene insulation. At poised anode potential, current output of MFC bioreactors was recorded on a computer through the potentiostat. When external resistance was connected to MFC/SMFC bioreactors, voltage output was recorded (at 10 minutes intervals) on a computer through a data logger (ADC-16, Pico Technology, UK). Voltage (V) output was converted to current (I) using Ohm's Law (V=IR). The electrode potentials (versus Ag/AgCl reference electrode) and pH of each bioreactor was measured during medium change. Diagrams of the laboratory scale MFC and SMFC bioreactors used during this research project are presented in Figure 3-4 to Figure 3-6.

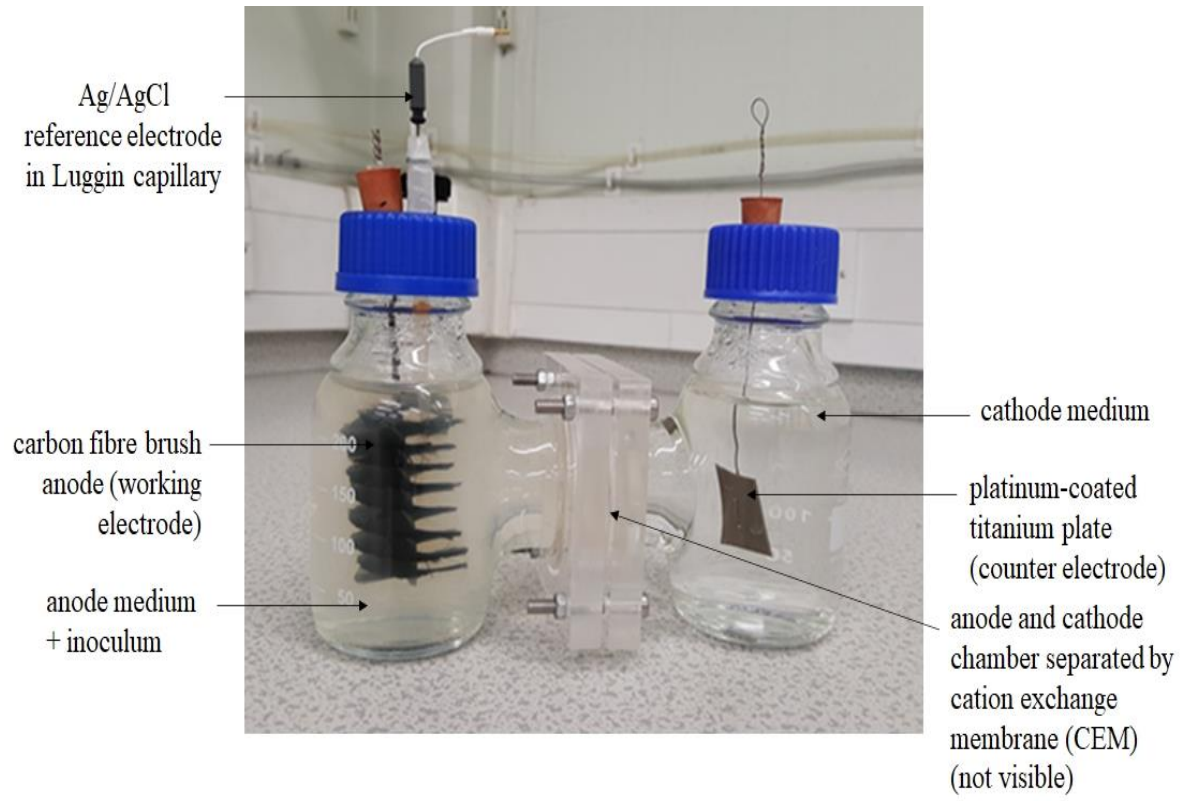


Figure 3-4. Laboratory Scale double-chamber microbial fuel cell employed for biofilm growth and development and hydrocarbon contamination experiments.

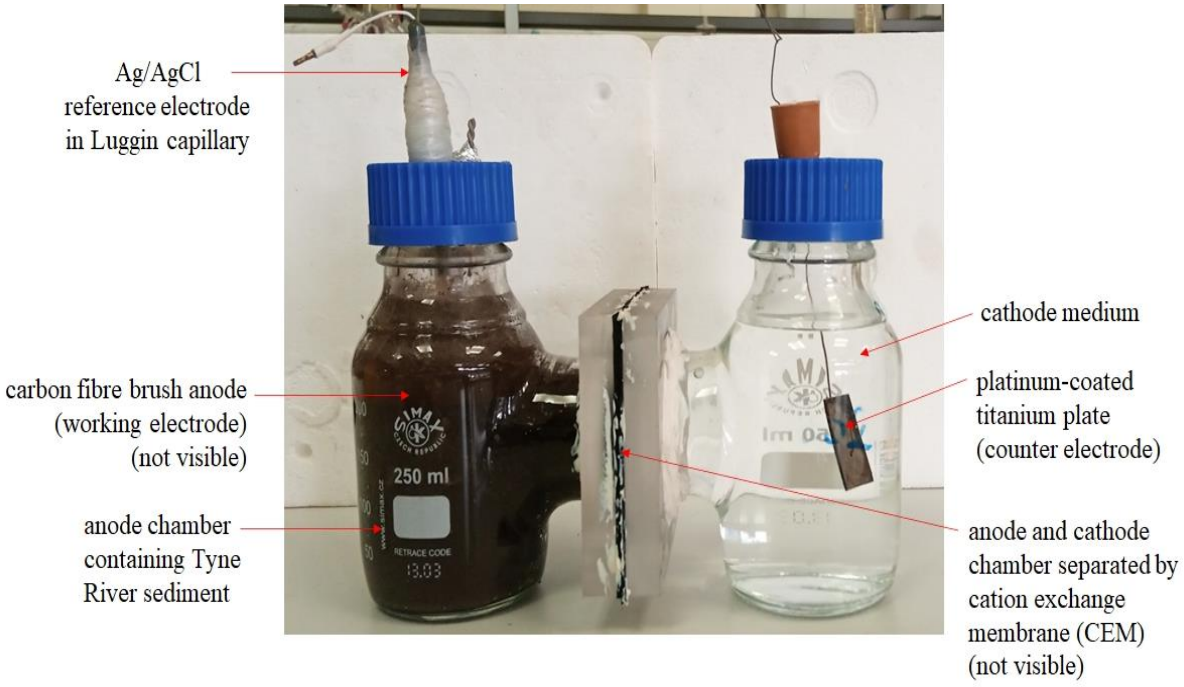


Figure 3-5. Experimental set-up for double chamber sediment microbial fuel cells (both anode and cathode were aligned vertical in their respective chambers).

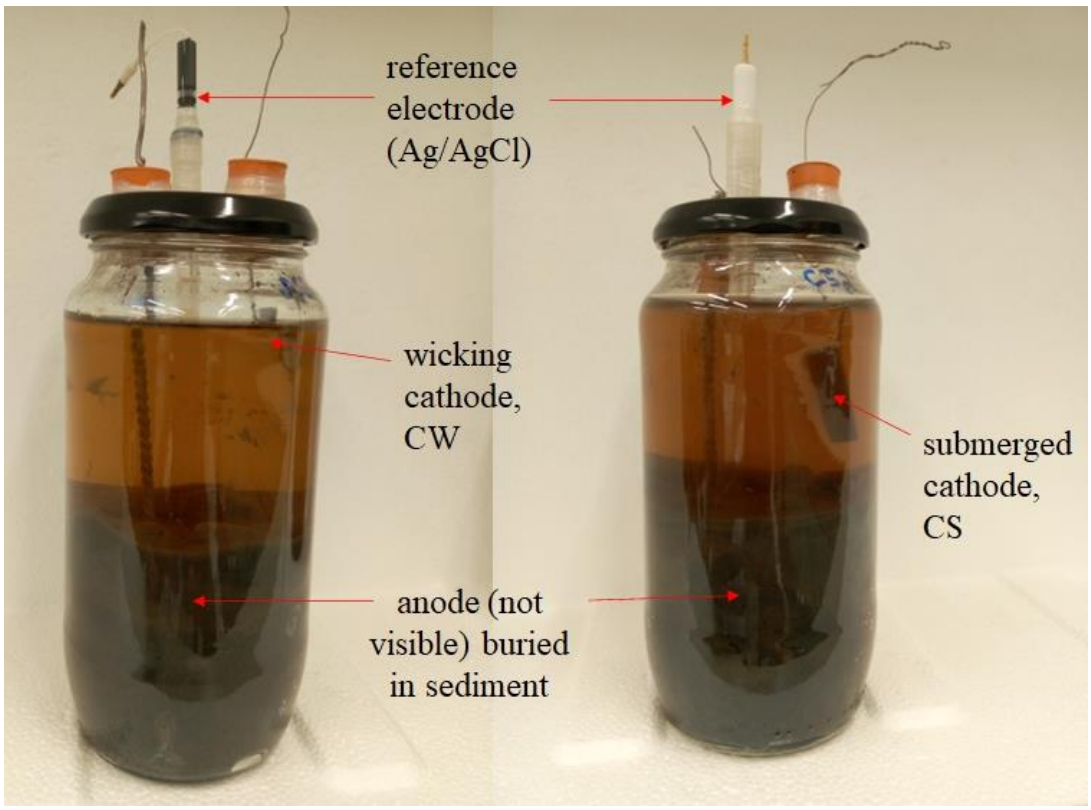


Figure 3-6. Experimental set-up for single chamber sediment microbial fuel cells showing wicking cathode (CW) and submerged cathode (CS) configurations.

3.2 Surfactant Concentration tests

3.2.1 Preparation of Tween 80 surfactant stock solution

Petroleum hydrocarbons have low aqueous solubility, hence anionic surfactant, polyoxyethylene (20) sorbitan monooleate (Tween 80), was added to MFC bioreactors to enhance bioavailability (Cheng and Wong, 2006; Iglesias *et al.*, 2014). Stock solution of 100 g/L Tween 80 was prepared by dissolving 9.35 ml Tween 80 in 90.65 ml de-ionized water (1 ml Tween 80 = 1.07 g). Five (5) different concentrations of Tween 80 (0.01, 0.05, 0.5, 1.0 and 2.0 g/L) were prepared from the stock solution as shown in Table 3-2.

Table 3-2. Preparation of various concentrations of Tween 80 surfactant.

S/N	Stock solution (g/l)	Volume of stock solution needed (ml)	Volume of stock solution needed (ul)	Final concentration of Tween 80 required (g/l)	Final volume of Tween solution to be prepared (ml)	Volume of deionized water (ml)
1	100	0.0010	1	0.01	10	9.99
2	100	0.0100	10	0.10	10	9.99
3	100	0.0500	50	0.50	10	9.95
4	100	0.1000	100	1.00	10	9.90
5	100	0.2000	200	2.00	10	9.80

3.2.2 Preparation of polycyclic aromatic hydrocarbon stock solution

To simplify determination of hydrocarbon degradation pathway, only 2 polycyclic aromatic hydrocarbons (PAHs) were selected for preparation of hydrocarbon solution, namely phenanthrene ($C_{14}H_{10}$) (98%, L01921, Alfa Aesar) and fluoranthene ($C_{16}H_{10}$) (98%, CAS No. 206-44-0, Sigma-Aldrich). A stock solution of 20 mg hydrocarbon in 2 ml dichloromethane (DCM) was prepared as shown in Table 3-3.

Table 3-3. Preparation of petroleum hydrocarbon solution for solubility test

S/N	Hydrocarbon	mg	ml	mg/L
1	phenanthrene	20	2	10000
2	fluoranthene	20	2	10000
	Total petroleum hydrocarbon in solution			20000

Five (5) different concentrations (50, 100, 500, 1000, 2000 ppm) of hydrocarbon solution were prepared from the stock solution as shown in Table 3-4.

Table 3-4. Preparation of various concentrations of petroleum hydrocarbon mixture. (1mg/ml = 1000ppm = 1000mg/l).

Stock solution (ppm)	Volume of stock solution needed (ml)	Concentration of hydrocarbon solution required (ppm)	Final volume of hydrocarbon solution (ml)	Volume of DCM used (ml)
20000	0.025	50	10	9.975
20000	0.050	100	10	9.950
20000	0.250	500	10	9.750
20000	0.500	1000	10	9.500
20000	1.000	2000	10	9.000

3.2.3 Emulsion stability tests

Emulsion stability test was conducted to determine lowest concentration of surfactant required to dissolve polyaromatic hydrocarbons and enhance its bioavailability in anode medium. The method used was adapted from Cheng and Wong (2006). One millilitre (1 ml) of a known hydrocarbon solution was transferred into 6 clean glass test tubes (Ø 15 mm) and placed in a fume cupboard for DCM solvent to evaporate. One millilitre (1 ml) of deionized water was added to test tube 1 as control experiment. One millilitre (1 ml) of known concentrations of Tween 80 solution (0.01, 0.05, 0.5, 1.0 and 2.0 g/L) was added to test tubes 2 to 6, respectively. Emulsion stability test for different concentrations of hydrocarbon was carried out using experimental matrix shown in Table 3-5. Contents of test tubes were mixed using a vortex mixer (Scientific Industries SI-0266, Fisher Scientific, UK) for 60 – 90 seconds. Test tubes were covered with parafilm to prevent spilling of contents during mixing. Test tubes were observed for colloidal dispersion of hydrocarbon in surfactant solution. All tests were conducted in duplicates.

Table 3-5. Experimental matrix for emulsion stability test using Tween 80 surfactant.

Hydrocarbon concentration (ppm)		**Tween 80 concentration (g/L)**					
		1 (control)	**2**	**3**	**4**	**5**	**6**
		0	0.01	0.1	0.5	1	2
A	50	1A	2A	3A	4A	5A	6A
B	100	1B	2B	3B	4B	5B	6B
C	500	1C	2C	3C	4C	5C	6C
D	1000	1D	2D	3D	4D	5D	6D
E	2000	1E	2E	3E	4E	5E	6E

3.3 Hydrocarbon contamination

3.3.1 Hydrocarbon contamination in water

For MFC contamination tests, two (2) different concentrations of hydrocarbon solution were prepared from a stock solution of 1000 mg hydrocarbon in 2 ml DCM using experimental matrix shown in Table 3-6 and Table 3-7.

Table 3-6. Preparation of petroleum hydrocarbon solution for contamination tests.

S/N	Hydrocarbon	mg	ml	mg/L
1	phenanthrene	500	2	250000
2	fluoranthene	500	2	250000
		Total petroleum hydrocarbon in solution		500000

1 ml of required hydrocarbon concentration in Tween 80 solution was then prepared as described in Section 0. The solution also contained 100 µl 5α -androstane (CAS No. 438-22-2, Sigma-Aldrich) as internal standard to monitor hydrocarbon degradation (Wang *et al.*, 2002).

Table 3-7. Experimental matrix for hydrocarbon contamination tests.

Stock solution (ppm)	Actual weight of sample in 2 ml of DCM (mg)	Actual weight of sample required (mg)	Volume of stock hydrocarbon solution required (ml)	Volume of DCM used to make up hydrocarbon solution (ml)
500000	1000	50	0.1	0.9
500000	1000	100	0.2	0.8

Hydrocarbon and Tween 80 solution was mixed using ultrasonic bath for 30 - 40 minutes to ensure homogenous mixture of hydrocarbon in solution. One millilitre (1 ml) hydrocarbon and Tween 80 solution was added to 1 litre anode medium as carbon source for MFC bioreactor. Once biofilm in MFC bioreactors were fully developed, bioreactors were contaminated sequentially with medium containing increasing concentrations of hydrocarbon.

3.4 Sample collection, preparation, and preservation

Aliquots of activated sludge inoculum, MFC effluent inoculum and Tyne River water were collected for characterization. Aliquots of sample removed from MFC anode chamber during 50% medium change were collected and preserved for various analysis. Samples taken from each bioreactor was replaced with 150 ml freshly prepared anode medium. The headspace in all bioreactors was purged with nitrogen for 3 – 5 minutes after each medium change. Excess sample was placed in wastewater bottle and autoclaved for 20 minutes at 15 p.s.i pressure and temperature 121˚C. Contents of wastewater bottle was discarded into labelled wastewater container for collection & removal by waste disposal company. Sample collection, preparation, and preservation was based on analysis to be performed, as follows:

3.4.1 Chemical Oxygen Demand

Five millilitres (5 ml) each of activated sludge inoculum, MFC effluent inoculum and Tyne River water were transferred to labelled 5 ml Eppendorf tubes (Fisher Scientific, UK). Samples were analysed immediately. Otherwise, samples were acidified with concentrated hydrochloric acid to pH below 2 and stored in refrigerator at 4^{o}C.

3.4.2 Metal analysis

One hundred millilitres (100 ml) of Tyne River water were filtered using 0.45 µm Polyethersulfonate (PES) syringe filter (Fisher Scientific, UK), and filtrate acidified with concentrated nitric acid until pH was below 2. Acidified samples were digested with concentrated nitric acid using nitric acid hydrochloric acid digestion Method 3030F (Baird, 2017). Samples were stored in refrigerator at 4^{o}C for analysis.

3.4.3 Volatile fatty acids (VFA)

Five millilitres (5 ml) of sample was filtered using 0.22 µm Polyethersulfonate (PES) syringe filter (Fisher Scientific, UK), and filtrate transferred to labelled 5 ml Eppendorf tubes (Fisher Scientific, UK). Samples were analysed immediately. Otherwise, samples were stored in refrigerator at -20^{o}C.

3.4.4 Total organic carbon (TOC)

Ten millilitres (10 ml) of sample was filtered using 0.22 µm PES syringe filter, and filtrate transferred to labelled 15ml sterile falcon tubes (Fisher Scientific, UK). Samples were stored in refrigerator at -20^{o}C. For SMFCs, dried sediment samples were used for TOC. Procedure for drying sediment samples is presented in 3.4.5.

3.4.5 Total petroleum hydrocarbon (TPH)

Ten millilitres (10 ml) wastewater sample was acidified with 0.2 ml concentrated sulphuric acid (H_2SO_4) or hydrochloric acid. Acidified samples were stored in refrigerator at 4^{o}C. All samples were preserved in sterile glass vials or glass reagent bottles pre-rinsed with DCM (Baird, 2017). Sediment samples were collected from SMFC days 0 and 35. Unused or unprocessed samples were stored in refrigerator at -20^{o}C. All samples were collected into pre-weighed glass beakers under anaerobic conditions. Weight of wet samples was calculated using the formula in equation 3-1:

$$W_w = W_{gw} - W_g \qquad 3\text{-}1$$

Samples were placed a freeze dryer for 24 to 48 hours to remove all moisture content, and dry weight of samples calculated as shown in equation 3-2:

$$W_d = W_{gd} - W_g \qquad 3\text{-}2$$

where W_g = weight of glass beaker only

W_w = wet weight of sample

W_d = dry weight of sample

W_{gw} = weight of glass beaker + wet sample

W_{gd} = weight of glass beaker + freeze-dried sample

Difference between wet and dry weight of samples was used to calculate moisture content of sediment, as shown in equation 3-3 below:

$$\%\ sediment\ moisture\ content = \frac{(W_w - W_d)}{W_w} \times \frac{100}{1} \qquad 3\text{-}3$$

Freeze-dried sediment samples were homogenised in a mortar cleaned with dichloromethane (DCM). The dried samples were ready for hydrocarbon extraction.

3.4.6 Extraction of petroleum hydrocarbon

The method used for extraction of petroleum hydrocarbon depends on sample under investigation and information required. Hydrocarbon can be extracted from water samples by conventional liquid-liquid extraction (LLE), continuous LLE or solid phase extraction (SPE) (Adeniji *et al.*, 2017). In conventional LLE, sample is extracted multiple times in a separating funnel followed by separation of organic phase from aqueous phase. Continuous LLE involves recycling organic solvent through sample and has good extraction efficiency but is time-consuming (18- 24 hours). SPE has higher extraction efficiency than LLE but is time-consuming. Although it is labour-intensive and requires large amounts of solvent, conventional LLE is a straightforward, economical, and effective method.

Hydrocarbon can be extracted from soil/sediment samples using several methods such as: mechanical shaking, ultrasonication, traditional Soxhlet apparatus or automated Soxhlet apparatus (Soxtec machine). Each of these methods have their advantages and disadvantages. For example, mechanical shaking is labour-intensive with low reproducibility. Ultrasonication requires less labour than mechanical shaking but involves multiple extraction, decanting and filtration steps. Traditional Soxhlet apparatus is time-consuming because it takes 16 – 48 hours for hydrocarbon to be extracted from only 6 samples at a time. Soxtec machine is an automated method of Soxhlet extraction and is based on the principle that soluble organic compound can be extract more easily by using hot solvent. Soxtec extraction is faster and uses less solvent

than Soxhlet extraction (Anderson, 2004). Soxtec extraction method involves a 3-step procedure (boil, rinse and evaporate). A time setting is programmed for each step based on solvent and sample characteristics. Prior to extraction of hydrocarbon from either water or soil/sediment sample, known concentrations of recovery (surrogate) standards are added to the sample under investigation to determine efficiency of hydrocarbon extraction from water or soil/sediment. For this research, liquid-liquid extraction and Soxtec machine were used to extract hydrocarbons from water and sediment samples, respectively.

Hydrocarbons were extracted from water samples using liquid-liquid extraction method reported in Adeniji *et al.* (2019) with some modifications. Clean, dry separating funnels (Pyrex, 100 ml) were rinsed three times with dichloromethane (DCM) to remove residual carbon. The separating funnels were held in position using clamps. For each sample, ten millilitres (10ml) was transferred into separating funnel and 50 µl each of recovery standards was added – squalene (≥98%, CAS No. 111-02-4, Sigma-Aldrich) for aliphatic hydrocarbons and 1,1-binapthalene (98%, Cat. No. A16852, Alfa Aesar) for aromatic hydrocarbons. Ten millilitres (10 ml) of a mixture of dichloromethane and hexane (2:1) was added to separating funnel and contents were shaken vigorously in a semi-inverted position to ensure proper mixing of sample with organic solvents. The stopcock of separating funnel was occasionally opened partially to release gas pressure. The separating funnel was clamped to a vertical position and left for 30 minutes to achieve a clear phase separation. The lower layer was collected into a clean dry labelled round bottomed flask (RBF) pre-rinsed with DCM. The process was repeated for three times to improve hydrocarbon recovery (Alegbeleye *et al.*, 2017). One sample blank (de-ionized water) was included for every 9 samples, and extracted as described above (Baird, 2017). For MFC samples, hydrocarbon extract was concentrated to about 0.5 ml and transferred to 2 ml gas chromatography vial (Screw Vials clear, 12x32mm 9-425, RESTEK). Internal standards (50 µl each) were added for quantification – Heptadecylcyclohexane (HDCH) (≥97%, CAS No. 19781-73-8, VWR) for aliphatic hydrocarbons and p-Terphenyl (≥99.5%, Cat. No. 92-94-4, Sigma-Aldrich) for aromatic hydrocarbons. Sample in GC vial was made up to 1 ml with DCM for analysis by gas chromatography.

Hydrocarbon was extracted from sediments samples using soxtec extraction. Materials for the procedure were pre-extracted in soxtec instrument (Soxtec™ 2050 Automatic Extraction System, FOSS) to remove residual organic material - cotton wool (Cat. No. 118-0300, VWR, UK), cellulose extraction thimble (Fisherbrand™, 33 x 80 mm, Fisher Scientific), aluminium oxide/alumina (CAS No. 1344-28-1, Sigma-Aldrich) and silicon dioxide/silica (Silica gel 60, VWR). Ten grams (10 g) of dried sediment sample from SMFCs were extracted in triplicates in soxtec instrument (Soxtec™ 2050 Automatic Extraction System, FOSS) using Soxtec

extraction method modified from Luthria *et al.* (2004). Dried sediment sample was placed in separate clean dry pre-extracted cellulose extraction thimble and 50 µl each of recovery standards – squalene (for aliphatic hydrocarbons) and 1,1-binapthalene (for aromatic hydrocarbons) – were added. All thimbles were numbered carefully using a pencil. Pre-extracted cotton was placed in mouth of thimble to absorb vapour during extraction process. Activated copper turnings and five to seven antibump granules (Scientific Laboratory) were added into the extraction solvent (125 ml of an azeotrophic mixture of dichloromethane and methanol - 93:7) to remove interference from sulphur in sediment samples and to prevent explosion of hot organic solvent, respectively (Qian *et al.*, 1998; Tolosa *et al.*, 2004). Extraction cups (containing extraction solvent and thimbles) were labelled to correspond with thimbles. Thimbles and extraction cups were inserted in Soxtec extraction unit and extraction procedure boil-rinse-evaporate-predry was run for 4 hours 40 minutes. When cool, solvent (containing hydrocarbon extract) was transferred to labelled pre-rinsed RBF using glass pipettes (Volac unplugged 230mm, Scientific Laboratories). Hydrocarbon extract was then processed for total petroleum hydrocarbon (TPH) analysis.

3.4.7 Extractable organic matter (EOM)

Total petroleum hydrocarbon (TPH) is a measure of volatile and extractable petroleum-based hydrocarbon found in environmental samples and comprises aliphatic and aromatic compounds. It can be calculated gravimetrically from extractable organic matter (EOM). TPH is not a precise measure of hydrocarbon content because highly volatile fractions can be lost during solvent concentration and hydrocarbon composition of sample cannot be determined by this method (Agency for Toxic Substances and Disease Registry, 1999). Gravimetric methods are not suitable for samples with low concentrations of hydrocarbon (Adeniji *et al.*, 2017).

EOM was performed with only hydrocarbon extracts from SMFC samples because these samples were expected to contain high concentrations of hydrocarbon. A rotary evaporator was used to concentrate hydrocarbon extract to about 2ml. The extract was transferred into a clean dry 10 ml measuring cylinder pre-rinsed with DCM, using a glass pipette (Volac unplugged 150mm, Scientific Laboratories), and made up to 10 ml with DCM. Using a 3 ml pre-weighed glass vial, 2 ml aliquot of extract was evaporated under a gentle stream of nitrogen and weighed to constant weight. Constant weight minus weight of empty vial was taken as weight of EOM in 2 ml aliquot. This weight was used to calculate total weight of EOM in volume of sample analysed. The remaining 8 ml aliquot of hydrocarbon extract was concentrated to about 2ml and adsorbed onto alumina in a 10 ml glass vial. Adsorbed aliquot was separated into aliphatic and aromatic fractions using column chromatography.

3.4.8 Column chromatography

Column chromatography serves two purposes: (i) sample clean up to remove interferences; and (ii) separation of sample into component fractions (Adeniji *et al.*, 2017). The underlying principle of column chromatography is adsorption of solute/interference onto a stationary phase (silica, alumina or Florisil) as a mobile phase (organic solvent) is passed through the length of the column (Khan *et al.*, 2005). Samples are fractionated based on their polarity. Fractions with lower affinity for stationary phase are eluted first. It is a simple and cost-effective method and is commonly used in environmental analysis (Khan *et al.*, 2005). United States Environmental Protection Agency (US EPA) methods 3611 (Alumina Column Cleanup and Separation of Petroleum Wastes), 3620 (Florisil Cleanup) and 3630 (Silica Gel Cleanup) are used column chromatography (United States Environmental Protection Agency, 1996).

Column chromatography was conducted as reported by Wang *et al.* (2012b) with some modifications. Clean dry columns (25mm x 250mm) plugged with pre-extracted cotton wool were pre-rinsed sequentially with DCM and hexane. Column was filled sequentially with slurry of silica and alumina to height of 12 cm and 4 cm, respectively. Slurries were prepared with hexane. Sample adsorbed onto alumina was transferred into column. Aliphatic and aromatic fractions were eluted with 70 ml hexane and DCM:hexane (50:50), respectively. Each fraction was collected in separate round bottom flasks and processed for gas chromatography analysis as described in section 3.5.5.

3.5 Chemical analysis

3.5.1 Chemical Oxygen Demand

Chemical oxygen demand (COD) was determined by using the cell test kits (COD cuvette test - ISO 15705, 0-1000 mg/L O_2) supplied by HACH, UK. The samples were prepared and added into the reagent vials according to the manufacturer's procedures and then measured by a spectrophotometer (DR6000 UV-VIS, HACH, UK).

3.5.2 Metal Analysis

Tyne river water samples were analysed for metal content using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Optima 7300DV, Perkin Elmer, UK). Liquid sample is aerosolized using a nebulizer, and introduced into a high energy plasma, mostly composed of argon gas. This causes the sample to disintegrate into individual elements, which absorb and emit optical signals at a characteristic wavelength. The wavelengths of each element are separated using an optical grating device and detected using a photomultiplier and charged coupled device. The detector calibrated with known quantities of the elements is used to

determine the composition of the sample. For Tyne River sample the following metals were analysed (wavelength shown in brackets): Magnesium, Zinc, Iron, Calcium, Sodium, Potassium and Lead (See Appendix D for sample and standard preparation).

3.5.3 Volatile fatty acids

Microbial degradation of petroleum hydrocarbon produces lower molecular weight carboxylic acids (volatile fatty acids, VFAs) as intermediates. Monitoring VFA concentration provides information on progress of anaerobic biodegradation of organic matter and confirms bacterial activity (Siedlecka *et al.*, 2008). Samples were prepared for VFA analysis as reported by Izadi (2020). Two millilitres (2 ml) of filtrate was transferred from labelled 5 ml Eppendorf tubes (see Section 3.4) into labelled IC tubes. Twenty microlitres (20 µl) of hydrochloric acid (HCl) was added to each tube and sonicated for 30 minutes to exclude interference from bicarbonate. Sample blanks comprised de-ionized water instead of sample. Samples were analysed for VFAs using a conductivity detector in ion chromatography instrument (Eco IC, Metrohm, Switzerland). Ten microlitres (10µl) of sample was injected into Eco IC using an autosampler (Metrosep organic acids 100/7.8). VFA analysis was conducted with 0.5mM H_2SO_4 as eluent, flow rate 0.5 ml min^{-1}, pressure 3.44 MPa. Sample peaks were automatically integrated. Calibration curve was prepared using standard solution with known concentrations of VFAs (formate, acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate and hexanoate). The calibration curve was used to quantify concentration of VFAs in samples collected from the MFCs.

3.5.4 Total Organic Carbon

Total organic carbon (TOC) in water samples were diluted as required to reduce interference of salt solution on the analysis or where high concentrations of hydrocarbon were expected in sample. Two millilitres (2ml) of filtrate transferred into labelled 15 ml sterile conical centrifuge tubes (Cat. No. 11849650, Fischer Scientific, UK) and made up to 10ml with de-ionized water. Two (2) sample blanks containing de-ionized water were also included as controls. Standard solutions with known concentrations of organic carbon (10 – 200 ppm) were prepared. The standard solutions and blank solutions (deionized water) were measured on the TOC analyser and a calibration curve prepared. The calibration curve was used to quantify concentration of organic carbon in samples. All MFC samples were analysed on a TOC analyser, TOC-5050A (Shimadzu, UK) fitted with an autosampler (ASI-5000A).

Large particles were removed from sediment samples and samples homogenised in DCM-cleaned mortar. TOC for sediment was determined by dry combustion method using LECO RC-612 multiphase carbon analyser (LECO Corporation, St. Joseph, MI, USA) with oxygen as carrier gas (Quijano *et al.*, 2016). 100 mg homogenised sediment was combusted using

temperature ramp from 100˚C to 1000˚C to convert sediment carbon to carbon dioxide (CO_2). CO_2 evolved was measured by an infrared detector and TOC is calculated as a percentage of sample weight.

3.5.5 Petroleum hydrocarbon analysis

Composition of fractionated or unfractionated hydrocarbon extracts can be identified by gas chromatography. Sample is injected into gas chromatograph (GC) fitted with nonpolar capillary column where separation into constituent compounds occurs. Each compound produces a response that is detected, amplified, and converted to a signal to generate a chromatogram. GC can be coupled with mass spectrometry (MS) detector for identification of compounds based on mass spectrum - mass to charge (*m*/*z*) ratio of charged fragments of the compound. MS can be used in full scan mode to acquire qualitative data on compounds in the sample, or in selected ion monitoring (SIM) to detect low concentrations of specific compound(s) of interest in complex matrix. Constituent compounds, presented as peaks on chromatogram, are identified by comparing their retention time with reference standards under same conditions (Khan *et al.*, 2005).

Hydrocarbon extracts (including aliphatic and aromatic fractions) from section 3.4.6 and 3.4.8 above were analysed by gas chromatography-mass spectrometry (GC-MS) as reported in Bebeteidoh *et al.* (2020) with minor modifications. The GC-MS was operated in full scan mode on an Agilent 7890B GC fitted with a split-splitless injector (280°C) and linked to an Agilent 5977B MSD (mass spectrometry detector). A 1µl aliquot of the sample in DCM was injected in pulsed split mode onto the GC by an Agilent 7683B autosampler. The split opened after 1 minute. Separation of the compounds was performed on an Agilent HP-5 fused silica capillary column (30m x 0.25mm ID x 0.25µm film thickness, dimethyl polysiloxane). The GC temperature programme was set from 50 - 310°C at 5°C min^{-1} and held at final temperature (310°C) for 10 minutes. The carrier gas used was Helium at a flow rate of 1ml min^{-1} and initial pressure of 50 kPa. The split flow rate after 1 minute was 20 ml min^{-1}. All samples were analysed in full scan mode at a scanning range 50-600 amu sec^{-1} with electron voltage 70eV, source temperature 230°C, quad temperature 150°C, multiplier voltage 1200V, and interface temperature 310°C.

Data acquisition and processing was performed using Chemstation software installed on a HP Z240 computer. Individual compounds presented as peaks on mass chromatograms and were identified and labelled after comparison of their relative retention time and elution order based on data from geochemistry literature. The peaks were also identified by comparison of their mass spectra with those in the NIST05 library (acceptable at > 90% fit). The NIST Mass Spectral Library is a collection of tools and peer-reviewed databases that have been developed

and endorsed by the National Institute for Standards and Technology USA. Peak areas were acquired by integration and quantified with a calibration curve. The hydrocarbon concentration obtained for water and sediment samples were used to prepare charts demonstrating consumption of hydrocarbon over time in MFC and SMFC bioreactors.

Hydrocarbon concentration (for both aliphatic/n-alkanes and aromatic/polyaromatic fractions) were quantified using predetermined Relative Response Factor (RRF) of recovery (surrogate) standard (RS) and internal standards (IS) added to samples (Rome *et al.*, 2012). Aliphatic fraction considered were n-alkanes (C10 – C35), while aromatic fractions considered were 16 polyaromatic hydrocarbons (PAHs). N-alkanes below C10 were not considered in this study due to their highly volatile nature. The 16 PAHs considered were: naphthalene (NAP), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benz[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[e]pyrene (BeP), benzo[a]pyrene (BaP), benzo[g,h,i]perylene (BghiP), dibenzo[a,h]anthracene (DBA) and indeno[1,2,3-c,d]pyrene (InP). RRF and hydrocarbon concentration was calculated using formulas shown in equations 3-4 to 3-9:

$$RRF = \frac{RS_{pa}}{IS_{pa}} \times \frac{IS_w}{RS_w} \qquad 3\text{-}4$$

$$RS_w = RS_v \text{ x } RS_{cn} \qquad 3\text{-}5$$

$$IS_w = IS_v \text{ x } IS_{cn} \qquad 3\text{-}6$$

$$R_c = RRF_S \times \frac{1}{RRF_{NS}} \times \frac{1}{(f_{ext})} \times 100 \qquad 3\text{-}7$$

$$S_{mw} = \frac{S_{pa}}{IS_{pas}} \times \frac{IS_{ws}}{(f_{ext})} \qquad 3\text{-}8$$

$$S_{cw} = S_{mw} \times \frac{1}{R_c} \times 100 \qquad 3\text{-}9$$

where:

RS_{pa}= peak area of recovery/surrogate standard

IS_{pa} = peak area of internal standard in organic solvent

RS_w= weight of recovery/surrogate standard (µg)

IS_w = weight of internal standard in organic solvent (µg)

RS_v= volume of recovery/surrogate standard (µl)

IS_v = volume of internal standard (µl)

RS_{cn}= concentration of recovery/surrogate standard (µg µl^{-1})

IS_{cn} = concentration of internal standard (µg µl^{-1})

R_c = percentage recovery (%)

RRF_S = relative response factor of standards in sample matrix

RRF_{NS} = relative response factor of standards without sample matrix

f_{ext} = fraction of extract used

S_{mw} = measured weight of sample (µg)

S_{pa} = peak area of sample

IS_{pas} = peak area of internal standard in sample

IS_{ws} = weight of internal standard in sample (µg)

S_{cw} = corrected weight of sample (µg/g)

TOC or petroleum hydrocarbon removal was calculated as shown in equation 3-10 and equation 3-11, respectively:

$$TOC\ removal\ (\%) = \frac{TOC_i - TOC_f}{TOC_i} \times \frac{100}{1} \qquad 3\text{-}10$$

$$hydrocarbon\ removal\ (\%) = \frac{PH_i - PH_f}{PH_i} \times \frac{100}{1} \qquad 3\text{-}11$$

where:

TOC_i = initial total organic hydrocarbon concentration

TOC_f = final total organic hydrocarbon concentration

PH_i = initial petroleum hydrocarbon (aliphatic/aromatic) concentration

PH_f = final petroleum hydrocarbon (aliphatic/aromatic) concentration

3.6 Electrochemical characterization

3.6.1 Parameter monitoring

Parameters monitored for each bioreactor during each medium change included pH, oxidation reduction potential (ORP), and electrode potentials. Electrode potentials and pH/ORP were measured using digital multimeter (ISO-TECH IDM201) and pH/ORP meter (Hanna Instrument HI9025), respectively. Parameter monitoring was conducted to understand impact of environmental parameters on electrochemical reactions occurring within the bioreactors. Current output calculated using the formula V= IR where V= voltage, I = current and R = resistance. Current density was normalized to cathode surface area. MFC and SMFC bioreactors, including controls, were characterized using cyclic voltammetry (CV). All

bioreactors were disconnected from external load and left for 30 - 60 minutes to stabilise prior to performing electrochemical measurements and tests.

3.6.2 Performance indicators for microbial fuel cells

Performance of microbial fuel cells (MFC) and sediment microbial fuels (SMFC) can be evaluated in terms of current density, charge output, coulombic efficiency, or energy recovery/energy efficiency. Limiting current density based on cathodic oxygen reduction reaction (ORR) was calculated using Fick's first law of diffusion:

$$\frac{I}{A4F} = -\frac{DC}{\delta} \qquad 3\text{-}12$$

where:

I = ORR mass transport limited oxygen reduction current (amperes)

4 = number of electrons transferred per mole of oxygen (O_2) reduced

F = Faraday constant (96485 C mol^{-1})

A = electrode area (cm^2)

D = oxygen diffusion coefficient (cm^2 s^{-1})

C = oxygen solubility (mol cm^{-3})

δ = diffusion layer thickness (cm)

Total charge (coulombs transferred) in the MFC/SMFC was calculated by integrating the current (I) measured at each time interval (t) (see equation 3-13) below:

$$Q = \int_{t_0}^{t_b} I\, dt \qquad 3\text{-}13$$

where:

Q= cumulative charge output (coulombs)

I = current (ampere)

t= time (seconds)

Coulombic efficiency (CE) is defined as the fraction (percentage) of electrons recovered as current versus the total number of electrons extractable from organic substrate. Coulombic efficiency for fed-batch MFCs was calculated using equation 3-14, as described in Logan (2008):

$$CE = \frac{M_s \int_{t_0}^{t_b} I\, dt}{F b_{es} v_{an}\, \Delta C} \qquad 3\text{-}14$$

where:

M_s= molecular weight of substrate (grams)

$\int_{t_0}^{t_b} I\ dt$= total coulombs transferred in the MFC over batch cycle (Coulombs)

F = Faraday's constant (96,485 Coulombs)

b_{es}= number of moles liberated per mole of substrate

v_{an} = volume of medium in anode chamber (litres)

ΔC =change in substrate concentration over batch cycle (grams/litre).

Energy efficiency (ϵ_E) is the ratio of power produced by MFC/SMFC over a time interval to heat of combustion of organic substrate added within the stated timeframe (Logan *et al.*, 2006). Energy efficiency of SMFCs was determined using Equation 3-15 below as described in (Logan, 2008):

$$\epsilon_E = \frac{\int_{t_0}^{t_b} E_{cell} I\ dt}{\Delta H\ n_s} \qquad 3\text{-}15$$

where:

E_{cell} = cell potential (V)

$\int_{t_0}^{t_b} I\ dt$= total coulombs transferred in SMFC over batch cycle (Coulombs)

ΔH = heat of combustion of organic substrate (joules/mole)

F = Faraday's constant (96,485 Coulombs)

n_s= number of moles of organic substrate.

Extractable electrons from polycyclic aromatic hydrocarbons (phenanthrene and fluoranthene) used in MFC bioreactors was determined using oxidation half-reaction of phenanthrene and fluoranthene as derived in Tsai *et al.* (2009):

$$\textit{Phenanthrene: } C_{14}H_{10} + 28H_2O \longrightarrow 14CO_2 + 66H^+ + 66e^- \qquad 3\text{-}16$$

$$\textit{Fluoranthene: } C_{16}H_{10} + 32H_2O \longrightarrow 16CO_2 + 74H^+ + 74e^- \qquad 3\text{-}17$$

For SMFC bioreactors, theoretical number of electrons extractable from aliphatic and aromatic hydrocarbons were derived from data obtained from (Tsai *et al.*, 2009; Audran *et al.*, 2018). Heat of combustion (ΔH)of components of petroleum hydrocarbon were obtained or calculated from data obtained from texts (Ruscic *et al.*, 2004; Sagadeev and Sagadeev, 2006; Roux *et al.*, 2008; Audran *et al.*, 2018). Data for extractable electrons and ΔH are presented in Appendix A.

3.6.3 Cyclic voltammetry

Cyclic voltammetry (CV) is a common and simple technique used to understand electrochemical behaviour of bio-electrochemical systems (BES), such as nature of redox reactions as well as electron transfer mechanisms and kinetics (Heering *et al.*, 1998; Elgrishi *et al.*, 2017). Using CV, it is possible to determine reversibility or irreversibility of electrode reactions, capacitive behaviour of electrode, potentials at which oxidation and reduction are

taking place, electron transfer co-efficient and rate constant of reaction occurring. Ratio of anodic to cathodic peak current (see equations 3-18 and 3-19) and peak potential separations between anode and cathode (see equation 3-20) are used to determine if a system is reversible (Zhao *et al.*, 2009).

$$\text{reversible system:} \quad \frac{I_{p,a}}{I_{p,c}} = 1 \qquad \textit{3-18}$$

$$\text{irreversible system:} \quad \frac{I_{p,a}}{I_{p,c}} \neq 1 \qquad \textit{3-19}$$

$$\Delta E_p = E_{p,c} - E_{p,a} = \frac{59\ mV}{n} \qquad \textit{3-20}$$

Where:

$I_{p,a}$ = anodic peak current

$I_{p,c}$ = cathodic peak current

ΔE_p = difference between peak potentials

$E_{p,a}$ = anodic peak potential

$E_{p,c}$ = cathodic peak potential

n = number electrons transferred in electrode reaction

CV is conducted using 3-electrode configuration. CV is a potentiodynamic technique where the potential of MFC working electrode is varied linearly over pre-defined potential range at a specified speed or scan rate (volts, V or millivolts, mV per second) (Scott, 2016b). Current generated during CV is recorded as a function of the applied potential, producing the characteristic sigmoidal shape of a typical cyclic voltammogram as shown in Figure 3-7. Comprehensive characterization of microbial biofilm can be obtained when CV is conducted under non-catalytic conditions and at low scan rates such as 1mV/s (Fricke *et al.*, 2008). Effect of electrode/electrolyte and substrate are ruled out by performing CV with plain electrode (without biofilm) in blank electrolyte and uninoculated fresh medium/substrate, respectively (Zhao *et al.*, 2009).

Cyclic voltammograms of MFC and SMFC bioreactors in this research project were obtained using a scan rate of 0.001 V s^{-1} over potential range of -0.6V to 0.5V vs Ag/AgCl, with a total of 3 scans per bioreactor (Fricke *et al.*, 2008). For blank test, CV was performed using plain carbon fibre brush in 0.05M phosphate buffer solution (PBS). The CV for control test was carried out using plain carbon fibre brush in 0.05M phosphate buffer solution (PBS) & anolyte containing acetate (1g/L) only (no bacteria/biofilm).

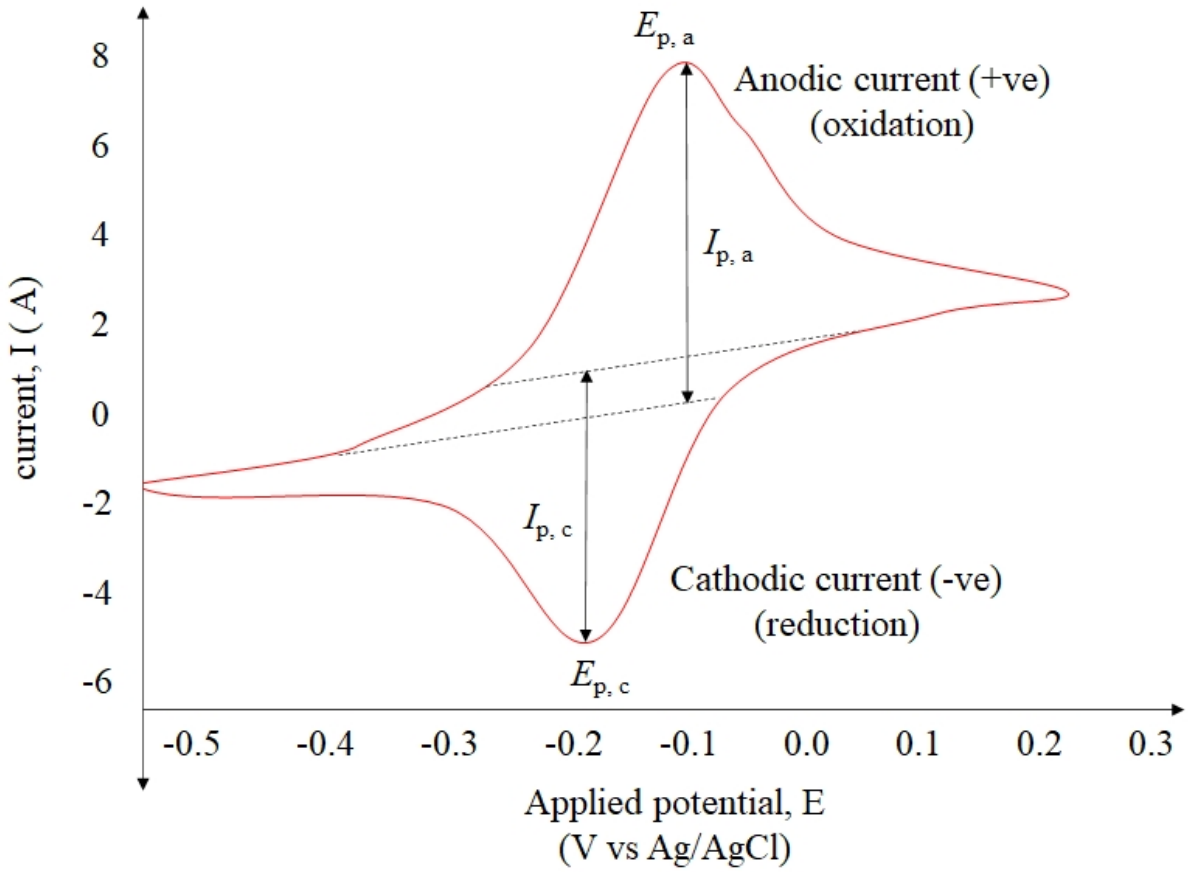


Figure 3-7. Illustration of a typical cyclic voltammogram of a reversible redox reaction in bio-electrochemical system showing peak potentials (anode: $E_{p,a}$ and cathode: $E_{p,c}$) and peak currents (anode: $I_{p,a}$ and cathode: $I_{p,c}$) (modified from Zhao et al. (2009)).

3.7 Microbial Community Analysis

Culture independent molecular biological techniques can be used to characterize microbial communities in environmental samples, in terms of microbial structure and diversity as well as interactions between the communities. Deoxyribonucleic acid (DNA) is first directly extracted from environmental sample by lysis of cell within environmental matrix (Bruce *et al.*, 1999). Microbial communities are identified by analysis of sequences obtained from environmental samples. Sequences from 16S ribosomal ribonucleic acid (16S rRNA) genes are frequently used because they are widespread in all cells, are highly conserved between species and possess species-specific hypervariable regions that facilitate identification of bacteria and archaea (Head, 1999; Obata, 2016). In addition, extensive database of rRNA sequences allows for rapid and detailed phylogenetic analysis of DNA extracts (Head, 1999). Polymerase chain reaction (PCR) amplification of 16S rRNA genes followed by cloning and sequencing can be used to identify whole microbial community. However, use of PCR with specific primers with or without oligonucleotide probes allows for rapid and detailed phylogenetic analysis.

Microbial community analysis was conducted only on bioreactors where there was significant difference in hydrocarbon removal efficiency between closed and open circuit cells. Hence,

Microbial community analysis was performed on MFC bioreactors with low external resistance (R_{ext} = 100 Ω), and single chamber SMFCs with vertical electrode arrangement.

3.7.1 Deoxyribonucleic acid (DNA) extraction

Microbial biofilm samples were obtaining by picking strands of carbon fibre from the top of the anode using sterile scalpel. Genomic DNA was extracted using FastDNA™ Spin Kit for Soil (MP Biomedical, UK) according to the manufacturer's instructions. Five hundred milligrams (500 mg) of sample, 978 µL sodium phosphate buffer and 122 µL MT buffer was added to Lysing Matrix tube and homogenised in FastPrep-24™5G instrument (MP Biomedical, UK) at 6 m/s for 40 seconds. Cells collected by centrifugation at 14,000 x g for 10 minutes using a microcentrifuge (Mikro 200R, Catalogue number 2423, Germany). The supernatant was transferred to 2 ml microcentrifuge tube, 250 µL Protein precipitate solution (PPS) added, and contents were mixed 10 times by inverting the tube. Tubes were incubated at room temperature for 10 minutes, then centrifuged at 14,000 x g for 5 minutes. Supernatant was transferred to 15 ml sterile tube, 1 ml Binding Matrix added, and contents inverted by hand for 2 minutes to allow binding of DNA. The 15 ml tube was placed in a rack for 3 minutes, then 500 µL supernatant was removed and discarded. Binding matrix was re-suspended in supernatant and 600 µL transferred to Spin™ Filter and centrifuged for 14,000 x g for 1 minute. Catch tube was emptied and process was repeated for remaining contents. Pellet was re-suspended after addition of 500 µL SEWS-M and centrifuged at 14,000 x g for 1 minute. Catch tube was emptied, replaced, and centrifuged a second time at 14, 000 x g for 2 minutes to 'dry' up residual SEWS-M solution. Spin™ Filter was removed, placed in clean catch tube, and air-dried for 5 minutes at room temperature. DNase/pyrogen free water (DES, 100 µL) was added, and mixture re-suspended for efficient elution of DNA. Mixture was centrifuged at 14,000 x g for 1 minute to transfer eluted DNA into clean catch tube. Final volume of DNA extract was 100 µL. Concentration and quality of genomic DNA extract was verified using Thermo Scientific NanoDrop™ 1000 spectophotometer (ThermoFisher, UK). The 260/280 absorbance ratio was used to confirm purity of DNA extract. A ratio of 1.8 to 2 is normally accepted as 'pure' (ThermoFisher Scientific, 2016). DNA extract was stored at -20˚C.

3.7.2 Polymerase Chain Reaction

The 16s rRNA gene fragments were amplified as described by Caporaso *et al.* (2011) using universal primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT -3′) targeting V4 hypervariable region for capturing bacterial communities. PCR reaction mix constituted 25 µL PCR mix made up of 13 µL PCR-grade water, 10 µL PCR master mix, 1 µL genomic DNA, 0.5 µL each of forward and reverse

primers. PCR was held at 94˚C for 3 minutes for initial denaturation; amplification proceeded for 35 cycles each 94˚C for 45 seconds (denaturing), 50˚C for 60 seconds (annealing), and 72˚C for 90 seconds (extension); and final extension was performed at 72˚C for 10 minutes. PCR amplicons were quantified with Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher/Invitrogen, catalogue number P11496); and cleaned using UltraClean PCR Clean-Up Kit (Mo Bio, CA, USA) following manufacturer's instructions.

3.7.3 Gene sequencing and Phylogenetic analysis

PCR amplicons were pyrosequenced at NU-OMICS DNA sequencing facility (Northumbria University, UK) using final library concentration of 4pM on Illumina MiSeq v2 500 cycle kit. Sequencing primers were as follows: read 1 - forward primer pad, primer linker and primer (TATGGTAATT GT GTGYCAGCMGCCGCGGTAA); read 2 - reverse primer pad, primer linker and primer (AGTCAGCCAG CC GGACTACNVGGGTWTCTAAT); and index sequencing primer (AATGATACGGCGACCACCGAGATCTACACGCT). Sequence data (fastq files) obtained from Illumina MiSeq were demultiplexed and denoised, and amplicon sequence variants (ASVs) resolved using Divisive Amplicon Denoising Algorithm 2 (DADA2) found in an open-source bio-informatics pipeline for performing microbial community analysis - Quantitative Insights Into Microbial Ecology (QIIME 2 v2018.2.0; available at https://qiime2.org) (Caporaso *et al.*, 2010; Callahan *et al.*, 2016; Bolyen *et al.*, 2019). Obtained ASVs were classified using Naïve-Bayes trained classifier and identified taxonomy assigned using SILVA reference database at 99% similarity. ASVs obtained were rarefied at sequencing depth of 450 reads (Bolyen *et al.*, 2019). Alpha and Beta diversity metrics were calculated using QIIME (Caporaso *et al.*, 2010).

3.8 Statistical analysis

All data from microbial fuel cells (MFC and SMFC) were checked for normality using Anderson-Darling Normality test and Test for equal variance using Minitab 17.0 software. One-way ANOVA with Games-Howell Pairwise comparisons *post-hoc* test was applied to assess influence of inoculum and external resistance on current output and PAH removal efficiency in all MFCs; and to evaluate differences in current output and PAH removal efficiency in all SMFCs. For the analysis, variances were not assumed to be equal. All tests were conducted at 95% confidence interval. Difference between means were considered significant at p-value $\leq$ 0.05.

Statistical analyses for metagenomic data were conducted using STAMP (STatistical Analysis of Metagenomic Profiles) (Parks *et al.*, 2014). Principal Component Analysis (PCA) was used to visualize dissimilarity between sequences from water and sediment samples. Kruskal Wallis

H-test with Tukey-Kramer *post-hoc* test was used for comparison of mean abundances between multiple groups. *Post-hoc* tests were performed by applying Storey's FDR correction (Parks, 2014; Parks *et al.*, 2014). Two-sided Welch's t-test and two-sided Fisher's exact test were used to compare means of two groups and samples, respectively (Parks, 2014; Parks *et al.*, 2014). For the latter, filtering was applied at q-value > 0.05 and difference between proportion < 1.00. Confidence interval of 95% was applied for all statistical analyses.

Chapter 4 Application of enriched bioanodes for enhancement of hydrocarbon removal efficiency in the presence of surfactant

4.0 Chapter summary

Bioanodes of double chamber microbial fuel cells (MFCs) were enriched with inoculum from two different sources (MFC effluent and activated sludge). Enriched bioanodes were employed for removal of polyaromatic hydrocarbons (PAHs) from hydrocarbon wastewater under different external resistance, R_{ext} (100 and 1000 Ω). Tween 80 surfactant (100 ppm) was applied to enhance bioavailability of PAHs. The influence of inoculum source and external resistance (R_{ext}) on anodic biofilm development and PAH removal efficiency were examined in this study. Anodic biofilms of all MFCs studied were able to tolerate low concentration of Tween 80 (100 ppm). MFCs inoculated with activated sludge (AS) started up faster and produced higher current output (59.44 ± 0.98 mA/m^2) than MFCs inoculated with MFC effluent (M) (1.65 ± 0.03 mA/m^2). Inoculum source did not appear to have any influence on overall PAH removal efficiency. Reducing external resistance from 1000 Ω to 100 Ω, increased current output in all MFCs, with higher coulombic efficiency (CE) in AS (27.5 ± 3 %) than M (2.3 ± 1.4 %) at PAH concentration of 50 ppm. Higher electrochemical removal of PAH and CE was achieved in AS under low R_{ext} (100Ω) (PAH removal = 84 ± 3 %; CE = 27.5 ± 2.9 %) than high R_{ext} (PAH removal = 72 ± 5 %; CE = 14.8 ± 2.6 %) at 50 ppm PAH. Cyclic voltammetry showed that stable anodic biofilm with higher bio-electrocatalytic properties were developed under low R_{ext} (100 Ω). New findings seen in this study were: (i) activated sludge inoculum supported faster start-up and current output in MFCs employed for PAH removal from hydrocarbon wastewater; (ii) activated sludge inoculum enhanced higher bio-electrocatalytic activity in anodic biofilms employed for PAH removal; and (iii) lowering external resistance in double chamber MFCs enhanced removal efficiency of complex substrates such as PAH from hydrocarbon wastewater.

4.1 Introduction

Petroleum hydrocarbons are crude oil derivatives comprising polar fractions (containing sulfur, nitrogen, and oxygen) and non-polar fractions (aliphatic and aromatic hydrocarbons). Aliphatic hydrocarbons consist of alkanes, alkenes, and alkynes whereas aromatic hydrocarbons consist of one or more benzene rings and comprise benzene, toluene, ethylbenzene, xylene (BTEX) and polycyclic aromatic hydrocarbons (PAHs) (Ite *et al.*, 2013; Kponee *et al.*, 2015). Highly recalcitrant nature of PAHs and their limited solubility in aqueous phase hinders their removal from water (Liang *et al.*, 2007). PAH can be removed using biological methods such as bioremediation (Kuppusamy *et al.*, 2017). However, bioremediation is a slow process and requires lengthy periods due to insufficient electron acceptors to drive the biodegradation process (Zabbey *et al.*, 2017). Bio-electrochemical systems (BES) use bioreceptors (e.g. micro-organisms) as catalysts to produce electrical energy from organic substrates. Interest in BES for environmental remediation started in 2001, with contaminants being used in place of organic substrate. However, BES use for hydrocarbon removal has only advanced in the last 12 years (Kronenberg *et al.*, 2017). Electrons generated by micro-organisms during anaerobic oxidation of PAH are transferred to anodes of BES such as microbial fuel cell (MFC). These electrons flow from anode via a conductive wire to the cathode where they are consumed in reduction reactions. Thus, chemical energy in PAH (and its metabolic products) is converted into electrical energy, thereby enhancing the rate of degradation of PAH in environmental matrix (Sherafatmand and Ng, 2015; Daghio *et al.*, 2017; Kronenberg *et al.*, 2017).

MFC technology has been applied in various studies to transform PAHs to less complex, even in some cases, environmental benign compounds. Feasibility of MFC for hydrocarbon removal has been highlighted in some studies (see Table 2-1 of Chapter 2). Some reported outcomes include treatment efficiencies (15 to 100%), current densities (4.89 mA/m^2 to 480 mA/m^2), power densities (0.19 mW/m^2 to 1089 mW/m^2), and treatment duration (120 hours to 770 days) (Morris *et al.*, 2009; Zhang *et al.*, 2010; Mohan and Chandrasekhar, 2011; Chandrasekhar and Venkata Mohan, 2012; Friman *et al.*, 2013; Rakoczy *et al.*, 2013; Adelaja *et al.*, 2014a; Adelaja *et al.*, 2015; Wei *et al.*, 2015; Daghio *et al.*, 2016; Venkidusamy *et al.*, 2016; Adelaja *et al.*, 2017; Chang *et al.*, 2017; Daghio *et al.*, 2018a; Mohanakrishna *et al.*, 2019; Zhou *et al.*, 2020). Furthermore, efficacy of MFCs for treatment of flowback and produced water in oil and gas industry has been demonstrated (Jain *et al.*, 2016; Sheikhyousefi *et al.*, 2017; Shrestha *et al.*, 2018; Zhang *et al.*, 2018; Mohanakrishna *et al.*, 2019; Feng *et al.*, 2020; Hemalatha *et al.*, 2020). Chemical oxygen demand (COD) removal efficiencies between 58% and 88% were

reported for double chamber MFCs using external resistance (R_{ext} =1000 Ω), except for Jain *et al.* (2016) where cathode potential was poised at 400 mV vs Ag/AgCl and COD removal was 91.25%. Mohanakrishna *et al.* (2019) reported higher COD removal in double chamber MFC (60.2%) compared with single chamber MFC (54.7%) using R= 100 Ω. Differences in COD removal and power output in double chamber MFCs from Shrestha *et al.* (2018) (88%, 3 mW/m^2) and Mohanakrishna *et al.* (2019) (60.2%, 1089 mW/m^2) was attributed to nature of inoculum used and presence of more active anodic biofilm, respectively. However, it is unclear if differences in external resistance (R_{ext} = 1000 Ω and R_{ext} = 100 Ω, respectively) may have played a role in different outcomes reported.

MFC optimization for maximum removal of contaminant coupled with high power output is desirable to enhance cost-effectiveness of MFC for environmental remediation. A robust anodic biofilm is crucial for high hydrocarbon removal efficiency coupled with electric current production (Mohan *et al.*, 2008a). The nature of anodic biofilm can be influenced by inoculum used for MFC start-up. High microbial diversity in wastewater has been linked with improved MFC performance due to more efficient organic substrate degradation or consumption (Adelaja *et al.*, 2014b; Heidrich *et al.*, 2018). Hence, it is expected that inoculum with higher microbial diversity would support development of robust anodic biofilm and consequently translate to enhanced hydrocarbon removal in MFC. Furthermore, external resistance applied has been shown to impact anode potential, anodic biofilm structure and diversity, cell voltage, coulombic efficiency as well as MFC current and power output (Menicucci *et al.*, 2006; Aelterman *et al.*, 2008b; Jadhav and Ghangrekar, 2009; Lyon *et al.*, 2010; Katuri *et al.*, 2011; Ren *et al.*, 2011; Rismani-Yazdi *et al.*, 2011; Zhang *et al.*, 2011b; González del Campo *et al.*, 2016; Pasternak *et al.*, 2018; Koók *et al.*, 2020; Cabrera *et al.*, 2021; Potrykus *et al.*, 2021). Reports of high COD removal efficiency using low R_{ext} (Jadhav and Ghangrekar, 2009; Katuri *et al.*, 2011) and low COD removal efficiency with low R_{ext} (González del Campo *et al.*, 2016) have been published. Moreover, these studies were conducted using simple organic substrates such as acetate, sucrose, glucose, and fructose. Clearly, different external resistances have different impacts on COD removal efficiency. Consequently, it is postulated that external resistance could impact removal efficiency of complex substrates such as PAHs. However, corelation between external resistance and hydrocarbon removal efficiency has not been established. As previously mentioned, PAH are not readily bioavailable because of their low aqueous solubility (Liang *et al.*, 2007; Lamichhane *et al.*, 2017); hence PAH bioavailability in aqueous medium could also be enhanced with surfactant application (Cheng and Wong, 2006).

The aim of the study was to understand the influence of inoculum source and external resistance on anodic biofilm development, current output, and PAH removal efficiency in the presence of surfactant. The objectives of the study were: (i) to enrich anodic biofilm with inoculum from different sources; (ii) to understand tolerance of anodic biofilm for low concentration of surfactant; (iii) to study impact of inoculum source on current output and PAH removal efficiency; and (iv) to examine effect of different external resistance on current output and PAH removal efficiency.

4.2 Experimental Procedure

Experimental set-up, operating conditions and experimental analysis for this chapter are described in sections 3.1 to 3.6.

4.3 Results and Discussion

4.3.1 Biofilm growth and development using different sources of inoculum

Biofilm growth and development was performed using acetate as substrate because acetate is an expected terminal metabolite of anaerobic degradation of polyaromatic hydrocarbon. As can be seen from Figure 4-1, bioreactors inoculated with MFC effluent (M) exhibited longer lag phase (11 days) than bioreactors inoculated with activated sludge (AS) (4 days). Even though AS started up faster than M, stable current output was achieved faster for M than AS. However, by the end of the experiment, maximum current output in MFCs inoculated with MFC effluent and activated sludge were similar (16.7 ± 0.0 A/m^2). Fast start-up in AS may have been due to high microbial diversity and high organic content (chemical oxygen demand, COD = 361.6 ± 1.3 mg/L) in activated sludge compared with MFC effluent (COD = 39.3 ± 0.2 mg/L). Nevertheless, similar maximum current output in M and AS agreed with previous studies where no difference in MFC performance was observed when bioanode was fully developed, regardless of inoculum source (Wang *et al.*, 2009; Yates *et al.*, 2012).

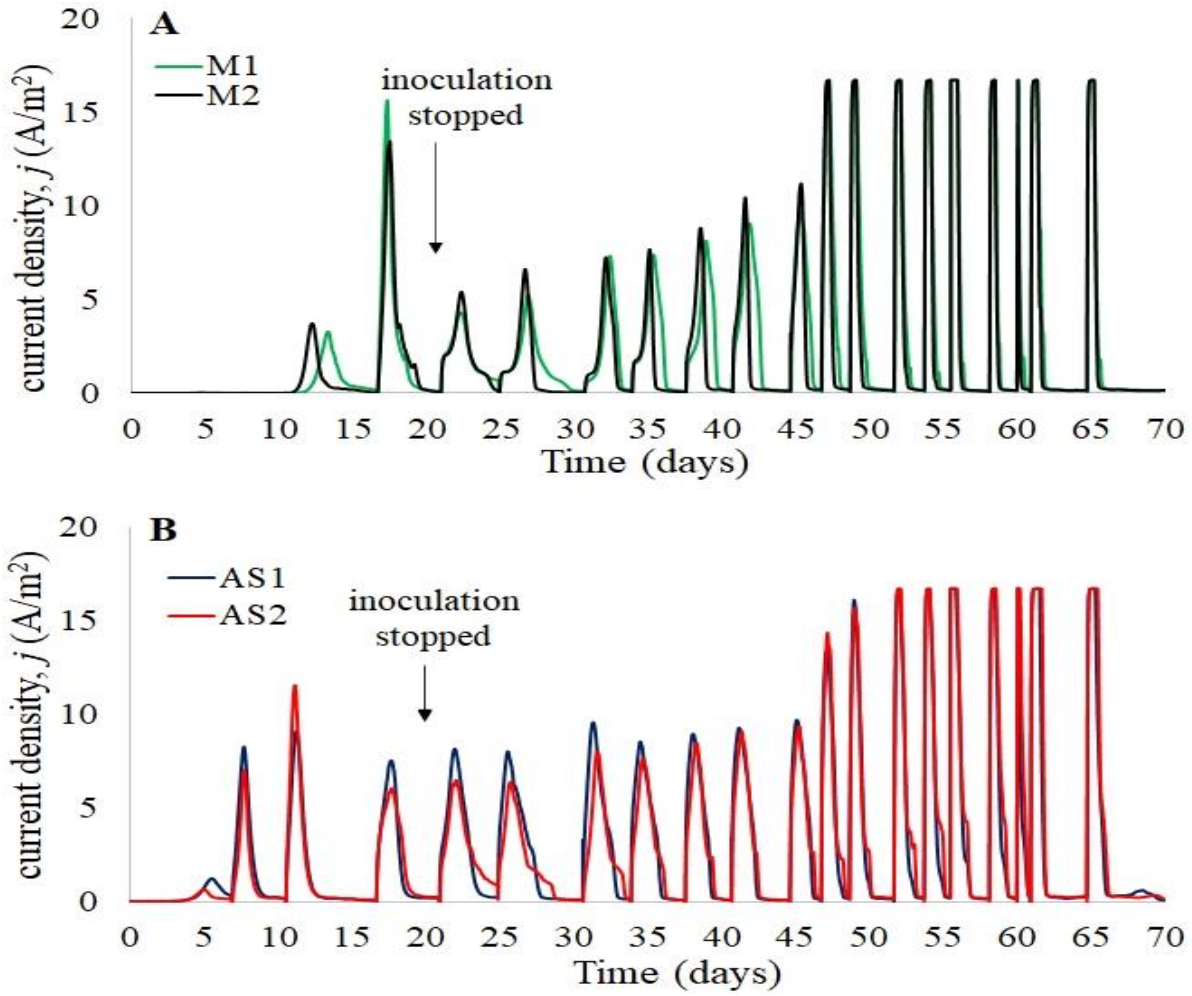


Figure 4-1. Current output in duplicate microbial fuel cells during biofilm enrichment on carbon fibre brush anodes using inoculum from (A) effluent from pre-existing microbial fuel cell and (B) activated sludge (carbon source = acetate, temperature = 30℃, poised potential = -0.2V vs Ag/AgCl reference electrode).

Similarity of cyclic voltammograms (CV) of anodic biofilms of MFCs inoculated with MFC effluent and activated sludge at zero substrate turnover suggested that similar redox systems were present in these bioreactors (see Figure 4-2). Bioreactors inoculated with MFC effluent exhibited 2 redox systems at: (1) -359 mV and (2) -266 mV (vs Ag/AgCl) which were close to formal potentials for cytochrome a3 and c, outer membrane-bound proteins commonly used by bacteria for direct electron transfer. Bioreactors inoculated with activated sludge exhibited one redox system at -340 mV vs Ag/AgCl (see point 3 in Figure 4-2). These CVs closely resembled those of *Geobacter sulfurreducens*, a well-documented electroactive bacterium capable of direct electron transfer (Fricke *et al.*, 2008; Marsili *et al.*, 2010). It could be inferred that anodic biofilm in all bioreactors were selectively enriched with electroactive bacteria. Biofilms from activated sludge bioreactors had higher electron holding capacity than biofilms from MFC effluent bioreactors which may explain why AS1 and AS2 initially produced higher current

output than M1 and M2. It was likely that activated sludge bioreactors had developed thicker and more active biofilms than MFC bioreactors.

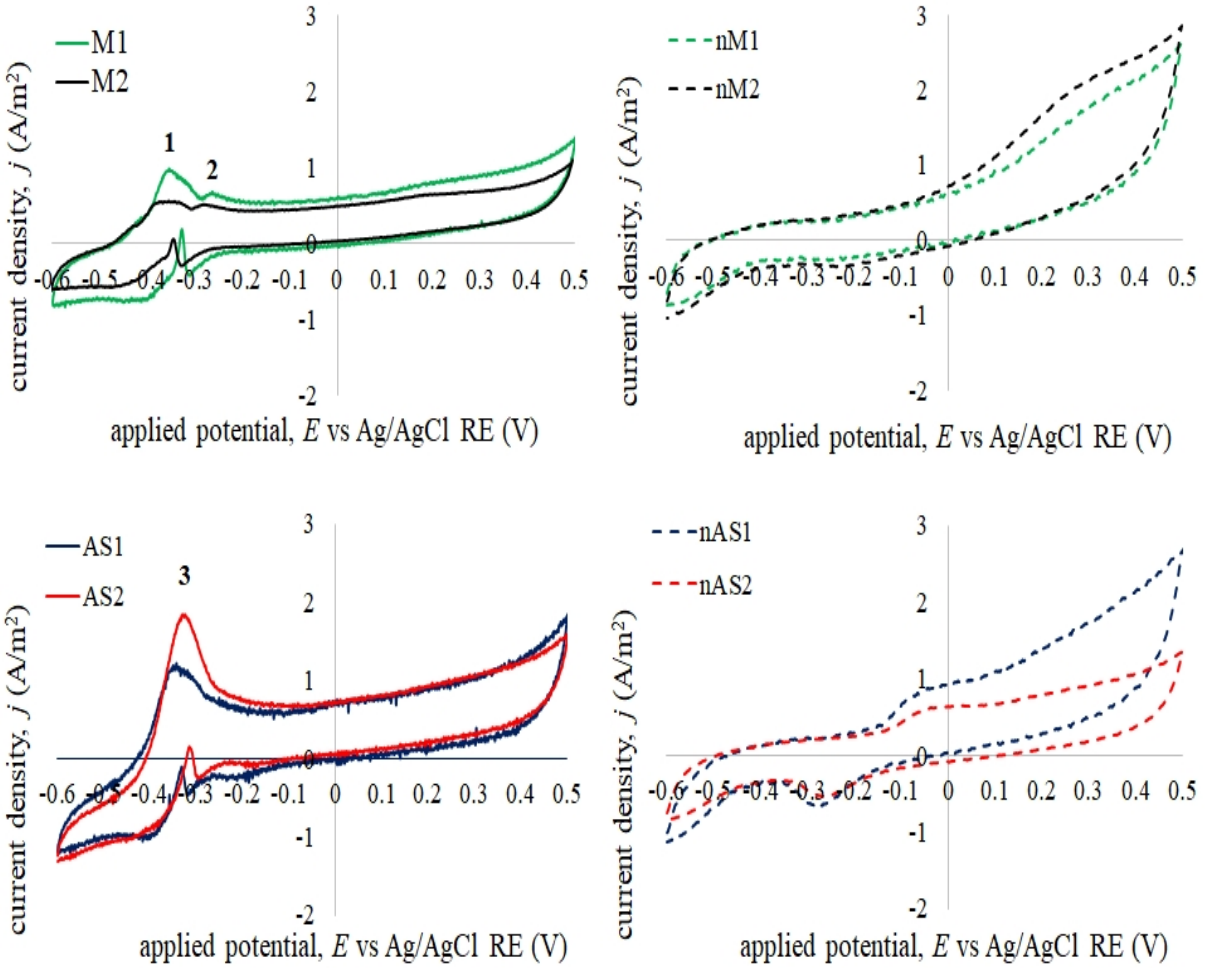


Figure 4-2. Cyclic voltammograms of acetate-fed microbial fuel cells during biofilm enrichment on carbon fibre brush anodes using inoculum from effluent from pre-existing microbial fuel cell (M = closed circuit, nM = open circuit) and activated sludge (AS =closed circuit, nAS = open circuit) Redox systems observed at -359 mV (1) and -266 mV (2) for M, and at -359 mV (3) and -266 mV for AS (temperature = 30℃, poised anode potential = -0.2V vs Ag/AgCl reference electrode, RE).

Following addition of polycyclic aromatic hydrocarbons (PAHs) (50 ppm) on day 66, current output in all bioreactors dropped to less than 1 A/m^2. Although there was marginal improvement in current when MFCs were fed with acetate (0.1 g/L), significant current output (up to 10 A/m^2) was restored only after all MFCs were re-inoculated and fed with acetate (1g/L). These observations suggested anodic biofilm either did not acclimate to hydrocarbon because hydrocarbons have more complex structure than acetate (Chae *et al.*, 2009; Pant *et al.*, 2010). It also suggested that hydrocarbon was likely not readily available to anodic biofilm. Consequently, all MFCs were started-up with polyaromatic hydrocarbon as substrate instead of acetate. Taking into consideration low aqueous solubility of hydrocarbons, surfactant was

applied was applied to enhance hydrocarbon bioavailability (Paria, 2008). From previous studies on surfactant-enhanced remediation polyaromatic hydrocarbons, Tween 80 was found to more biodegradable and less toxic than anionic and cationic surfactants, and more effective than other nonionic surfactants (Wen *et al.*, 2011; Iglesias *et al.*, 2014; Lamichhane *et al.*, 2017). Emulsion stability tests were conducted to determine minimum concentration of Tween 80 required to enhance hydrocarbon bioavailability.

4.3.2 Biofilm response to surfactant

For emulsion stability tests, solubility of polyaromatic hydrocarbon in the presence of surfactant was determined by formation of colloidal solution (see section 3.2.3). As shown in Table 4-1, the minimum concentration of Tween 80 required to solubilize the lowest concentration of hydrocarbon (50ppm) was 0.1 g/L. Hence, Tween 80 concentration of 0.1 g/L was selected to minimize competitive inhibition of hydrocarbon biodegradation. Minimum concentration of Tween 80 was used to prevent micro-organisms from switching to Tween 80 as carbon source instead of PAHs.

Table 4-1. Results of emulsion stability tests showing solubility of different concentrations of hydrocarbon solution (containing phenanthrene and fluoranthene) (50, 100, 500, 1000, 2000 ppm) in presence of various concentrations of Tween 80 surfactant (0.01, 0.1, 0.5, 1, 2 g/L). Distilled water containing no surfactant was used in control experiments.

Hydrocarbon concentration (ppm)		**Tween 80 concentration (g/L)**					
		1 (control)	**2**	**3**	**4**	**5**	**6**
		0	0.01	0.1	0.5	1	2
A	50	N	N	Y	Y	Y	Y
B	100	N	N	PS	Y	Y	Y
C	500	N	N	N	PS	N	PS
D	1000	N	N	N	N	N	N
E	2000	N	N	N	N	N	N

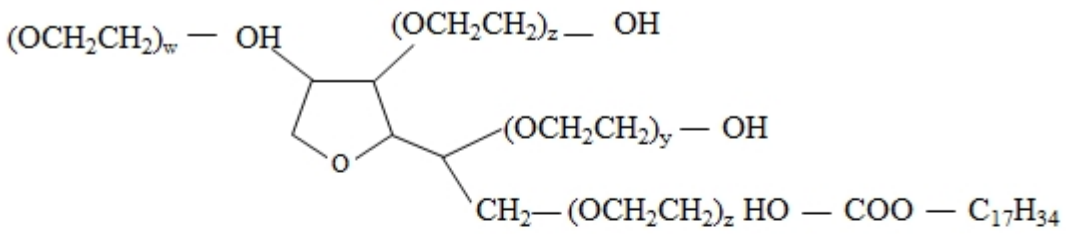
PS = partially soluble. N = not soluble. Y = completely soluble.

Although higher concentrations of Tween 80 were required to completely solubilize higher concentrations of hydrocarbon, high concentrations of Tween 80 could lead to disruption of biofilm or bacterial degradation of Tween 80 thereby inhibiting PAH biodegradation (Xiao-Hong *et al.*, 2010; Schreiberová *et al.*, 2012). Tween 80 concentrations ranging from 0.08 to 3

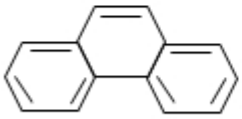
g/L have been shown to have no negative effect on anodic biofilm (Cheng and Wong, 2006; Wen *et al.*, 2011).

Bioreactors were first fed on only Tween 80 (under R_{ext} = 1000 Ω) to observe biofilm response to surfactant as well as surfactant contribution to current output. Anodic biofilms in all MFCs were able to tolerate low concentrations of Tween 80 (0.1 g/L or 100 ppm). During stage 1 feeding cycle (Tween 80, 100 ppm), average current density in MFCs inoculated with activated sludge (59.44 ± 0.98 mA/m^2) was observed to be significantly higher than average current density in MFCs inoculated with MFC effluent (1.65 ± 0.03 mA/m^2). High microbial diversity and synergistic relationships within bacteria in activated sludge inoculum, compared with MFC effluent inoculum, may have enhanced quick adaptation and subsequent breakdown of Tween 80, culminating in higher current density. This observation ties in with previous studies where high microbial diversity in wastewater fed MFCs corelated with improved performance (Heidrich *et al.*, 2018).

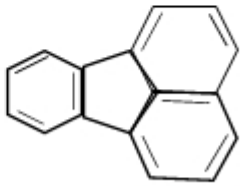
Polyoxyethylene (20) sorbitan monooleate (Tween 80/T80, $C_{64}H_{124}O_{26}$) is a non-ionic surfactant derived from polyoxyethylene sorbitan (hydrophilic segment) and oleic acid (lipophilic segment). Tween 80 is highly soluble and dispersible in water and has a critical micelle concentration (CMC) of 13 mg/L and hydrophile-lipophile balance (HLB) of 15. Critical micelle concentration is the minimum concentration of surfactant required for formation of micelles; formation of micelles increase solubility of hydrophobic compounds in aqueous systems (Mulligan *et al.*, 2001; Makkar and Rockne, 2003). HLB is the emulsifying potential of a surfactant based on an arbitrary scale of 0 – 20; and surfactants with high HLB values (>10) have high water solubility (Volkering *et al.*, 1997; Myers, 2006). As can be seen in Figure 4-3, the open and extended structure of the hydrophilic and lipophilic segments of Tween 80 make it more amenable to bacterial attack compared with phenanthrene (PHE) and fluoranthene (FLT) where high energy is required to break C=C bonds and open the aromatic rings. Surfactants such as Tween 80 enhance biodegradation either by increase hydrocarbon diffusion into aqueous phase for bacterial uptake or direct mass transfer from micelles into bacterial cell (Li and Chen, 2009). Various concentrations of Tween 80 have been successfully employed for enhanced degradation of phenanthrene and/or fluoranthene, for example 5 g/L (Di Gennaro *et al.*, 2008), 1 – 5 g/L (Rodríguez-Escales *et al.*, 2013), 0.5 – 2 g/L (Aryal and Liakopoulou-Kyriakides, 2013), 0.5 g/L (Hickey *et al.*, 2007) and 0. 01 to 0.08 g/L (Li and Zhu, 2012). Hence, Tween 80 concentration used in this study (0.1 g/L) was within tolerable limits for anodic biofilm and established limits for enhancing PAH solubility.



Polyoxyethylene (20) sorbitan monooleate
(polysorbate 80/Tween 80/T80, $C_{64}H_{124}O_{26}$)
$w + x + y + z = 20$



Phenanthrene
$C_{14}H_{10}$



Fluoranthene
$C_{16}H_{10}$

Figure 4-3. Structure of parent organic compounds used as substrate in microbial fuel cells (MFCs) treating hydrocarbon contaminated wastewater. Adapted from Yang et al. (2010).

MFCs in this study were operated under anaerobic conditions. Under anaerobic conditions, Tween 80 can undergo hydrolysis and fermentation to generate methane via several metabolites including ethanol, lactate, formate, acetate, carbon dioxide, and hydrogen (Yeh and Pavlostathis, 2005). Tween 80 biodegradation is initiated by bacterial attack on the central point between the hydrophilic and lipophilic segments, or on the far end of the hydrophilic or lipophilic segment (Kim and Weber, 2003). This initial attack produces sorbitan, polyethylene glycol (PEG) units and long chain fatty acid (stearic acid). Sorbitan is hydrolysed and fermented to produce organic acids and alcohol via sorbitol, while PEG units are successively depolymerized to produce ethanol. Ethanol is converted to acetate and hydrogen, both of which may be used for methane (CH_4) production. Stearic acid undergoes β-oxidation to produce acetyl-CoA which is then converted to acetate. As shown in Figure 4-4, these different metabolic routes generate electron donors that can be utilized by electroactive bacteria as carbon and energy source with concomitant production of electric current.

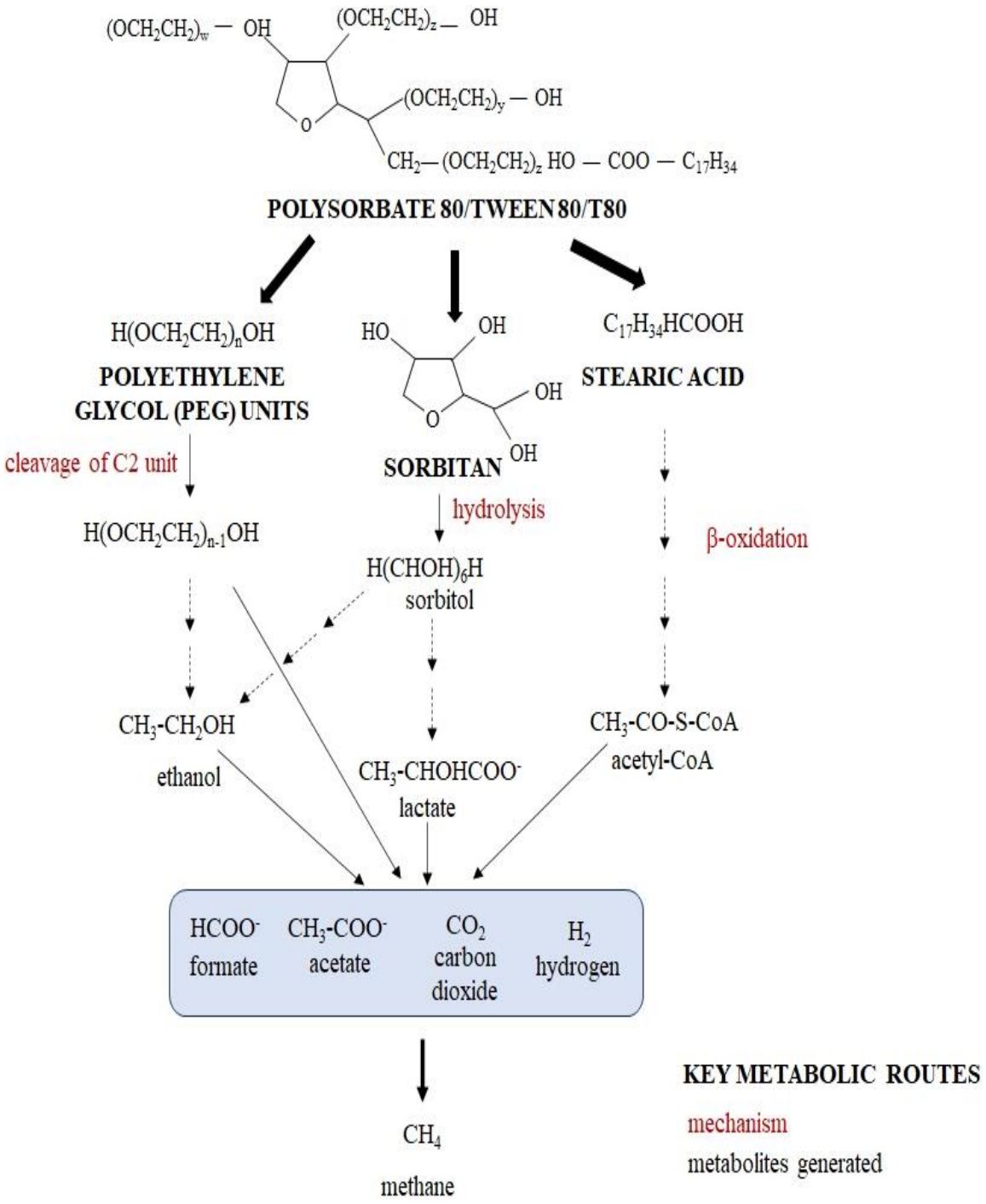


Figure 4-4. Anaerobic degradation pathway of Polyoxyethylene (20) sorbitan monoleate (Tween 80) surfactant. Adapted from Yeh and Pavlostathis (2005).

4.3.3 Effect of inoculum on current density and polyaromatic hydrocarbon removal

As previously mentioned in section 3.1.4, MFC bioreactors were fed with increasing concentrations of polycyclic aromatic hydrocarbon (PAH) in stage 2 (100 ppm Tween 80 + 50 ppm PAH) and stage 3 (100 ppm Tween 80 + 100 ppm PAH) of the experiment. When the feeding cycle was switched from stage 1 to stage 2, the average current density remained higher

in MFCs inoculated with activated sludge (57.00 ± 0.62 mA/m^2) when compared with MFCs inoculated with MFC effluent (13.09 ± 0.20 mA/m^2). Nevertheless, current density for activated sludge bioreactors steadily declined with increase in PAH concentration while current output for MFC effluent bioreactors increased with increase in PAH concentration. Current decline in MFCs can occur when there is depletion of substrate near the anode (Reimers *et al.*, 2001; Hong *et al.*, 2009). In this study, substrate limitation was unlikely because all MFCs were batch-fed. Current decline in MFCs inoculated with activated sludge was likely caused by negative impact of increased concentration of substrate on anodic biofilm. Increase in concentration of organic substrate has been demonstrated to have a detrimental effect on MFC anodic biofilm and electrogenic activity leading to reduced current output (Chandrasekhar and Venkata Mohan, 2012; Venkidusamy *et al.*, 2016). Some electrons could also have been diverted to other processes such as fermentation and methanogenesis.

For duration of the experiment, highest average current density for MFCs inoculated with activated sludge was generated in stage 1 (100 ppm Tween 80) whereas for MFCs inoculated with MFC effluent, highest average current density occurred in stage 3 (100 ppm Tween 80 + 100 ppm PAH). After 64 days of operation, similarities in current output were observed in MFCs inoculated with activated sludge (30.41 ± 0.46 mA/m^2) and MFC effluent (29.93 ± 0.37 mA/m^2). Statistical analysis was conducted using one-way ANOVA and showed that at 95% confidence interval, there was a significant difference in current density between MFC effluent bioreactors and activated sludge bioreactors during feeding stages 1 and 2, based on inoculum used ($p < 0.05$). For feeding stage 3, one-way ANOVA showed there was no significant difference in current density in these bioreactors ($p = 0.416$). P-value measures the probability that an observed difference between two or more samples/groups occurred randomly. The lower the p-value, the higher the significance of the observed difference.

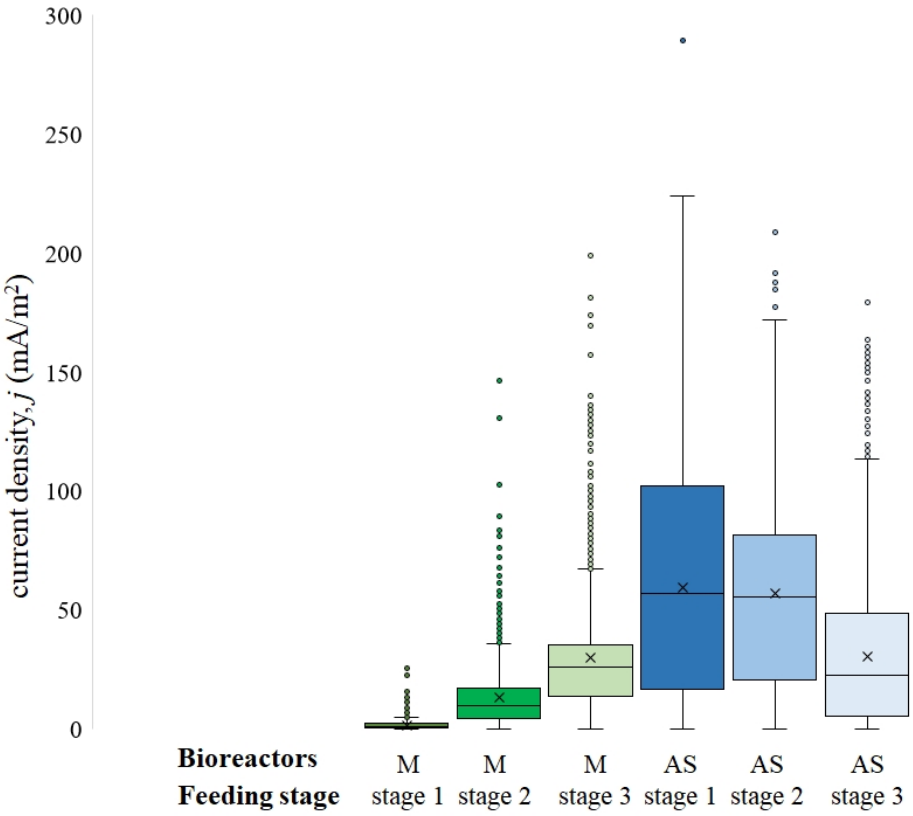


Figure 4-5. Effect of inoculum on current output in double chamber microbial fuel cells used for treatment of hydrocarbon contaminated wastewater in the presence of 0.1 g/L Tween 80 surfactant at temperature = 30℃ (M = inoculated with MFC effluent. AS = inoculated with activated sludge. Stage 1 = 100 ppm Tween 80 surfactant. Stage 2 = 100 ppm Tween 80 + 50 ppm polyaromatic hydrocarbon. Stage 3 = 100 ppm Tween 80 + 100 ppm polyaromatic hydrocarbon. External resistance, R_{ext} = 1000 Ω).

MFC bioreactors using polyaromatic hydrocarbons as substrate started up faster when activated sludge was used as inoculum. This may have been related to high microbial diversity in activated sludge inoculum, capable of utilizing different components of the hydrocarbon substrate. Contrary to previous observations when acetate was used as carbon source and poised anode potential was applied, simultaneous start-up of MFC effluent and activated sludge bioreactors on Tween 80 surfactant (using external resistance) suggested that even though anode potential influenced energy gain potential of anodic biofilm, anode potential alone did not necessarily determine start-up time. Energy gain potential is the theoretical amount of energy that bacteria can derive from Gibb's free energy of oxidation of organic substrate, and which the bacteria utilize for growth and cellular maintenance (Schröder, 2007; Torres *et al.*, 2009). In this study, simultaneous start-up for MFC effluent bioreactors and activated sludge bioreactors indicated inoculum had no impact on start-up time. However, wide margin in current output between MFC effluent bioreactors and activated sludge bioreactors in stages 1 and 2 pointed to influence of inoculum on current output. Microbial population in each

inoculum was not determined therefore, it could not be ascertained if the observed difference was due to variance in concentration of micro-organisms in the inocula. Differences in current output for MFC bioreactors and activated sludge bioreactors also suggested that organic substrate was being consumed at different rates in these systems hence, all bioreactors were investigated for total organic carbon (TOC) removal. For ease of discussion, MFCs inoculated with MFC effluent inoculum are referred to as MFC effluent bioreactors while MFCs inoculated with activated sludge inoculum are referred to as activated sludge bioreactors.

Differences in TOC removal rates were observed in all bioreactors. As can be seen from Figure 4-6, TOC removal rates varied across different treatment cycles of the experiment. TOC removal in all bioreactors during stage 1 (Tween 80 cycle) suggested that Tween 80 was readily utilized by anodic biofilm as carbon source. Except for one outlier (nAS2), MFC effluent and activated sludge bioreactors exhibited similar TOC removal rates for stage 1. Nevertheless, TOC removal was on the average slightly better in activated sludge bioreactors than MFC effluent bioreactors with increase in PAH concentration showing up to 92% rate of increase in TOC removal rate from 18.5% (average M1 and M2) to 35.4% (average AS1 and AS2) in stage 3 (100 ppm PAH). The lowest TOC removal rates were recorded during stage 3 (100 ppm PAH cycle) in most MFCs. In general, TOC removal rates between main cells (M and AS) and their counterparts at open circuit potential (OCP) (nM and nAS) were similar throughout the experiment.

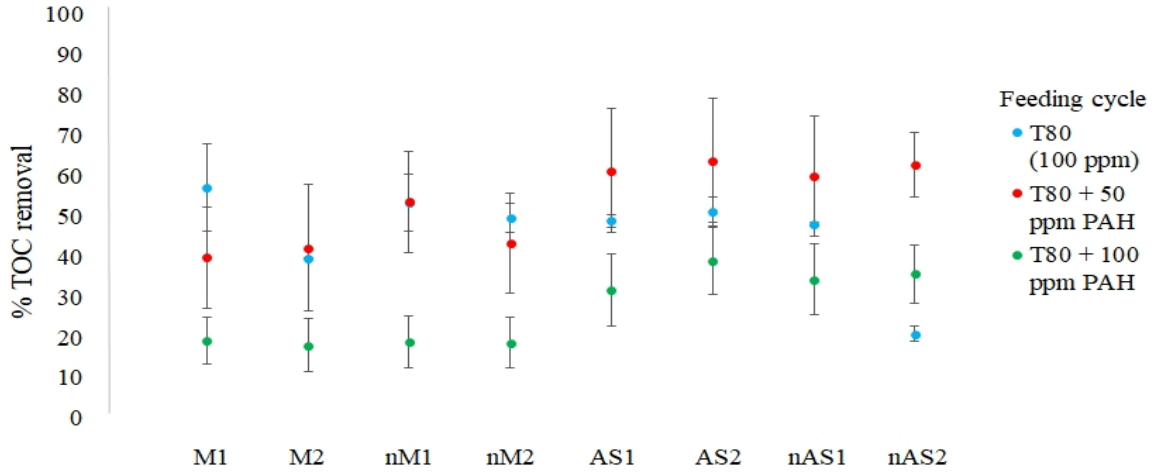


Figure 4-6. Total organic carbon (TOC) removal in double chamber microbial fuel cells used for treatment of hydrocarbon contaminated wastewater (temperature = 30℃, external resistance, R_{ext} = 1000 Ω, M = closed circuit, inoculated with MFC effluent, AS = closed circuit, inoculated with activated sludge, nM = open circuit, inoculated with MFC effluent, nAS = open circuit, inoculated with activated sludge).

TOC removal rates in activated sludge bioreactors appeared to correspond with current output especially during Tween 80 cycle, while the reverse was observed in MFC effluent bioreactors.

Negligible current was generated in MFC effluent bioreactors during Tween 80 cycle with concomitant TOC removal between 40 – 57%. This supports the hypothesis of diversion of electrons by oxidation of organic substrate. These electrons were likely being channelled by alternative electron acceptors into other processes such fermentation or methanogenesis. In a recent study, Ai *et al.* (2022) demonstrated that under anaerobic conditions, Tween 80 enhanced hydrolysis and acidogenic fermentation of activated sludge, resulting in increased generation of volatile fatty acids (VFAs). The most abundant VFAs generated were acetate and butyrate. Increase in microbial diversity was observed in this study in which both Tween 80 and organic matter in activated sludge were consumed by microbial community. Although Tween 80 degradation efficiency was almost 85%, Tween 80 contribution to sludge fermentation and VFA production was only 4 %, and VFA production declined overtime as organic matter was consumed (Ai *et al.*, 2022). In activated sludge bioreactors, it was likely that high current output during Tween 80 cycle was mainly due to fermentation of organic matter in activated sludge inoculum. Low contribution of Tween 80 to VFA production could be possible as Tween 80 reportedly has theoretic chemical oxygen demand (ThCOD) of 2.01 g O_2/g (Yeh *et al.*, 1998). For this study, effective mass of Tween 80 per bioreactor was 0.03 g. This also explains why both TOC removal and current output decreased over time as organic matter decreased and PAH became the primary carbon source for anodic biofilm. In addition, higher TOC consumption in activated sludge bioreactors may have been due to higher microbial diversity suggesting that TOC is being consumed by both electrochemically active/electroactive bacteria (EAB) and non-EAB. This could have explained why TOC removal was enhanced at R_{ext} = 1000 Ω during 50 ppm PAH cycle. Differences in production and consumption rates of volatile fatty acids such as formate, propionate, and acetate from Tween 80 may explain variation in TOC removal rates as well as fluctuations in current output throughout the duration of the experiment.

Initial low current output in MFC effluent bioreactors made it unlikely that Tween 80 was being directly consumed by electrochemical active bacteria (EAB). As previously explained, fermentative, and methanogenic bacteria were likely responsible for Tween 80 degradation to generate simpler carbon compounds like acetate which were then consumed by EAB (Yeh and Pavlostathis, 2005; Morris *et al.*, 2009; Heidrich *et al.*, 2018). While both MFC effluent and activated sludge bioreactors displayed similar rates of TOC removal (during stage 1 i.e. Tween 80 cycle), MFC effluent bioreactors exhibited lower current output in relation to TOC removed. It is most probable that slow turnover of electron donors from degradation of Tween 80 in the presence of low microbial diversity (based on MFC effluent inoculum likely consisting

primarily of EAB) may have resulted in the disparity between TOC removed and current output seen in MFC bioreactors during Tween 80 cycle.

Even though activated sludge bioreactors exhibited higher TOC removal, similar removal rates in both closed and open circuited bioreactors suggested that hydrocarbon removal was probably dominated by fermentation. High external resistance has been shown to cause shift in microbial metabolism to fermentation (Rismani-Yazdi *et al.*, 2011). It was also possible that oxygen ingress into the bioreactors, perhaps during medium change/feeding cycle change, may have inhibited some electroactive bacteria. Previous studies have shown that low external resistance may enhance electron transfer rates and treatment efficiency (Katuri *et al.*, 2011), hence further investigation was conducted to effect of external resistance on current output and polyaromatic hydrocarbon removal.

4.3.4 Effect of external resistance on current output

Regardless of external resistance applied, MFC effluent and activated sludge bioreactors started up at the same time. Similar start up time at same resistance has been reported in another study (Ren *et al.*, 2011). Anodic biofilm start-up time was halved when external resistance (R_{ext}) was lowered from 1000 Ω to 100 Ω. Except for one duplicate (AS2), higher current output was displayed in activated sludge bioreactors when external resistance was lowered to 100 Ω (see Figure 4-7). In AS2, higher current output was generated in stage 1 (Tween 80 cycle) at R_{ext} = 1000 Ω. This was evident when cumulative charge output was calculated for all bioreactors.

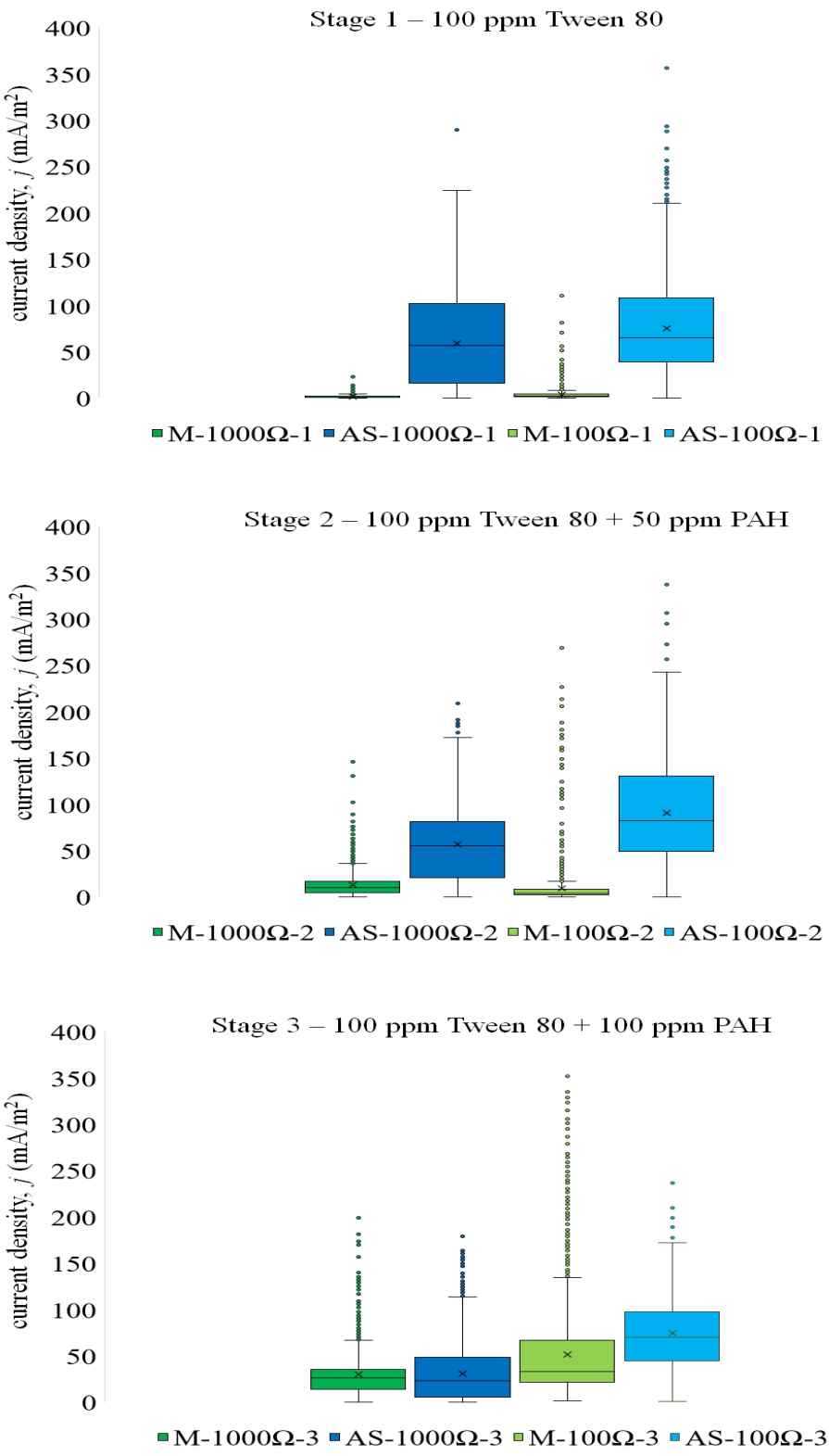


Figure 4-7. Effect of external resistance on current output in double chamber microbial fuel cells used for treatment of hydrocarbon contaminated wastewater in the presence of 0.1 g/L Tween 80 surfactant at temperature = 30℃ (M = inoculated with MFC effluent. AS = inoculated with activated sludge. Stage 1 = 100 ppm Tween 80 surfactant. Stage 2 = 100 ppm Tween 80 + 50 ppm polyaromatic hydrocarbon. Stage 3 = 100 ppm Tween 80 + 100 ppm polyaromatic hydrocarbon).

As can be seen from Table 4-2, the charge output for activated sludge bioreactors was 2 - 5 times higher for than MFC effluent bioreactors. Activate sludge bioreactors showed high cumulative charge in each of the three test stages while steady increase in charge output was recorded for MFC effluent bioreactors as test proceeded from stage 1 to 3. On the average cumulative charge output increased in activated sludge bioreactors (in stage 2) when R_{ext} was reduced from 1000 Ω (57 ± 13 C) to 100 Ω (102 ± 9 C). Variance in cumulative charge passed between repeats made it difficult to draw conclusion on effect of applied external resistance on cumulative charge. Variance between the duplicate activated sludge bioreactors (AS1 and AS2) was indicative of changes in electron transfer rates, likely due to the highly sensitive and dynamic nature of anodic biofilms. Anodic biofilms have been demonstrated to be continuously evolving even after voltage stabilization has been achieved (Paitier *et al.*, 2017). Low reproducibility was likely caused by low number of replicates used in the experiment. The number of replicates set-up was limited by availability of laboratory facility for potentially bio-hazard experiments, and limited number of channels that could be accommodated on the equipment.

Table 4-2. Cumulative charge output (in Coulombs, C) in microbial fuel cells treating hydrocarbon wastewater (duration of each stage = 21 days. M = inoculated with MFC effluent, AS = inoculated with activated sludge. T80 = Tween 80 surfactant. PAH = polycyclic aromatic hydrocarbon).

treatment cycle	**MFC effluent inoculum**				**activated sludge inoculum**			
	100 Ω		**1000 Ω**		**100 Ω**		**1000 Ω**	
	M1	**M2**	**M1**	**M2**	**AS1**	**AS2**	**AS1**	**AS2**
Stage 1: 100 ppm T80	2.35	5.01	2.09	1.50	90.54	52.70	25.28	101.01
Stage 2: 50 ppm PAH	5.02	15.35	18.37	8.01	107.73	95.40	48.27	66.16
Stage 3: 100 ppm PAH	67.89	104.58	84.66	95.46	104.03	170.67	26.34	168.65

Furthermore, as seen in Figure 4-7, current output on average increased with lower R_{ext} for a given sample. A 10-fold decrease in R_{ext} (from 1000 Ω to 100 Ω), resulted in 1.3 - 1.7 times higher average current output in both M and AS bioreactors. One-way ANOVA ($p < 0.05$) confirmed that there was a significant difference in current output based on R_{ext} applied. Higher current output at low R_{ext} is expected as for a given cell voltage lower external resistance allows for larger current flow (Ohm's law). The higher current will consequently result in lower cell voltage and higher anode potential and could result in better biofilm establishment. According to Zhang *et al.* (2011b), faster start-up time and higher current output at lower resistance could

be attributed to increase in biomass and extracellular polymeric substances (EPS) content of biofilms. EPS are high molecular weight natural polymers secreted by bacteria. EPS control functional and structural integrity of biofilms. MFCs with low R_{ext} undergo low cell polarization, hence improved current output (Aelterman *et al.*, 2008b). Increase in R_{ext} has been shown to decrease current output, electron transfer rate and EAB growth rate (Katuri *et al.*, 2011), which would explain why lower current is produced at higher R_{ext}. Nevertheless, marginal increase in maximum current density was contrary to observations from another study where 10-fold decrease in R_{ext} resulted in 3-fold increase in current output (Jadhav and Ghangrekar, 2009). It was generally understood that current output in the bioreactors was also affected by the complexity of substrate used in the study as well as other factors such as cathode performance, electrolyte conductivity and distance between anode and cathode. Coulombic efficiency can be used to evaluate conversion of chemical energy in organic substrate to electric current or charge.

4.3.5 Coulombic efficiency

One of the objectives of microbial fuel cells is to extract as much electrons as possible from organic substrate. As previous mentioned in section 3.6.2, the efficiency of conversion of extracted electrons to electricity is determined by coulombic efficiency (CE). As shown in Table 4-3, CE for M bioreactors was less than 4 % when PAH concentration was 50 ppm. Electron recovery improved (up to 8.61%) with increase in PAH concentration to 100 ppm; this corresponded with increased current output (see Figure 4-5). In contrast, AS displayed higher CE than M. CE appeared to improve at low R_{ext} where highest CE recorded was during 50 ppm PAH cycle (AS1 = 29.5%). Nevertheless, electron recovery diminished when PAH concentration increased from 50 to 100 ppm.

Table 4-3. Evaluation of coulombic efficiency (%) in double chamber microbial fuel cells treating hydrocarbon wastewater. (M = inoculated with MFC effluent, AS = inoculated with activated sludge. PAH = polycyclic aromatic hydrocarbons – phenanthrene and fluoranthene).

treatment cycle	**MFC effluent inoculum**				**activated sludge inoculum**			
	100 Ω		**1000 Ω**		**100 Ω**		**1000 Ω**	
	M1	**M2**	**M1**	**M2**	**AS1**	**AS2**	**AS1**	**AS2**
50 ppm PAH	1.32	3.34	3.24	1.41	29.56	25.42	12.92	16.67
100 ppm PAH	6.43	8.61	7.87	8.50	9.38	12.06	2.19	15.79

Variations in CE in MFCs can be linked to nature of substrate, with CE decreasing with increasing complexity of substrate (Rabaey *et al.*, 2005b; Velasquez-Orta *et al.*, 2011b; Nimje *et al.*, 2012). Low CE has previously been reported in double chamber MFCs using carbon fibre brush anode and water produced from oil and gas wastewater containing petroleum hydrocarbons as substrate (Shrestha *et al.*, 2018). Despite improvement in MFC performance over time, CE of 6% was displayed compared with 14% from a mixture of produced water and municipal wastewater and 39% from municipal wastewater only. Previous studies have reported inverse relationship between CE and R_{ext} (Rabaey *et al.*, 2005b; Ren *et al.*, 2011), however, this trend was only observed in AS bioreactors. Although high CE may be interpreted as high electron transfer efficiency, this is not necessarily true (Rabaey *et al.*, 2004). In AS bioreactors, high CE may have been due to high electron transfer rates caused by relative abundance of mixed consortia. High abundance of bacteria likely resulted in increased PAH biodegradation and conversion of metabolic intermediates into electron donors for electroactive bacteria (EAB), culminating in high current output. Low CE accompanied by high current output was suggestive of substrate consumption by non-electrochemical processes or non-EAB community (Katuri *et al.*, 2011). Low CE in both M and AS also suggested incomplete oxidation of PAH or diversion of electrons by alternative electron acceptors into other processes such fermentation or methanogenesis. This is expected given that full oxidation of PAH is very difficult to achieve even with most advanced catalyst. It is difficult to separate the various processes contributing to oxidation current, for example from surfactant or hydrocarbon oxidation; even for given hydrocarbon to what extent it is been oxidised and how much of that is via electron transfer reaction or via biochemical reaction. Total organic carbon (TOC) and PAH removal rates were investigated to understand substrate consumption pattern in the MFCs.

4.3.6 Influence of external resistance on polycyclic aromatic hydrocarbon removal

Despite similarities in average Total organic carbon (TOC) removal rates for MFC effluent (27 ± 2.3%) and activated sludge (33.2 ± 1.0%) bioreactors at low resistance (R_{ext} = 100Ω) and low PAH of 50 ppm, there was a significant difference between MFC effluent and activated sludge bioreactors in current output or total charge passed by factor of ca.10 (Table 4-2) and columbic efficiency also by factor of ca.10 (Table 4-3), for which MFC effluent bioreactors were lower in both. Overall, TOC removal rates were similar for closed circuit (M and AS) and open circuit (nM and nAS) bioreactors at both low and high external resistance (100 and 1000 Ω) except during stage 3 (100 ppm PAH cycle) at low resistance (R_{ext} = 100 Ω). During this cycle, closed circuit bioreactors exhibited higher removal rates than open circuit bioreactors. TOC removal

rates were observed to be significantly higher (by 50%) at lower R_{ext} (100 vs 1000 Ω) in the case of MFC effluent bioreactors but were similar in the case of activated sludge bioreactors (see Table 4-4).

Table 4-4. Variation of total organic carbon (TOC) removal with external resistance in double chamber microbial fuel cells used for treatment of hydrocarbon contaminated wastewater (Feeding stage 3: 100 ppm Tween 80 + 100 ppm polyaromatic hydrocarbon, PAH) (temperature = 30℃, M = closed circuit, inoculated with MFC effluent, AS = closed circuit, inoculated with activated sludge MFCs, nM = open circuit, inoculated with MFC effluent, nAS = open circuit, inoculated with activated sludge).

External resistance, R_{ext} (Ω)	**TOC removal (%) MFC effluent bioreactors***				**TOC removal (%) Activated sludge bioreactors***			
	M1	**M2**	**nM1**	**nM2**	**AS1**	**AS2**	**nAS1**	**nAS2**
100	30 ± 3	38 ± 7	16 ± 7	18 ± 2	44 ± 2	34 ± 5	13 ± 5	31 ± 5
1000	19 ± 6	18 ± 7	19 ± 6	19 ± 8	32 ± 9	39 ± 8	34 ± 9	36 ± 7

*TOC removal rates are presented as average of replicate analysis ± standard error

Increasing R_{ext} reduces energy gain by anodic biofilm and triggers modification of anodic community structure. This results in a shift to fermentative metabolism, accompanied by increased VFA production and lower CE (Jadhav and Ghangrekar, 2009; Rismani-Yazdi *et al.*, 2011). Consequently, higher organic substrate can be removed under such conditions. Hence, high TOC removal at high R_{ext} was possibly due to predominance of fermentation and explained why there were similar removal rates between closed and open circuit MFC bioreactors. Higher removal rates during 100 ppm PAH cycle at R_{ext} = 100 Ω may have been a function of increased biocatalytic activity of stable biofilm formed at low R_{ext}. More active and robust biofilms have been formed under low external resistance (Zhang *et al.*, 2011b; Koók *et al.*, 2020). This was evident in significant difference between closed and open circuit MFC effluent bioreactors. Total organic carbon removal possibly occurred via two competitive processes - biochemical degradation/fermentation and bio-electrochemical involving external electron transfer. There were also two sources of organic carbon: Tween 80 surfactant and PAH. In the absence of PAH, conversion of Tween 80 is high as seen in the large drop in TOC when switching from stage 1 (T80 only) (40.0 ± 5.1 %) to stage 2 (T80 + PAH) (27.0 ± 1.7 %). This process is biochemical/fermentation dominant and is associated with low current generation in cells as seen in Figure 4-5. When 50 ppm PAH is added the TOC reduction seen is a combination of fermentation removal of T80 and electrochemical removal of PAH with the latter being less complete (harder to fully oxidise) resulting in lower TOC removal. Though some studies have

reported improved substrate removal at low R_{ext} (Katuri *et al.*, 2011; Pasternak *et al.*, 2018), probable oxygen ingress into the activated sludge bioreactors may have caused marginal difference in TOC removal rates between closed and open circuit cells. As earlier mentioned, TOC removal represented removal of both surfactant and polyaromatic hydrocarbon, thus hydrocarbon analysis was conducted to determine actual change in concentration of polyaromatic hydrocarbons.

At low R_{ext} (100 Ω), within the error of experiments (between 2 repeats), there was no significance difference between PAH removal between 50 and 100 ppm PAH (see Figure 4-8A). At high R_{ext} (1000 Ω), PAH removal efficiency increased consistently in all bioreactors with increase in PAH concentration (see Figure 4-8B). One-way ANOVA ($p > 0.05$) showed that there was no significant difference in hydrocarbon removal efficiency between main and open circuit bioreactors under high R_{ext}. In previous study, decrease in hydrocarbon removal declined with increased concentration (Venkidusamy *et al.*, 2016). However, hydrocarbon concentrations used in this study was 16 – 160 times lower than used by Venkidusamy *et al.* (2016); hence, within tolerable limit for anodic biofilm. Similarities in PAH removal (at R_{ext} = 1000 Ω) in closed and open circuit bioreactors were indicative of dominance of fermentation as the primary mechanism of PAH degradation in these MFCs. Maximum CE values attained under high R_{ext} for M (8.50%) and AS (16.67%) were suggestive of low contribution of electrons generated from PAH oxidation to current output. Low CE at high R_{ext} agreed with previous studies where CE declined with increase in R_{ext} and fermentative metabolism became primary means of organic carbon removal (Katuri *et al.*, 2011; Ren *et al.*, 2011; Rismani-Yazdi *et al.*, 2011; Potrykus *et al.*, 2021). High PAH removal rates coupled with low CE were also suggestive of incomplete oxidation of PAH.

At low R_{ext} (100 Ω), similar PAH removal was achieved during 50 ppm PAH cycle (M = 86 ± 4 %; AS = 84 ± 3 %) and during 100 ppm PAH cycle (M = 84 ± 9 %; AS = 82 ± 2 %) (see Figure 4-8), even though CE was higher for AS during 50 ppm PAH cycle (M = 2.3 ± 1.4 %; AS = 27.5 ± 2.9 %) than during 100 ppm cycle (M = 7.5 ± 1.5 %; AS = 10.7 ± 1.9 %). Maximum CE values attained for MFC effluent bioreactors was 8.61% in contrast to the robust, thicker and more active anodic biofilms established at low R_{ext} (Zhang *et al.*, 2011b). At low R_{ext}, internal resistance is low and electron transfer is more efficient, hence higher treatment efficiencies can be achieved. MFC effluent bioreactors (at low R_{ext}) also displayed low anode potentials (-0.385 and -0.476 V vs Ag/AgCl, respectively) which supported existence of stable anodic biofilm. However, it was possible that increase in bacterial biomass either hampered access to substrate or led to dead microbes in inner biomass layer, thereby reducing substrate

consumption. In addition, lower respiration rate at -0.400 V vs Ag/AgCl despite increase in biomass has previously been reported (Aelterman *et al.*, 2008a). Reduced PAH consumption could also have been caused by negative interactions within microbial communities (Adelaja *et al.*, 2014a). Nonetheless, increase in current densities overtime in MFC effluent bioreactors indicated continuous evolution of microbial community structure including electroactive bacteria on anodic biofilm (Paitier *et al.*, 2017).

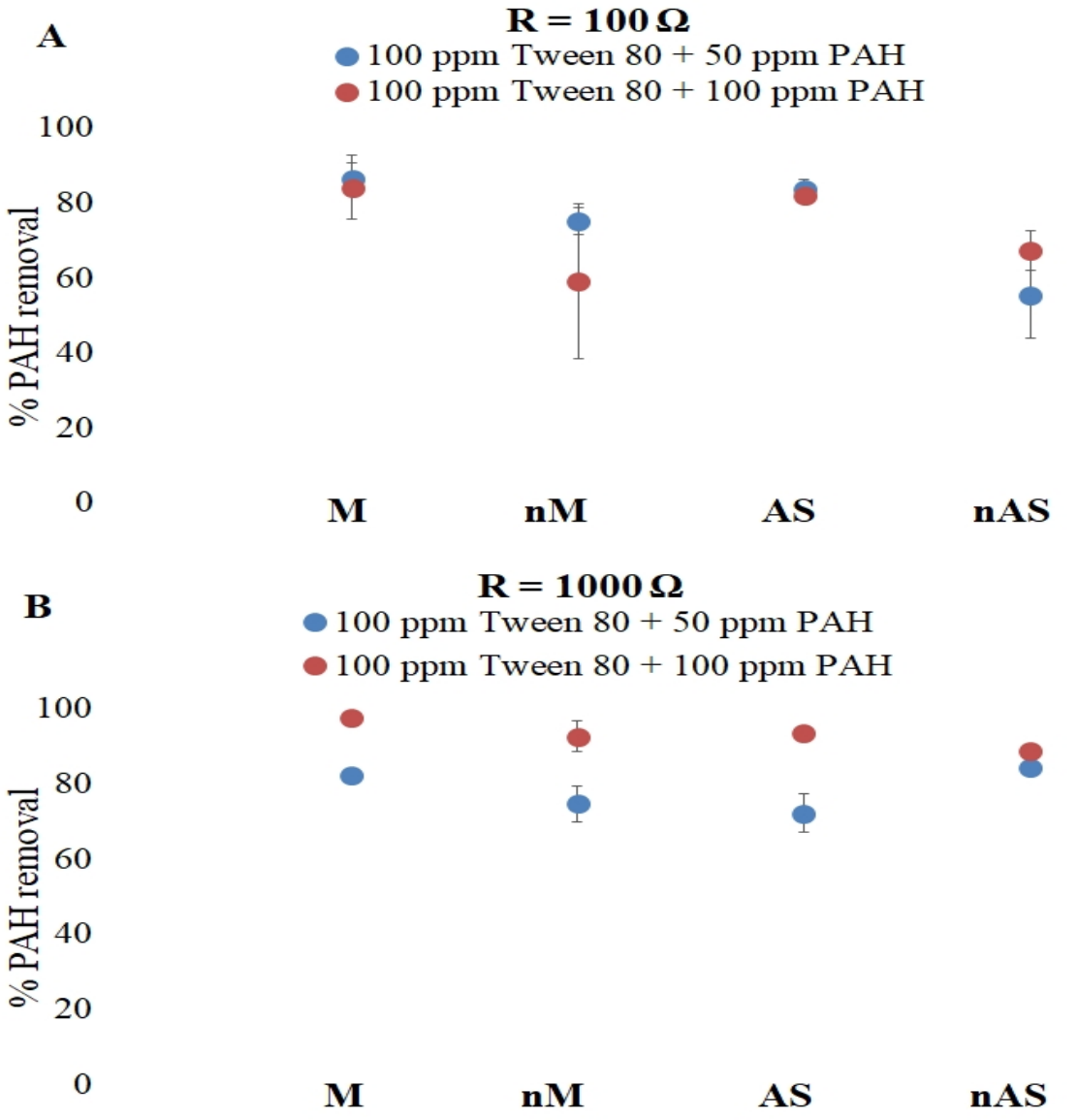


Figure 4-8. Comparison of polyaromatic hydrocarbon (PAH) removal rates with different sources of inoculum and external resistance in double chamber microbial fuel cells at 30℃ (M and AS = closed circuit; nM and nAS = open circuits; M and nM = inoculated with MFC effluent; AS and nAS = inoculated with activated sludge. 0.1 g/L (100 ppm) Tween 80 surfactant was used to enhance PAH bioavailability).

During stage 3 (100 ppm Tween 80 + 100 ppm PAH), PAH removal efficiencies in closed circuit MFCs in this study were observed to be higher under high R_{ext} (M = 98 ± 0 %, AS = 94

± 1 %) than at low R_{ext} (100Ω) (M = 84 ± 9 %, AS = 82 ± 2 %). High PAH removal efficiencies under high R_{ext} corresponded with previous studies where PAH removal efficiency 82 - 90% were been reported (Morris and Jin, 2007; Adelaja *et al.*, 2017). Nevertheless, treatment efficiencies (under high R_{ext}) did not agree with results obtained from other studies where closed circuit MFCs exhibited higher hydrocarbon removal than open circuit MFCs (Morris *et al.*, 2009). Absence of marked difference between closed and open circuit MFCS may be indicative of oxygen ingress into the system. It also suggested that hydrocarbon removal may have been driven by fermentative and hydrolytic bacteria rather than EAB or electrochemical oxidation, which agrees with higher PAH removal with decrease in current (higher R_{ext}) and lower CE. This is also in agreement with decrease in TOC removal from stage 1 to 3 in the treatment cycle i.e. from Tween 80 only stage to 50 to 100 ppm added PAH. This suggests that the microbes on anodic biofilm were selective towards oxidation of Tween 80 over PAH.

Under anaerobic conditions, PAH degradation is initiated by fumarate addition, carboxylation, or hydroxylation to its aromatic ring. Anaerobic PAH degradation progresses at a lower rate than aerobic degradation and is coupled to fermentation, methanogenesis, denitrification, sulphate reduction or metal reduction. Reduced metabolic products from these processes are used as electron acceptors and shuttle electrons generated by PAH degradation between EAB and MFC anode. Electron acceptors with higher redox potential are first consumed. These processes may be executed by the same or different bacteria; hence a plethora of microbes may be involved in degradation of a single PAH compound. Thus, syntrophic co-operation within microbial communities is necessary to support, if possible, complete oxidation of complex substrate such as PAH. Breakdown of high molecular weight (HMW) PAHs can generate methylated derivatives of PAHs of lesser C-ring number e.g. anaerobic degradation of chrysene produced methylated phenanthrene compounds (Liang *et al.*, 2014). The first phase of anaerobic PAH degradation generates benzoyl-CoA as central intermediate. The second phase of degradation involves the conversion of benzoyl-CoA to acetyl-CoA which is subsequently metabolised via Citric Acid cycle (Fuchs *et al.*, 2011).

Although, much remains to be understood about anaerobic degradation pathway for phenanthrene, phenanthrene can be degraded under nitrate or sulphate-reducing conditions (Rockne and Strand, 1998). Tsai *et al.* (2009) has shown that under sulphate reducing conditions phenanthrene can be degraded via *p*-cresol and phenol to acetic acid. Figure 4-9 shows how phenanthrene might be biodegraded anaerobically to provide electron donors for EAB. In this study, alternative electron acceptors such as sulphate and nitrate were supplied via Wolfe's mineral and vitamin solution (see Appendix B and Appendix C) contained in anode medium.

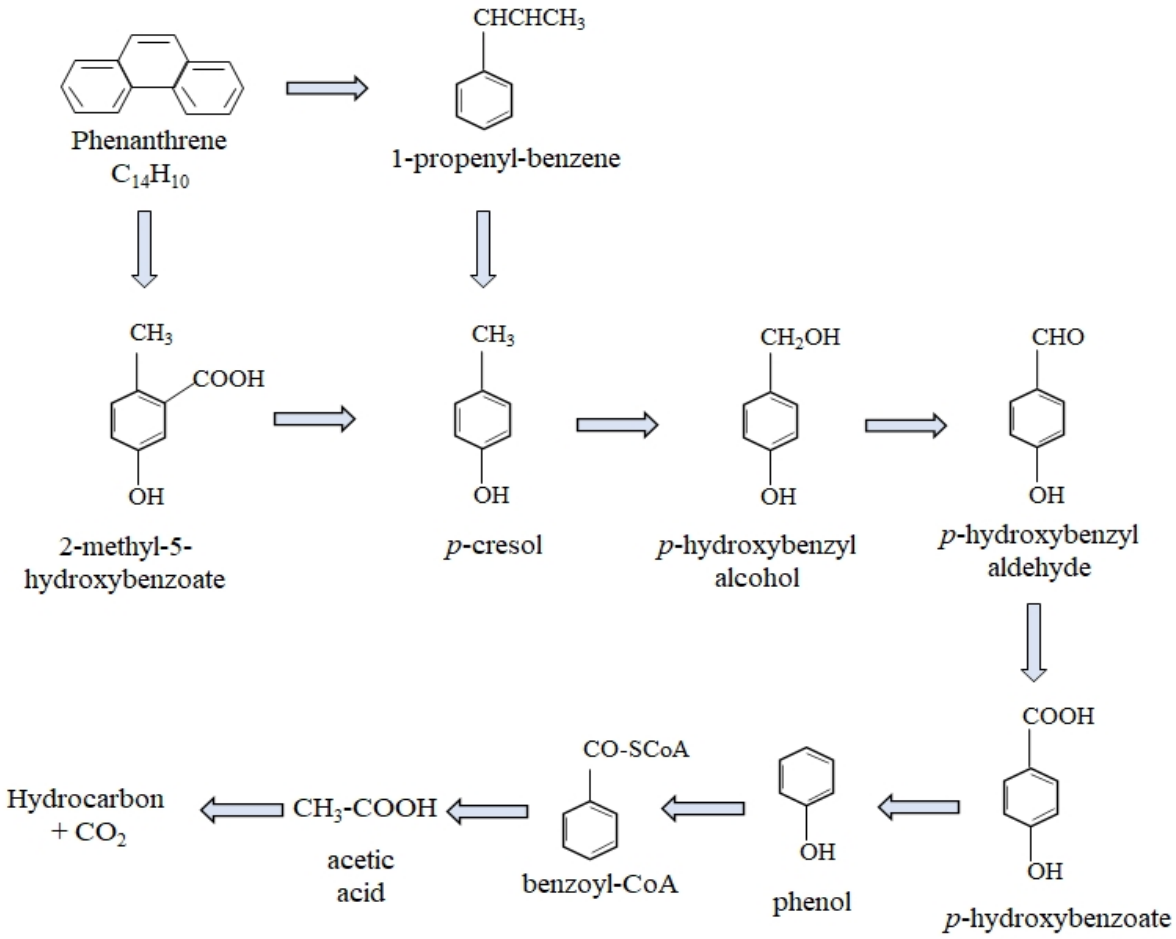


Figure 4-9. Proposed anaerobic pathway for phenanthrene. Adapted from Tsai et al. (2009) and Fuchs et al. (2011).

Surfactants enhance hydrocarbon degradation and current production by improving cell permeability. Improved cell permeability reduces charge transfer resistance increases the electron transfer rate and number, thereby increasing current and power output (Wen *et al.*, 2011). However, hydrocarbon degradation could be impeded if Tween 80 was preferential utilized as substrate, if it is toxic to microbes or if it blocks bacterial contact with hydrocarbon molecules (Li and Chen, 2009; Xiao-Hong *et al.*, 2010). Exact mechanism is determined by type of microbes involved and surfactant concentration. In mixed PAH systems, certain PAH may be preferentially consumed over another. For example, despite improved degradation of PHE and FLT with T80, PHE was preferentially degraded by *Sphingomonas* EPA 505; both substrates appeared to compete for the same enzymatic sites (Luning Prak and Pritchard, 2002).

4.3.7 Variation in volatile fatty acid production, pH, and anode potential (E_a)

Volatile fatty acids (VFAs) such as acetate, formate, propionate, and butyrate were detected at both high R_{ext} and low R_{ext} during stage 1 (T80 only day 0 to 21) and first 2 weeks of stage 2

(50 ppm PAH, day 21 – 34). No VFAs were detected during stage 3 (100 ppm PAH). Under high R_{ext} (1000 Ω) acetate concentrations were high in bioreactors on day 0-2, ranging from 2.11 to 7.78 ppm except in one outlier sample (nAS2) where up to 25 ppm acetate was detected and decreased rapidly to negligible values by day 21 (Appendix E). When using low R_{ext} (100 Ω) the decline in acetate concentration was slower and acetate was still detected during stage 2 (day 25) and was accompanied by increase in formate concentration. Within the experimental error (from two repeats), there was no significance difference between the samples from MFC effluent and activated sludge bioreactors. The most detected VFAs were acetate and formate. Longer chain VFAs such as propionate, butyrate, and isovalerate also accumulated in nAS2 (16.4, 24.9 and 23.9 ppm, respectively) but subsequent decline corresponded with increased TOC removal rates and current output during 50 ppm PAH cycle.

Metabolic products of anaerobic biodegradation of T80, as previously discussed, include formate, acetate, carbon dioxide, hydrogen, and methane. Highest concentration of VFAs occurred during Tween 80 cycle and corresponded with highest TOC removal rates, thereby confirming complete degradation of Tween 80 via fermentation (Siedlecka *et al.*, 2008). Even though VFA production was high, this did not result in significant current output during Tween 80 cycle, and implied that acetate produced may have been diverted into other processes like methanogenesis. Presence of longer VFAs suggested that either fermentation of Tween 80 was proceeding at a slower rate or alternative organic moieties were being consumed. However, subsequent rapid disappearance of these VFAs indicated that the latter was the case. Rapid decline and subsequently no detection of VFAs during 50 and 100 ppm PAH cycles could have signified rapid utilization of VFAs, because this corresponded with increased current output. Metabolic products of hydrocarbon breakdown can contribute more protons to anodic medium (Chandrasekhar and Venkata Mohan, 2012; Potrykus *et al.*, 2021). It is also possible that hydrogen generated via PAH degradation may have impeded Tween 80 biodegradation by diverting acetate to methane production (acetoclastic methanogenesis) (Yeh and Pavlostathis, 2005), hence reduction in acetate concentration, and current output, during these cycles overtime. As earlier demonstrated, hydrocarbon removal in MFCs in this study was dominated by fermentation and, likely, methanogenesis. VFA production may lower pH which may have consequences on anodic biofilm metabolism hence, pH and anode potential in the bioreactors was monitored over the duration of the experiment.

Generally, pH in the bioreactors remained between 6.5 and 7 with very slow steady decline over the 64 days of three stages of treatment cycle (see Figure 4-10). Under low external resistance (R_{ext} = 100 Ω), pH varied between 5.9 and 7, and rapidly decline over the treatment

period suggestive of rapid proton transfer across the membrane to cathode chamber for oxygen reduction. MFC anolyte comprised phosphate buffer which may explain why pH remained stable despite PAH consumption. Moreover, Veer Raghavulu *et al.* (2009) showed that EAB achieve more efficient electron transfer and higher coulombic efficiency under acidic conditions. Nonetheless, recorded pH values were within normal range (6-8) for EAB activity. Fluctuations in anode potential were recorded throughout the duration of the experiment as shown in Figure 4-10.

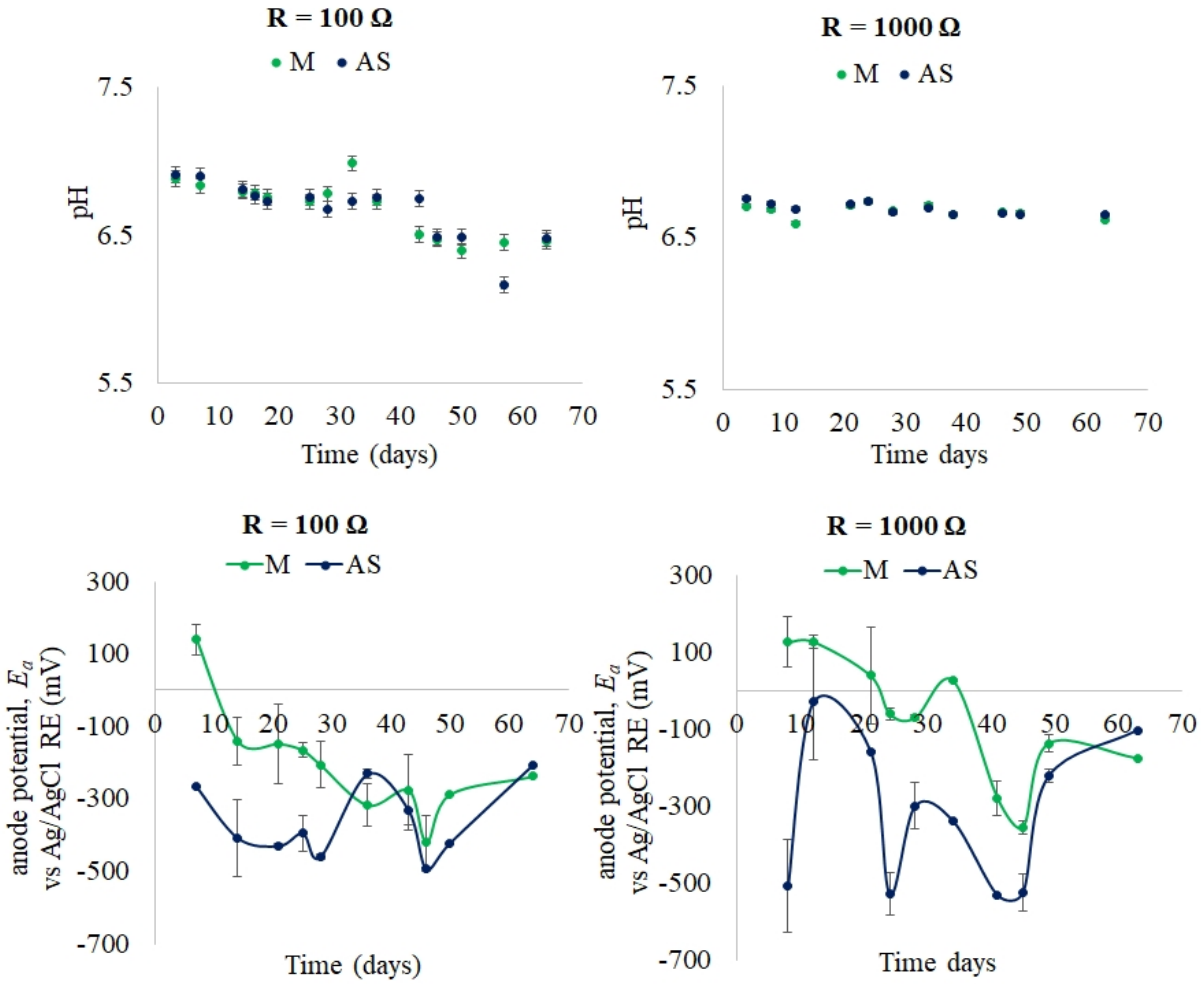


Figure 4-10. Change in pH and anode potential over time in double chamber microbial fuel cells used for hydrocarbon removal from wastewater under different external resistances: R=1000 Ω and R =100 Ω. (M = inoculated with MFC effluent, AS = inoculated with activated sludge).

Anode potentials became lower (more negative) with time as biofilm become more established. On average, after one week lower anode potentials were recorded under low R_{ext} (100 Ω) than at high R_{ext} (1000 Ω) suggesting that more active biofilm was developed capable of efficient electron transfer and hence improved substrate consumption (Aelterman *et al.*, 2008a). Lower anode potentials were recorded in activated sludge bioreactors during T80 100 ppm phase (stage

1: day 0 -21). Differences in anode potentials became smaller between MFC effluent bioreactors with time i.e. during 100 ppm PAH phase (stage 3: day 43 - 64). It appeared that electrode colonization was faster in bioreactors inoculated with activated sludge than those inoculated with MFC effluent, which was why initial current output was more rapid and higher in activated sludge bioreactors. Control of anode potential by R_{ext} was observed in this study. More negative anode potentials (-418 to -495 mV vs Ag/AgCl RE) were observed at low R_{ext} compared with high R_{ext} (-356 to -478 mV vs Ag/AgCl RE). This observation corresponded with previous observations by González del Campo *et al.* (2016) and implied that more active anodic biofilm was established when R_{ext} was low.

4.3.8 Cyclic voltammetry of MFC under low external resistance

Electrochemical behaviour of anodic biofilms was determined using cyclic voltammetry (CV) of all MFCs under high and low external resistances at the end of each treatment cycle (zero turnover). CV was conducted using a scan rate of 0.001 V s^{-1} over potential range of -0.6V to 0.5V vs.Ag/AgCl (saturated NaCl), with a total of 3 scans per bioreactor (Fricke *et al.*, 2008). In view of enhanced TOC removal and coulombic efficiency under low R_{ext}, development of CV profile over the duration of the experiment was centred on bioreactors operated under this condition. CV profiles for bioreactors operated under high R_{ext} are presented in Appendix G. Anodic and cathodic peak values increased with decrease in R_{ext} (e.g. 0.4 – 1.2 A/m^2 at 100 Ω and 0.2 – 0.8 A/m^2 at 1000 Ω), with higher peak values observed in activated sludge bioreactors compared with MFC effluent bioreactors regardless of external resistance applied. CV profiles indicated that reducing R_{ext} improved biofilm coverage on anode which was responsible for improved performance. Generally, differences between replicate CVs for both MFCs inoculated with activated sludge and MFC effluent agreed with variance in cumulative charge passed between repeats as seen in section 4.3.4. This variance between the bioanodes of replicate MFCs was indicative of variations in electron transfer rates, for example in stage 2, as seen in higher coulombic efficiency in M2 (3.34 %) and AS1 (29.56 %) compared with M1 (1.32 %) and AS2 (25.42 %), respectively.

As shown in Figure 4-11, in potential range between -100 and -200 mV, peak oxidative and reductive current densities attained under low R_{ext} were 400 & -500 mA/m^2 (M1) and 650 & -800 mA/m^2 (M2) (stage 3: 100 ppm PAH phase). No peaks were observed for CV obtained at the end of T80 cycle, which indicated that TOC removal was governed by fermentative and methanogenic processes. It appears that over time, limited electron donors were available to electroactive bacteria on anodic biofilm, thus limited charge output was observed during 50

ppm and 100 ppm PAH cycles. Redox peaks for M1 and M2 were observed only during 50 and 100 ppm PAH cycles; increase in PAH concentration was accompanied by marginal increase in redox peak. M1 and M2 were characterized by reversible redox peaks, indicating fast electron transfer. This confirmed the contribution of bio-electrocatalytic activity of anodic biofilm, albeit limited, to electric current production in M1 and M2 (Fricke *et al.*, 2008). Development of CV profile across the different treatment cycles was characterized by increase in biofilm electron-holding capacity and indicated gradual improvement and stability of anodic biofilm. Increase in biofilm electrocatalytic activity has been linked with increased biofilm density/thickness and transfer proteins (Zhang *et al.*, 2011b). Formal peak reduction potentials were observed at -0.149 to -0.18 V vs Ag/AgCl. There were no redox peaks in open circuit bioreactors inoculated with MFC effluent (nM) indicating that PAH removal in these bioreactors was not due to electrooxidation (see Figure 4-11).

As can be seen in Figure 4-12, wide peak separation in AS indicated irreversible processes, hence slow electron transfer. Peak value was highest during T80 cycle (0.8 – 1.2 A/m^2) in comparison with 50 or 100 ppm PAH cycle (0.5 – 0.7 A/m^2), which suggested high biofilm activity during stage 1 (Tween 80 cycle). Electroactive bacteria would have been dependent on fermentative products from T80 degradation. Electron-holding capacity declined as PAH concentration increased. This could have been due to reduced availability of electron donors from T80/PAH breakdown because PAH is less oxidizable than T80. In addition, reduction peak potential shifted from -0.35 V vs Ag/AgCl (AS1 and AS2) during T80 cycle to -0.32 V (AS1) and -0.28 V vs Ag/AgCl (AS2) during 50 and 100 ppm PAH cycle. A different reduction peak potential was observed in nAS2 (-0.12 V vs Ag/AgCl). This indicated that not only were different redox shuttles responsible for electron transfer but that different shuttles were likely involved when substrate was switched from only T80 and a mixture of T80 and PAH. These were not the same formal reduction potentials observed under high R_{ext}, namely -0.32 V vs Ag/AgCl (AS1 and AS2) during T80 cycle and -0.18/-0.2 V vs Ag/AgCl during PAH cycles. It suffices to say that change in R_{ext} probably stimulated modification of electron transfer mechanisms occurring on anodic biofilm (Aelterman *et al.*, 2008a). Redox potentials identical to known electron shuttles supported the assumption that electron transport within the MFCs was primarily via indirect method, especially in AS. Use of electron shuttles may contribute to slow electron transfer as these shuttles diffuse between bacteria and anode (Schröder, 2007; Torres *et al.*, 2010; Zhang *et al.*, 2011b).

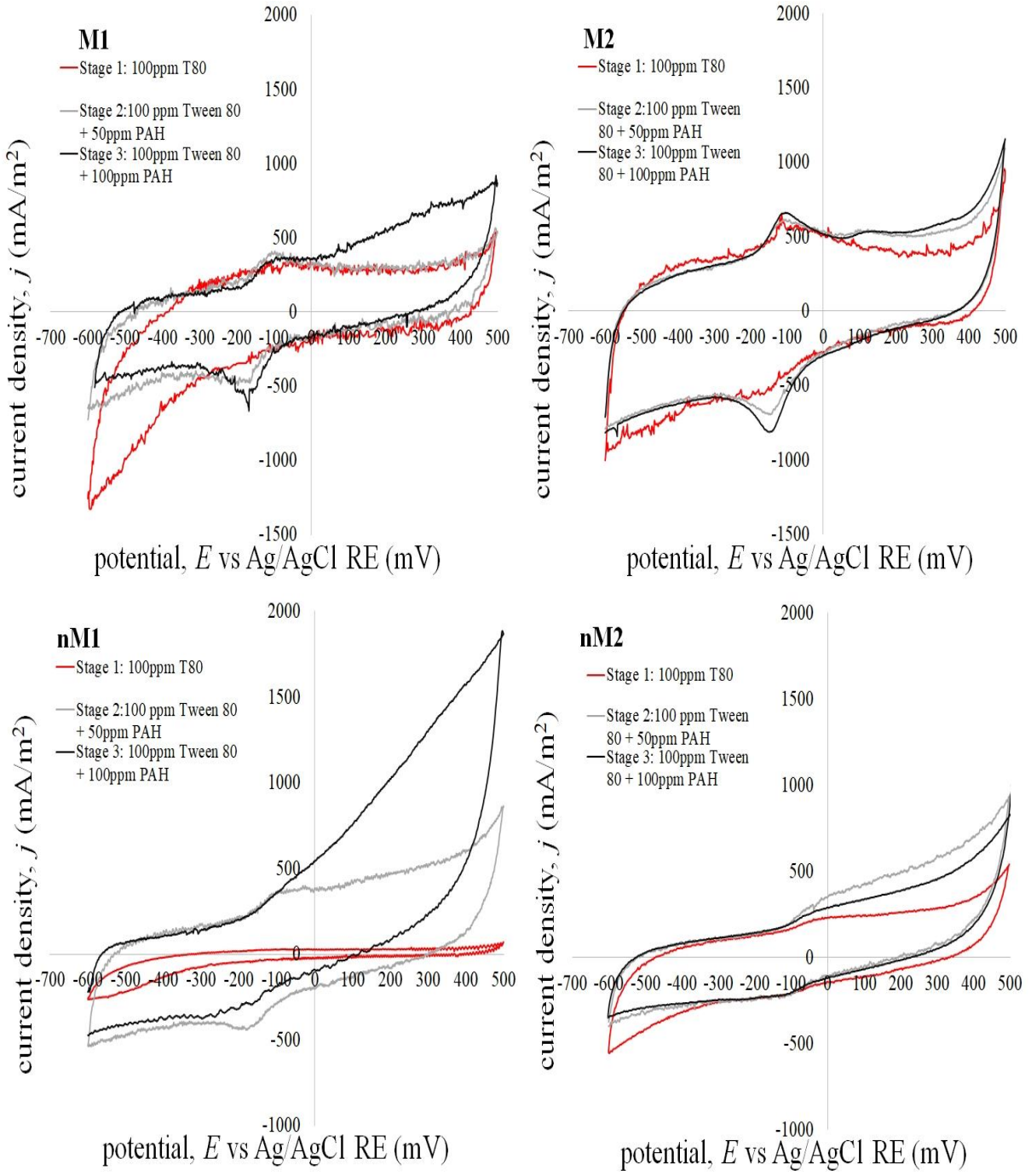


Figure 4-11. Evolution of cyclic voltammograms of anodic biofilms in double chamber microbial fuel cells used for treatment of hydrocarbon contaminated wastewater at external resistance R_{ext} = 100 Ω (scan rate = 0.001 Vs^{-1}, temperature 30℃, M = closed circuit bioreactors inoculated with MFC effluent, nM = open circuit bioreactors inoculated with MFC effluent).

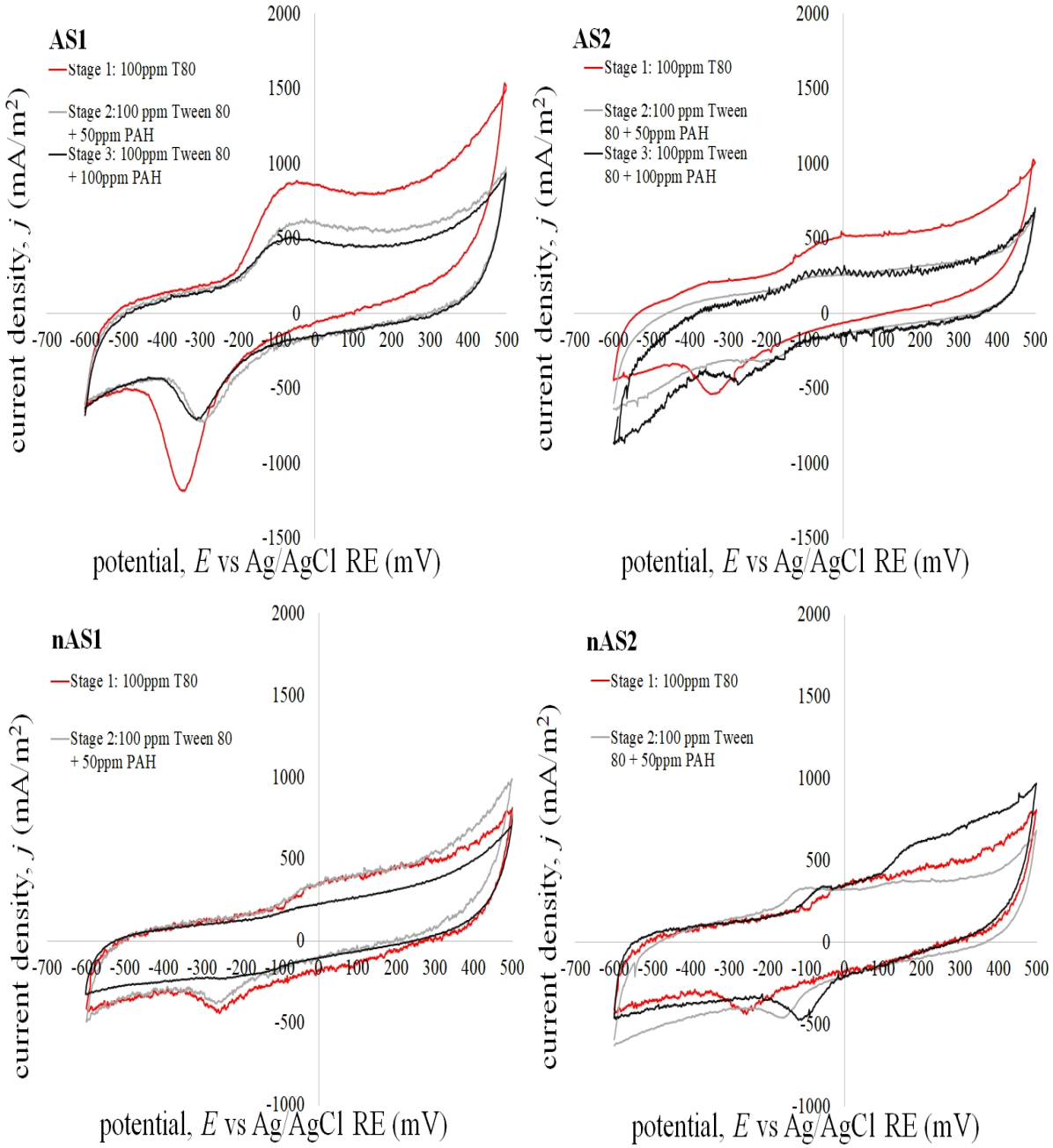


Figure 4-12. Evolution of cyclic voltammograms of anodic biofilms in double chamber microbial fuel cells used for treatment of hydrocarbon contaminated wastewater at external resistance R_{ext} = 100 Ω (scan rate = 0.001 Vs^{-1}, temperature 30°C, AS = closed circuit inoculated with activated sludge, nAS = open circuit inoculated with activated sludge).

4.4 Conclusion

The influence of inoculum source and external resistance on anodic biofilm development, current output, and polycyclic aromatic hydrocarbons (PAHs) removal efficiency in the presence of surfactant was studied using double chamber microbial fuel cells (MFC) inoculated with two different sources of inoculum (MFC effluent and activated sludge). This study showed that anodic biofilms in all MFCs were able to tolerate low concentrations of Tween 80 (0.1 g/L

or 100 ppm). However, MFCs inoculated with activated sludge were quicker to adapt to low concentrations of anionic surfactant (polyoxyethylene (20) sorbitan monooleate - Tween80/T80) and produced higher current density (59.44 ± 0.98 mA/m^2) in comparison to MFCs inoculated with MFC effluent (1.65 ± 0.03 mA/m^2). High organic content and high microbial diversity in activated sludge inoculum, compared with MFC effluent inoculum, were thought to be responsible for rapid and significant current output in MFCs inoculated with activated sludge when fed with only Tween 80. In the case of MFCs inoculated with MFC effluent, slow turnover of electron donors from degradation of Tween 80 in the presence of low microbial diversity was the probable cause of the disparity between total organic carbon removed and current output seen in MFC bioreactors during Tween 80 cycle. Activated sludge inoculum was demonstrated to support higher current output at start-up but this effect diminished when PAH concentration was increased from 50 ppm to 100 ppm. Current output increased in all MFCs when external resistance was lowered from 1000 Ω to 100 Ω. Control of anode potential by external resistance was seen in this study. High external resistance resulted in diversion of electrons from PAH oxidation to fermentation and hydrolysis, with no significant different in PAH removal between closed circuit and open circuit MFCs across all bioreactor sets. Thus, inoculum source did not appear to have direct influence on overall PAH removal efficiency. Active anodic biofilms with higher bio-electrocatalytic activity were established under low external resistance. Highest coulombic efficiency was achieved under low R_{ext} (100 Ω) (AS = 27.5 ± 3.0 %) than under high R_{ext} (1000 Ω) (AS = 14.5 ± 2.6 %) at 50 ppm PAH cycle. Results presented in this chapter indicate that PAH degradation was dominated by fermentation, electrochemical oxidation of PAH from hydrocarbon contaminated wastewater could be improved marginally under low external resistance, and that MFCs inoculated with activated sludge (AS) exhibited higher bio-electrocatalytic activity. Further analysis of microbial community profile of anodic biofilm would provide more clarity on interactions between fermentative/hydrolytic bacteria and electroactive bacteria in simultaneous hydrocarbon removal and electricity production. This study showed that MFC could be used to remove petroleum hydrocarbon from contaminated water. However, as previously mentioned in section 2.2.4, if hydrocarbon contaminated water runs off or is accidentally discharged into the environment (water/soil/sediment), the hydrocarbons sink through the water column and accumulate in sediments where they sorb onto organic matter. Removal of hydrocarbon from such soil/water system would require sediment MFC.

Chapter 5 Performance evaluation of sediment microbial fuel cells (SMFCs) for removal of polycyclic aromatic hydrocarbons from contaminated sediment

5.0 Chapter summary

Different reactor and electrode configurations of sediment microbial fuel cells (SMFCs) were investigated for enhancement of petroleum hydrocarbons removal from hydrocarbon-contaminated sediment obtained from River Tyne. Single chamber (anode and cathode in same reactor) and double chamber (anode and cathode in two reactors separated by ion exchange membrane) bioreactors were studied. Two cathode configurations were evaluated to trade-off between oxygen and ion transport on performance: submerged cathode vs wicking cathode. Wicking cathode configuration was utilized in single chamber SMFCs to improve cathodic oxygen reduction rection. Although polyaromatic hydrocarbon (PAH) removal was achieved in closed circuit double chamber SMFCs (R) (33.5 ± 8.7 %), higher removal rates were obtained in its open circuit counterpart (nR) (43.2 ± 2.2 %), this implied possible oxygen ingress into the system. Current density and charge output in single chamber SMFC with vertical electrode arrangement was 3 times higher using wicking cathode, CW (166 ± 78 mA/m^2 & 298 ± 140 C) in comparison with submerged cathode, CS (54 ± 21 mA/m^2 & 97 ± 38 C). Similar PAH removal was achieved in CW (51 ± 3 %) and CS (46 ± 2 %). Low coulombic efficiency in CW (< 2 %) indicated that there was limited electrochemical oxidation of PAH in this SMFC. The novelty of this study was demonstration that wicking cathode could be used to enhance current/charge output in single chamber sediment microbial fuel cells using vertical electrode arrangement with marginal improvement in electrochemical oxidation of PAH.

5.1 Introduction

Petroleum hydrocarbons are pervasive in the environment because of their widespread use as fuel for domestic heating, automobiles, and industrial appliances. They contain a mixture of simple and complex chemicals, many of which are hazardous and threaten public health and ecological habitat. Petroleum hydrocarbons are classified into 4 main fractions: aliphatics (n-alkanes, cycloalkanes), aromatics, NSO (nitrogen-sulphur-oxygen) compounds and asphaltenes/resins (Leahy and Colwell, 1990). Petroleum hydrocarbons from industrial and municipal runoff get into water bodies. These eventually sink down the water column into sediment. Sediments therefore become reservoirs of these recalcitrant chemical compounds. Although bioremediation has been employed for removal of persistent hydrocarbon pollutants from soil and sediments, this process progresses slowly due to diminishing electron acceptors within anoxic soil/sediment layer (Daghio *et al.*, 2017). As mentioned earlier, in this thesis we will explore if the anode of SMFC can be used as electron sink to drive hydrocarbon biodegradation, therefore speeding up its removal from the environment.

Like other BES, SMFC performance is influenced by type of electrode materials used, bioreactor configuration, temperature, salinity, pH, soil organic matter, and indigenous microbial community. Several studies have reported varying degrees of success in treatment of hydrocarbon contaminated sediment using bio-electrochemical systems such as sediment microbial fuel cells (SMFCs). Treatment duration have varied between 25 to 250 days with variable total petroleum hydrocarbon (TPH) removal efficiencies from 11% to 89.7% (Wang *et al.*, 2012b; Li *et al.*, 2014; Lu *et al.*, 2014b; Li *et al.*, 2015; Zhang *et al.*, 2015; Li *et al.*, 2016a; Li *et al.*, 2016c; Li *et al.*, 2018b; Li *et al.*, 2019; Zhang *et al.*, 2020a). Varying current densities (35.2 mA/m^2 to 203 mA/m^2) and power densities (3.4 to 87.85 mW/m^2) have also been reported (Morris and Jin, 2012; Li *et al.*, 2014; Lu *et al.*, 2014a; Lu *et al.*, 2014b; Sherafatmand and Ng, 2015; Li *et al.*, 2016b; Li *et al.*, 2016c; Xu *et al.*, 2017).

Despite potential benefits of concomitant removal of contaminant and electricity generation, there are several challenges to effective petroleum hydrocarbon removal from sediments. Electrodes alignments and distance has large effect on MFC performance and TPH removal. For example, increasing anode depth in sediment may enhance performance but high internal resistance can occur in SMFC due to spatial separation of anode and cathode increasing distance of ion travel between them (Sajana et al., 2016; Girguis et al., 2020). Absence of membrane between cathode and anode as well as reduced distance between electrodes while might reduce cell internal resistance, can lead to oxygen diffusion into anode zone and decrease SMFC performance (Hong et al., 2009). Double chamber configuration has been shown to enhance

power output than single chamber configuration (Mohanakrishna *et al.*, 2019). Presence of electron acceptors such as nitrate and sulphate in the anaerobic SMFC enhance hydrocarbon degradation. Hence, nutrient limitation can also impact on SMFC performance (Sherafatmand and Ng, 2015). Mass transport limitations of hydrocarbons in soil/sediment and oxygen from air to cathode may increase overpotentials, and reduce system efficiency (Li and Yu, 2015). Porous woven carbon fibre felt has been shown to improve soil conductivity and enhance mass transport of hydrocarbons (Li *et al.*, 2016c).

Previous study using vertical versus horizontal anode arrangement, showed that hydrocarbon removal from contaminated sediment enhanced with horizontal anode arrangement in comparison with vertical anode arrangement (Zhang *et al.*, 2015). However, only 12.5% of total petroleum hydrocarbon (TPH) was removed after 135 days of operation. Shorter treatment periods are desirable to boost appeal of SMFC as eco-solution for environmental remediation. Distance between anode and cathode also affects the level of separation and degree of interference between them. For example, low concentrations of dissolved oxygen in overlying water while required for cathode to operate, it can reduce hydrocarbon removal efficiency due to anode's non-anaerobic conditions (Wang et al., 2012a; Sajana et al., 2016). Cathode biofouling from sediment particles may also limit oxygen reduction (Song and Jiang, 2011). Catholyte with wicking cathode was shown to have higher dissolved oxygen than submerged cathode (Morris and Jin, 2012). It was hypothesized that wicking cathode configuration would enhance oxygen reduction reaction (ORR) at cathode and therefore drive faster removal of hydrocarbon from contaminated sediment. This can be due to faster oxygen mass transfer over shorter distance between cathode and solution/air interface as well as higher interface surface area between the cathode and air interface.

The aim of the study was to investigate the effect of reactor and electrode configurations on the performance of sediment microbial fuel cell (SMFC) for both power production and removal of petroleum hydrocarbon from sediments. The objectives of the study were: (i) to assess hydrocarbon removal from sediment using double chamber SMFC; (ii) to assess hydrocarbon removal from sediment using single chamber SMFC with vertical electrode alignment; and (iii) to improve cathodic oxygen reduction reaction using wicking cathode.

5.2 Experimental Procedure

Experimental set-up, operating conditions and experimental analysis for this chapter are described in sections 3.1 to 3.6.

5.3 Results and Discussion

5.3.1 Current production in double chamber sediment microbial fuel cells

Organic matter consumption through electrochemical reactions (involving direct electron transfer) in double chamber sediment microbial fuel cells was inferred by current production. Stable current production at start-up was suggestive of the existence of electroactive bacteria and that electron donors were likely present in Tyne River sediment. Previous studies have established the presence of hydrocarbon-degrading microbial consortia in Tyne River sediment comprising *Deltaproteobacteria* (*Desulfuromonadaceae*, *Desulfuromondales*, *Desulfobulbaceae*), *Gammaproteobacteria*, *Chloroflexi*, *Firmicutes* (Logan, 2009; Sherry *et al.*, 2013; Viggi *et al.*, 2017). Notably, some members of these classes have been identified as electroactive bacteria. Electric current (50 - 130 mA/m^2) was generated within a few hours of start-up, using Tyne River water as catholyte. Low ionic conductivity may result in low electric current output, however, high level of ions (i.e. sodium, calcium, and magnesium) as well as electricity conductivity of Tyne River water were not indicative of low conductivity. Although chemical oxygen demand (COD) of Tyne River water was low (64.4 mg/L), it was suspected that organic matter present in the water might have hampered the oxygen reduction reaction at the cathode by consuming dissolved oxygen in the water or biofouling the cathode, resulting in low current production (see Table 5-1 for characterization of Tyne River water and sediment). Electric current generation was enhanced (up to 300 mA/m^2) after replacement of catholyte with 0.05M phosphate buffer solution (PBS) on day 15. Cathode was thoroughly rinsed with deionized water and replaced in cathode chamber of double chamber SMFC. Maximum current density generated during operational period was 526 mA/m^2 and 432 mA/m^2 for R1 and R2 respectively.

Table 5-1. Characterization of Tyne River water and sediment (Source of data: Baker (2002); Siavalas et al. (2013); Sherry et al. (2020); This study).

Tyne River Water		**Tyne River Sediment**	
pH	6.93	pH	6.91
Chemical oxygen demand (mg/L)	64.4	Total organic carbon (TOC) (% weight)	7.2
Electrical conductivity (µS/cm)	241	Inorganic carbon (% weight)	0.2
Calcium (mg/L)	123	Chloride (mg/L)	9518
Magnesium (mg/L)	223	Salinity (mg/L)	17195
Sodium (mg/L)	890	Iron (mg/kg)	39000
Potassium (mg/L)	133	Sulphur (mg/kg)	9200
Zinc (mg/L)	1.1	Magnesium (mg/kg)	8500
Iron (mg/L)	13	Sodium (mg/kg)	5600
Lead (mg/L)	0.1	Potassium (mg/kg)	6100

Fluctuations in current output were observed throughout the experiment even after catholyte replacement. Current fluctuation in SMFC is not unusual especially where complex substrate is used as carbon source. As previously discussed in section 2.3, complex hydrocarbons are biodegraded by a consortium of microbes leading to generation of different metabolic intermediates or electron donors. The rate at which these electron donors are generated is dependent on the metabolic route utilised by the relevant microbe(s). Thus, electron donors supply may not be constant. Consequently, rate of electric current production would not be constant leading to fluctuations in current output corresponding to periods of availability of electron donors. Subsequently, current output would likely be limited by rate of mass transfer of these electron donors from the microbes responsible for generating them to microbes on the anode (Lu *et al.*, 2014a; Liu *et al.*, 2015). In addition, higher output was recorded during oxygen sparging and pointed to likelihood of inadequate dissolved oxygen in catholyte. This suggests that cathode was operating at current density close to that of oxygen mass transfer rate. This was expected given that the cathode was submerged in catholyte where oxygen diffusion coefficient (D) at 30˚C in water is 23 $x10^{-6}$ cm^2 s^{-1} and oxygen solubility (C) is limited to ca. 200 uM bar^{-1} at 30˚C or solubility from air saturated solution is ca. 40 $x10^{-6}$ M or 40 $x10^{-9}$ mol cm^{-3}. Using Fick's first law of diffusion (see equation 3-12 in section 3.6.2), where I = ORR

mass transport limited oxygen reduction current in amperes, 4 = number of electrons per mole of oxygen (O_2) reduced, Faraday constant (F) = 96485 C mol^{-1}, electrode area (A) = 6 cm^2, D is in cm^2 s^{-1} and C is in mol cm^{-3}, δ is diffusion layer thickness in cm in order of 0.001-0.01 cm. This gives a total current of 2-0.2 mA. This translates to current density in the order of 0.033-0.33 mA cm^{-2} or 330-3300 mA m^{-2}. This suggested that under the operating regime oxygen mass transport would influence bioreactor performance and that oxygen sparging and use of wicking cathode can have effect on oxygen mass transport by reducing diffusion layer thickness and consequently increasing current density.

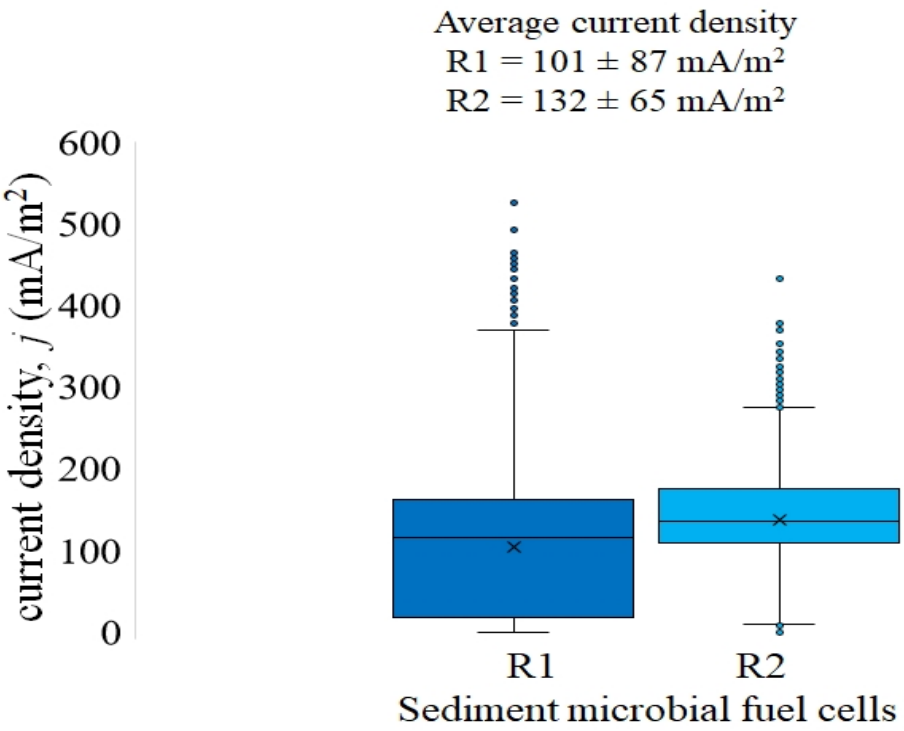


Figure 5-1. Current output in replicate double chamber sediment microbial fuel cells (R1 and R2) used for treatment of hydrocarbon contaminated sediment from Tyne River (temperature = 30°C, R_{ext} = 1000 Ω)(A = Cathode medium change on day 15; Grey arrows represent oxygen sparging).

5.3.2 Hydrocarbon removal in double chamber sediment microbial fuel cells

Hydrocarbon removal efficiency was analysed with references to aliphatic and aromatic fractions. Aliphatic and aromatic fractions of petroleum hydrocarbon in the double chamber SMFC bioreactors were consumed at different rates, with aromatics achieving higher removal efficiencies than aliphatics. This could be because aromatics are converted to aliphatic as they are degraded. As shown in Figure 5-2, on average (between two repeats) aliphatic fraction removal in open circuit bioreactors (nR1 and nR2) was at least equal to or higher than that of closed circuit bioreactors (R1 and R2). Aromatic hydrocarbons were consumed faster than aliphatic hydrocarbons in all bioreactors. Similarly, on average (between two repeats) aromatic

fraction removal in nR1 and nR2 was at least equal to or higher than that of R samples. Average aromatic removal in nR samples was 43 % while for R samples ca. 34%. Hydrocarbon removal efficiency seems to be unrelated to current production. For example, equal or higher removal rates were seen in open circuit SMFCs (nR1 and nR2), in comparison to closed circuit SMFCs (R1 and R2). This suggested that hydrocarbon removal mechanism was dominated by biochemical instead of bio-electrochemical processes and that power generation resulted from oxidation of other organic compounds in the sediment than hydrocarbons. For example, organic compounds derived from vitamins included in the anode medium at the start of the experiment. This was contrary to previous studies where closed circuit SMFCs enhanced hydrocarbon removal over open circuit SMFCs (Chandrasekhar and Venkata Mohan, 2012; Sherafatmand and Ng, 2015).

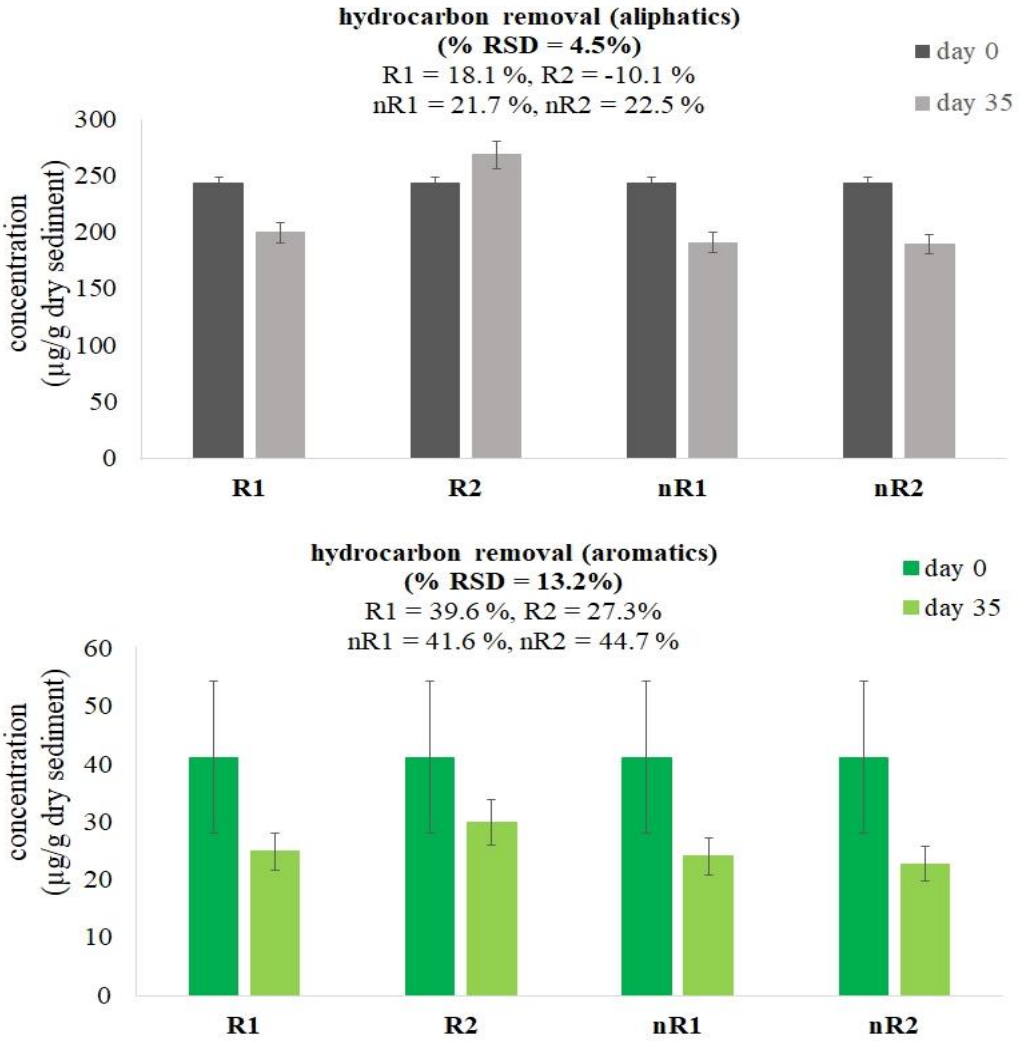


Figure 5-2. Degradation of aliphatic and aromatic hydrocarbon fractions in double chamber SMFC (temperature = 30^{o}C, external resistance = 1000 ohms; R= closed circuit cells, nR= open circuit cells. RSD = relative standard deviation).

Although aliphatics are relatively more abundant than other hydrocarbon fractions, they are more easily degraded (Salleh *et al.*, 2003; Head *et al.*, 2006). Higher consumption of aromatics

than alkanes was not unusual, and this has been reported in other studies (Jones *et al.*, 1983; Lavania *et al.*, 2012). The limited decrease or increase in aliphatics in R samples in comparison to decrease in aromatic hydrocarbon can be explained: (1) additional hydrocarbon input may be from phytoplankton or microbial biomass (Duran and Cravo-Laureau, 2016); (2) degradation of higher molecular weight (HMW) hydrocarbon fractions including aromatics could lead to accumulation of lower molecular weight (LMW) fractions (Foght, 2008); (3) degradation of one hydrocarbon fraction could lead to increase in relative abundance of lesser degraded or undegraded fraction (s) (Head *et al.*, 2006); and, (4) additional hydrocarbon input may be from degradation of other hydrocarbon fractions not considered in this study, namely, asphaltenes (i.e. phenols, ketones and esters) and resins (i.e. pyridines, quinolines and amides) – some of these fractions contain nitrogen-sulphur-oxygen (NSO) (Leahy and Colwell, 1990). Although asphaltenes and resins are highly resistant to biodegradation, extensive biodegradation of asphaltenes and resins have been achieved under optimized experimental conditions (Bertrand *et al.*, 1983; Lavania *et al.*, 2012). This study focused on only aliphatic (nC10 – nC35) and aromatic (PAHs) fractions of petroleum hydrocarbons. Hydrocarbon biodegradation involves sequential reactions that produce lower molecular weight metabolic intermediates, with complete oxidation producing carbon dioxide and water and partial oxidation resulting in accumulation of intermediates (Foght, 2008). Ring cleavage may also convert aromatic compounds to or generate linear compounds (Harayama *et al.*, 2004; Foght, 2008; Rabus *et al.*, 2016).

Biodegradation profile of individual components of aliphatic and aromatic hydrocarbon fractions is presented in Figure 5-3. Analysis of individual components of aliphatic hydrocarbon fractions showed that lower fraction hydrocarbons e.g. C10 - C13 were being consumed at higher rate than those of higher fractions (C15-35) in all SMFCs (see Figure 5-3A). Additionally, it can also be observed that on average open circuit samples (nR1 and nR2) had slightly better n-alkanes and aromatic hydrocarbon removal rates (across the studied range) than those of R samples (Figure 5-3A &B). These observations did not appear to correlate with postulated accumulation of LMW hydrocarbons from degradation of HMW hydrocarbons. All PAHS were degraded in bioreactors to varying degree. Degradation rates were related to number of C rings. For example, 2-ring PAH achieved 89 – 91 % removal across R and nR. Up to 42 % of 3- and 4-ring PAHs were degraded in R bioreactors in comparison to 38.7 % in nR. On the average, all 5-ring PAHs in R bioreactors were resistant to biodegradation, except BkF; wheeas all 5-ring PAHs in nR bioreactors were biodegraded (18.9 %). Only one 6-ring PAH was degraded in nR (1.6 %). Notably, pyrene (C16) had the highest concentration of 9.2 µg/g and showed an average of 62 % degradation to ca. 3 to 4 µg/g in both R and nR bioreactors.

Pyrene can be degraded anaerobically to produce range of aromatics with final degradation product including C6 aliphatic compound (Zada *et al.*, 2021). Since only aliphatics from C10 and above were considered, it cannot be concluded that degradation of HMW PAH could have contributed to limited decrease in aliphatic concentrations observed. Moreover, aromatic hydrocarbons comprised only 15.5 % of total hydrocarbon analysed.

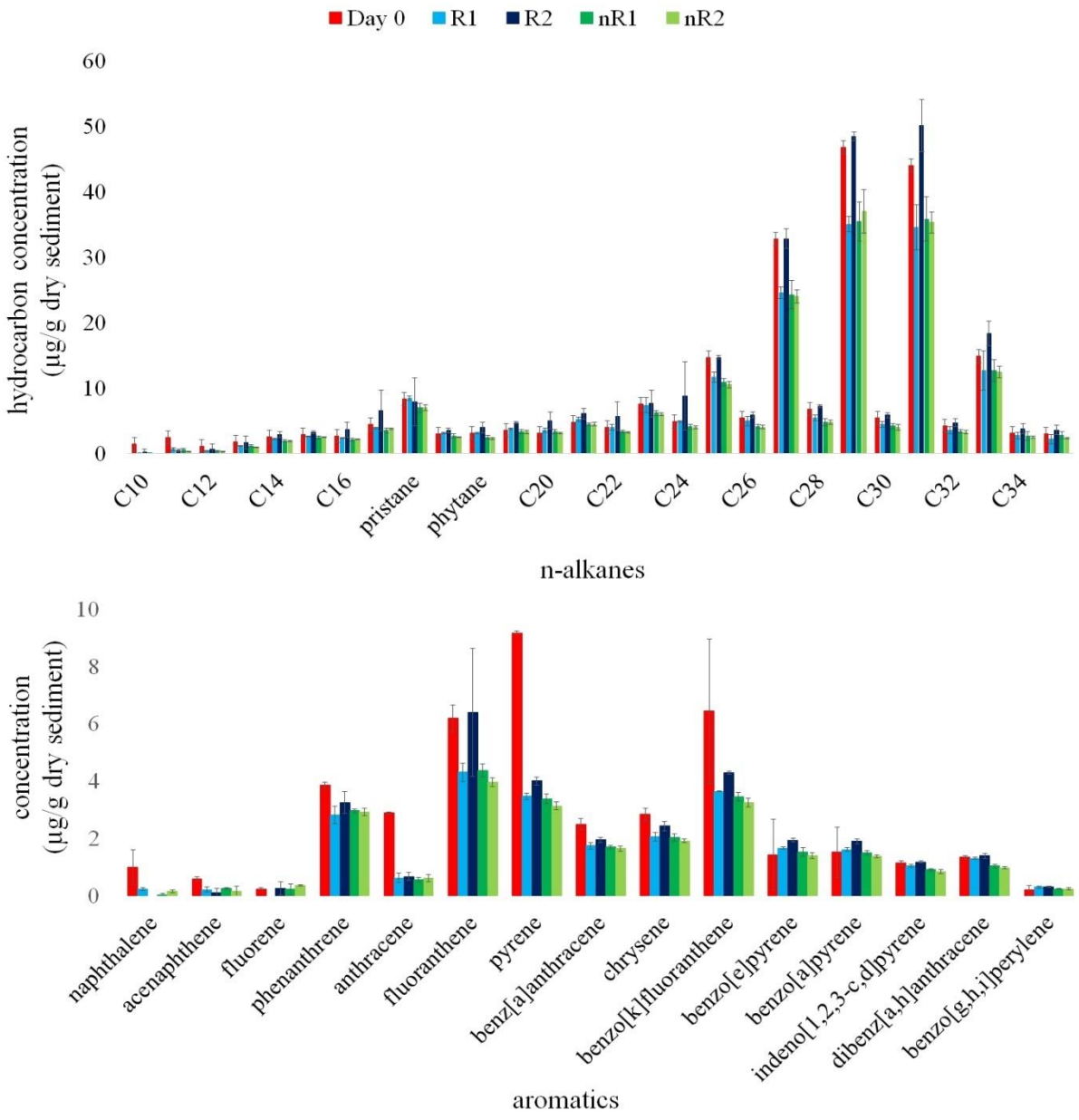


Figure 5-3. Hydrocarbon biodegradation profile for (A) n-alkanes (C10-C35) and (B) polyaromatic hydrocarbons (PAHs) in Tyne River sediment treated in double chamber sediment microbial fuel cell (temperature = 30^{o}C, external resistance = 1000 ohms; R= closed circuit cells, nR= open circuit cells*).*

Petroleum hydrocarbon indices such as Pr/Ph, nC17:Pr and nC18:Ph can be used to characterize and deduce biodegradation of alkanes (Gong *et al.*, 2020). Pristane (Pr) and phytane (Ph) are acyclic isoprenoids found in marine sediments. Pr and Ph are diagenetic products of oxidation

and reduction of phytol side chain of chlorophyll, respectively. They are indices of degree of diagenesis of petroleum hydrocarbons, with higher values reflecting significant thermal alteration (Ten Haven *et al.*, 1987). On the other hand, nC17:Pr and nC18:Ph ratios decrease as biodegradation of alkanes increase. As presented in Table 5-2, variation in these indicators were small and sometimes within experimental error to make any additional significant conclusion. As discussed above changes in concentration of aliphatics above C15 was limited in comparison to aliphatics below C14 and hence no significant changes were expected using indicator relying on C17/18.

Table 5-2. Petroleum hydrocarbon characterization indices for aliphatic fraction (alkanes) in double chamber sediment microbial fuel cells treating hydrocarbon-contaminated sediments (values shown as mean ± standard deviation).

	Day 0 (initial sediment)	R1	nR1
Pr/Ph	2.73 ± 0.47	2.66 ± 0.47	2.77 ± 0.47
nC17:Pr	0.54 ± 0.04	0.46 ± 0.04	0.51 ± 0.04
nC18:Ph	1.00 ± 0.22	0.98 ± 0.22	1.06 ± 0.22

It can be concluded that hydrocarbon removal was mainly occurring via biochemical rather than bioelectrochemical routes with no significant enhancement from the latter to the overall removal rate. Under anaerobic conditions, alkane degrading microbes rely on other electron acceptors such as nitrate, sulphate, or metal ions (e.g. ferric iron). Slow growth rate of these microbes (between hours to months) implies that alkane degradation also proceeds at a slow rate (Rojo, 2009). Hydrocarbons can be removed by abiotic processes such as volatilization/evaporation, adsorption to particulate organic or inorganic matter, dispersion into water column and photo-oxidation (Duran and Cravo-Laureau, 2016). In MFCs, LMW aliphatics can be removed by volatilization (Mohan and Chandrasekhar, 2011).

5.3.3 Coulombic and Energy efficiency in double chamber sediment microbial fuel cell

In view of likelihood of mixed processes contributing to hydrocarbon removal in DC SMFC, coulombic efficiency of closed circuit bioreactors (R1 and R2) was calculated to determine percentage of electrons extracted converted to electric current (see section 3.6.2 for formula). As can be seen in Table 5-3, contribution of total hydrocarbon consumed to coulombic and energy efficiency in both R1 and R2 are very low. CE of less than 2 % in R1 was indicative of low efficiency of electron transfer from hydrocarbon. This is additional evidence that hydrocarbon removal occurred by other processes than electrochemical oxidation.

Table 5-3. Coulombic and energy efficiency in double chamber sediment microbial fuel cell.

	R1	R2
Charge output (coulombs, C)	182.67	235.40
CE (%)	0.96	1.23
Energy output (joules, J)	19.86	24.73
$\boldsymbol{\epsilon_E}$ (x 10^{-6} %)	1.12	1.39

Anaerobic hydrocarbon biodegradation involves syntrophic co-operation between different bacteria. For example, fermentative bacteria breakdown complex HMW hydrocarbon via cascades of reactions into simpler LMW compounds that are preferred by EAB. Competition between groups of bacteria for fermentative products, partial oxidation of HMW hydrocarbons, and accumulation of dead-end metabolites may result in lower amount of substrate available for EAB to use for electric current generation (Rabus *et al.*, 2016; Yin *et al.*, 2021). This results in lower coulombic efficiency. Substantial low values for ϵ_E were indicative of poor energy recovery from petroleum hydrocarbons in sediments. Low energy recovery may also result from high energy required for initial activation of hydrocarbon oxidation and for ring cleavage of aromatic hydrocarbons during biotransformation into aliphatic intermediates. Alkanes were possibly being removed by abiotic processes.

5.3.4 Electrochemical characterization of double chamber sediment microbial fuel cells

Electrode potentials. Maximum voltage output (vs Ag/AgCl) was higher for open circuit cells nR1 (777 mV on day 25) and nR2 (755 mV on day 11) than closed circuit cells R1 (762 mV on day 18) and R2 (664 mV on day 14). Anode potentials for nR bioreactors remained stable during the operational period. As expected anode potentials were more negative for open circuit bioreactors (nR) than closed circuit bioreactors (R), because polarisation of the anode results in increase of its potential (Rimboud *et al.*, 2014). Evidence suggesting contribution of other biotic and abiotic hydrocarbon removal implied that this biofilm likely comprised more non-EAB communities such as hydrolytic and fermentative bacteria. Increase in anode potentials for R1 and R2 occurred after catholyte replacement (day 15). This was expected as synthetic catholyte results in improved cathode performance and higher cell current. Higher cell current would result in larger anode polarisation and consequently higher potential. Some metabolic intermediates of hydrocarbon biodegradation can also be toxic to anodic biofilm (Rodriguez Martinez *et al.*, 2008; Sherry *et al.*, 2014). It was also likely that anodic biofilm self-regulated anode potential, based on available electron acceptor, to favour proliferation of electroactive

bacteria (Rabaey *et al.*, 2004). This would increase biofilm energy gain and sustain non-rate-limiting electron transfer processes (Finkelstein *et al.*, 2006).

Cyclic Voltammetry (CV) conducted at low scan rates enable multiple enzymatic turnovers so that redox systems within anodic biofilm can be detected. Information obtained from CV can be used to determine reversibility of reactions, formal reduction potential of active redox systems identified and study redox processes. Classic sigmoidal shape of CV and peak potential separations for R1 and nR2 as shown in Figure 5-4 were indicative of reversible redox reactions, and fast electron transfer processes. Reductive/oxidative peak potentials for R1 (-0.250/-0.050 V vs Ag/AgCl), and nR2 (-0.250/-0.050 V vs Ag/AgCl) were similar, suggesting that R1 and nR2 comprised of the same fermentative micro-organisms. Half wave potential (E1/2) potential of R1 was -0.150 V vs Ag/AgCl and for nR2 was -0.130 V vs Ag/AgCl. There are several reported electron transport moieties with similaer E1/2 values e.g. phenazine redox mediators –pyocyanine PYO (-0.116 V vs Ag/AgCl), phenazine-1-carboxamide, PCN (-0.140 V vs Ag/AgCl) and 1-hydroxy-phenazine 1HP (-0.174 V vs Ag/AgCl) (Bosire *et al.*, 2016; Bosire and Rosenbaum, 2017). Phenazines are electron shuttles that can be secreted by different bacteria and are linked to indirect electron transfer (Schröder, 2007; Agostino *et al.*, 2017). Bacteria are known to use outer membrane-bound cytochromes for direct electron transfer (Schröder, 2007).

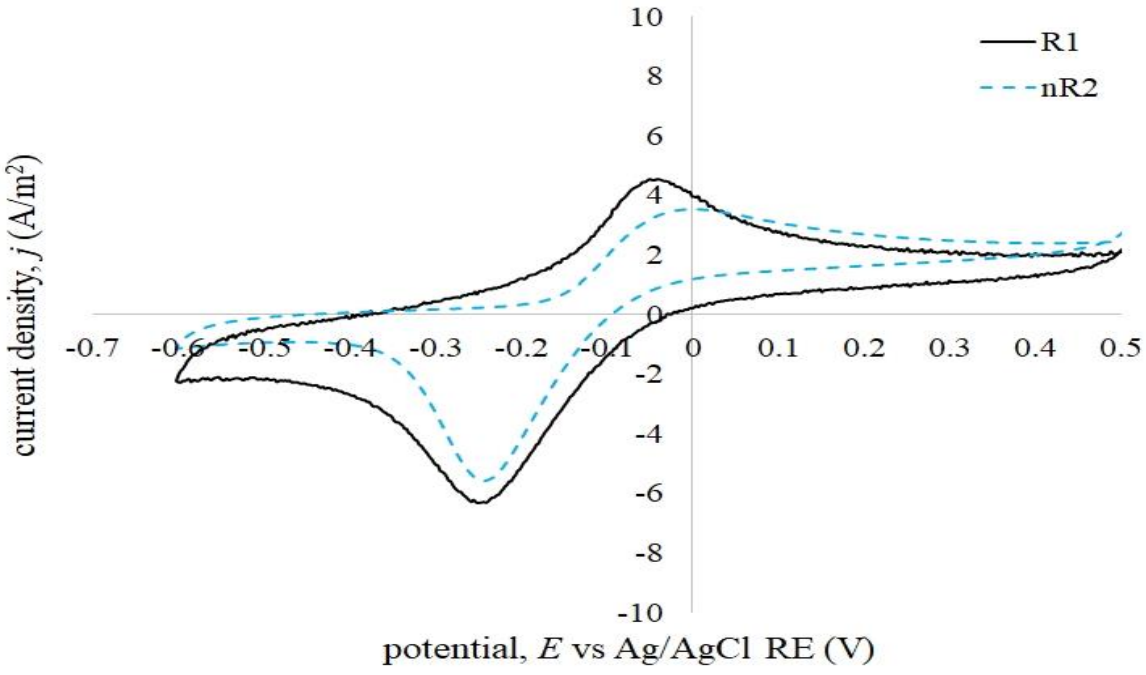


Figure 5-4. Cyclic voltammograms on day 34 for double chamber sediment microbial fuel cells – DC SMFC (scan rate = 0.001 Vs^{-1}, temperature = 30℃).

Total Gibb's free energy available from organic substrate in SMFC is used for bacterial metabolism and conversion to electricity. The portion of energy available for conversion to

electricity depends on how much energy is utilized for bacterial metabolism. Anodic biofilm utilize microbial redox systems or microbial terminal electron donors (MTEDs) (such as fumarate, cytochromes and phenazines), to transport electrons generated from substrate oxidation to anode. Anodic biofilm maximize metabolic energy gain by adapting their metabolic pathway and electron transfer mechanisms according to anode potential. This means that to conserve energy, bacteria will utilize redox systems with potentials close to anode potential. If difference between anode potential and substrate potential is high, more energy is used by bacteria and less is available for conversion to electricity. Conversely, if the potential difference is low, bacteria energy gain would be low but more Gibb's free energy would be available for conversion to electricity. In summary, while clear fast redox oxidation can be seen from CV studies which agrees with current measurement from MFC tests, given the low CE of less than 2% of electrochemical reaction, no signficant change was detected in total hydrocarbon removal rate which is dominated by biochemical process. The domination by biochemical process may also have been related to high external resistance applied; high external resistance may result in shifts in microbial metabolism to fermentation and reduced respiration and ineffective electron transfer activity (Lyon *et al.*, 2010; Rismani-Yazdi *et al.*, 2011). Even in the detailed analysis of various hydrocarbon species, no signficnant difference was seen between nR and R samples to attribute the oxidation to specific hydrocarbon species. Given the low bioelectrochemical removal of hydrocarbons using double chamber SMFCs, the bioreactor was modified to single chamber SMFC (without membrane), and external resistance was lowered from 1000 Ω to 200 Ω. These modifications were expected to improve SMFC performance by reducing internal resistance and balancing out microbial diversity (between fermentative and anode-respiring micro-organisms) on bioanode, respectively.

5.3.5 Current production in single chamber microbial fuel cells with vertical electrode configuration

Experiments were conducted using single chamber SMFC with vertically arranged electrodes and included a comparison between two wicking cathodes (CW) repeats and two submerged cathodes (CS) repeats. To eliminate oxygen limitation at cathode, all SMFCs were air-sparged through-out the duration of each experiment. Continuous air-sparging resulted in electrolyte evaporation and necessitated frequent replenishing with deionized water.

All SMFCs started up simultaneously, however, current quickly declined in CW1 on days 1 to 4. Different current outputs were obtained for CW and CS. Average current density in CW (166 ± 78 mA/m^2) was 3.1 times higher than that in CS (54 ± 21 mA/m^2). Peak current output occurred around days 6 to 7 in all bioreactors, after which current dropped. Stable current output

was observed CW2 from days 16 to 35. Observed spikes on current profile may have been caused by oxygen ingress into overlying water during replenishment of evaporated catholyte. At $p < 0.05$, one-way ANOVA showed that there was significant difference in current output between CW and CS. This highlights the importance of oxygen transport to the cathode particularly when current densities are increasing > 330 mA/m^2 as discussed previously.

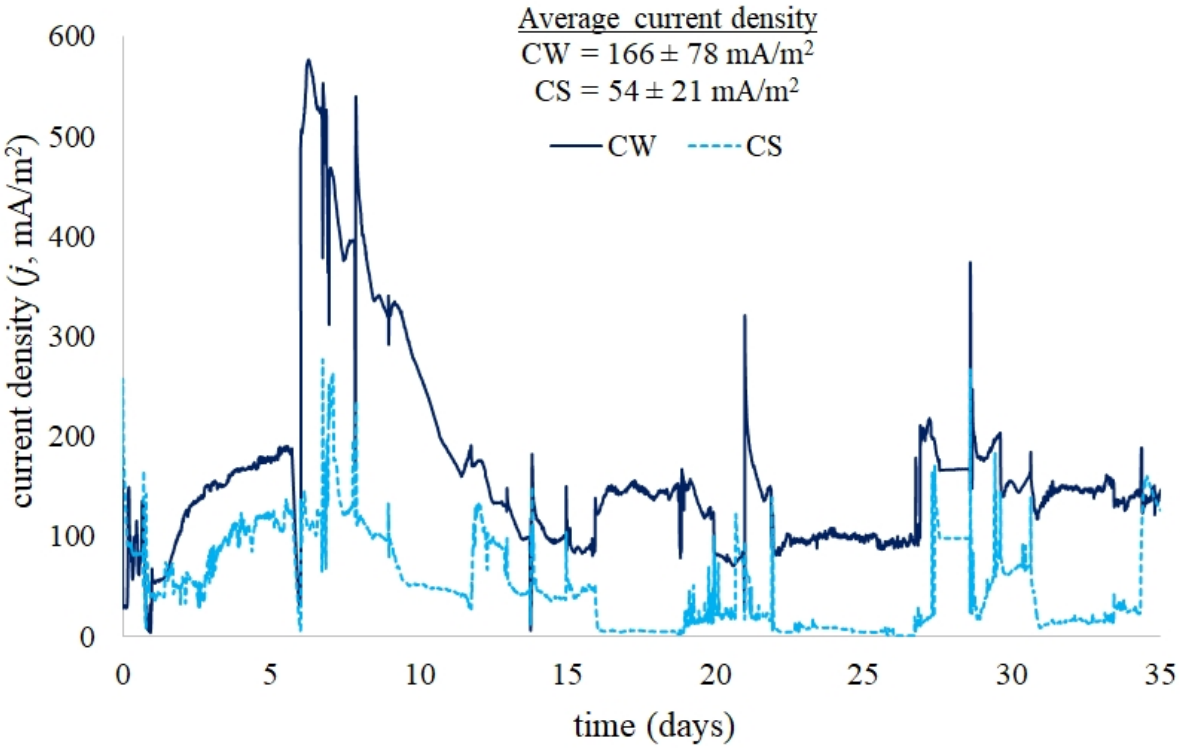


Figure 5-5. Current output in single chamber sediment microbial fuel cells (SC SMFC) used for treatment of hydrocarbon contaminated sediment (CW = wicking cathode; CS = submerged cathode; temperature = 30^{o}C; external resistance = 200 ohms).

Average current output of CW (166 ± 78 mA/m^2) obtained in this study was higher compared with other studies, namely, 70.4 ± 0.2 mA/m^2 (Lu *et al.*, 2014b) and 85.9 ± 0.1 mA/m^2 (Lu *et al.*, 2014a). Observation of peak current on days 6 and 7 and followed by current decline could have been due to limited availability of electron donors because oxygen supply remained constant during the experiment. Similar observations have been made in other studies. For example, in a study conducted for 137 days, peak current output was achieved on day 5 (569 ± 2 mA/cm^3) and subsequent decline in current output was attributed to limited supply of biodegradable hydrocarbons and other nutrients (Wang *et al.*, 2020a). in another study, fluctuations in current output observed in all SMFCs was attributed to the heterogenous nature of the hydrocarbons and irregular mass transport within the sediment (Lu *et al.*, 2014b). As seen earlier in Table 5-1, this Tyne River sediment comprised 7.2% organic carbon and other nutrients, some of which have accumulated from plant and material decomposition. Hence, organic carbon in sediment may also include carbon that are not petroleum hydrocarbon (Wang

et al., 2020a). In addition, biodegradation of petroleum hydrocarbons generates metabolites (including electron donors) that must be transported from point of production to point of utilization. Tyne River sediment comprised mainly silt (55.1 %) and clay (33.6 %) (see Table 5-1), suggesting low permeability which could impede mass transport of ions, hydrocarbons, and other nutrients. Low mass transport could also result from adsorption of hydrocarbon to sediment organic matter or clay content (Duan *et al.*, 2015; Kuppusamy *et al.*, 2017). The heterogeneous nature of the sediment implied that these compounds are unevenly transported within the sediment. This may cause in voltage/current fluctuations and result in performance variations, such as seen in Figure 5-5. These fluctuations were reflective of complex interactions likely occurring within different constituents of real environmental medium (Wang *et al.*, 2015). The effect of air flow at the cathode would result in enhanced oxygen mass transfer and reduction of diffusion layer and hence improved current density. Oxygen flow would also have convection effect on electrolyte contributing to enhancement of oxygen and substrate mass transport to the anode too.

5.3.6 Hydrocarbon biodegradation in single chamber sediment microbial fuel cell with vertical electrode configuration

Sediment samples from single chamber SMFC were analysed to determine hydrocarbon removal efficiency. As observed in double chamber SMFC, different removal efficiencies were observed for different hydrocarbon fractions. Open circuit SMFCs (OC) and control (XX, no electrodes) exhibited higher n-alkanes removal than closed circuit SMFCs (with wicking cathode, CW and with submerged cathode, CS). This agrees with previous results seen in this chapter. As can be seen in Figure 5-6, , n-alkanes removal efficiency was in the order: XX > OC > CW > CS. Statistical analysis using one-way ANOVA ($p = 0.585$) showed that there was no significant difference in n-alkane removal between closed circuit and open circuit bioreactors. Conversely, CW and CS appeared to be more efficient in removal of PAH than OC. The order of PAH removal was as follows: CW > CS > XX > OC. Statistical analysis has showed (one-way ANOVA) that there is significant difference in PAH removal between closed circuit (CW and CS) and open circuit (OC) SMFCs. Increase in relative abundance of PAH was observed in OC.

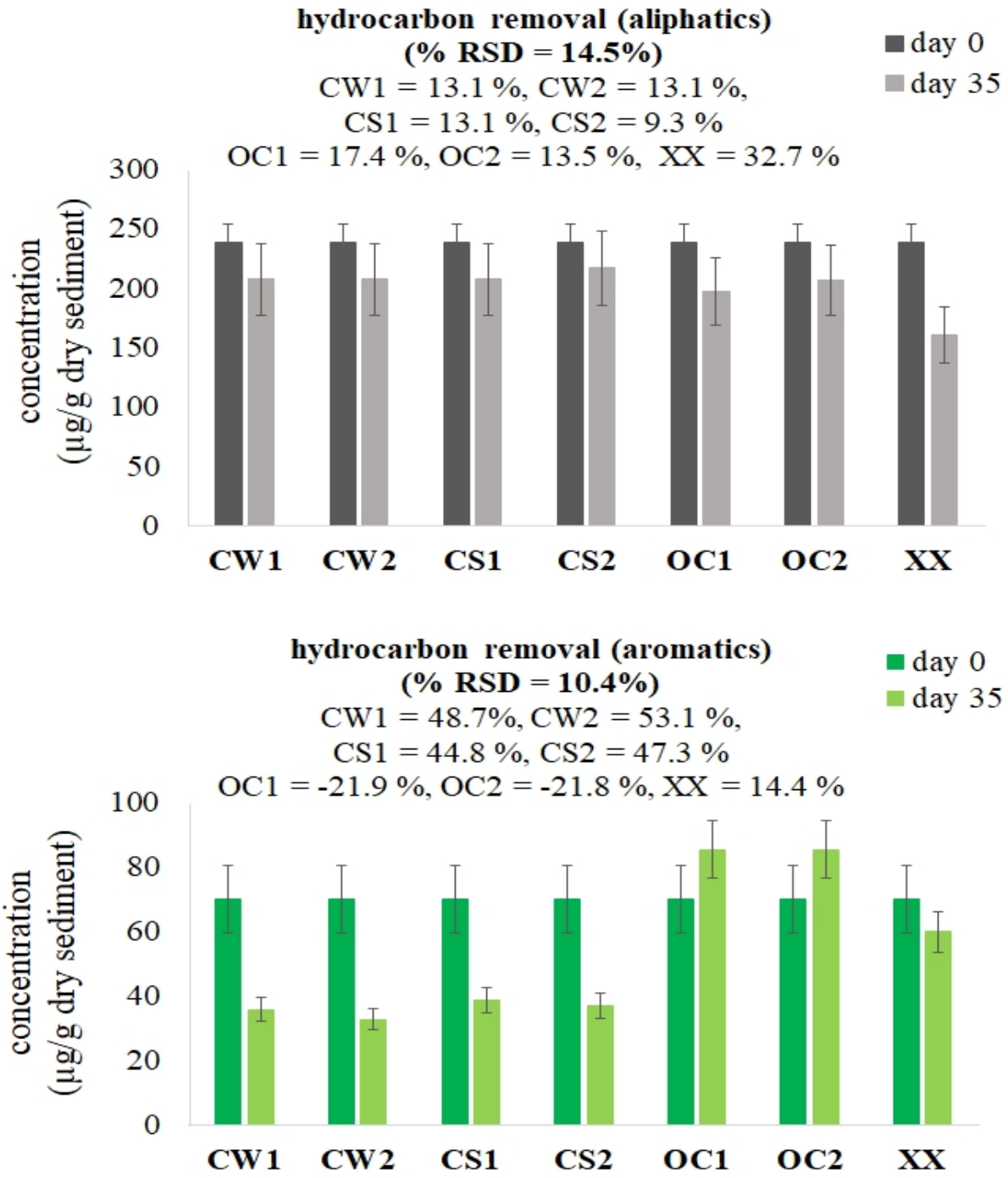


Figure 5-6. Degradation of aliphatic and aromatic hydrocarbon content in single chamber sediment microbial fuel cell (SC SMFC) (temperature = 30°C, external resistance = 200 ohms; CW= closed circuit with wicking cathode, CS= closed circuit with submerged cathode, OC= open circuit with submerged cathode. RSD = relative standard deviation).

Marginally better n-alkane removal efficiency was seen in XX sample compared with CW and CS. This is also consistent with OC samples having similar or slightly better n-alkane removal efficiency than CW and CS. Despite variation in generated current CW and CS showed similar n-alkane removal efficiency. These three observations suggest that alkane removal was not likely governed by electrochemical oxidation. SMFCs are reportedly expected to enhance electrochemical hydrocarbon removal compared to removal by abiotic and other processes. (Xu

et al., 2017). As earlier mentioned in section 2.2.5, hydrocarbon degradation in sediment is limited by insufficient electron acceptors for electrons generated during hydrocarbon degradation. SMFC anode in sediment serves as an additional electron acceptor. Through biocatalytic activity of biofilm on surface of SMFC anode, electrons generated during anaerobic oxidation of hydrocarbons are collected and transferred via an external circuit to the cathode where they are used to reduce oxygen. It was likely that abiotic processes such as dissolution or volatilization may have contributed to higher removal of hydrocarbons in XX, considering that bioreactors were maintained in enclosed box at 30^{o}C. It was also probable that alkane removal was facilitated by fermentation rather than electrochemical oxidation. The higher removal rates in XX samples could be due to lower oxygen content in the single chamber electrolyte maintaining the anaerobic conditions required by biofilm for efficient hydrocarbon removal.

On the other hand, CW and CS samples showed higher PAH removal compared to XX and OC samples (Figure 5-6). Although CW achieved higher current density than CS, there did not appear to be significant difference in PAH removal between CW and CS. Significant difference in PAH removal in CW and CS compared with OC and XX suggested that PAH removal was enhanced by electrochemical oxidation, but PAH oxidation current was only small fraction of oxidation current. This agrees with previous results reported in this chapter showing lower columbic efficiency of the oxidation process with respect to hydrocarbon oxidation. It seemed that anodic biofilm within CW and CS appeared to favour or be selective towards PAH removal over n-alkanes. Although several studies involving hydrocarbon removal using SMFCs have reported higher alkane removal over PAH (Wang *et al.*, 2012b; Li *et al.*, 2014; Li *et al.*, 2015; Li *et al.*, 2016a; Li *et al.*, 2016b; Li *et al.*, 2016c), few reports of higher PAH removal over alkanes has also been reported: 7% alkane and 13% PAH removed within 21 days (Zhang *et al.*, 2020a); and 34% alkane and 44% PAH removal within 223days (Li *et al.*, 2019). Higher PAH removal than alkane removal as seen in this study agrees with some results obtained from our double chamber SMFC tests. No difference in PAH removal between closed and open circuit cells was observed in double chamber SMFCs, whereas significant difference between closed and open circuit cells was seen in single chamber SMFCs. This could be due to difference in anode environment (bacteria colony developed and oxygen levels). In single chamber SMFC there is higher oxygen content in the electrolyte/anolyte and near biofilm compared to membrane separated anaerobic anode in double chamber fuel cell tests.

Individual components of aliphatic and aromatic fractions were further analysed to clarify biodegradation pattern. Biodegradation profile of individual components of aliphatic and aromatic hydrocarbon fractions for this experiment are presented in Figure 5-7. Individual n-

alkanes from C10 to C35 were biodegraded in all SMFCs. The highest overall removal rate of aliphatic hydrocarbons seen in XX samples appeared to be across the studied C10-35 range with significance advantage over other samples in the C22-35 range. All 16 PAHs were biodegraded in CW and CS samples, and at significantly higher rate than those of OC and XX samples. The limited increase or decrease in various PAH concentration in OC and XX samples was within 20%, which is within experimental error. This makes it difficult to draw strong conclusion that degradation in PAH occurred in OC and XX. It was difficult to conclude with certainty if the increase seen, was within experimental error of measurement or caused by accumulation of degradation product of higher molecular weight PAHs. However, measurement of both repeats e.g. OC1 and OC2 showed an increase from initial PAH concentration > 15%, which suggested that the likely reason was accumulation. A comparison of PAH removal rates under open circuit conditions between single chamber (Figure 5-7) and double chamber (Figure 5-2), showed that removal rates decreased significantly when using single chamber reactor. This can be caused by effect of higher oxygen content in the electrolyte affecting the bacteria colony and its ability to biochemically remove PAH. On the other hand, polarised cells did not show significant difference in PAH removal rate.

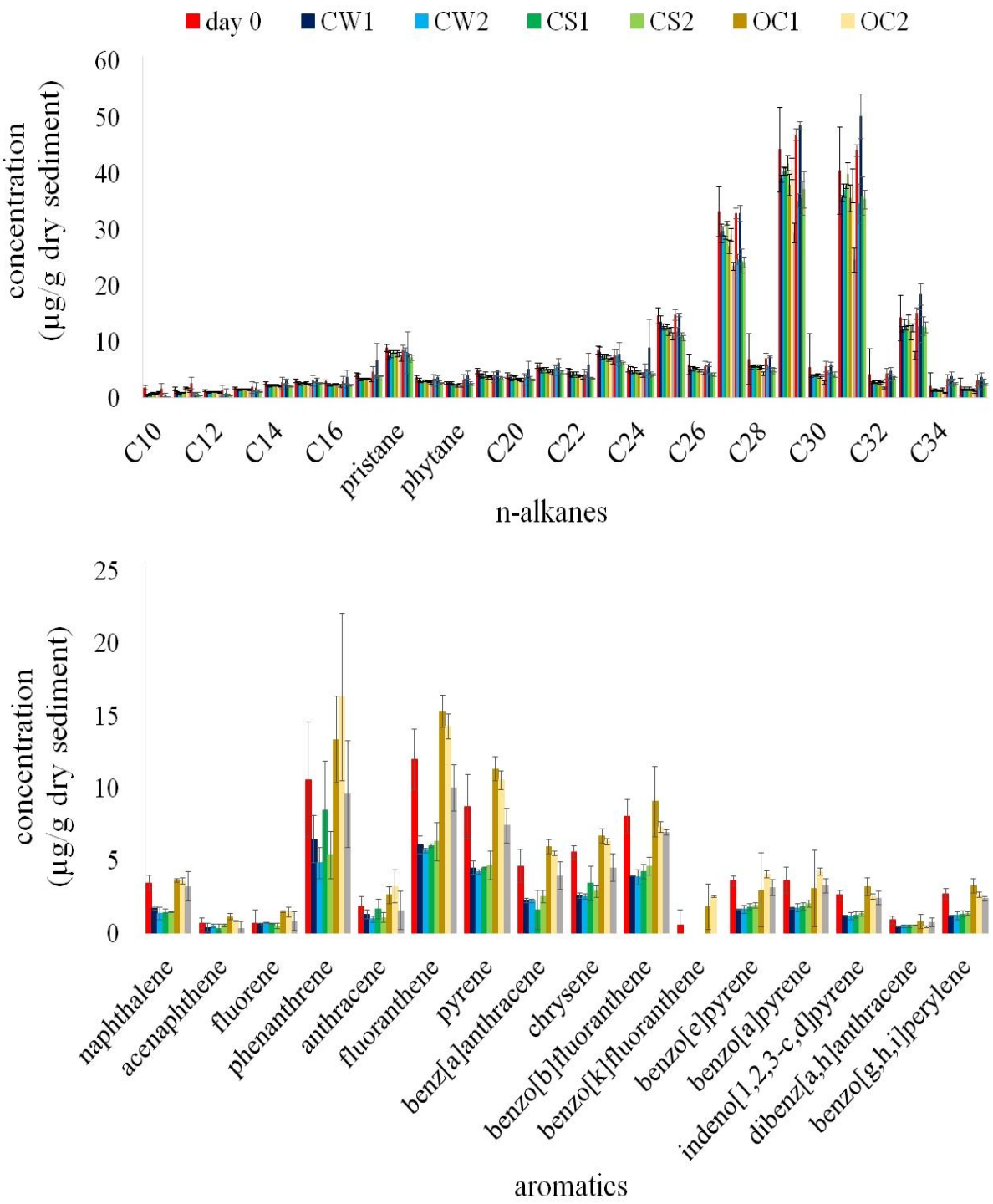


Figure 5-7. Hydrocarbon biodegradation profile for (A) n-alkanes (C10-C35) (B) n-alkanes (C25 -C33) and (C) polyaromatic hydrocarbons (PAHs) in single chamber sediment microbial fuel cell treating hydrocarbon-contaminated sediment using vertical electrode configuration (SC SMFC) (temperature = 30°C, external resistance = 200 ohms; CW= closed circuit with wicking cathode, CS= closed circuit with submerged cathode, OC= open circuit with submerged cathode, XX = biotic control, no electrodes).

Low molecular weight hydrocarbons compounds are generally easier to degrade than high molecular weight hydrocarbons. Hence, higher removal rates of lower molecular weight n-alkanes in all SMFCs was expected. The relative increase in C11 in both OC samples was likely

due to breakdown of longer chain alkanes to short chain alkanes rather than being an outlier or error in measurement as it was consistent across both OC1 and OC2 repeats. Higher PAH removal than alkane removal using vertical anode arrangement in this study corresponded with higher PAH removal (10%) than alkane removal (9%) reported in a previous study (Zhang *et al.*, 2015). In the previous study, however, 5- and 6-ring compounds were more resistant to biodegradation than 2- and 3-ring compounds. In open circuit SMFCs, only HMW PAHs with 5- and 6-rings were degraded – this pointed to likelihood that SMFC conditions supported removal of HMW PAH. This observation suggested that HMW PAHs could have been degraded and converted to LMW PAHs, resulting in accumulation of LMW PAHs (2- and 3-ring compounds) in OC samples. 5-ring PAH were reportedly transformed to 4- and 3- ring PAHs (Liang *et al.*, 2014; Zhou *et al.*, 2020). One study reported -13.88 ± 0.9 % (accumulation) alkane and 75.54 ± 1.5 % PAH removal in single chamber SMFC (electrode-membrane assembly with open-air cathode) with initial TPH concentration of 413 ± 2 mg/g, and operated at 29 ± 2 ˚C for 17 days (Mohan and Chandrasekhar, 2011). However, Mohan and Chandrasekhar (2011) attributed accumulation of alkanes to biotransformation of higher molecular weight (HMW) aromatics to lower molecular weight (LMW) aliphatics, and significant removal of 4- , 5- and 6-ring compounds alongside accumulation of 2- and 3-ring compounds. This observation was consistent for both SMFC and non-SMFC bioreactors studied by Mohan and Chandrasekhar (2011). HMW PAHs can also be converted to LMW PAHs, which could result increase in 3- and 4-ring compounds. For example, Figure 5-8 shows how BaP can be degraded via different metabolic routes to generate BaA, ANT, CHR, PYR and PHE. Higher removal of PAHs in bio-electrochemical systems has been attributed to self-induced bio-potential mediated reactions that involve cleavage of aromatic compounds into linear or aliphatic compounds (Chandrasekhar and Venkata Mohan, 2012). Degradation of PAH is dependent on oxygen level in electrolyte and time, where longer duration allows for degradation of HMW PAH to aliphatic hydrocarbons while shorter period converts them only to LMW PAH as has been suggested here.

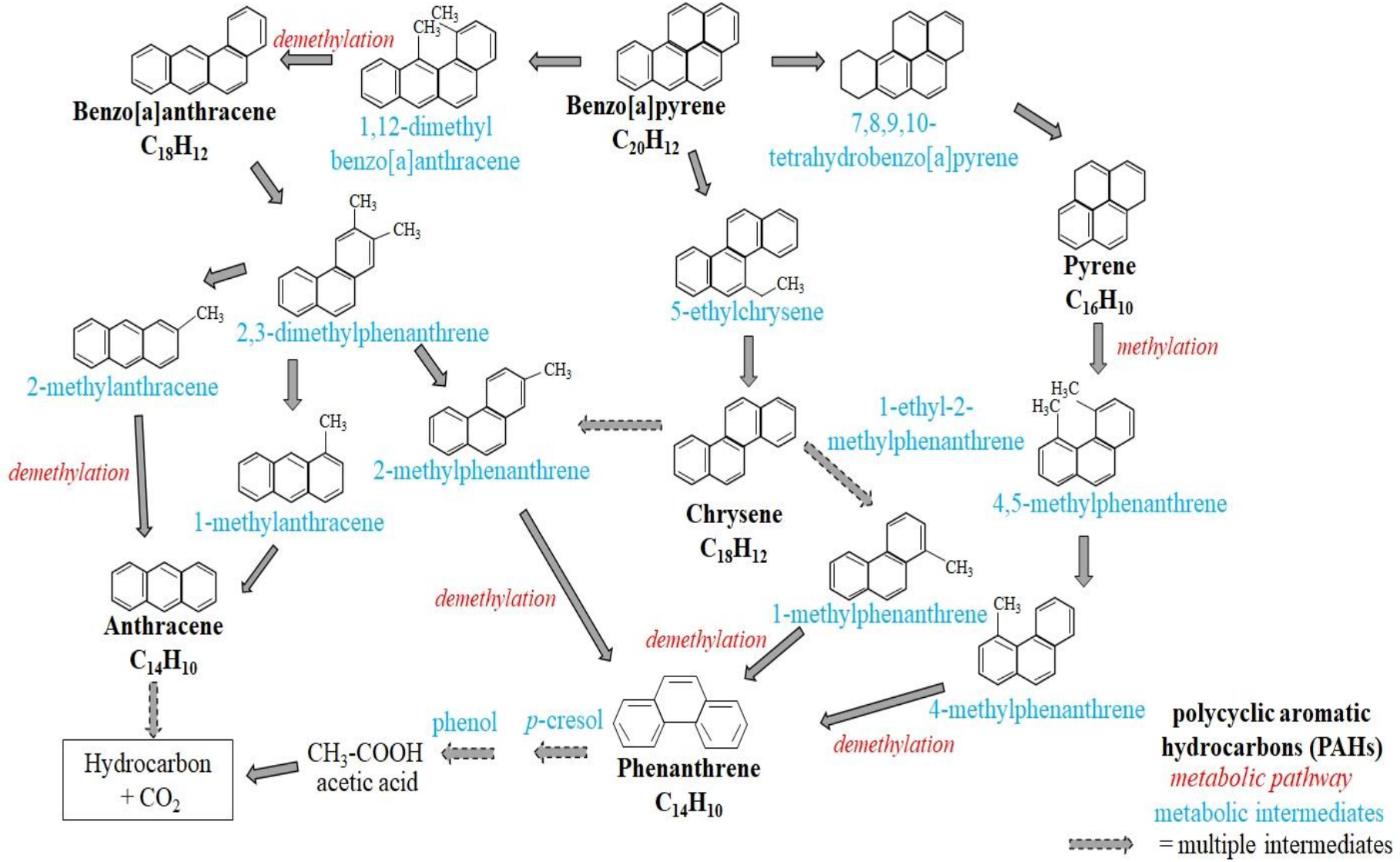


Figure 5-8. Putative metabolic pathways of anaerobic biodegradation of polycyclic aromatic hydrocarbon compounds (adapted from Liang et al. (2014); Zhou et al. (2020)).

5.3.7 Coulombic and Energy efficiency in single chamber sediment microbial fuel cell with vertical electrode configuration

In view of previous observations with double chamber SMFC as well as hydrocarbon removal in biotic control XX compared with SMFCs, contribution of total petroleum hydrocarbon consumed to electric current and power production was calculated using coulombic and energy efficiency. As can be seen in Table 5-4, CE was on average less than 1 % for CS and less than 2 % for CW samples. Higher CE in CW corresponded with highest hydrocarbon removal as well as higher current and charge output. However, energy efficiency was substantially low in all SMFCs.

Table 5-4. Coulombic and energy efficiency in single chamber sediment microbial fuel cell with vertical electrode arrangement.

	CW1	CW2	CS1	CS2
Charge output (coulombs, C)	198.63	396.72	69.49	123.86
CE (%)	0.96	1.92	0.34	0.60
Energy output (joules, J)	5.03	13.23	0.81	2.00
ϵ_E (x 10^{-7} %)	2.8	7.2	0.4	1.1

Low CE and energy efficiency in SC SMFC despite considerable removal of hydrocarbon (e.g. PAH) up to 50% was probably due to incomplete oxidation hydrocarbon compounds, and limited liberation of electron donors from these high energy organic substrates. Precise mechanisms of anaerobic degradation of complex substrates such as petroleum hydrocarbons is not clearly understood and is largely influenced by existent microbial consortia and prevailing environmental conditions. Furthermore, there is likelihood of oxygen diffusion into anode compartment in single chamber SMFC due to absence of membrane and so anode conditions may not be 100% anaerobic (electrolyte near cathode was air saturated). Nevertheless, impact of oxygen was likely to be limited due to high salinity (17195 mg/L) of Tyne River sediment (see Table 5-1) which would not support high levels of dissolved oxygen. Another factor that can influence energy efficiency is how much of liberated electrons from oxidation reaches the electrode and how much is utilised directly by nearby reduction reaction e.g. involving other terminal electron acceptors in solution. In SMFC, microbial utilization of hydrocarbon as carbon or energy source is exploited for hydrocarbon removal. Anaerobic hydrocarbon degraders are reportedly able to use a limited range of hydrocarbons - either aliphatic

hydrocarbons within limited chain lengths or a few aromatic hydrocarbons. It is not commonplace for a microbe to utilize both aliphatic and aromatic hydrocarbons, neither is it commonplace to find one microbe capable of utilizing a parent hydrocarbon compound as well as its metabolic intermediates. Anaerobic hydrocarbon oxidation to carbon dioxide is coupled with reduction of electron acceptor and can be executed within a single species; however, anaerobic oxidation of hydrocarbon to methane involves syntrophic co-operation of bacteria (activate hydrocarbon oxidation) and methanogens (use metabolic intermediates of hydrocarbon oxidation i.e. hydrogen, acetate) (Widdel *et al.*, 2010). Methanogens and EAB compete for acetate resulting in loss of potential energy from acetate that has been utilized by methanogens for methane production. The complexity of these interactions results in diversion of electrons liberated from oxidation of primary hydrocarbon compounds into several biological processes. Additionally, some microbes can degrade a compound as non-growth substrate while depending on another compound as growth substrate via cometabolism (Nzila, 2013). Microbes that use recalcitrant HMW PAHs as carbon source are rare, however, HMW PAHs such as PYR, BaA, BaP, and CHR can be removed via cometabolism (Baboshin *et al.*, 2008; Nzila, 2013). Further energy losses are incurred when a compound is used as growth substrate. This phenomenon buttresses the fact that SMFC support syntrophic associations or interactions that enhance removal of recalcitrant contaminants such as PAHs.

5.3.8 Environmental changes in single chamber sediment microbial fuel cells with vertical electrode configuration

Oxidation reduction potential (ORP) and pH were recorded during the measurements. Changes in pH also affects ORP measurement where increase in pH results in -59mV/pH unit as estimated by Nernst equation. As can be seen in Figure 5-9A and B, this occurred within first 14 days of operation for single chamber SMFC. Even though maximum current output was achieved between days 6 and 7 in all SMFCs, maximum pH was reached between day 6 and day 14. Increase in pH in BES is often associated with accumulation of nitrogen-sulphur-oxygen (NSO) fractions and metabolic intermediates (e.g. bicarbonate) that are basic in nature, or accumulation of hydroxyl radicals due to cathode reaction in water; hence pH tends towards alkaline (Zhao *et al.*, 2016; Widdel and Musat, 2019). Nevertheless, these systems are capable of self-buffering resulting in marginal pH variations over the treatment duration. For single chamber SMFC with wicking cathode (CW) and single chamber SMFC with submerged cathode (CS), significant decline in current coupled with marginal pH variations suggested reduced microbial electrochemical substrate oxidation in these bioreactors. This was supported by similarity in pH profile of these bioreactors to that of biotic control XX from days 20 to 35.

The decrease in ORP can directly be linked to potential shift caused by pH shift i.e. increase of pH for example from 6.5 to 7.5 in CW2 (day 0 to 5) is expected to cause a negative shift of 59 mV in ORP. This was seen with change in ORP from 25 mV to -35 mV (day 0 to 5).

Figure 5-9C shows recorded anode potentials (E_a) in single chamber SMFCs. Anode potential reached an average of -492 ± 23, -503 ± 4 and -494 ± 11 mV (vs Ag/AgCl RE) for CW, CS and OC respectively, within 6 days of start-up. This suggested high electroactive bacteria activity and was consistent with current output within same period. Oxidation of hydrocarbon and reduction of anode by electroactive and non-electroactive bacteria in sediment is controlled by the respiratory pathway that can be optimized by bacteria at existing anode potential (Torres *et al.*, 2009). More negative anode potential at rest (when not polarised) is known to support faster biofilm growth and higher current output (Logan, 2009). Anode potentials became more positive in CW and CS reaching between -391 ± 56 and -308 ± 47 mV (vs Ag/AgCl RE) by day 35. This is expected as single chamber SMFCs with wicking cathode (CW) and submerged cathode (CS) were polarised (connected by external resistor to cathode) and hence anode potential will increase with current flow due to anodic overpotential (to overcome activation energy of oxidation reaction and transport of active species). Changes in anode potential can induce changes in respiratory pathway utilized by anodic biofilm as bacteria will adapt energy-conserving pathways (Finkelstein *et al.*, 2006). This in turn may have influenced substrate oxidation efficiency and led to fluctuations in current output as was observed in CW and CS samples.

Maximum cell voltage in CW (585 ± 7 mV) coincided with peak current output and was higher than achieved in other studies: 190 mV (Morris and Jin, 2012), 323 mV (Venkidusamy *et al.*, 2016) and 208.4 ± 2.7 mV (Li *et al.*, 2019). However, total petroleum hydrocarbon (TPH) concentration in these previous studies was higher ranging from 8,000 to 64,228 mg/kg. Electrogenic activity has been shown to decline with increase in hydrocarbon concentration (Chandrasekhar and Venkata Mohan, 2012; Venkidusamy *et al.*, 2016). Additionally, presence of small oxygen content in solution when using single chamber MFC would result in mixed potential at the anode (oxidation of organics and reduction of oxygen) pushing the anode potential to higher values (towards oxygen reduction reaction potential).

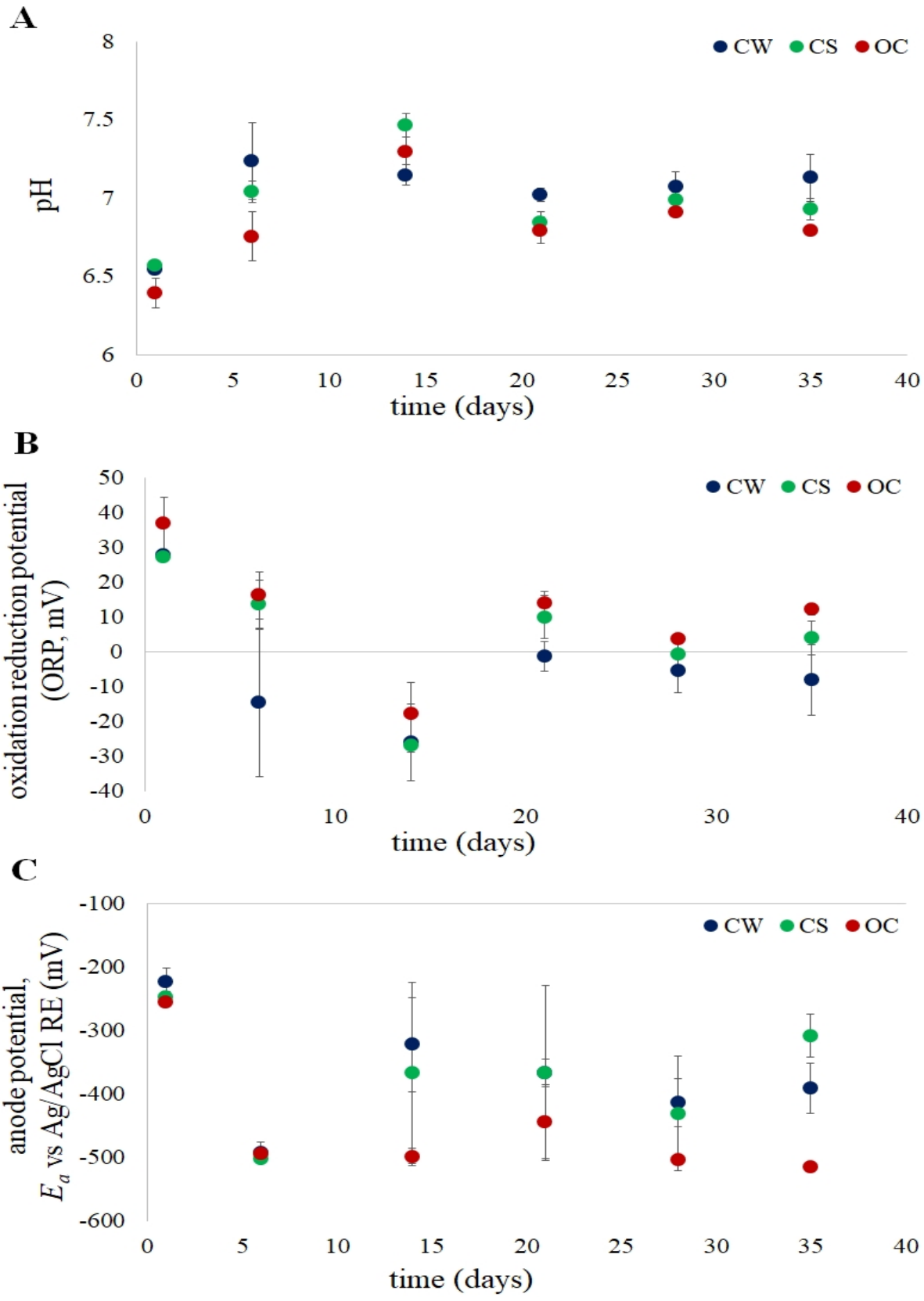


Figure 5-9. Changes in environmental parameters in single chamber sediment microbial fuel cells (SC SMFC) used for treatment of hydrocarbon contaminated sediments: (A) pH, (B) oxidation reduction potential (ORP), (C) anode potentials (temperature = 30°C, resistance = 200 ohms, CW = wicking cathode; CS = submerged cathode; OC = control at OCP, submerged cathode).

5.3.9 Electrochemical characterization of single chamber sediment microbial fuel cells with vertical electrode configuration

Variations in cyclic voltammetry (CV) profiles were observed in closed circuit single chamber SMFCs (with submerged cathode - CS, with wicking cathode - CW) and open circuit SMFCs (OC). As can be seen in Figure 5-10, sigmoidal shape of CVs and ratio of anodic to cathodic peak current for CW2, CS1, and OC2 were suggestive of semi-reversible electrode reactions. In addition, low peak magnitude (2 to 3 A/m^2) suggested depleted substrate and appeared consistent with reduced current output by day 35. Peak values were in same order of magnitude to these obtained from DC MFC ca. 5 A/m^2 (Figure 5-4) but lower. Complex substrates like petroleum hydrocarbons require consortium of bacteria for complete oxidation (Heider and Fuchs, 1997; Rabus *et al.*, 2016). Different fractions and their metabolic intermediates are consumed at different rates, and slow mass transport of these fractions and intermediates coupled by minimal electrolyte flow in sediment seemed the more probable reason for increased diffusion resistance and consequently low peak currents in the order of 1-10 A/m^2 seen in CVs (Lu *et al.*, 2014b; Gambino *et al.*, 2017). Oxidation and reduction peak potentials (vs Ag/AgCl) were as follows: CW2 (0.090/-0.290 V), CS1 (-0.120/-0.380 V), and OC2 (-0.060/-0.360 V). These differences are larger than expected differences from variation in pH (59 mV/pH unit) and may have resulted from differences in microbial communities enriched on anodic biofilm. Sediment is known to contain high diversity of microbes; hence it is possible that different groups of bacteria were enriched in each bioreactor, more so influenced by anode potential and surrounding oxygen levels. Additionally, in presence of O_2 in solution mixed potential will occur at anode (from substrate oxidation and ORR) and anode potential will shift to higher potentials with increase in O_2 content in solution as discussed earlier. Higher oxygen concentration near the anode will result in larger reduction peak in comparison to oxidation peak as ORR will contribute to reduction peak in addition to that of active redox moiety at anode. However, as can be seen from Figure 5-10, reduction peak current was only marginally bigger than anodic oxidation peak in the studied samples (by 5-25%) or 0.2-1.6 A/m^2 increase. This suggests that anode conditions might not be 100% anaerobic. As discussed previously ORR limiting current at air saturation is within the range of 0.33-3.3 A m^{-2}. This suggests that O_2 concentration near the anode is below that of saturation (from air). This observed shift coincided with decrease in pH. Order of decrease in pH was: CW2>CS1>OC2.

E1/2 potentials of CS1 (-0.250 V vs Ag/AgCl) and OC2 (-0.210 V vs Ag/AgCl) were close to fumarate/succinate redox couple (- 0.280 V vs Ag/AgCl) (Dumas *et al.*, 2008). E1/2 potentials of CW2 (-0.100 V vs Ag/AgCl) were close to some known phenazine redox mediators –

phenazine-1-carboxylate PCA (-0.240 V vs Ag/AgCl), pyocyanine PYO (-0.116 V vs Ag/AgCl), phenazine-1-carboxamide, PCN (-0.140 V vs Ag/AgCl) and 1-hydroxy-phenazine 1HP (-0.174 V vs Ag/AgCl) (Bosire *et al.*, 2016; Bosire and Rosenbaum, 2017). Furthermore, onset redox potentials around -0.400 V vs Ag/AgCl are in line with oxidation of malate (to oxaloacetate).

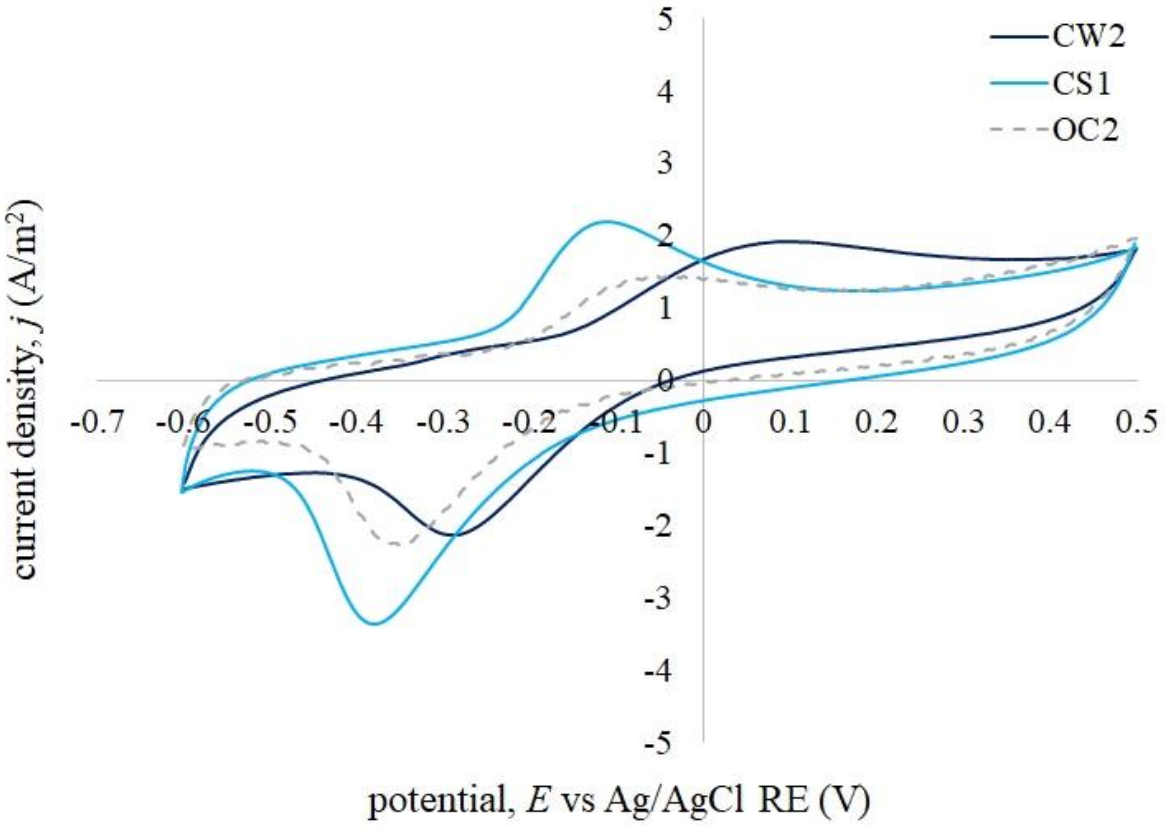


Figure 5-10. Cyclic voltammograms on day 35 for single chamber microbial fuel cells using vertical electrode configuration (scan rate = 0.001 Vs^{-1}).

Fumarate addition is known as one of the dominant mechanisms for used by anaerobic hydrocarbon-degrading bacteria for activation of biodegradation of alkanes and PAHs (Gieg and Suflita, 2005). Fumarate is produced from aminoacids and other metabolites within the bacteria and is useful for generation of energy for bacterial growth. Fumarate reduction is coupled with proton transport across bacterial cytoplasmic memebrane (Kröger, 1978). Electron shuttles such as phenazines and ubiquinone are involved in mediated electron transfer (Schröder, 2007; Fricke *et al.*, 2008; Martin *et al.*, 2013). Identification of E1/2 potentials close to redox systems involved in both direct and indirect electron transfer indicated that anodic biofilm comprised microbial consortia capable of utilizing different electron transfer mechanisms. Predominance of E1/2 potentials closer to electron shuttles suggested extracellular electron transfer was a significant mechanism used by anodic biofilm during hydrocarbon removal. This observation was suggestive of bacteria utilization of fermentative products of

hydrocarbon degradation. Several bacterial species are capable of synthesizing and utilizing electron shuttles (Rabaey *et al.*, 2004; Rabaey *et al.*, 2005a; Marsili *et al.*, 2008a). Electron shuttling is also vital for interspecies electron transfer observed in syntrophic microbial interactions (Liu *et al.*, 2020). Few EAB can utilize complex substrate such as hydrocarbons, hence EAB rely on metabolic intermediates produced by fermentative bacteria (Chaudhuri and Lovley, 2003). Multiple electron transport processes or systems within hydrocarbon contaminated sediment reflected high microbial diversity often associated with marine sediments and substantiated involvement of several microbial consortia in biotransformation of various components of petroleum hydrocarbon in the sediment.

5.4 Conclusion

Double and single chamber sediment microbial fuel cells were explored to optimize removal of petroleum hydrocarbons (aliphatic and aromatic fractions) from contaminated sediment. Generally, aliphatic fraction (n-alkanes) removal in open circuit SMFCs compared to closed circuit SMFCs, which suggested that alkane removal was not governed by electrochemical oxidation. Similar polyaromatic hydrocarbons (PAH) removal was observed in closed circuit double chamber sediment microbial fuel cells (41 – 45 %) and closed circuit single chamber sediment microbial fuel cells using vertical electrode arrangement (44 – 54 %). No significant difference in PAH removal was observed between closed and open circuit double chamber SMFCs which suggested that PAH removal mainly occurred via other biochemical processes such as fermentation and methanogenesis. On the contrary, closed circuit single chamber SMFCs were able to remove PAH while no PAH was removed in its counterpart open circuit cells. This showed that microbial electrochemical oxidation of PAH occurred in these SMFCs, albeit to a limited degree as characterized by low coulombic efficiencies (< 2 %) across the SMFC bioreactors. Wicking cathode used in single chamber SMFC achieved higher charge output (297 ± 140 C) compared to submerged cathode (98 ± 38 C). The succinct role of bioreactor and electrode configurations in performance of SMFCs for removal of contaminants from environmental medium was evidenced in this study. Wicking cathode improved oxygen reduction reaction leading to enhanced charge output in comparison to submerged cathode. PAH removal was also marginally enhanced when wicking cathode was used in single chamber SMFC compared with use of submerged cathode. Nonetheless, this study showed that biotransformation of petroleum hydrocarbons by fermentative and hydrolytic microbial consortia was primarily responsible for generation of electron donors used by electroactive bacteria in SMFC for current production. Microbial community analysis on SMFC bioanodes would be required to verify this.

Chapter 6 Microbial community analysis of microbial fuel cells used for treatment of hydrocarbon-contaminated media.

6.0 Chapter summary

Microbial community analysis was conducted on bioanode samples taken from double chamber microbial fuel cell (MFC) (low external resistance, 100 Ω) and single chamber sediment microbial fuel cell (SMFC) used for treatment of hydrocarbon-contaminated wastewater and sediment, respectively. The aim of the study was to correlate microbial community dynamics with bioreactor performance and to understand relationship between MFC/SMFC bioanodes and hydrocarbon removal. Individual members of phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*) found in both MFCs and SMFCs were remarkably distinct and had potentially diverse roles within the bioreactors. *Chloroflexi*, *Planctomycetes,* and *Epsilonbacteraeota* also occurred in single chamber SMFC bioanodes. Putative electroactive bacteria (EAB) *Acidovorax* and *Geobacter* were enriched in closed circuit MFCs while *Pseudomonas, Sphingobium,* and *Comamonas* were more abundant in open circuit MFCs, some members of which include hydrocarbon degraders. Although similar microbial groups were found in both closed and open circuit MFCs, bacterial enrichment in each MFC was linked to their specific function in the bioreactor. This new finding was observed from the presence of *Pseudomonas* (some members of which are putative EAB) in both closed and open circuit MFCs, thereby highlighting its versatile role in MFCs. Highly diverse but evenly distributed taxa were found on single chamber SMFC bioanodes. Another novelty of this study was the significant enrichment (145 to 317%) of putative fermentative anaerobe *Anaerolineaceae* in all single chamber SMFCs within 35 days of operation. Other enriched families included *Bacteroidetes* and *Clostridiaceae 1,* some members of which are capable of anaerobic fermentation. Relative abundance of these families correlated with low coulombic efficiency (< 2 %) obtained in closed circuit single chamber SMFCs seen earlier in Chapter 5.3.7.

6.1 Introduction

Micro-organisms utilize inorganic or organic compounds to meet their energy and growth requirements. In bio-electrochemical systems (BES), such as microbial fuel cells (MFCs) or sediment microbial fuel cells (SMFCs), electrons and protons are generated from oxidation of organic compounds (Daghio *et al.,* 2017). Electric current is produced when electrons are transferred from the organic compounds to the anode of the microbial fuel cell and then, via an external circuit, from anode to cathode (Schröder, 2007). Protons combine with oxygen and electrons in the cathode chamber to produce water (Logan *et al.,* 2006). Electric current production is facilitated by anode-respiring bacteria or electroactive bacteria (EAB) in an anodic biofilm. These micro-organisms transfer electrons directly or indirectly to the anode via either electron transport chains that include outer membrane cytochromes, electrically conductive pili or endogenous or exogenous diffusible electron shuttles such as flavins (Venkata Mohan *et al.*, 2014). In MFCs/SMFCs, anodic biofilms comprise associations of both EAB and non-EAB micro-organisms (Logan, 2009). Consortia of micro-organisms enable breakdown of complex organic matter in wastewater and sediment, via hydrolysis and fermentation to long chain fatty acids, volatile fatty acids and eventually acetate, and hydrogen (Harayama *et al.,* 2004; Yeh and Pavlostathis, 2005; Tsai *et al.*, 2009; Fuchs *et al.*, 2011). These reduced products of microbial metabolism are utilized by EAB as electron donors. Some members of well-known EAB from the family *Geobacteraceae* (e.g. *Geobacter metallireducens* and *Geobacter chapellei*) can couple oxidization of fermentation products, most commonly acetate, with dissimilatory iron, Fe (III) or manganese, Mn (IV) reduction to generate electricity (Coates *et al.*, 2001a; Lovley *et al.*, 2004; Lovley, 2006). *Geobacter* species can either use Fe (III) or Mn (IV) as electron acceptor or can transfer electrons via direct interspecies electron transfer (DIET) with *Methanothrix* (formerly *Methanosaeta*) or *Methanosarcina* (Rotaru *et al.*, 2014; Holmes *et al.*, 2017; Lovley and Holmes, 2022). Micro-organisms from different phyla such as *Proteobacteria*, *Firmicutes* and *Acidobacteria* have demonstrated capability of electric current generation (Logan, 2009). The diversity of micro-organisms found in MFCs/SMFCs reflect not only the range and complexity of organic compounds that can be used as feed in these systems, but also various roles played by these micro-organisms in metabolism of organic compounds and electricity production. Syntrophic relationships between electroactive and non-electroactive bacteria therefore plays a crucial role in contaminant removal during bioremediation under anoxic conditions.

6.1.1 Microbial communities in microbial fuel cells (MFCs)

Anode potential exerts a major influence on anodic microbial communities in microbial fuel cells (Finkelstein *et al.*, 2006; Aelterman *et al.*, 2008a). Micro-organisms maximize energy gain by self-regulating redox potentials of their terminal reductases close to redox potentials of terminal electron acceptor (TEA). Hence, microbial communities are selectively enriched based on their ability to utilize available TEA effectively. Torres *et al.* (2009) demonstrated that diversity of anode-respiring bacteria increased when anode potential was increased from -0.15 V to + 0.37 V vs standard hydrogen electrode (SHE). High anode potential induced changes in extracellular electron transfer (EET) mechanism, and only bacteria capable of using TEA with reduction potential close to anode potential were able to colonize the anode. Microbial communities in MFCs not only diverge from the original community present in the start-up inoculum, but dynamically evolve throughout the duration of operation. In one study for instance, even though *Geobacter*, *Pseudomonas* and *Shewanella* were the first to be established on an MFC anode, the latter two micro-organisms were not sustained; rather *Sphingomonas* and *Rhizobium* became predominant after voltage stabilization (Paitier *et al.*, 2017). *Geobacter sulfurreducens* and *Bacteroidetes* were key micro-organisms found in double chamber MFCs fed with acetate, lactate and glucose, while *Firmicutes* occurred only in glucose fed MFCs as oxygen scavengers (Jung and Regan, 2007). Micro-organisms closely related with *Pseudomonas* and *Stenotrophomonas* (both belonging to the *Gammaproteobacteria*) were enriched in diesel fed MFCs, while low abundance of *Bacteriodetes*, *Clostridia* and *Actinobacteria* were observed (Venkidusamy *et al.*, 2016). Facultative *Gammaproteobacteria* capable of nitrate reduction (including *Citrobacter* sp., *Pseudomonas* sp., and *Stenotrophomonas* sp.) were dominant on the anode in a MFC developed for diesel removal (Morris *et al.*, 2009).

6.1.2 Microbial communities in sediment microbial fuel cells (SMFCs)

Active microbial species have been shown to be influenced by the sediment source used as an inoculum in SMFC. Different classes of proteobacteria appear to be selectively enriched on anaerobic anodes using different soils and sediments. For example, *Betaproteobacteria* and *Gammaproteobacteria* were enriched on anaerobic anodes using soil contaminated with diesel and engine oil (Lu *et al.*, 2014a) and *Gammaproteobacteria* were enriched on anaerobic anodes using petroleum hydrocarbon contaminated soil (Zhang *et al.*, 2020a). *Deltaproteobacteria* were enriched in marine, salt-marsh and freshwater SMFC anodes, with *Betaproteobacteria* enriched only in freshwater SMFC anodes (Holmes *et al.*, 2004). Anodes from marine and salt-marsh SMFCs were dominated by *Desulfuromonas* species while anodes from freshwater

SMFC were dominated by *Geobacter* sp or *Pelobacter propionicus* (Holmes *et al.*, 2004). EAB in sediments appeared to be dominated by *Deltaproteobacteria* with *Geobacteraceae* accounting for more than 50% of microbial community on SMFC bioanode, after 3 - 7 months of operation (Holmes *et al.*, 2004; Lovley, 2006). Other bacteria classes found on the anodes of sediment MFC include: *Alphaproteobacteria, Gammaproteobacteria,* and bacteria from the order *Cytophagales,* and phylum *Firmicutes* (Holmes *et al.*, 2004).

BES-enhanced petroleum hydrocarbon degradation has been shown to induce changes in microbial community structure in original sediment, with decreased microbial diversity in anode (compared with original sediment) after BES remediation, due to selection of hydrocarbon-degrading bacteria (Li *et al.*, 2017; Zhang *et al.*, 2020a). Bacterial clusters after remediation were shown to be distinct from uncontaminated sediment and original hydrocarbon contaminated sediment and were selectively enriched based on metabolic function (Lu *et al.*, 2014a; Li *et al.*, 2017). For example, after treatment in BES abundance of *Alphaproteobacteria* declined from 46 % in original contaminated soil to an average of 26 % on BES anodes (Lu *et al.*, 2014a). EAB such as *Comamonas testosteroni*, *Pseudomonas putida* and *Ochrobactrum anthropi* capable of hydrocarbon degradation were selectively enriched on the anode (Lu *et al.*, 2014a). Although, *Proteobacteria* increased from 20.77 % in raw sediment to 40.12 % in SMFC, this class of bacteria comprised more of *Gammaproteobacteria* in raw sediment compared with *Deltaproteobacteria* on SMFC anode (Xu *et al.*, 2017). *Pseudomonas* dominated in SMFC anode using soil amended with Fe (III) oxide while *Geobacter* dominated in SMFC anode using unamended soil (Xu *et al.*, 2017). Higher organic matter removal in SMFC amended with Fe (III) oxide was attributed to presence of iron (Fe) as alternative electron acceptor. Fe (III) oxide induced changes in microbial community, which demonstrated that nature of alternative electron acceptors available in SMFC can also influence SMFC anodic community structure.

In summary, microbial communities of MFC and SMFC anodes are highly diverse. Microbial community structure in these BES are influenced by several factor including: MFC/SMFC anode potential, alternative electron acceptors available, nature of substrate, environmental conditions within aqueous medium (for MFC) or sediment (for SMFC). The complex interactions of anodic microbial communities with these factors as well as interactions between various groups within these communities are pivotal to determination of MFC/SMFC performance.

The aim of microbial community analysis was to correlate microbial community composition with MFC/SMFC performance in terms of current and power output; and to understand

connection between anodic biofilm structure and hydrocarbon removal in MFCs and SMFCs in this study.

6.2 Experimental Procedure

Microbial community analysis was conducted only on bioreactors where there was significant difference in hydrocarbon removal efficiency between closed and open circuit cells. Hence, microbial community analysis was performed on MFC bioreactors with low external resistance (R_{ext} = 100 Ω), and single chamber SMFCs with vertical electrode arrangement. Materials and methods for microbial community analysis are described in sections 3.7 to 3.8.

6.3 Results and Discussion

6.3.1 Alpha diversity

16S rRNA gene sequences recovered from both MFCs and single chamber SMFCs bioanodes were considered together to understand commonalities between microbial communities found in different bioelectrochemical systems (BES). For ease of discussion, MFC effluent inoculum (IM) and activated sludge inoculum (IAS) refer to inoculum composition before addition into the MFCs. As can be seen in Table 6-1, amplified sequence variants (ASVs) were highest for activated sludge inoculum (IAS) (199) and lowest for MFC effluent inoculum (IM) (25), with similar observations in MFCs inoculated with activated sludge (AS1 and nAS2) and MFCs inoculated with MFC effluent (M2 and nM2), respectively. Higher ASVs in activated sludge inoculum compared with MFC effluent inoculum suggested there were higher number of different taxonomical groups in activated sludge. Synergistic interactions within these groups in activated sludge inoculum could have supported establishment of a robust anodic biofilm capable of efficient breakdown of Tween 80 surfactant and polyaromatic hydrocarbons (PAH) – phenanthrene and fluoranthene. Consequently, this would explain why faster start-up and higher current output was achieved during stage 1 (100 ppm Tween 80 surfactant feeding cycle) in MFCs inoculated with activated sludge (3.76 ± 0.26 mA/m^2) compared with MFCs inoculated with MFC effluent (75.37 ± 0.96 mA/m^2) (see Figure 4-7). Correlation of ASVs with current density appeared to be more obvious in MFCs inoculated with MFC effluent. Higher ASVs in MFCs inoculated with MFC effluent (69) (compared with the original MFC effluent inoculum, ASVs = 25) by the end of stage 3 (100 ppm Tween 80 surfactant + 100 ppm PAH) corresponded with higher current density (51.73 ± 1.00 mA/m^2) during this stage compared with stage 1 (3.76 ± 0.26 mA/m^2). Current density in MFCs inoculated with activated sludge declined by the end of stage 3 (74.67 ± 0.71 mA/m^2, ASVs = 138), as ASVs also declined

compared with original activated sludge inoculum (75.37 ± 0.96 mA/m^2, ASVs = 199). Coulombic efficiency also followed a similar trend, declining from stage 2 to 3 in MFCs inoculated with activated sludge (29.56 – 9.38 %) and increasing from stage 2 to 3 in MFCs inoculated with MFC effluent (3.34 – 8.61 %). However, microbial community analysis was only conducted on anodic biofilm at end of stage 3. Future work on microbial community dynamics with change in feeding cycle would be necessary to further understand correlation of ASVs with current density and coulombic efficiency. ASVs in activated sludge inoculum (199) in this study were low (indicating low diversity) compared with 1,183 – 3,567 (indicating high diversity) from a previous study (Zhang *et al.*, 2012) where similarities/differences between activated sludge from different locations was compared.

Alpha diversity indices (Shannon index, Pielou's evenness and Faith phylogenetic distance) displayed similar trend with ASVs. Although, alpha diversity indices were generally higher for MFCs inoculated with activated sludge, there was no significant difference in alpha diversity between MFCs inoculated with activated sludge and those inoculated with MFC effluent (Kruskal Wallis, p = 0.121). There was also no significant difference between closed circuit MFCs (AS1 and M2) and open circuit MFCs (nAS2 and nM2) (Kruskal Wallis, p = 0.439). Shannon index for MFC effluent inoculum (1.66) was lower than obtained for anode sample of an acetate-fed MFCs (3.98 – 4.58) (Yates *et al.*, 2012). This indicated that there was less microbial diversity in MFC effluent inoculum compared with the anode sample of the acetate-fed MFC. This observation highlighted that inoculum obtained directly from MFC anode would likely have higher diversity and support better performance.

Table 6-1. Alpha diversity of microbial communities in double chamber microbial fuel cells (MFCs) treating hydrocarbon contaminated wastewater (see footnote for description of sample IDs).

sample ID*	Reads	ASVs	Shannon index	Pielou's evenness	Faith_pd
IAS	34354	199	7.23	0.94	19.65
AS1	66703	138	6.27	0.88	12.56
nAS2	24892	106	5.59	0.85	7.99
IM	31015	25	1.66	0.36	3.72
M2	21683	69	4.91	0.82	6.14
nM2	38090	62	4.70	0.80	5.25
	216737	**598**			

*IAS = Activated Sludge inoculum, AS1= closed circuit MFC inoculated with activated sludge, nAS2 = open circuit MFC inoculated with activated sludge, IM = MFC Effluent inoculum, M2 = closed circuit MFC inoculated with MFC effluent, nM2 = open circuit MFC inoculated with MFC effluent

For single chamber sediment microbial fuel cells (SMFC), ASVs were between 213 – 277 for original sediment (Tyne River sediment) and all SMFCs (see Table 6-2). ASVs were lower for

some SMFCs than original sediment. Shannon index (7.44 ± 0.22), Pielou's evenness (0.94 ± 0.01), and Faith pd (17.83 ± 1.28), were similar for Tyne River sediment and all SMFCs (see Table 6-2). A comparison of alpha diversity obtained in MFCs and single chamber SMFCs showed that there was a significant difference (Kruskal Wallis, p = 0.003) in microbial communities on bioanodes of these bio-electrochemical systems.

Table 6-2. Alpha diversity indices of microbial communities in single chamber sediment microbial fuel cell (SMFC) treating hydrocarbon contaminated sediment (see footnote for description of sample IDs).

sample ID*	Reads	ASVs	Shannon index	Pielou's evenness	Faith_pd
TRS	36711	227	7.44	0.94	17.68
CS2	90139	277	7.86	0.96	20.66
OC1	91782	266	7.58	0.94	16.99
XX	50464	244	7.48	0.94	17.91
CS1	36146	234	7.35	0.93	17.96
CW1	30966	237	7.40	0.94	16.37
OC2	30869	221	7.07	0.91	18.05
CW2	20402	213	7.36	0.95	17.05
	387479	**1917**			

* TRS = Tyne River sediment (original sediment), CW1, CW2 = duplicate closed circuit SMFCs using wicking cathode, CS1, CS2 = duplicate closed circuit SMFCs using submerged cathode, OC1, OC2 = duplicate open circuit SMFCs using submerged cathode, XX= control (no electrodes)

Alpha diversity indices such as Shannon's index and Pielou's evenness provide information on richness (number of different species) and evenness (relative abundance of different species) of microbial community structure, respectively. Shannon index is a measure of the number and frequency of species, with high values indicating heterogeneity (diversity of species) in microbial community (Thukral, 2017). Pielou's evenness, is typically between 0-1, with values closer to 1 indicating equal number of different species within a microbial community. Faith phylogenetic diversity (Faith_pd) indicates branch length spanned by microbial community – lower values of Faith_pd indicate that members of a microbial community are closely related.

MFC effluent inoculum had lower number of different species, most of which were closely related species, compared with bioanodes of MFCs inoculated with the effluent (M2 and nM2) which had a higher number of diverse species that were evenly distributed but less closely related than those in the inoculum. MFC effluent inoculum was obtained from an acetate fed MFC. Therefore, this inoculum was expected to be dominated by electroactive bacteria. Consequently, most of ASVs (76.1%) in MFC effluent inoculum belonged to family *Pseudomonodaceae*. Some members of this family e.g. *Pseudomonas aeruginosa* are electroactive bacteria (EAB) (Rabaey *et al.*, 2004). Low diversity has been demonstrated in acetate fed MFC where 99% of microbial community comprised electroactive bacteria from

family *Geobacteriaceae*. (Torres *et al.*, 2009). Conversely, activated sludge inoculum had higher number of less closely related but evenly distributed species than bioanodes of MFCs inoculated with activated sludge (AS1 and nAS2). Activated sludge inoculum comprised *Proteobacteria* (39.4%), *Bacteroidetes* (26.7%), *Actinobacteria* (9.31%), *Nitrospirae* (6.1%), *Firmicutes* (5.7%), *Planctomycetes* (3.6%), *Acidobacteria* (1.9%) and *Spirochaetes* (1.2%). Similar diverse phyla were obtained in same activated sludge inoculum used in another study: *Proteobacteria* (27.9%), *Planctomycetes* (16.7%), *Chloroflexi* (16.1%), *Bacteroidetes* (11.8%), *Actinobacteria* (8.9%), *Verrucomicrobia* (6.9%), and *Firmicutes* (1.9%) (Khan *et al.*, 2021).

For all closed circuit MFCs (both MFC effluent and activated sludge bioreactors), similarities in Shannon index (5.59 ± 0.96) and Pielou evenness (0.85 ± 0.04) indicated that species in these MFCs were moderately diverse but evenly distributed. However, higher Faith_pd in MFCs inoculated with activated sludge compared with MFCs inoculated with MFC effluent suggested that species in MFCs inoculated with activated sludge were not as closely related compared with species in MFCs inoculated with MFC effluent. As previously indicated, these differences were not significant. This observation was further strengthened by similarities in cumulative charge output at the end of the MFC operational period of 64 days. Cumulative charge output (expressed in coulombs, C) in closed circuit MFCs, inoculated with MFC effluent (M2 =104.58 C) and inoculated with activated sludge (AS1=104.03), were similar from day 42 to 64 compared with day 0 to 21 (M2 = 5.01 C and AS1 = 90.54 C). It also agreed with previous studies where no difference in MFC performance was observed when bioanode was fully developed, regardless of inoculum source used (Wang *et al.*, 2009; Yates *et al.*, 2012).

Number of ASVs obtained for original sediment and single chamber SMFCs in this study were low compared with values obtained from other studies on sediment/soil microbial fuel cells (see Table 6-3). This was indicative of high diversity and low abundance of species in microbial community in Tyne River sediment (original sediment) and single chamber SMFC compared with other studies. Nevertheless, Shannon index and Pielou's evenness in this study was comparable values obtained in other studies (see Table 6-3) and showed that microbial community in sediment/soil comprise evenly distributed numbers of different species.

Table 6-3. Comparison of amplified sequence variants (ASVs) and alpha diversity obtained from various studies on sediment/soil microbial fuel cells.

S/N	Amplified Sequence Variants (ASVs)*		Shannon index		Goodness coverage (Evenness)		Reference
	Sediment	SMFC bioanode	Sediment	SMFC bioanode	Sediment	SMFC bioanode	
1	532 - 729	523 - 747	3.79 - 4.74	4.15 – 4.68	0.98 – 0.99	0.98 – 0.99	(Li *et al.*, 2016b)
2	7173	6543 - 7044	7.28	7.06 – 7.17	0.94	0.91 – 0.96	(Xu *et al.*, 2017)
3	2002 - 2135	667 - 1535	9.02 – 9.32	6.35 – 7.90	0.9024 – 0.9374	0.9065 – 0.9741	(Shi *et al.*, 2019)
4	4200	3504 - 4001	9.651	8.899 – 9.350	0.978	0.981 – 0.984	(Liang *et al.*, 2020)
5	227	213 -277	7.44	7.07 – 7.86	0.94	0.91 – 0.96[#]	This study

*****reported as operational taxonomic units (OTUs) in referenced studies
[#]reported as Pielou's evenness

Highest number of ASVs as well as highest Shannon and Faith_pd in CS2 (closed circuit single chamber SMFC with submerged cathode) indicated that this SMFC comprised high diversity of species with more evolutionarily divergent taxa. Closed circuit single chamber SMFCs with wicking cathode exhibited highest charge output (CW2 = 396.72 C and CW1 = 198.63 C) and had more closely related taxa than closed circuit single chamber SMFCs with submerged cathode (CS1 = 69.49 C and CS2 = 123.86 C). Relative abundance of *Gammproteobacteria* in closed circuit single chamber SMFCs with wicking cathode was 14.9 ± 0.05 % and 12.4 ± 0. 5 % in closed circuit single chamber SMFCs with submerged cathode. Several electroactive species such as *Escherichia*, *Pseudomonas*, *Shewanella* and *Klebsiella* are found in phyla *Gammaproteobacteria* (Logan, 2009).

Number of amplified sequence variants (ASVs) obtained in single chamber SMFCs was 3.21 times higher than ASVs obtained in MFCs. Sediment/soil have been reported to possess higher microbial community abundance and diversity than wastewater or aqueous environment. High number of ASVs in activated sludge was expected as activated sludge has been known to encompass high diversity of microbes (Zhang *et al.*, 2012), possibly due to high organic content of activated sludge. Higher Shannon index and Faith-pd values in single chamber SMFCs

bioanode communities showed that single chamber SMFCs comprised higher abundance and diversity of species that were more divergent compared to MFCs.

MFCs and SMFCs are bioelectrochemical systems operated under same priniciples – microbes on anode convert chemical energy in organic compounds to electrical energy. At phylum level, there were communities common to both MFC and SMFC bioreactors, albiet in varying proportions. Both MFCs and SMFCs were dominated by *Proteobacteria* (26 - 93%), *Actinobacteria* (2.4 -19.6%), *Firmicutes* (0.7 -17%) and to a lesser extent especially in MFCs, *Bacteroidetes* (2.9 - 26%) (see Appendix I). However, certain groups were distinct in each set of bioreactors. For instance, *Chloroflexi, Epsilonbacteraeota, Planctomycetes* were found mostly in single chamber SMFCs, and activated sludge inoculum with less than 1% in MFCs. *Nitrospirae, Spirochaetes* and *Tenericutes* were found only in activated sludge inoculum, Tyne River sediment and single chamber SMFC bioreactors. Members of these phyla have been associated with hydrocarbon contaminated environments, especially *Proteobacteria, Bacteroidetes, Firmicutes* and *Chlorolfexi* (Morris *et al.*, 2009; Yan *et al.*, 2012; Venkidusamy *et al.*, 2016).

6.3.2 Comparative analysis of microbial community structure in microbial fuel cells (MFCs) and sediment microbial fuel cells (SMFCs)

Principle component analysis (PCA) was used to visualize dissimilarities and identify patterns within MFC effluent inoculum/activated sludge inoculum/Tyne River sediment and MFC/ SC SMFC bioanode microbial communities. Distinct clusters were observed for MFCs and single chamber SMFCs in PCA plot (Figure 6-1). Activated sludge inoculum and MFC effluent inoculum were also distinct from MFCs clusters. Microbial community of Tyne River sediment and all single chamber SMFC bioanodes formed one cluster, indicating that similar microbial community structure was present in both Tyne River sediment and single SMFC bioanodes. Two-sided Welch t-test ($p \leq 0.05$) visualized by PCA plot indicated there was a significant difference between microbial communities in MFCs and SMFCs ecosystems.

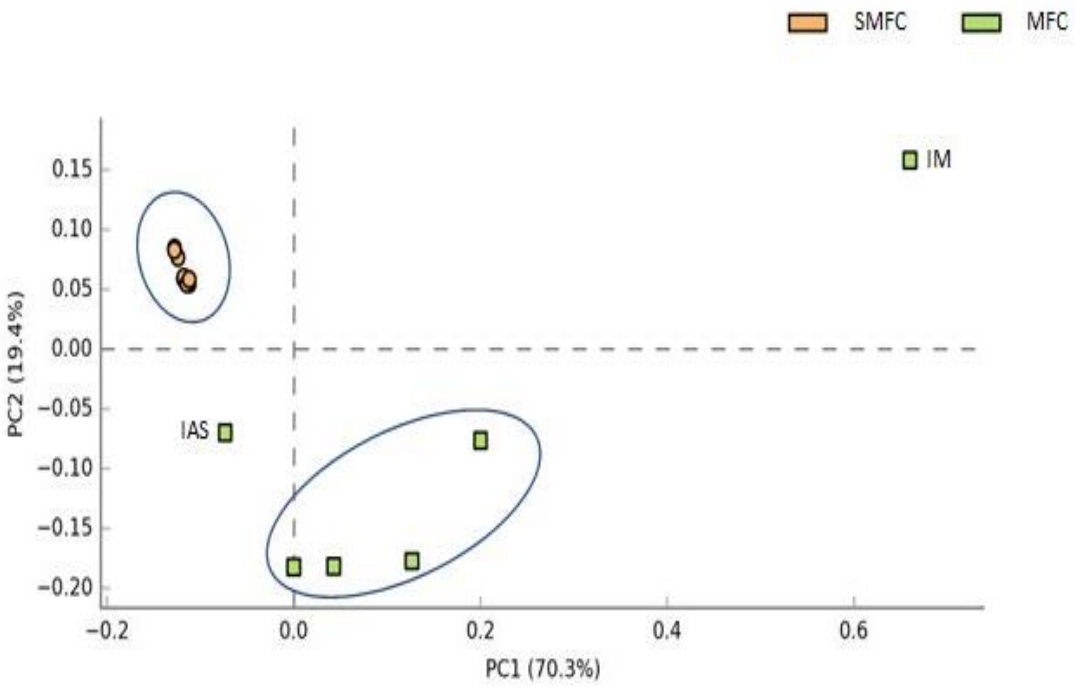


Figure 6-1. Principal Component Analysis (PCA) plot of bacterial taxa at family level in inoculum and bioanodes of bioelectrochemical systems (MFC = microbial fuel cells, SC SMFC = sediment microbial fuel cells, IAS = activated sludge inoculum, IM = MFC effluent inoculum. IAS and IM were based on inoculum composition before addition to MFCs).

Microbial community in MFCs and single chamber SMFCs are diverse. SMFCs have been reported to be dominated by *Deltaproteobacteria* (Holmes *et al.*, 2004), while MFCs mainly comprise *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* or *Deltaproteobacteria* (Phung *et al.*, 2004; Logan and Regan, 2006a). Microbial diversity could also be influenced by bioreactor configuration, inoculum, and substrate. For example, MFC was dominated by *Betaproteobacteria* (including *Leptothrix* sp., *Cytophaga* sp.) when enriched with river water but dominated with *Alphaproteobacteria* (including *Aminobacter aminovorans*, *Methylotrophic proteobacterium*) when enriched with artificial wastewater (Phung *et al.*, 2004). Deoxyribonucleic acid (DNA) bands observed during denaturing gradient gel electrophoresis (DGGE) analysis, showed that anodes of MFCs fed with simple substrate (acetate) had less bands (denoting lower diversity) compared with MFCs fed with complex substrate (glucose + glutamate) (Lee *et al.*, 2003). Similarly, microbial diversity (represented by observed taxonomic units, OTUs) in MFCs spiked with phenanthrene (13) and pyrene (17) increased to 21 and 39, respectively, when the MFCs were spiked with mixed solutions of naphthalene-phenanthrene and naphthalene-pyrene (Zhou *et al.*, 2020). The foregoing considerations may explain why there was higher microbial diversity in Tyne River sediment/single chamber SMFCs compared with MFC effluent inoculum/MFCs may have been due to higher concentration/composition of organic compounds (including hydrocarbons) in

SMFC compared to MFCs. For example, MFCs contained one surfactant (Tween 80) and two polyaromatic hydrocarbons (phenanthrene and fluoranthene) (100 – 200 ppm), whereas single chamber SMFCs contained n-alkanes (C10 to C35, 239 ± 46 ppm) and 16 PAH compounds (70 ± 4 ppm).

6.3.3 Microbial communities associated with MFC bioanodes.

Clustering of communities from MFCs despite being set up with different inocula indicated there were some similarities in microbial communities in these MFCs (see Figure 6-2). Difference in microbial community structure in MFC bioanodes compared with original inocula used agreed with observed changes in alpha diversity indices. Clustering of communities in both closed and open circuit MFCs was likely due to presence of similar families on their MFC anodes. Comparison of MFC samples at family level using Kruskal Wallis H-test at $p \leq 0.05$ showed communities in MFC effluent inoculum (IM) and activated sludge inoculum (IAS) were significantly different from MFC bioanode communities.

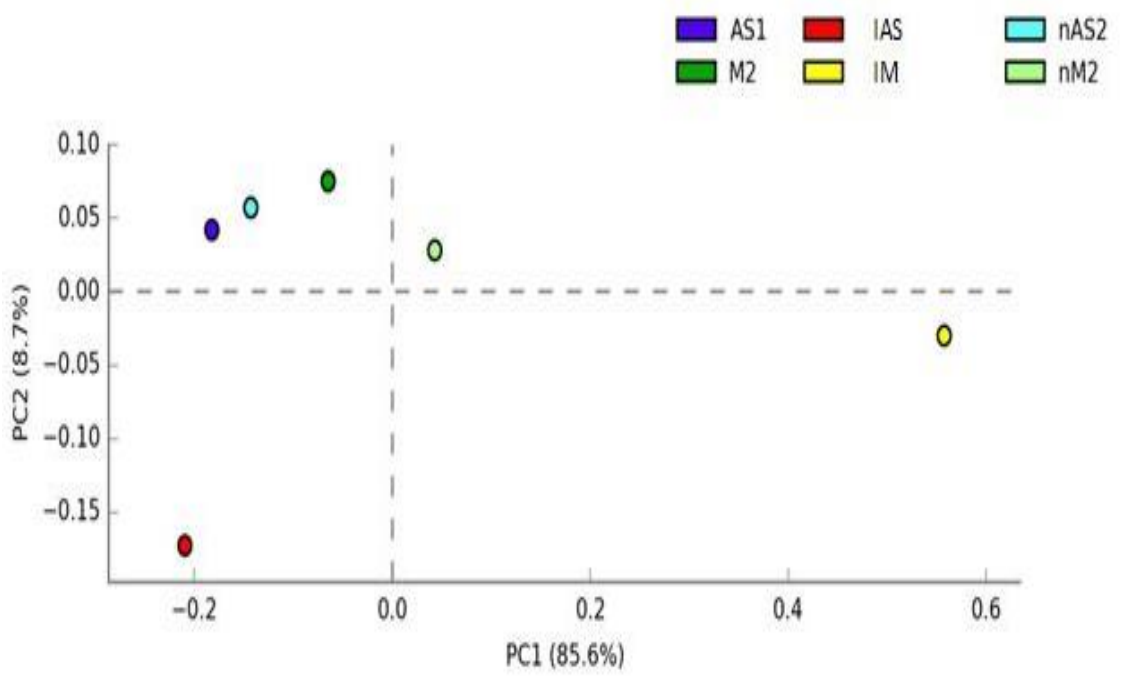


Figure 6-2. Principal Component Analysis (PCA) plot of bacterial taxa at family level in microbial fuel cell (MFC) bioanodes used for treatment of hydrocarbon-contaminated wastewater (IM = MFC effluent inoculum, M2 = closed circuit MFC inoculated with MFC effluent, nM2 = open circuit MFC inoculated with MFC effluent, IAS = Activated Sludge inoculum, AS1= closed circuit MFC inoculated with activated sludge, nAS2 = open circuit MFC inoculated with activated sludge).

Families common to all MFCs included: *Burkholderiaceae, Rhodocyclaceae, Dysgonomonadaceae, Rhizobiaceae, Mycobacteraceae, Desulfovibrionaceae, Geobacteraceae, Xanthobacteraceae,* and *Beijerinckiaceae* (see Figure 6-3). The most abundant families in all MFCs were *Burkholderiaceae* (14 – 23.7 %), *Rhodocyclaceae* (5.6 –

13 %), and *Mycobacteraceae* (7.9 – 8.9 %). Members belonging to *Burkholderiaceae* and *Rhodocyclaceae* have been reported as aromatic hydrocarbon degraders (Pérez-Pantoja *et al.*, 2010; Zhong *et al.*, 2011; Adelaja *et al.*, 2014a; Wei *et al.*, 2015; Vaidya *et al.*, 2020; Yan *et al.*, 2020). *Burkholdericeae* (e.g. *Comamonas*) seen in this study contains some members of that are aerobic hydrocarbon degraders while others are facultative anaerobes (e.g. *Acidovorax*) or electroactive bacteria (e.g. *Comamonas testosteroni*) (Lu *et al.*, 2014a; Sheikhyousefi *et al.*, 2017). Identification of some possibly strict aerobic hydrocarbon degraders in these MFCs implied that there was some oxygen diffusion from cathode chamber into the anode chamber of the MFCs. One study showed that growth of family *Mycobacteraceae* could be enhanced in the presence of Tween 80 surfactant, leading to enhanced degradation of high molecular weight PAHs (Wang *et al.*, 2016a).

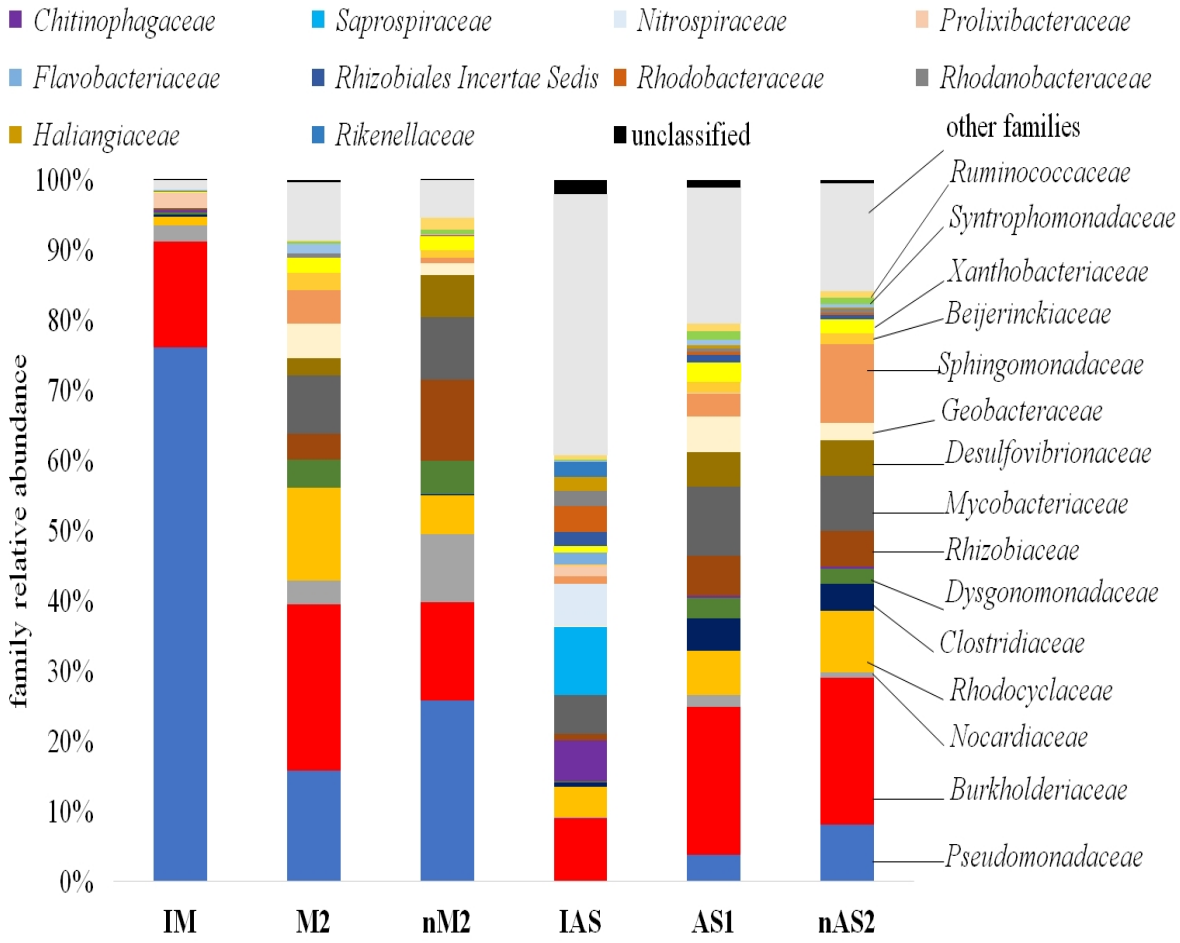


Figure 6-3. Relative abundance (> 1%) of microbial communities at family level in microbial fuel cells (MFCs) treating hydrocarbon-contaminated wastewater (IM = MFC effluent inoculum, M2 = closed circuit MFC inoculated with MFC effluent, nM2 = open circuit MFC inoculated with MFC effluent, IAS = Activated Sludge inoculum, AS1= closed circuit MFC inoculated with activated sludge, nAS2 = open circuit MFC inoculated with activated sludge).

Syntrophic relationships within MFC bioanode was seen from the different families, some members of which may have been responsible for playing diverse roles within the MFCs, for

example: sulphur oxidation - *Rhizobiaceae* (Yin *et al.*, 2021); anaerobic fermentation - *Dysgonomonadaceae, Clostridiaceae* (Morris *et al.*, 2009; Pannell *et al.*, 2016; Sheikhyousefi *et al.*, 2017; Feng *et al.*, 2020); electron transfer – *Beijerinckiaceae, Geobacteraceae* (Strik *et al.*, 2011; Koók *et al.*, 2020); sulphate reduction – *Desulfovibrionaceae* (Petropoulos *et al.*, 2021); anaerobic hydrocarbon degraders – *Sphingomonadaceae, Xanthobacteraceae, Nocardiaceae, Flavobacteriaceae,* (Zhong *et al.*, 2011; Venkidusamy *et al.*, 2016; Koók *et al.*, 2020; Tucci *et al.*, 2022); nitrification - *Nitrospiraceae* (Zhang *et al.*, 2020a); and *Syntrophomonadaceae* – syntrophic fatty acid degraders (Reimers *et al.*, 2006). *Chitinophagaceae* can degrade phenanthrene, but its proliferation could be hampered by some nonionic surfactants (i.e. Brij 30) (Wang *et al.*, 2016a; Zhou *et al.*, 2020).

To ascertain if polarisation had impacted anodic biofilm community, all closed circuit MFCs and open circuit MFCs were compared using a profile scatter plot of families with relative abundance of > 1% (total of 21 families) (see Figure 6-4). Two-sided Welch's t-test ($p > 0.05$) showed that there was no significant difference between these families. The profile scatter plot was also used to visualize enriched families in all closed and open circuit MFCs - enriched families are shown on either side of the grey dashed line. It showed that there were more enriched families in open circuit MFCs (enriched families are indicated on left side of the grey-dashed line) than closed circuit MFCs. *Pseudomonadaceae, Rhizobiaceae, Sphingomonadaceae, Desulfovibrionaceae* and *Norcardiaceae* were enriched in open circuit MFCs whereas closed circuit MFCs were enriched with *Burkholderiaceae, Rhodocyclaceae, Mycobacteriaceae* and *Geobacteraceae*. The latter observation agreed with the relative abundance of *Burkholderiaceae, Rhodocyclaceae, Mycobacteriaceae* and *Geobacteraceae* which was higher in closed circuit MFCs (22.4 ± 1.8 %, 9.7 ± 4.8 %, 9.0 ± 1.0%, 5.0 ± 0.0 %) than open circuit MFCs (17.5 ± 5.0 %, 7.2 ± 2.3 %, 8.4 ± 0.7 %, 2.1 ± 0.4 %).

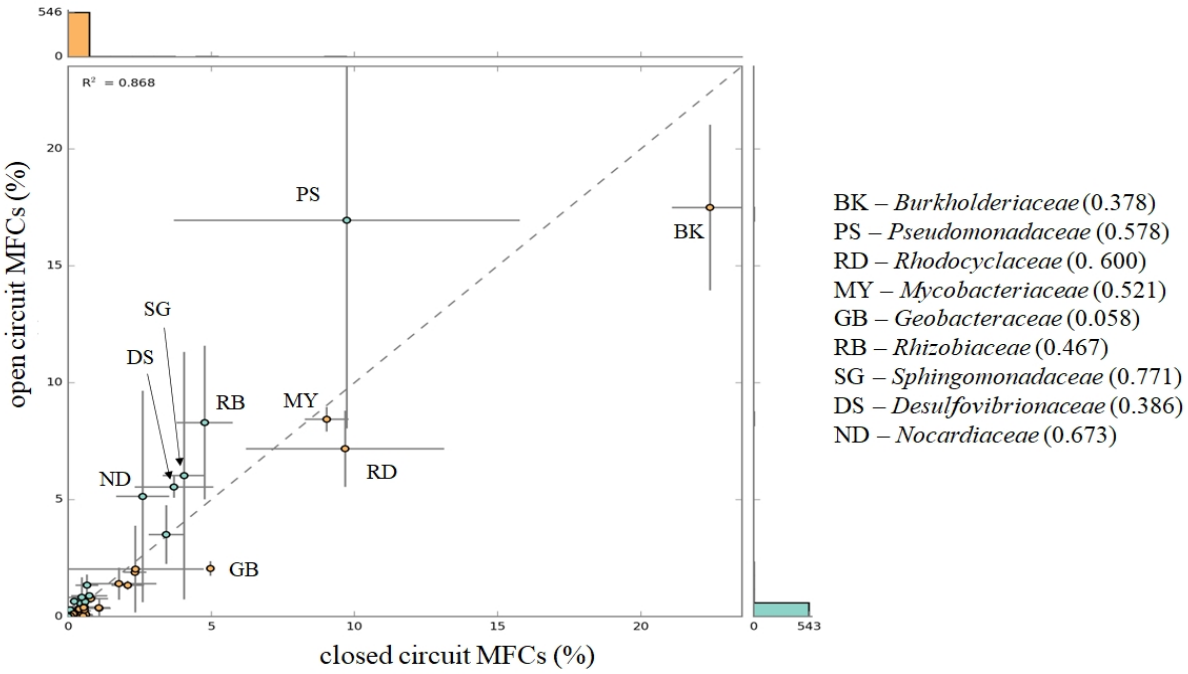


Figure 6-4. Profile scatter plot comparing the relative proportions (> 1 %) of 21 families of microbial communities within closed circuit microbial fuel cells (M2 and AS1) and open circuit microbial fuels (nM2 and nAS2) used for treatment of hydrocarbon contaminated wastewater at temperature 30°C (R^2 = 0.868) (p-values based on two-sided Wech's t-test are indicated in brackets besides each family).

Some members of *Geobacteraceae*, *Burkholderiaceae* (*Comamonas testosteroni*), and *Rhodocyclaceae* are electroactive bacteria (Achenbach *et al.*, 2001; Bond *et al.*, 2002; Bond and Lovley, 2003; Lu *et al.*, 2014a). For example, *Dechloromonas* sp. CS-1 and *D. agitata* CKB (family *Rhodocyclaceae*) have recently been described as electroactive bacteria (Torres-Rojas *et al.*, 2020; Torres-Rojas *et al.*, 2022). Similarities in relative abundance of family *Geobacteraceae* in closed circuit MFCs (M2 and AS1) correlated with similarities in cumulative charge output (expressed in coulombs, C) in closed circuit MFCs, M2 (104.58 C) and AS1(104.03) by day 64, when the bioanode samples were collected for microbial analysis. In addition, higher relative proportions of *Pseudomonadaceae* were observed in open circuit MFCs (16.9 ± 12.5 %) compared with closed circuit MFCs (9.7 ± 8.5 %). Although some members of family *Pseudomonadaceae* are capable of electron transfer using phenazine electron shuttles (Rabaey *et al.*, 2005a), higher proportions of this family in open circuit MFCs relative to closed circuit MFCs suggested that this group of bacteria may have had little or no involvement in electron transfer activities. Although generally known for aerobic bacteria, some members of *Pseudomonadaceae* are capable of hydrocarbon degradation under anoxic conditions (Berdugo-Clavijo and Gieg, 2014). Some members of *Burkholderiaceae* are hydrocarbon-degrading bacteria that can be found in MFC bioreactors for treatment of hydrocarbon contaminated water (Rakoczy *et al.*, 2013).

Extended error bar plots are useful in identifying statistically significant features between two or more microbial communities (Parks *et al.*, 2014). Extended error bar plots of closed circuit (M2) and open circuit (nM2) MFCs inoculated with MFC effluent showed that there was a high number of bacteria that were significantly different between closed and open circuit MFC effluent bioreactors. *Acidovorax*, *Sphingobium*, *Dechlorosoma*, and *Geobacter* were more abundant in closed circuit MFC compared to open circuit MFC (see Figure 6-5). *Pseudomonas*, *Rhodococcus*, *Comamonas*, and *Ochrobactrum* were the significantly enriched in open circuit MFC inoculated with MFC effluent. At $q \leq 0.05$, two-sided Fisher's Exact tests showed there were statistically significant differences in relative abundance of specific members of the microbial community between closed and open circuit MFC effluent bioreactors at genus level.

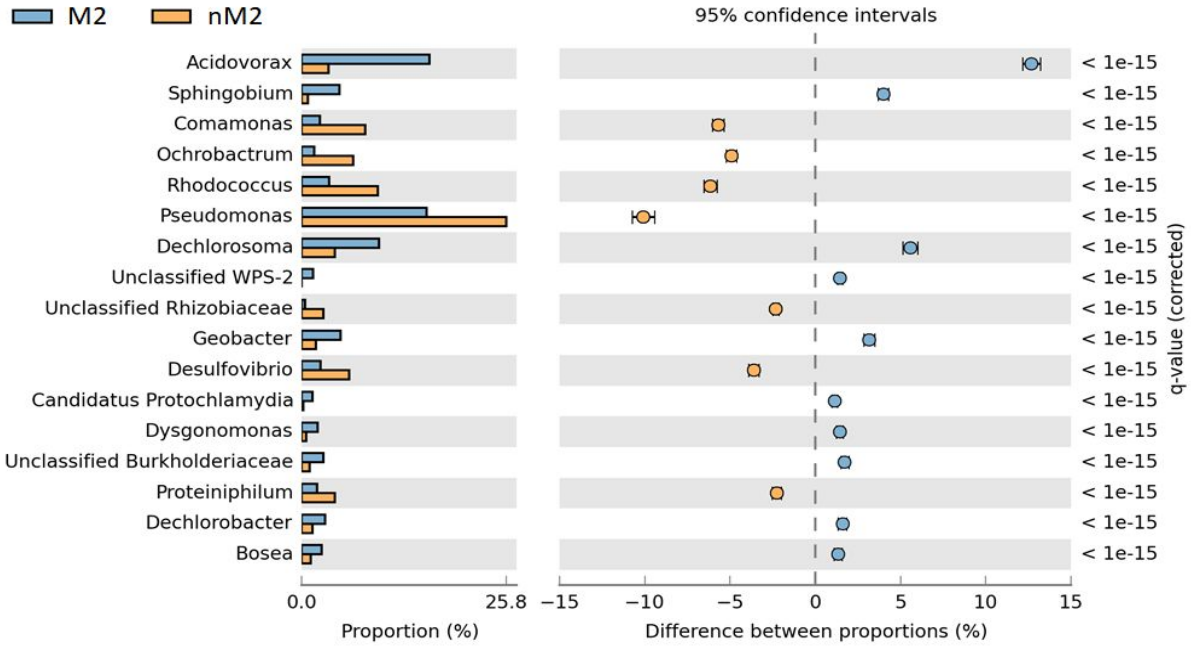


Figure 6-5. Extended error bar plot showing significant differences between bioanode of closed circuit (M2) and bioanode of open circuit (nM2) MFCs inoculated with MFC effluent (differences shown at genus level).

Dechlorosoma, *Acidovorax* and *Geobacter* are known electroactive bacteria (Achenbach *et al.*, 2001; Bond *et al.*, 2002; Bond and Lovley, 2003; Lu *et al.*, 2014a; Sheikhyousefi *et al.*, 2017; Wang *et al.*, 2020b). Some members of genus *Dechlorosoma* (*Dechlorosoma suillum*) are gram-negative, facultative anaerobes with c-type cytochromes, that can use consume simple organic acids using a variety of electrons acceptors ranging from oxygen, to nitrate or perchlorate (Achenbach *et al.*, 2001). Enrichment of these genera in closed circuit MFC effluent bioreactor likely contributed electric current production in the MFC. Members of these enriched genera also include some putative hydrocarbon degrading bacteria (Coates *et al.*, 2001b). For example, four strains of *Acidovorax* (P3, P4, NA2, and NA3^{T}) were demonstrated to have high tolerance for phenanthrene (Singleton *et al.*, 2018). *Dechloromonas* (predominant member of

Rhodocyclaceae seen in this study) has been demonstrated to anaerobically degrade benzene under nitrate reducing conditions (Chakraborty and Coates, 2005). In another study, facultative anaerobes *Dechloromonas* strains (RCB and JJ) were able to mineralize benzene anaerobically under nitrate reducing conditions (Coates *et al.*, 2001b). While members belonging to *Pseudomonas, Comamonas,* and *Ochrobactrum* have been identified as electroactive bacteria (Zuo *et al.*, 2008; Zhou *et al.*, 2016), their relative abundance in open circuit MFCs suggests their involvement in hydrocarbon degradation. *Rhodococcus* contains members capable of degrading aromatic hydrocarbon compounds in the presence of surfactant such as Tween 80 (Schreiberová *et al.*, 2012).

In closed circuit MFC inoculated with activated sludge (AS1) *Acidovorax, Mycobacterium,* and *Geobacter* were present at higher relative abundance compared to open circuit MFC inoculated with activated sludge (nAS2) (see Figure 6-6). At $q \leq 0.05$, two-sided Fisher's Exact t-test showed there were statistically significant differences between closed and open circuit activated sludge bioreactors in relative abundance of these genera. Interestingly, open circuit MFC had higher relative abundance of *Pseudomonas, Sphingobium* and *Dechlorosoma* than closed circuit, even though closed circuit MFC exhibited higher PAH removal (80 ± 5.6%) than open circuit MFC (62 ± 13.3 %). Some members of *Pseudomonas* and *Dechlorosoma* are putative hydrocarbon-degrading bacteria. These genera include members that are capable of electron transfer, however, their relative abundance in open circuit MFC suggests they were likely involved in hydrocarbon degradation. *Mycobacterium* has several metabolic pathways for fluoranthene degradation (Kweon *et al.*, 2007) and was enriched in closed circuit MFC inoculated with activated sludge.

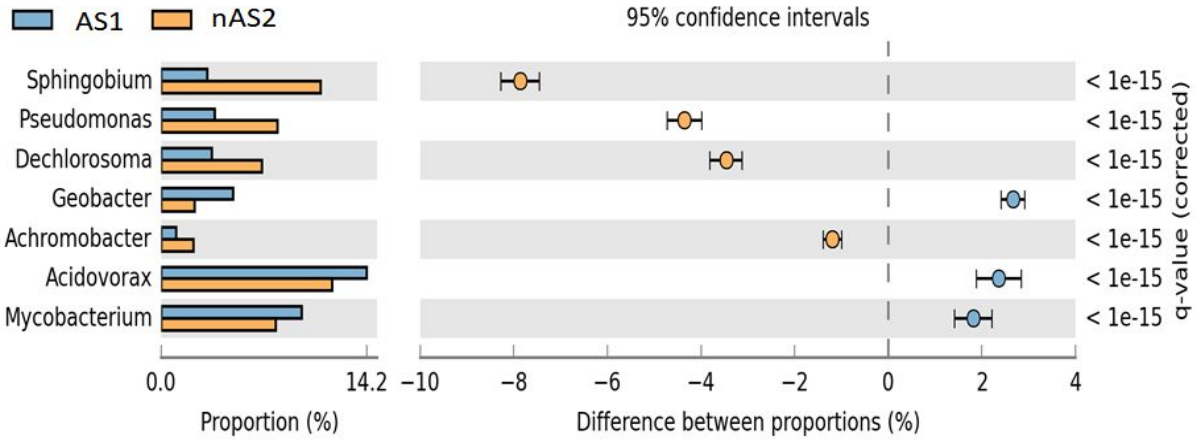


Figure 6-6. Extended error bar plot showing significant differences between bioanode of closed circuit (AS1) and bioanode of open circuit (nAS2) MFCs inoculated with activated sludge (differences shown at genus level).

Relative abundance of *Pseudomonas* was higher in closed circuit MFCs inoculated with MFC effluent (20.8 ± 7.1 %) compared with closed circuit MFCs inoculated with activated sludge

(5.9 ± 3.1 %). As seen in Figure 6-7, extended error bar plots of MFC inoculated with MFC effluent (M2) and MFC inoculated with activated sludge (AS1) showed that there was a higher number of bacteria that were significantly different in comparison to differences between closed and open circuit MFCs inoculated with either MFC effluent (see Figure 6-5) or activated sludge (see Figure 6-6). *Pseudomonas* and *Dechlorosoma* were predominantly enriched in closed circuit MFC inoculated with MFC effluent compared with closed circuit MFC inoculated with activated sludge, followed by *Acidovorax, Sphingobium, Rhodococcus,* and *Proteiniphilum. Proteiniclasticum* was significantly enriched in closed circuit MFC inoculated with activated sludge relative to closed circuit MFC inoculated with MFC effluent.

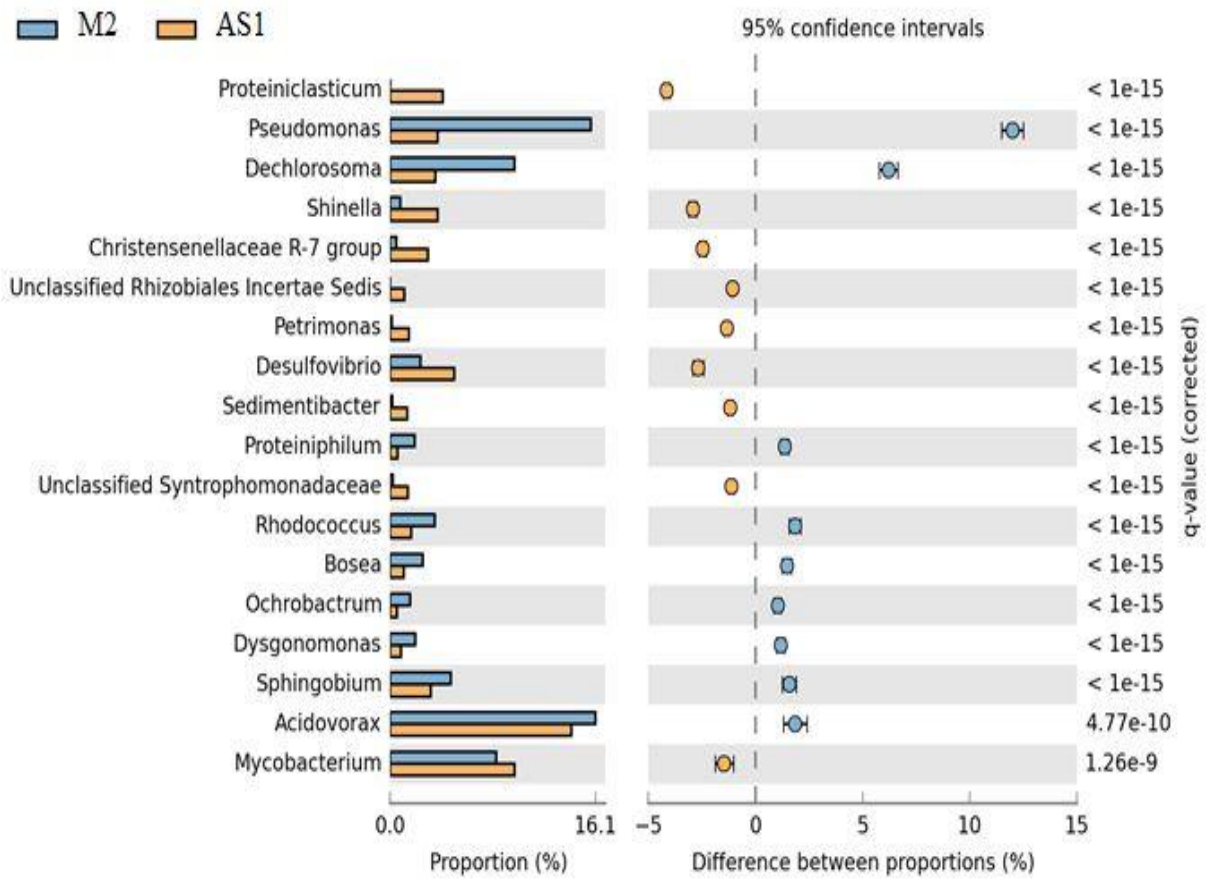


Figure 6-7. Extended error bar plot showing significant differences between bioanode of closed circuit MFCs inoculated with MFC effluent (M2) and bioanode of closed circuit MFC inoculated with activated sludge (AS1) (differences shown at genus level).

Although, there was no significant difference in the relative abundance of *Geobacter* between closed circuit MFC inoculated with MFC effluent and closed circuit MFC inoculated with activated sludge, closed circuit MFC inoculated with MFC effluent had higher abundance of genera that may include some putative electroactive bacteria than AS1 e.g. *Ochrobactrum, Dechlorosoma* and *Acidovorax* (Achenbach *et al.*, 2001). Other non-electroactive bacteria that were enriched may have been involved in various roles. *Pseudomonas* can degrade phenanthrene and fluoranthene under nitrate reducing conditions (Liang *et al.*, 2014; Zhang *et*

al., 2020b). As earlier stated, *Pseudomonas* can degrade hydrocarbons under both aerobic and anaerobic conditions (Coates *et al.*, 2001b; Morris *et al.*, 2009; Berdugo-Clavijo and Gieg, 2014; Venkidusamy *et al.*, 2016). *Rhodococcus* can generate volatile fatty acids from organic substrate degradation while *Desulfovibrio* are sulphate reducing bacteria (Hemalatha *et al.*, 2020). *Dysgonomonas* and facultative anaerobe, *Proteiniphilum*, have been linked with petroleum hydrocarbon in MFC (Li *et al.*, 2018b). Enrichment of several genera with diverse roles (including current production, sulphate reduction, hydrocarbon degradation, fermentation) pointed to possible synergistic associations within MFCs for removal of complex substrates such as PAHs. This appeared to corelate with high PAH removal in closed circuit MFC inoculated with MFC effluent (93 ± 3.2 %) and closed circuit MFC inoculated with activated sludge (80 ± 5.6 %). Even though closed circuit MFC inoculated with MFC effluent had higher relative abundance of genera with members capable of hydrocarbon degradation than AS1, there was only a marginal difference in PAH removal between closed circuit MFC inoculated with MFC effluent and closed circuit MFC inoculated with activated sludge. While sequence reads provided information on relative abundance of microbial taxa, they do not provide information on their absolute abundance. Therefore, it could not be ascertained if quantitatively, closed circuit MFC inoculated with MFC effluent had a higher number of PAH degraders compared with closed circuit MFC inoculated with activated sludge.

Proteiniclasticum is a known electroactive bacteria (Dai *et al.*, 2022). Notably, relative abundance *Proteiniclasticum* in closed circuit MFC inoculated with activated sludge (3.8 %) was significantly higher relative to closed circuit MFC inoculated with MFC effluent (0.02 %). This appeared to correlate with coulombic efficiency which was higher in closed circuit MFC inoculated with activated sludge (9.38 %) than closed circuit MFC inoculated with MFC effluent (8.61 %) by day 64 when the bioanode samples were obtained. The exact species of *Proteiniclasticum* present in this study could not be ascertained and was only identified as uncultured anaerobic bacterium.

6.3.4 Microbial communities associated with single chamber SMFC bioanode.

The microbial consortium in single chamber SMFC anodes comprised mainly bacteria (98.4 – 99.5%) with a much lower proportion of archaea (0.5 - 1.6%). Despite the similarity in alpha diversity of the single chamber SMFC bioanodes (see Table 6-2), differences were detected in bacterial community structure in SMFC bioanodes relative to the original sediment (Tyne River sediment). PCA plot at family level showed that bacterial taxa within SMFC bioanodes diverged from those found in the original sediment. Comparison of SMFC samples at family level using Kruskal Wallis H-test at $p < 0.05$ showed there was significant difference between

communities in Tyne River sediment and SMFC bioanode communities. Closed circuit SMFCs with wicking cathode (CW1, CW2) and submerged cathode (CS2) as well as one open circuit SMFC (OC1) and control (XX) formed a cluster distinct from Tyne River sediment. Also, one closed circuit duplicate with submerged cathode (CS1) and one open circuit duplicate (OC2) formed a cluster distinct from both Tyne River sediment and other SMFCs (see Figure 6-8). Observations on the PCA plot were indicative of the dynamic nature of the SMFC anodic biofilm after 35 days of operation.

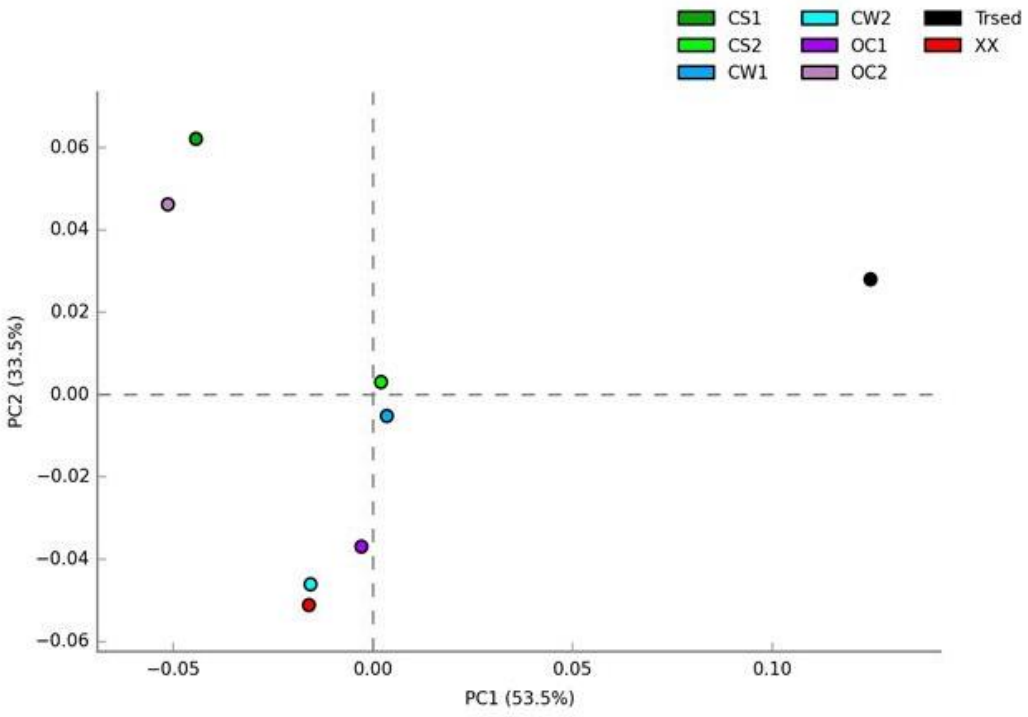


Figure 6-8. Principal Component Analysis (PCA) plot of bacterial taxa at family level in SMFCs treating hydrocarbon-contaminated sediment (Trsed = original Tyne River sediment, CW=closed circuit, wicking cathode; CS=closed circuit, submerged cathode; OC=open circuit, submerged cathode; XX= control, no electrodes).

Tyne River sediment were dominated by *Flavobacteriaceae* (10.5 %), *Sva1033* (5.4 %), *Desulfobulbaceae* (5.3%), *Woeseiaceae* (5.0 %), *Sulfurovaceae* (4.9 %), *Anaerolineaceae* (3.7 %), *Desulfobacteraceae* (3.4 %), and *Halieaceae* (2.9%). After 35 days of operation of SMFCs, relative abundance of these families decreased except for *Anaerolineaceae* which increased (9.4 – 15.3 %) in both closed and open circuit SMFCs as well as non-SMFC control (XX) (see Figure 6-9). This was an interesting observation because it constituted the most significant increase in relative abundance in relation to other families. There was also increase in relative abundance of *Bacteroidetes vadinHA17* (from 1.6 % in sediment to 1.7 to 3.9 % in SMFCs) and *Clostridiaceae 1* (from 0.8 % in sediment to 1.5 to 3.2 % in SMFCs). One closed circuit SMFC with submerged cathode (CS1) and one open circuit SMFC (OC2) were distinguished

from other SMFCs and control by the enrichment of *Rikenellaceae* (CS1= 8.1%, OC2 = 7.4 %) in these SMFCs, relative to original Tyne River sediment.

Although higher PAH removal was achieved in single chamber SMFC with wicking cathode (CW = 51 ± 3 %) compared with single chamber SMFC with submerged cathode (CS = 46 ± 2 %), total petroleum hydrocarbon (TPH) removal was similar in both SMFCs, namely, 22 ± 0.7 % for CW and 19 ± 2 % for CS. However, cumulative charge output (expressed as coulombs, C) and coulombic efficiency (%) for single chamber SMFC with wicking cathode (298 ± 140 C, 1.4 ± 0.8 %) was higher than single chamber SMFC with submerged cathode (97± 38 C, 0.5 ± 0.2 %). A comparison of all genera in microbial communities within closed circuit SMFCs with wicking cathode (CW) and submerged cathode (CS) indicated that there was no significant difference between microbial communities in these SMFCs (Fisher's Exact test, $p > 0.05$) (Appendix J). A similar observation was made when closed circuit SMFC (CW) was compared with open circuit SMFC (OC) (Fisher's Exact test, $p > 0.05$) (Appendix K). Clustering of closed circuit SMFC (CS1) with open circuit SMFC (OC2) was similar to observations reported by Zhao *et al.* (2016) from another study involving SMFCs. In the study involving removal of organic matter using SMFC, closed circuit and open circuit SMFCs formed distinct clusters when visualized using Weighted UniFrac Principal Coordinates Analysis (PCoA), with exception of one closed circuit (S1B) and one control bioreactor (C1). However, S1B and C1 did not cluster together. Zhao *et al.* (2016) reported that S1B had low bacterial diversity and consequently failed to produce power. Further discussion on clustering of CS1 and OC2 is presented later in this section. First, key features of microbial community structure in the single chamber SMFCs in this study is discussed.

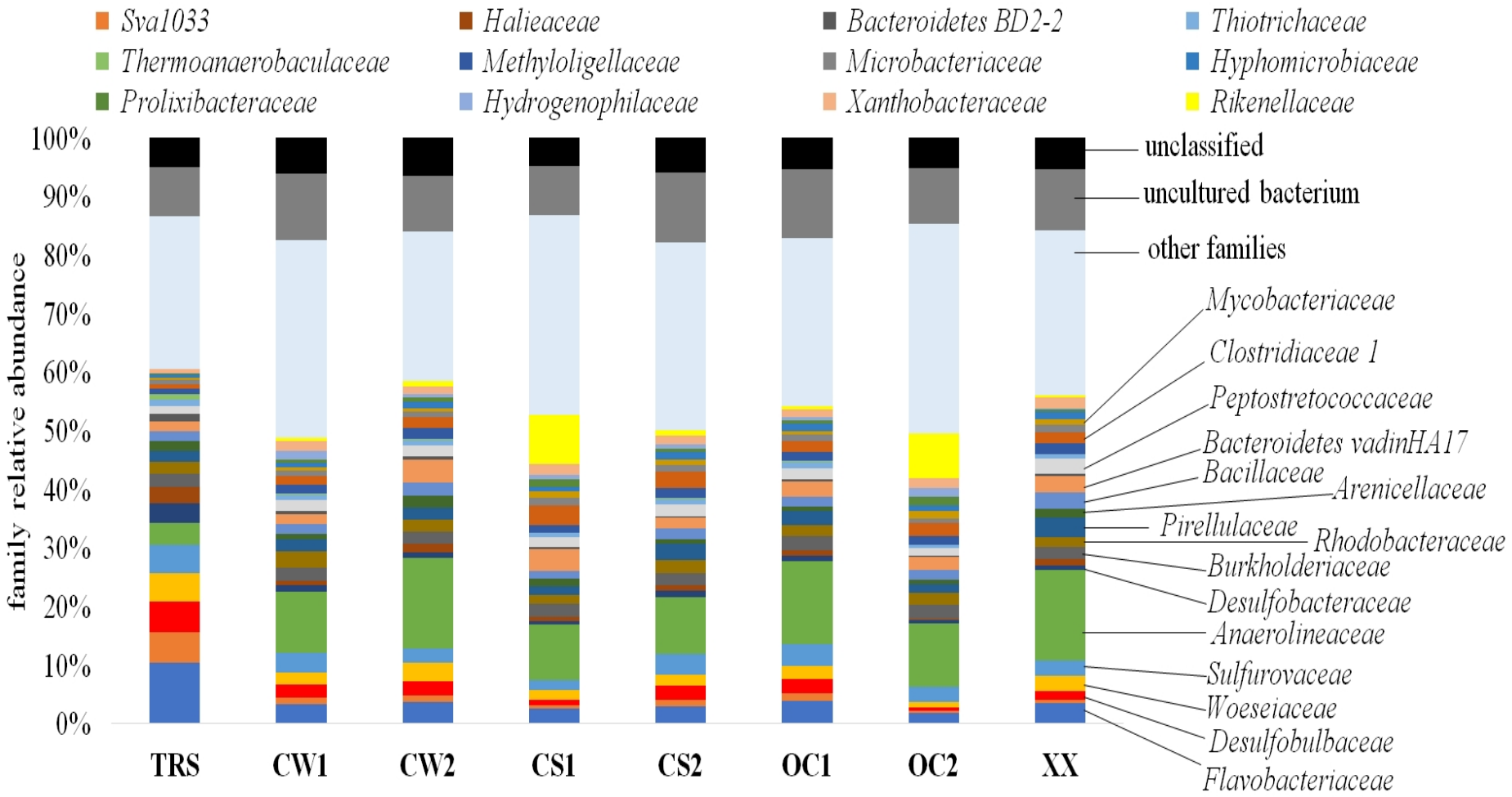


Figure 6-9. Relative abundance (> 1%) of microbial communities at family level in sediment microbial fuel cells (SMFCs) treating hydrocarbon contaminated sediment (TRS = Tyne River sediment (original sediment), CW1, CW2 = duplicate closed circuit SMFCs using wicking cathode, CS1, CS2 = duplicate closed circuit SMFCs using submerged cathode, OC1, OC2 = duplicate open circuit SMFCs using submerged cathode, XX= control, no electrodes).

Relative decrease in sulphate reducing bacteria such as *Desulfobulbaceae* and *Desulfobacteraceae* (class *Deltaproteobacteria)* was accompanied by relative increase in *Anaerolineaceae* (phylum *Chloroflexi*) and *Clostridaceae 1* (phylum *Firmicutes*). This observation was in agreement with another study involving BES where *Chloroflexi* and *Firmicutes* were enriched relative to *Deltaproteobacteria* after 175 days in electrochemical snorkels and controls containing crude oil (Viggi *et al.*, 2017), and in SMFC for bioremediation of petroleum hydrocarbon contaminated soil, operated for 64 days (Lu *et al.*, 2014a). It was also comparable with studies on non-BES oil-degrading microcosms operated for 686 days under sulphate reducing conditions (Sherry *et al.*, 2013). In other BES studies, *Deltaprotetobacteria* (44 -57%) was enriched in electrochemical oil snorkels that were operated for 417 days (Cruz Viggi *et al.*, 2015). Also, *Chloroflexi* and *Firmicutes* decreased and *Deltaproteobacteria* was enriched in SMFC after 60 days (Xu *et al.*, 2017).

Sulphate reducing bacteria such as *Desulfobulbaceae, Desulfovibrionaceae* and *Desulfobacteraceae*can have been implicated in hydrocarbon degradation in contaminated sediments (Sherry *et al.*, 2013; Daghio *et al.*, 2018a; Daghio *et al.*, 2018b). Occurrence of sulphate reducing bacteria (class: *Deltaproteobacteria*) in Tyne River sediment has been linked with syntrophic anaerobic oxidation of petroleum hydrocarbons or fermentation products with sulphate reduction, even though enrichment of SRB was modest (Sherry *et al.*, 2013; Head *et al.*, 2014). *Desulfobulbaceae* is involved in sulphur recycling during hydrocarbon degradation under sulphate reducing conditions. During hydrocarbon degradation in bio-electrochemical systems (BES), sulphate reducing bacteria (SRB) reduce sulphate to sulphide, which is then oxidized to elemental sulphur. Elemental sulphur is reduced back to sulphide by sulphur oxidizers belongings to families *Desulfobulbaceae* and *Desulfuromonadaceae* (Daghio *et al.*, 2018a). Daghio *et al.* (2018a) demonstrated that low sulphide oxidation could occur at low anode potential e.g. - 0.205 V vs Ag/AgCl (reference electrode), and in the absence of sulphide, BES current has been shown to decline after initial 7 days (Daghio *et al.*, 2018b). Peak current in closed circuit single chamber SMFCs (CW and CS) occurred on day 6/7, after which current declined until the end of the experiment. Anode potential in CW and CS increased from about – 0. 500 V vs Ag/AgCl to between - 0.225 to - 0.397 V vs Ag/AgCl on day 14 in all SMFCs except CS1. Low sulphur cycling and therefore decline in alternative electron acceptors to support hydrocarbon degradation, could have led to decline in current.

Anaerolineaceae are chemoorganotrophs found in anaerobic sludge, marine sediment, and deep terrestrial aquifers. Members of this family are strict fermentative anaerobes and have been linked with degradation of iso-alkanes under methanogenic conditions (Abu Laban *et al.*, 2015). Members of the family *Anaerolineaceae* have also been found in BES using PAHs

(naphthalene, phenanthrene and pyrene) as substrates (Zhou *et al.*, 2020). In a biochar-amended soil MFC using aged petroleum hydrocarbon contaminated soil, relative abundance of *Anaerolineaceae* increased by 20 -91% relative to the unamended soil (Li *et al.*, 2019). Increase in *Anaerolineaceae* in oil-degrading microcosms relative to control microcosms was observed by day 302 after sulphate was depleted (Sherry *et al.*, 2013). It is postulated that selective enrichment of *Anaerolineaceae* over sulphate reducing bacteria in this study was induced by reduction in sulphate, likely due to sulphate consumption by sulphate reducing bacteria for growth and maintenance. So far, this study is the only study that have shown significant enrichment of *Anaerolineaceae* (145 to 317% increase) in single chamber SMFCs (with wicking cathode configuration) used for hydrocarbon removal relative to original sediment.

Clostridium has been linked with biodegradation of petroleum hydrocarbons under anaerobic conditions (Jain *et al.*, 2016; Li *et al.*, 2018b). Fermentative bacteria such as *Clostridiaceae* can oxidize aromatic hydrocarbon or hydrocarbon intermediates coupled with iron or thiosulphate reduction (Wiegel, 2015; Sherry *et al.*, 2020). *Clostridiaceae* has also been linked in particular with methanogenic hydrocarbon degradation (Gieg *et al.*, 2008). In anoxic sediments, terminal electron acceptors are selected by microbes based on their reduction potential. Terminal electron acceptors with higher reduction potentials are utilized first (Daghio *et al.*, 2017). Therefore, microbial consortia capable of hydrocarbon-degradation under methanogenic conditions could be selected under sulphate-depleted conditions. Hydrocarbon degradation under methanogenic conditions is much slower than under sulphate-reducing conditions (Aitken *et al.*, 2013), hence rate of generation of metabolic intermediates could have declined. Consequently, the substrate limitation for electroactive bacteria would have led to low current output. Enrichment of known fermenters *Anaerolineaceae* and *Clostridiaceae* supports likelihood of fermentation as the primary means of hydrocarbon degradation in the SMFCs in this study and correlates with low coulombic efficiency (< 2 %) obtained in all closed circuit SMFCs.

Clustering of communities from closed circuit SMFC, CS1 and open circuit SMFC, OC2 could be attributed to a distinct feature of these two SMFCs, initially observed at class level. Closed circuit SMFC, CS1 and open circuit SMFC, OC2 had the highest relative abundance of phylum *Bacteroidia* (18 ± 2 %) and family *Clostridia* (12 ± 2 %) compared with other SMFCs (7 ± 0.8 %, 8 ± 2 %, respectively). Also, the lowest relative abundance of *Deltaproteobacteria* (4 ± 0.2 %) and *Anaerolineae* (11 ± 1 %) compared to other SMFCs (7 ± 0.8 %, 14 ± 3 %, respectively) was observed in closed circuit SMFC, CS1 and open circuit SMFC, OC2. At family level, *Rikenellaceae* (class *Bacteroidia*) was selectively enriched in only in CS1 (8%) and OC2 (7.3%). Some members of this family are reported to be anaerobes that can ferment

carbohydrates to acetate and hydrogen (Su *et al.*, 2014; Ali *et al.*, 2022). In one study, this family was more dominant on bioanode of SMFC control (Ali *et al.*, 2022). This could have been one reason why closed circuit SMFC, CS1 exhibited poor performance with charge output (69.49 C).

Extended error bar plots of closed circuit (CS1) and open circuit (OC2) SMFCs showed that there were some bacteria that were significantly different between CS1 and OC2 at genus level (see Figure 6-10). Unclassified *Bacteroidetes vadinHA17*, *Proteiniclasticum*, *ADurb.Bin120* and unclassified *Clostridiales* were significantly enriched in closed circuit SMFC, CS1. Some members of *Proteiniclasticum* and *Clostridiales* are electroactive bacteria (Miyahara *et al.*, 2013; Dai *et al.*, 2022). No electric current was generated in open circuit SMFC, OC2. Closed circuit SMFC, CS1 was the lowest performing closed circuit SMFCs, exhibiting cumulative charge output and coulombic efficiency of 69.49 C and 0.34 % compared with other closed circuit SMFCs (CS2 = 123.86 C, 0.60 %; CW1 = 198.63 C, 0.96 %; CW2 = 396.72 C, 1.92 %).

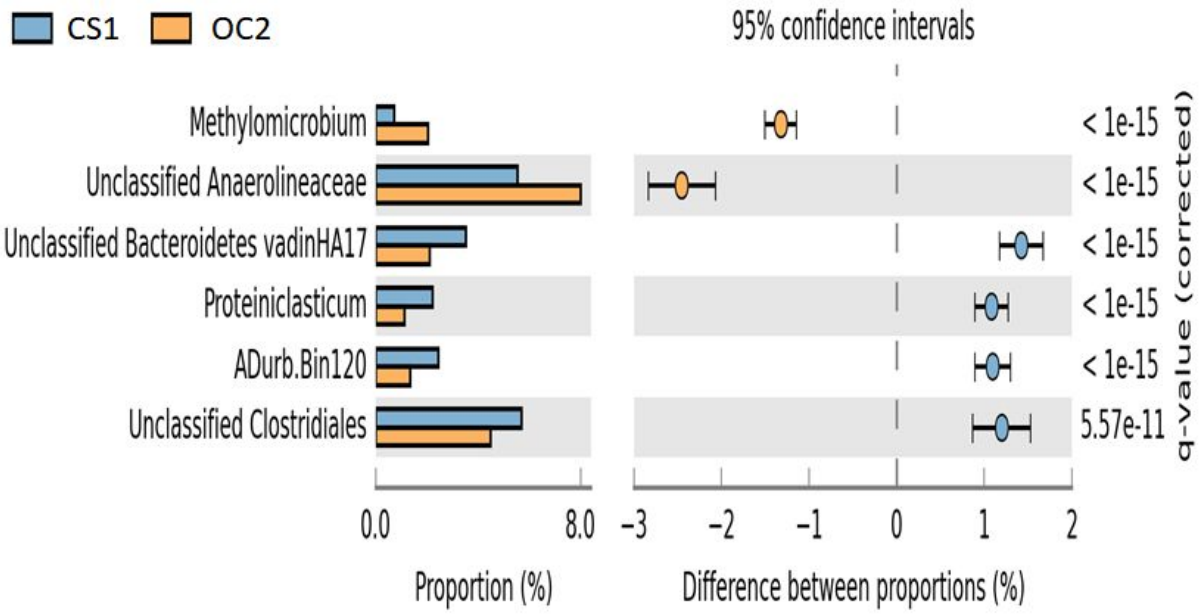


Figure 6-10. Extended error bar plot showing significant differences between bioanode of closed circuit SMFC (CS1) and bioanode of open circuit SMFC (OC2) used for treatment of hydrocarbon contaminated sediment (differences shown at genus level).

6.3.5 Archaeal Community Structure

Only 2.1 % relative abundance was observed in activated sludge inoculum. In MFCs treating hydrocarbon wastewater, Archaea was found in low relative abundance (0.01 – 0.03 %), namely, *Methanosarcinaceae* (open circuit MFC inoculated with MFC effluent), *Methanobacteriaceae* (closed circuit MFC inoculated with activated sludge) and both *Methanobacteriaceae* and *Methanoregulaceae* (open circuit MFC inoculated with activated sludge). Archaea communities in the single chamber SMFCs ranged between 0.45 – 1.57 %.

Archaeal communities at family level in closed circuit single chamber SMFCs (CW1, CW2, CS1 and CS2) were dominated by hydrogenotrophic *Methanobacteriaceae* (14.8 ± 5.0%), acetoclastic *Methanosarcinaceae* (14.1 ± 1.3%), and acetoclastic *Methanosaetaceae* (6.8 ± 1.4%) (see Figure 6-11). Archaeal communities at family level in open circuit single chamber SMFCs (OC1 and OC2) were dominated by *Methanosarcinaceae* (18.8 ± 4.4 %), *Methanobacteriaceae* (12.4 ± 1.1 %), and *Methanosaetaceae* (12.2 ± 0.2 %). Less than 4 % relative abundance of *Nitrososphaeraceae* was observed in both closed and open circuit single chamber SMFCs.

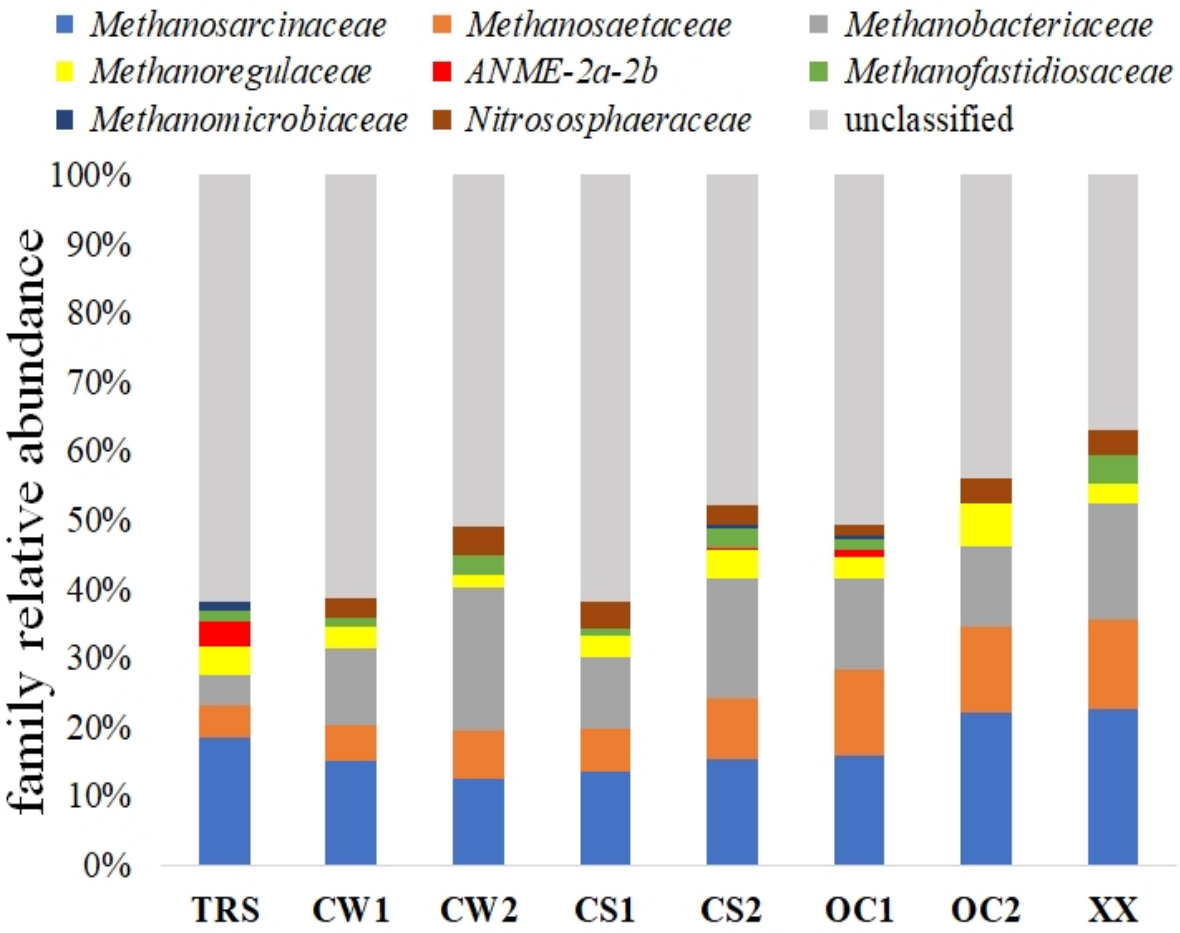


Figure 6-11. Relative abundance (> 1%) of archaeal communities at family level in sediment microbial fuel cells (SMFCs) treating hydrocarbon contaminated sediment (TRS = Tyne River sediment (original sediment), CW1, CW2 = duplicate closed circuit SMFCs using wicking cathode, CS1, CS2 = duplicate closed circuit SMFCs using submerged cathode, OC1, OC2 = duplicate open circuit SMFCs using submerged cathode, XX= control, no electrodes).

Low abundance of archaea corresponded with similar findings in electrochemical snorkels where relative abundance of archaea communities in ranged from 0.5 – 2.2 % (Röling *et al.*, 2004; Viggi *et al.*, 2017). Both acetoclastic and hydrogenotrophic methanogens can occur in syntrophic association with anaerobic hydrocarbon degradation (Wei *et al.*, 2015). Acetoclastic and hydrogenotrophic methanogens use acetate and hydrogen as electron acceptors,

respectively. These methanogens compete with electroactive bacteria for electron donors such as acetate, thereby reducing electron recovery and coulombic efficiency. In this study, low relative abundance of archaea implied that they did not play significant role in SMFCs.

6.4 Conclusion

Microbial community composition on microbial fuel cells (MFC) and sediment microbial fuel cells (SMFC) bioanodes were investigated to understand the connection between anodic biofilm microbial structure and MFC/SMFC performance, in terms of current output and hydrocarbon removal in selected MFCs and SMFCs in this study. As expected, microbial community on MFC/SMFC bioanodes were distinct from microbial community in original inoculum or sediment. Similar bacterial classes (*Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*) were represented in bioanode samples, yet individual members of these classes found in both MFCs and SC SMFCs were distinct. MFC bioanodes comprised members of groups with potentially diverse roles within the bioreactors. Electroactive bacteria *Acidovorax,* and *Geobacter* were enriched in closed circuit MFCs. Facultative anaerobe *Pseudomonas* was enriched in all MFCs. Some members of *Pseudomonas* are capable of hydrocarbon degradation. Closed circuit MFC using activated sludge was enriched with electroactive *Proteiniclasticum* relative to closed circuit MFC inoculated with MFC effluent. Highly diverse but evenly distributed taxa were found on SMFC bioanode microbial community. Putative fermentative anaerobe *Anaerolineaceae* was significantly enriched in all SMFCs relative to other families. Other enriched taxa included fermentative *Clostridiaceae 1*. Enrichment of anaerobic fermenters correlated with low coulombic efficiency (< 2 %) obtained in all closed circuit SMFCs. As postulated in chapters 5, fermentation appeared to be the primary means of hydrocarbon degradation in the single chamber SMFCs in this study. Non-performing closed and open circuit SMFCs were enriched with microbes that are known to use metabolites from hydrocarbon degradation. Microbial diversity seen in MFC/SMFC was indicative of potential diverse roles played by different members of the community and their synergistic interactions during degradation of complex substrates such as petroleum hydrocarbon.

Chapter 7 Conclusion and Future work

The aim of the research project was to develop and optimize performance of microbial fuel cells (MFCs) for bioremediation of water and soil/sediment contaminated with petroleum hydrocarbons. The research objectives were to:

i. Apply selected inocula to enrich and develop active anodic biofilms able to degrade petroleum hydrocarbons.

ii. Design and investigate different bioreactor configurations for biodegradation of petroleum hydrocarbons.

iii. Characterise microbial community and their changes on bioanode in MFC/SMFC for bioremediation of petroleum hydrocarbons.

7.1 Conclusion

In chapter 4, hydrocarbon removal from synthetic hydrocarbon wastewater was studied. Double chamber microbial fuel cells (MFC) were used investigated the influence of inoculum source and external resistance on MFC anodic biofilm development, current output, and polyaromatic hydrocarbons (PAH) (phenanthrene and fluoranthene) removal efficiency in the presence of anionic surfactant (polyoxyethylene (20) sorbitan monooleate - Tween80) (0.1 g/L or 100 ppm). Anodic biofilms developed by activated sludge and MFC effluent inocula were adaptable to low concentrations of Tween 80. Remarkably, this study showed that inoculum source influenced current density and charge output in MFCs at start-up. MFCs inoculated with activated sludge started up faster and produced significantly higher current output (AS = 59.44 ± 0.98 mA/m^2) during stage 1 feeding cycle (100 ppm Tween 80 only) compared with MFCs inoculated with MFC effluent (M = 1.65 ± 0.03 mA/m^2). This effect was diminished during stage 3 feeding cycle (100 ppm Tween 80 + 100 ppm PAH) where both bioreactors displayed similar current output (AS = 30.41 ± 0.46 mA/m^2; M = 29.93 ± 0.37 mA/m^2). Current output increased in all MFCs when external resistance was lowered from 1000 Ω to 100 Ω. High external resistance resulted in diversion of electrons from PAH oxidation to fermentation and hydrolysis, with no significant different in PAH removal between closed circuit and open circuit MFCs. During stage 2 feeding cycle (100 ppm Tween 80 + 50 ppm PAH), higher electrochemical PAH removal and coulombic efficiency (CE) was achieved in MFCs inoculated with activated sludge with low external resistance (PAH removal = 84 ± 3 %; CE = 27.5 ± 2.9 %) than with high external resistance (PAH removal = 72 ± 5 %; CE = 14.8 ± 2.6 %). Active anodic biofilms with higher bio-electrocatalytic activity were established under low

external resistance. Results presented in this chapter indicate that electrochemical oxidation of PAH from hydrocarbon contaminated wastewater was marginally improved under low external resistance, and that bioanodes of MFCs inoculated with activated sludge (AS) displayed higher bio-electrocatalytic activity.

The aim of experiments discussed in chapter 5 was to evaluate performance of double and single chamber sediment microbial fuel cell (SMFC) with vertical electrode arrangement for removal of petroleum hydrocarbon from contaminated sediment. For the single chamber SMFCs, two cathode configurations (submerged cathode vs wicking cathode) were evaluated to trade-off between oxygen and ion transport on both current output and hydrocarbon removal. For double chamber SMFCs, marginally higher PAH removal in open circuit cells (43 ± 2 %) compared with closed circuit cells (34 ± 9 %) was indicative of possible oxygen ingress into the system. Current density and charge output in single chamber SMFC with vertical electrode arrangement was 3 times higher using wicking cathode, CW (166 ± 78 mA/m^2, 298 ± 140 C) compared with submerged cathode, CS (54 ± 21 mA/m^2, 97 ± 38 C). However, similar PAH removal efficiencies were obtained in these single chamber SMFCs: CW (51 ± 3 %) and CS (46 ± 2 %). This study showed that microbial electrochemical oxidation of PAH occurred in the single chamber SMFCs, albeit to a limited degree as characterized by low coulombic efficiencies (< 2 %) across the single chamber SMFCs. In this study, SMFCs stimulated removal of PAH over alkanes during treatment of hydrocarbon-contaminated sediment and wicking cathode was shown to enhance current/charge output. Nonetheless, cyclic voltammetry showed that biotransformation of petroleum hydrocarbons by fermentative and hydrolytic microbial consortia played a significant role in generation of electron donors used by electroactive bacteria in SMFC for current production.

The discourse of chapter 6 was microbial community structure of bioanodes of double chamber microbial fuel cells (MFC) (under low external resistance) and single chamber sediment microbial fuel cells (SMFC) (vertical electrode configuration) used for treatment of hydrocarbon contaminated wastewater and sediment, respectively. The aim of the chapter was to understand the connection between anodic biofilm microbial structure and MFC/SMFC performance, in terms of current output and hydrocarbon removal. Microbial communities found in the bioreactors were significantly different from inoculum or initial sediment used to start up the bioreactors. Though both MFCs and SMFCs were dominated by *Proteobacteria, Actinobacteria*, *Firmicutes* and *Bacteroidetes,* MFC and SMFC clusters were distinct and indicated that different genera within these larger groups were responsible for current output and hydrocarbon degradation. Putative electroactive bacteria (EAB) *Acidovorax,*

Dechlorosoma, and *Geobacter* were enriched in closed circuit MFCs while *Pseudomonas, Comamonas, Mycobacterium,* and *Sphingomonas* were enriched in open circuit MFCs. Some members of the enriched genera in open circuit MFCs are hydrocarbon-degraders. A significant finding in the study was the presence of putative electroactive bacteria such as *Pseudomonas* in both closed and open circuit MFCs, which was indicative of its versatile role in MFCs. This finding showed that bacterial enrichment in each MFC was linked to their specific function in the bioreactor. Significant enrichment of EAB *Proteiniclasticum* in MFC inoculated with activated sludge in comparison to MFC inoculated with MFC effluent corelated with higher coulombic efficiency seen in MFC inoculated with activated sludge. Highly diverse but evenly distributed taxa were found on SMFC bioanode microbial community. SMFCs were dominated by *Actinobacteria, Chloroflexi*, *Firmicutes* and *Proteobacteria*. *Proteobacteria* was not enriched relative to other phyla. Putative fermentative anaerobes belonging to families *Anaerolineaceae, Bacteroidetes vadinHA17,* and *Clostridiaceae 1* were enriched relative to other families. This supported claims in previous chapter 5, of fermentation as the primary means of hydrocarbon degradation in the SMFCs in this study and correlated with low coulombic efficiency (< 2 %) obtained in all closed circuit SMFCs. Another new finding in this study was significant enrichment (145 to 317 %) of putative fermentative anaerobe *Anaerolineaceae* in single chamber SMFCs within 35 days of operation. Non-performing closed and open circuit SMFCs were enriched with microbes capable of utilizing metabolic products of hydrocarbon degradation. In summary, microbial diversity seen in MFC and single chamber SMFC was indicative of potentially diverse roles played by different members of the community and importance of syntrophic interactions during degradation of complex substrates such as petroleum hydrocarbons.

7.2 Future Work

In this study, bioanodes of microbial fuel cells (MFCs) inoculated with activated sludge (undefined mixed culture) were demonstrated to have higher bio-electrocatalytic activity than bioanodes of MFCs inoculated with effluent from an acetate fed MFC (defined mixed culture). This resulted in higher current output and marginal improvement in electrochemical oxidation of polyaromatic hydrocarbons (PAH) from hydrocarbon contaminated wastewater in MFCs inoculated with activated sludge. Although, PAH accumulation was observed in one closed circuit double chamber SMFCs, marginal improvement in electrochemical oxidation of PAH was seen in closed circuit single chamber sediment microbial fuel cell (SMFC) using wicking cathode configuration. Nevertheless, several limitations were observed that require further investigation to substantiate results obtained in this study:

i. This study did not consider if there was a correlation between microbial population (number of bacteria) in each inoculum sample and current output or contaminant utilization (PAH removal). Future work will need to consider inclusion of microbial count for incoula samples. In addition, using inocula with same number of bacteria per inoculum would provide clarification on whether the effect seen was due to composition of inoculum or due to number of bacteria in the inoculum;

ii. As seen from microbial community analysis, MFC effluent had low microbial diversity in comparison with activated sludge inoculum or with bioanode of an acetate fed MFC from another study. This suggested that inoculum obtained directly from MFC anode would likely have higher diversity and support better performance than MFC effluent. For future work, MFC anode inoculum is recommended for use rather than MFC effluent inoculum;

iii. MFC effluent inoculum was obtained from MFC bioreactor that was fed solely on acetate (a simple substrate with known concentration) whereas activated sludge was obtained from a wastewater treatment plant (containing a mixture of complex substrates/organic matter). It was likely that differences in performance based on inoculum could have been due to composition of inoculum derived from the parent bioreactor. Future work will be needed to compare inoculum with similar composition but obtained from different sources. For example, comparison between activated sludge inoculum (natural source) and inoculum from bioanode of hydrocarbon fed MFC (artificial source). Tyne river sediment, which was proven to contain hydrocarbon-degrading microbes, could also be used as natural source of inoculum;

iv. Change in current output, charge output and organic carbon removal was seen across the 3 feeding stages as concentration of total organic carbon increased (Stage 1: 100ppm Tween 80 only. Stage 2; 100 ppm Tween 80 + 50 ppm PAH. Stage 3: 100 ppm Tween 80 + 100 ppm PAH). However, microbial community analysis only captured in community in MFC effluent and activated sludge inocula before addition to the MFCs and bioanode community at the end of stage 3 feeding cycle. Future work on microbial community dynamics of MFC anodic biofilm with change on feeding cycle would provide better understanding of bacteria adaptation to nature and concentration of organic substrate throughout duration of the wastewater treatment;

v. Bioanode samples for microbial community analysis was limited to the top of the anode. Different bacteria may occur at different length of the vertically configured anode. Future work could be done on stratification of bioanode samples (top, middle and

bottom) to compare change in bioanode microbial community with bioanode stratum; and,

vi. PAH accumulation seen in one closed circuit double chamber SMFC could be further investigated to pin-point reasons for accumulation. To investigate PAH accumulation, the method for PAH analysis could be adjusted to include the following considerations: determine biomass content of sample to distinguish between carbon content from hydrocarbon and non-hydrocarbon source; use selective ion scan mode (SIM) to trace identity and concentration of specific PAHs before and after sample treatment; determination of initial and final concentration of all fractions of petroleum hydrocarbon, namely, aliphatics, aromatics, NSO (nitrogen-sulfur-oxygen) and resins/asphaltene fractions.

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Chapter 8 APPENDICES

Appendix A Heat of combustion for petroleum hydrocarbons

Heats of combustion (- ΔH_c°)for n-alkanes was calculated using ideal combustion reaction (1) and Prosen &Rossini formula (2) shown below and as described in Sagadeev and Sagadeev (2006) and Audran *et al.* (2018).

1. $C_nH_{2n+2} + (1.5n + 0.5)\ O_2 \longrightarrow n CO_2 + (n + 1)\ H_2O + \Delta H_c^\circ$
2. Prosen & Rossini formula: $-\Delta H_c^\circ$ (kJ/mol, 25°C) = 658.74*n* + 242.29

A1. Calculated heats of combustion for n-alkanes

n-alkanes	number of carbon atoms	heat of combustion (calculated) $-\Delta H_c^\circ$ (kJ/mol)
C10	10	6829.69
C11	11	7488.43
C12	12	8147.17
C13	13	8805.91
C14	14	9464.65
C15	15	10123.39
C16	16	10782.13
C17	17	11440.87
C18	18	12099.61
C19	19	12758.35
C20	20	13417.09
C21	21	14075.83
C22	22	14734.57
C23	23	15393.31
C24	24	16052.05
C25	25	16710.79
C26	26	17369.53
C27	27	18028.27
C28	28	18687.01
C29	29	19345.75
C30	30	20004.49
C31	31	20663.23
C32	32	21321.97
C33	33	21980.71
C34	34	22639.45
C35	35	23298.19

A2. Calculated heats of combustion for polycyclic aromatic hydrocarbons.

polycyclic aromatic hydrocarbon (PAH)	molecular weight	number of carbon atoms	number of carbon rings	extractable electrons* (theoretical)	heat of combustion (calculated) $-\Delta H_c^\circ$ (kJ/mol)
naphthalene	128	10	2	48	5077.92
acenaphthene	152	12	3	58	6150.67
fluorene	166	13	3	62	6544.13
phenanthrene	178	14	3	66	6937.59
anthracene	178	14	3	66	6937.59
fluoranthene	202	16	4	74	7724.51
pyrene	202	16	4	74	7724.51
benzo[a]anthracene	228	18	4	84	8797.26
chrysene	228	18	4	84	8797.26
benzo[b]fluoranthene	252	20	5	92	9584.18
benzo[k]fluoranthene	252	20	5	92	9584.18
benzo[e]pyrene	252	20	5	92	9584.18
benzo[a]pyrene	252	20	5	92	9584.18
dibenzo[a,h]anthracene	278	22	5	102	10656.93
benzo[g,h,i]perylene	276	22	6	100	10371.10
indeno[1,2,3-c,d]pyrene	276	22	6	100	10371.10

*extractable electrons were calculated based on complete oxidation of PAHs as described in Ruscic *et al.* (2004); Roux *et al.* (2008) and Tsai *et al.* (2009).

Appendix B Composition and preparation of Wolfe's mineral solution (1 litre)

$MgSO_4 \cdot 7H_2O$ ---------- 3.00 g
Nitrilotriacetic acid ---------- 1.50 g
NaCl ---------- 1.00 g
$MnSO_4 \cdot H_2O$ ---------- 0.50 g
$CaCl_2$ ---------- 0.10 g
$CoCl_2 \cdot 6H_2O$ ---------- 0.10 g
$FeSO_4 \cdot 6H_2O$ ---------- 0.10 g
$ZnSO_4 \cdot 7H_2O$ ---------- 0.10 g
$AlK(SO_4)_2 \cdot 12H_2O$ ---------- 0.10 g
$CuSO_4 \cdot 5H_2O$ ---------- 0.10 g
H_3BO_3 ---------- 0.10 g
$Na_2MoO_4 \cdot 2H_2O$ ---------- 0.10 g
Na_2SeO_3 ---------- 0.10 g
$NaWO_4 \cdot 2H_2O$ ---------- 0.10 g
$NiCl_2 \cdot 6H_2O$ ---------- 0.10 g

Wolfe's mineral solution was prepared by dissolving 1.5 g nitroloacetic acid in 500 ml of deionized water. Potassium hydroxide (KOH) was used to adjust the pH of the solution to 6.5. The remaining components were added, and the solution made up to 1,000 ml (1 litre) with deionized water.

Appendix C Composition and preparation of Wolfe's vitamin solution (1 litre)

Pyridoxine HCL ---------- 10.0 mg
p-Aminobenzoic acid ---------- 5.0 mg
Lipoic acid ---------- 5.0 mg
Nicotinic acid ---------- 5.0 mg
Riboflavin ---------- 5.0 mg
Thiamine HCL ---------- 5.0 mg
Calcuim DL-pantothenate ---------- 5.0 mg
Biotin ---------- 2.0 mg
Folic acid ---------- 2.0 mg
Vitamin B12 ---------- 0.1 mg

Wolfe's vitamin solution was prepared by adding all components to a beaker containing 800ml of deionized water. All components were mixed to dissolve completely and the solution made up to 1,000 ml (1 litre) with deionized water.

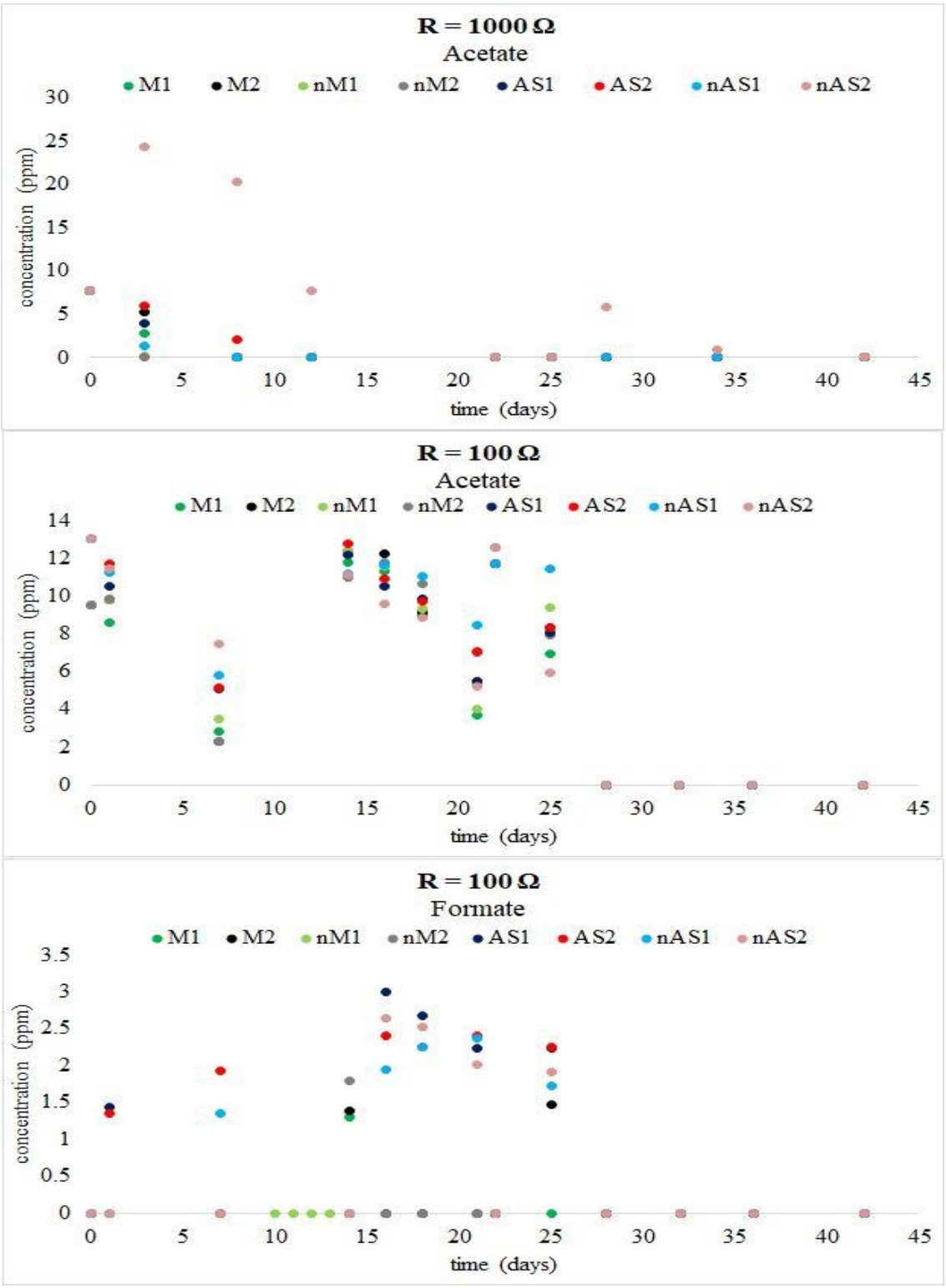
Appendix D Sample and Standard Preparation for Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

Volumetric glassware was soaked in dilute nitric acid overnight to prevent contamination of standards and samples. Standards were prepared from purchased 1000 ppm stock solutions. A blank (deionized water) and 5 standards (0.1, 1, 10, 50, 100 ppm) were used to produce a calibration curve for the ICP-OES machine. The concentrations of the standards were kept close to the expected concentration of the element in the sample. Final dilutions of sample and standards was kept below 5% acid concentration. The metals analysed, wavelengths used and calibration concentration are show in Table D1 below:

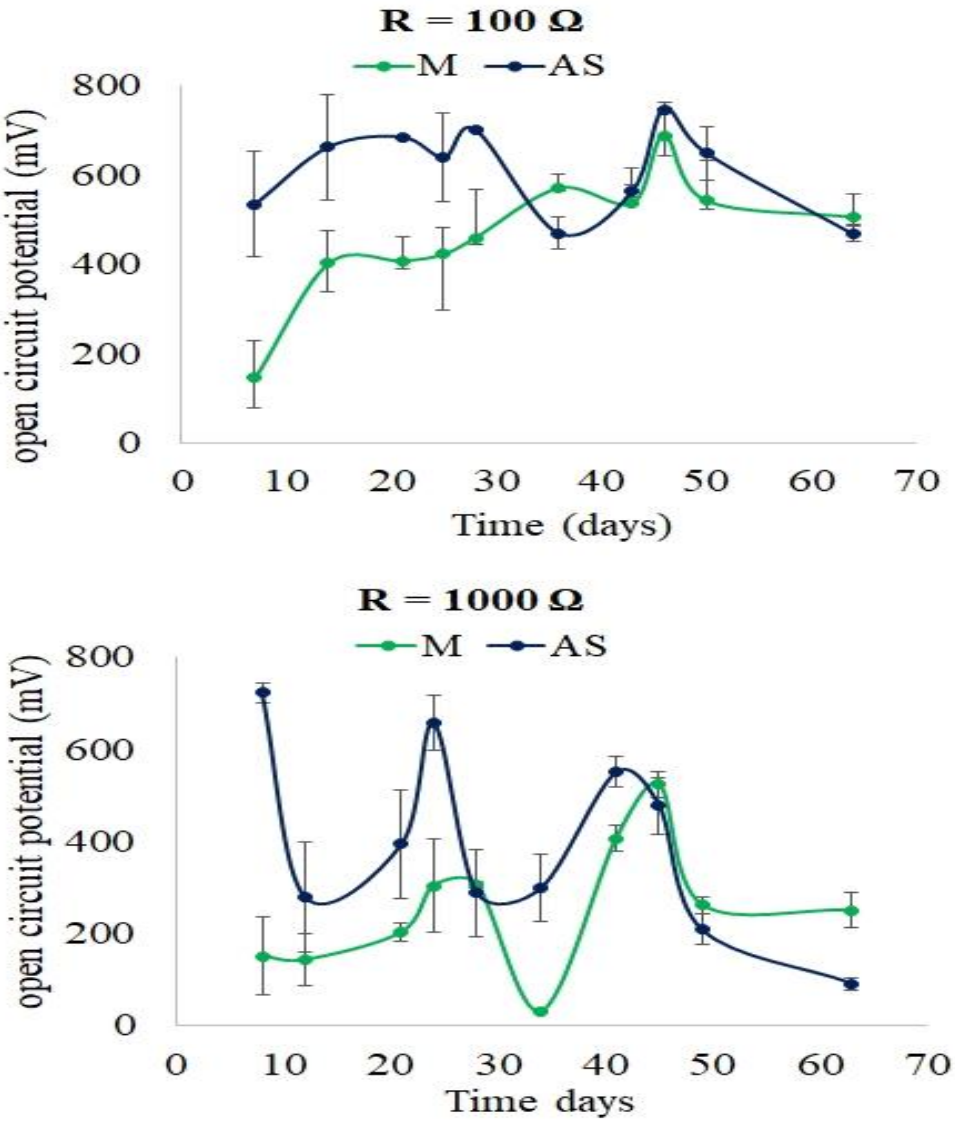
D1. Wavelengths and calibration concentrations for analysis of metals in Tyne River water.

element	suggested wavelength nm	alternative wavelength nm	detection level mg/L	calibration concn mg/L	upper concn limit mg/L
Zn	213.86	206.2	0.002	5	100
Pb	220.35	217	0.04	10	100
Fe	259.94	238.2	0.007	10	100
Mg	279.08	279.55	0.03	10	100
Ca	317.93	315.89	0.01	10	100
Na	589	589.59	0.05	10	100
K	766.49	769.9	0.1	10	100
concn = concentration					

Appendix E Volatile fatty acid production in double chamber microbial fuel cells used for hydrocarbon removal from wastewater under high (R=1000 Ω) and low external resistance (R=100 Ω) (M = inoculated with effluent of pre-existing MFC, AS = inoculated with activated sludge, nM and nAS = open circuit cells for M and AS, respectively).

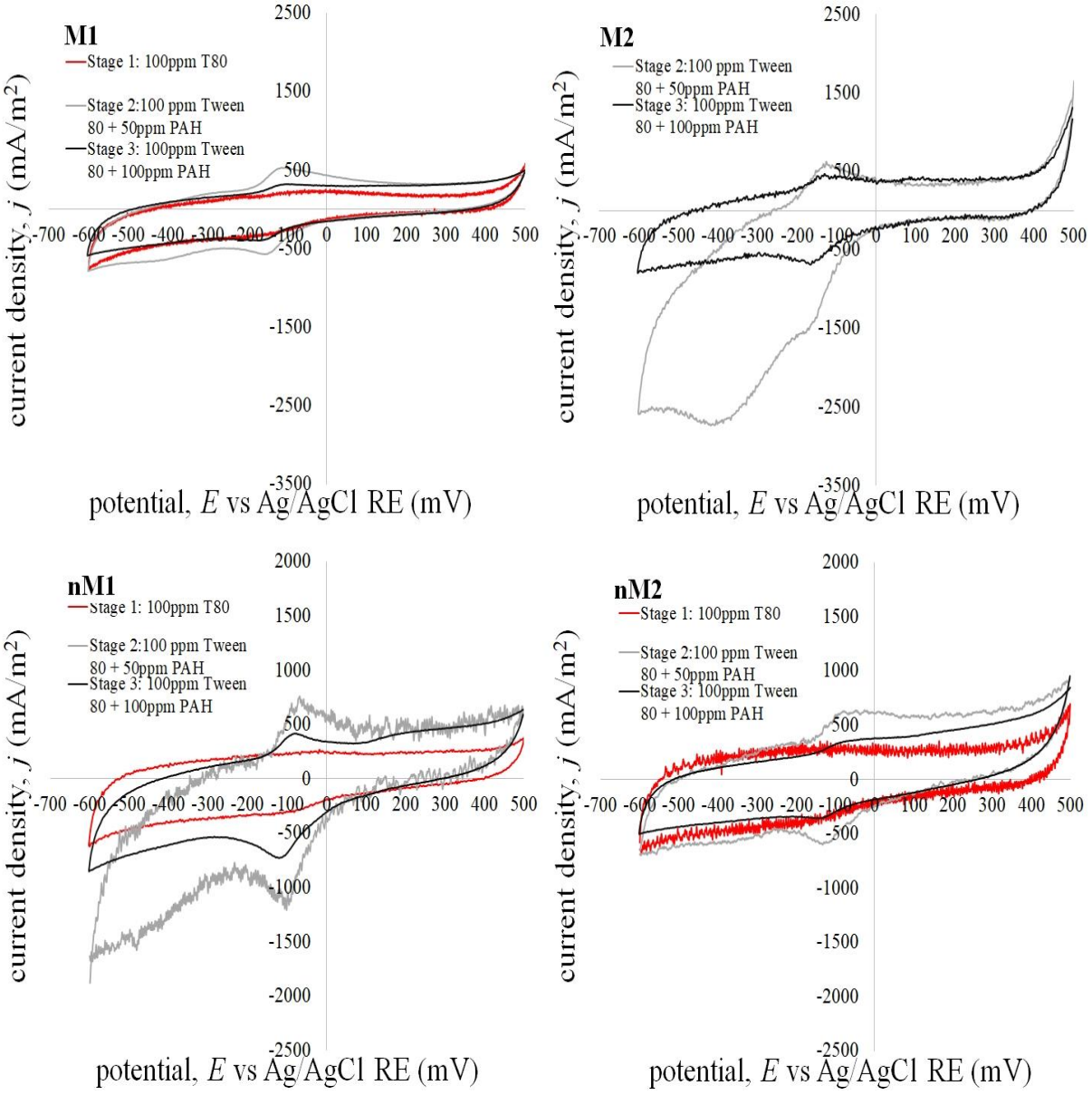


Appendix F Change in open circuit potential over time in double chamber microbial fuel cells used for hydrocarbon removal from wastewater using two different external resistances, R_{ext}: 1000 Ω and 100 Ω (M = inoculated with effluent from pre-existing MFC, AS = inoculated with activated sludge).

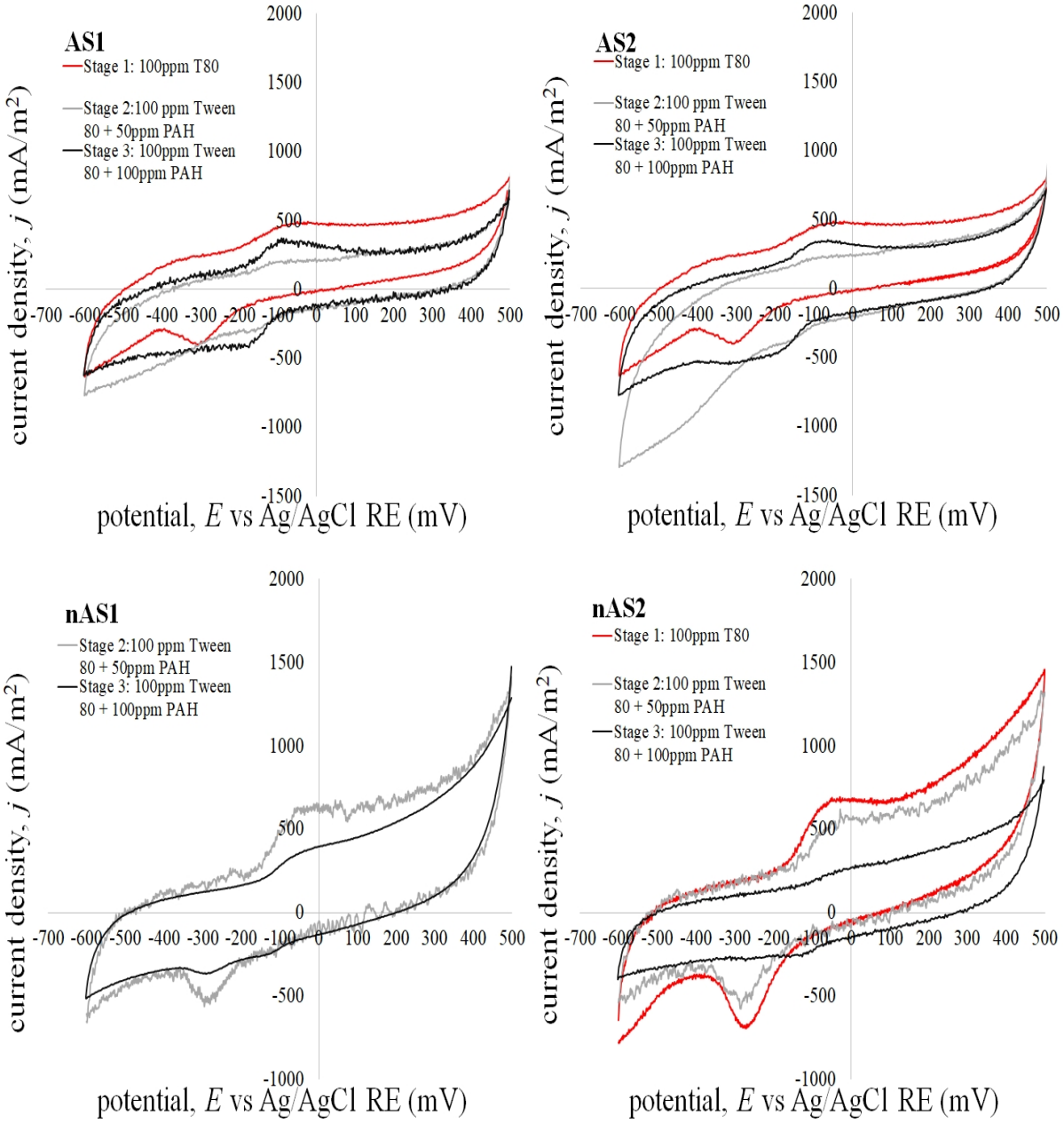


Appendix G Evolution of cyclic voltammograms of anodic biofilms in double chamber microbial fuel cells at closed (M and AS) and open circuits (nM and nAS) used for treatment of hydrocarbon contaminated wastewater at external resistance R_{ext} = 1000 Ω (scan rate = 0.001 Vs^{-1}, temperature 30℃ , M and nM inoculated with effluent from pre-existing MFC; AS and nAS inoculated with activated sludge).

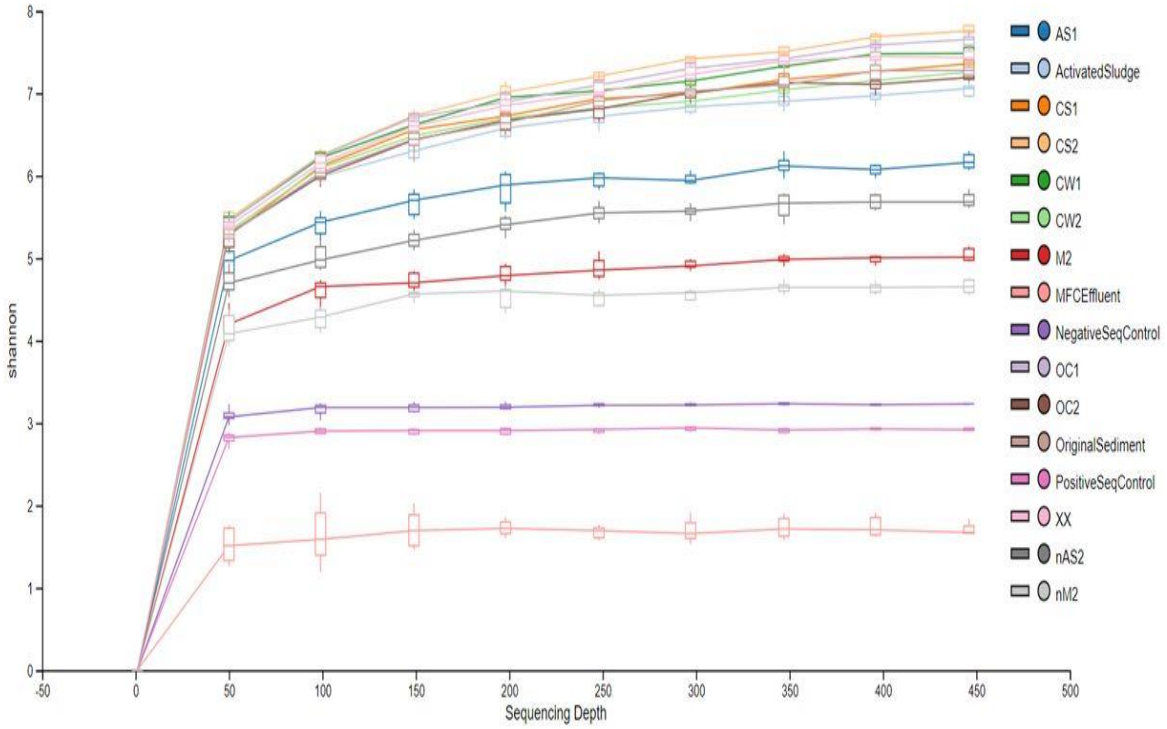
G1. Microbial fuel cells inoculated with MFC effluent from pre-existing acetate fed MFC (scan rate = 0.001 Vs^{-1}).



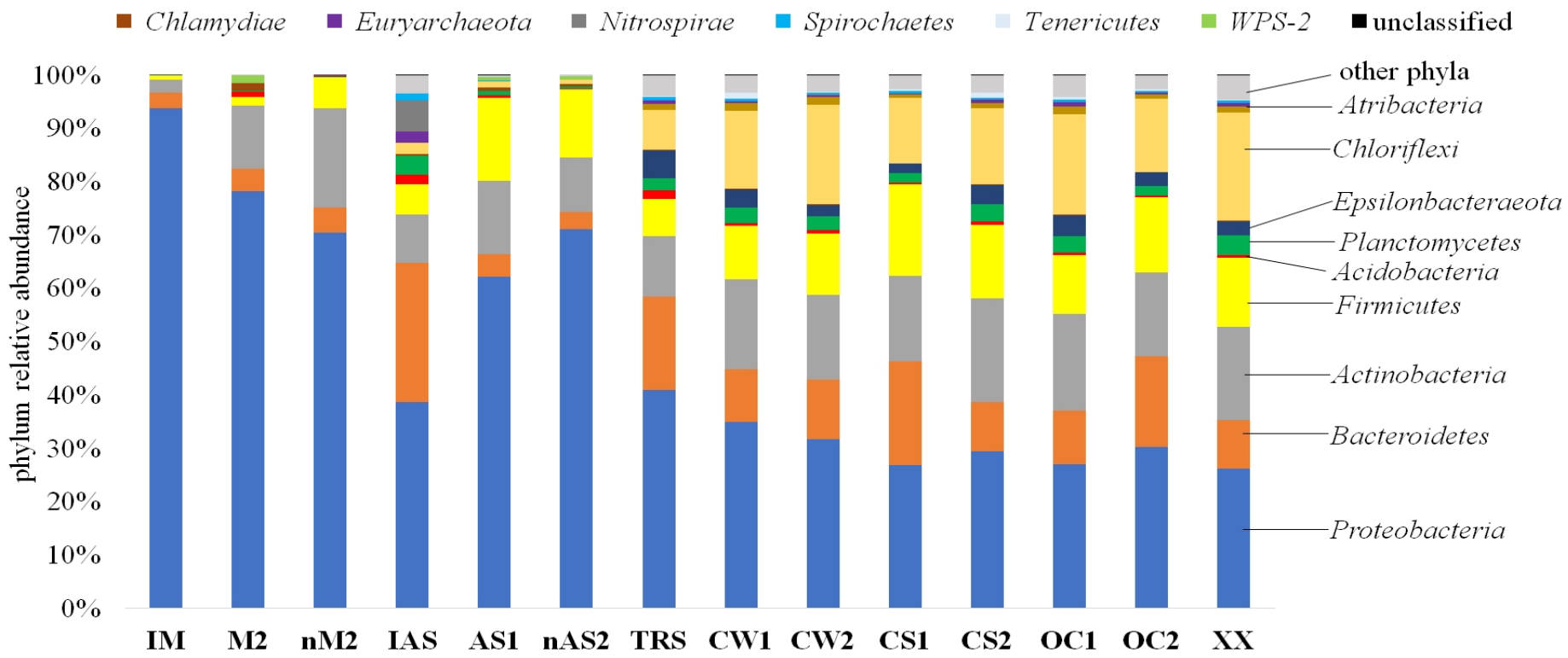
G2. Microbial fuel cells inoculated with activated sludge (scan rate = 0.001 Vs^{-1}).



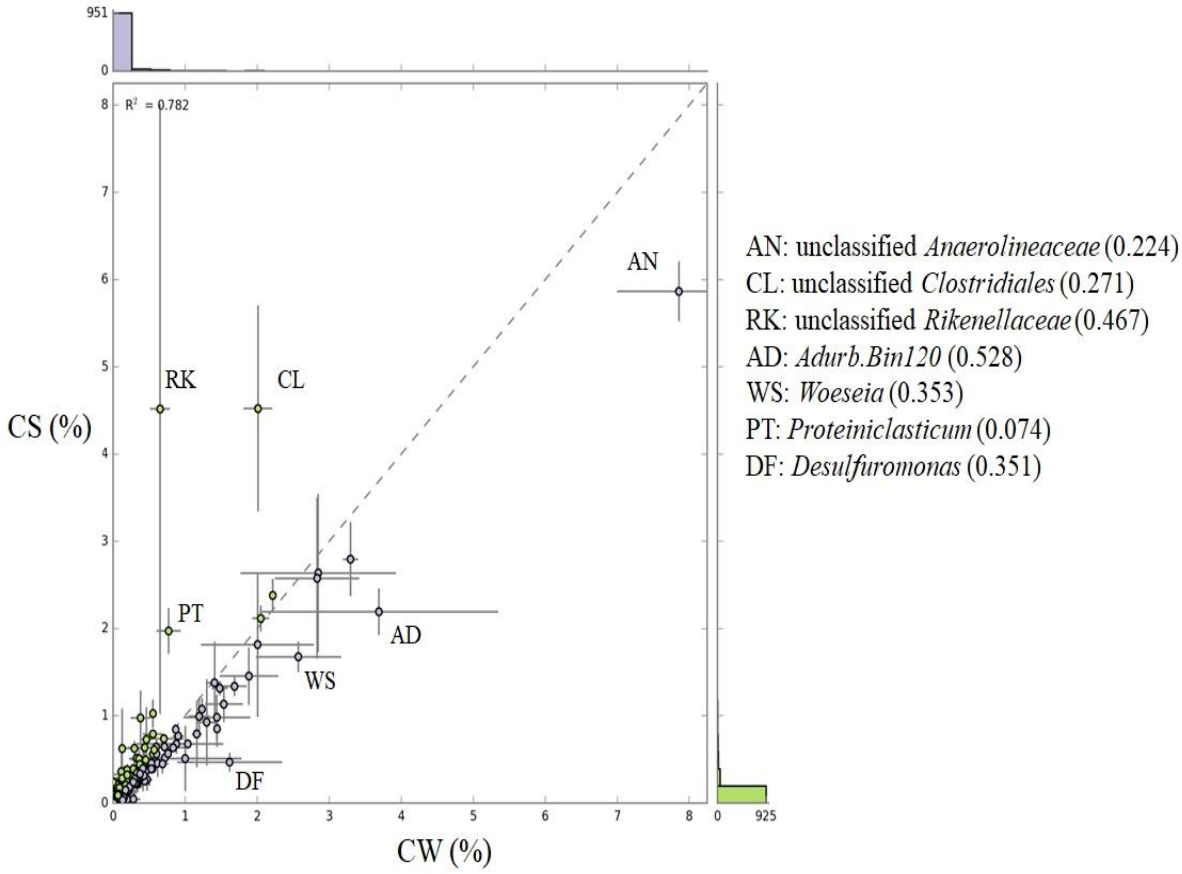
Appendix H Alpha rarefaction curve based on pyrosequencing of bacterial sequences for anodic biofilms of microbial fuels cells and sediment microbial fuels cells treating hydrocarbon contaminated wastewater and sediment.



Appendix I Relative abundance of microbial phyla in microbial communities in bio-electrochemical systems used for removal of petroleum hydrocarbons from contaminated wastewater and sediment (> 1% abundance at phylum level) (IM = MFC effluent inoculum, M2 = closed circuit MFC inoculated with MFC effluent, nM2 = open circuit MFC inoculated with MFC effluent, IAS = Activated Sludge inoculum, AS1= closed circuit MFC inoculated with activated sludge, nAS2 = open circuit MFC inoculated with activated sludge. TRS = Tyne River sediment (original sediment), CW1, CW2 = duplicate closed circuit SMFCs using wicking cathode, CS1, CS2 = duplicate closed circuit SMFCs using submerged cathode, OC1, OC2 = duplicate open circuit SMFCs using submerged cathode, XX= control, no electrodes).

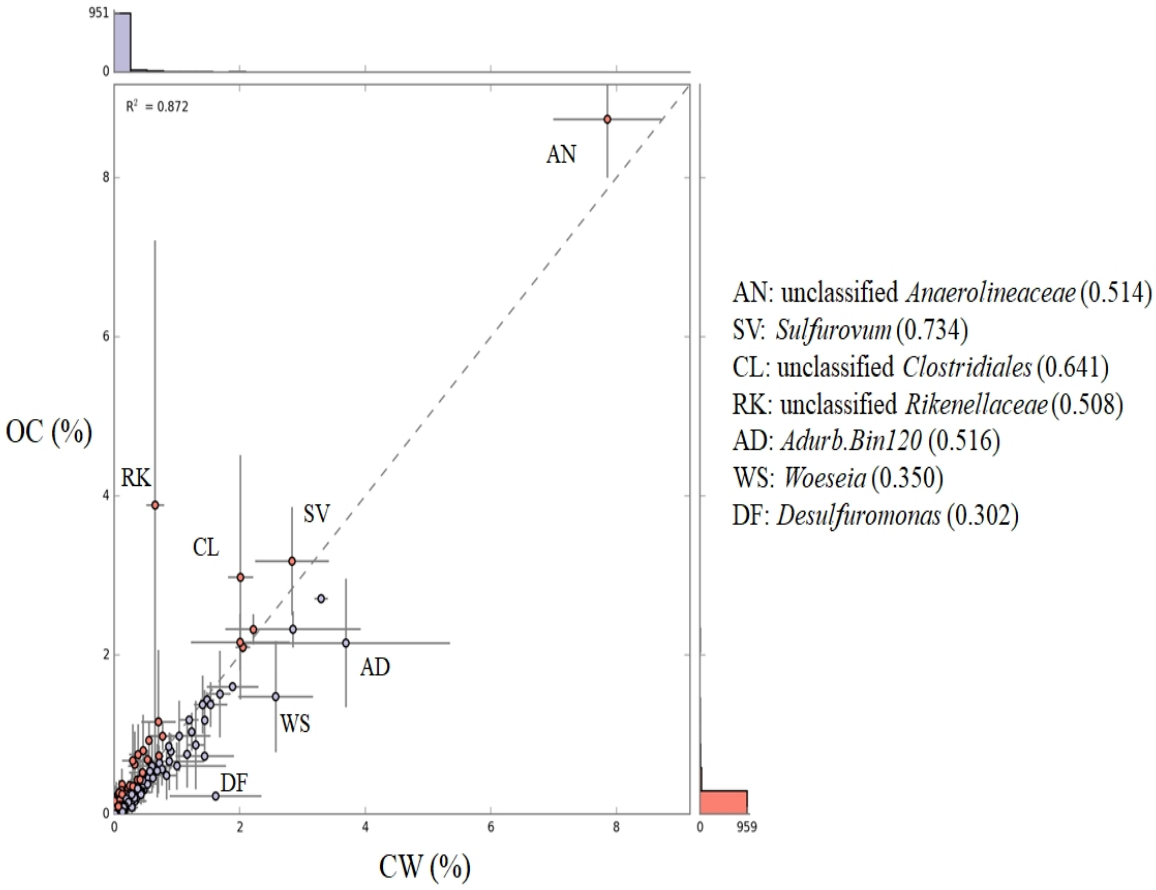


Appendix J Profile scatter plot showing the relative proportion of all genera of microbial communities within closed circuit single chamber sediment microbial fuel cells with wicking (CW) and submerged cathode (CS) (R^2 = 0.782)*.



*(p-values based on two-sided Wech's t-test are indicated in brackets besides each family).

Appendix K Profile scatter plot showing the relative proportion of all genera of microbial communities within closed circuit single chamber sediment microbial fuel cells with wicking cathode (CW) and open circuit single chamber microbial fuel cells with wicking cathode (OC) (R^2 = 0.872)*.



*(p-values based on two-sided Wech's t-test are indicated in brackets besides each family).