Waste Stabilization Pond Ecology -A Molecular Approach

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Abstract

Waste Stabilization Ponds (WSPs) are used to treat wastewater largely in developing countries, though their ecology is not well understood. Past studies have used taxonomic microscopy methods to assess the photosynthetic organisms vital for WSPs functioning. There has been little use of molecular methods based on evolutionary classification in this field. This thesis sets out to develop molecular methods to study the ecology of these systems.

Efficient, non-biased DNA extraction is vital for reliable molecular analysis. Commercially available DNA extraction kits were tested for efficiency when used on WSP samples. Qiagen's Blood and Tissue kit was recommended for use.

The proportion of non-photosynthetic to photosynthetic organisms making up the WSP community was investigated. Fluorescence in Situ Hybridisation (FISH) and flow cytometry based methods were devised to this end. FISH had low efficiency due to variable algal cell wall permeability. Flow cytometry proved to be an effective way to sort photosynthetic organisms from non-photosynthetic, though fixation of samples reduced efficiency.

Flow cytometric counting and a PCR and DGGE approach optimised to assess microalgae and cyanobacteria were used in two case studies. The first showed a significant difference between the community found in two pond systems in Brazil, one fed with domestic wastewater and the other with mixed industrial - domestic wastewater. The second assessed the effects of engineered baffles on communities in facultative ponds (in Colombia) across the diurnal cycle. The baffled pond had lower diversity, but more of the species identified were photosynthetic.

The PCR-DGGE based method was compared to traditional microscopy techniques with the help of a taxonomic specialist. Little agreement between the methods was seen at species level. The molecular analysis, including the primers chosen and the available database sequences favoured the Chlorophyceae and the cyanobacteria, the microscopy methods favoured the larger Euglenophyceae and other microalgae with morphologically distinct characteristics.

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Abbreviations

ANOSIM- Analysis of Similarity

ANOVA- Analysis of Variance

AS- Activated Sludge

BLAST- Basic Local Alignment Search Tool

BOD- Biochemical Oxygen Demand

CAGECE- Campanhia de Água e Esgoto do Ceará (Ceará state water company, Brazil)

CCAP- Culture Collection of Algae and Protozoa- The Scottish Association for Marine Science, Oban, Scotland

CEG- School of Civil Engineering and Geosciences, Newcastle University

CFD- Computational Fluid Dynamics

CINARA- Research and development institute in water supply, environmental sanitation and water resources conservation, Cali, Colombia

CLSM- Confocal Laser Scanning Microscope

COD- Chemical Oxygen Demand

CRC-Energy Efficiency Scheme- Carbon Reduction Commitment Energy Efficiency Scheme

DGGE- Denaturing Gradient Gel Electrophoresis

DNA- Deoxyribonucleic acid

DO- Dissolved oxygen

FACS- Fluorescence Activated Cell Sorting

FCS- Forward Scatter

FISH- Fluorescence In Situ Hybridisation

HRAP- High-Rate Algal Pond

HRT- Hydraulic Retention Time

LSU- Large SubUnit

MAST- Department of Marine Science and Technology, Newcastle University

MDS- Multi-Dimensional Scaling

MLSS- Mixed liquor suspended solids

MR-Marechal Randon Treatment Plant (Brazil)

NCBI- National Centre for Biotechnology Information, US National Library of Medicine, Maryland, USA

OTU- Operational Taxonomic Unit

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

q-PCR- Real time Polymerase Chain Reaction

PFA- Paraformaldehyde

QBT- Qiagen's Blood and Tissue Kit

QPM- Qiagen's Plant Mini Kit

rRNA- Ribosomal ribonucleic acid

RuBisCO- Ribulose- 1,5, bisphosphate carboxylase oxygenase

SIDI- Treatment plant in Fortaleza, Brazil

SSC- Side Scatter

SSU- Small SubUnit

TKN- Total Kjeldahl Nitrogen

TSS- Total Suspended Solids

TS- Total Solids

UC- MoBio's UltraClean DNA extraction kit

WSP- Waste Stabilization Pond

Culture Collections

ACOI- Coimbra Collection of Algae- Coimbra, Portugal

CCALA- Culture Collection of Autotrophic organisms, Academy of Sciences of the Czech Republic

CCAP- Culture Collection of Algae and Protozoa- The Scottish Association for Marine Science, Oban, Scotland

FDCC- Freshwater Diatom Collection- formerly Loras College, now part of UTEX

IAM Culture Collection at the Institute of Molecular and Cellular Biosciences, the University of Tokyo, Japan

KMMCC- Korea Marine Microalgae Culture Collection

MCC-Culture Collection Melkonian, Botanical Institute, University of Cologne, Germany

NCMA- Pravasoli-Guillard National Centre for Marine Algae and Microbiota- Maine, USA, formerly CCMP

NIES- Microbial Culture Collection- National Institute of Environmental Sciences, Tsukuba, Japan

SAG- Sammlung von Algenkulturen- Georg-August Universität, Gottingen, Germany

UTEX- The Culture Collection of Algae- University of Texas, at Austin

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Chapter 1. Introduction

1.1 Scope and Aims of the study

The efficient treatment of wastewater has long been seen as important by those concerned with public health and environmental issues. Currently in the UK systems that treat the wastewater to the required discharge standard in the fastest time and have the smallest land requirements are the most common. However in recent years economic and environmental issues have come to the fore and alternatives to the traditional energy expensive methods have become the focus of research. Waste Stabilization Ponds (WSPs) are a low energy alternative primarily used in developing countries, which if optimised may have a larger place in the UK water industry. There were just 39 WSP systems reported in 2003 (Abis and Mara, 2003), treating wastewater from between 3-1,000 people.

WSPs are biological treatment systems, relying on communities of bacteria and algae for effective wastewater treatment. In facultative ponds microalgae produce oxygen through photosynthesis, allowing aerobic bacteria to remove BOD. In return bacteria provide carbon dioxide to the algae as a carbon source for photosynthesis. The main mechanism of nitrogen removal in maturation ponds is biological uptake into algae and the sedimentation of this biomass (Camargo Valero *et al.*, 2009a), though additional mechanisms such as nitrification and denitrification occur more when algal biomass levels are lower (Camargo Valero *et al.*, 2009b). The importance of photosynthetic organisms to the treatment process has been clearly highlighted, but there are relatively few studies on the ecology of WSP systems.

Ecological surveys of WSPs have been carried out using traditional taxonomic methods, based on microscopy. There are limitations of such techniques, in terms of time and the extensive taxonomic knowledge of the target organisms required. Molecular biology methods, now common place in the analysis of bacterial wastewater treatment systems, have been used to provide insight into WSP ecology in only a very limited number of studies, (Yu and Mohn, 2001; Moura *et al.*, 2009; Belila *et al.*, 2012). Camargo Valero *et al.* (2009b) used Denaturing Gradient Gel Electrophoresis (DGGE) to detect and identify bacteria associated with nitrogen cycling. Ghosh and Love (2011) focused on photosynthetic organisms, by targeting a gene encoding part of the RuBisCO enzyme (Ribulose Bisphosphate Carboxilase Oxygenase), responsible for carbon dioxide

fixation. In this study they detected greater species diversity than has been previously estimated in microscopy studies.

The aim of this thesis is to develop a molecular biology based procedure to assess algal ecology in mixed cultures with application in wastewater bioremediation using WSPs.

1.1.1 Objectives

The objectives of the thesis are to;

- Recommend a suitable DNA extraction method for WSP researchers, by testing commercially available kits on WSP samples and pure microalgal cultures (Chapter 3)
- Optimise DGGE methods for the detection of eukaryotic microalgae and cyanobacteria
- Devise a method to quantify the proportions of eukaryotic microalgae, cyanobacteria and non-photosynthetic organisms within a WSP sample (Chapters 4 and 5)
- Devise a method for the separation and concentration of photosynthetic organisms in a WSP sample to improve community assessment (Chapter 5)
- Compare traditional taxonomic methods to optimised PCR-DGGE based molecular methods for a range of WSP samples. This was done in collaboration with a microalgal taxonomy specialist, Victor Ceron, from CINARA, Universidad del Valle, Cali, Colombia (Chapter 6).
- Use the devised methods in two case studies to assess both method performance and the effects of pond conditions on the algal community detected (Chapter 7).
    - Case study 1 will compare two treatment plants in Ceará, Northeast Brazil, one treating industrial and the other domestic wastewater.
    - Case study 2 will assess the effects of baffles on facultative pond communities over the course of a day, and with varying depth, in Ginebra, Colombia.

1.2 Waste Stabilization Ponds

This chapter aims to review the key literature on WSP ecology and understanding.

WSP systems provide a cost effective and low energy wastewater treatment method. They are used around the world due to their simple construction from readily available materials and low maintenance requirements. They are common in developing countries and in countries were land is readily available, such as the USA, Australia and central Europe. The systems are composed of a series of earth-bottomed ponds, through which wastewater passes at a slow rate, see photograph in Figure 1-1.

Figure 1-1 CerritoWSP system in Colombia, the focus of Chapter 6 (photograp Victor Ceron).

Traditionally, WSP systems have an anaerobic pond, followed by a secondary facultative pond and then a number of maturation ponds (Figure 1-2). The facultative pond in Figure 1-2 is classed as 'secondary' as it receives wastewater that has already undergone some treatment (the anaerobic pond). Facultative ponds are also sometimes used to receive raw untreated wastewater; these are referred to as primary facultative ponds. Anaerobic ponds contain no dissolved oxygen due to their high organic loading

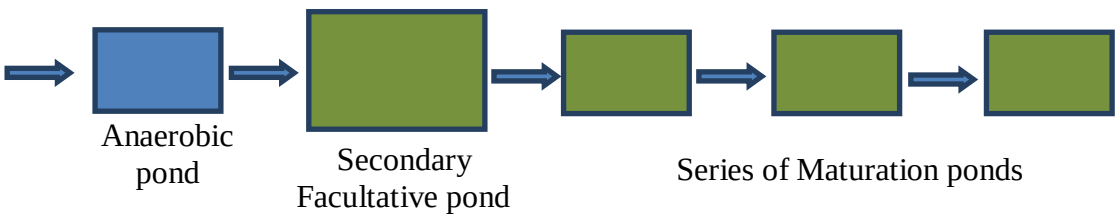


Figure 1-2 The traditional format of a WSP system.
This is not to scale. Green represents expected presence of photosynthetic organisms within ponds.

rates. Anaerobic bacteria work to reduce the BOD content of the water and the pond acts as a settling tank to remove large solids and further BOD.

Facultative ponds either receive raw wastewater or anaerobic pond effluent. They are designed to be stratified with an aerobic surface layer supporting photosynthetic algae and cyanobacteria. Photosynthetic organisms release oxygen into the pond for facultative bacteria to use for BOD removal (Mara, 1997). Facultative ponds also have an anaerobic layer at the bottom created by sedimentation of biomass, where further breakdown occurs. Some nitrogen (N) and phosphorus (P) removal can take place in facultative ponds.

The final ponds in the treatment system are the maturation ponds. These ponds are rich in algae and are thus well oxygenated. It is in these ponds that the majority of N and P removal takes place. The main reduction in faecal coliforms and pathogenic bacteria occur at this stage of the treatment process, though some will already have been removed in the facultative pond. Faecal bacteria removal occurs at high pHs, of over 9 occurring due to the high dissolved oxygen levels generated by algal photosynthesis outweighing bacterial respiration during daylight hours (Curtis and Mara, 1994). Maturation ponds are designed to be shallow for high levels of algal growth. The decreased depth promotes light penetration and both visible light (Curtis *et al.*, 1992) and UV light aid the removal of faecal bacteria and pathogens (Mara, 1997).

Over time there have been a number of adapted systems built based on traditional WSP treatment. Advanced pond systems often include High Rate Algal Ponds (HRAP). HRAPs are designed to increase the algal concentration within the pond. They are typically ‘raceway’ shaped ponds with paddlewheel mixing and are shallower than other pond types (typically between 0.2 and 0.8 meters deep). The algae photosynthesise in the shallow water where light can readily penetrate. Increased photosynthetic rates result in increased oxygen concentrations, used by bacteria for BOD removal. The algae also remove N and P which they require for the production of amino acids, DNA and other metabolic products. HRAPs have been shown to improve effluent quality and reduce its variability, when used in sequence with a facultative pond, the HRAP, and algal settling pond and a maturation pond, compared to conventional WSP systems (Craggs *et al.*, 2003). The algal biomass produced in the high rate pond can then be settled out to remove the N and P (Oswald and Asce, 1990) and providing a potentially

useful by-product. HRAPs have also been adapted, using selective biomass recycling to promote the growth of highly settleable algal species (Park *et al.*, 2011a).

In the UK the use of WSPs is restricted to rural sites treating wastewater from small communities, due to their large land area requirement. They are more common in mainland Europe, particularly in France and Germany, where land is more readily available. WSPs are also more uncommon in the UK as the rate of removal of BOD and nutrients is lower in temperate regions than in hotter and sunnier climates. Abis and Mara (2003) showed that the nutrient removal of a pilot scale facultative pond in the UK varied depending on the season and that the algal population was not maintained over the winter months. However, Johnson *et al.* (2007) suggest that systems such as maturation ponds, rock filters and reed beds can function at low temperatures. Optimisation based on a clear understanding of the microbial processes involved could be used to reduce the land requirements and make WSPs a more viable option for the UK.

1.3 Why are they interesting and important?

Despite the low uptake of this technology in the UK it has huge potential and benefits that if optimised could vastly improve the sustainability of the UK water industry.

1.3.1 Energy and carbon neutral/negative wastewater treatment?

WSP systems are highly sustainable systems requiring little energy input and with many potential energy outputs. Conventional wastewater treatment plants require energy for mechanical aeration, production and application of chemicals for disinfection and for sludge treatment. WSP systems in contrast rely on photosynthetic organisms powered by sunlight to produce the oxygen required by aerobic bacteria to breakdown organic matter. Disinfection in WSP systems is also achieved passively by sun light penetration, with no chemical or energy requirements. The energy and carbon required for the construction of WSP systems is also minimal in comparison to other treatment methods, as WSP systems are often unlined ponds and require very little concrete or specialist parts. WSPs also have low levels of sludge production, though large quantities of algal biomass are produced. All these factors keep the cost of starting up and running WSP systems low. Sato *et al.* (2007) found that WSPs cost less per unit volume in India than a UASB treatment system. The majority of the costs associated with the WSP were for

the initial land purchase and manpower costs. Repair costs and electricity costs were minimal.

Traditionally the algal biomass in WSPs leaves the system in the effluent (Figure 1-3). In order for WSPs to be used to meet UK discharge standards for sensitive receiving waters and to be able to convert the biomass into an energy source the biomass would need to be harvested before discharge. There are many harvesting mechanisms, as outlined by Christenson and Sims (2011). Harvesting adds to the energy, cost and maintenance requirements of treatment, but could be lucrative if biofuels or other energy products were produced from the biomass.

Figure 1-3 A photograph of effluent containing algal biomass leaving the Marechal Randon WSP system in Ceará, Brazil.
(Photograph taken by Lucy Eland)

There are a number of ways in which biomass can be reused and transformed. Algal biomass can be used as a fertiliser or soil amendment (Benemann *et al.*, 2003), for feed stock or be further processed into bio oil (Craggs *et al.*, 2012) and biogas. Anaerobic digestion of algal biomass can be carried out on pond algae to produce methane (Salerno *et al.*, 2009). Biodiesels can also be produced from microalgae, as many

species are rich in lipids. This area of research has focused predominantly on microalgae cultured in media with added nutrients, though this is not economically viable for fuel production and economically is better suited to high value products, such as carotenoids and aquaculture feedstock (Borowitzka, 1992; Sun *et al.*, 2011). Wastewater is rich in primary nutrients, carbon, nitrogen and phosphorus required for algal growth. The coupling of wastewater treatment with biofuel production could offset the costs associated with both processes and is seen as having the potential to be the most competitive way to produce a competitive biofuel (Christenson and Sims, 2011). For more information on this area see Olguin (2012).

1.3.2 Public health

WSP systems have been shown to drastically reduce the levels of pathogenic bacteria contained in their effluent (Curtis and Mara, 1994). This can also be achieved when other treatment methods are used but only if an additional disinfection stage is added. Commonly used disinfection techniques require chemicals, for chlorination, or extra energy inputs for UV or ozone sterilization. All of these methods add complexity to maintenance and also cost to the treatment process, as control of disinfection biproducts becomes necessary. WSPs on the other hand rely on a number of natural processes to disinfect the wastewater. These mechanisms include sedimentation, biological disinfection and damage linked to sunlight (Curtis and Mara, 1994; Bolton *et al.*, 2010).

Faecal coliform counts are commonly used as a proxy for bacterial pathogens. Faecal coliforms are present in higher concentrations in raw wastewater than pathogens and are much easier to detect. Faecal coliforms are thought to accurately represent pathogens that have a similar DNA composition and life history to *Escherichia coli*. There has been some doubt about the use of faecal coliforms as an indicator of other pathogens, including O1 and O139 forms of *Vibrio cholerae*, the organisms responsible for cholera and *Campylobacter*, a major cause of diarrhoea in developing countries (Curtis and Mara, 1994). *Campylobacter* has been shown however to be removed at a faster rate than other faecal coliforms (Curtis, 1985). Curtis *et al.* (2003) showed that in Dhaka, Bangladesh, an endemic cholera area where outbreaks of the disease are common, a WSP system treating approximately 18 % of the city's wastewater was able to effectively remove *V. cholerae* O1 and O139 at a faster rate than faecal coliforms where removed. This suggests that faecal coliform counts are a good proxy for these

pathogens, as the pathogens are more rapidly removed than faecal coliforms, providing a safety factor.

1.3.3 Organics removal

WSP systems have the ability to remove organic load (BOD) from wastewater. Anaerobic treatment ponds greatly reduce the organic load of the wastewater before it passes to the facultative ponds. In systems that have primary facultative ponds and no anaerobic pond the anaerobic portion at the bottom of the facultative pond is where the majority of organics removal will take place (Shilton and Walmsley, 2005). UNEP (1999) quoted the soluble BOD_5 removal of well managed WSP systems as 70-90%, comparable to other biological treatment methods, such as activated sludge.

1.3.4 Nutrient removal

Nitrogen and phosphates need to be removed from wastewater in order to prevent eutrophication and acidification of effluent receiving waters. UNEP (1999) quoted the nitrogen removal of well-maintained WSPs as 50-70% and a phosphorus removal rate of 20-50%. The phosphorus removal is dependent on the removal of algae from the effluent before discharge. This suggests that in a system where microalgae are harvested for the production of energy products the effluent should be sufficiently depleted in these core nutrients to prevent eutrophication in receiving waters. The harvested microalgae that contain the nutrients also have potential use as solid fertiliser to be applied to agricultural land (Benemann *et al.*, 2003).

1.3.5 Maintenance

One of the key reasons for the uptake of WSP technologies in developing countries is the cost and relative ease of their construction and maintenance. After design, the building of a WSP requires minimal materials, predominantly for inlets and outlets, with the main structure being an unlined ‘hole in the ground’. This keeps the costs much lower than for those conventional systems currently used in the UK where aeration systems or chemical processes are costly. Maintenance is still required on a daily to weekly basis depending on the size of the WSP system. Tasks include daily cleaning of intake screens and grit channels and checking any pumps. The embankments must be maintained and any build-up of solids near outlets and inlets removed. In general the

removal of sludge from the pond bottoms is not required on a regular basis, the time between cleaning determined by the pond volume, the population served by the pond and the sludge accumulation rate (Mara, 1997).

1.4 Why is the study of WSP ecology important?

The ecology of WSP systems is vital for the functioning of the ponds, with photosynthetic organisms driving oxygen production for use by aerobic bacteria. Figure 1-4 shows the major processes occurring for the removal of organics in a facultative pond and highlights the important role of both bacteria and photosynthetic organisms.

The removal of nutrients and its links to WSP ecology is more complex and for a time controversial and so has been the subject of much research. This research is reviewed below.

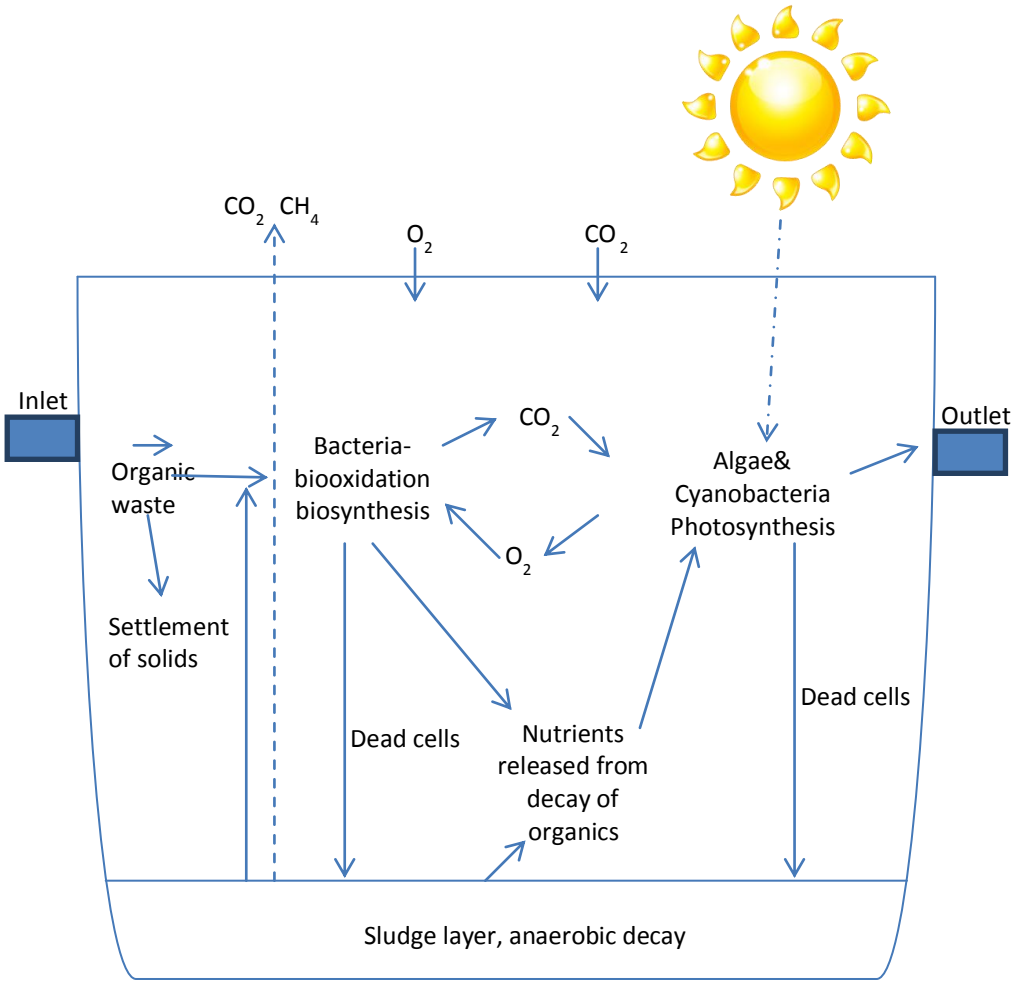


Figure 1-4 Summary of the role of bacteria and algae in the cycling of soilds and organics in facultative ponds.
Diagram modified form Walmsley and Shilton (2005).

1.4.1 Nutrient removal dependent on ecology

There are a number of pathways that have been proposed for nitrogen transformation and removal from wastewater in WSP systems. Possible pathways include volatilization of ammonia to the atmosphere, sedimentation of organic nitrogen, biological nitrogen uptake by bacteria and algae, denitrification and nitrification and mineralization (Ferrara and Avci, 1982; Pano and Middlebrooks, 1982; Lai and Lam, 1997; 2007a).

There has been much debate on pathways for nitrogen removal in WSP systems. In the past papers used mathematical modelling of the reactions within ponds, based on limited data and some laboratory based studies, often with conflicting conclusions on the mechanisms at work. For example Pano and Middlebrooks (1982) produced models which supported volatilization as the chief mechanism. Another research paper by Ferrara and Avci (1982) appearing in the same journal and based on one of the same ponds, reached very different conclusions, as highlighted in a discussion article later that year (DiGiano, 1982). They concluded that the main mechanism was the settling of organic nitrogen and cell uptake of ammonia, and that volatilization was negligible. The differences in results can be explained by the differences in assumptions and equations used in each of the models. Pano and Middlebrooks (1982) model was based on first order equations, with rate determined as a function of temperature, HRT and pH. While high values for these parameters do increase the potential for volatilization they could lead to an increase in algal uptake, so the equation is really predicting overall N removal rather than specifying a particular mechanism. Using modelling Reed (1985) also suggested that nitrification coupled with denitrification was possible, but not likely and supported volatilization and settling out of organic N, such as that taken up by algae and higher plants. Answering the research question therefore required a different approach based on measurement and experimental findings rather than theoretical modelling.

Lai and Lam (1997) conducted a study at the Werribee WSP system in Australia, using measurements of Total Kjeldahl Nitrogen (TKN), total ammonia nitrogen and nitrite and nitrate concentrations in the influents and effluents of each pond in the system. Their work supported the models of Ferrara and Avci (1982), were volatilization was negligible and ammonia uptake by algal cells accounts for some of the removal. However, it differs from the model by naming nitrification as the predominant reaction occurring within the pond system, based on the high nitrite and nitrate levels recorded.

The exception is that in the presence of low ammonia concentrations algae will begin to take up nitrates preferentially. This finding was also supported by a similar study by Hurse and Connor (1999), though they did not consider uptake into algae as a possible mechanism.

More recently studies have been carried out in which the pathways can be measured more directly. Camargo Valero and Mara (2007b) used apparatus to collect gas coming out of the WSP in the UK, to measure the ammonia volatilization occurring. This accounted for just 3% of the total nitrogen that was removed by the pond and therefore was not the main mechanism at work. Biological algal uptake was highlighted as the main pathway, however their study was only performed in the summer, so conclusions were limited to this period and to temperate regions, as other studies concluded seasonality, temperature and pH all effect the mechanisms used (Zimmo *et al.*, 2004; Van der Linde and Mara, 2010). Zimmo *et al.* (2004) used a similar method to detect ammonia volatilization and concluded that ammonia volatilization was negligible, in this case accounting for removal of less than 1.1% of the influent total nitrogen. This conclusion was also supported by Senzia *et al.* (2002), though a less direct method was used in this study. These two papers also concluded that the main removal mechanism for nitrogen was the sedimentation of organic nitrogen, predominantly from decaying algae. In addition Senzia *et al.* (2002) recorded that 50% of the nitrogen within the effluent was contained in microorganisms, suggesting that if a method could be found to remove this, the N removal efficiency of the system could be greatly increased.

More recently it has become possible to directly trace the transformation and then removal of nitrogen species in WSPs. Camargo Valero and Mara (2007a) pioneered the use of ^{15}N isotope tracer studies to determine the proportion of ammonium-nitrogen in different fractions within the pond. 69 % of the ^{15}N recovered was in the organic fraction, with 5 % in the inorganic. The experiments showed clearly that inorganic forms entering the pond were being transformed into organic forms, and then removed by sedimentation. The proportion of nitrogen within the algal biomass in the sludge layer was lower than would be expected from theoretical and laboratory based testing. This suggests that anaerobic digestion is occurring in the sludge layer with the dead algal biomass being decomposed and releasing nitrogen back into the water column in the form of inorganic ammonium (Camargo Valero *et al.*, 2009a).

The ^{15}N isotope tracing method was also used to address another possible mechanism for the transformation and removal of nitrogen that had often been discounted in many earlier studies. Nitrification has often been regarded as an unlikely mechanism for removal of ammonium, due to the small quantities of nitrates and nitrites found in treatment ponds and their effluents. The idea was at first discarded by (Camargo Valero and Mara, 2007a), however they later confirmed that it does occur by using ^{15}N labelled ammonia and ^{15}N labelled nitrite as tracers and a molecular study (Camargo Valero *et al.*, 2009b). They conclude that the coupling of nitrification with denitrification or algal uptake masks the mechanisms detection in traditional studies. Nitrate produced in the nitrification process can be utilised by algae, being taken up through their cell walls and processed into usable compounds by nitrate reductase enzymes, though algae preferentially take up ammonia if it is present, as it requires less processing thereby making it more energetically efficient. The researchers, however, do still support their earlier conclusions that algal uptake of ammonia nitrogen is the main mechanism, but that nitrification-denitrification does play a role, especially when conditions for algal growth are poor.

In conclusion, though the mechanisms have been much disputed, it is clear that algal uptake of ammonia, followed by sedimentation is an important process for the removal of nitrogen in WSPs. Other mechanisms such as coupled nitrification and denitrification are also key to its efficient removal. Despite this debate in uncovering the mechanism involved, little work has been done to identify the micro algae that are involved and those that are most efficient in terms of nitrogen removal.

1.5 The ecology of Waste Stabilization Ponds

The ecology of WSP systems is complex. There are many groups of organisms present, both photosynthetic and non-photosynthetic. The non-photosynthetic fraction of the community includes bacteria, protists and larger eukaryotic organisms, such as insects. The photosynthetic population can be broadly divided into the prokaryotic cyanobacteria and the eukaryotic microalgae. A number of studies have been carried out using microscopy to assess the diverse range of photosynthetic organisms present. Microalgae come from a range of algal phyla, the Euglenophyta, Chlorophyta (green algae), Heptokontophyta (predominantly diatoms), Cryptophyta and Cyanophyta (prokaryotic cyanobacteria). A summary of the algae identified in the literature to genus

level can be seen in Table 1-1. The literature suggests that the algae from the phylum Chlorophyta are detected most often in WSPs.

There are a number of genera that are seen across a large number of the studies. These include *Nitzchia* and *Navicula*, (Bacillariophyta), *Euglena* and *Phacus* (Euglenophyceae), and *Chlamydomonas*, *Chlorella*, *Scenedesmus* and *Micractinium* (Chlorophyta). *Chlamydomonas* and *Euglena* are tolerant to anoxic conditions and also pollution tolerant. It is also possible however that there are other organisms in common between ponds that are more difficult to identify, because they have less morphologically distinct characteristics.

Quantification of organisms has been carried out in many of the studies, though in different ways. Park *et al.* (2011a) concentrated on the quantification of dominant organisms and recorded only the presence and absence of rarer organisms. Ceron (personal communication) recorded only presence-absence data for organisms. Wiedeman (1965) defined species as predominant, co-dominant and present. Papers in the literature identify organisms to genus level or a mixture of genus and species level, highlighting the issues of defining to species level based solely on morphological, visual traits. von Sperling *et al.* (2008) confirmed this by showing values for the number of algae in the samples that could not be positively identified, ranging from between 2.7 and 15.3 per cent.

The cyanobacteria have also been identified as being present in WSP systems, though a smaller range of species were detected, as seen in Table 1-2. Wiedeman (1965) showed that in a WSP system in Texas cyanobacteria were actually the dominant organisms during the summer months, with *Oscillatoria* making up 90% or more of the total population of two of the ponds and *Merismopedia* being dominant in the third pond, that received water from the two previous ponds at the end of the treatment process. Furtado *et al.* (2009) assessed cyanobacteria in a facultative pond, using cell isolation, morphological identification and 16SrRNA sequencing of isolated cells. They found 10 species of cyanobacteria and cell counting showed cyanobacteria being the dominant photosynthetic organisms within the pond, also at greater than 90%. Several papers that report microalgae make no mention of cyanobacteria, although it is unclear whether this was because they were not the focus of the study or because they were not detected (Garcia *et al.*, 2000; Costa *et al.*, 2009; Godos *et al.*, 2009; Park *et al.*, 2011a). von

Sperling *et al.* (2008) noted that cyanobacteria were not detected in the polishing ponds in their study. The literature is inconclusive on the role and proportions of cyanobacteria compared to eukaryotic microalgae in pond systems, though literature suggests that cyanobacteria are most common in ponds with low organic loads (Amengual-Morro *et al.*, 2012). It is important to address this knowledge gap given the potential for cyanobacteria to produce harmful toxins that could pollute receiving waters and effect the flora and fauna of WSPs (Furtado *et al.*, 2009).

1.5.1 Algal taxonomy

The algal phyla are generally named based on their colours, for example; Cyanophyta the Blue-Green algae, Rhodophyta, the red-algae and the Chlorophyta the green algae. The algae are classified based on the pigments that they contain (a summary of the pigments within cells of the algal classes can be seen in Chapter 4, Table 4-1). Traditionally a number of other morphological and biochemical traits are used to classify the algae, such as cell wall structure and components, the presence or absence of flagella, patterns of nuclear division and cell division and the types of storage products used by the cells. For algal identification, down to genus and species level a number of other morphological characteristics are also considered, including; the shape and size of the cell, whether it forms colonies and how many cells these contain, the colour, the presence of gas vesicles, the presence of 'eyespots' and 'pyrenoids', to name but a few.

The eukaryotic microalgae and prokaryotic cyanobacteria were traditionally grouped together into one class based on morphology in taxonomic schemes, such as that by Eichler (1883). Since this scheme was published knowledge of the cell and its structure has improved and the distinction between eukaryotic and prokaryotic cells seen. This highlighted that the microalgae (eukaryotic) and cyanobacteria (prokaryotic) are distinct and more distantly related than first thought.

Table 1-1 Summary of eukaryotic microalgae identified and reported in WSP microalgal ecology surveys. Division and class groupings were confirmed using 2 sources (van den Hoek et al., 1995; Guiry and Guiry, 2013).

			References											
Division (phylum)	Class	Genus	Barthel *et al.* (2008)	Bernal *et al.* (2008)	Ceron (pers.com)	Costa *et al.* (2009)	El-Deeb Ghazy *et al.* (2008)	Garcia *et al.* (2000)	Godos *et al.* (2009)	Mara (1997)	Park *et al.* (2011a)	Shanthala *et al.* (2009)	von Sperling *et al.* (2008)	Wiedeman (1965)
Heptokontophyta	Bacillariophyceae	*Achnanthes sp.*							x			x		
		Amphipleura sp.			x									
		Cyclotells sp.								x		x		
		Cymbella sp.										x		
		Dyatoma sp.										x		
		Frigilaria sp.										x		
		Gomphonema sp.										x		
		Melosira sp.												x
		Navicula sp.			x			x		x		x		
		Nitzschia sp.	x		x			x	x			x		
		Pinularia sp.										x		
		Rhopalodia sp.			x									
		Stauronesis sp.										x		
		Synedra sp.										x		
		Thalassiosira sp.									x			
	Chrysophyceae	*Mallomonas sp.*											x	
		Synura sp.		x										
	Eustigmatophyceae	*Chlorobotrys sp.*				x								
Dinophyta Cryptophyta	Cryptophyceae	*Cryptomonas sp.*					x						x	x
		Chroomonas sp.			x									x
		Rhodomonas sp.										x		
	Dinophyceae	*Peridinales*											x	
Euglenophyta	Euglenophyceae	*Astasia sp.*										x		
		Chromulina sp.										x		
		Cryptomonas sp.										x		
		Euglena sp.		x	x	x	x			x		x	x	x
		Lepocinclis sp.		x								x		x
		Phacus sp.			x		x			x		x	x	x
		Trachelomonas sp.			x							x	x	
		Vacularia sp.										x		

References														
Division (phylum)	Class	Genus	Barthel *et al.* (2008)	Bernal *et al.* (2008)	Ceron (personal com.)	Costa *et al.* (2009)	El-Deeb Ghazy *et al.* (2008)	Garcia *et al.* (2000)	Godos *et al.* (2009)	Mara (1997)	Park *et al.* (2011a)	Shanthala *et al.* (2009)	von Sperling *et al.* (2008)	Wiedeman (1965)
Chlorophyta	Chlorophyceae	*Ankistrodesmus*							x	x	x	x		x
		Carteria sp.		x		x				x				
		Chlamydomonas sp.		x	x	x	x		x	x	x	x	x	x
		Chlorella sp.	x			x		x	x	x				x
		Chlorococcum sp.			x								x	x
		Chlorogonium sp.								x			x	
		Chordatella sp.												x
		Coelastrum sp.			x					x	x	x		x
		Desmodesmus sp.			x						x			
		Dictyosphaerium sp.						x		x	x	x		x
		Dunaliella sp.				x								
		Dysmorphococcus sp.				x								
		Golenkinia sp.												x
		Gonium sp.									x			
		Haematococcus sp.					x							
		Kirchneriella sp.									x			
		Microspora sp.							x					
		Monoraphidium sp.				x					x		x	
		Oocystis sp.				x			x	x		x		x
		Pandorina sp.					x			x	x			x
		Pediastrum sp.					x			x	x	x		x
		Planktosphaeria sp.										x		
		Polyedriopsis sp.												x
		Polytoma sp.		x										
		Polytomella sp.		x										
		Protoderma sp.							x					
		Pyrobotrys sp.								x		x		
		Radiococcus sp.									x			
		Scenedesmus sp.	x			x		x		x		x	x	x
		Selenastrum sp.							x	x				
		Stigeoclonium sp.										x		

		Tetraedron sp.									x		x
		Tetraspora sp.									x		
	Trebouxiophyceae	*Coronastrum sp.*			x								
		Eremosphaera sp.			x								
		Micractinium sp.				x	x	x		x	x	x	x	x
		Planctonema sp.				x								
		Siderocelis sp.					x							
	Ulvophyceae	*Ulothrix sp.*												x
	Uncertain classification	*Actinastrum sp.*			x						x	x		x
		Arthrodesmus sp.				x								
		Closterium sp.			x							x	x	
		Cosmarium sp.				x						x		
		Euastrum sp.										x		
		Mesotaenium sp.				x								
		Staurastrum sp.												x

Table 1-2 Summary of Cyanobacteria identified and reported in WSP microalgal ecology surveys.

Division (phylum)	Class	Genus	Barthel *et al.* (2008)	Bernal *et al.* (2008)	Ceron	El-Deeb Ghazy *et al.* (2008)	Furtado *et al.* (2009)	Mara (1997)	Shanthala *et al.* (2009)	Wiedeman (1965)
Cyanophyta	Cyanophyceae	*Anabaena sp.*	x		x			x		x
		Anacystis sp.								x
		Aphanocapsa sp.		x			x		x	
		Chlorococcus sp.							x	
		Cyanobium sp.		x						
		Geitlerinema sp.		x	x					
		Glaucospira sp.		x						
		Gleocapsa sp.			x				x	
		Lyngbya sp.					x		x	
		Merismopedia sp.			x		x		x	x
		Microcystis sp.							x	
		Oscillatoria sp.				x		x		x
		Phormidium sp.		x			x			
		Planktothrix sp.	x							
		Pseudoanabaena sp.		x			x			
		Romeria sp.		x						
		Spirulina sp.			x			x		
		Synechococcus sp.			x					
		Synechocystis sp.		x	x		x			

There are a wide variety of morphologies of microalgae, however many species within groups look very similar, depending on single differences or even the absence of a characteristic or lifestage (Proschold and Leliaert, 2007) for their identification. This can make identification problematic. For example the genus *Chloromonas* is traditionally separated from the genus *Chlamydomonas* on the basis that pyrenoids (a spherical structure containing RuBisCOs enzymes) in the chloroplasts are absent in *Chloromonas* species and present in *Chlamydomonas* species. It has been shown that some of the *Chloromonas* species have strains both with and without pyrenoids, and that this is not a legitimate character by which to classify them. 18SrRNA sequences confirm that these groups require reorganisation (Proschold *et al.*, 2001). It has also been shown that culture conditions can determine the presence or absence of characteristics traditionally used to positively identify species. For example *Chlorella vulgaris* and *Micractinium pussilum* both have smooth cell walls when cultured under anoxic conditions (Luo *et al.*, 2006). It was only when a grazer was added to the culture that the *M. pussilum* formed colonies and the characteristic cell wall spines, used to identify the species.

The definition of a species and of traditional taxonomic groups based on morphological characteristics also poses problems with identification. Natural classification groups are not accurately considered in taxonomies based on morphological characteristics. Woese *et al.* (1990) suggested a new way to classify organisms based on molecular sequences and evolutionary history rather than relying on the morphological approach. This molecular approach has since been used to improve systematics within the eukaryotic group and will be discussed below.

18SrRNA phylogenetic trees have been used to evaluate the relationship between living organisms from known phyla. van den Hoek *et al.* (1995) produced a tree of life based on 18SrRNA and 16SrRNA gene sequences, shown in Figure 1-5. This tree clearly shows that the 'algae' are an 'unnatural' taxonomic group, not clustering together or diverging from a common ancestor at the same time. For example the Euglenophyta have also been shown to be more closely related to non-photosynthetic kinetoplastid protozoa (including the organism *Trypanosoma*, that causes sleeping sickness), than they are to the Chlorophyta (Sogin *et al.*, 1986a), that are thought to have given rise to the higher plants. When multiple sequences from organisms of the same phyla are

compared the organisms are clustered together, suggesting that though traditional methods of grouping organisms together into phyla was done based on morphology, it is still accurate at phylum level when genetic relationships are accounted for.

The taxonomy of the algae within phyla is still undergoing changes and is constantly in debate, for example the Phylum Chlorophyta was traditionally divided into classes and orders based on thallus organisation. They were grouped according to complexity seen, such that the single celled coccoid and flagellate Chlorophyta were seen as being the primitive ancestors of the more 'complex' filamentous forms (Bold and Wynne, 1985).

More recent evidence has shown that evolutionary lineages do not agree with the original method of grouping, with many genus' with different levels of thallus organisation being grouped together according to evolutionary phylogeny. This led Mattox and Stewart (1984) to propose a new classification system based on the ultrastructure of the basal body of flagellated cells and also took the pattern of cytokinesis during cell division into account. This was felt to be an over simplification by Van den Hoek *et al.* (1988), who proposed adding a number of other characteristics to the ultrastructure classification system, such as life histories, the structure of vegetative cells and cell wall composition. With the application of molecular methodologies to Chlorophyta systematics (originally proposed for assessing phylogeny by (Woese and Fox, 1977), the lineages of green algae have been further adjusted. This has resulted in five main lineages within the phyla being accepted, a summary of the development of Chlorophyta systematics can be seen in Proschold and Leliaert (2007).

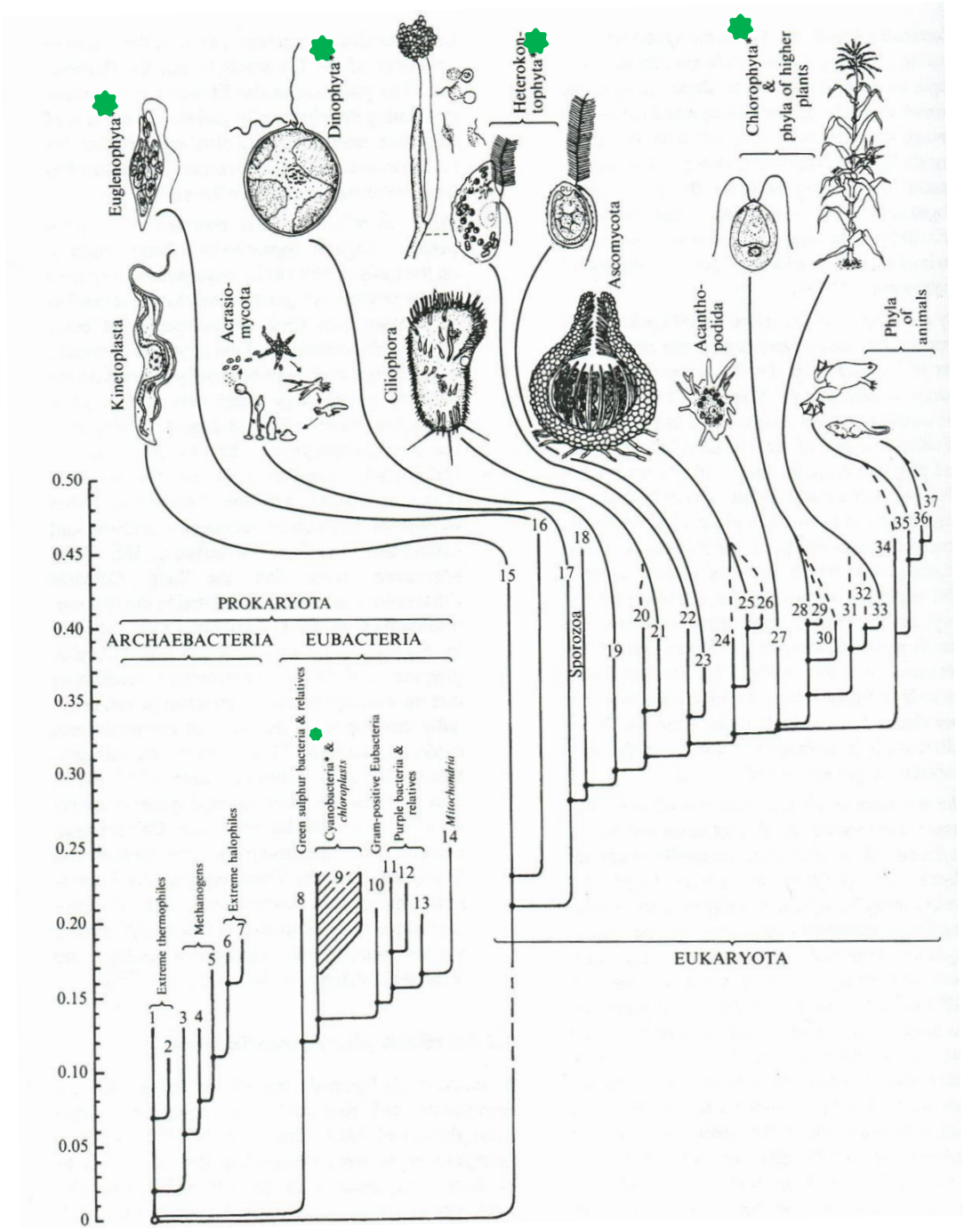


Figure 1-5 A phylogenetic tree of the Archaebacteria, Eubacteria and Eukaryota. * marked phyla are algae. The tree is based on the similarities in the 16S rRNA and 18S rRNA nuclueotide sequences from representative species. *The vertical axis gives an estimate of the evolutionary distance between taxa. 1- Sulfolobus solfatarius. 2-Thermoproteus tenax. 3- Methanococcus vannielii. 4-Methanobacterium formicicum. 5- Methanospirillum hungatei. 6- Halobacterium volcanii. 7- Halococcus morrhua. 8-Thermomicrrobium roseum. 9- Cyanobacteria and chloroplasts. 10- Bacillus subtilis. 11-Pseudomonas testosterone. 12-Escherichia coli. 13- Agrobacterium tumefaciens. 14-mitochondrion of maize. 15-Trypanosoma brucei. 16-Euglena gracilis. 17-Dictyostelium discoideum. 18-Plasmodium berghei 19-Prorocentrum micans. 20- Paramecium tetraurelia. 21-Stylonichia pustulata. 22-Achlya bisexualis. 23-Ochromonas danica. 24- Saccharomyces cerevisiae. 25-Neurospora crassa. 26-Podospora anserine. 27-Acanthomoeba castellani 28- Chlamydomonas reinhardtii. 29-Volvox carteria. 30Nanochlorum eukaryotum. 31-Glycine max (soy). 32- Oryza sativa (rice). 33-Zea mais (maize). 34-Artemia salina (shrimp). 35-Xenopus laevis. 36-Rattus norvegicus (rat). 37-Oryctolagus coniculus (rabbit) Figure reproduced from van den Hoek et al. (1995).*

1.5.2 Microscopy used for WSP ecology surveys

Current knowledge of the ecology of WSP systems has been gained predominantly by microscopy. This traditional technique relies heavily on the expertise and training of taxonomic specialists. There are a range of information guides and keys available to aid identification, however, few of these are comprehensive and guides are not available that cover all regions and habitats. There is also potentially a 'time-lag' between new classification being proposed in journal papers and it being used in taxonomic keys for microscopy.

Inexperienced users may also miss some of the small but significant differences between organisms present in mixed samples. Microscopy techniques are also limited by the image resolution and the morphology of the organisms being investigated, with smaller organisms, such as chlorella and the single celled cyanobacteria requiring 100 times magnification for defining features to be seen (Shubert, 2003).

Microscopy is also time-consuming, making it especially impractical when large numbers of samples need to be examined. Damage to microalgal cells caused during fixation and viewing under the microscope can also lead to difficulties in identification. Many species with delicate structures and flagellated forms can be misidentified or eliminated from the analysis entirely if damaged or destroyed. Cells with different morphologies are more easily identified using different microscopy methods, for example staining is required to make pyrenoids and mucilage sheaths viable and shock treatments are required to cause some cells to go into a reproductive state or to produce more of their defining pigment, before identification can be completed (John *et al.*, 2002). This makes a one size fits all approach for a mixed sample difficult to achieve and may lead to errors in identification.

1.6 Molecular Methods as an alternative

Modern molecular methods can be used as an alternative to microscopy for assessing microalgal community structure. These techniques provide unequivocal identification of organisms based on evolutionary markers, as well as having a higher sample throughput. The introduction of Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988) and the discovery that rRNA molecules could be used as identification markers (Olsen *et al.*, 1986), paved the way for the development of a host of techniques to the study of

bacterial 16S rRNA genes in environmental samples. They have been applied extensively to study bacterial communities in diverse environments (Sogin *et al.*, 2006; Truu *et al.*, 2009; van Elsas and Boersma, 2011). More recently they have been applied to eukaryotes to assess community dynamics in marine ecosystems (Larsen *et al.*, 2001; Stoeck *et al.*, 2007; Potvin and Lovejoy, 2009), and to study harmful algal blooms (Tengs *et al.*, 2001; Connell, 2002; Galluzzi *et al.*, 2005; Vila *et al.*, 2005). Their application to eukaryotic organisms in mixed systems is somewhat more problematic due to the huge range of morphologies and cell walls of eukaryotic cells and their more complex chromosomal and gene arrays.

In contrast, the study of microalgae within natural and engineered freshwater systems using molecular biology techniques is in its infancy. A few studies have been carried out on photoautotrophic picoplankton communities from lakes using fluorescence in situ hybridisation (FISH) (Lepere *et al.*, 2010) and clone libraries, based on the 18S rRNA gene (Lefranc, 2005; See *et al.*, 2005), though the majority of freshwater studies focus on cyanobacteria (Zwart *et al.*, 2005; Ye *et al.*, 2011).

Very few studies have used molecular approaches to study microalgal communities in wastewater treatment. Moura et al (2009) and Yu and Mohn (2001) focused on general bacterial populations, within WSP systems, while Camargo Valero *et al.* (2009b) and Shipin *et al.* (2005) looked specifically at the nitrogen processing organisms, using PCR-DGGE-sequencing and a FISH based approach respectively. Furtado et al (2009) isolated and cultured cyanobacteria, before using 16S rRNA gene sequencing to assess their identity.

There has only been one study thus far to have assessed eukaryotic microalgae in wastewater treatment plants (Ghosh and Love, 2011). They assessed photosynthetic organisms by producing clone libraries targeting the RuBisCO gene that encodes for an enzyme vital for the fixation of carbon. This study detected greater species diversity than previously estimated by microscopy studies. Whilst this outcome is likely to be due to the increased resolution of molecular methods, it is imperative to consider possible sources of bias when using molecular techniques. These biases will be discussed in further detail and be addressed in later chapters. A comparison between traditional microscopy and molecular techniques will be the focus of Chapter 6. The theory of molecular methods and their use will be discussed in more detail in Chapter 2.

Chapter 2. General methods

2.1 Introduction

A range of molecular methods and techniques were used and adapted throughout this thesis. The theory and uses of these techniques have been explained in the first half of this chapter. The second half of the chapter is dedicated to describing how the methods were carried out in the laboratory, with any adaptations made highlighted in later chapters.

2.2 Molecular methods

2.2.1 Introduction

Molecular methods exploit the characteristics of DNA and the genomes of organisms to provide us with information about the ecology of microorganisms. A number of these methods were optimised and used throughout this project to assess the diversity of WSP samples, identify the dominant organisms that they contain and attempt to quantify these organisms.

The techniques used take advantage of molecular biomarkers, in this case ribosomal RNA. Ribosomes are structures within cells that play a vital role in decoding the genome and protein synthesis. Due to their essential function they are present in all living cells. Ribosomes are made up of proteins and rRNA. The genes that code for these ribosomal RNAs (rRNA) are largely conserved due to their essential function, but contain variable regions (Woese, 1987). These variable regions allow for the detection of different organisms at many taxonomic levels (Amann *et al.*, 1995). The variation in rRNAs has been shown to correspond to evolutionary relationships, with species sharing close ancestry having more homologous sequences (Ludwig and Schleifer, 1994). This makes rRNA genes ideal targets for molecular methods.

There are significant differences between microalgae and cyanobacteria, on both a genomic level and in terms of cell structure (Brock, 2006). These differences are important to consider when choosing molecular methods. Microalgae are eukaryotic and cyanobacteria are prokaryotic. Prokaryotic cells are simpler in structure, lacking a defined nucleus to contain their DNA. Eukaryotic cells are more complex with a defined

nucleus and membrane bound organelles. Eukaryotic microalgae contain mitochondria, involved in the respiration process and chloroplasts responsible for photosynthesis.

The structure of ribosomes also differs in prokaryotic and eukaryotic cells, though they are both made up of a Small-Subunit (SSU) and a Large-subunit (LSU). In prokaryotes the LSU is composed of 23S and 5S rRNA molecules and 31 proteins. The SSU contains a 16S rRNA molecule and 21 proteins. Eukaryotic ribosomes are much larger. 28S, 5.8S and 5S rRNA molecules and 50 proteins make up the LSU and 18S rRNA and 33 proteins make up the SSU (Lodish *et al.*, 2004). The 16S and 18S rRNA molecules are commonly used as molecular markers, for prokaryotes and eukaryotes respectively. These markers have been widely sequenced in a large number of organisms, with databases of sequences widely available to the public on the internet making them a good target gene for assessing diversity in environmental samples.

Figure 2-1 summarises the molecular methods that were investigated for use on photosynthetic communities within WSP system samples. The techniques can be divided into two categories, those that are qualitative, such as PCR and DGGE and those that are quantitative, for example Fluorescence in Situ Hybridisation (FISH) and flow cytometry.

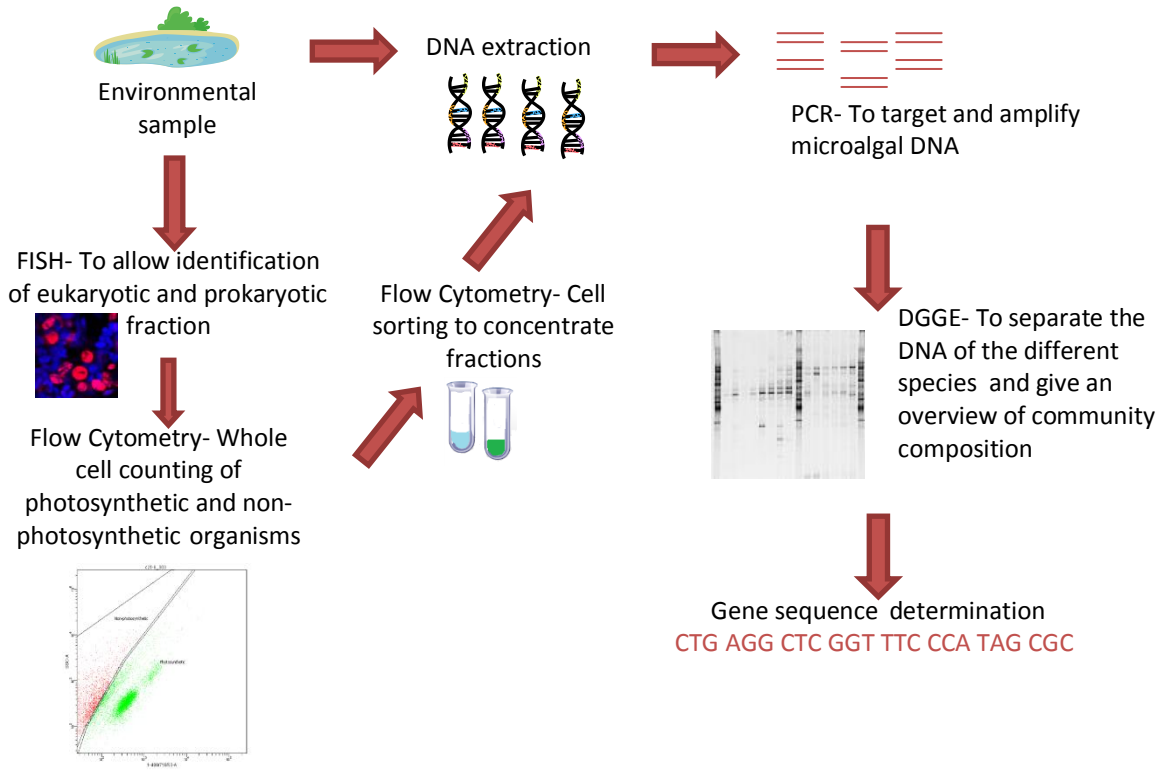


Figure 2-1 Summary of the molecular techniques that were used throughout this research thesis.

2.2.2 DNA extraction

The majority of molecular techniques require DNA to be extracted from cells as a first step. DNA extraction involves the lysis of cells to spill the DNA content into solution and the removal of inhibitors, prior to further downstream analysis. DNA extraction needs to be carried out using a method that is able to lyse as many of the cells and cell types in the sample as possible, to ensure all organisms have released their DNA for consideration in downstream analysis (Head *et al.*, 1998). On the other hand harsh extraction methods, can result in DNA shearing and the presence of short sequences that may form chimeric products during DNA amplification (Wintzingerode *et al.*, 1997).

There are four main mechanisms of DNA extraction, often used in combination in commercially available kits. Mechanical lysis involves physical breaking of the cells, usually with small beads and physical shearing. Chemical lysis can be carried out with detergents or other chemicals to break down components of the cell walls. Freeze-thaw lysis in which cells are rapidly frozen (usually at -80°C or using liquid nitrogen) and thawed can also be used to disrupt and burst the cells open to release DNA. Enzymes, such as Proteinase K can be used to break down proteins in cell walls, and also break down proteins that have the potential to interfere with PCR and denature nucleases that degrade DNA. Optimisation is particularly important when a range of cell wall configurations are likely to be present within the same sample, as in WSPs. This is the basis for experiments carried out in Chapter 3.

2.2.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a method by which target genes, for example a section of the 16S or 18S gene, can be amplified from the mixture of total DNA that was extracted from the sample. PCR is designed to mimic the natural process if DNA replication and involves a series of temperature controlled steps. The denaturing step breaks the hydrogen bonds that hold the two strands of the double helix of DNA together. The temperature is then lowered to allow a specially designed primer molecule to bind to the target DNA fragment of interest. DNA polymerase enzymes then work to copy and extend the DNA fragment, known as the extension step. The fragment of DNA between the two primers is thus replicated exponentially with each successive cycle of the reaction. A schematic of the process can be seen in Figure 2-2.

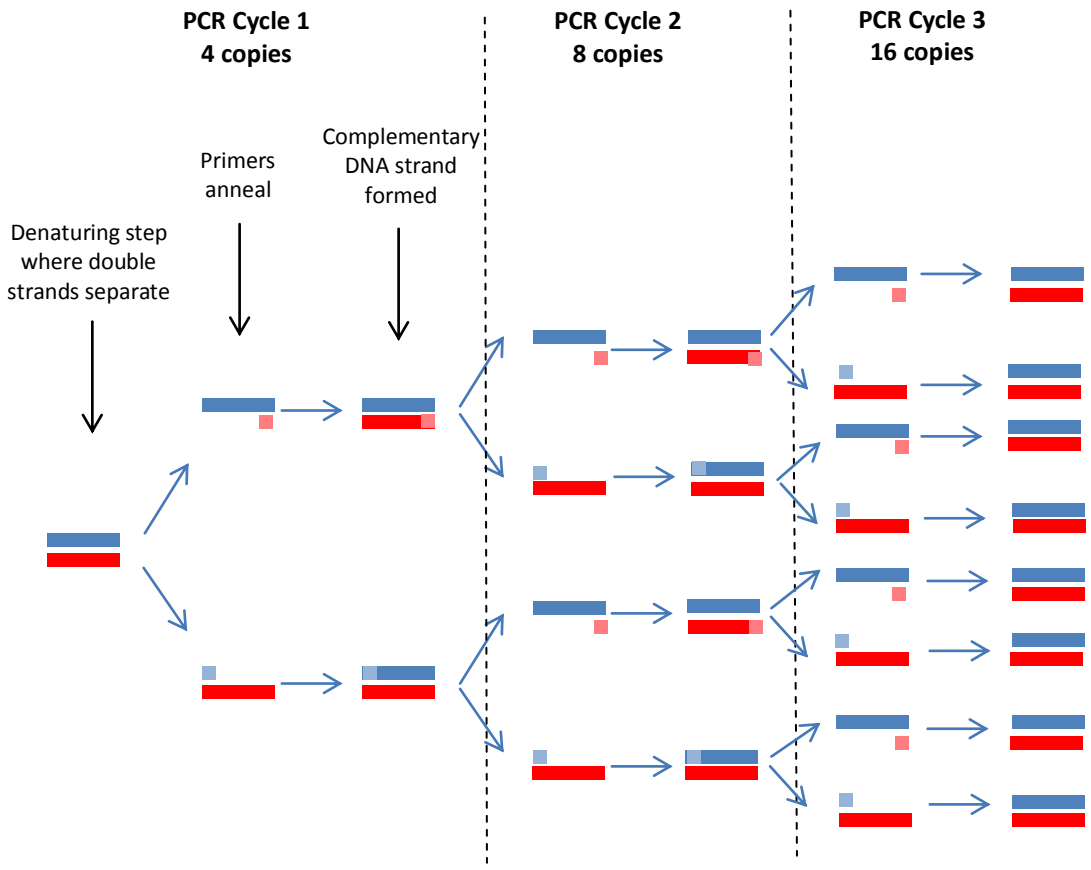


Figure 2-2 The stages of a PCR reaction and the doubling of gene copy number per cycle.

PCR primers are synthetic oligonucleotides, usually between 16-20 base pairs in length. They are designed to complement and target a specific region within or flanking genes within the DNA of the target organism. They can be designed to target a species or genus, by targeting areas of a gene that are highly variable and differ in organisms closely related to the target organisms. On the other hand they can be designed, as is the case with the primers used in this study, to target the majority of eukaryotes or the majority of cyanobacteria, by annealing to a target region that is stable over evolutionary time and so highly conserved.

Agarose gel electrophoresis allows the separation of DNA fragments based on their size. It has been used in this thesis to check all PCRs for amplification of the correct size product and to assess any contamination of the PCR process. DNA molecules have a negative charge, due to their phosphate backbone, when a charge is applied DNA molecules travel across the agarose gel matrix towards the positive anode. Shorter DNA molecules are able to travel faster through the pores in the matrix, due to their smaller

size, with larger molecules being slowed by the matrix (Sambrook *et al.*, 2001b). The gel is treated with a DNA stain, so that DNA positions can be viewed under UV light.

2.2.4 Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) is a qualitative technique that allows PCR amplified products of the same size to be separated based on their sequences (Muyzer *et al.*, 1993b). The gel is formed with a gradient of denaturant concentration (urea and formamide) from high at the bottom to low at the top. The levels of Guanine (G) and Cytosine (C) that the DNA fragment contains and the sequence composition determines the distance that the DNA will travel down the gel before denaturation of the double stranded DNA occurs. Guanine and Cytosine are held together by three hydrogen bonds in the double helix structure, Adenosine and Thymine are held by two, making the number of G-C linkages and the order of the sequences the limiting factor for denaturing speed and therefore distance of travel through the gradient. The resulting gel shows a banding pattern, when stained and viewed under UV light that represents the different sequences contained within the PCR amplified sample.

Though DGGE is a powerful technique for assessing the diversity of the target organisms within a sample, there are some important points and limitations to consider. In theory two bands that have travelled the same distance through the gel and denatured in the same position, should be from the same organism and have the same sequence. However this is not always the case, the same GC content does not automatically mean an identical sequence. To account for this, the term species cannot be used when referring to bands, instead they are described as Operational Taxonomic Units (OTUs). The intensity of DGGE bands has often been used as a proxy for the abundance of the OTU within the sample. This is not a relationship that can be reliably applied. For example in bacteria an average of 2.3 copies of the target gene is present per cell. For eukaryotic organisms the rRNA gene copy numbers vary in different species. These copies are arranged as a tandem array of repeats with the other rRNA genes (for e.g. 28S, 5S and ITS) and can occur across multiple chromosomes.

Zhu *et al.* (2005) calculated 18S rRNA gene copy numbers for a range of microalgae using qPCR and flow cytometry methods. The copy numbers ranged between one for *Nannochloropsis salina* (phylum Ochrophyta) and 30,545 for *Akashiwo sanguinea*

(phylum Dinophyta), with an average of 1922 copies per cell, across 18 algal strains (personal correspondence with Daniel Vaulot, corresponding author). They also found that copy number shows a strong positive correlation with cell length, as shown in Figure 2-3. DGGE band intensity values are therefore unlikely to be linearly related to the number of cells, with varied gene copy numbers. The variable 18S rRNA gene copy numbers in eukaryotes should be considered when using any technique that relies on the gene copy number, as a proxy for cell number, such as qPCR.

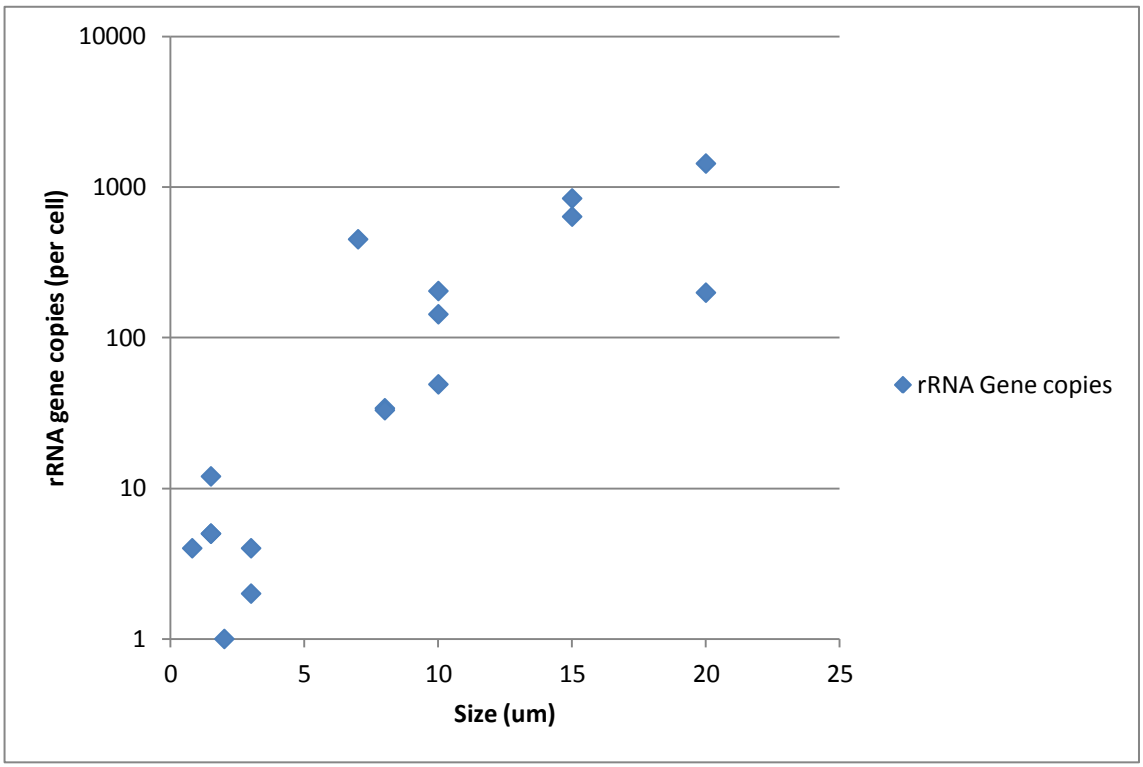


Figure 2-3 The relationship of algal cell size to 18SrRNA gene copy number found using qPCR by Zhu et al. (2005).
Data provided through personal correspondence with authors. Microalgae in reported in WSPs can be between 2 and 150µl in length.

Also of consideration is the detection limit of DGGE. The limit was reported to be approximately 10^7 cells/ml or organisms making up at least 1% of the sample population (Akarsubasi *et al.*, 2009), when studying bacterial communities in a wastewater treatment plant. However Kan *et al.* (2006) estimated a detection limit in the range of 2.5 x 10^3 to 1x 10^4 (0.1 to 0.4% of the total cell counts), when looking at three cyanobacterial strains. The authors also note that the detection limit 'appeared to be affected by gene copy number', having chosen cyanobacteria with between 2 and 9 rRNA operon copy numbers. These studies suggest that the DGGE detection limit is

variable and should be estimated where possible to give a better idea of the errors involved.

In recent years software has been developed for the analysis of DGGE images. BioNumerics (Applied Maths, USA) allows gels to be corrected in case of any unevenness caused by inconsistent pouring and for the comparison of the lanes in more than one gel, if a suitable marker has been run in both gels. In the DGGE runs carried out in this project, one of two makers was used. A bacterial marker designed (Fiona Read, CEGs) from cloned organisms containing 11 strong bands and maintained at the same DNA concentration was used for both bacterial and cyanobacterial gels. A eukaryotic marker was also produced from a pond sample in the initial phase of the laboratory work. This marker contains 11 bands and is used for all of the eukaryotic gels runs. Primer 6 software (Clarke and Gorley, 2006) has also been developed for ecological data that can be used to compute statistics and produce graphical representations of the similarities of biotic communities from the DGGE data.

Bands from a DGGE gel can be cut out and used in a further amplification step (PCR). These bands can then be checked for purity, cleaned up to remove inhibitors and any primer dimer from the PCR. Band DNA can then be used for Sanger sequencing, to determine the sequence of base pairs in the band fragment (Ferris *et al.*, 1996). Sequencing from DGGE bands can pose difficulties, in terms of contamination and also in positively relating the sequence back to the DGGE band targeted.

Despite the limitations of DGGE, it provides a good overview of the community within samples and allows different samples, for example in a time series to be compared simply and quickly (Head *et al.*, 1998).

With the advent of Next Generation Sequencing methods, DGGE has lost favour, though it still provides an excellent starting point with which to assess whether further sequencing effort and expense is going to be useful when addressing a research problem. Caporaso *et al.* (2012) addressed whether increasing the number of sequencing reads in Next Generation Sequencing significantly changed the levels of diversity seen. In fact they say that with just 10-100 reads the beta diversity conclusions were the same as those drawn from sequencing with read numbers 2 order of magnitudes higher. This

suggest that in terms of beta diversity DGGE, though limited in terms of sequence number is still likely to yield valid estimates of relative diversity.

The molecular work in this thesis will focus on the use of PCR-DGGE for WSPs.

2.3 Quantitative molecular methods

Quantitative methods provide additional information on the structure of the microbial community. FISH was used along with flow cytometry to assess the proportions of eukaryotic, cyanobacteria and non-photosynthetic bacteria within WSP samples.

2.3.1 Fluorescence In-Situ Hybridisation

FISH is a technique in which fixed whole cells within a sample are labelled by oligonucleotide probes attached to fluorescent markers, first used by Delong *et al.* (1989). Target organism specific oligonucleotide probes are designed to attach to the rRNA molecules in the ribosomes. The abundance of ribosomes makes the fluorescence probes within the cells detectable using microscopy or flow cytometry techniques. There are four steps in FISH; fixation and permeabilization, hybridisation, washing and visualisation.

Cells within a sample are first permeabilized and fixed. Chemicals such as alcohols and aldehydes are used to preserve the cells within a sample and in the process render the cells permeable to the fluorescent probes that will label them. Different cell types having different cell wall structures may require different permeabilization conditions; this is the focus of Chapter 4.

After the cells are permeabilized a hybridisation step is carried out. A small DNA fragment (oligonucleotide) called a probe is designed to match the rRNA sequence of the target organism and is made with a fluorescent marker attached. This probe is mixed with the cells, permeates the cell walls and binds to the target rRNA inside. The hybridisation conditions and the specificity of the probes are critical for the success of the technique and this was also investigated in Chapter 4. Once hybridisation has occurred, the cells are washed to remove any unbound probe. The cells can then be visualised and counted using either a fluorescence microscope or flow cytometer.

2.4 Laboratory protocols and conditions

2.4.1 Sample collection

A number of WSP sites were sampled during this study, each with different access for collecting samples. Samples from SIDI and Marechal Randon treatment plants in Brazil were collected using a sample container connected to a long pole. The pole was stretched across the pond to sample from as far from the edge as possible. The sampling site in Ginebra, Colombia, had access bridges so that sampling could be carried out further from the pond edges. Samples were taken by dipping the sample bottle directly into the water at the point of interest whilst kneeling on the access bridge. At the Larchfield site, access directly to the pond was more difficult and potentially dangerous, so samples were collected from cascades that recirculated and recycle the wastewater through the pond system. All samples were collected in sterile 1 litre Duran bottles or 0.5 litre Nalgene bottles, washed in distilled water and autoclaved at 121°C for 15 minutes. Samples were collected from the water surface unless otherwise stated. Any samples that were collected at depth were collected using a Kemmerer sampler bottle and then put into a 0.5 litre sterile Nalgene container. Samples were kept on ice, returned to the laboratory and then either fixed or centrifuged and stored.

2.4.2 Algal culturing

Pure algal cultures were used in some of the experiments throughout the thesis. The origins, strain details, culture media and conditions can be seen in Table 2-1. All cultures were seeded into sterile culture media, using sterile pipette tips or a sterile loop (if culture was provided on agar). Cultures were grown using a bug-stop bung as a barrier to prevent contamination and a metal stirrer was introduced to keep the culture well mixed and improve aeration. All cultures were grown at room temperature, with a 12:12 hour light-dark cycle using strip lights. When required cultures were harvested using sterile pipette filter tips.

2.4.3 Fixation

Samples were treated using a range of fixatives, including ethanol, paraformaldehyde and gluteraldehyde and also by freezing. Paraformaldehyde and gluteraldehyde fixed

samples were used for FISH and for flow cytometry. Frozen samples and ethanol fixed samples were used for DNA extractions, for use in downstream PCR and DGGE.

Paraformaldehyde fixation

Fixation was carried out on return to the laboratory. 1ml of refrigerated sample was added to a sterile 2ml Eppendorf tube. This was centrifuged for 3 minutes at 13,000g. The supernatant was removed and the cell pellet washed with phosphate buffered saline (PBS). 1ml of PBS was added and vortexed before centrifuging again at 13,000 x g for 3 minutes. The supernatant was removed and the pellet resuspended in 0.25ml of PBS. 0.75ml of 4% PFA fixative was added and vortexed. The cell suspension was then incubated overnight (approximately 15 hours) at 4°C. After fixation the cells were centrifuged at 13,000 x g for 3 minutes and the supernatant removed. The cells were once again washed by adding 1ml of PBS, vortexing and then centrifuging. The cells were finally resuspended in a 1:1 mix of PBS and absolute ethanol. These fixed cells were then stored in a freezer at -20°C until use.

Table 2-1 Algal cultures, their origins and growth conditions.
Recipes for the algal media used can be seen in Appendix. 1 (CCAP, 2010)

Algal species and strain number	**Class-order**	**Origin**	**Culture medium**	**Culture collection or source**
Chlamydomonas reinhardtii CCAP- 11/45	Chlorophyceae-Volvocales	Edgewood Park, Connecticut, USA	3N-BBM+V Agar slope	CCAP
Chlorella vulgaris CCAP- 211/80	Trebouxiophyceae-Chlorellales	Molkerteich, Elsnigk, Sachsen-Anhalt, Germany	3N-BBM+V Agar slope	CCAP
Pandorina morum CCAP-60/2	Chlorophyceae-Volvocales	Priest Pot, Cumbria, England	3N-BBM+V Liquid media	CCAP
Scenedesmus quadricauda CCAP- 276/21	Chlorophyceae-Chlorococcalles	Priest Pot, Cumbria, England	EG:JM Liquid media	CCAP
Navicula pelliculosa CCAP-1050/9	Bacillariophyceae, Naviculales	Oyster pond, Marthas vineyard, Massachusetts, USA	F2 liquid medium with air bubbling	CCAP
Anabaena cylindrica CCAP-1403/2A	Cyanophyceae	Freshwater pond, Surrey, England	JM liquid medium	CCAP
Synechococcus sp. CCAP-1479/13	Cyanophyceae	Freshwater, North Basin, Windermere, Cumbria, England	BG11 liquid medium	CCAP
Microcystis aeruginosa CCAP-1450/4	Cyanophyceae	Freshwater, Little Rideau Lake, Ontario, Canada	BG11 liquid media	CCAP
Dunaliella viridis	Chlorophyceae, Volvocales		JM liquid medium	MAST
Tetraselmis sp.	Chlorophyceae, Volvocales		JM liquid medium	MAST

The PFA fixative was prepared fresh or prepared then frozen and defrosted immediately before use. 50ml of PFA fixative was produced by heating 44.5ml of sterile distilled water to 60°C and adding 5ml of 10X PBS and one drop of 10M NaOH. 2g of powdered paraformaldehyde was then dissolved into the heated liquid and then stored on ice and pH adjusted to 7.2. The fixative was then filtered through a 0.2µm filter to remove any debris or contamination ready for use.

Gluteraldehyde fixation

Gluteraldehyde was used as an alternative fixative for flow cytometry. 0.1ml of concentrated gluteraldehyde was added to samples of 1.9ml volume to make a concentration of 1.25%. The sample and gluteraldehyde were vortexed thoroughly and then frozen.

Ethanol fixation

Ethanol fixation when done was carried out in the field. Sterile sample bottles were filled up to half their capacity with absolute ethanol and then the sample added. This results in a sample to ethanol ratio of 1:1 (v/v). Samples were then stored at -20°C on return to the laboratory.

Freeze

Samples for DNA extraction (between 50ml and 80mls in total) were placed in 50ml sterile centrifuge tubes and centrifuged for 3392 x g (4200rpm) for 2 hours or 7690 x g for 10 minutes. The supernatant was then removed and the pellets transferred into sterile 2ml Eppedorf tubes. The pellets were then frozen at -20°C until DNA extraction could be carried out. DNA extraction of these samples was usually carried out within a week of the sampling day.

2.4.4 Sample and extracted DNA storage

All samples and DNA extracts were stored at -20°C in a freezer until use. Samples and DNA extractions were transported from Colombia and Brazil by courier on dry ice. Spectrophotometer readings were taken using a Nano Drop ND-1000 (Nano Drop Technologies, Inc., Wilmington, USA) at absorbance 26Onm of the DNA extractions

carried out in Brazil to assess degradation. No significant difference was seen in readings from before and after transportation.

2.4.5 DNA extraction

Following testing of a number of DNA extraction protocols (Chapter 3) all subsequent extractions were carried out using Qiagen's Blood and Tissue Kit, using the Tissue protocol from the manufacturer's instruction.

All solutions used except the ethanol were provided in the kit. Spin column tubes and collection tubes were also provided. The defrosted pellets of algal material were centrifuged at 13,000 x g for 3 minutes to remove any liquid and 180µl of Buffer ATL was added along with 20µl of proteinase K solution. The mixture was vortexed thoroughly to mix and incubated in a shaking incubator at 56°C overnight.

After incubation samples were vortexed for 15 seconds and 200µl of Buffer AL and 200µl of absolute ethanol added before mixing thoroughly by vortexing. The mixture is then applied to the DNeasy Mini Spin column provided and centrifuged at 6000 x g for 1 minute. In some cases this step needed to be repeated to make sure that all of the liquid had passed through the spin column membrane. The flow through and the collection tube are then discarded. The spin column is then placed in a fresh collection tube and 500µl of Buffer AW1 added and centrifuged at 6000 x g for 1 minute. The flow through and collection tube are once again discarded and the spin column placed in a new collection tube. This time 500µl of AW2 is applied to the column and centrifuged at 20,000 x g for 3 minutes. The flow through and collection tube are then discarded and the spin column placed in a sterile 2ml micro centrifuge tube. 200µl of elution buffer AE was then added to the membrane of the spin column, incubated at room temperature for 1 minute and then centrifuged for 1 minute at 6000 x g to elute the DNA. This step was then repeated and the two elutions collected together in the same tube.

2.4.6 Polymerase Chain Reaction

PCR was used to amplify target DNA from DNA extractions throughout the project. A range of primers from the literature were used and with each of these a tailored PCR program. Details of the primers and thermo cycler programmes used can be seen in Table 2-2. More information on the primer choice and evaluation will appear in the

experimental chapters. A premixed solution, MegaMix Blue (Microzone, UK) was used to standardise PCR, reduce errors and eliminate the need to add a DNA loading buffer for agarose gel electrophoresis. MegaMix Blue contains recombinant Taq polymerase (the thermo-tolerant enzymes catalysing the extension step of PCR), 220µM dNTPs (nucleotide building blocks) and blue loading dye in a 2.75mM $MgCl_2$ buffer. Reaction volumes were 50µl, made up of 47µl of MegaMix Blue, 1µl of DNA extraction from the sample and 2µl of primer mix (1µl forward primer and 1µl of reverse at 10pmol/µl). Positive and negative controls were included. Positive controls contained 1µl of a DNA extraction known to contain DNA from the target organism and previously proven to produce a PCR fragment of the correct sized fragment. The negative control contained 1µl of filter sterile deionised water in place of the sample.

2.4.1 Agarose Gel Electrophoresis

The PCR products were examined by agarose gel electrophoresis to assess whether the correct size DNA gene fragment had been amplified and to highlight any reaction contamination. All PCR products produced were tested in this way.

1.5g of agarose was added to 100ml of 1xTAE buffer (2M Tris-Acetate, 0.05 M EDTA, pH 8.3) and heated to melt the agarose. 20ul of Nancy- 520 DNA stain (Sigma-Aldrich) was added and the mixture poured into the gel casting tray, containing a comb for well formation. After leaving the gel to solidify it was transferred into the electrophoresis tank and the comb removed. The tank was filled with 1x TAE buffer and 7ul of PCR product added to each well. A PCR marker (Hyperladder II, Bioline, UK) was also added to allow size comparisons to be made. Electrophoresis was run for 45 minutes at 100 Volts. The gel was then visualised under ultra violet illumination, using a Dual Intensity Transilluminator (Genetic Research Instrumentation Ltd, UK) and photographed. If the correct fragment size was seen for the sample and the negative lane remained free of bands, then the PCR product was used in downstream analysis, such as DGGE.

Table 2-2 PCR reaction conditions and details of primers used.

Primer	Sequence (5'-3')	Specificity	Fragment size	Cycle details	Reference
F357 GC	*CGCCCGCCGCGCCCCGCGCCCGGCC CGCCGCCCCCGCCCCCC* TACGGGAGGCAGCAG	Bacteria	200 base pairs	95°C for 1 min (95°C for 30 secs, 65-53°C 30 secs, 72°C for 30 secs) x 24 cycles (95°C for 30 secs, 53°C for 1 min, 72°C for 1 min) x 15 cycles 72°C for 10 mins	Muyzer *et al.* (1993a) Zwart *et al.* (2005)
R518	ATTACCGCGGCTGCTGG	Bacteria			
Cya-b-F371	CCTACGGGAGGCAGCAGTGGGGAA TTTTCCG	Cyanobacteria	435base pairs	95°C for 1 min (95°C for 30 secs, 65-53°C 30 secs, 72°C for 30 secs) x 24 cycles (95°C for 30 secs, 53°C for 1 min, 72°C for 1 min) x 15 cycles 72°C for 10 mins	Zwart *et al.* (2005)
Cya-R783	GACTACWGGGGTATCTAATCCCW	Cyanobacteria			
F357 GC*	*CGCCCGCCGCGCCCCGCGCCCGGCC CGCCGCCCCCGCCCCCC* TACGGGAGGCAGCAG	Bacteria	200 base pairs	95°C for 3mins (94°C for 1 mins, 65°C for 1 min, 72°C for 1 min) x 20 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) x 5 cycles 72°C fir 5 mins	Zwart *et al.* (2005)
R518*	ATTACCGCGGCTGCTGG	Bacteria			
Euk1A	CTGGTTGATCCTGCCAG	Eukarya	560 base pairs	94°C for 130 secs (94°C for 30secs, 56°C for 45 secs, 72°C for 130 secs) x 35 cycles 72°C for 8 mins	Diez *et al.* (2001)
Euk516r GC	ACCAGACTTGCCCTCC *CGCCCGGGGCGCGCCCCGGGCGGG CGGGGGCACGGGGGG*	Eukarya			
Euk1A	CTGGTTGATCCTGCCAG	Eukarya	1265 base pairs	95°C for 3 mins (95°C for 1 mins, 54°C for 1 min, 72°C for 90 secs) x 20 cycles 72°C for 6 mins	Lim *et al.* (1993)
Euk1209r GC *	*CGCCCGGGGCGCGCCCCGGGCGGG GCGGGGGCACGGGGGG* GGGCATCACAGACCTG	Eukarya			
Euk1A	CTGGTTGATCCTGCCAG	Eukarya		95°C for 3 mins (95°C for 1 mins, 54°C for 1 min, 72°C for 90 secs) x 20 cycles 72°C for 6 mins	(Baker *et al.*, 2003)
U906R GC *	*CGCCCGGGGCGCGCCCCGGGCGGG GCGGGGGCACGGGGGG* CAATTCMTTTAA	Universal			

** When used as second stage of nested protocol with cyanobacterial PCR product*

** Probe Base web program used to find the primer (Loy et al., 2007)*

Sequences in italics are GC clamps, added to the primers when the PCR product was used for DGGE, to aid travel of DNA through the gel matrix.

2.4.2 Denaturing Gradient Gel Electrophoresis

The diversity of the predominant members of the eukaryotic, bacterial and cyanobacterial communities were evaluated using DGGE. DGGE was carried out using a BioRad system and a Power Pac 3000. Electrophoresis was run in 0.75mm thick, polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) (where 100% denaturing agent is defined as 7mol L-1 urea and 40% deionized formamide). The gradient was optimised to allow for maximum band separation and to suit the primer sets used for the different taxonomic groups. Details of the gradients and gel concentrations for each primer set can be seen in Table 2-3. Electrophoresis was carried out at a total of 900 Volt hours (a constant 200Volts for 4.5 hours). Gels were stained using SybrGold for half an hour and visualised with a UV transilluminator with the program Quantity One (BioRad).

A DGGE marker was run either 3 or 4 times on each gel, at either side of the sample lanes and in the middle. This provides a predictable ladder pattern that can be seen on each DGGE image. These lanes are used for image processing.

Table 2-3 DGGE gel gradient conditions optimised for different PCR primer sets

Primer set	**Specificity**	**Acrylamide concentration (%)**	**Gradient (%)**	
			Low	High
F357GC & R518	Bacteria	8	20	50
Nested Cya-b-T371 & Cya- R783 products with F357GC & R518	Cyanobacteria	8	20	50
Euk 1A & Euk 516r GC	Eukarya	6	15	40
Nested Euk 1A &Euk1209r products with Euk1A & Euk516r GC	Eukarya	6	15	40

2.4.3 Band cutting and sequencing

Bands were excised from DGGE gels after staining and imaging. The gel was placed on a flat UV transilluminator (UVP, UK) and the bands cut out using sterile syringe needles. Bands were stored in 50µl of TE buffer and frozen at -20°C. Bands were melted into the TE buffer on a 95°C hot plate and PCR carried out following the standard procedure using the same primers used in the original amplification. The PCR amplified DNA was then examined using agarose gel electrophoresis to determine if the amplification of the band was successful and the product the expected size. The PCR products were then cleaned up using Qiagen's PCR purification kit.

10ul of this cleaned up product was then sent to GeneVision Ltd (Newcastle, UK) for sequencing. Sequencing was carried out using the Sanger sequencing method. Sequences were returned from the company as files that were opened using Chromas Lite software (Technelysium Pty Ltd, Australia). Sequences had their ends trimmed and were compared to the nr database in the NCBIs BLAST search tool online (Altschul *et al.*, 1990).

DGGE image processing

DGGE images were prepared for analysis using BioNumerics software (Applied Maths, Belgium). In this software sample lanes from a number of DGGE gels can be aligned to each other based on the marker lanes. The software corrects for any irregularities in gradient forming and a normalised image produced. After normalisation, bands were matched, using the 'Auto detect bands' function, though these assignments were manually confirmed. The data generated from the gel, namely the band height quantification values were exported.

Band height quantification values from the DGGE image was imported into Primer6 software (Clarke and Gorley, 2006) for further processing. Diversity statistics were generated using the DIVERSE function with the non-transformed band height quantification data. Species richness (S) was calculated using presence absence data from normalised and Pielou's evenness index (J') using band height data from normalised DGGE gel images. Each band was deemed to represent a unique operational taxonomic unit (OTU). Pielou's evenness index is a measure of equitability and was calculated using equation 1 below, where H'max is the maximum possible value of Shannon Wiener diversity (H').

$$J' = \frac{H'}{H'max} = \frac{H'}{logS}$$

Non-metric multivariate analysis was carried out in the form of MDS (Multidimensional Scaling) and ordination plots produced using presence-absence data generated from the DGGE images and Bray-Curtis similarities. Ordination plots visually represent the similarity of samples, with special proximity representing similarity. Stress values (given for each MDS ordination plots) represent the degree to which the spatial representation is successful, a value close to zero being excellent, 0.1 being good and

anything over 0.3 suggesting poor or no better than random distribution(Clarke and Gorley, 2006). Cluster analysis calculated using the group average method in Primer6, was overlaid onto MDS ordination plots in the form of contours to represent sample similarity.

One way analysis of similarity (ANOSIM) was used to assess the difference between samples as required (Clarke, 1993).

Primer 6's BEST- BioEnv function was used to assess the relationship between the collected environmental data and the observed biologically pattern seen in samples. This methodology is covered in more details in the chapters in which it is used (Chapter 7).

2.4.4 Fluorescence In-Situ Hybridisation

Two general methods were used for FISH described in in Chapter 4, a tube-based method and a slide-based method. The general principle of the two methods is the same, with the same buffers and wash solutions being used. Details of the probes, conditions and any adaptations will be detailed in Chapter 4.

Samples used for FISH were fixed with 4% paraformaldehyde, as described earlier. Microscope slides were prepared in advance for both of the methods. The slides were cleaned by immersing them in a 10% solution of Potassium Hydroxide (w/v) in 95% ethanol for an hour. Slides were then washed thoroughly in distilled water and air dried. A gelatine coating solution (0.1% gelatin, 0.01% $CrK(SO_4)_2$ in distilled water) was heated to 70°C in a water bath. Slides were immersed in the solution for 3 minutes, dried for 5 minutes and this step repeated a total of four times. Slides were then dried and stored in the dark until use. Hybridisation buffers and wash buffers were made according to Table 2-4 and Table 2-5 respectively.

Table 2-4 Hybridisation buffer concentrations for FISH dependant of formamide conditions required

	Formamide concentration					
	20%	**30%**	**40%**	**50%**	**60%**	**70%**
4.5 M NaCl (ml)	0.2	0.2	0.2	0.2	0.2	0.2
200 mM Tris HCl, pH 7.2 (ml)	0.1	0.1	0.1	0.1	0.1	0.1
10% SDS (µl)	10	10	10	10	10	10
Deionised formamide (FA) (ml)	0.2	0.3	0.4	0.5	0.6	0.7
Filter sterilised water (ml)	0.5	0.4	0.3	0.2	0.1	0
Total volume (approx.) (ml)	1	1	1	1	1	1

Table 2-5 Wash buffers for FISH
With concentrations dependent on the amount of formamide used in the preceding hybridisation step

Equivalent % formamide	**20%**	**30%**	**40%**	**50%**	**60%**	**70%**
Equivalent conc. NaCl (nM)	225	112	56	28	14	7
4.5M NaCl (ml)	1	0.5	0.25	0.125	0.063	0.031
0.5 M EDTA, pH 8.0 (µl)	200	200	200	200	200	140
200 mM Tris-HCl, pH 7.2 (ml)	2	2	2	2	2	2
10% SDS (µl)	200	200	200	200	200	200
Make up to total vol. with filter sterilised water (ml)	20	20	20	20	20	20

Two negative controls were hybridised alongside the samples, one of these was used to observe auto fluorescence (no probe added) and another to observe non-specific binding of the probe, containing non target organisms (in this case activated sludge). A nonsense probe designed not to bind to any known organism, was used in the initial FISH test, but its use was discontinued to reduce the number of tests required during method development.

The slide-based method was carried out as follows; PFA fixed cells stored in ethanol and PBS were washed and resuspended in PBS. 10µl of the sample was then added to a well of the gelatine coated slide and air dried for 2 hours at 37°C. The sample on the slide was serially dehydration in ethanol (50, 80 and 96% ethanol) for three minutes at each concentration an air dried. Formamide hybridisation buffer (9µl) and 1µl of probe was added to the well. For the 'no probe' negative 10µl of hybridisation buffer was used. Slides were then incubated at 46°C for 1.5 hours in an isotonic chamber. The slide was then flushed with the corresponding wash solution and immersed for 15 minutes at 48°C. This step was repeated and the slide rinsed in sterile water and air-dried. Citifluor antifadant (Citifluor Ltd, London, UK) and a coverslip were added.

The tube-based method was carried out as follows; 200µl of the PFA fixed sample was added to a sterile 2ml tube and centrifuged at 13,000 x g. The supernatant was then removed and the sample washed using 500µl of PBS, mixing, centrifuging for a further 3minutes and removing the supernatant. The pellet was then resuspended in 500µl of ethanol for serial dehydration (using the same concentrations as the slide-based method). Each solution was used for 3 minutes followed by 3 minutes centrifuging at 13,000 x g, removing and discarding the supernatant between each successive dehydration step. After the ethanol was removed 38µl of hybridisation buffer and 2µl of probe were added. The tube was then mixed and placed in a heating block at 46°C for 2

hours. Cells were then pelleted out by centrifugation at 13,000 x g for 3 minutes and the supernatant discarded. 500µl of wash buffer was added and mixed with the sample, before incubating for 15 minutes at the hybridisation temperature. The centrifuging and wash steps were repeated. The sample was then centrifuged again to remove the wash buffer and washed in 500µl of ice-cold filter sterilised water. After further centrifuging and removal of supernatant the pellet was resuspended in 50µl of ice-cold filter sterilised water. A 10µl spot of sample was added to the well of a gelatine coated slide and allowed to dry. Citifluor antifadant and a coverslip were added. Slides were viewed on a Leica TCS SP2 UV, a confocal laser scanning microscope (CLSM) (BioImaging suite, Newcastle University Medical School).

Chapter 3. Chapter 3- Evaluation of DNA extraction methods for freshwater eukaryotic microalgae

3.1 Introduction

The DNA extraction method used can have a major impact on downstream community analysis of samples. Eukaryotic microalgae have a large range of cell wall structures, which create challenges for the unbiased, uniform and universal extraction of nucleic acids from such communities. Some microalgae have simple glycoprotein cell walls, while others contain decay resistant algaenans or silica compounds. It is therefore extremely important to identify DNA extraction methods that are effective for a broad range of cell types for total community DNA analysis. Table 3-1 summarises the cell wall composition of freshwater algal groups highlighting those reported to be present in WSPs.

Simonelli et al. (2009) tested eight protocols, including four commercially available kits on ten cultured marine microalgae to determine which protocol gave the best results in terms of DNA quantity and quality. They concluded that Qiagen's Blood and Tissue (QBT) kit, Qiagen's Plant Mini (QPM) kit and the Ultra Clean (UC) soil DNA isolation MoBio kit stood out as being the most effective in terms of extracting DNA that could be used to produce PCR products from a range of pure cultures. These three favoured kits have been used successfully in a number of mostly marine algal studies; QBT in Shi et al. (2009), Maloy et al. (2009) and Ghosh and Love (2011), QPM in Bowers et al. (2000), Dorigo et al. (2002) and Galluzi et al. (2005) and UC in Simonelli et al. (2009), and Nejstgaard et al. (2008). While these methods are valid for marine samples they might not necessarily be applicable to freshwater eukaryotic microalgal communities, due to inherent differences in community structure (and therefore cell wall types). The levels of inhibitory substances common in WSP, such as humic acids (Amir *et al.*, 2006), also have the potential to inhibit downstream processes such as PCR (Wilson, 1997).

In this chapter the application of the three commercially available kits outlined above for the extraction of DNA from freshwater eukaryotic algae were investigated, in both pure cultures and mixed natural consortia in WSP samples. DNA extraction was evaluated in terms of total DNA yield and purity, as well as the success in the amplification of targeted fragments of the 18S rRNA gene by PCR.

Table 3-1 Typical cell wall structures of the major classes and orders of microalgae (van den Hoek *et al.*, 1995)

Division (phylum)	Class	Orders	Cell wall structure and materials	Representatives known to be present in WSPs
Heterokontophyta	Chrysophyceae		varied- some naked, felt like mesh of microfibrils and covered with silica scales	
	Xanthophyceae		cellulose microfibrils, sometimes impregnated with silica	
	Eustimatophyceae		polysaccharide cell walls	
	Bacillariophyceae		silica cell wall, frustule- like a box with an overlapping lid. 2 types pennate and centric, frustrule of pennate diatom like a box and lid, centric diatoms frustule like a petri dish	*Nitzschia sp.*
Haptophyta	Haptophyceae		cell wall made up of granules of cellulose, some also have calcified scales	
Cryptophyta	Cryptophyceae		cell wall made up of proteins (stiff) in a series of rectangular plates, some fibrillar material sometimes present	*Cryptomaonas erosa*
Dinophyta	Dinophyceae		layer of flat vesicles, often containing cellulose plates	
Euglenophyta	Euglenophyceae		pellicle made up predominantly of proteins wound around the cell in a helix, below the pellicle is an array of microtubules, mucilage consisting of glycoproteins and complex polysaccharides	*Euglena valiabilis, E.sanguinea, E.gracilis, E.clavata, E.sp., E.clara, Phacus triquetre, Phacus sp., Lepocynclis ovum*
Chlorarachniophyta	Chlorarachniophyceae		naked, no cell wall	
Chlorophyta	Prasinophyceae		e.g. Pyramimonas- no typical cell wall, have layers of scales	
		Volvocales	e.g. Chlamydomonas- fibrous glycoproteins (no polysaccharides) forming crystalline surface layer, protein fraction- hydroxyproline, carbohydrate fraction- galactose, arabinose, mannose & glucose	*Chlamydomonas reinhardii, C.caeca, Pandorina morum, Haematococcus pluvialis, Eudorina elegans, Coccomonas sp., Polytomella sp., P.tetraolare, Carteria sp.*
		Chlorococcales	firm polysaccharide walls, lack glycoprotein lattice, some spores have cellulose wall inside the glycoprotein envelope. Some chlorococcales have sporopollenin. Scenedesmus cell wall has inner layer of sporopollenin-like substance. *Pediastrum* cell walls contain silica and an outer layer of sporopollenin-like material	*Chlorella sp., Oocystis sp., Micractinium pusillium, M.sp, Pediastrum clanthatum, Scenedesmus sp.*
	Cladophorophyceae		principle polysaccharide in cell wall is highly crystalline cellulose I, arranged in a fibrillar pattern. Branched arabino-galactan makes up most of amorphus outer fraction.	
	Bryopsidophyceae		fibrillar layer mostly mannan, xylan and glucan (cellulose). Cellulose in these species not highly crystalline.	
	Zygnematophyceae		3 layers: outermost layer of mucilage (composed of complex polysaccharides) the 2 layers of microfibrillar cellulose. The fibres in the 2 layers are aligned differently. Main fibrillar component is crystalline cellulose	
	Charophyceae		crystalline cellulose microfibrils in a crossed fibrillar pattern	

The diversity of dominant species in natural mixed cultures was also assessed using DGGE.

In addition, the effect of ethanol fixation on DNA extraction and subsequent PCR, was investigated, as fixation is often used in the field to preserve cell morphology and community composition when samples cannot be immediately frozen. Ethanol is the simplest and safest fixative, which has previously yielded PCR products from some marine algae (Marin *et al.*, 2001), in contrast to other common fixatives such as formalin and Lugol's solution, which in some cases have been shown to interfere with subsequent PCR reactions (Ahokas and Erkkila, 1993; Wilson, 1997; Marin *et al.*, 2001; Godhe *et al.*, 2002). A summary of available fixatives tested on microalgae found in the literature can be seen in Table 3-2.

Table 3-2 Fixatives commonly used in microalgal studies and their reported effects on PCR amplification. Red = no visible PCR product (using agarose gel electrophoresis), yellow, unreliable PCR (either faint or non-reproducible amplification), green= positive PCR

Fixative	**PCR reaction**	**Reference**
Lugol's solution		Godhe *et al.* (2002), Marin *et al.* (2001)
Gluteraldehyde (5%)		Marin *et al.* (2001)
Neutral formalin (4%)		Marin *et al.* (2001)
Formalin-methanol		Godhe *et al.* (2002)
Frozen methanol		Marin *et al.* (2001)
Frozen ethanol		Marin *et al.* (2001)
Ethanol dilution- 75-80%		Godhe *et al.* (2002)
Ethanol dilution->80%		Godhe *et al.* (2002)

3.2 Materials and Methods

3.2.1 Sample collection from WSP

Samples were collected from a WSP system that serves Larchfield community in Teesside, UK. The samples were collected from a cascade that feeds wastewater from one pond to another. 12 samples of 100ml and 12 samples of 250ml were collected and frozen at -20°C on return to the laboratory. Another six 250ml samples were collected.

These samples were fixed with 250ml of 98-100% ethanol in the field and then frozen at -20°C on return to the laboratory.

Tropical samples were collected from two WSP systems in Fortaleza, Ceará, in the northeast of Brazil. One of the systems, SIDI served the industrial district of the city, with a mixed influent, approximately 50% from industrial sources and 50% from domestic sources. The other system, Marechal Randon, was fed purely domestic wastewater. The sampling sites and positions can be seen in Figure 7-1. Tropical samples were collected in the same way as UK samples from all of the ponds in both systems, though none of the samples were fixed with ethanol.

3.2.2 Sample preparation

Samples were defrosted and then centrifuged at 3,392x g (4,200rpm) for 2 hours or 7,690x g, 10 minutes, which were shown to give the highest percentage removal of cells (approximately 99.8%) in trials using different centrifugation times (data not shown). Cell counting was carried out on WSP and pure culture samples using a Sedgwick rafter counting slide.

3.2.3 Eukaryotic algal cultures

Five cultured algal species were used in this study (Table 3-3). The species were cultured in a variety of media as advised by CCAP (Culture Collection of Algae and Protozoa- Scottish Marine Institute) (see Appendix 1) . All the cultures were grown at room temperature using a dark:light cycle of 8:16 hours. One of the cultures, the diatom *Navicula pelliculosa* was obtained from MAST at Newcastle University.

Table 3-3 Algal species chosen as references.
The species taxonomic classification, characteristics and culture conditions. Media recipes in Appendix 1.

Algal species and strain number	Class-order	Origin	Culture medium	Characteristic cell features	Assumed ease of lysis
Chlamydomonas reinhardtii CCAP- 11/45	Chlorophyceae-Volvocales	Edgewood Park, Connecticut, USA	3N-BBM+V Agar slope	Layered glycoprotein cell wall	Medium
Chlorella vulgaris CCAP- 211/80	Trebouxiophyceae - Chlorellales	Molkerteich, Elsnigk, Sachsen-Anhalt, Germany	3N-BBM+V Agar slope	Glucose and mananose or glucosamine cell walls	Medium
Pandorina morum CCAP-60/2	Chlorophyceae-Volvocales	Priest Pot, Cumbria, England	3N-BBM+V Liquid media	Globular colonies of 16-32 cells	Easy
Scenedesmus quadricauda CCAP- 276/21	Chlorophyceae-Chlorococcalles	Priest Pot, Cumbria, England	EG:JM Liquid media	Cell walls contain decay resistant algaenans	Difficult
Navicula pelliculosa CCAP-1050/9	Bacillariophyceae, Naviculales	Oyster pond, Marthas vineyard, Massachusetts, USA	F2 liquid medium with air bubbling	Silica cell wall	Difficult

The five species were chosen as they are known to be present in WSPs (Mara, 1997; El-Deeb Ghazy *et al.*, 2008). They encompass a range of algal groups and differ in terms of cell wall characteristics that may affect the relative efficiency of DNA extraction.

3.2.4 DNA extraction

Three kits commonly used for marine algal samples were used on each of the pure culture samples, the non-fixed WSP samples, and the ethanol fixed WSP samples. Only the QBT kit was used to extract DNA from the tropical WSP samples. Each of the WSP sample extractions were carried out 4 times, in duplicate from 100ml of sample (containing approximately 1.6 x 10^6 cells) and in duplicate 250ml of sample (approximately 4.1x10^6 cells).The kits were Qiagen DNeasy® Plant Mini kit (QPM), Qiagen DNeasy® Blood and Tissue kit (QBT), and MoBio UltraClean™ Soil DNA Isolation kit (UC).

The UC kit was used following the manufacturer's instructions to maximise DNA yields with minor modifications, as follows. A HybaidRiboLyser was used for the mechanical lysis step in place of the MoBioVortexer and adaptor, which were unavailable in the laboratory. In a previous trial at Newcastle University both of these machines were shown to yield similar results for the extraction of DNA from bacteria (data not shown). DNA was eluted into 50µl of elution buffer (10mM Tris at pH 8).

Extraction using QPM was carried out following the manufacturer's instructions with minor modifications. This method includes freezing in liquid nitrogen and bead-beating with Tungsten Carbide beads. A Mikro-dismembrator U (B.Braun Biotech International) was used instead a Tissue Lyser Adapter Set for the bead-beating step. Two elutions of DNA in 100µl of Buffer AE, (10mM Tris Cl, 0.5mM EDTA at pH 9.0) were collected, giving a total of 200µl.

Extraction using QBT was carried out according to manufacturer's instructions using the Animal Tissue protocol. Lysis was carried out by incubation with proteinase K for approximately five hours at 56°C. RNase A and liquid nitrogen were not used. Two elutions of DNA in 200µl of Buffer AE, (10mM Tris Cl, 0.5mM EDTA at pH 9.0) were collected, giving a total of 400µl. The extracted DNA elutions were frozen at -20 °C until PCR was carried out.

3.2.5 DNA quantification and purity

A Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Inc.) was used to quantify the DNA present in all of the DNA extracts. The quality or purity of the elution in terms of the presence of humic acids (indicated by the absorbance ratio at 260 nm/230 nm) and protein contaminants (indicated by the absorbance ration at 260 nm/280 nm) was also assessed using the Nano Drop.

3.2.6 Amplification of 18S rRNA gene fragments

Amplification of 18S ribosomal RNA gene fragments was carried out in duplicate by PCR using primers Euk 1A and Euk516r that target members of the *Eukarya* domain (Diez *et al.*, 2001). A GC clamp was added to the 5'-end of Euk516r for subsequent DGGE analysis. PCR was carried out according to the method set out in Chapter 2.

3.2.7 Diversity analysis

The diversity of the predominant members of the eukaryotic communities was evaluated using DGGE. DGGE was carried out according to the standard method stated in Chapter 2, using a 6% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) with a linear gradient of denaturing agents from 15% to 40%.

3.2.8 Statistical analyses

Statistical analyses were carried out using MiniTab v15 software. Two way-ANOVA tests were carried out to assess the effects of the extraction kits on the different samples. The same tests were carried out to compare the different contaminant levels, 260:230 ratios, and 260:280 ratios. Samples were grouped prior to statistical testing into pure cultures, WSP samples, and fixed samples. Quality data was tested for normality and conformed. Quantity data was transformed using transformations recommended in Box-Cox Transformation test.

BioNumerics (Applied Maths, Belgium) was used to define and normalise bands within the DGGE gel and to perform cluster analysis. The Shannon-Wiener diversity index was calculated as follows using the relative intensity of bands in each sample (quantified in BioNumerics) as a proxy for the proportional abundance of each band, each of which was deemed to represent a unique operational taxonomic unit (OTU).

$$H' = -\sum p_i \ln p_i$$

Where H' = Species Diversity Index

p_i = the relative contribution of band i intensity to the total band intensities for the whole lane

3.3 Results

3.3.1 Total DNA yield

The quantity of DNA extracted by the three kits and the presence of contaminants are shown on Figure 3-1. As the elution volumes of the kits differed, the total DNA extracted per kit was calculated to make a valid comparison among kits. These values are used in all further statistical analysis.

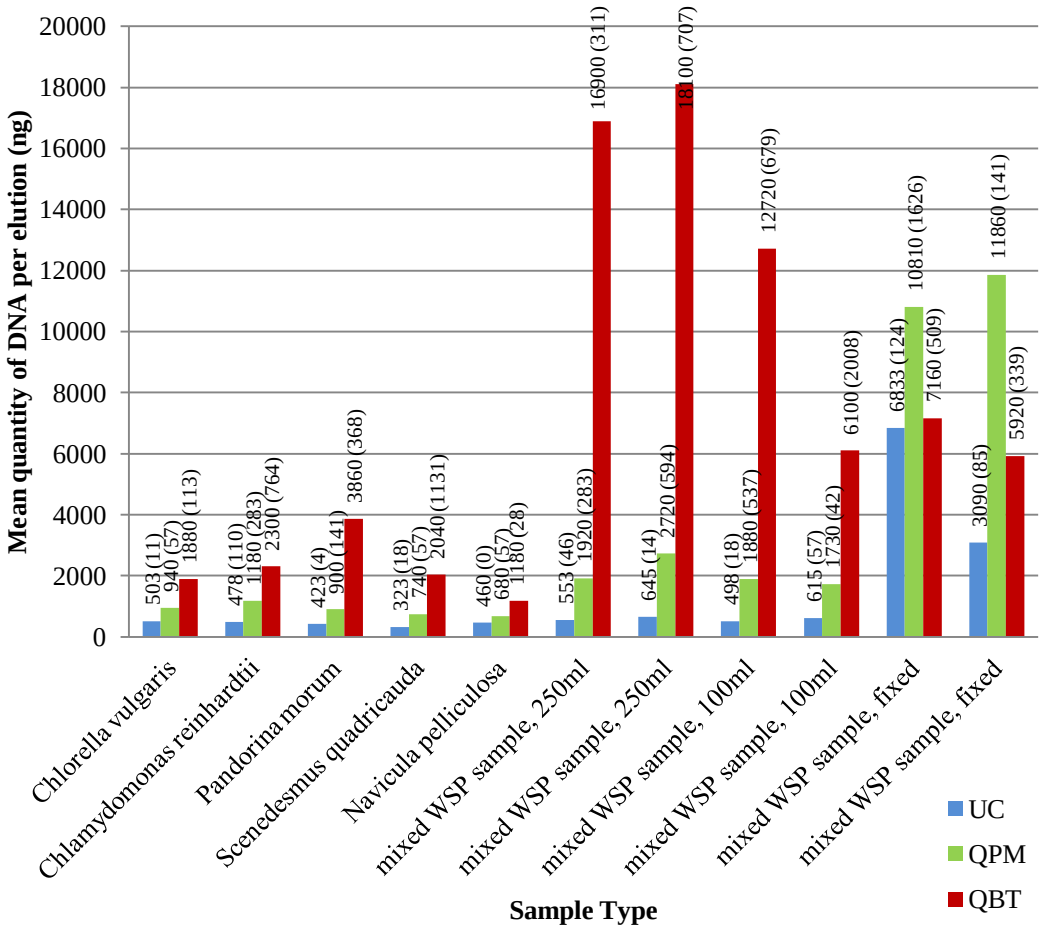


Figure 3-1 Total quantity of nucleic acids extracted by each of the DNA extraction kits per elution (ng) Total quantity of DNA per elution is the mean of two NanoDrop reading on the final elution mixture multiplied by the elution volume. The starting cell concentration of pure cultures was lower than that of WSP samples. Numbers in brackets are standard deviations of two nanodrop readings.

All extractions yielded DNA, though this varied greatly in both quality and quantity, with DNA yields from 323ng for pure culture *S. quadricauda* using the UC kit to 18100ng for one of the WSP samples, using the QBT kit. A summary of the data for the quantity of DNA eluted by each of the three kits for the samples is shown in Figure 3-1. Two Way ANOVA tests carried out on the quantity of DNA extracted from non-fixed WSP samples indicated that there was a significant difference (95% confidence) between the 3 kits tested (p-value = <0.001). QBT extracted significantly higher quantities of DNA of the three kits and UC the lowest. The same pattern was seen with the pure culture samples with a p-value of <0.001. Samples of 250ml did not yield significantly more DNA than samples of 100ml. In order to confirm the success of QBT in terms of DNA yield, DNA from a number of different tropical WSP samples was extracted using this kit. The results, in Table 3-4, confirm that a consistently high yield of DNA could be obtained across a range of pond types in systems treating both domestic and mixed industrial wastewater.

DNA yields for ethanol fixed WSP samples, however, showed a different pattern, with a much increased yield for the UC kit, compared to the non-fixed samples. There were, however, no significant differences (95% confidence) between quantities of DNA extracted when comparing the three kits.

3.3.2 Quality of the DNA extracted

The ratio of absorbance at 260 nm and 280 nm wavelengths can be used as an indicator of the presence of DNA compared to contaminants that absorb light at 280nm wavelengths, typically proteins. DNA extractions with a 260/280 ratio of above 1.80 are deemed to be of high quality and suitable for use in downstream applications. In reality many DNA extractions from environmental samples do not meet this standard and further purification methods are commonly used before the DNA is used downstream. 260/280 ratio values ranged from 1.0 to 3.4, with 39% of samples tested achieving a value of 1.8 or greater (Table 3-4). The UC kit achieved values greater than 1.8 with 64% of samples, QBT with 45% and QPM with only 9%. For samples from the tropical WSP systems tested only with the QBT kit, all extractions achieved the 1.8 threshold (Table 3-5). ANOVA tests indicated that the 260/280 ratio did not vary significantly with the three kits for pure cultures (p-value=0.06), WSP samples (p-value=0.107) and for fixed WSP samples (p-value=0.250).

Similarly, 260/230 ratio can be used to quantify the level of other contaminants, such as humic acids commonly present in the DNA elutions of environmental samples. A chemical commonly used in DNA extraction kits, guanidine thiocyanate, can also absorb light at 230nm. This chemical is not highlighted as a component of any of the three kits tested though the full chemical content of all of the buffers in the kits is not published. Values of 2 or more would be considered high quality samples, less than this indicates the presence of contaminants that absorb light at 230 nm, such as carbohydrates, guanidine thiocyanate, phenols and humic acids (Sambrook *et al.*, 2001a). In all samples, 260/230 ratios were below two, with a range of 0.08 to 1.25. In pure culture tests, there were no significant differences in quality between kits (p-value= 0.433). For non-fixed WSP samples, the kit used did show significant differences, with QBT outperforming the other kits (p-value=0.009). For the fixed samples, the kit used had a significant impact on the 260/230 ratio, and QPM outperformed the other kits (p-value= 0.007). The volume of sample or the pure culture microalgal species tested had no significant impact on the 260/230 ratio.

Table 3-4 260/230 ratios, corresponding to possible humic acid contamination and the 260/280 ratios, corresponding to possible protein contamination, for each of the three kits.

Sample	**260/230 Ratio**			**260/280 Ratio**		
	UC	**QPM**	**QBT**	**UC**	**QPM**	**QBT**
C. vulgaris	0.08 (0.014)	0.17 (0.014)	0.17 (0.014)	3.4 (0.5)	1.5 (0.085)	1.5 (0.085)
C. reinhardtii	0.21 (0.035)	0.24 (0.042)	0.17 (0.042)	2.2 (0.007)	1.2 (0.014)	2.8 (1.2)
P. morum	0.13 (0)	0.21 (0.028)	0.31 (0.014)	2.3 (0.45)	1.1 (0.028)	1.4(0.049)
S. quadricauda	0.23 (0.014)	0.18 (0.021)	0.19 (0.078)	2.9 (1.2)	1.3 (0.21)	1.4 (0.21)
N. pelliculosa	0.19 (0.007)	0.2 (0.007)	0.16 (0.007)	2.4 (0.049)	1.0 (0.035)	1.7 (0)
mixed WSP sample, 250ml	0.36 (0.06)	0.405 (0.04)	0.96 (0.04)	1.79 (0.08)	1.965 (0.3)	2.025 (0.021)
mixed WSP sample, 250ml	0.185 (0.01)	0.46 (0.01)	0.97 (0.03)	1.855 (0.04)	1.21 (0.7)	1.98 (0.04)
mixed WSP sample, 100ml	0.32 (0.13)	0.37 (0.04)	0.92 (0.06)	1.565 (0.03)	1.565 (0.09)	1.865 (0.02)
mixed WSP sample, 100ml	0.75 (0.03)	0.255 (0.01)	0.62 (0.01)	1.715 (0.01)	1.51 (0.2)	2.045 (0.11)
mixed WSP sample, fixed	0.28 (0.01)	0.675 (0.01)	0.255 (0.01)	2.31 (0.01)	1.485 (0.04)	1.725 (0.02)
mixed WSP sample, fixed	0.135 (0.04	0.68 (0)	0.25 (0.01)	1.715 (0.7)	1.505 (0.01)	1.665 (0.08)

Values of 2 or more are considered 'high quality' for the 260/230 ratio and of 1.8 or more for the 260/280 ratio. The values in brackets are the standard deviations of two Nano Drop readings taken per elution.

Table 3-5 Quantity (ng/elution) of DNA extracted from tropical WSP samples using the QBT extraction kit.
Data is shown for two systems, one treating purely domestic wastewater and the other treating a mixed industrial and domestic effluent. Values in brackets are standard deviations.

Sample	Mean Quantity of DNA (ng/elution)		Mean 260/280[1]		Mean 260/230[1]	
	Domestic	Industrial	Domestic	Industrial	Domestic	Industrial
Anaerobic pond	24020 (10222)	41162 (27008)	1.9 (0.08)	1.8 (0.24)	0.91 (0.16)	0.34 (0.18)
Facultative pond	14011 (2481)	16423 (2930)	2 (0.02)	2.1 (0.15)	0.73 (0.02)	0.66 (0.2)
Maturation pond 1	22072 (3195)	16705 (1667)	2 (0.06)	2 (0.18)	0.84 (0.15)	0.7 (0.27)
Maturation pond 2	20445 (6062)	11477 (2287)	2 (0.04)	2.2 (0.13)	0.77 (0.14)	0.49 (0.12)
Maturation pond 3[2]		7796 (2724)		2.3 (0.16)		0.49 (0.12)

[1]*The 260/280 and 260/230 ratios indicating quality of the extraction in terms of protein and humic acid contamination respectively.*

[2]*The system treating domestic wastewater did not have a third maturation pond. Discharge occurred after maturation pond 2.*

3.3.3 PCR amplification of 18S rRNA gene fragments

The successful amplification on the DNA extracted is vital if the kit is to be used as the starting point for further molecular techniques. Agarose gel electrophoresis, with appropriate markers, was used to determine if amplification of the target 560 base pair fragment was successful. For the pure cultures, the QPM kit failed to extract DNA of sufficient quality for PCR amplification in three of the species tested, namely *C. vulgaris*, *P. morum* and the diatom *N. pelliculosa* (Figure 3-2). The UC and QBT kits were both successful in extracting PCR amplifiable DNA from all five of the cultured species, despite their varied cell wall structures and components.

In the frozen WSP samples, PCR amplification was more successful; all of the kits provided DNA that resulted in a positive PCR product of the desired size. These results show that all kits extracted DNA of sufficient quality to carry out PCR amplification from frozen algal samples (Figure 3-3a).

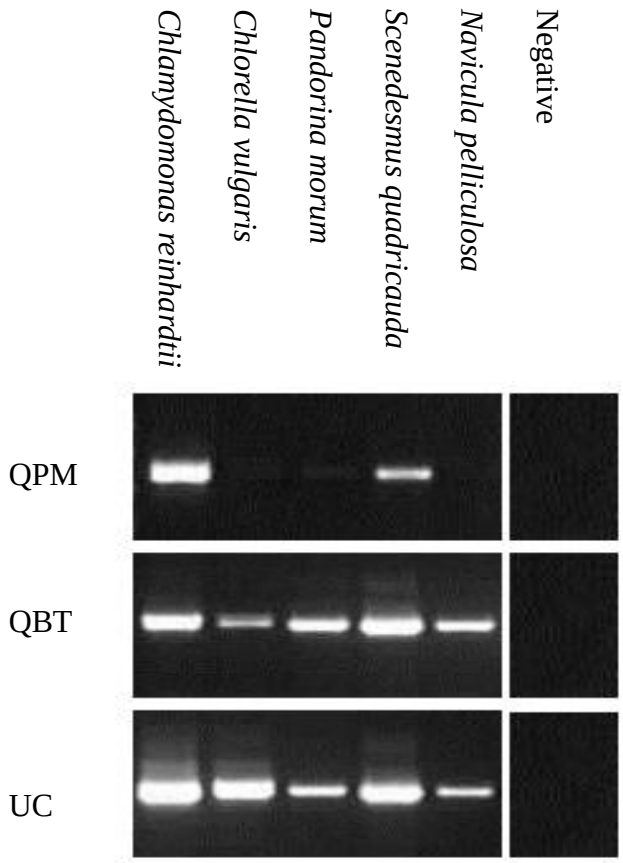


Figure 3-2 Agarose gel showing PCR amplification of products approximately 560 basepairs in length, from the DNA extracted from pure cultures with QPM, UC and QBT kits.

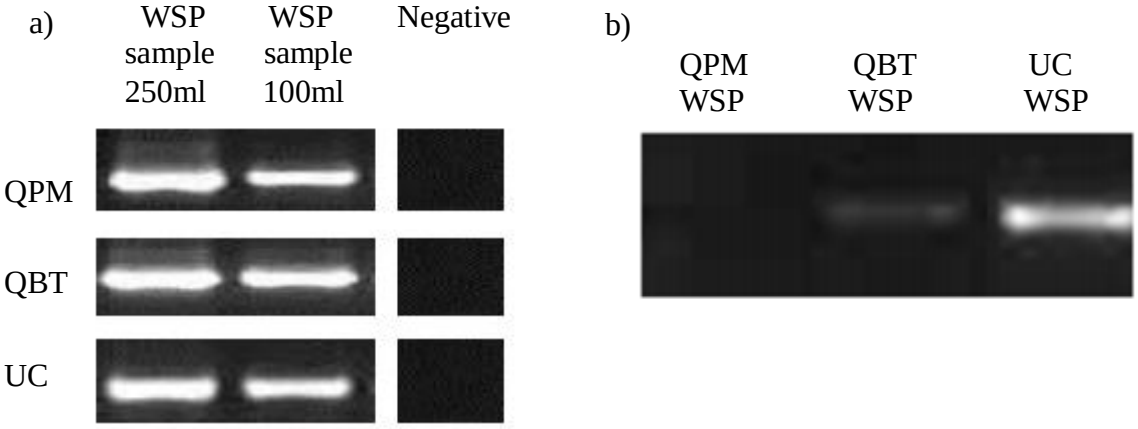


Figure 3-3 a) Agarose gel showing PCR products from frozen WSP samples, comparing the success of three DNA extraction kits, QPM, QBT and UC, and a negative control. b) Agarose gel showing PCR performed using DNA extracted from ethanol-fixed cells using kits QPM, QBT and UC.

Fixing WSP samples with ethanol appears to have a strong negative impact on the quantities of DNA eluted and on the success of the PCR reaction (Figure 3-3b). DNA from the QPM kit failed to yield any PCR product in both of the fixed samples. PCR of DNA extracted using the QBT kit resulted in faint bands compared to other bands on the same gel, suggesting that very little PCR product was produced.

3.3.4 Assessment of diversity using DGGE

DGGE was carried out on the PCR-positive samples to assess the diversity of eukaryotes within the mixed culture WSP samples (Figure 3-4) and evaluate whether the extraction methods tested were biased towards cells with specific wall types. Both the band richness (number of bands) and Shannon-Wiener diversity index were used to evaluate the diversity of the predominant eukaryotic community members (Table 3-6). There was no significant difference in the Shannon-Wiener index ($P = 0.82$) or band richness ($P = 0.18$) for DNA extracted from 250ml and 100ml of sample. The Shannon-Wiener index was similar for all of the non-fixed samples, although it was highest for the 100ml WSP sample when the QBT kit was used.

OTU band richness for those samples using DNA extracted by the UC and QBT kits was just two and one respectively. As expected, no bands were seen in those samples of DNA extracted using the QPM kit, which had been derived from a PCR-negative reaction. Ethanol fixation clearly had a negative effect on both DNA extraction and PCR amplification, which also resulted in a greatly reduced observable eukaryotic diversity of the samples analysed.

Table 3-6 Shannon Wiener Index of Diversity and number of bands per sample in the DGGE gel, using BioNumerics.

Sample	Shannon Wiener Diversity Index			Number of bands		
	QPM	QBT	UC	QPM	QBT	UC
WSP 250ml	2.24	2.22	2.29	13	14	15
WSP 100ml	2.11	2.39	2.17	14	15	15
Fixed WSP	0	0	0.40	0	1	2

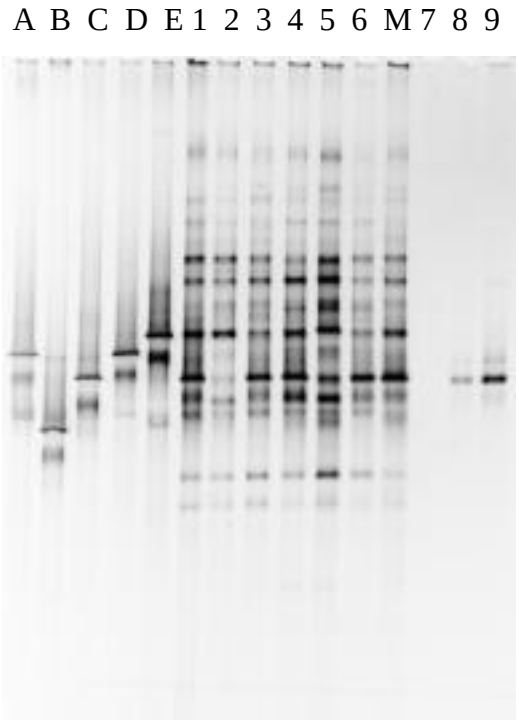


Figure 3-4 DGGE gel of 18S rRNA products.
Lanes 1-6 are non-fixed WSP samples; 1-250ml sample QPM, 2-250ml sample QBT, 3-250ml sample UC, 4-100ml sample QPM, 5-100ml sample QBT, 6-100ml sample UC. 7-9 are WSP samples fixed with ethanol; 7-QPM, 8-QBT and 9-UC. M is the reference lane for use in BioNumerics. Note that bands represent eukaryotic species, not only microalgae due to the generality of the primer set. A-E are pure cultures as extracted by UC. A- *Chlorella vulgaris*, B- *Pandorina morum*, C- *Chlamydomonas reinhardtii*, D- *Scenedesmus quadricauda*, E- *Naviculla peliculosa*

3.3.5 Discussion

Overall, QBT outperformed both QPM and UC for the mixed freshwater microalgal consortia from WSP samples that have been frozen shortly after collection. QBT extracted significantly more DNA than the other kits and this elution was less contaminated according to 260/280 ratio. QBT was one of the two best performing kits in terms of PCR detection of target gene fragments from pure algal cultures. The QBT kit used on a 100ml WSP containing a mixed consortium of microalgae gave the highest observed diversity, although the diversity was found to be similar in all three kits.

The main difference among the protocols of the three kits tested is on the physical, chemical and enzymatic methods of cell lysis used. I speculate that this is the cause of the difference in the extraction efficiencies of the three kits. The QBT protocol involves the incubation of the microalgal cells with the enzyme proteinase K, a general protein

degrading enzyme, which acts to break down cell walls and denature protein contaminants that lead to inhibition of PCR. Its performance in this trial was shown to be widely successful. QBT performed poorly for ethanol fixed samples, this may be due to its reliance on proteinase K. Ethanol is known to cause the cross linking of proteins and thus may be damaging the enzymes before extraction can occur, as well as making cell walls more resistant to protein degradation. The UC kit relied solely on mechanical lysis and was the least successful of the three kits tested. This suggests that bead beating alone may not have been powerful enough to break down the cell walls and release the DNA from some of the tougher cell types present, such as *N. pelliculosa*, which has silica-based cell walls. QPM performed well, though not as well as QBT, suggesting that the addition of freeze-thaw lysis improved the effectiveness of bead beating. Further testing to determine whether the lysis technique applied was the most important contributing factor to the effectiveness of the extraction protocol as done in Miller (1999) would validate this.

QBT is also a very practical kit to use on many levels. It has a much simpler protocol, also agreed by Nejstgaard et al (2008), requires less steps than the other kits and does not involve the use of either liquid nitrogen (as in QPM) or any expensive laboratory equipment, such as a bead-beater. Instead, it makes use of basic laboratory equipment, such as a micro-centrifuge and an incubator. This makes it extremely useful when conducting WSP studies and processing samples in laboratories not especially set up for microbiological studies and were the purchase of expensive equipment is not possible. The cost of the three kits was similar.

DNA extraction was shown to be most effective using the QBT kit on pure culture samples and mixed consortia of microalgae from geographically distinct zones in WSPs having distinctly different environmental and operational conditions, and which may therefore be likely to harbour different microalgal species. Indeed, DNA extracted from tropical WSP samples showed the greatest DNA yields and higher quality, with less contamination, particularly from proteins, than other samples in this study.

There is much evidence to show that changes in culture conditions or environmental conditions, such as nutrient levels, temperature and light conditions has wide reaching effects on lipid contents (Pribyl *et al.*, 2012) and growth and community composition of algal species. Although a range of representatives from commonly occurring algal

groups, with varying morphologies was included in the testing, there are a number of other algal groups, such as Euglenophytes that were not included. These groups could potentially react differently to the tested species in terms of DNA extraction efficiency.

Despite the clear result that the QBT kit outperformed the other kits tested in many ways, there remain some limitations to molecular work on microalgae. DGGE is a semi-quantitative technique relying on PCR which can be biased by the presence of inhibitors (Wilson, 1997). Separate bands in a gel are assumed to be from different species, though it has been known for multiple bands to show the same sequence when further analysed (Janse *et al.*, 2004). In Figure 3-4, multiple bands are seen in supposedly 'pure cultured' algae, this may be due to the different migration of sequences within the same organism. The dominant bands in each of the pure culture lanes was isolated and sequenced and found to match database sequences from their named species. One of the bands seen in the chlorella vulgaris culture lane matched the sequence for a protest common in freshwater, suggesting culture contamination. Additionally, one species may be represented by more than one band as 18SrRNA gene copy number can vary among microalgal species (Zhu *et al.*, 2005). Therefore the number and intensity of the bands within the DGGE cannot be said to be directly proportional to the concentration of the species of interest in the sample. Therefore some caution needs to be applied when interpreting the Shannon Wiener Index of diversity. Organisms that occur at low relative abundance are also likely to be underestimated or missed completely as DGGE has been shown to be able to detect only organisms that make up at least 1% of the DNA within a sample (Akarsubasi *et al.*, 2009). This limitation could be overcome by using a technique that has a lower detection threshold, such as pyrosequencing. At the time of the experiment there were clear cost implications in applying such techniques, though costs are now coming down.

The fixation of WSP microalgal cells with ethanol was shown to have a negative impact on DNA extraction and PCR amplification. The texture of the pellet of microalgal sample centrifuged from the fixed solutions was noticeably gelatinous. This gelatinous material appeared to clog up the spin filters of the three kits, which may have contributed to the lower levels of amplifiable DNA seen.

3.3.6 Conclusions

In this chapter the effectiveness of a range of commercially available kits for extracting DNA from Waste Stabilization Pond system algae was tested. The viability of extracted DNA for downstream PCR and community analysis, as well as the quality and quantity of the DNA was assessed.

In conclusion

- QBT, the Qiagen Blood and Tissue kit will be used throughout this thesis to extract DNA from Waste Stabilization Pond samples. The kit was able to extract DNA from all the tested pure culture strains and from a diverse range of organisms in the community and thus should help to reduce bias from DNA extraction in downstream community analyses. A 100ml sample or approximately $1.6x10^6$cells are recommended for use with this kit, as additional cells do not significantly improve outcomes and make sample processing more time consuming.
- The fixation of algal samples with ethanol has a detrimental effect on the extraction of viable DNA when using these commercial kits. Freezing the samples as soon after collection as possible is an effective alternative that does not affect extraction efficiency.

This Chapter is the basis for a paper published in the journal 'Water Research', in October 2012 (Eland *et al.*, 2012).

Chapter 4. Development of FISH-Flow Method

4.1 Introduction

The aim of this chapter was to devise a method to sort mixed prokaryotic from eukaryotic photosynthetic organisms in WSPs and other environmental samples. The proportions of eukaryote to prokaryote phototrophs within a sample is not easily quantifiable and dictates the way molecular analysis can be carried out, effecting choices of genes to target and primer sets to use.

There is a need to be able to support non-quantitative and semi-quantitative techniques with numerical data, providing knowledge of abundance and population sizes and thus give a more complete assessment of the diversity and community structure of photosynthetic organisms within the environment. FISH is a quantitative technique (Amann *et al.*, 1995) that has been used extensively to enumerate bacteria. It has been successfully used to quantify microalgae in marine samples, and is often coupled with flow cytometry to provide a rapid counting method (Simon *et al.*, 1995; Biegala *et al.*, 2005).

The role of cyanobacteria within pond systems is not often considered, with Table 1-1 highlighting the relative lack of knowledge about their presence in WSP communities. In many microalgal microscopy studies cyanobacteria are disregarded and their relative proportion in comparison to eukaryotic microalgae remains unknown. Riano *et al.* (2012) found that in an algal bioreactor treating high strength wastewater, the algal species introduced to the system were gradually replaced by Cyanophyceae, suggesting they are an important group to consider in highly loaded systems. Furtado *et al.* (2009) investigated the presence of cyanobacteria in a Brazilian facultative pond. They showed that in this system cyanobacteria were the dominant organisms, and that microcystins are produced within and could be cause for concern in WSPs and their effluents. Cyanobacterial dominance, particularly *Oscillatoria* species, was also observed by Pastich *et al.* (2013) in two Brazilian wastewater treatment systems. Microcystins are cyanotoxins that are toxic to plants and animals and cause liver damage in humans (Nishiwaki-Matsushima *et al.*, 1992). It follows that as these compounds are toxic to other eukaryotes, they may have a negative impact on the eukaryotic algae within WSPs, in turn affect treatment efficiency, though this has not been shown in the literature. The proportion of non-photosynthetic to synthetic organisms within ponds is

also relatively unknown. The balance of oxygen production and consumption between photosynthetic organisms and non-photosynthetic organisms (shown in Figure 1-4) is a major driver of bio oxidation for organics removal. And as such changes in the proportions of these vital organisms is likely to affect treatment efficiency.

4.1.1 Photosynthesis and pigments

Photosynthesis is the conversion of light energy, water and carbon dioxide into glucose with the release of oxygen. Photosynthetic pigments within the organism's cells absorb light photons and transfer electrons for use in the light reaction of photosynthesis. The most common photosynthetic pigment on earth is chlorophyll a, though there are a number of other pigments used across the algal and cyanobacterial groups. A summary of the pigments found in the major algal groups can be seen in Table 4-1

Table 4-1 Photosynthetic pigments typically present in the major algal groups reported in WSP literature. Table adapted from van den Hoek et al. (1995).
* Pigment occurs rarely or in small amounts
** Pigment is present
*** Important pigment

	Cyanophyta	**Bacillariophyceae**	**Cryptophyta**	**Euglenophyta**	**Chlorophyta**
Chlorophylls					
Chlorophyll a	***	***	***	***	***
Chlorophyll b				***	***
Chlorophyll c_1		***			*
Chlorophyll c_2		***	***		*
Chlorophyll c_3		***			*
Phycobilins					
Phycocyanin	***		***		
Allophycocyanin	***				
Phycoerythrin	***		***		
Phycobilisomes	***				
Carotenes					
α-carotene			***		*
β-carotene	***	***	*	***	***
γ-carotene				*	*
ε-carotene		**	*		

Photosynthetic pigments give characteristic auto fluorescent signals when excited by light that can be detected by photosensitive receptors such as those in our eyes or in detectors on microscope cameras and flow cytometers. Light, at lower wavelengths,

excite electrons to a higher quantum state in auto fluorescent molecules, such as a photosynthetic pigment, which then emit light (at a longer wavelength than the absorbed light) as they release the energy as they return to their original quantum state, causing fluorescence of that molecule.

The different photosynthetic pigments shown in Table 4-1 all have different spectral signatures, and fluoresce at different wavelengths. It is possible to use these differences to count cells based on their pigment content. Flow cytometry uses lasers of different wavelengths and detectors to determine the optical properties of cells within a sample (Shapiro, 2002). Cells are passed through the laser's beam in single file, allowing individual cells to be characterised, counted and, with some flow cytometers, sorted. There are a number of potential ways in which flow cytometry could be used to sort cells, based on their auto fluorescence or using fluorescent probes specific to certain organisms within the population.

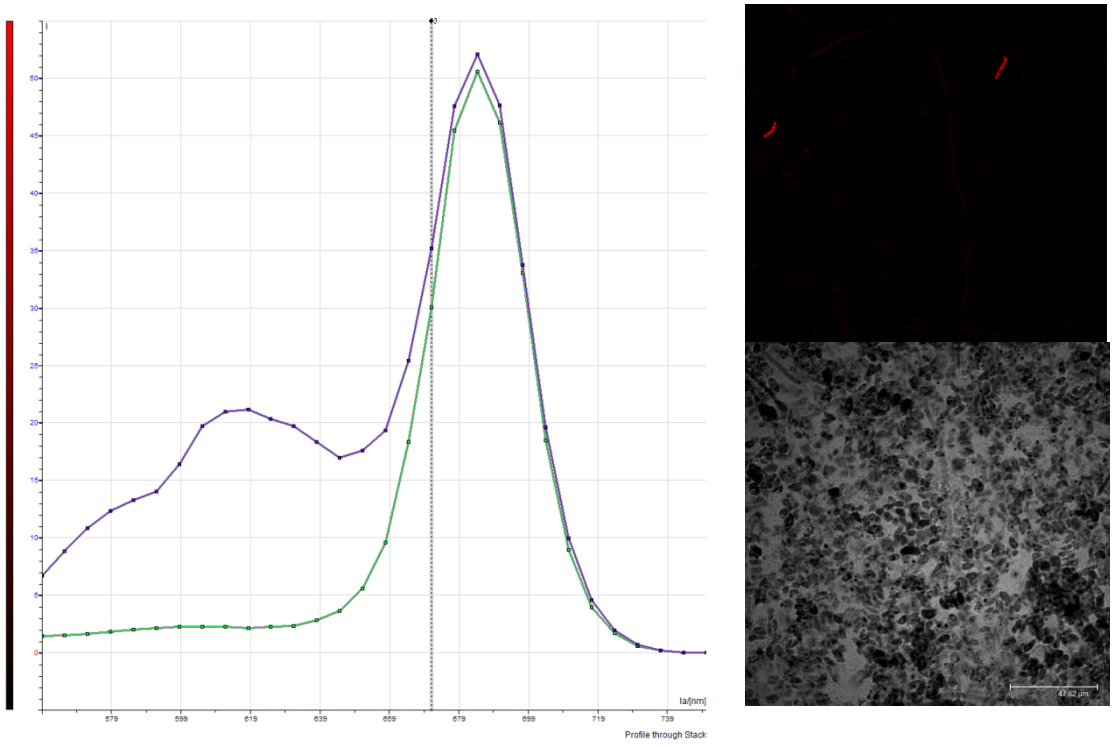


Figure 4-1 Lambda scan to show autofluorescence peaks of eukaryotic microalgae (green line), and a cyanobacteria cell (purple line).
The upper right image is from 619nm stack image and shows cyanobacterial cells (red). The lower right image is a bright field image of the same group of cells, many more chains of cyanobacteria can be seen on this scan than on the image taken at 619nm.

Lambda scans were performed using a Leica TCS SP2 UV confocal laser scanning microscope (CLSM; Newcastle Unviersity BioImaging Facility) on a mixed microalgal culture, using the 543nm laser channel. In this method a series of images, called a stack, are taken while the sample is illuminated with light of a set wavelength. Each of the images in the stack records a different emission wavelength. This was performed to determine if a cyanobacterial cell and a eukaryotic microalgal cell within a mixed algal culture have significantly different signals. The 543nm scan shown in Figure 4-1 shows a typical chlorophyll peak for the eukaryotic cell (green line) and an additional peak, corresponding to the expected phycoerythrin signal from the cyanobacterial cell (purple line). It should however be noted that not all of the cyanobacteria on the microscope slide showed auto fluorescence in this region. This is to be expected in a mixed culture, where there are cells of different species and in different growth stages (Simon *et al.*, 1995; Ueno, 2009). This makes using purely auto fluorescence for sorting of cyanobacteria from eukaryotic microalgae unlikely to work in this case.

Preliminary scans using a flow cytometer (BD LSRII) on pure cultured microalgae, *Chlorella vulgaris, Scenedesmus quadricauda* and *Naviculla pelliculosa* and cyanobacteria; *Synechococcus sp*. and *Anabeana cylindrica* and *E.coli* as a negative (non-photosynthetic), showed little distinction between eukaryotic microalgae and cyanobacteria in terms of auto fluorescence (Figure 4-2).

An alternative method that has the potential to make it easier to distinguish between cyanobacteria and eukaryotic algae is the use of FISH in conjunction with flow cytometry. Due to the auto fluorescence seen in photosynthetic organisms it is important that the fluorophore attached to a FISH probe fluoresces at a different wavelength to the photosynthetic pigments within the sample. For the development of the method the Cy3 fluorophore, also known as fluorescein isothyocianate (FITC) (Simon et al 1997) was chosen. Cy3 when excited at 554nm has an emission wavelength of 568nm providing a signal distinct from the 619 nm emission typical of the autofluorescence.

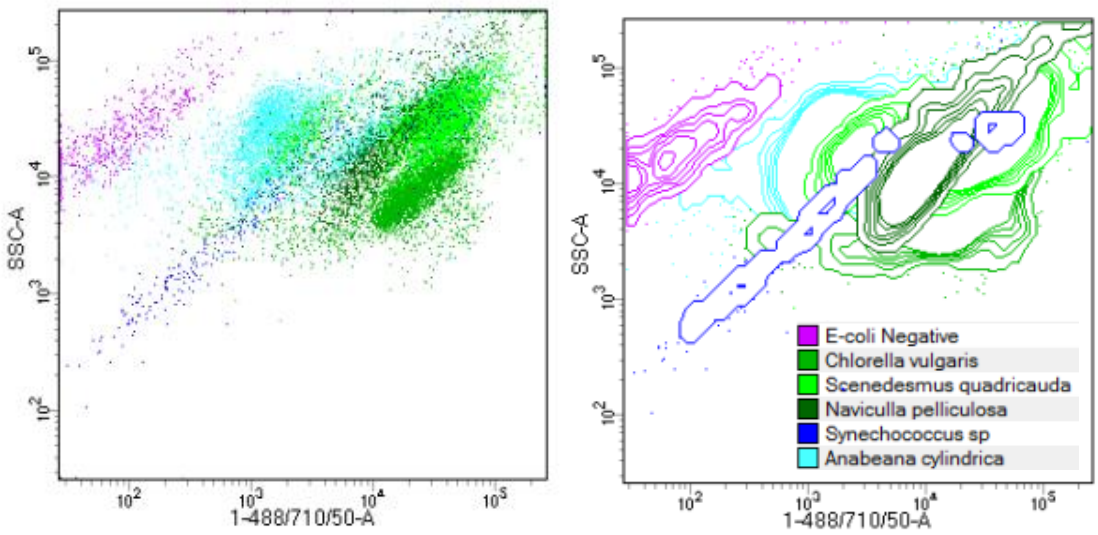


Figure 4-2 FACs DIVA software generated dot plot (left) and contour plot (right).
Showing autofluorescence of E.coli, microalgae and cyanobacteria cultures detected using side scatter (SSC) and the 488nm laser and 710nm detector.

A combined FISH-flow method has been used in a number of marine algal samples, predominantly to identify and count species that have been known to cause blooms or produce toxins (Adachi *et al.*, 1996; Simon *et al.*, 1997; Chen *et al.*, 2008a).

In order to be able to distinguish between cyanobacteria and eukaryotic microalgae with the use of fluorescent oligonucleotide probes one of the target groups needs to be labelled. Knapp and Graham (2004) saw that the presence of 'prokaryotic-like DNA sequences' in microalgae lead to overestimates of bacterial diversity when using bacterial targeting probes in aquatic environments. Biegala *et al.* (2005) reported that when using bacterial probes, they saw high levels of binding to picoeukaryotes. Two reasons were given for this, the presence of 16S rRNA like sequences in the organelles of microalgae or due to the presence of bacterial cells within the microalgal cells, either symbiotically or antagonistically.

There are a number of available FISH probes that target cyanobacteria listed on ProbeBase (Loy *et al.*, 2007). When using Green Genes Primer Checker 4261 out of 6748 cyanobacteria hits were seen for Probe CYA361. On closer inspection 1194 of the matches were to chloroplast rRNA sequences, from eukaryotic algae. The same was true of the other available cyanobacterial probes (see Table 4-2). There use for separating eukaryotic microalgae from cyanobacteria is therefore unlikely to be successful, as false positives may occur. Given these findings FISH probes that target the eukaryotic

fraction of the photosynthetic community were used throughout this chapter. This approach was also taken by Medlin and Strieben (2010), Tobe *et al.* (2006) and a number of others, to avoid false positives.

Table 4-2 Cyanobactreial probe matches to the Green Genes database when using the program Primer Checker

Probe	Reference	Matches to cyanobacteria (Hits)	Mishits to chloroplasts	Eukaryotic algae chloroplast Mishits
CYA361	Schonhuber *et al.* (1999)	4281/6748	1194/2547	Cercozoa (6/6), Chlorophyta (152/376), Cryptophyta (51/61), Euglenozoa (31/176), Haptophyceae (110/127), Rhodophyta (85/91), Stramenopiles 183/370), Streptophyta (558/1258)
CYA664	Schonhuber *et al.* (1999)	2019/6748	37/2547	Chlorophyta 13/376), Glaucocystophyceae (6/8), Rhodophyta (12/91), Streptophyta (3/1258)
CYA762	Schonhuber *et al.* (1999)	2161/6748	465/2547	Chlorophyta (20/376), Euglenozoa (2/176), Rhodophyta (31/91), Streptophyta (40/1258)

4.2 Method Development

4.2.1 Algal Culture

All of the method development presented in this section was carried out of a mixed microalgal culture. This culture has been grown in large polythene reactors in modified Bolds Basal Media (3N-BBM+V) with air mixing and a 12:12 hour light and dark cycle maintained using vertically hung strip lights (this culture was grown and maintained by Stephen Edwards, CEGs). The original inoculum for this culture was from the Larchfield WSP system (described in Chapter 3). The culture contains a mixture of eukaryotic microalgae and cyanobacteria of different morphologies and species. The most visible cyanobacteria were filamentous forms. 2% PFA fixation was carried out prior to hybridisation on the samples, unless otherwise stated, the slide-based method was adopted. The slide method allowed for easier preparation of multiple reaction conditions and there appeared to be less cell loss than the tube-based method (see Chapter 2).

4.2.2 Probe selection

A search of the literature showed a small number of possible probes that could be suitable for the proposed FISH-Flow method. Euk 516 was initially selected as this probe was used previously (Beardsley *et al.*, 2005) and was also the reverse probe used in the DNA extraction PCR tests carried out in Chapter 3. Euk 1209 was also selected as

it was successfully used as a positive probe by Medlin and Strieben (2010). The specificity of the two probes was analysed using the TestProbe feature and SILVAs REFNR database (Table 4-3) (Quast *et al.*, 2013).

Table 4-3 Results of TestProbe analysis for microalgae containing taxonomic groups, within the SILVA REFNR database.

				% of sequence matches within each designated group for a given probe	
				Euk 516	Euk 1209R
Archaea				8.1	0
Bacteria				0	0
Eukaryota				88	89
	Cryptophyta			73	99
	Eugenozoa			33	93
		Euglenida		96	96
		Kinetoplastida		0.17	94
	Haptophyceae			99	99
	Viridiplantae			95	95
		Chlorophyta		90	91
			Chlorophyceae	98	99
			Trebouxiophyceae	99	99
		Streptophyta		98	97
			Charophyceae	97	98
			Chloropkybophyceae	100	100
			Klebsormidiophyceae	92	100

This table was constructed based on the taxonomic structure and categories used in the SILVA database.

4.2.3 Formamide concentration

Different formamide concentration were used (20, 30, 40 and 50 %) to optimise hybridisation stringency of the probe to eukaryotic algal cells. The tube-based FISH method was used (Chapter 2). This initial test showed a large peak in fluorescence at 570nm, corresponding to Cy3 emission for the sample hybridised with 50% formamide. There appeared to be no probe hybridisation at any other formamide concentrations. Another range of formamide concentrations, 40, 50, 60 and 70% were tested to see if a higher concentration would further improve hybridisation. This test showed hybridisation at 50% was not reliable as fluorescence at the expected Cy3 emission wavelength was not seen again (Figure 4-3)

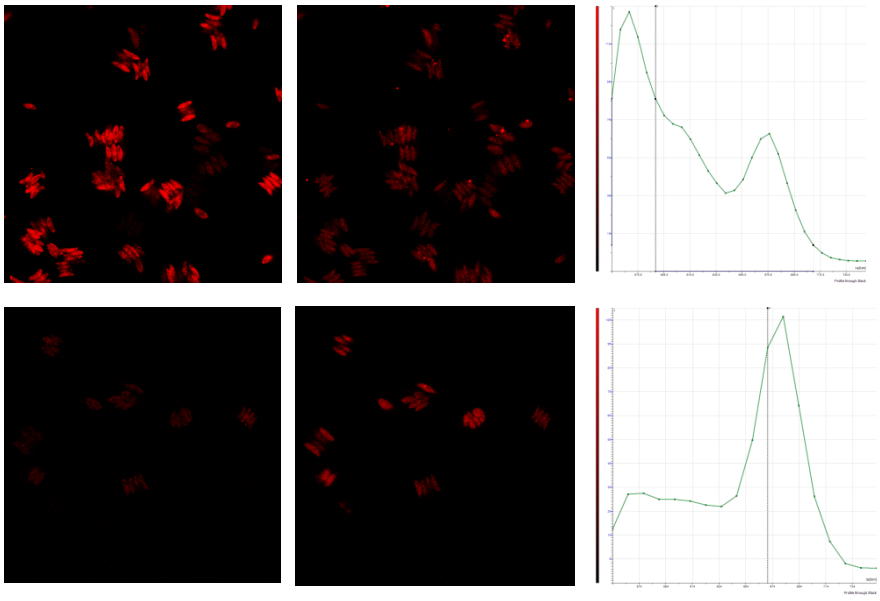


Figure 4-3 543nm micrographs of algal cells hybridised with Cy3-labelled EUK516 using 50% formamide detected at emission wavelengths characteristic for Cy3 (left) and chlorophyll a (centre), together with the emission spectra (right).

Fixation

To improve the hybridisation process a number of pre-treatments were tested. Fixation is the initial step for FISH, and serves to both fix and permeabilize the cells ready for hybridisation. A range of alcohols and aldehydes can be used. In previous hybridisations (see above) 2% paraformaldehyde fixation was carried out overnight. The effect of the following pre-treatments on hybridisation were investigated; PFA fixation for 2 hours, overnight fixation and using ethanol instead of PFA fixation. There appeared to be no noticeable difference between the three methods (data not shown). In subsequent further tests 2% paraformaldehyde with overnight fixation was used.

Sonication and enzyme lysis to increase permeabilization

In order to increase permeability of algal cells to the probe, pre-treatment methods to disrupt the cell walls were tested. The sonication of cells has been used in a number of studies (Biegala *et al.*, 2003 ; Lam and Cowen, 2004) to break up clumps of cells and make cell walls more permeable to probes. Cells were sonicated in Eppendorf tubes before being applied to the slides (slide method detailed in Chapter 2 used). Sonication for 10 seconds, repeated 8 times (Biegala *et al.*, 2003) did not appear to increase the number of algal cells that were successfully hybridised (data not shown).

Alternative pre-treatment methods were then tested. A number of enzymes that have potential to act on different parts of microalgal cell walls were tested at a number of concentrations to see if they improved the number of successfully hybridised algal cells.

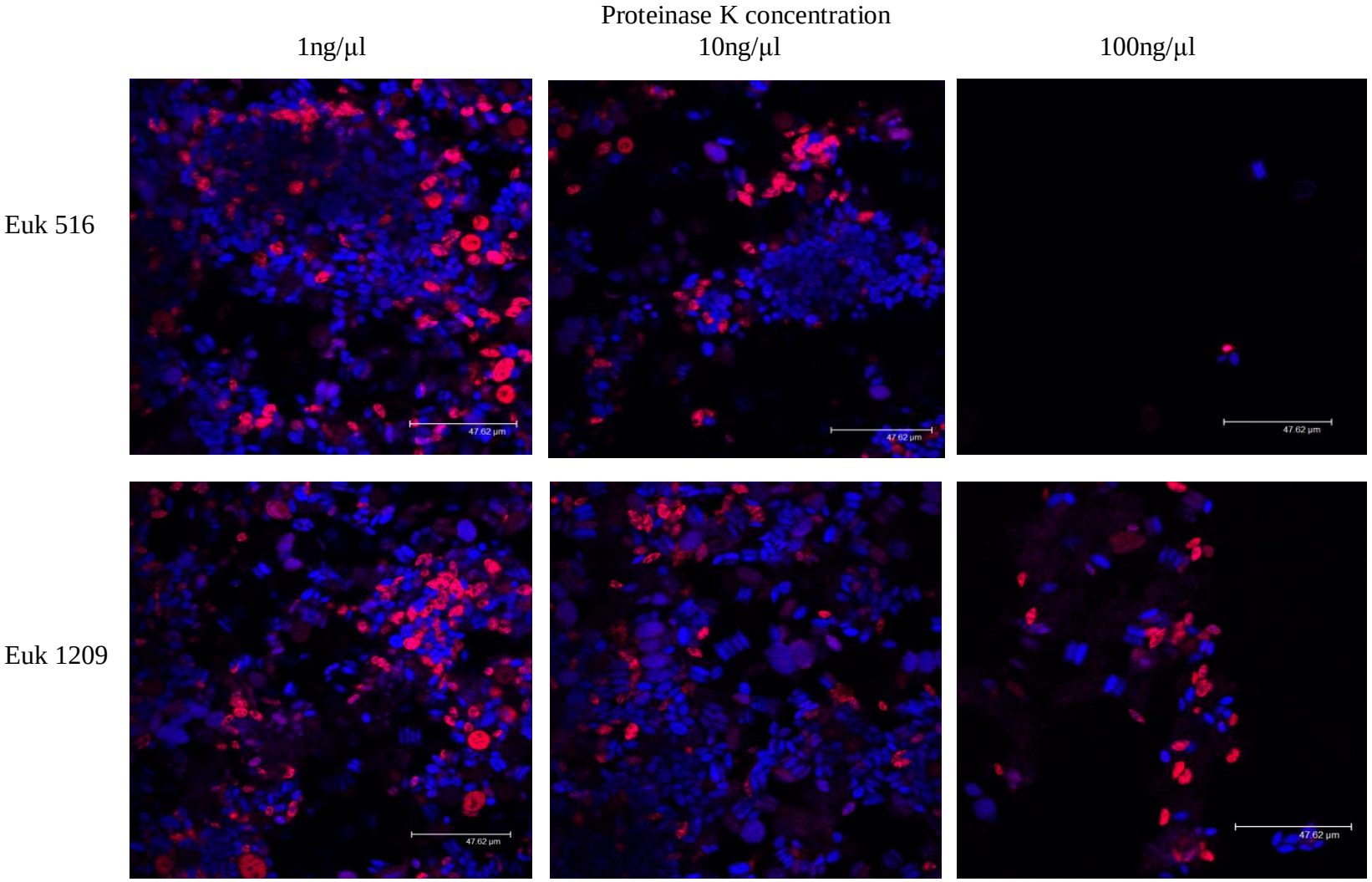
Proteinase K used in the DNA extraction kit recommended in Chapter 3 was also investigated. It is a broad spectrum protease enzyme, with the ability to break down a range of proteins. Many algal groups have protein components in their cell walls, making its use at high concentrations for DNA extractions ideal. At lower concentrations the action of proteinase K may increase cell wall permeability and improve hybridisation of the Cy3 probe. Proteinase K was tested at a range of concentrations, 1µg/ml, 10µg/ml and 100µg/ml (Protocol, 2010).

Cellulase is an enzyme that breaks down cellulose into beta-glucose; it is found in the cell walls of some Heterokontophyta, Chlorophyta and Dinophyta. Cellulose was used by Palacios and Marin (2008) to aid the disruption of the cell walls of a species of thecate dinoflagellate. Cellulose was tested at three concentrations, 0.5, 1 and 2% (w/v in water) on mixed microalgae from an exponentially grown laboratory culture.

Cellulose addition had a very limited effect on the number of successfully hybridised algal cells (data not shown), regardless of concentration. Proteinase K lead to improvement of probe uptake for both Euk 516 and 1209 probes, however, this only appears to have occurred in a third to a half of the total eukaryotic cells, Figure 4-4. The use of enzymes also led to an increase in cell loss from the slides surface.

In the majority of cells that took up the probe, the signal from the fluorophore was weaker than the auto fluorescence of the pigments within the cells (Figure 4.4) This suggests that the signal from the fluorophore may need to be improved if it is to be used for quantification. Biegala *et al.* (2003) used ‘tyramide signal amplification (TSA)-FISH, in which the fluorophores signal is increased to overcome the variation in signal strength across cells in different growth phases. In FISH the probe hybridises to the rRNA in the ribosome, rather than the rRNA coding gene in the nucleus, and as such the level of hybridisation varies as the number of ribosomes in the cell changes with algal growth phase. TSA was reported to increase signal strength from probes in algal cultures by between 10 and 20 times (Not *et al.*, 2002)

Figure 4-4 lambda scan overlay, showing two slices from the emission spectrum.
The chlorophyll peak at 670λ (blue) and the Cy3 peak at 568λ in pink. A mixture of blue (auto fluorescence) and pink (Cy3) can be seen in some cells (they appear purple).



4.2.5 Hybridisation issues

Hybridisation of microalgal cells was improved by the addition of a pre-treatment step, using enzymes, though quantification of the cells would still not be possible using the procedure, as only a fraction of the cells were hybridised. In order to investigate the cause of this, samples were hybridised in the usual way with enzyme addition, but without the cell-washing step. Slides were then viewed with the CLSM to assess whether the low hybridisation rates were due to probes failing to enter the cells or probes entering the cells, and failing to hybridise.

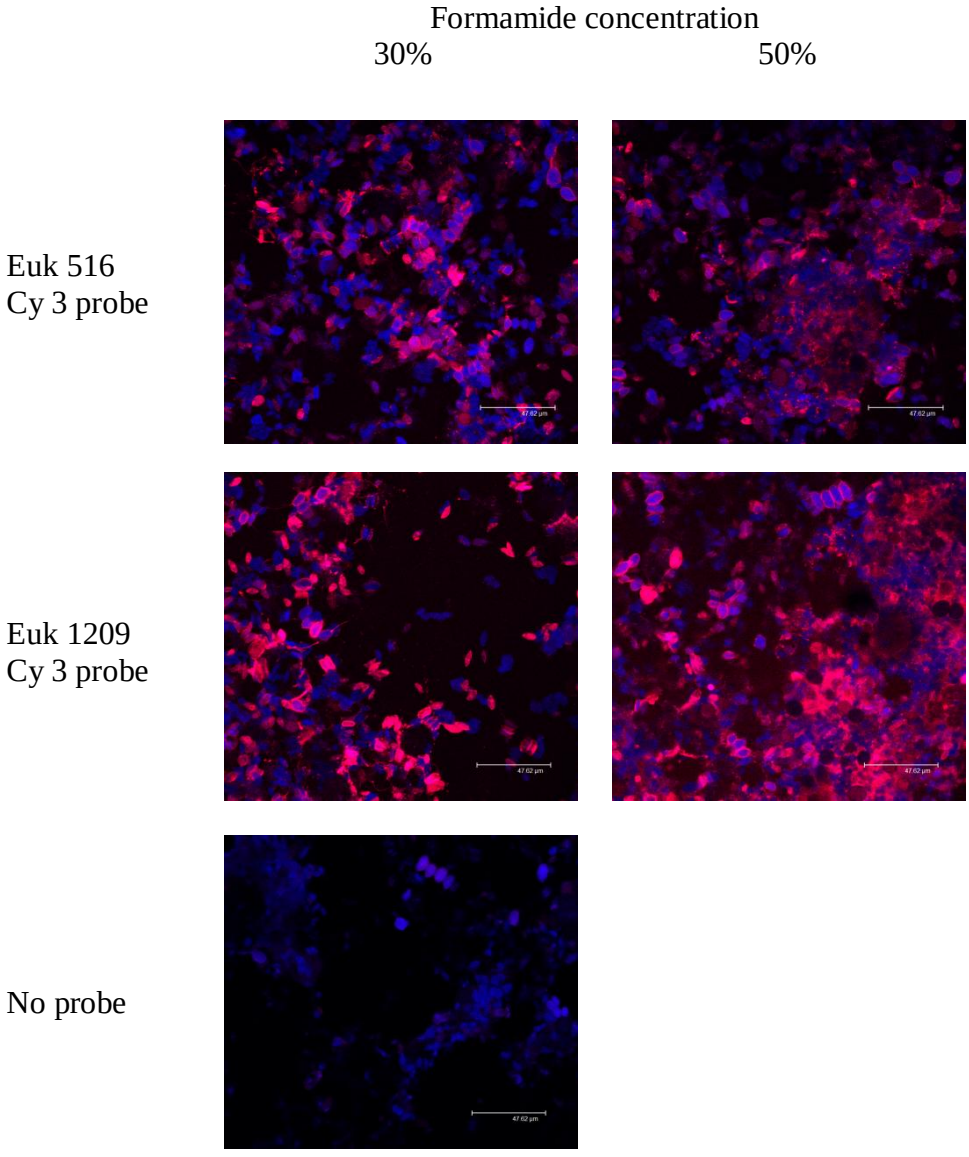


Figure 4-5 lambda scan overlay
Showing two slices from the stack, the chlorophyll peak at 570nm (blue) and the Cy3 peak at 568nm in pink. The probe has been allowed to hybridise under standard hybridisation conditions, but no washing step completed.

Figure 4-5 shows that the probe had entered into some of the cells, however on the majority of cells Cy3 fluorescence aggregated around cell surfaces, and did not penetrate through the cell walls. The concentration of formamide used in this test does not appear to make a difference to the number of successfully hybridised cells. Probes Euk 516 and Euk 1209 were both tested without the washing steps and there appears to be no obvious difference in the ability of the two probes to hybridise with algal cells. *Scenedesmus quadricauda* (four oblong cells in a colony) did not appear to have taken up the probes in any of the tests, though there appeared to be no obvious pattern with other cell types. *S. quadricauda* has a firm polysaccharide cell wall, with an inner layer of a sporopollenin-like material, a decay resistant and chemically stable material. This substance is also present in a number of other algal species of Chlorophyceae including *Chlorella* species, known to be common in ponds and being present in the cultures sample used in this method test.

4.3 Conclusions

The use of FISH to label and visualise the eukaryotic organisms within WSP samples was shown to be ineffective. The penetration of the fluorescent probes into the cells was limited by the cell wall permeability. This cell wall permeability varied from cell to cell, though some species showed a greater resistance to permeabilization, such as the *S.quadricauda*.

Chapter 5. Flow cytometry-sorted DGGE to assess photosynthetic community diversity

5.1 Introduction

A method for quantifying and sorting photosynthetic and non-photosynthetic communities was developed and assessed as an alternative method to their quantification by FISH and flow cytometry (Chapter 4). This method was developed in order to overcome the following;

- Limitations of detecting photosynthetic eukaryotes over the other non-photosynthetic organisms when using a general eukaryotic primer
- Limitations of detecting cyanobacteria over plastids within the eukaryotic fraction and other bacteria, given the similarity of their 16S rRNA sequences
- Limitations of the use of FISH (see Chapter 4)
- And the limitations of other potential gene targets, such as RuBisCO (discussed below)

Both photosynthetic and non-photosynthetic eukaryotes are detected when using the eukaryotic primers (Euk 1A and Euk 516r), sequencing of DGGE bands from Chapter 3 confirmed this (data not shown). The algae were historically grouped based on morphology by Eichler (1883) and later found to be an 'unnatural' group rather than one based on shared evolutionary history (see discussion in Chapter 1). Therefore, finding a PCR primer-set that targets all of the microalgal groups, but not other non-photosynthetic eukaryotes is difficult. The use of alternative primers such as those targeting functional genes specific to photosynthetic organisms could be a viable alternative strategy. Primers that target the gene responsible for the formation of the RuBisCO enzyme, a key enzyme in the Calvin cycle and carbon fixation can be used. This approach was used by Ghosh and Love (2011) to investigate WSP diversity. The main drawback of this approach is the lack of publicly available reference sequences in online nucleic acid databases with which to compare sequences because of the limited numbers of RuBisCO gene studies carried out. The databases available for the identification of gene sequences contain vastly more 18S rRNA and 16S rRNA sequences than they do RuBisCOs sequences. The NCBI nucleotide database contains 457678, 5118742, and 109534 sequences, respectively, for 18S rRNA, 16S rRNA and RuBisCO genes. Furthermore, RuBisCO genes are not exclusively present in only

photosynthetic organisms. Chemoautotrophic proteobacteria also use RuBisCO enzymes for carbon fixation (Badger and Bek, 2008). In addition, there are several related but different forms of RuBisCO enzymes, each of which would require a different primer-set in order to cover all of the algal groups.

A method by which to sort photosynthetic organisms from mixed microbial communities was devised in order to improve community analysis using molecular methods and as an alternative to targeting different genes.

Flow cytometry is a technique used for the rapid sorting and counting of cells based on their optical properties. In flow cytometry cells are suspended in sheath fluid (usually sterile PBS) and passed through lasers and detectors in single file. Laser beams of a single wavelength are focused onto the stream and when a cell passes through the light, the light is scattered. In addition, fluorescent chemicals in the cells can be excited by the light energy and emit fluorescence at a longer wavelength than the original laser light (Shapiro, 2002). A range of detectors are positioned close to the liquid channel to detect the scattered light and fluorescence at different wavelengths. There are two types of scatter detectors, ones that are in line with the light beam, detecting forward scatter (FSC) and one that is perpendicular to the beam, detecting side scatter (SSC). The combination of SSC and FSC can be used to determine cell sizes. The fluorescence detectors detect fluorescence at specific wavelengths if emitted by any excited chemicals within cells (Shapiro, 2002). Photosynthetic organisms contain photosynthetic pigments that are excited by light and the lasers within a flow cytometer. The presence or absence of chlorophyll *a* auto fluorescence was used as the basis by which to sort cells in this Chapter. Software on the flow cytometer can be set up to partition cells or 'events' based on their optical properties. In this way the photosynthetic cells can be sorted from the non-photosynthetic cells. The occurrence of pigments of different types in microalgae and cyanobacteria can be seen in Table 4-1.

The samples were sorted into photosynthetic and non-photosynthetic fractions using flow cytometry, which were then assessed using PCR-DGGE to establish whether sorting can distinguish such fractions and allow further insight into the structure of communities.

Flow cytometry is used extensively to assess pico and micro algae in the world's oceans (Larsen *et al*., 2001, Rutten *et al*, 2005, Silvoic *et al*, 2012).

In order to count and sort cells in flow cytometry a gating strategy needs to be devised. This gating strategy allows the user to determine which organisms are sorted or counted into a fraction. Gating in marine algae is usually based on the auto fluorescent properties of the cells of interest, for example Silovic *et al.* (2012) were interested in Synechococcus (Cyanophyceae) and picoeukaryotes and chose gates for counting these groups of organisms based on the side-scatter (SSC), chlorophyll and phycoerythrin content of the cells. The study gives little detail of how gating was determined or whether any attempt was made to confirm if counting was accurate. The use of reference organisms to confirm that the data obtained by flow cytometry is accurate and that gating is not resulting in false positives is important.

The method development and assessment carried out in this chapter makes use of samples from two WSP systems in Brazil. Samples were from two full-scale WSP systems in the city of Fortaleza, in Ceará, northeast Brazil. One of the systems, Marechal Randon, treated domestic wastewater from a suburban community. The other treatment system, SIDI, was much larger and treated a mixture of domestic and industrial wastewater at a ratio of 1:1. Samples from these two treatment systems were used for flow cytometer sorting and counting, and the sorted concentrated fractions used for subsequent DGGE and sequencing.

5.2 Methodology

5.2.1 Sampling

One litre grab samples were collected from two sampling points per pond (both facultative and maturation ponds in the SIDI and Marechal Randon treatment systems). One sample was taken close to the influent entry point and another close to where the effluent leaves each pond. The positioning of the sampling points can be seen in Figure 7-1. 80ml samples were used for DNA extraction and 5 x 1ml samples were fixed with 4% PFA, using the standard method set out in Chapter 2.

5.2.2 Cell sorting and counting

Negative and positive samples were used to set up the 'gating' of the FACS Aria II cell sorter using FACs DIVA software. On the initial run all of the available channels and lasers on a LSRII flow cytometer (BD Biosciences, Oxford) were utilised to determine which lasers and detectors to use, see Figure 4-2 and Appendix 4. Gating was designed based on the signals recorded from negative and positive samples. The negative samples were a pure laboratory culture of *Escherichia coli* and an activated sludge samples from Tudhoe Mill sewage treatment works, Spennymoor, County Durham (Northumbrian Water Ltd). The positive samples were pure cultures of algae grown in sterile conditions at MAST and CEGs. Details of the cultures and their growth conditions can be seen in Table 2-1. The gating strategy was also validated and modified using a mixed microbial consortium from a WSP. The sorting strategies were assessed using PCR with specific primers for photosynthetic eukaryotes, cyanobacteria and general bacteria. FACs DIVA software was used to produce dot plots, contour plots and to access the count data. Each dot appearing on the dot plots represent an 'event' recorded on the flow cytometer. Contour plots help to highlight areas where the majority of the cell population falls in terms of optical characteristics.

5.2.3 DNA extraction

DNA was extracted from pellets of sorted material from the flow cytometer. Both the negatively and the positively gated fractions were centrifuged and the supernatant removed. The cells were then washed by mixing with 1ml of PBS, centrifuging and removing the supernatant. DNA extraction was carried out using the QBT kit (see Chapter 3). An 80 ml unsorted frozen sample acted as a control and allowed assessment of any diversity not represented after the cell sorting in either the negative or the positively gated fraction.

5.2.4 PCR amplification

PCR was carried out on each DNA extraction using three sets of primers. A modified version of Muyzer *et al.* (1993a) bacterial primers (F357GC and R518) were used to target the 16S rRNA of bacteria (Zwart *et al.*, 2005). This primer pair was also used as a secondary nested primer following amplification of cyanobacterial 16S rRNA fragments with Cya-bF371 and Cya-R783 (Zwart *et al.*, 2005). Using F357GC and R518 to re-

amplify amplicon products of the cyanobacterial fragments allowed the bacterial and cyanobacterial diversity to be directly compared, thus highlighting which of the bacterial population were cyanobacteria. The eukaryotic microalgae 18SrRNA were targeted by PCR using Euk1A and Euk516rGC primers.

A nested approach was adopted for assessment of the photosynthetic eukaryotes due to the poor DNA yield from these populations after sorting (see Results). A first round of PCR using Euk1A and Euk1209 was carried out followed by PCR amplification using Euk1A and Euk 516rGC primers.

More information about the primers can be found in Table 2-2. PCR was carried out using the standard method detailed in Chapter 2.

5.2.5 Diversity analysis

The diversity of the bacterial, cyanobacterial and eukaryotes within the two sorted fractions and the unsorted pond sample were evaluated using DGGE, using the standard methods set out in Chapter 2. BioNumerics software was used to align and normalise the gels, band matching was performed. Primer 6 software was then used to count OTU richness.

5.2.6 Sequencing

The dominant bands seen in the DGGE gel for the positive and negative fractions, for both cyanobacteria and eukaryotes, were excised, reamplified and purified following the method set out in Chapter 2. Bands were then sent for sequencing (GeneVision, Newcastle, UK) and the results analysed by BLAST and the RDP classifier (for the prokaryotes). Eukaryotic bands were BLAST searched against the nr nucleotide database and the prokaryotic bands against the rRNA reference (ref-seq) database.

5.3 Results

Gating was set up to reflect the major patterns seen in the negative and positive samples, a number of these are shown in Figure 5-1.

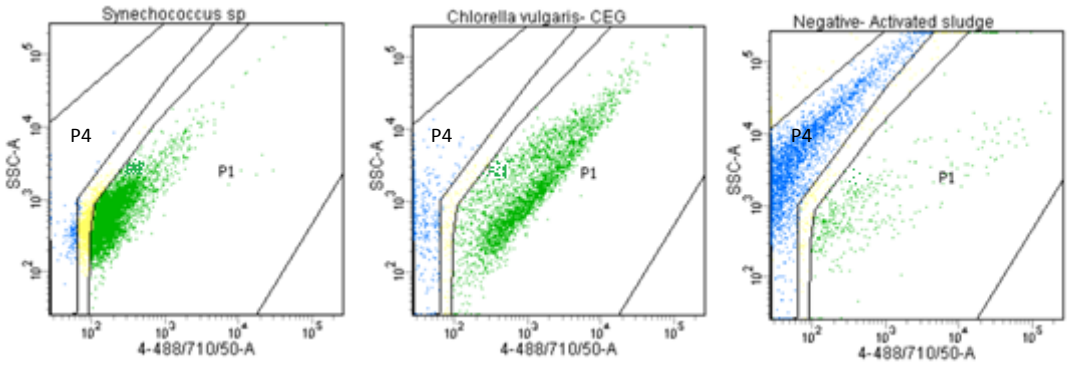


Figure 5-1 FACs DIVA software generated dot plots showing the side scatter (SSC-A) and fluorescence intensity at 488nm excitation with a 710nm detector (488/710/50-A) signal, for two algal cultures. Synechococcus sp. (left) Chlorella vulgaris (centre) and Activated sludge (right).
Two gates were set up based on the positions of the populations within these plots. P1 shows the gates for sorting cells into the positive channel (green cells) (photosynthetic), P4 show the negative gate (Blue cells) (non-photosynthetic).

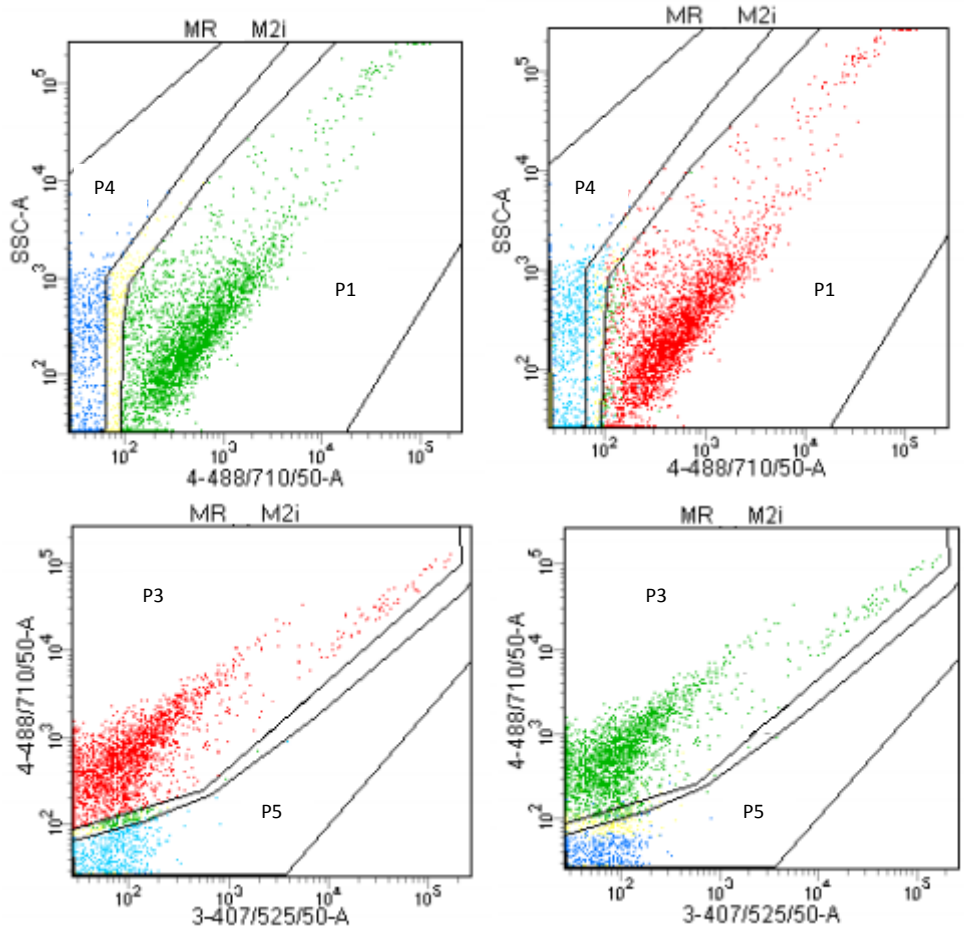


Figure 5-2 FACs DIVA dot plots showing two possible gating strategies on a Maturation pond sample (MR M2i).
Top left shows gating strategy 1 (SSC-A vs 4-488/710/50-A) with gates P1 (in green)(photosynthetic) and P4 (non-photosynthetic)(in dark blue), the top right shows gating strategy 1, but colours show where cells gated by gate strategy 2 would be. The bottom left shows gating strategy 2 (4-488/710/50-A vs 3-407/525/50-A) with gates P3 (red)(photosynthetic) and P5 (pale blue)(non-photosynthetic). The bottom right shows gating strategy 2, but colours show gating strategy 1 colours.

From the initial run of the positive and negative cultures two alternate methods were devised for gating, one using SSC (side scatter) versus the 488 nm excitation wavelength laser detected using an emission wavelength of 710 nm (also referred to as 488/710/50-A) and the other using 4-488/710/50-A and 3-407/525/50-A (a 407 nm excitation wavelength laser detected using an emission wavelength 525nm detector). In order to test which of these was most effective at sorting the photosynthetic and non-photosynthetic fractions, a PBS washed, paraformaldehyde fixed sample from Marechal Randons second maturation pond (sample MR-M2i) was sorted using both of these gating strategies. These two gating strategies can be seen in Figure 5-2. If gating strategy 1 (Top left) is assumed to be the 'true' strategy, by shifting to gating strategy 2 you would be miss sorting a number of cells as 'false negatives' (i.e. the green cells falling outside of gate P3 (bottom left). If gating strategy 2 (bottom left) is 'true' then by adopting gating strategy 1 the number of false positives increases. In this situation the most conservative strategy, which favours false positives was chosen, as sequencing provides the opportunity to identify false positives.

PCR was then carried out using eukaryotic, bacterial and cyanobacterial primers, as outlined earlier in the methods section. Agarose gel electrophoresis results showed amplification of cyanobacterial 16SrRNA in the positive fraction of the SSC vs 4-488/710/50-A gated sample and not in the negative fraction. On the other hand the alternative gating strategy showed amplification of cyanobacterial 16SrRNA in both the positive and the negative, suggesting that this latter strategy was not as effective as the former at sorting the photosynthetic and non-photosynthetic organisms. The results of this amplification test can be seen in Table 5-1.

Table 5-1 PCR amplification success as assessed by agarose gel electrophoresis.
Green (the presence of a strong band) and failure - red (no band present) or partial success- yellow (faint band present) of the FACs sorted positive and negative gated fractions using three primer sets and two different gating strategies on samples of a mixed microbial consortium from Marechal Randon WSP. *This table refers to the gating strategy shown in Figure 5.2.*

		Organisms DNA detected		
Gating strategy	**Fraction (+/-)**	**Eukaryotes**	**Bacteria**	**Cyanobacteria**
SSC-A vs 4-488/710/50-A	Positive			
	Negative			
4-488/710/50-A vs 3-407/525/50-A	Positive			
	Negative			
Positive PCR control				
Negative PCR control				

A test sample was run to check that the gating was appropriate for other WSP samples. There appeared to be two populations of cells, as would be expected when using the SSC-A vs 4-488/10/50 gating strategy, but the gating boundary determined using the positive (P1) and negative cultures (P4), appeared to cut across one of the populations rather than pass between the two (as shown in Figure 5-3).

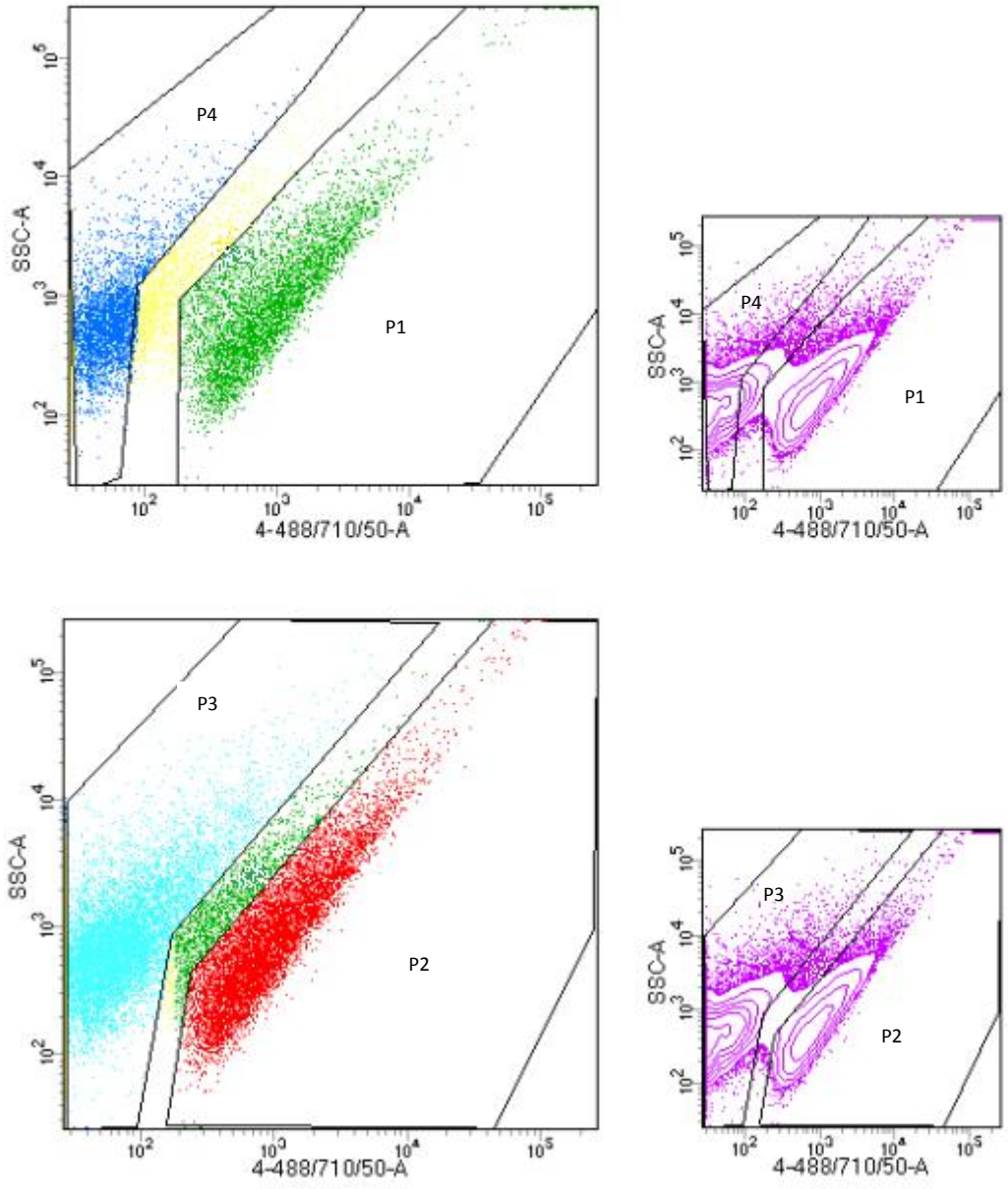


Figure 5-3 FACs DIVA dot plots (left) and contour plots (right).
The gating was moved to the right for the test (bottom), so that the gates separated the two dense areas of population seen in the contour plots rather than cutting across them as the initial gating strategy (top) did. P1-green, P4-dark blue, P2-red, P3-pale blue and any cells not falling within the other gates are yellow. Contour plots show the density of cells detected, the centre circle representing the area of highest cell density.

In order to assess whether the gate needed to be moved a trial sample was sorted, such that 1 million cells were counted into the positive fraction and 1 million into the negative fraction using the gating strategy determined by the positive and negative cultures. A second run was carried out using the same sample but sorting with an altered gating strategy, moving the gate to sit between the two populations seen on the contour plot (P2 and P3, Figure 5-3). As in the previous test, the fractions were used for DNA extraction and PCR. This alternative strategy (with gates P2 and P3, bottom of Figure 5.3) resulted in the loss of cyanobacterial detection by PCR from the photosynthetic fraction, suggesting an increase in 'false-negatives'. The more conservative strategy (P1 and P4, top of Figure 5-3) was therefore adopted as there was less evidence from the PCR data to suggest 'false-negatives' were occurring.

Table 5-2 PCR amplification success as assessed by agarose gel electrophoresis.
Green (the presence of a strong band) and failure - red (no band present) or partial success- yellow (faint band present) of the FACs sorted positive and negative gated fractions using three primer sets and two different gating strategies on samples of a mixed microbial consortium from Marechal Randon WSP.

		Organisms DNA detected		
Gating strategy	**Fraction (+/-)**	**Eukaryotes**	**Bacteria**	**Cyanobacteria**
Adjusted based on populations	Positive			
	Negative			
Negative and positive control based	Positive			
	Negative			
Positive PCR control				
Negative PCR control				

After this optimisation, all of the samples were sorted according to the P1-P4 gating strategy, such that 1 million cells were sorted in to the negative fraction and one million cells into the positive fraction. The first 100,000 cells or 'events' were also counted, to give a proportion of positive to negative events giving the ratio of photosynthetic to non-photosynthetic organisms within the sample.

A trial of the eukaryotic primer set on the FACs sorted WSP samples, showed that the template DNA concentration extracted from the sorted cells was inadequate for downstream analysis. PCR reactions were positive, though fainter than the initial unsorted samples, when amplification was checked with agarose gel electrophoresis. On a test DGGE however there was a very limited banding pattern for the sorted samples compared to the unsorted sample. In order to determine whether it was an issue of extracting DNA from PFA samples or simply a matter of DNA quantity, samples were collected from a microalgal reactor system (original inoculum from Larchfield

community WSP). The reactors were set up and maintained by Kasim Mohammed (PhD student, CeG, Newcastle University). Reactor A had a MLSS concentration of 50mg/l and Reactor B a concentration of 300mg/l. Two samples were taken from each Reactor, one frozen for DNA extraction and the other fixed with 4%PFA following the same method used for the Brazilian WSP samples. DNA extraction was carried out on each of the four samples and followed by PCR reaction, agarose gel electrophoresis and DGGE. The resulting checks suggested that the quantity of initial material had a more pronounced effect upon the PCR and DGGE outcome than the fixation method. Both the PFA fixed and the non-fixed samples yielded DNA sufficient for use in DGGE. However these samples may differ from the Brazilian samples as the PFA fixatives were made in different laboratories and Brazilian samples were stored in the freezer for a longer period of time.

5.3.1 PCR amplification

PCR amplification was carried out with varying degrees of success, on the DNA extracted samples and the sorted fractions of the samples (Table 5-3). Eukaryotic (using Euk1A and Euk516r primers) 18SrRNA gene fragments were amplified in all of the 'whole' samples and in all of the sorted samples from the Marechal Randon treatment system. In this sample, eukaryotes were also amplified in the negative-gated samples, suggesting either a high proportion of non-photosynthetic eukaryotes were present or that the gating was not accurate. Only four of the positive-gated samples from the SIDI site showed a band of the correct size during agarose gel electrophoresis, whereas none of the negative-gated fractions gave a positive PCR result. A nested approach was adopted to improve the amplification of eukaryotic DNA following an assessment of the influence of the fixation method on DNA extraction yield. This resulted in all of the samples giving a fragment of the expected size whether from the original (whole), the positive-, or negative-gated fractions. This suggests that the levels of eukaryotic organisms in the SIDI treatment pond are much lower than in the Marechal Randon pond, and that there are more photosynthetic eukaryotes than non-photosynthetic ones.

The cyanobacterial PCR amplification (using primers Cya-b-F371 and Cya-R783) of the positive-gated fraction gave a PCR product of the expected size for the Marechal Randon site, but not for the SIDI site. In contrast, no PCR products were observed in the

negative-gated (non-photosynthetic) fraction of the samples from either site. A nested approach was used in an attempt to improve the sensitivity of the method.

After flow cytometry (Figure 5-4), the SIDI treatment plant had more cells clustered close to the left side of the P1 gate (positive), suggesting the presence of small cells with low fluorescence signals. The Marechal Randon samples plot had a greater spread of cell sizes and fluorescent intensities, with many more cells in the upper half of the negative (blue) gate. These organisms are large, but with low fluorescence, potentially representing large non-photosynthetic eukaryotes. The cells in the positively gated area (green) were also more varied than those in the SIDI samples suggesting the presence of larger cells with greater fluorescence.

Table 5-3 PCR amplification success of the FACs sorted trial samples positive and negative fractions using five different primer sets.
Green (the presence of a strong band in agarose gel electrophoresis failure- red (no band) or partial success- yellow (faint band).

		Eukaryotic			Nested eukaryotic			Bacterial			Cyanobacterial			Nested cyanobacterial		
WSP system	Sample point	Whole	Positive	Negative	Whole	Positive	Negative	Whole	Positive	Negative	Whole	Positive	Negative	Whole	Positive	Negative
Marechal Randon	F1															
	F2															
	M1i															
	M1e															
	M2i															
	M2e															
SIDI	F1i															
	F1e															
	M1i															
	M1e															
	M2i															
	M2e															
	M3i															
	M3e															

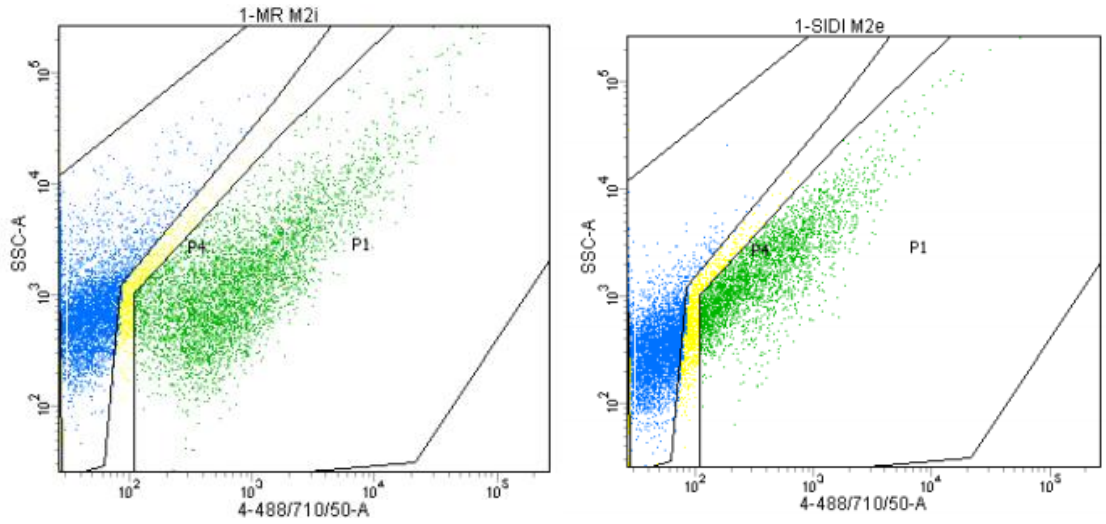


Figure 5-4 FACs DIVA dot plots for two Brazilian WSP samples, from the Marechal Randon system (left) and the SIDI treatment system (right).

5.3.2 Diversity analysis

The OTU richness of the samples was not always improved by the flow cytometry sorting method (see Figure 5-5). The Marechal Randon OTU richness was on average reduced by the treatment. In the SIDI treatment system, there was an increase in the OTU richness in the sorted fractions compared to the original sample, however, this seemed to be variable across the samples. There were no significant differences between the eukaryotic communities from the negative-gated and positive-gated fraction in both of the treatment systems (Figure 5-6).

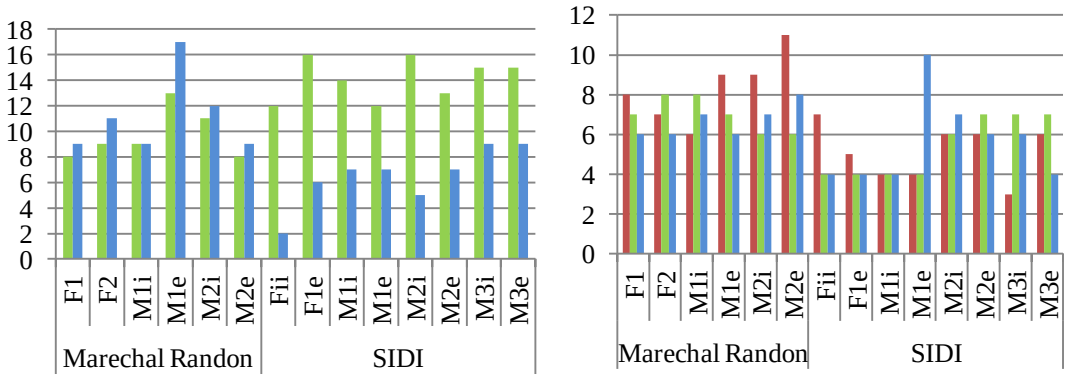


Figure 5-5 OTU richness calculated by Primer 6, DIVERSE, based on bands present in the DGGE. Cyanobacteria (left) and Eukaryotes (right).
Green bars represent the positive gated fraction, blue bars the negative-gated fraction and red the original sample (not sorted)

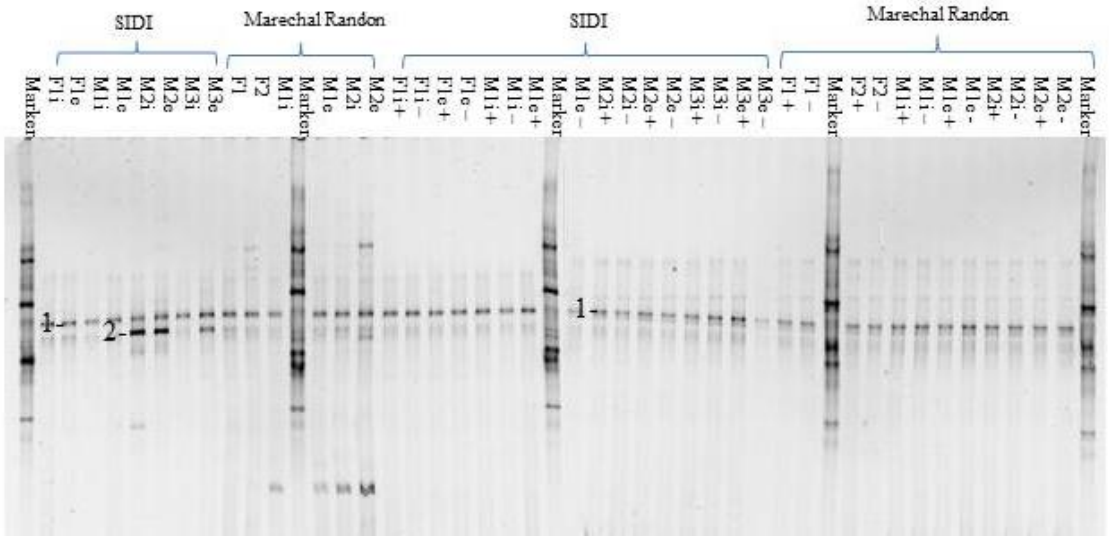
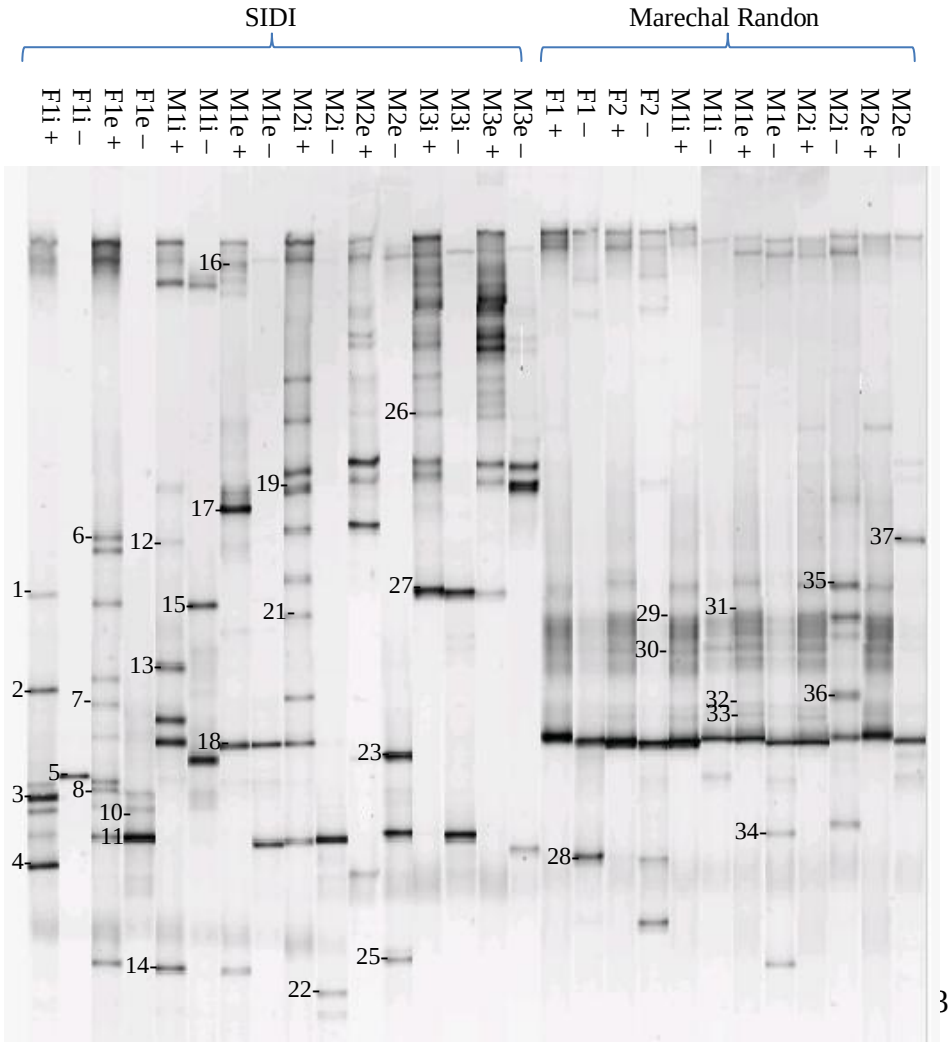


Figure 5-6 DGGE gel image, original samples (on the left) and the positive- (+) and negative-gated (-) fractions for each of the WSP samples, using a nested eukaryotic PCR method.

The OTU richness of the cyanobacteria showed very different patterns for the two treatment systems. The negative-gated fraction (non-photosynthetic) of the Marechal Randon system samples appeared to have a similar number of cyanobacterial OTUs as the positive-gated fraction. The DGGE gel image (Figure 5-7), however, showed that the most intense bands were seen in both the positive- and the negative-gated fractions. There were a number of OTUs that appeared in the negative-gated fraction but not the positive-gated fraction, for example Band 28 in F1-ve and F2 –ve, Band 34 in M1e –ve and Band 37 in M2e –ve. Two of these bands when sequenced matched closely with delta proteobacteria (band 28) and clostridia, (band 34). This suggests that the gating was correctly sorting these non-photosynthetic organisms into the negative fraction. However, the band 37 sequence matched that from a cyanobacteria, suggesting that the gating was not correctly sorting all photosynthetic organisms (false negative). 5 bands were sequenced from the negative-gated sorted samples, 3 were from cyanobacteria. There were, however, a number of bands that were present across the whole set of samples, but that were more intense in the positive-gated fraction, all of which were found to have sequences that closely matched cyanobacteria.

Figure 5-7 DGGE gel image using a cyanobacterial nested PCR strategy.
The bands labelled on the gel were sequenced and the top match by BLAST using the NCBI RefSeq database are included on the right. Those that are cyanobacterial are green and the rest in black.



1- Stanieria cyanosphaera, Thermosynechococcus elongatus, Cyanothece sp.
2- Pseudanabaena sp.
3- Calothrix sp. , Chroococcidiopsis thermalis, Anabaena cylindrica , Nostoc punctiforme, Nostoc azollae
4-Thermosynechococcus elongatus
5- Stigmatella aurantiaca , Myxococcus stipitatus
6 & 12- Stanieria cyanosphaera , Cylindrospermum stagnale, Cyanobacterium stanieri, Calothrix sp. , Cyanobacterium aponinum, Thermosynechococcus elongatus, Cyanothece sp, Nostoc sp., Anabaena variabilis
7- Gelria glutamica
8- Gelria glutamica
10- Gelria glutamica
11 & 34- Gelria glutamica
13- Cyanobium gracile, Prochlorococcus marinus subsp. marinus
14- Geopsychrobacter electrodiphilus
15- Medicago truncatula
16- Geitlerinema sp., Pseudanabaena sp.
17- Geitlerinema sp., Pseudanabaena sp.
18- Oscillatoria nigro-viridis, Oscillatoria acuminata, Trichodesmium erythraeum, Planktothricoides raciborskii
19- Stanieria cyanosphaera , Pleurocapsa sp., Geitlerinema sp. , Cyanobium gracile, Pseudanabaena sp. , Prochlorococcus marinus subsp. Marinus, Thermosynechococcus elongatus , Cyanothece sp., Laceyella sacchari
21, 29 & 31- Oscillatoria nigro-viridis, Oscillatoria acuminata, Trichodesmium erythraeum, Planktothricoides raciborskii
22- Clostridium estertheticum
23- Pseudanabaena sp.
25- Clostridium estertheticum
26- Cyanobium gracile, Pseudanabaena sp. , Prochlorococcus marinus subsp. marinus
27- Stanieria cyanosphaera, Thermosynechococcus elongatus , Cyanothece sp.
28- Geopsychrobacter electrodiphilus
30- Oscillatoria nigro-viridis, Oscillatoria acuminata, Trichodesmium erythraeum, Planktothricoides raciborskii
32 & 36- Oscillatoria nigro-viridis, Oscillatoria acuminata, Trichodesmium erythraeum, Planktothricoides raciborskii
33- Oscillatoria nigro-viridis, Oscillatoria acuminata, Trichodesmium erythraeum, Planktothricoides raciborskii
35- Oscillatoria nigro-viridis, Oscillatoria acuminata, Trichodesmium erythraeum, Planktothricoides raciborskii
37- Leptolyngbya sp, Synechocystis sp

The OTU richness cyanobacteria in the SIDI treatment system, showed markedly different results. The OTU richness values were significantly higher in the positive-gated fraction than in the negative-gated fraction. The DGGE gel (Figure 5-7) confirmed that there was little similarity between OTUs in the negative and positive fractions. 5 of the 7 dominant bands sequenced from the negative fractions of the SIDI sample matched closely to non-cyanobacterial organisms, confirming that the sorting method was not concentrating the cyanobacteria into the negative fraction (false negative).

The apparent difference in cyanobacterial sorting efficiency between the two treatment systems was unexpected. There may be less species of cyanobacteria present in the Marechal Randon treatment plant than the SIDI system. In Marechal Randon the pond water appeared green in all of the ponds, due to high levels of microalgae that may have been out-competing the cyanobacteria for light and nutrients. We speculate that in the SIDI system conditions were less favourable for microalgal growth, due to lower nutrient levels (Table 7-1) and the presence of industrial chemicals. These conditions may have provided niches suitable for cyanobacteria. The loading rates, hydraulic retention times and current sludge accumulation were not assessed, though have the potential to affect the ecology of the systems.

The methodology did not appear to successfully sort the photosynthetic from the non-photosynthetic eukaryotes in the samples. This may be as a result of bias in the DNA extraction. The samples that were sorted were fixed with paraformaldehyde shortly after collection to preserve them. Paraformaldehyde causes the cross-linking of proteins present in the cell walls, making them more difficult to break open.

It is also possible that cells were lost, or DNA was sheared, during storage or processing, which involved many centrifuging and washing steps. Any cells that were damaged during sample processing would release their DNA into the buffer. It is likely that these fragments would end up in the negative gate, as this is defined as being the smaller sized ‘events’ with low fluorescence signals detected by the 710 nm wavelength detector.

Chapter 6. Comparing Molecular techniques to traditional Microscopy

6.1 Introduction

Traditionally microscopy has been used to characterize photosynthetic organisms present in WSPs. There are, however, problems associated with the use of these techniques; they are very time consuming, especially when large number of samples need to be processed, and depend on the expert knowledge of taxonomic specialists. Misidentification of species may also occur when species are morphologically similar to one another and when identifications rely on the absence of cell features for positive identification (Proschold *et al.*, 2001), see discussion of algal taxonomy in Chapter 1.

There have been many reworking's of algal classification, since the 'algae' are an unnatural group, made up of a diverse range of organisms, often more closely related to organisms within other groups than to the rest of the 'algae'. Originally the algae were grouped together by Eichler (1883) and the name and grouping has persisted. Typically in microscopy algae are categorized according to morphological traits, such as cell walls and the nature of their storage products, and the photosynthetic pigments that they contain e.g. Chlorophyta (green algae), Rhodophyta (red algae). With the advent of molecular techniques, evolutionary genetic characteristics have begun to be taken into account, leading to some changes in classification (Proschold and Leliaert, 2007).

Molecular methods have been developed for use on WSP samples throughout this thesis. The performance of these methods in terms of their ability to characterize communities, assess the dominant organisms and lead to a greater understanding of pond ecology in relation to WSP conditions should be considered. The current established method for assessing the ecology of ponds, microscopy, provides a bench mark alongside which molecular methods can be evaluated.

With this in mind the aim of this chapter was to compare the data generated using traditional microscopy methods with a PCR-DGGE and sequencing molecular approach. Nine samples from a range of treatment ponds and eutrophic environments were assessed using the two methods.

This chapter had a number of aims:

- To assess whether most abundant photosynthetic organisms within a pond are the same across the two methodologies and whether the detection technique used introduces bias towards a particular taxonomic group
- To compare the two techniques and see if there is a correlation between samples tested by them
- To evaluate whether the technique used for detection affects diversity or number of organisms (OTUs) seen

6.2 Methods

6.2.1 Sample collection

Samples were collected from 5cm below the surface of 9 ponds, in Colombia and Brazil. A summary of the ponds sampled can be seen in Table 6-1. Samples of 250ml and 200ml were collected and frozen at -20^{0}C on return to the laboratory. In addition samples were taken for taxonomic identification. These samples were fixed with formaldehyde solution at 4% v/v in the field and then frozen at 4^{0}C on return to the laboratory.

Table 6-1 Details of the varied sampling points chosen for analysis, selected to assess the robustness of the methods applied to different WSP samples.
Conventional refers to a pond without baffles. N/A- not applicable, N/P- not provided

Sample	Site	Country	Pond Type	Waste source	Pond design	Area (m2)	Depth (m)	HRT (days)	Applied organic load (kg BODd-1)
Cerrito 1	Cerrito Line 1 3°44'04"N 76°31'55"W	Colombia	Facultative	Industrial/ domestic	Baffles	10092	1.5	4	656
Cerrito 2	Cerrito Line 2 3°44'04"N 76°31'55"W	Colombia	Facultative	Industrial/ domestic	Baffles	10092	1.5	4	656
Ginebra 1	Ginebra 3º43'50'' N 76º16'20'' W	Colombia	Facultative	Domestic	Conventional	6844	1.75	6	77
Ginebra 2	Ginebra 3º43'50'' N 76º16'20'' W	Colombia	Maturation	Domestic	Conventional	832	0.9	0.7	9.07
Ginebra 3	Ginebra 3º43'50''N 76º16'20''W	Colombia	Facultative	Domestic	Baffles	98.04	1.32	4.3	6.22
San Pedro	San Pedro 3º56`01" N 76º26`26"W	Colombia	High-rate	Leachate	Baffles	1.4	0.5	2	0.0091
Uni Valle	Uni Valle 3º22`34" N 76º31`55"W	Colombia	Natural	None	None	14	0.60	N/A	N/A
Marechal Randon	Marechal Randon 3°46'44" S 38°38'11" W	Brazil	Facultative	Domestic	Conventional	15600	N/P	N/P	N/P
SIDI	SIDI 3°51'19" S 38°37'24" W	Brazil	Facultative	Industrial/ Domestic	Conventional	200450	2m	16.6	N/P

6.2.2 DNA extraction and PCR amplification

DNA was extracted from 80ml samples using a DNeasy® Blood and Tissue kit, as recommended in Chapter 3.

PCR was carried out using 3 primer sets; general eukaryotic primers Euk1A and Euk 516r (Diez *et al.*, 2001), cyanobacterial primers Cya-b-F371and Cya-R783 and general bacterial primers F357GC and R518 (Zwart *et al.*, 2005), following the method in Chapter 2.

6.2.3 Community analysis

Eukaryotic and cyanobacterial organisms within the communities were compared using DGGE, (BioRad system) following protocols in Chapter 2. Bands were excised, reamplified and cleaned up before sending for Sanger sequencing. Sequences were viewed in Chromas and NCBI-BLAST was used to determine sequence matches from the database. The RDP classifier was used also to identify the cyanobacterial 16S rRNA sequences. Eukaryotic sequences were aligned against their nearest neighbours and 18S rRNA sequences for a number of other microalgae species commonly found in WSP literature. The majority of chosen sequences came from cultured species maintained by algal culture collections. A list of culture collections referred to can be seen in the abbreviations section).

6.2.4 Taxonomic methods

Victor Ceron, a collaborator from CINARA, Colombia, who is trained in algal taxonomy carried out the microscopy analysis on the formaldehyde fixed samples.

Two optical microscopes were used to identify the photosynthetic organisms, an inverted Nikon microscope and a Zeiss Axioimager (Carl Zeiss). 40 times magnification was used for identification. Cells were found with the view finder, focused on and identified to class level (Bicudo and Menezes, 2006). Specific guides for the different algal groups were then used to refine the identification, including; the Cryptophyceae class (Castro and Bicudo, 2007), the *Chlorococalles* order (Comas, 1996), the Scenedesmaceae family (Godinho, 2009), the *Phacus* genus (Pochmann, 1942) and the Euglenophyceae (Tell and Conforti, 1986; da Silva, 1998). Where taxonomy was

uncertain guidance was sought from a number of taxonomic experts (e.g. James Duke, National University of Colombia, Amazonia).

Counting was carried out following the method recommended in Utermohl (1958) and Venrick (1995). The sample was settled, and the settled biomass was collected into a Pasteur pipette, which was then deposited in the chamber of a Sedgewick-rafter cell counter with a 1ml capacity (Venrick, 1995). The algal cells were quantified by counting the number of individuals in 5-10 fields of view for each of the species previously identified (Woelkerling *et al.*, 1976; Gomez *et al.*, 2009). The number of individuals per millilitre of each species using the objective 40x was obtained using Equation 1 (Venrick, 1995; Gomez *et al.*, 2009).

Equation 1.

$$C = \frac{N}{Vb}$$

Where C is the number of cells per millilitre, N is the number of cells counted in the swept volume (Vb in ml) of the chamber. Vb was calculated using Equation 2.

Equation 2.

$$Vb = \frac{(Ab)V}{A1}$$

Where Ab is the area swept (µl), V is the volume of the Rafter cell and A1 is the total area of the counting chamber.

6.2.5 Comparison of the two data sets

Data from DGGE was compiled, combining the BioNumerics character table and sequencing data. For more details on BioNumerics see Chapter 2. Taxonomic identification and quantification data using microscopy was imported along with the DGGE data into Primer 6 software (Clarke and Gorley, 2006). The microscopy data set was divided into cyanobacterial and eukaryotic portions in order to compare it to the DGGE data. Only those DGGE data pertaining to algae were used in the analysis. These originated from the centre of the gel targeting eukaryotic organisms, as sequencing showed that this portion of the gel contained all of the microalgal sequences identified

(Figure 6-3). Primer 6 was used to calculate the Shannon-Wiener Diversity Index and to assess OTU species richness in the two data sets.

Taxonomic equivalent comparisons were made using presence-absence data at the genus level for the two different methods. Primer 6 was used for cluster and non-metric multidimensional scaling (MDS) analyses to produce an ordination plot to spatially represent the similarity between the samples detected by the two techniques. One way ANOSIM tests were also carried out. Global R values close to zero show that there is on average no difference in the community structure between groups and within groups, showing that the two samples from the same pond processed in different ways were no more similar than samples from one pond to the next.

6.3 Results and discussion

6.3.1 Species richness

Overall it appeared that molecular analyses consistently detected greater OTU richness than microscopy. For instance, OTU richness detected for eukaryotic microalgae was consistently higher in the molecular analysis, than in the microscopy analysis (Figure 6.1), using species level data (for microscopy) and numbers of bands (in the central region of the DGGE gel). Eukaryotic microalgae were undetected in the SIDI sample by microscopy compared to the three OTUs detected using DGGE. Cyanobacteria were undetected by microscopy in 6 of the 9 samples. DGGE on the other hand showed much higher OTU richness (based on band presence and absence in the DGGE) in all samples using the cyanobacterial primer set, with an average of 11.7 OTUs. However, sequencing showed this was likely to be an overestimate of cyanobacterial OTUs, with 8 of the 17 sequences observed sharing high similarity with sequences in the public database from 16S-like plastid DNA from a eukaryotic source (Figure 6-1). This is a common issue when detecting cyanobacteria using 16SrRNA gene targets (Knapp *et al.*, 2008) using molecular methods, as they have closely shared evolutionary history with chloroplasts in algae (Giovannoni *et al.*, 1988). This suggests that microscopy underestimates the presence of cyanobacteria, while molecular methods overestimate them. The use of DGGE bands as a proxy for species, as has been done for ease in Figure 6.1 and the calculation of the diversity statistics should be treated with caution. In theory each band should represent a different sequence with different denaturant properties, though in practice the presence of chimeric PCR products or different

versions of the gene being present in the same species may lead to OTU number being an overestimation of species number. Underestimation of species number can also occur if the region of the gene targeted by PCR is not variable enough for closely related species to have unique sequences. The OTU number (or band) number is the closest approximation available in this case when only a small number of the DGGE bands were successfully sequenced.

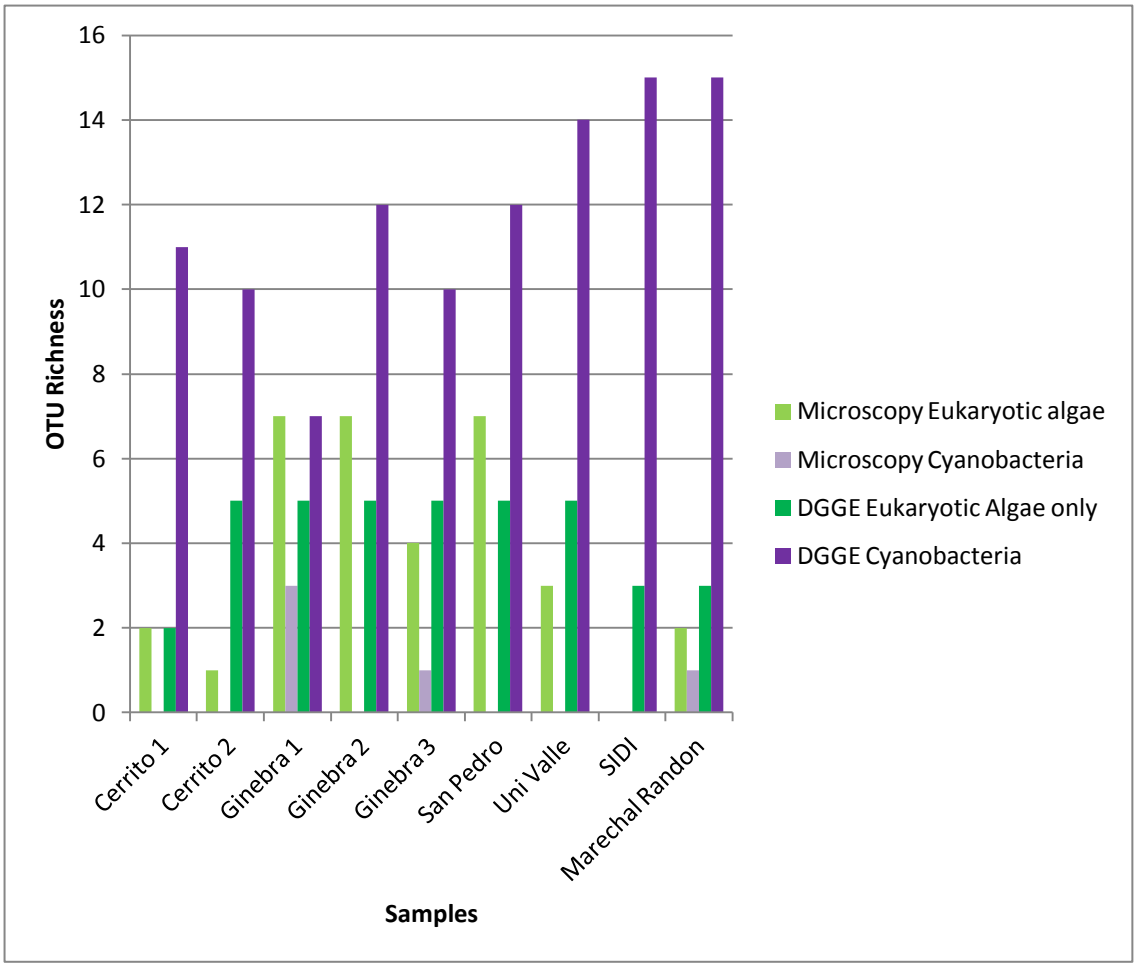


Figure 6-1 Operational Taxonomic Unit (OTU) richness of eukaryotic algae and cyanobacteria as determined by DGGE analysis and microscopy data at species level.
For the purpose of this figure species and OTU are assumed to be equivalent (see discussion for more details of this)

6.3.2 Diversity indexes

The Shannon Wiener Diversity Index (Figure 6-2) followed much the same pattern as the OTU species richness. Cyanobacterial diversity detected in DGGE was higher than that detected by microscopy, with an average of 2.44 and 0.0507 respectively . Eukaryotic algal diversity was more similar between the two techniques, with average diversities of 1.17 and 0.84 for the DGGE and microscopy respectively.

6.3.3 Identification

Microalgae and cyanobacteria identified and quantified by microscopy (Table 6-2) and by DGGE (Figure 6-3, Figure 6-4 and Figure 6-5). More in depth sequence search results can be seen in Appendix 5 (eukaryotes) and Appendix 6 (cyanobacteria).

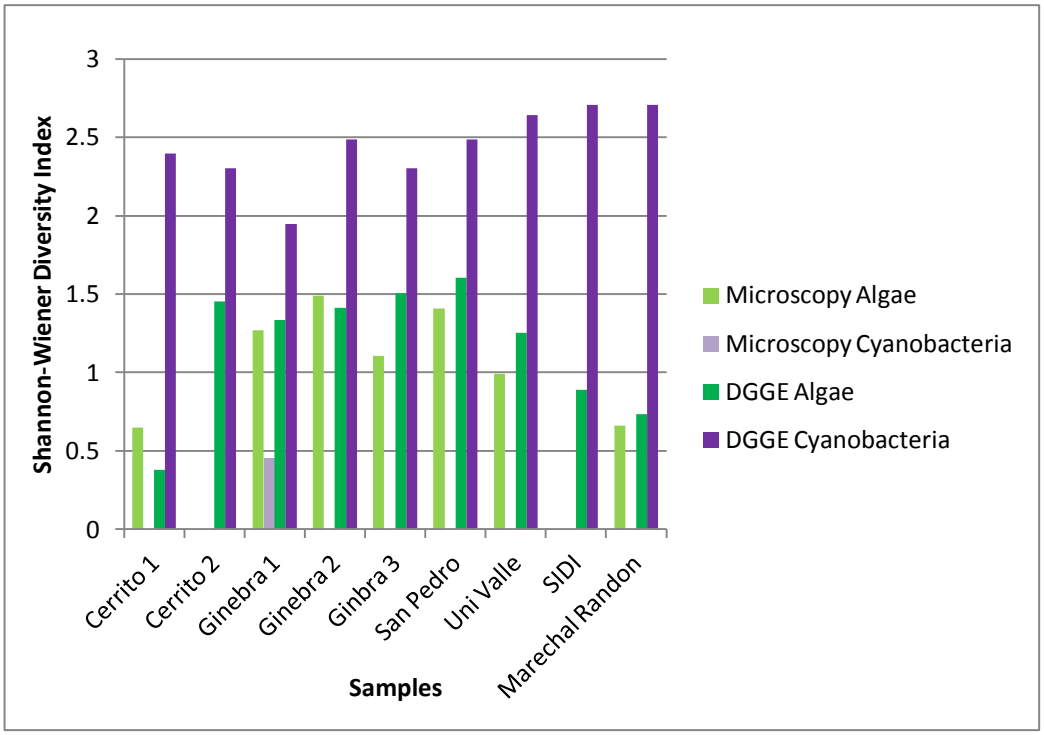


Figure 6-2 Shannon- Wiener Diversity Index of eukaryotic algae and cyanobacteria as determined by microscopy and DGGE analysis for 9 samples.

A number of algal genera were detected by both microscopy and molecular methods, though not in all samples (**Error! Reference source not found.** and Figure 6-3). *esmodesmus*, *Scenedesmus* and *Chlorella* species were all detected in both analysis, though the exact species detected varied and they were not always detected in the same samples. In **Error! Reference source not found.** those genera that were detected in the ame sample by both of the methods are highlighted in red. This only occurs 7 times, across all genera and all samples, suggesting a low similarity between the results for the two techniques. There are a number of possible reasons for this lack of similarity. The lack of distinguishing features by which to classify the microalgae based on morphology and the reliance of morphological taxonomy on features that are culture dependent may lead to error in identification (see discussion in Chapter 1). There are also potential problems related to the molecular methodology. The sequence data and the resulting phylogenetic tree for eukaryotic

Table 6-2 Algal and cyanobacterial species identified and quantified in 9 samples, using microscopic identification-based methods (data provided by Victor Ceron).

		Distribution and abundance of microalgal species. Cells detected (cell/ml)								
Class	**Species**	**Cerrito 1**	**Cerrito 2**	**Ginebra 1**	**Ginebra 2**	**Ginebra 3**	**San Pedro**	**Uni Valle**	**SIDI**	**Marechal Randon**
Cyanophyceae	*Merismopedia trolleri*	0	0	1050	0	0	0	0	0	0
	Phormidium willei	0	0	100	0	0	0	0	0	0
	***Phormidium* sp.**	0	0	50	0	200	0	0	0	0
	Oscillatoria sp.	0	0	0	0	0	0	0	0	350
Chlorophyceae	***Scenedesmus acuminatus***	0	0	0	0	2250	74400	9700	0	0
	Desmodesmus quadricauda	0	0	0	0	0	81300	9600	0	0
	Desmodesmus nanus	0	0	0	0	0	0	2950	0	0
	Demosdesmus spinosus	0	0	0	0	4900	0	0	0	0
	***Chlorella* sp.**	0	0	1350	0	2900	116200	0	0	1700
	Chlorella vulgaris	0	0	0	0	0	16200	0	0	0
	Pandorina sp.	6200	0	0	0	0	0	0	0	0
	Chlamydomonas gyrus	0	0	0	0	0	9250	0	0	0
	Chlamydomonas sagittula	0	0	9100	0	0	0	0	0	0
	Chlamydomonas gloeopara	0	0	14350	0	0	0	0	0	0
	***Chlamydonomas* sp.**	0	0	2850	0	0	6300	0	0	0
	Chlamydomonas obergurlii	0	10900	0	0	0	0	0	0	0
Euglenophyceae	*Lepocinclis salina*	0	0	0	4250	0	0	0	0	0
	Phacus tortus	0	0	0	950	0	0	0	0	0
	Phacus ephippion	0	0	0	7500	0	0	0	0	0
	Phacus longicauda	0	0	0	700	0	0	0	0	2850
	Euglena proxima	3400	0	1600	0	0	0	0	0	0
	Euglena hemichromata	0	0	0	2600	0	0	0	0	0
	Euglena anabaena	0	0	0	1100	0	0	0	0	0
	Euglena subehrenbergii	0	0	0	100	0	0	0	0	0
Cryptophyceae	*Chilomonas insignis*	0	0	0	0	0	1	0	0	0
	Chroomonas sp.	0	0	0	0	14350	0	0	0	0
Bacillariophyceae	*Gomphonema sp*	0	0	100	0	0	0	0	0	0
	Pinnularia sp.	0	0	50	0	0	0	0	0	0
Total		9600	10900	30600	17200	24600	303651	22250	0	4900

microalgae show that region of the 18SrRNA gene may not be long or variable enough to make distinguishing between species and even genera of eukaryotic algae. This is especially true of the Chlorophyceae class (Figure 6-4). The constant updating of algal classifications also leads to potential errors in both taxonomic and database-reliant methods. The sequence databases used are also incomplete with many microalgal genomes left unsequenced. This leads to a large number of 'Uncultured…' entries or less than 100% matches to similar closely related species, rather than an exact match to the species present.

Microalgae from the *Euglenophyceae* class were detected by microscopy but not by DGGE. Three species of the *Phacus* genus and four species from the genus *Euglena* (from the *Euglenophyta* group) were detected using microscopy but not by DGGE. In Ginebra 2, Euglenophyta made up 100% of the 17200 organisms counted, whilst in the molecular analysis the community was shown to contain organisms from the classes Cyanophyceae, Chlorophyceae and Bacillariophyceae.

In addition to this 5 species of the genus *Chlamydomonas* (within the Chlorophyceae class *(Chlorophyta* group) were detected by microscopy (present in pond Ginebra 1, San Pedro and Cerrito 2), none of which appeared in sequencing of the DGGE bands.

There are six potential reasons that these organisms were not seen in the molecular method.

i. It is possible that some algal species have been misidentified by microscopy, for the reasons described in Chapter 1.
ii. The eukaryotic primer was chosen as it has broad coverage of the eukaryotes. It was not specifically designed to target all algal species. Assessment of the primers (using SILVA, TestPrime) showed 88.2% coverage for Eukaryotes. Allowing for 1 sequence mismatch, primers had a coverage of 93.7% for the Chlorophyta and 84.6% for Euglenophyceae sequences contained within the database. Lower coverage of the Euglenophyceae and lower overall numbers of Euglenophyceae sequences in the database may explain their absence from molecular analysis.

Table 6-3 Simplified data prepared from DGGE sequencing and microscopy data. Data converted to presence-absence and genus level to allow comparison using Primer 6 software

		Microscopy									Molecular								
		Cerrito 1	Cerrito 2	Ginebra 1	Ginebra 2	Ginebra 3	San Pedro	Uni Valle	SIDI	Marechal Randon	Cerrito 1	Cerrito 2	Ginebra 1	Ginebra 2	Ginebra 3	San Pedro	Uni Valle	SIDI	Marechal Randon
Cyanophyceae	Merismopedia	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Phormidium	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Oscillatoria	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	1	1
	Uncultured cyanobacteria	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
	Fischerella	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0
Chlorophyceae	Tetranesphris	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	Kirchneriella	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
	Scenedesmus	0	0	0	0	1	1	1	0	0	0	1	1	1	0	1	0	0	0
	Desmodesmus	0	0	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	1
	Chlorella	0	0	1	0	1	1	0	0	0	0	1	0	0	0	1	1	1	1
	Pandorina	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Chlamydomonas	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Pyrobotrys	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1
	Ourococcus / Monoraphidium	0	1	1	0	0	1	0	0	0	0	1	0	1	1	1	1	0	0
Chrysophycea	Ochromonas	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
Euglenophyceae	Lepocinclis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Phacus	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	Euglena	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cryptophyceae	Chilomonas	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Chroomonas	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillariophyta	Gomphonema	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pinnularia	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Attheya/ Thalassiosira	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1

iii. A number of the bands in the DGGE gel were not successfully sequenced, and may account for the missing organisms. However Euglenophyceae sequences have not been seen in any of the other sequencing efforts within this thesis, so perhaps this is an unlikely explanation.

iv. DNA extraction is also a source of error in molecular methods. The extraction method chosen was tested on a range of algae (Eland *et al.*, 2012) including a common *Chlamydomonas* species, though none of the pure cultures tested in Chapter 3 where from the Euglenophyceae. The Euglenophyta do not have cell walls but have a tough protein based coating called a pellicle. The pellicle structure is composed of approximately 80% protein (van den Hoek *et al.*, 1995) and is highly organized. It would therefore be harder to digest for proteinase K.

v. 18S rRNA gene copy numbers vary in eukaryotes and this may create bias towards those organisms with larger copy numbers. The Euglenophyceae are an interesting case, and rare among eukaryotic microalgae in not having a tandem repeat of rRNA genes within their chromosomes. Instead they have 1 copy of the rRNA genes encoded for in an extra-chromosomal plasmid structure in the cells cytoplasm (Charette and Gray, 2009). These plasmids are present in high copy numbers (estimates of 800-4000 plasmids per cell)(Ravel-Chapuis, 1988). This alternative structure may be the cause of their non-detection by the molecular method.

vi. Phylogenetic analysis of the 18S rRNA gene region targeted in this study shows that it is sufficiently variable to provide a stable tree with Euglenophyta, Bacillariophyta and Chlorophyta forming distinct clusters. However the distances between sequences within the Chlorophyta cluster were small, having many highly similar sequences. This suggests that the gene region targeted is not necessarily good for distinguishing between different Chlorophyta genera or species. Thus, highly similar sequences are less likely to form clearly separate bands on the DGGE gel. This may have resulted in mis-assignment of DGGE sequences within the Chlorophyta group and the discrepancy observed between microscopy and DGGE. For example, the band found to be a 98% match for *Pyrobotrys stellate*, may in fact be from a *Chlamydomonas* species. BLAST searching revealed that the bands sequence was a 97% match to a

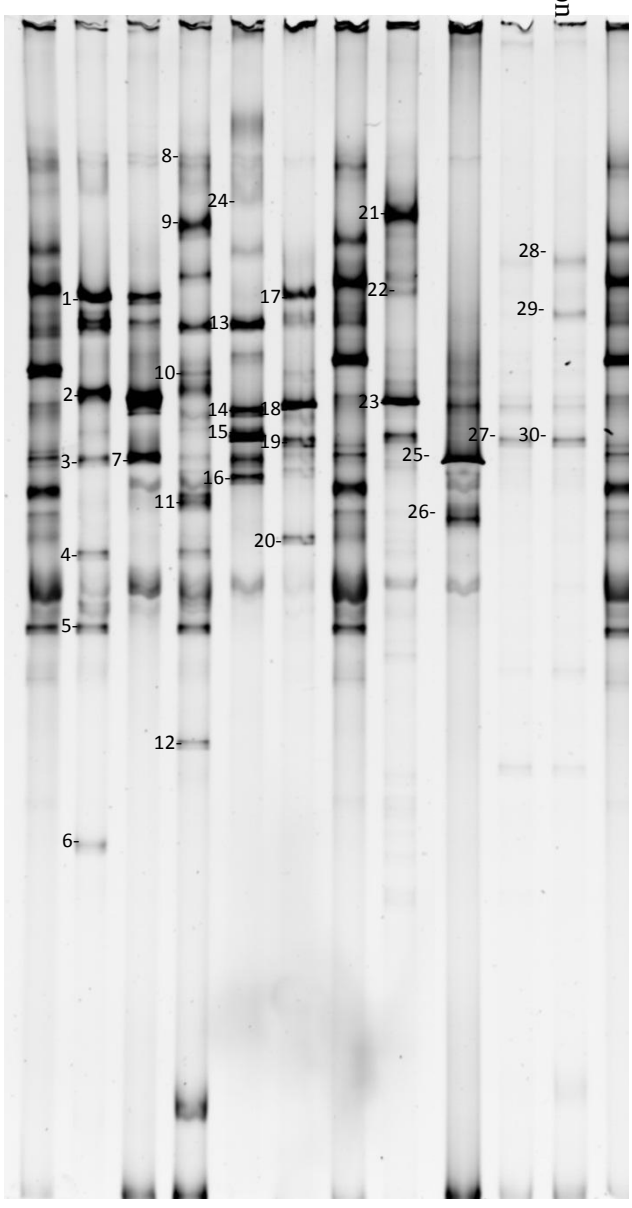
Chlamydomonas species. Both organisms belong to the Volvocales order of Chlorophyceae and are closely related. In order to improve this, a more specific Chlorophyta primer set targeting a region of high variability within this group could be used.

A number of eukaryotic microalgae were detected by DGGE that were not seen in the microscopy analysis, including sequences closely related to *Pyrobotrys stellate*, *Kirchneriella obesa* and *Tetranephris brasiliensis (*Figure 6-3*).* As explained in vi) above *P. stellate* is closely related to Chlamydomonas and with the low variability of the target region of the 18SrRNA, it is difficult to say to which genus this sequence corresponds. *T. brasiliensis* is a relatively newly identified and classified species, with the genus only being first described in 1977, in Brazil (Ramano Leite and Bicudo, 1977). Its recent identification mean that it is not commonly seen in algal taxonomy books. It has a relatively simple structure and its identification relies on its lack of pyrenoids and appendages from the cell surface that could result in it being misidentified. It is also found in a colony of four cells that 'radiate from a common centre'.

Cyanobacterial identification using microscopy proved challenging. This is in part due to the low relative abundance of cyanobacteria compared with eukaryotic microalgae and also their smaller size. Though the molecular method described highlighted the presence of a large variety of cyanobacterial species, the cyanobacteria only represent a relatively small proportion of the DNA within the sample. The cyanobacteria have very small genome sizes compared to the microalgae and have low copy numbers of the 16S rRNA gene targeted. This led to the use of a nested approach to increase amplification for visualization with DGGE. This analysis has also highlighted the lack of cyanobacterial genome sequencing done to date, with the majority of sequences being identified as 'Uncultured'.

There are also issues with microscopy related to scaling that could affect the detection of cyanobacteria more than eukaryotic microalgae. The detection of cyanobacteria requires higher magnification due to their smaller cell sizes. In addition to this in the molecular method DNA extraction is carried out on 50ml of sample, microscopy

methods on the other hand involve the viewing of a number of fields of view, made up of a much smaller sample volume.



1- *Paramecium* (*Alveolata*)
2- *Tetrahymena* (*Alveolata*)
3- *Tubulinida* (*Amoaebozoa*)
4- *Tokophyra lemnarum* (*Alveolata*)
5- *Lepadella patella* (*Rotifera*)
6- Uncultured *eukaryote*
7- *Tubulinida sp*. (*Amoebozoa*)
8- *Oxytrichia longa* (*Alveolata*)
9- *Oxytrichia longa* (*Alveolata*)
10- Uncultured *ciliate* (*Alveolata*)
11- Uncultured *alveolata*
12- Uncultured freshwater *eukaryote*
13- *Cyclidium glaucoma* (*Alveolata*)
14- *Scenedesmus sp*. (*Chlorophyta*)
15- *Scenedesmus sp*. (*Chlorophta*)
16- *Tetranesphris brasiliensis* (*Chlorophyta*)
17- *Paramecium tetraurelia* (*Alveolata*)
18- *Pyrobotyrys stellate* (*Chlorophyta*)
19- *Chlorella sp*. (*Chlorophyta*)
20- *Ochromonas vasocystis* (*Chrysophyceae*)
21- Uncultured *chytridiomycota* (*Fungi*)
22- *Paramecium multimicronucleatum* (*Alveolata*)
23- *Desmodesmus sp*. (*Chlorophyta*)
24- *Gaertneriomyces spectabile* (*Fungi*)
25- *Kirchneriella obesa* (*Chlorophyta*)
26- *Kirchneriella obesa* (*Chlorophyta*)
27- *Chlorella sp*. (*Chlorophyta*)
28- Uncultured *ciliate* clone (*Alveolata*)
29- *Opisthonecta minima* (*Alveolata*)
30- *Chlorella sp*. (*Chlorophyta*)

Figure 6-3 DGGE gel image for the Eukaryotic primer set (Euk 1A and Euk 516r).
Bands excised for sequencing are numbered and the nearest match according to BLAST analysis and Figure 6.4 shown on the right. Bands in green are algal species.

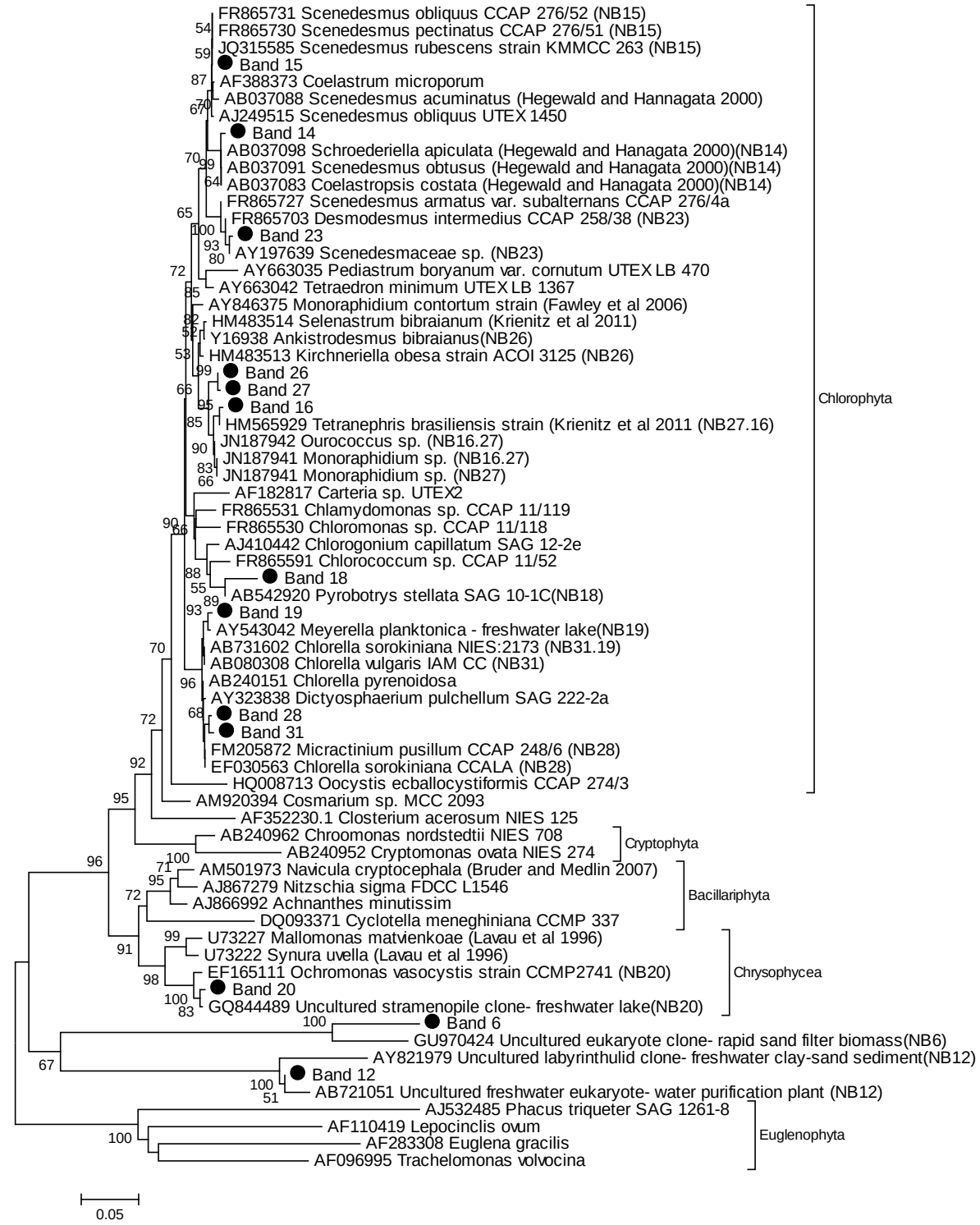
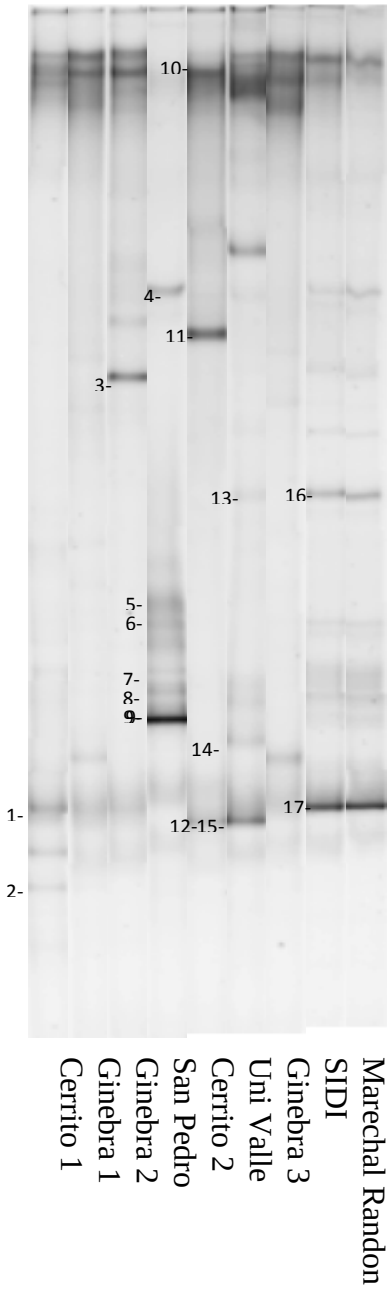


Figure 6-4 A neighbor joining phylogenetic tree showing excised microalgal DGGE bands. Nearest neighbors from BLAST (NB) were added along with a number of sequences from the Silva database corresponding with algal genera shown in previous studies to be common in WSPs. Euglenophyta sequences were used as the out-group. In order to reduce error from poor database entries, sequences selected were in most cases from culture collections (Name and no.) or published peer-reviewed sources. Uncultured sequences are followed by their cited source. Bootstrapping values greater than 50% are shown on nodes (100 replicates). BLAST results in Appendix 5.



1- Uncultured cyanobacteria (Sludge digester)
2- Uncultured bacteria (Brewery wastewater clarifier)
3- Uncultured bacteria (microbial mat in evaporation pond)
4- Uncultured Attheya/Uncultures Thalassiosira chloroplast Aquatic sediment)
5- Uncultured Attheya/Uncultures Thalassiosira chloroplast Aquatic sediment)
6- Uncultured Attheya/Uncultures Thalassiosira chloroplast Aquatic sediment)
7- Uncultured Attheya/Uncultures Thalassiosira chloroplast Aquatic sediment)
8- Uncultured Attheya/Uncultures Thalassiosira chloroplast Aquatic sediment)
9- Uncultured Attheya/Uncultures Thalassiosira chloroplast Aquatic sediment)
10- Uncultured Fischerella sp. (hot spring mat)
11- Uncultured bacterium/cyanobacterium (river water)
12- Oscillatoria sp.
13- Plant chloroplast
14- Uncultured bacteria/ cyanobacteria
15- Oscillatoria sp.
16- Chlorella variabilis chloroplast
17- Planktothrix sp. (Freshwater lake)

Figure 6-5 Cyanobacterial DGGE gel showing sequenced bands (numbered).
The most likely match appears to the right (more details on the sequencing can be seen in Appendix 6). Photosynthetic organisms are highlighted in green. A number of the bands sequenced were found to be from chloroplasts (of eukaryotic microalgae) suggesting an overestimate of the number of cyanobacterial species.

In order to directly compare the two methodologies the complexity of the data was reduced down to genus level and each band given a genus name based on the nearest neighbour BLAST search, the RDP classifier and the phylogenetic trees produced using MEGA 6. A general consensus approach was adopted though in most instances the different search methods agreed.

The condensed data table used to produce the MDS ordination plots can be seen in Table 6.3. The MDS ordination plot (Figure 6-6) confirms that the two techniques do not detect the same organisms or diversity within the samples. One way ANOSIM tests for the methodology had a Global R of 0.328 and a p-value of 0.002 and the test for the samples a Global R of -0.177 and p-value of 0.87. This showed that the method used to analyse the sample had a significant effect on the variation seen.

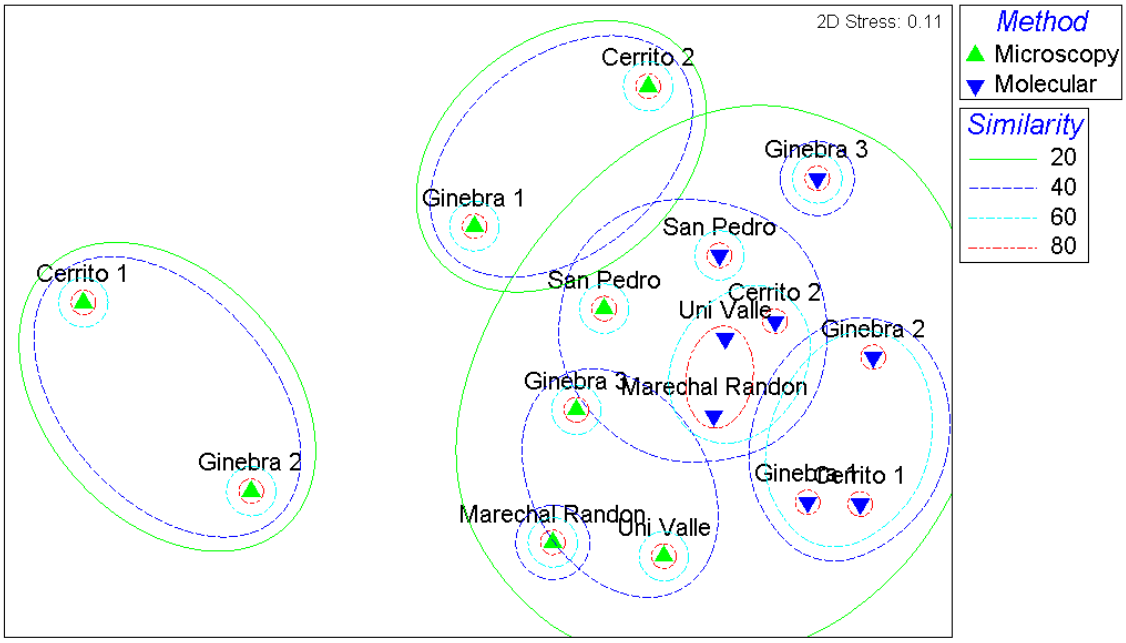


Figure 6-6 Multi-dimensional Scaling analysis ordination plot, with overlay of cluster analysis. Showing 20, 40, 60 and 80 similarity contour for samples 1-7 and 9 for data detected using microscopy (green) and molecular (blue) techniques. Sample 8 had to be removed as it was so dissimilar from the other samples (in the microscopy analysis, with no organisms seen) that distances were too great to show on the same plot.

6.4 Conclusions

The results suggest that a multi-faceted approach to ecological assessment is required to evaluate the photosynthetic organisms present in WSPs. Taxonomic microscopy techniques are well established and detect a range of eukaryotic microalgae, including the Euglenophyceae. However they are time consuming and require a high level of training and specialist knowledge and literature. The molecular methods tested show a more broad analysis of diversity and are much faster, requiring less specific species knowledge. The main problem seen with the molecular work was the broad nature of the primer set used the incomplete nature of the 18S databases available with which to compare the sequences, the oversight of Euglenophyceae and the 1% detection limit of DGGE. More comprehensive molecular sequencing methods, such as Next Generation Sequencing are available that may allow us to uncover more about the ecology of WSPs.

6.5 Recommendations

Additional work on isolating organisms from WSP systems, identifying them using taxonomic methods and sequencing their genomes would help to make positive identification of organisms by molecular methods more robust. In order for molecular methods to become more widely used in WSP ecology research a collaborative effort between phycologists, taxonomists and molecular biologists is required to optimize the methods and improve the gene databases.

Chapter 7. Community composition of WSP systems in South America- Case studies

7.1 Case study 1- Comparing the ecology of two Brazilian Waste Stabilization Pond systems- Domestic vs. Industrial/Domestic mixed wastewater

7.1.1 Introduction

As countries develop and rapid expansion of industry occurs, methods that can effectively treat industrial wastewater are required to mitigate against environmental damage. Waste Stabilization Pond systems have long been used in developing countries for domestic wastewater treatment. Hybrid systems treating a mixed influent of domestic and industrial wastewater are becoming more common as industry expands.

WSPs are widely used across the world as a passive wastewater treatment, but are often treated as a 'black box' with little known about their ecology. Currently systems are designed empirically based on organic loading, but a better understanding of the biological process involved in treatment may help to better optimise designs. Integral to WSP systems are microorganisms including non-photosynthetic bacteria (both anaerobic and aerobic depending upon pond conditions), photosynthetic bacteria and photosynthetic eukaryotes (Mara, 1997). Photosynthetic organisms make up the vast majority of biomass in facultative and maturation ponds in domestic wastewater fed WSPs. The effect of high strength industrial wastewater on these communities is unknown, as is the effect on their ability to treat the wastewater effectively.

The aim of this study was to use the molecular methods refined throughout the thesis to compare the ecology of two systems in the northeast of Brazil.

Campanhia de Água e Esgoto do Ceará, CAGECE the Ceará state water company manages treatment of wastewater and water provision in and around Fortaleza, a large city in the northeast of Brazil. The city's economy is based predominantly on the textile and leather production industries and one of the systems, SIDI (Figure 7-1, left), being investigated deals with wastewater from these industries. This wastewater is likely to contain a mixture of inorganic and organic compounds including dyes, dying aids and sizing agents from the textile industry and tanins, chlorides, proteins, non-ionic

surfactants and oils from the tanneries (Naumczyk and Rusiniak, 2005; Aber and Sheydaei, 2012). The manufacturers of metal products, plastics, concrete and ceramics, food products, soft drinks, PVC and cardboard also contribute to the wastewater stream, as do two industrial scale laundries and an industrial poultry abattoir. This wastewater stream is mixed at a ratio of 1:1 with domestic wastewater in order to supplement the nutrients and allow growth of organisms involved in the treatment process. The other treatment plant, Marechal Randon, treats domestic wastewater from a suburb of the city and has no industrial inputs.

Marechal Randon (Figure 7-1, right) consists of an anaerobic pond, two parallel facultative ponds and two maturation ponds in series. The industrial system, SIDI, contains an anaerobic treatment pond followed by one facultative and three maturation ponds in series.

Communities of cyanobacteria and eukaryotic microalgae were examined using Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing. The proportion of bacteria to phototrophs was also assessed using Flow Cytometry.

Figure 7-1 Satellite images (Google Maps) of the two WSP systems, SIDI on the left and Marechal Randon on the right.
Arrows show the location of the inlet and outlet pipes, yellow dots and writing in white shows the sample collection points and names. SIDI is located at 3°51'19" S, 38°37'24" W and Marechal Randon at 3°46'44" S, 38°38'11" W.

7.2 Methods

7.2.1 Sampling

Samples for subsequent DNA extraction were collected from two sampling points per pond in the two systems, one close to the influent and another close to the effluent. Figure 7-1 shows the layout of the two systems and the sampling points.. Samples were collected from the surface of the pond in sterile 1 litre Duran bottles and transported on ice to the laboratory. A team from CAGECE collected and processed samples for physical, chemical and microbiological analysis from the influent and effluent flows of each of the ponds, using standard methods (Eaton *et al.*, 2005). The pH of the influent and effluent streams were also measured in situ. Samples were centrifuged at 7690 x g for 10minutes, and the pellet removed for DNA extraction. An aliquot of 5mls of each sample was fixed overnight with 4% paraformaldehyde (PFA) and stored in 50:50 PBS and Ethanol at -20°C, for flow cytometry.

7.2.2 Cell Counting

PFA fixed samples were used for flow cytometry. Phototrophic and the non-phototrophic fractions were distinguished and counted using a FACs ARIA flow cytometer (Flow Cytometry Core Facility, Newcastle University) on the basis of the auto fluorescence of photosynthetic pigments (Rutten *et al.*, 2005). Side scatter (SSC) and the 488/710/50 laser and detectors were used (Chapter 5). Activated sludge was used as a negative control and several pure algal cultures as positive controls.

7.2.3 DNA extraction and PCR amplification

DNA was extracted from 80ml samples using a DNeasy® Blood and Tissue kit (Qiagen, UK), following the manufacturers animal tissue protocol, as previously described (Eland *et al.*, 2012) (Chapter 3).

Amplification of 18S and 16S gene fragments was carried out in duplicate by PCR. A GC clamp was added to the 5'-end of the forward primers for PCR in order for DGGE analysis to be carried out. PCR was carried out using three primer sets Euk 1A and Euk 516r (Diez *et al.*, 2001) to target 18S rRNA genes in eukaryotic microalgae and Cya-b-

F371 and Cya-R783 and modified 2/3 (Muyzer *et al.*, 1993b) called F357GC and R518 to target cyanobacterial 16S rRNA. Cya-b-F371 and Cya-R783 amplification product was nested with the F357GC and R518 primers (Zwart *et al.*, 2005). Details of the primers and reaction conditions are in Table 2-2.

7.2.4 Community analysis

The predominant eukaryotic and cyanobacterial organisms within the communities were compared using DGGE. DGGE was carried out following the methodology set out in Chapter 2.

Dominant bands were excised from the DGGE gel after imaging using sterile needles and stored in TE buffer. Band DNA was amplified using the initial primers and PCR programs. A QIAquick PCR purification Kit (Qiagen, UK) was used to clean up DNA before sending for Sanger sequencing (GeneVision, Newcastle, UK). Sequences were aligned and identified using NCBI BLAST (Altschul *et al.*, 1990) against the nucleotide collection (nr/nt) databases and additionally cyanobacterial sequences were entered into the RDP classifier (Wang *et al.*, 2007).

BioNumerics (Applied Maths, Belgium) was used to define and normalise bands within the DGGE gel and to perform cluster analysis. Gene copy numbers in eukaryotes are more variable than those in prokaryotes (Zhu *et al.*, 2005), so band intensity may be skewed by species with higher copy numbers. To reduce the effects of this band presence- absence data was used in Primer 6 software (Clarke and Gorley, 2006) to analyse the similarity between samples for both communities.

Species richness (S) was calculated using presence absence data from normalised and Pielou's evenness index (J') using band height data from normalised DGGE gel images. Each band was deemed to represent a unique operational taxonomic unit (OTU). Pielou's evenness index us a measure of equitability and was calculated using equation 1 below, where H'max is the maximum possible value of Shannon Wiener diversity (H').

$$J' = \frac{H'}{H'max} = \frac{H'}{logS}$$

MDS (Multidimensional Scaling) analysis was carried out and ordination plots produced using presence-absence data generated from the DGGE images. Two way crossed ANOSIM analysis was used to assess the difference between samples from the two sites and between samples from different stages in the treatment systems, for both eukaryotic and cyanobacterial community data.

7.3 Results and Discussions

7.3.1 Treatment performance

Chemical, physical and biological data for the treatment systems can be seen in Table 7-1. The wastewater influent in SIDI is characterised by low ammonia level. At 10.47mg $N\text{-}NH_3$/l and high total solids (1852 mg/l), whilst the domestic system has comparatively high ammonia (51.68 mg $N\text{-}NH_3$/l) and phosphate levels (6.421 mg $P\text{-}PO_4^{-3}$/L). For both treatment systems a percentage decrease in faecal coliforms of 99.99% was achieved, Total suspended solids also decrease in both SIDI and Marechal Randon, by 70% and 81.2% respectively. The starting BOD was not supplied for the SIDI treatment system, but COD reduced by 65.86%, throughout the system, with the biggest reduction occurring in the anaerobic pond. COD reduction of 75.87% and BOD reduction of 88.96 was achieved by the domestic system (MR). The data shows that the Marechal Randon system effectively removes ammonia, with a reduction of 98.27%, but is less efficient at phosphate removal (42.21% decrease). The SIDI treatment data suggests the opposite with a 14.61% increase in ammonia and an 82.78% reduction in orthophosphate, by the end of maturation pond 2, though effluent data was not provided. The low levels of oxygen seen throughout the SIDI treatment process are indicative of ponds containing low levels of algae, since algae provide much of the oxygen in these passive non mixed treatment systems.

Table 7-1 Chemical, physical and biological data for treatment processes.
ND- Not detected, NP- Not provided

		Raw sewage Influent	Anaerobic output	Facultative output	Maturation 1 output	Maturation 2 output	Maturation 3 output
pH	MR	7.31	7	7.65	8.12	7.95	
	SIDI	8.56	7.62	8.09	8	7.74	7.45
Total suspended solids (mg/l)	MR	405	31	71	90	76	
	SIDI	176	110	70	85	66	52
Total solids (mg/l)	MR	1185.5	592.5	578	606.5	580.5	
	SIDI	1852	1565	1217	1321.5	1193.5	NP
Ammonia (mg $N\text{-}NH_3$/l)	MR	513.68	31.69	15.78	13.04	8.91	
	SIDI	10.47	13.47	14.47	11.83	12	NP
Orthophosphate (mg $P\text{-}PO_4^{-3}$/l)	MR	6.421	3.79	3.099	2.813	3.711	
	SIDI	2.56	1.478	0.125	0.109	0.106	NP
Nitrate (mg $N\text{-}NO^{-3}$/l)	MR	0.027	0.07	0.056	0.085	1.608	
	SIDI	0.024	ND	ND	ND	ND	ND
Nitrite (mg $N\text{-}NO^{-2}$/l)	MR	0.005	ND	ND	0.018	0.71	
	SIDI	ND	ND	ND	ND	ND	ND
Total BOD (mg O_2/l)	MR	626.6	158.15	120.36	126.12	69.19	
	SIDI	NP	NP	NP	NP	89.41	129.55
Total COD (mg O_2/l)	MR	704.5	194.3	174.1	194.3	170	
	SIDI	513.2	362	277	244.4	166.3	175.2
Dissolved Oxygen (mg O_2/l)	MR	NP	NP	8.7	8.5	13.4	
	SIDI	NP	NP	ND	0.19	0.79	1.9
Total coliforms (cells/100mls)	MR	9.9×10^7	2.2×10^6	1.0×10^6	5.1×10^5	8.6×10^4	
	SIDI	6.1×10^7	3.0×10^6	2.4×10^7	3.6×10^5	5.3×10^5	1.9×10^5
E.coli (cells per 100mls)	MR	2.9×10^7	9.6×10^5	1.1×10^5	9.3×10^3	2.4×10^3	
	SIDI	1.1×10^7	8.3×10^5	3.8×10^6	8.1×10^4	4.2×10^3	$<1.0 \times 10^2$

7.3.2 Cell Counts

The industrial treatment system, SIDI, was shown to have overall higher non-photosynthetic counts than the domestic system, Marechal Randon (Figure 7-2). This result is supported by on site assessment of the ponds, with the domestic system appearing green and the industrial systems early facultative and early maturation ponds appearing black and pink respectively. The black colouration can be explained by the high proportion of indigo dye chemical that could be seen in clumps within the samples collected. The pink colouration may be as a result of growth of purple sulphur bacteria, common in ponds with anoxic conditions and sulphides (Belila *et al.*, 2013).

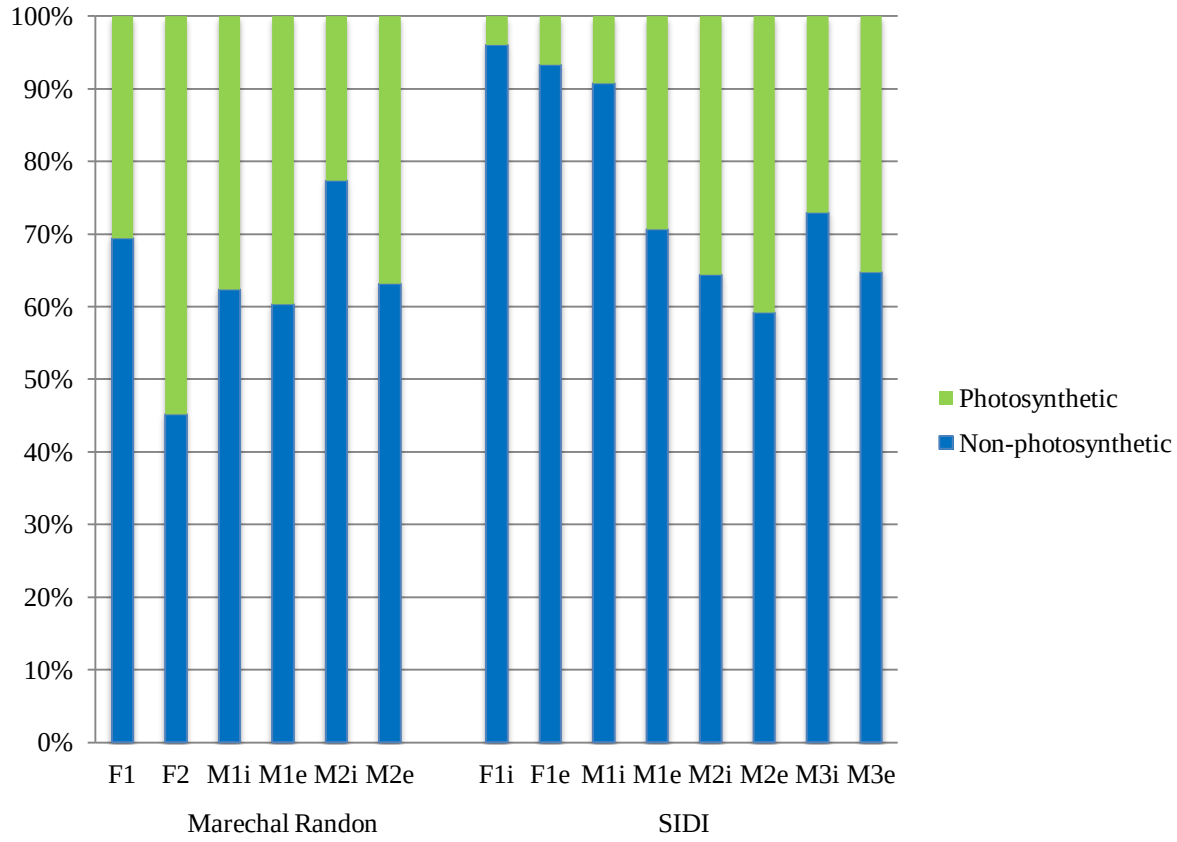


Figure 7-2 Flow cytometry counts of photosynthetic and non- photosynthetic 'events' in WSP samples. Samples named as follows; F1- Facultative pond 1, F2- Facultative pond 2, F1i- Facultative pond 1, influent sample, F1e- Facultative pond 1, effluent sample, M1i- Maturation pond 1, influent sample, M1e- Maturation pond 1, effluent sample, M2i- Maturation pond 2, influent sample, M2e- Maturation pond 2, effluent sample, M3i- Maturation pond 3, influent sample, M3e- Maturation pond 3, effluent sample.

7.3.3 Community Analysis

The eukaryotic communities were found to be most similar in samples taken from the same pond system, with all Marechal Randon samples clustered the 60% similarity contour,

Figure 7-3a. The eukaryotic community is more variable in the SIDI system forming two clusters. Broadly those samples from earlier in the system are more dissimilar than all of the later samples and those from the Marechal Randon system (within the 40% similarity contour). This suggests that the nature of the wastewater entering the treatment system and the inoculum used to start the system has a greater effect on the species that dominate than the whether the pond is facultative or for maturation, as shown in Curtis *et al.* (2006) in activated sludge systems. The pattern is similar, though

slightly less pronounced, for the cyanobacterial community, with the two systems being largely dissimilar from one another,

Figure 7-3b. ANOSIM confirmed this with an R value of 0.667, when testing the difference between site groups for the cyanobacterial community (significance level of 3.7%) compared to an R value of 0.688 for between site groups for the eukaryotic community (significance level of 3%). ANOSIM R values were much lower for the between treatment stage group of samples, suggesting that this factor accounts for less variability than the site. A more systematic study would be required to confirm whether stochastic immigration or environmental niche effects are driving community differences.

Pielou's evenness index (J') for cyanobacteria tended to be relatively constant throughout the systems (

DGGE gel images were assessed and 9 of the dominant eukaryote bands (Appendix 7) and 17 of the dominant cyanobacterial bands were sequenced (Appendix 8). Sequencing of these bands has shown a huge range of eukaryotic diversity from microalgae such as *Chlorella sorokiniana* and *Parachlorella kessleri* and ciliates such as *Opisthonecta minima* and rotifers like *Brachionus calyciflorus*. Chlorella species appear to be common across both pond systems and in all stages of the treatment (Figure 7-5). Parachlorella was seen in all of the Industrial treatment systems samples, but only at low levels in the domestic treatment system.

Table 7-2). In the domestic system it decreased in the final pond to 0.7872. Cyanobacterial species richness (S) also showed an upward trend throughout the SIDI system, ranging from 6 OTUs in the first facultative sample to 17 OTUs in the final maturation sample. This dramatic increase in cyanobacterial band richness was not seen in the Marechal Randon samples, with a smaller range of between 8 and 12 OTUs, the smallest value occurring in the final pond. Eukaryotic band richness showed a similar pattern, with a wide range from 3 bands to 14 in the SIDI system and only 7 to 13 in the Marechal Randon system. This suggests that there is more variability in community structure in samples across the SIDI pond system than the Marechal Randon system.

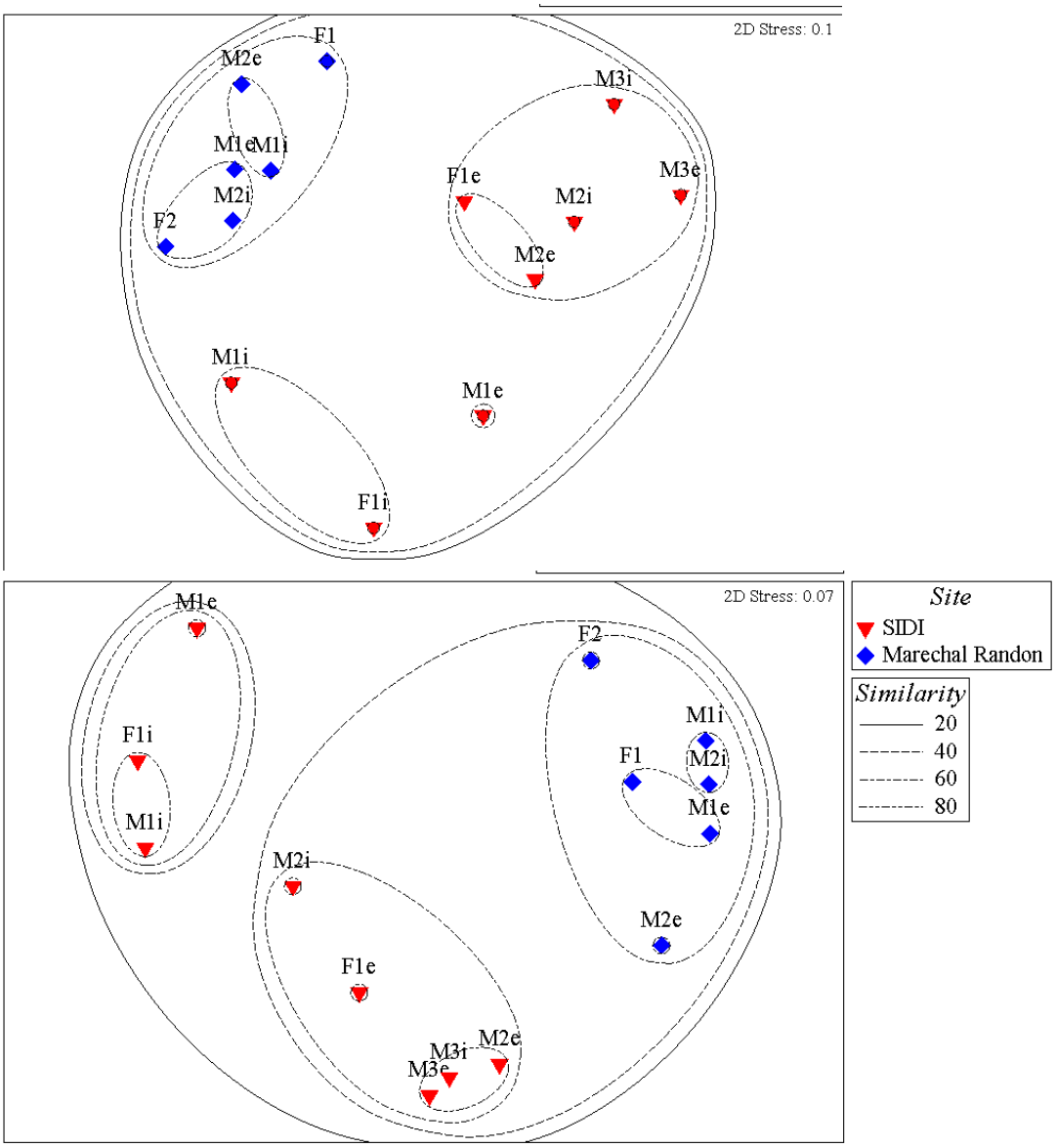


Figure 7-3 Primer 6 Non-metric multidimensional scaling (MDS) ordination plots.
Based on similarity between a) Eukaryotic community in samples, b) Cyanobacetrial community in samples, based on DGGE data. Contours represent the degree of similarity expressed as a percentage.

DGGE gel images were assessed and 9 of the dominant eukaryote bands (Appendix 7) and 17 of the dominant cyanobacterial bands were sequenced (Appendix 8). Sequencing of these bands has shown a huge range of eukaryotic diversity from microalgae such as *Chlorella sorokiniana* and *Parachlorella kessleri* and ciliates such as *Opisthonecta minima* and rotifers like *Brachionus calyciflorus*. Chlorella species appear to be common across both pond systems and in all stages of the treatment (Figure 7-5).

Parachlorella was seen in all of the Industrial treatment systems samples, but only at low levels in the domestic treatment system.

Table 7-2 Pielou's evenness index (J') and Species (OTU) richness (S) for the cyanobacterial and eukaryotic communities in treatment system samples.

	SIDI cyanobacteria		SIDI eukaryotes		Marechal Randon cyanobacteria		Marechal Randon eukaryotes	
	J'	S	J'	S	J'	S	J'	S
F1 or F1i	0.8748	6	0.8662	3	0.8156	11	0.9002	9
F2 or F1e	0.8406	14	0.6781	9	0.8403	10	0.6497	7
M1i	0.8374	8	0.9703	4	0.8148	10	0.9598	10
M1e	0.8751	12	0.8529	3	0.8144	10	0.7912	10
M2i	0.911	14	0.7977	7	0.8292	12	0.7751	12
M2e	0.8708	12	0.7814	13	0.7872	8	0.7234	13
M3i	0.8637	12	0.8431	14				
M3e	0.8775	17	0.8706	14				

Planktothrix rubescens or *P.agardhii* related cyanobacterial bands (Figure 7-4) (12 & 17) were seen to be the dominant cyanobacteria in the facultative ponds of the SIDI plant and throughout all of the Marechal Randon system. Bands matching the *Arthrospira* in the database (bands 13 & 14) were found in the SIDI treatment system (particularly in the first half of the treatment process). This group of organisms is commonly found where pH and dissolved solid levels are high (Mara, 1997).

Sequencing of cyanobacterial bands also highlighted problems commonly seen in the molecular identification of photosynthetic prokaryotes (Knapp and Graham, 2004). The shared evolutionary history of cyanobacteria and eukaryotic chloroplasts, results in 16S rRNA genes being present in the chloroplasts of eukaryotes (Giovannoni *et al.*, 1988). Around half of the bands seen in the DGGE had sequences whose best match in the database was an algal chloroplast or plastid.

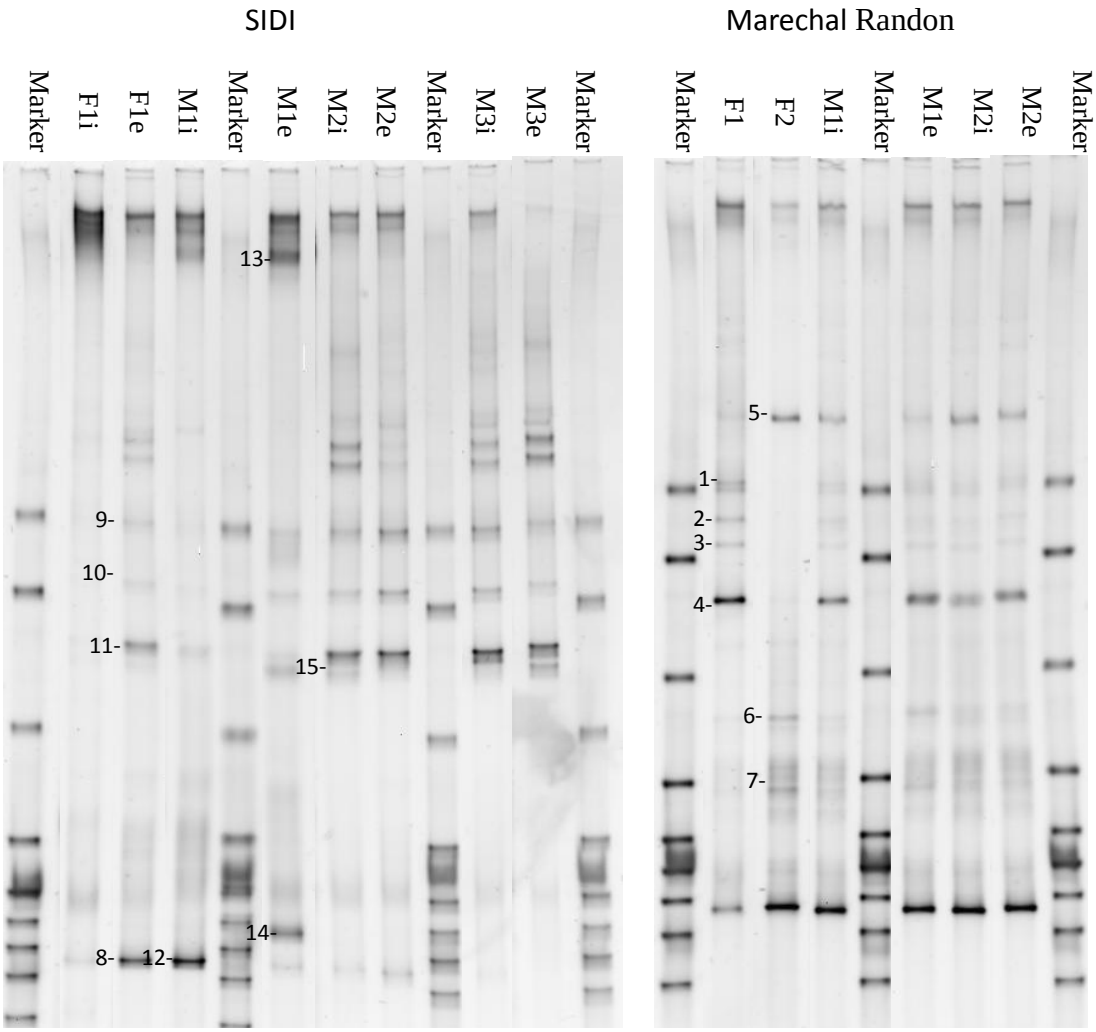


Figure 7-4 Denaturing gradient gel electrophoresis image, showing cyanobacterial community. Bands that were isolated and sequenced are numbered.

DGGE is a good method with which to produce a broad assessment of community diversity and how this changes or is affected by external factors. Though it is a well-established technique its limitations should be considered. The detection limit of DGGE is thought to be 1%, that is organisms making up less than that percentage of the population are largely undetected (Akarsubasi *et al.*, 2009).

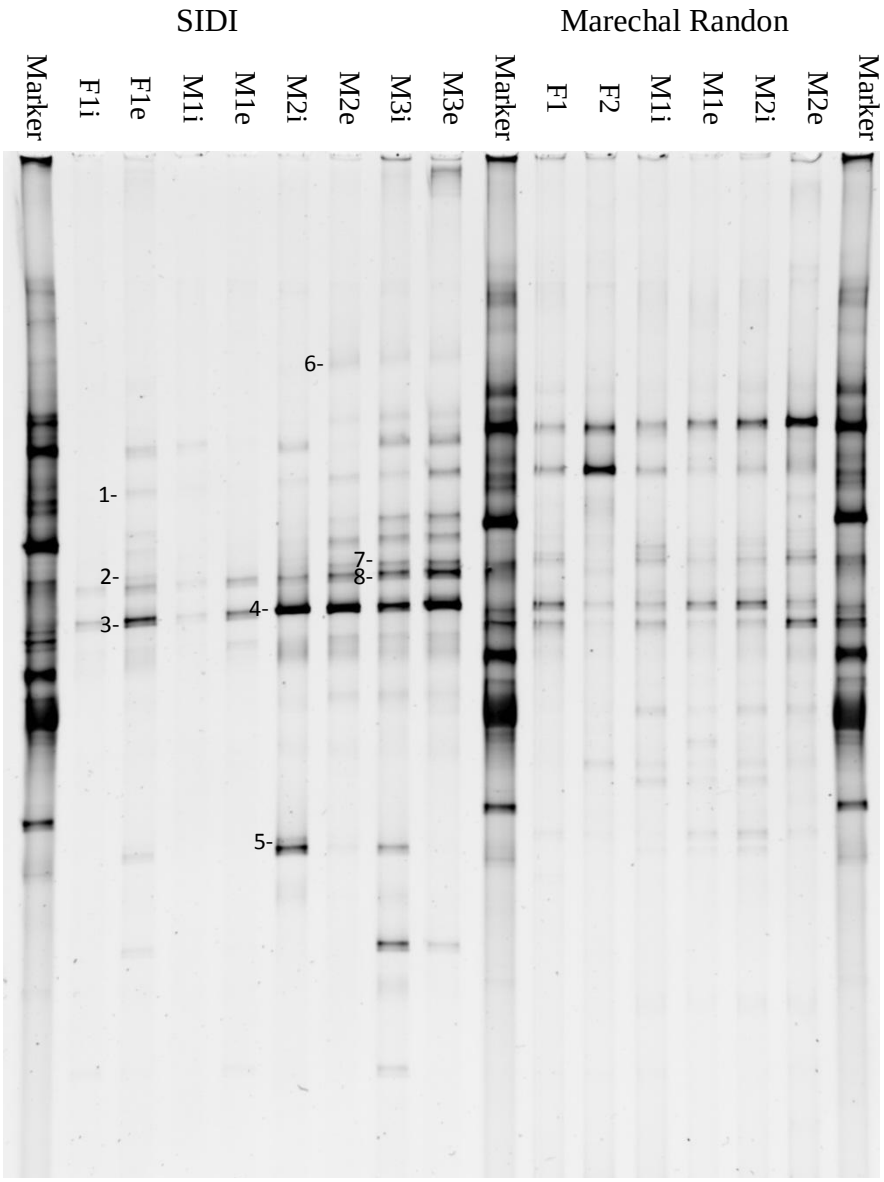


Figure 7-5 DGGE image, showing eukaryotic community.
Bands that were isolated and sequenced are numbered. Details of the sequences obtained can be seen in Appendix 7.

7.4 Conclusions

The ecology of the two wastewater treatment systems have inherent differences, with the proportions of non-photosynthetic to photosynthetic organisms and also the patterns of diversity in succession throughout the pond series. There are however some OTUs common across both systems, with sequencing showing Chlorella species to be the most common in both these Brazilian treatment pond systems. In order to establish a direct link between the community differences and the wastewater source treated an extended study including more sites and accounting for initial inoculum use to seed the ponds

would be required. Data on theoretical and actual organic loads and HRTs were not available, though may provide further insight into the pressures on the photosynthetic communities.

Further work on improving the database used for comparing 18S rRNA sequences will be required if molecular biology techniques are to become common place in WSP research. This will require close work with taxonomic specialists to sequence microalgae found in WSPs.

7.5 Case study 2- The effect of hydraulic engineering interventions on the ecology of photosynthetic communities

7.5.1 Introduction

Engineering solutions to increase the wastewater treatment efficiency of ponds have long been used (Shilton and Sweeney, 2005). Baffles to increase hydraulic efficiency of ponds have been used in both facultative (Zanotelli *et al.*, 2002) and maturation ponds (Lloyd *et al.*, 2003; Ouali *et al.*, 2012) to great effect. The reduction in hydraulic short circuiting and the resultant increase in hydraulic retention time have been shown to improve treatment efficiency and reduce *E.coli* and *enterococci* counts in effluents from maturation ponds, compared to the same ponds without baffles (Ouali *et al.*, 2012). Facultative ponds with baffles have been shown to have greater phosphorus removal potential (Zanotelli *et al.*, 2002). Tracer studies, using Rhodamine, on ponds before and after the addition of baffles have been carried out to show the improved hydraulic efficiency and the reduction in 'dead zones'(Shilton and Harrison, 2003). Shilton and Harrison (2003) produced guidelines to improve hydraulic efficiency of WSPs based on data that considered baffles as well as inlet and outlet design and positioning. The addition of two baffles (70% of the pond width) to the CFD model showed a significant improvement in faecal coliform reduction. Shorter baffles were also tested and found to confer a similar advantage to the 'traditional' baffles.

The effect of baffles on the microalgal and cyanobacterial community within a WSP has never previously been studied. The aim of this case study was to assess the effects of baffles on the photosynthetic community, both the eukaryote microalgae and the cyanobacteria. The community present in the water column over the course of a day and at two different pond depths was also investigated.

7.5.2 The System

The Station for Research and Technology Transfer in Wastewater Treatment (ARD) and reuse is located in Ginebra, a town in the Valle del Cauca region of Colombia, located 3° 43' 50'' north latitude and 76° 16' 20'', at 1040 meters above sea level. Ginebra has an average temperature of 23°C and an average annual rainfall of 1280 mm.

Wastewater from ananaerobic ponds flows into a series of experimental secondary facultative ponds set up in parallel. Both ponds received an applied organic load of 6.2 kg BOD d^{-1} and had a theoretical HRT of 4.3 days.

7.6 Methods

7.6.1 Sampling

Samples were collected at 18:00 on the first sampling day and then at 06:00, 09:00 and 12:00 on the following day.

Two 500ml samples were collected at each sampling point, in sterile containers, one from approximately 5cm below the surface of the lagoon and another using a Kemmerer sampler bottle at a depth of 40cm, where light penetration was seen to be at its limit (Figure 7-6). 50ml of each sample was centrifuged, and the pellet collected for DNA extraction in sterile 2ml Eppendorf tubes. They were frozen in dry ice, in a cool box, to rapidly freeze them prior to transportation back to the Environmental Biotechnology Laboratory, Universidad del Valle. 3ml of each sample was split between 3 Eppendorf tubes and stored in the freezer until fixation with paraformaldehyde. The remainder of the sample in the 500ml bottle was fixed with sulphuric acid in order to preserve it for measurement of physicochemical parameters, and placed in a fridge at 4'C.

Two sampling points were chosen in each pond and samples taken here at two different depths, as shown in Figure 7-7.

7.6.2 Physical and Chemical Parameters

A number of parameters were measured in situ, including temperature, light intensity, pH and dissolved oxygen (DO) concentration. Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Phosphate (PO_4), Total Kjeldahl Nitrogen (TKN), Ammonium (NH_4) and Nitrates ($N\text{-}NO_3$) were all measured in the CINARA laboratories in the week following the sampling, using standard methods (Eaton *et al.*, 2005).

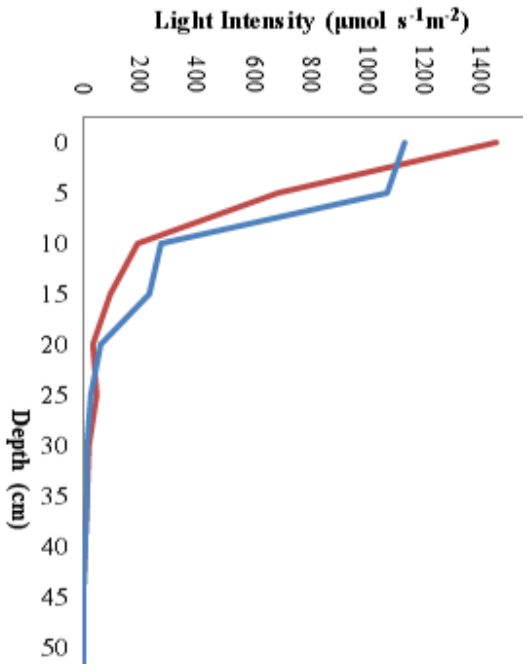


Figure 7-6 Light penetration profiles with increasing depth, readings taken at midday, at point B1 (red) and C1 (blue)(sampling points shown in Figure 7.7).
The uneven curve in pond C1 was due to the effect of variable cloud cover during measurement.

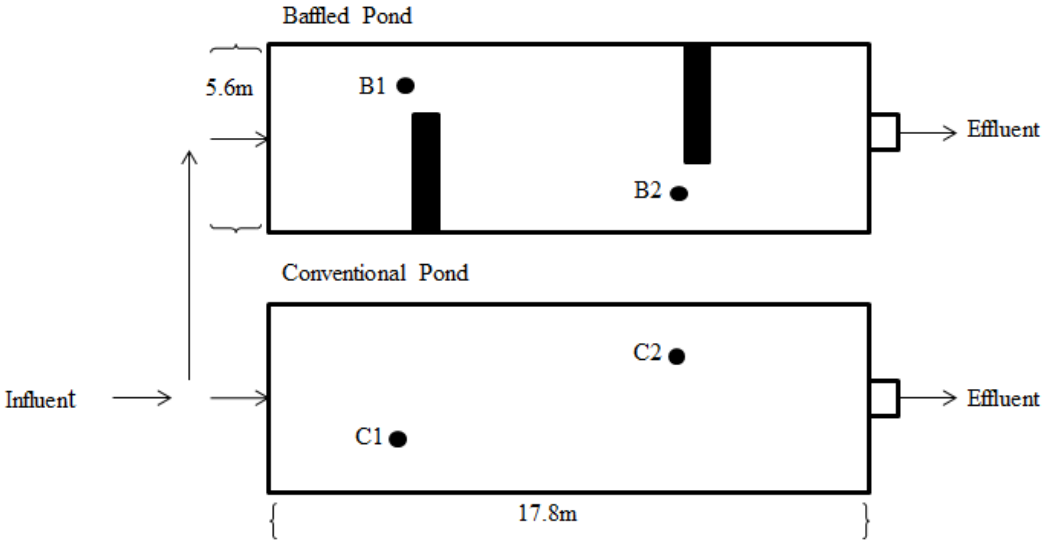


Figure 7-7 sampling points in the baffled facultative pond (top) and the conventional pond (bottom). Bamboo ladders were placed across the width of the pond for easy access, samples were taken where there was no shade from the ladder.

A light intensity profile was also constructed using measurements taken from points B1 and C1 at midday (12:00) on the second day of sampling. Light intensity was also measured at each sampling position at each sampling interval.

7.6.3 DNA extraction and PCR amplification

DNA was extracted from pellets of material removed from 50ml samples by centrifugation, using Qiagen's Blood and Tissue kit, following the standard method (Chapter 2). Amplification of DNA was carried out in duplicate by PCR using the methods and primer set described in Case study 1.

7.6.4 Diversity analysis

The diversity of the photosynthetic community was evaluated using DGGE, as described in Chapter 2. Gels were produced for both the cyanobacterial and eukaryotic microalgal fractions. Gel bands were isolated, the DNA within them re-amplified and Sanger-sequencing carried out to identify the dominant organisms present.

DGGE was used to examine microbial community structure in a subset of samples covering all of the potentially important variables: pond type, sample position, depth and time. The gel images were analysed using BioNumerics and Primer 6.

Primer 6 was used to analyse the DGGE gel image data. The DIVERSE statistics, cluster analysis, multi-dimensional scaling analysis and ANOSIM completed in Case study 1 was calculated. One-way ANOSIM was used to test the similarity between samples within groups compared to that of the whole community (pond type, depth and time). One-way ANOSIM tests for each of the variables were carried out for the bacteria, cyanobacteria and the eukaryotes. Global-R values of zero mean that the samples between and within the test group have the same mean community structure, values closer to one show that the samples within a group are more similar to each other than to samples from other groups. In addition, multivariate analyses of physical (temperature and light intensity) and chemical data (pH, DO, BOD, COD, PO_4, TKN, NH_4 and N-NO_3) were correlated with those of microbial community similarity using Bio-Env analysis within the BEST tool (Primer 6). This analysis assesses the extent to which the 'environmental' data correlates or predicts the observed similarity patterns observed between different microbial communities.

7.6.5 Cell Counting

Cell counting was carried out using the flow cytometry method defined earlier in the thesis. Activated sludge was used to set the negative or non-photosynthetic gate and microalgal and cyanobacterial pure cultures (Figure 5-1) were used to set the positive or photosynthetic gate (described in Chapter 5). Samples fixed in paraformaldehyde were washed, filtered and passed through the LSR II (BD).

7.7 Results and Discussions

7.7.1 Proportions of photosynthetic and non-photosynthetic cells

The proportion of photosynthetic to non-photosynthetic organisms seen in the samples was assessed with flow cytometry (Figure 7-8). The baffled pond showed a greater percentage of photosynthetic organisms both on the surface and at depth, compared to those detected in the conventional pond where ratios of photosynthetic to non-photosynthetic organisms were more variable. This was expected since the baffles are designed to disrupt any differential/short-circuiting flows, and natural stratification from passive diffusion gradients, typical of conventional ponds. Lloyd *et al.* (2003) showed that the addition of long baffles to partition a maturation into channels, had the effect of increasing the HRT of the pond and reducing hydraulic short circuiting. The differences between the baffled and conventional pond were maintained throughout the day (Figure 7-8). In the conventional pond, the greatest ratio of photosynthetic to non-photosynthetic organisms was observed at 9am in the surface samples and at 6pm in the depth samples. This may be due to motile cyanobacteria and algae moving to areas of lower light intensity during the brightest part of the day (negative phototaxis), away from the surface at 12 noon compared with at 6am and 9am when light intensity is lower. Phototaxis is seen in Euglenophyceae, the *Chlamydomonas* genus and also in cyanobacteria (van den Hoek *et al.*, 1995).

Flow cytometry counts the number of "events", which are often equated to cells, though in practice colony forming microalgae and cyanobacteria are also highly likely to be counted as one event. This may lead to some biases in cell counts, if conditions favoured colonial forms over unicellular algae.

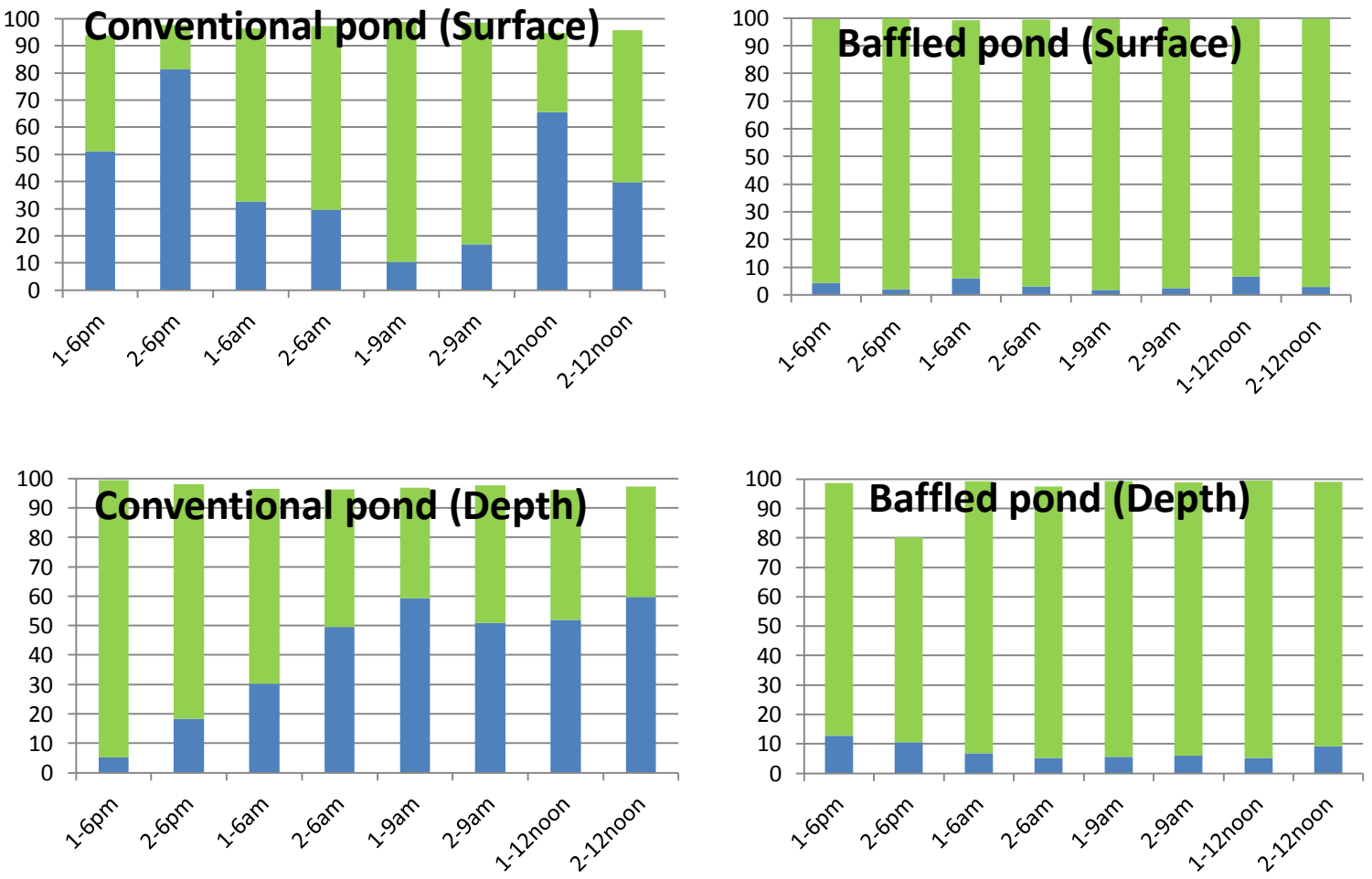


Figure 7-8 The proportion of photosynthetic (green) to non-photosynthetic organisms (blue) detected as cell (event) counts using flow cytometry for surface samples (top) and samples from 40cm depth (bottom) of a conventional pond (left) and baffled pond (right). The numbers before the time represent the sampling point (1 or 2) as shown in Figure 7.7.

7.7.2 Community analysis for reduction of variables

Cluster analysis and MDS plots suggested that there were minimal differences in the similarity of eukaryotic communities between samples taken from two different positions within a pond (Figure 7-9). This was confirmed using one way ANOSIM (Global-R value = -0.077, p-value = 0.756). In all further analyses samples from only one of the positions (position 1) were included in the DGGE analysis.

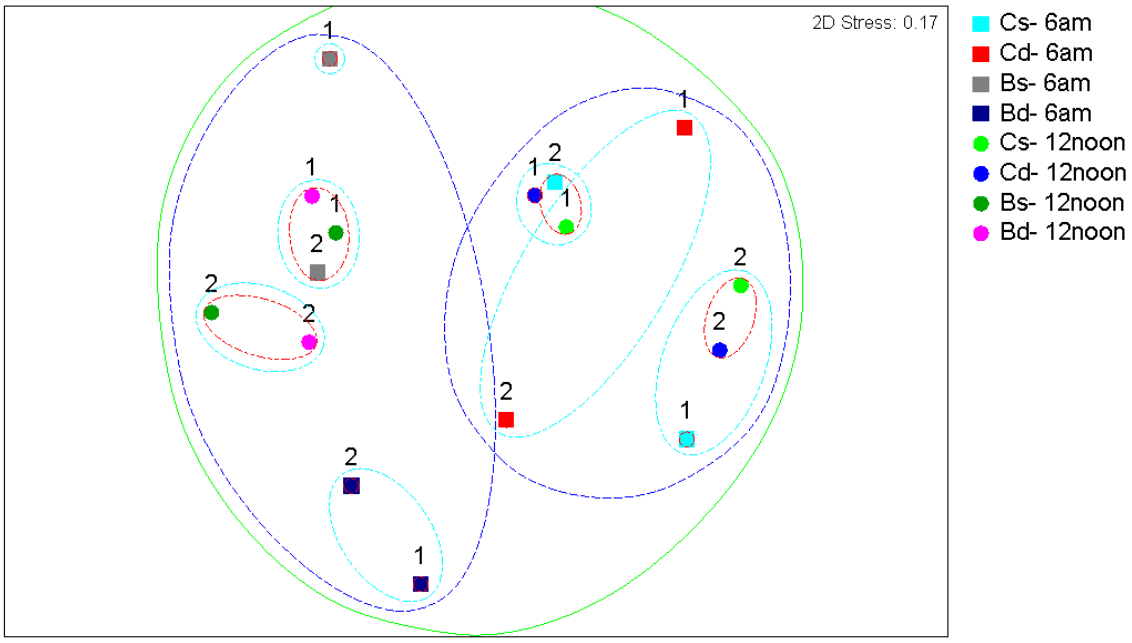


Figure 7-9 MDS plot showing the pairs of samples (from point 1 and point 2) for each of the sampling times.

7.7.3 Community Analysis

MDS ordination plots showed that the predominant eukaryotic communities (Figure 7-10a) within a pond were significantly more similar than between ponds. The close clustering of the samples from the baffled pond, suggest that the community seen in this pond was less variable over time and depth. In the conventional pond samples the community shows less similarity with a wide spatial spread of samples on the ordination plot. ANOSIM (shown in Table 7-3) confirms that similarity of samples was significantly higher within ponds than between them. Eukaryotic communities from the baffled pond showed less spread (spatially on the ordination plot) and also lower OTU richness than those from the conventional pond, with average OTU richness values of

8.125 and 11.25 respectively. The range of values was only between 7 and 8 for the baffled pond, with little difference between surface and depth samples. The conventional pond had a eukaryotic OTU range of between 8 and 18, with surface samples containing consistently more eukaryotic OTUs than at depth.

The bacterial community also show no overlap between samples on the ordination plot (Figure 7-10b) from the two ponds and this was confirmed as being significant by ANOSIM (Table 7-3). However the close clustering of the baffled pond samples seen in the eukaryotes was not seen in the bacteria. The bacterial OTU richness is relatively stable across all of the samples and at all depths, with a range for both of the ponds of 8-13 OTUs.

The cyanobacterial communities on the other hand less were affected by the pond type, with the samples from the two ponds not forming distinct groups in the ordination plot (Figure 7-10c). For cyanobacteria the time of day has a greater effect on the community, with community similarity being highest between samples taken at 12noon and 6pm (with all but one sample, the 6pm depth sample) having greater than 60 % similarity). Time was shown to be significant using ANOSIM (Table 7-3). Cyanobacterial OTU richness showed low variation across all of the depths and time periods in both ponds, except in the conventional pond at 6pm when the depth sample had an OTU of 16 (the highest cyanobacterial OTU richness seen).

Table 7-3 ANOSIM Global R and P-values calculated from the DGGE image data, using BioNumerics and Primer 6 software. Significant values are in bold.

	Pond type		Depth		Time	
Organisms Targeted	Global-R	p-value	Global-R	p-value	Global-R	p-value
Eukaryotes	**0.95**	**0.002**	0.02	0.452	0.131	0.889
Bacteria	**0.668**	**0.001**	0.011	0.484	0.124	0.174
Cyanobacteria	0.015	0.347	0.022	0.301	**0.385**	**0.001**

It is interesting that the increased hydraulic mixing seen in the baffled pond affects the patterns of eukaryotic diversity so strongly, but that it does not appear to effect cyanobacteria.

The baffled ponds eukaryotic community according to the DGGE profile (Figure 7-12) appears to be dominated by eukaryotic microalgae rather than non-photosynthetic

forms. In the conventional pond this is the other way around. Hydraulic mixing of the water assists microalgae to overcome light attenuation. When water is still microalgae at the surface attenuate light and prevent it from reaching the microalgae deeper in the water column. This results in a very narrow zone where algae are able to photosynthesise. In well mixed waters, the algae are moved around throughout the water column allowing more algae to photosynthesise, grow and multiply in the wider zone, without becoming light limited (Dobson and Frid, 1998).

Cyanobacteria may be less affected by light attenuation problems in waters that are not mixed, as cyanobacteria have the ability to move in the water column to where conditions suit them (van den Hoek *et al.,* 1995), a trait only shared by a small number of motile algal species. Cyanobacteria also survive better in areas where light intensity is not too high, so light attenuation by algal species in the surface layer has less affect in them.

Table 7-4 Pielou's evenness index and OTU richness, for eukaryotes, bacteria and cyanobacteria, for each of the sampling points.

		Eukaryotes		Bacteria		Cyanobacteria	
		J'	S'	J'	S'	J'	S'
Conventional	s-6pm	0.8372	13	0.8407	12	0.8073	11
	d-6pm	0.8221	8	0.8889	13	0.8119	16
	s-6am	0.845	12	0.8356	12	0.7124	10
	d-6am	0.8857	8	0.8483	10	0.8311	8
	s-9am	0.894	14	0.839	11	0.722	9
	d-9am	0.7464	9	0.8286	11	0.7979	10
	s-12noon	0.8889	18	0.7813	13	0.8163	12
	d-12noon	0.6904	8	0.856	8	0.7948	11
Baffled	s-6pm	0.8821	10	0.9156	13	0.766	10
	d-6pm	0.7591	7	0.9081	11	0.8015	10
	s-6am	0.8754	8	0.8851	12	0.8237	12
	d-6am	0.833	7	0.8716	12	0.7811	9
	s-9am	0.8714	7	0.8807	11	0.7966	12
	d-9am	0.8809	8	0.9097	10	0.754	9
	s-12noon	0.7831	10	0.8681	13	0.8332	11

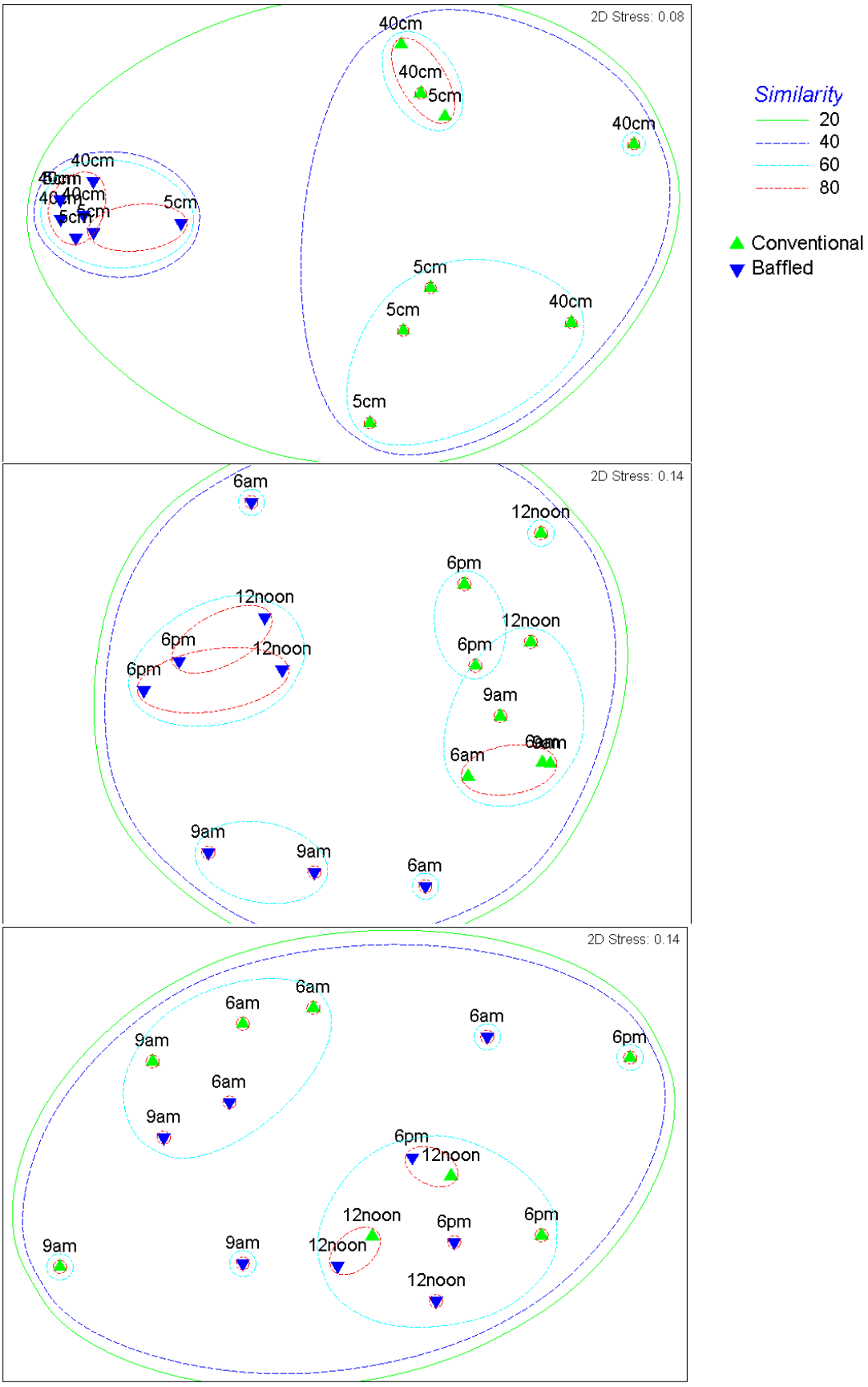


Figure 7-10 MDS ordination plots, with cluster analysis overlays. a) Eukaryotes (top), b) Bacteria (centre), c) Cyanobacteria (bottom)

7.7.4 BEST- Analysis of environmental variables and community similarity

Table 7-5 Summary of Bio-Env analysis of environmental variables and eukaryotic, bacterial and cyanobacterial DGGE OTU community presence-absence data.
Spearman's Rho values shown for most significant variable combinations. Light intensity data was not used in the analysis, as the data could not be transformed to fit the assumptions of the test. None of the combinations were found to be statistically significant (significant = p value of less than 0.1).

Organisms Targeted	No. of variables	Best variable combination	Global R/ spearmans rho	P-value
Eukaryotes	1	NH4	0.121	0.63
Bacteria	5	COD, NH4, N-NO3, pH, Temperature	0.247	0.14
Cyanobacteria	3	PO4, TKN, Temperature	0.248	0.30

The chemical and physical conditions found within the ponds also have the potential to affect the microbial community composition. Bio-Env analysis was carried out to test how effectively the physical and chemical parameters measured at each of the sampling points correlated with the variation observed in community composition similarity. Chemical and physical data used for this analysis can be seen in Appendix 11. None of the chemical or physical parameters used in the analysis were shown to be significant in terms of driving community patterns in any of the three organism types. The best variable combinations for each of the organism groups can be seen in Table 7-1. Though the test shows that even the best combinations of environmental variables were not significant, it appears that the nutrient levels, the nitrogen species measured and to a lesser extent phosphorus, possessed the highest correlation with the community similarities.

Though dissolved oxygen (mg/l) was not correlated with the observed diversity patterns, the data was interesting for a different reason. In both ponds the peak of dissolved oxygen occurred at 12noon in the surface samples, corresponding with the peak in light intensity. In both of the ponds dissolved oxygen values were at super saturation levels, 20.55mg/l for the conventional pond and 22.21mg/l in the baffled pond. Mara (1997) reported 2 pond systems in Kenya having DO readings above the detection limit of the DO meter used in the study (above 20mg/l). By 6 am the DO levels had been reduced to anoxic levels (0.46mg/l for C1s and 0.38mg/l for B1s).

When comparing DO reading to the light intensity data (Figure 7-11) there appeared to be a lag, with the largest increase in light intensity occurring between 6am and 9am and the largest increase in DO occurring between 9am and 12noon. The surface of the baffled pond (green line in Figure 7-11) was more oxygenated for a greater part of the day than the conventional pond, which may affect treatment efficiency, though this was not tested.

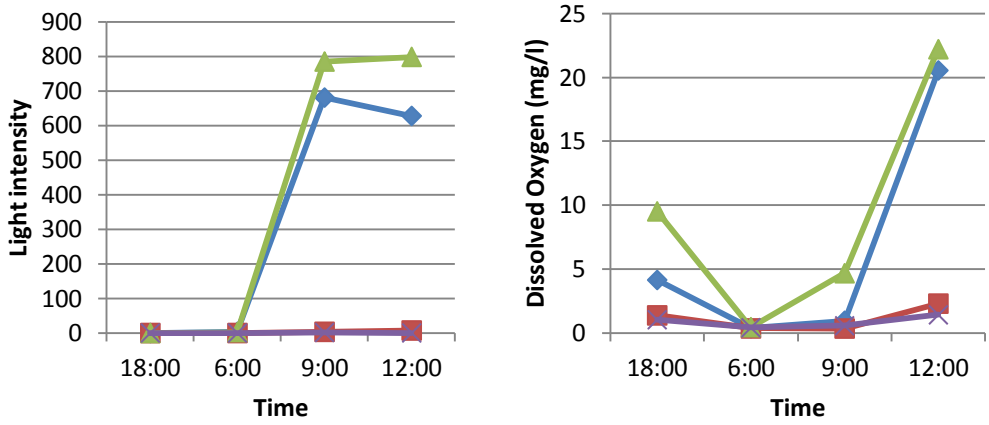


Figure 7-11 Light intensity and dissolved oxygen reading for the two ponds.
Blue- Conventional pond surface samples, Red- Conventional 40cm depth samples, Green- Baffled pond surface samples, Purple- Baffled 40cm depth samples.

7.7.5 Sequencing and organism identification

Observations from previous experiments (Chapter 6) showed that the central portion of the DGGE gel were most likely to contain sequences that are microalgal in origin. In this region the baffled samples appear to have more bands, with less bands in the outlying regions that correspond to sequences often obtained from protists and alveolates (Chapter 6). The baffled pond samples have a series of 'dominant' bands within the central region of the DGGE gel, which were not observed in the corresponding gel region of the conventional pond. This, taken with the high proportions of photosynthetic organisms seen in the baffled system, suggests that the photosynthetic microalgae dominated over other eukaryotes within this pond.

Only 40% (8/20) of the bands on the DGGE of eukaryotic organisms matched with non-photosynthetic organisms (Figure 7-12). Bands with labels coloured green were confirmed as eukaryotic microalgae by sequencing All of the microalgal bands

sequenced matched most closely with the Chlorophyceae species with a high level of similarity: bands 5, 6, 7, 8, 9, 14, 15, 16, 17 and 19 matched with *Kirchneriella obesa*, *K. dianae* and *Ankinstrodesmus bibraianus*. Bands 13 and 20 sequences both matched with *Pyrobotrys stellata*. Band 15 was the most dominant band in all of the baffled pond samples regardless of depth or time of the day. Band 13 was present in all of the conventional pond samples, but less prominent in the samples from 6pm, and was the dominant band from 12 noon. The bands in the baffled ponds showed little difference in pattern at the surface and at depth, as a result of the hydraulic mixing. The OTUs from the conventional ponds appeared to be both fewer in number and of lower intensity in the samples from 40cm below the surface compared to the surface samples. This confirms what was seen in the diversity statistics (Table 7-34).

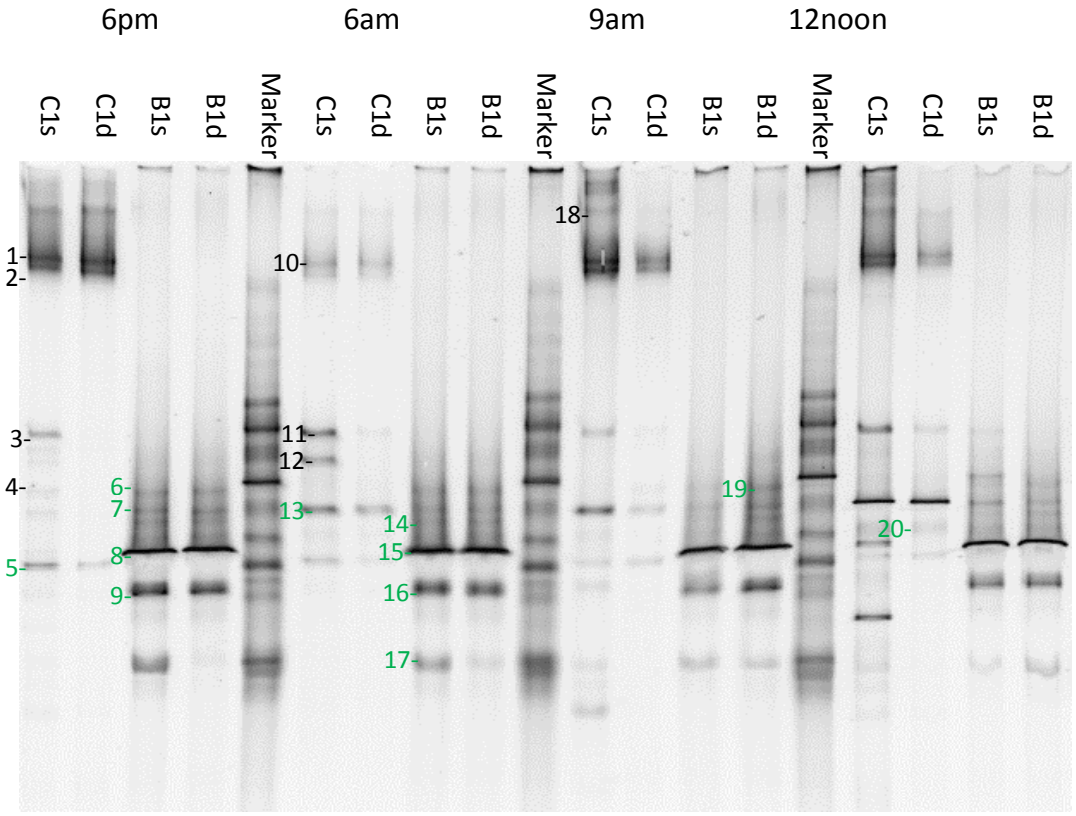


Figure 7-12 Eukaryotic DGGE gel.
Bands identified using BLAST against the NCBI nucleotide database, more details on sequence matching in Appendix 9. Bands with a green label matched microalgal sequences in the database.

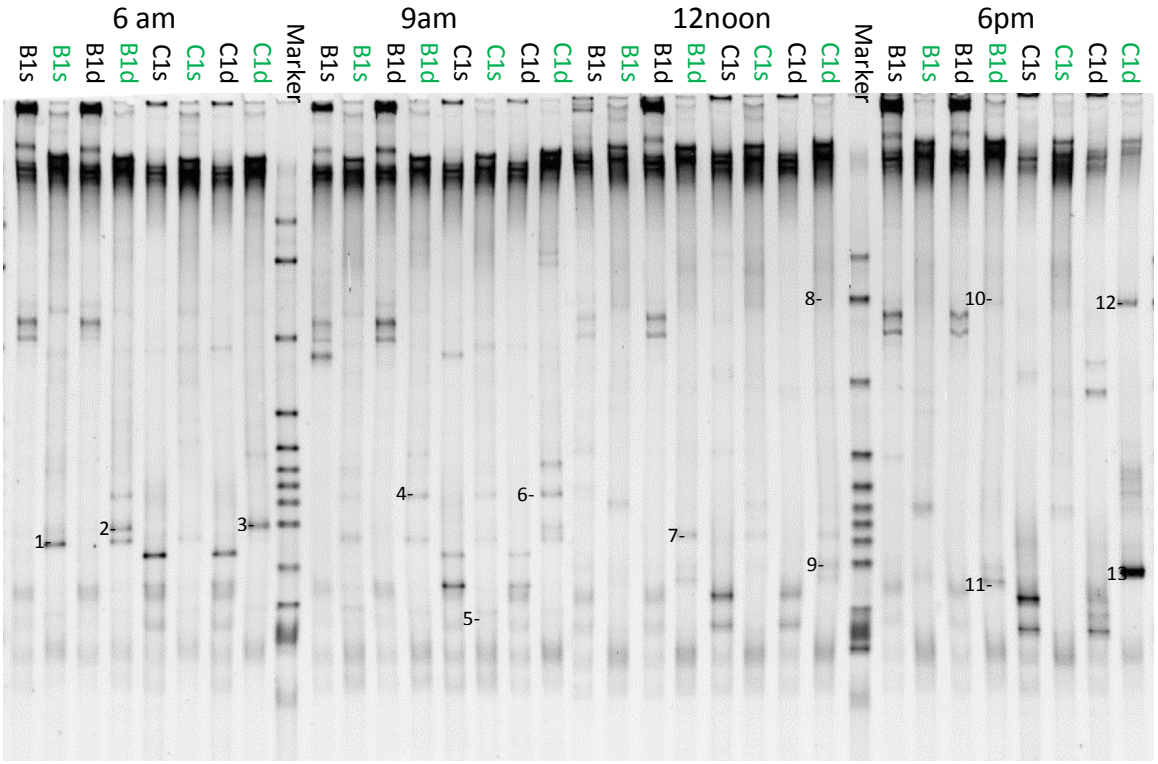


Figure 7-13 Cyanobacterial (green) and bacterial(black) DGGE gel.
Bands identified using BLAST against the NCBI nucleotide database, more details on sequence matching in Appendix 10. Top match for bands; 1- Chroococcidiopsis thermalis, 2- Plant chloroplast, 3- Plant chloroplast, 4- Cyanobacterial sp. 5- Uncultured bacteria, 6- Planktothrix sp., 7- Planktothrix sp., 8- Uncultured bacteria, 9- Plant chloroplast/ cyanobacteria 10- Uncultured bacteria, 11- Chroococcidiopsis sp. 12- Plant chloroplast, 13- Plant chloroplast.

In the DGGE analysis of the cyanobacterial community, only 46% (6/13) band sequences matched with cyanobacterial sequences in the database (Figure 7-13). The sequence amplified from band 4 matched to a range of cyanobacteria, suggesting that the sequence variability in the portion of the 16S rRNA gene targeted may not be large enough to differentiate between all the different genera of the cyanobacteria. *Planktothrix* species were detected in a number of the samples in both the baffled and conventional ponds. The band sequence was shown to match with two species from this genus, *P.rubescens* and *P.agardhii,* both have been shown to produce microcystin toxins in freshwater systems *(Jacquet et al., 2005; Tonk et al., 2005)*. Tonk *et al.* (2005) showed that in laboratory scale tests *P.agardhii* becomes more toxic at high light intensities. The possibility of cyanotoxins being produced by cyanobacteria in WSP systems in the tropics, is an under researched area, but one in which molecular identification of cyanobacteria may have an important role, given the failure of traditional microscopy techniques to detect them seen in Chapter 6.. *Chroococcidiopsis*

species were also detected across both ponds, these cyanobacteria are known for being adaptive to harsh environments (Lee, 1999) and have not before been identified in WSP systems. This may be due to their small size and unicellular nature making them difficult to identify by traditional microscopy.

Two of the bands matched most closely with unidentified bacteria in the database, these bands were submitted to the RDP classifier, band 5 was classified as a Geobacteraceae, a deltaproteobacteria, and band 10 was classified as an Aquificae. The Geobacteraceae are a group of organisms known to be involved in metal and sulphur reduction in the environment. The Aquificae are the dominant carbon fixing organisms in hot springs (Hamamura *et al.*, 2013). The rest of the sequences were found to be most closely related to plant chloroplast sequences.

7.8 Conclusions

The addition of baffles to facultative pond affects the ecology of these systems. Eukaryotic organisms appear to be the most affected by the presence of the baffles, with samples from the surface and at depth from throughout the day appearing to have high levels of similarity. Cyanobacterial populations appeared to be affected more by the diurnal cycle than by the presence or absence of baffles. Cyanobacteria known to be responsible for cyanotoxin production were identified by sequencing in both of the ponds, though whether or not they are producing toxins in these systems is unknown. This is a potential problem for WSPs that requires further investigation, as cyanotoxins in receiving waters can be damaging to human health and fish populations.

Photosynthetic organisms appear to dominate over the non-photosynthetic organisms regardless of depth in the baffled pond, presumably due to the reduction in short circuiting and better mixing occurring in the baffled pond, allowing photosynthetic organisms to be mixed reducing photo limitation and improving their efficiency to utilise light

Chapter 8. General Discussion, Conclusions and Future work

8.1 Discussion

The study of photosynthetic organisms in WSPs is critical to the understanding of how wastewater is treated, given their roles in oxygen production for organics removal (Walmsley and Shilton, 2005) and nitrogen removal (Camargo Valero *et al.*, 2009a). Traditional microscopy has been shown to provide an incomplete understanding of photosynthetic communities that does not account for the evolutionary relationship of organisms and tends to be bias against cyanobacteria and towards morphologically distinct forms (Chapter 6).

Molecular methods, such as those developed for use on WSP samples in this thesis have the potential to improve our understanding of pond dynamics, giving more in depth information about the organisms present, their abundance and potentially the roles that they play in WSP treatment using functional gene studies. Pearson (2005) called for the application of molecular techniques to WSP ecology, to detection microalgal organisms and to see if organisms are adapting in form as conditions change, as algae do in culture (Luo *et al.*, 2006) or dying off.

The initial step in many molecular methods is to disrupt cells and extract DNA. Chapter 3 (Eland *et al.*, 2012) tested DNA extraction kits, in attempt to ensure that bias from extraction was kept to a minimum. The recommendation made echoes the results of (Simonelli *et al.*, 2009), who selected the QBT kit for use on microalgae in the stomachs of copepods in the marine environment.

The molecular methods developed during the thesis led to some interesting findings about WSP ecology. Furtado *et al.* (2009) showed that cyanobacteria capable of producing cyanotoxins were present in the WSP studied. Using DGGE and band sequencing cyanobacteria from the genus *Planktothrix*, were shown to be present in the Colombian facultative pond (Case study 2). This genus of cyanobacteria have been linked to cyanotoxin production in freshwater systems and has not been reported before in WSPs (Jacquet *et al.*, 2005).

At the 10th IWA specialist group conference on wastewater pond technology, the importance of the hydrodynamics of WSPs and its effects on their efficiency was highlighted as a key research theme for the future. The effects of hydrodynamics on photosynthetic pond ecology has not been studied in detail in the literature (one unpublished study by Ceron, et al). Case study 2 showed that hydraulic mixing, caused by baffles had a distinctive effect on the eukaryotic community within the pond, making the community less variable throughout the photic zone and the diurnal cycle. This pattern was not seen in the cyanobacteria with community similarity varying diurnally and little difference between communities from the two ponds.

8.2 Conclusions

The conclusions of this study in relation to the main objectives set out in Chapter 1 are presented below.

8.2.1 Recommend a suitable DNA extraction method for WSP researchers

This objective was addressed in Chapter 4 and is the subject of a journal paper (Eland *et al.*, 2012). The effectiveness of a range of DNA extraction kits for use on eukaryotic organisms in WSP samples and microalgal cultures was evaluated.

- The Qiagen Blood and Tissue kit was recommended for use, as it was able to extract DNA from all of the microalgal pure culture strains tested and from a diverse range of organisms within a WSP sample
- The use of approximately 1.68 10^6 algal cells for DNA extraction was recommended, based on tests of extraction at two different volumes. Additional sample did not significantly improve DNA extraction outcomes and added to sample processing time and sampling effort.
- The fixation of WSP samples with ethanol had a detrimental effect on the extraction of PCR viable DNA. The ethanol may be causing cross linkages in

the protein structures of the cells making DNA extraction by proteinase K more difficult.

- In later Chapters it became apparent that this extraction kit, though shown to be the most effective kit, may be unable to break open Euglenophyceae cells, leading them to be undetected in all samples assessed.

8.2.2 Optimise DGGE methods for the detection of eukaryotic microalgae and cyanobacteria

The optimisation of DGGE for eukaryotic microalgae was carried out throughout the thesis. Broad eukaryotic 18S rRNA gene targeting primers were used to ensure that a large proportion of known microalgae groups were covered, as algae are an unnatural taxonomic group and as such primers that target only the microalgae are not available. DGGE gradients were optimised for the production of clear images, and to maximise band separation, but prevent band loss. The recommended conditions can be seen in Chapter 2.

The cyanobacteria were targeted using a nested PCR strategy as described by Zwart *et al.* (2005). DGGE gel gradients were optimised for use, however sequencing of the bands highlighted the cyanobacterial primers amplified eukaryotic plastids during PCR. This issue was addressed in Chapter 5, with the use of flow cytometry.

8.2.3 Devise a method to quantify the proportions of eukaryotic microalgae, cyanobacterial and non-photosynthetic organisms in WSP samples

Chapter 4 attempted to address this objective, employing the use of FISH to target eukaryotic organisms. The use of FISH probes to visualise eukaryotic organisms was shown to be inefficient. The penetration of fluorescence probes into the cells was limited by cell wall permeability, which varied from cell to cell. Species such as *Scenedesmus quadricauda* showed greater resistance to hybridisation, due to the presence of decay resistant materials in their cell walls. CLSM on unwashed cells showed pooling of the probe around the outside of the cell walls and not inside the cell.

In Chapter 5 a flow cytometry method was developed for the sorting and counting of photosynthetic from non-photosynthetic organisms. This method provided count data

for the photosynthetic and non-photosynthetic fraction, but was unable to distinguish the cyanobacteria from the eukaryotic microalgae due to their overlapping auto fluoresce signals.

8.2.4 Devise a method for the separation and concentration of photosynthetic organisms in a WSP sample to improve community assessment

The Flow-DGGE method in Chapter 5 was devised to meet this objective. The use of flow cytometry to separate and concentrate photosynthetic organisms was shown to be highly dependent on the flow cytometry gating strategy. The optimisation work in this Chapter highlighted the need for greater emphasis to be put on the use of multiple pure cultures for determining gating. The adjustment of gates to try to maximise false negatives is the approach that is recommended given the ability of molecular methods to identify these in later analysis.

The success of this technique for the concentration of photosynthetic eukaryotes was difficult to discern, given the use of paraformaldehyde fixation of the samples prior to the flow sorting. Fixation of the samples was unavoidable, given the huge distance that samples had to be transported, the requirements for storage and the fact that use of live algae was prohibited for use on the flow cytometer, which was part of a clinical facility.

For the cyanobacteria this technique was shown to yield more information on their diversity and the species present within WSP samples than methods that simple use unsorted samples. The method was particularly effective for the industrially treated WSP pond samples from Brazil.

8.2.5 Compare traditional taxonomic methods with molecular microbial ecology method

This was the subject of Chapter 6 and was the focus of a collaboration with Victor Ceron (CINARA, Uni del Valle, Colombia). The main conclusions from this chapter were;

- There was a lack of similarity between samples that were assessed using the two methodologies

- Molecular methods highlighted greater overall diversity and OTU numbers than the microscopy study, this being especially noticeable in the cyanobacterial analysis
- Microscopy analysis was time-consuming and required specialist knowledge and the consultation of a number of experts, in order to identify organisms.
- The Euglenophyceae were not detected by the molecular methodology though they were recorded as being among the most abundant organisms of microscopy studies in a number of samples. A number of explanations for this were discussed though the most likely of these is that these organisms are resistant to the DNA extraction method recommended in Chapter 3 coupled with their unusual rRNA gene structure (in plastids in the cytoplasm, rather than on chromosomes in the nucleus, as in other algal groups) and low gene copy numbers.
- An approach that combines microscopy and molecular methods, is likely to be ideal given the constant nature of change in algal systematics and the current biases in the two methodologies.

8.2.6 Use of the methods in two case studies to assess method performance and the effects of pond conditions on the algal community detected

Two case studies were carried out. In case study 1 two WSP systems in Brazil were assessed. The main conclusions were;

- That the ecology within the two systems were dissimilar to one another, in terms of patterns of diversity and succession through the ponds and in the proportions of photosynthetic to non-photosynthetic cells.
- The main difference between the two systems which may explain their ecological differences was the source of the influent wastewater (domestic or domestic and industrial mixed). The evidence to support this was inconclusive as the initial inoculum used in the ponds was not accounted for in the analysis.

In case study 2, the effects of baffles for improved hydrodynamic efficiency was assessed. The communities within two pilot scale facultative ponds, one

conventional and one with baffles were examined. The main conclusions drawn from this analysis were as follows;

- The introduction of baffles to facultative ponds has a significant effect on the ecology of the pond, particularly the eukaryotic organisms.
- The cyanobacterial population in the two ponds was more affected by the diurnal cycle than by the presence of baffles. Cyanobacteria that are known to produce cyanotoxins were identified in both pond systems. The presence of baffles appeared to make no difference to the presence of these organisms.
- The reduction in hydraulic short circuiting and increase in vertical mixing in the baffled pond resulted in higher proportions of photosynthetic compared to non-photosynthetic organisms being present at the surface and at depth.

8.3 Future work

The further development of the molecular techniques to overcome some of the potential sources of bias and limitations of the methods discussed throughout the chapters would improve the analysis and the validity of ecological findings. Areas for development and improvement include;

- Targeting a longer and more variable region of the 18S rRNA gene when assessing eukaryotic diversity, as the Chlorophyceae were difficult to distinguish from one another, even at genus level
- Using a combination of primers that target different algal classes to provide more in depth data on these groups. This would result in algae only analysis that does not include the non-photosynthetic eukaryotes targeted by the more general eukaryotic probes.
- The use of fresh unfixed samples for the Flow-DGGE technique would improve the efficiency of DNA extraction from the sorted cells and allow a better assessment of the methodology to be conducted.

- Investigate the cause of the lack of Euglenophyceae detected by molecular methods in samples where Euglenophyceae were identified as the dominant organism by microscopy. This would include;
    - Conducting a DNA extraction test on pure culture Euglenophyceae algae to see if the tough protein pellicle layer is the cause of their absence

8.4 The work conducted during the production of this thesis has highlighted many potential areas for future work on WSP systems and their ecology. These include;

- The role of the sulphur cycle in WSPs and exploring the link between this cycle, the ecology of the ponds and treatment failure.
- The presence of cyanobacteria, including their roles in nutrient processing and their potential cyanotoxin production
- The use of molecular methods for assessing grazers and predatory eukaryotes in WSP systems. Park *et al.* (2011b) discussed the detrimental effect of grazers on algal populations in HRAPs. The use of general Eukaryotic primers (such as Euk1A and Euk516r) has been shown to amplify DNA from a range of these organisms.
- Assessing the effects of optimisation strategies for improving wastewater treatment on the community photosynthetic community and determining how this links to treatment efficiency.

Appendix 1- Recipes for algal culture media (all recipes from CCAP culture collection website)

3N-BBM+V (Bold Basal Medium, modified with Nitrogen (3-fold) and Vitamins)

Stock solutions in g/1000ml of distilled water			for 1 litre of medium
1)	25g	$NaNO_3$	30ml
2)	2.5g	$CaCl_2.2H_2O$	10ml
3)	7.5g	$MgSO_4.7H_2O$	10ml
4)	7.5g	$K_2HPO_4.3H_2O$	10ml
5)	17.5g	KH_2PO_4	10ml
6)	2.5g	NaCl	10ml
7)	Trace element solution		6ml
8)	Vitamin B_1		
9)	Vitamin B_{12}		

Make up to 1 litre with distilled water.

For Trace element solution (7)

Add 0.75g of Na_2EDTA to 1000ml of distilled water and the following minerals (in this order)

- $FeCl_3.6H_2O$ 97mg
- $MnCl_2.4H_2O$ 41mg
- $ZnCl_2$ 5.0mg
- $CoCl_2.6H_2O$ 2mg
- $Na_2MoO_4.2H_2O$ 4mg

For Vitamin B_1 (8)

0.12g Thiaminhydrochloride in 100ml distilled water. Filter sterilise.

For Vitamin B_{12} (9)

0.1g Cyanocobalamin in 100ml distilled water, add 1ml of this solution to 99ml of distilled water and filter sterilise.

EG (Euglena gracilis Medium)

Stock		per litre
1.	$CaCl_2$ stock solution: $CaCl_2$	1g

Medium	per litre
• Sodium acetate trihydrate	1g
• "Lab-Lemco" powder (Oxoid L29)	1g
• Tryptone (Oxoid L42)	2g
• Yeast extract (Oxoid L21)	2g
• $CaCl_2$ stock solution	10ml

Add constituents above and make up to 1 litre with deionised water

JM (Jaworski's Medium)

Stocks	per 200ml
1. $Ca(NO_3)_2.4H_2O$	4g
2. KH_2PO_4	2.48g
3. $MgSO_4.7H_2O$	10g
4. $NaHCO_3$	3.18g
5.	
a. EDTAFeNa	0.45g
b. $EDTANa_2$	0.45g
6.	
a. H_3BO_3	0.496g
b. $MnCl_2.4H_2O$	0.278g
c. $(NH_4)6Mo_7O_{24}.4H_2O$	0.20g
7.	
a. Cyanocobalamin	0.008g
b. Thiamine HCl	0.008g
c. Biotin	0.008g
8. $NaNo_3$	16g
9. $Na_2HPO_4.12H_2O$	7.2g

Medium	per litre
Stock solutions	1ml each

Make up to 1 litre with deionised water

EG:JM medium

1:1 mixture of EG and JM media, mixed together and autoclaved to sterilise

F-2 Medium

Stocks	per litre
1. Trace elements (chelated)	
a. Na_2 EDTA	4.16g
b. $FeCl_3.6H_2O$	3.15g
c. $CuCO_4.5H_2O$	0.01g
d. $ZnSO_4.7H_2O$	0.022g
e. $CoCl_2.6H_2O$	0.1g
f. $MnCl_2.4H_2O$	0.18g
g. $Na_2MoO_4.2H_2O$	0.006g
2. Vitamin mix	
a. Cyanocobalamin (Vitamin B_{12})	0.0005g
b. Thiamine HCl (Vitamin B_1)	0.1g
c. Biotin	0.0005g

Medium	per litre
• $NaNO_3$	0.075g
• $NaH_2PO_4.2H_2O$	0.00565g
• Trace element stock solution (1)	1ml
• Vitamin mix stock solution (2)	1ml

Make up to 1 litre with filtered natural seawater. Adjust pH to 8.0 with 1M NaOH or HCl. Sterilise by autoclaving.

BG11 (Blue-Green Medium)

Stocks		per litre
1.	$NaNO_3$	15g
		Per 500ml
2.	K_2HPO_4	2g
3.	$MgSO_4.7H_2O$	3.75g
4.	$CaCl_2.2H_2O$	1.8g
5.	Citric acid	0.30g
6.	Ammonium ferric citrate green	0.30g
7.	EDTA Na_2	0.05g
8.	Na_2CO_3	1g
9.	Trace metal solution	per litre
	a. H_3BO_3	2.86g
	b. $MnCl_2.4H_2O$	1.81g
	c. $ZnSO_4.7H_2O$	0.22g
	d. $Na_2MoO_4.2H_2O$	0.39g
	e. $CuSO_4.5H_2O$	0.08g
	f. $Co(NO_3)2.6H_2O$	0.05g

Medium	Per litre
• Stock solution 1	100ml
• Stock solutions 2-8	10ml of each
• Stock solution 9	1ml

Make up to 1 litre with deionised water and adjust pH to 7.1 with 1M NaOH or HCl

Appendix 2 Summary of the similarities for the dominant excised eukaryotic bands from Chapter 5.

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class
1	*Dictyosphaerium sp.* CCAP 222/40 GQ487253 [*]	99	CCAP- culture collection	Chlorophyta	Trebouxiophyceae
	Parachlorella kessleri strain HY-6 JQ797561 [*]	99	Fresh water lake	Chlorophyta	Trebouxiophyceae
2	*Chlorella sorokiniana* strain MIC-G5 JF834706	99	Fresh water pool	Chlorophyta	Trebouxiophyceae

Appendix 3 Summary of the similarities for the dominant excised cyanobacterial bands from Chapter 5 (using ncbi-refseq database BLAST).

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class	RDP classifier
1	*Stanieria cyanosphaera* PCC 7437 NR_102468	97	Culture collection , pond water	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[83%] Chloroplast[69%] Chloroplast[69%] Bacillariophyta[44%]
	Thermosynechococcus elongatus NR_074328	97	Culture collection	Cyanophyta	Cyanophyceae	
	Cyanothece sp. ATCC 51142 NR_074316	97	Culture collection	Cyanophyta	Cyanophyceae	
2	*Pseudanabaena sp*. PCC 7367 NR_102446	91	Culture collection	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[87%] Chloroplast[86%] Chloroplast[86%] Streptophyta[84%]
3	*Calothrix sp*. PCC 7507 NR_102891	99	Culture collection sphagnum bog	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[92%] Cyanobacteria[89%] Family I[55%] GpI[55%]
	Chroococcidiopsis thermalis PCC 7203 NR_102464	99	Culture collection, soil sample	Cyanophyta	Cyanophyceae	
	Anabaena cylindrica PCC 7122 NR_102457	99	Culture collection, pond water	Cyanophyta	Cyanophyceae	
	Nostoc punctiforme PCC 73102 NR_074317	99	Culture collection	Cyanophyta	Cyanophyceae	
	Nostoc azollae NR_074259	99	Culture collection	Cyanophyta	Cyanophyceae	
4	*Thermosynechococcus elongatus* NR_074328	93	Culture collection	Cyanophyta	Cyanophyceae	Bacteria[100%] "Proteobacteria"[36%] Deltaproteobacteria[30%]
5	*Stigmatella aurantiaca* DW4/3-1 strain	94	Culture collection	Proteobacteria	Deltaproteobacteria	Bacteria[99%] "Proteobacteria"[24%]

	NR_102818					Deltaproteobacteria[20%] Bdellovibrionales[17%] Bdellovibrionaceae[17%] Vampirovibrio[17%]
	Myxococcus stipitatus DSM 14675 strain NR_102512	94	Culture collection	Proteobacteria	Deltaproteobacteria	
6	*Stanieria cyanosphaera* PCC 7437 strain NR_102468	93	Culture collection, pond at botanical garden	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[63%] Chloroplast[55%] Chloroplast[55%] Bangiophyceae[40%]
	Cylindrospermum stagnale PCC 7417 strain NR_102462	93	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Cyanobacterium stanieri PCC 7202 strain NR_102450	93	Culture collection, alkaline pond	Cyanophyta	Cyanophyceae	
	Calothrix sp. PCC 6303 strain NR_102449	93	Culture collection, lake water	Cyanophyta	Cyanophyceae	
	Cyanobacterium aponinum PCC 10605 strain NR_102443	93	Culture collection, fresh water	Cyanophyta	Cyanophyceae	
	Thermosynechococcus elongatus BP-1 strain NR_074328	93	Culture collection	Cyanophyta	Cyanophyceae	
	Cyanothece sp. ATCC 51142 strain NR_074316	93	Culture collection	Cyanophyta	Cyanophyceae	
	Nostoc sp. PCC 7120 strain NR_074310	93	Culture collection	Cyanophyta	Cyanophyceae	
	Anabaena variabilis ATCC 29413 strain NR_074300	93	Culture collection	Cyanophyta	Cyanophyceae	
7	*Gelria glutamica* NR_041819	89	Culture collection	Fermicutes	Clostridia	Bacteria[100%] Firmicutes[54%] Clostridia[45%] Thermoanaerobacterales[18%] Thermoanaerobacteraceae[18%]
8	*Gelria glutamica* NR_041819	89	Culture collection	Fermicutes	Clostridia	Bacteria[100%] Firmicutes[54%] Clostridia[44%] Thermoanaerobacterales[17%] Thermoanaerobacteraceae[17%] *Gelria*[15%]
10	*Gelria glutamica* NR_041819	93	Culture collection	Fermicutes	Clostridia	Bacteria[99%] Firmicutes[41%] Clostridia[32%] Clostridiales[16%] Clostridiales_Incertae Sedis III[3%] Tepidanaerobacter[3%]
11	*Gelria glutamica* NR_041819	89	Culture collection	Fermicutes	Clostridia	Bacteria[100%] Firmicutes[51%] Clostridia[36%] Thermoanaerobacterales[13%] Thermoanaerobacteraceae[13%] *Gelria*[12%]
12	*Stanieria cyanosphaera* PCC 7437 strain NR_102468	93	Culture collection, pond at botanical	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[70%]

			garden			Chloroplast[62%] Chloroplast[62%] Bangiophyceae[33%]
	Cylindrospermum stagnale PCC 7417 strain NR_102462	93	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Cyanobacterium stanieri PCC 7202 strain NR_102450	93	Culture collection, alkaline pond	Cyanophyta	Cyanophyceae	
	Calothrix sp. PCC 6303 strain NR_102449	93	Culture collection, lake water	Cyanophyta	Cyanophyceae	
	Cyanobacterium aponinum PCC 10605 strain NR_102443	93	Culture collection, fresh water	Cyanophyta	Cyanophyceae	
	Thermosynechococcus elongatus BP-1 strain NR_074328	93	Culture collection	Cyanophyta	Cyanophyceae	
	Cyanothece sp. ATCC 51142 strain NR_074316	93	Culture collection	Cyanophyta	Cyanophyceae	
	Nostoc sp. PCC 7120 strain NR_074310	93	Culture collection	Cyanophyta	Cyanophyceae	
	Anabaena variabilis ATCC 29413 strain NR_074300	93	Culture collection	Cyanophyta	Cyanophyceae	
13	*Cyanobium gracile* PCC 6307 strain NR_102447	99	Culture collection, lake water	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[90%] Cyanobacteria[89%] Family II[78%] GpIIa[77%]
	Prochlorococcus marinus subsp. marinus str. CCMP1375 strain NR_074172	99	Culture collection	Cyanophyta	Cyanophyceae	
14	*Geopsychrobacter electrodiphilus* NR_042768	93	Culture collection	Proteobacteria	Deltaproteobacteria	Bacteria[100%] Firmicutes[50%] Clostridia[40%] Clostridiales[24%] Eubacteriaceae[21%] Alkalibacter[21%]
15	*Medicago truncatula* XM_003610179	97	Culture collection	Streptophyta	Eudicotyledons	Bacteria[100%] Cyanobacteria/Chloroplast[100%] Chloroplast[99%] Chloroplast[99%] Streptophyta[99%]
16	*Geitlerinema sp.* PCC 7407 strain NR_102448	95	Culture collection	Cyanophyta	Cyanophyceae	Bacteria[98%] Cyanobacteria/Chloroplast[48%] Chloroplast[36%] Chloroplast[36%] Chlorophyta[29%]
	Pseudanabaena sp. PCC 7367 strain NR_102446	95	Culture collection	Cyanophyta	Cyanophyceae	
17	*Geitlerinema* sp. PCC 7407 strain NR_102448	96	Culture collection	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[96%] Chloroplast[93%] Chloroplast[93%] Chlorophyta[74%]
	Pseudanabaena sp. PCC 7367 strain NR_102446	96	Culture collection	Cyanophyta	Cyanophyceae	

18	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	96	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[99%] Cyanobacteria/Chloroplast[71%] Cyanobacteria[69%] Family XII[34%] GpXII[34%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	96	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain NR_074275	96	Culture collection	Cyanophyta	Cyanophyceae	
	Planktothricoides raciborskii NIES-207 strain NR_040858	96	Culture collection, lake water	Cyanophyta	Cyanophyceae	
19	*Stanieria cyanosphaera* PCC 7437 strain NR_102468	93	Culture collection, pond water	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[78%] Chloroplast[67%] Chloroplast[67%] Chlorophyta[32%]
	Pleurocapsa sp. PCC 7327 strain NR_102466	93	Culture collection, spring water	Cyanophyta	Cyanophyceae	
	Geitlerinema sp. PCC 7407 strain NR_102448	93	Culture collection	Cyanophyta	Cyanophyceae	
	Cyanobium gracile PCC 6307 strain NR_102447	93	Culture collection, lake water	Cyanophyta	Cyanophyceae	
	Pseudanabaena sp. PCC 7367 strain NR_102446	93	Culture collection	Cyanophyta	Cyanophyceae	
	Prochlorococcus marinus subsp. marinus str. CCMP1375 strain NR_074172	93	Culture collection	Cyanophyta	Cyanophyceae	
	Thermosynechococcus elongatus BP-1 strain NR_074328	93	Culture collection	Cyanophyta	Cyanophyceae	
	Cyanothece sp. ATCC 51142 strain NR_074316	93	Culture collection	Cyanophyta	Cyanophyceae	
	Laceyella sacchari strain DSM 43356 NR_041997	93	Culture collection	Fermicutes	Bacilli	
21	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	92	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[90%] Cyanobacteria/Chloroplast[55%] Cyanobacteria[43%] Family XII[17%] GpXII[17%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	92	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain NR_074275	92	Culture collection	Cyanophyta	Cyanophyceae	
	Planktothricoides raciborskii NIES-207 strain NR_040858	92	Culture collection, lake water	Cyanophyta	Cyanophyceae	
22	*Clostridium estertheticum* NR_044758	91	Culture collection	Fermicutes	Clostridia	Bacteria[96%] "Proteobacteria"[29%]

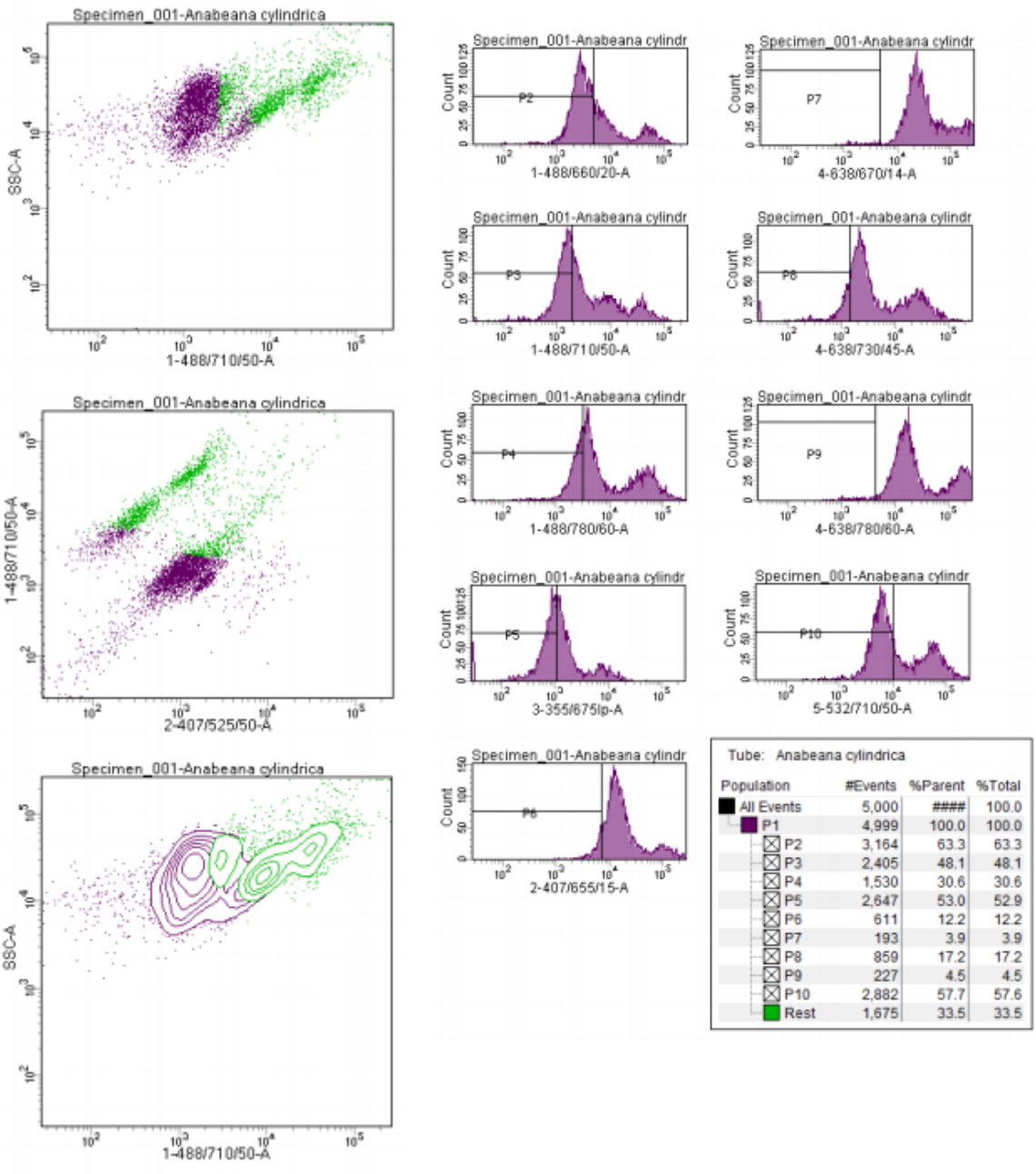
						Deltaproteobacteria[23%] Desulfuromonadales[7%]
23	*Pseudanabaena sp*. PCC 7367 strain NR_102446	91	Culture collection	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[68%] Chloroplast[48%] Chloroplast[48%] Chlorarachniophyceae[11%]
25	*Clostridium estertheticum* NR_044758	94	Culture collection	Fermicutes	Clostridia	Bacteria[100%] "Proteobacteria"[27%] Deltaproteobacteria[25%] Desulfuromonadales[20%] Desulfuromonadaceae[19%] Malonomonas[19%]
26	*Cyanobium gracile* PCC 6307 strain NR_102447	96	Culture collection, pond water	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[84%] Chloroplast[72%] Chloroplast[72%] Bacillariophyta[65%]
	Pseudanabaena sp. PCC 7367 NR_102446	96	Culture collection	Cyanophyta	Cyanophyceae	
	Prochlorococcus marinus subsp. marinus str. CCMP1375 NR_074172	96	Culture collection	Cyanophyta	Cyanophyceae	
27	*Stanieria cyanosphaera* PCC 7437 NR_102468	95	Culture collection, pond water	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[84%] Chloroplast[72%] Chloroplast[72%] Bacillariophyta[65%]
	Thermosynechococcus elongatus NR_074328	95	Culture collection	Cyanophyta	Cyanophyceae	
	Cyanothece sp. ATCC 51142 NR_074316	95	Culture collection	Cyanophyta	Cyanophyceae	
28	Geopsychrobacter electrodiphilus NR_042768	91	Culture collection	Proteobacteria	Deltaproteobacteria	Bacteria[99%] Firmicutes[47%] Clostridia[39%] Natranaerobiales[21%] Natranaerobiaceae[21%] Dethiobacter[21%]
29	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	95	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[98%] Cyanobacteria/Chloroplast[67%] Cyanobacteria[61%] Family XII[22%] GpXII[22%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	95	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain NR_074275	95	Culture collection	Cyanophyta	Cyanophyceae	
	Planktothricoides raciborskii NIES-207 strain NR_040858	95	Culture collection, lake water	Cyanophyta	Cyanophyceae	
30	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	96	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[99%] Cyanobacteria/Chloroplast[71%] Cyanobacteria[69%] Family XII[34%] GpXII[34%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	96	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain	96	Culture collection	Cyanophyta	Cyanophyceae	

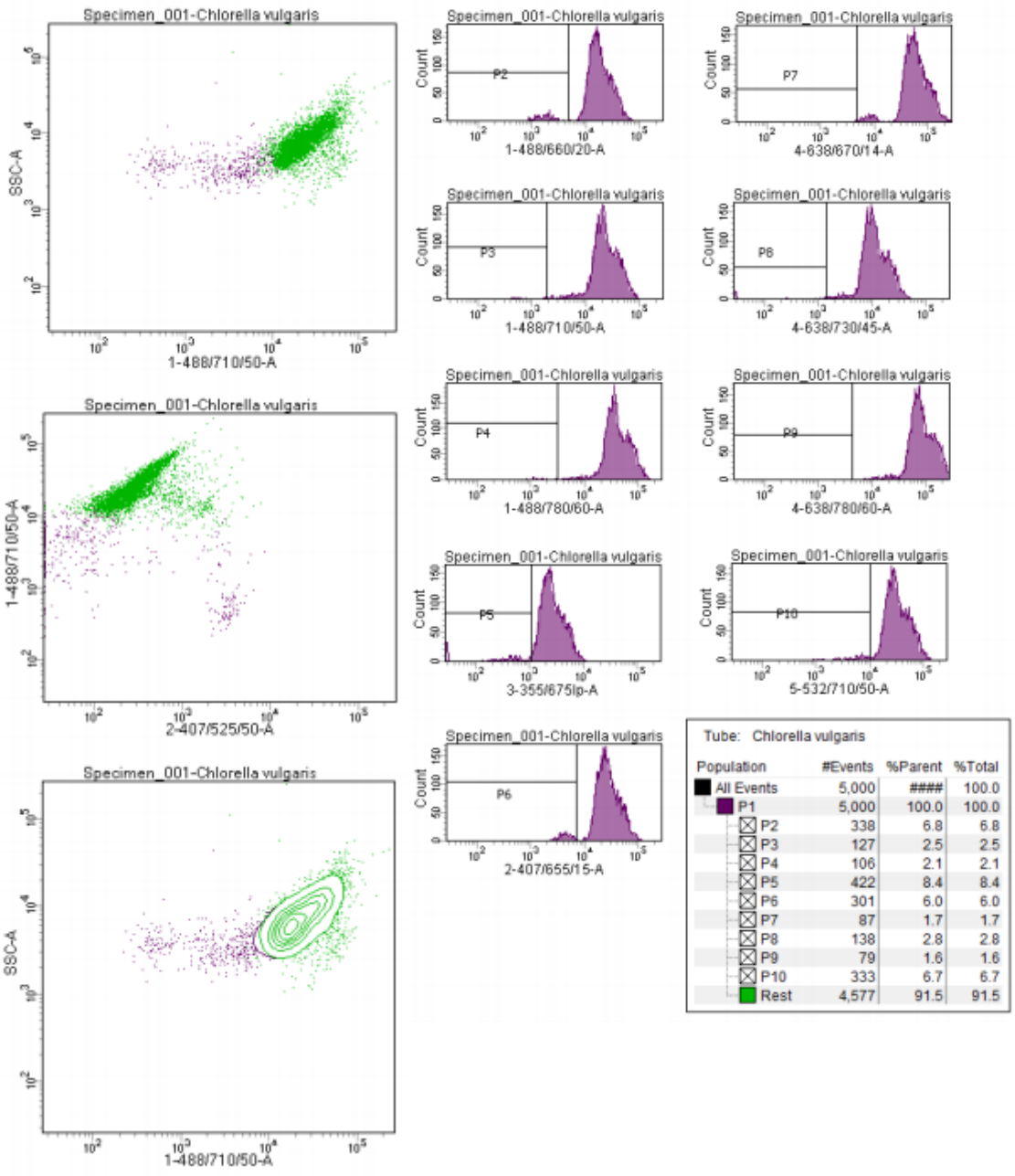
	NR_074275					
	Planktothricoides raciborskii NIES-207 strain NR_040858	96	Culture collection, lake water	Cyanophyta	Cyanophyceae	
31	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	94	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[98%] Cyanobacteria/Chloroplast[45%] Cyanobacteria[35%] Family XII[13%] GpXII[13%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	94	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain NR_074275	94	Culture collection	Cyanophyta	Cyanophyceae	
	Planktothricoides raciborskii NIES-207 strain NR_040858	94	Culture collection, lake water	Cyanophyta	Cyanophyceae	
32	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	96	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[99%] Cyanobacteria/Chloroplast[72%] Cyanobacteria[70%] Family XII[34%] GpXII[34%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	96	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain NR_074275	96	Culture collection	Cyanophyta	Cyanophyceae	
	Planktothricoides raciborskii NIES-207 strain NR_040858	96	Culture collection, lake water	Cyanophyta	Cyanophyceae	
33	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	96	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[64%] Cyanobacteria[60%] Family XII[38%] GpXII[38%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	96	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain NR_074275	96	Culture collection	Cyanophyta	Cyanophyceae	
	Planktothricoides raciborskii NIES-207 strain NR_040858	96	Culture collection, lake water	Cyanophyta	Cyanophyceae	
34	*Gelria glutamica* NR_041819	89	Culture collection	Fermicutes	Clostridia	Bacteria[100%] Firmicutes[54%] Clostridia[43%] Thermoanaerobacterales[16%] Thermoanaerobacteraceae[16%] Gelria[14%]
35	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	96	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[99%] Cyanobacteria/Chloroplast[71%] Cyanobacteria[69%] Family XII[34%] GpXII[34%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	96	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain	96	Culture collection	Cyanophyta	Cyanophyceae	

	NR_074275					
	Planktothricoides raciborskii NIES-207 strain NR_040858	96	Culture collection, lake water	Cyanophyta	Cyanophyceae	
36	*Oscillatoria nigro-viridis* PCC 7112 strain NR_102469	96	Culture collection, soil	Cyanophyta	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[64%] Cyanobacteria[60%] Family XII[38%] GpXII[38%]
	Oscillatoria acuminata PCC 6304 strain NR_102463	96	Culture collection, soil	Cyanophyta	Cyanophyceae	
	Trichodesmium erythraeum IMS101 strain NR_074275	96	Culture collection	Cyanophyta	Cyanophyceae	
	Planktothricoides raciborskii NIES-207 strain NR_040858	96	Culture collection, lake water	Cyanophyta	Cyanophyceae	
37	*Leptolyngbya sp.* PCC 7376, NR_102456	94	Culture collection, limestone cave	Cyanophyta	Cyanophyceae	Bacteria[100%] "Proteobacteria"[29%] Deltaproteobacteria[25%] Bdellovibrionales[19%] Bdellovibrionaceae[18%] Vampirovibrio[18%]
	Synechocystis sp. PCC 6803 strain NR_074302	94	Culture collection	Cyanophyta	Cyanophyceae	

Appendix 4 FACs DIVA dot plots and histograms for algal pure cultures.

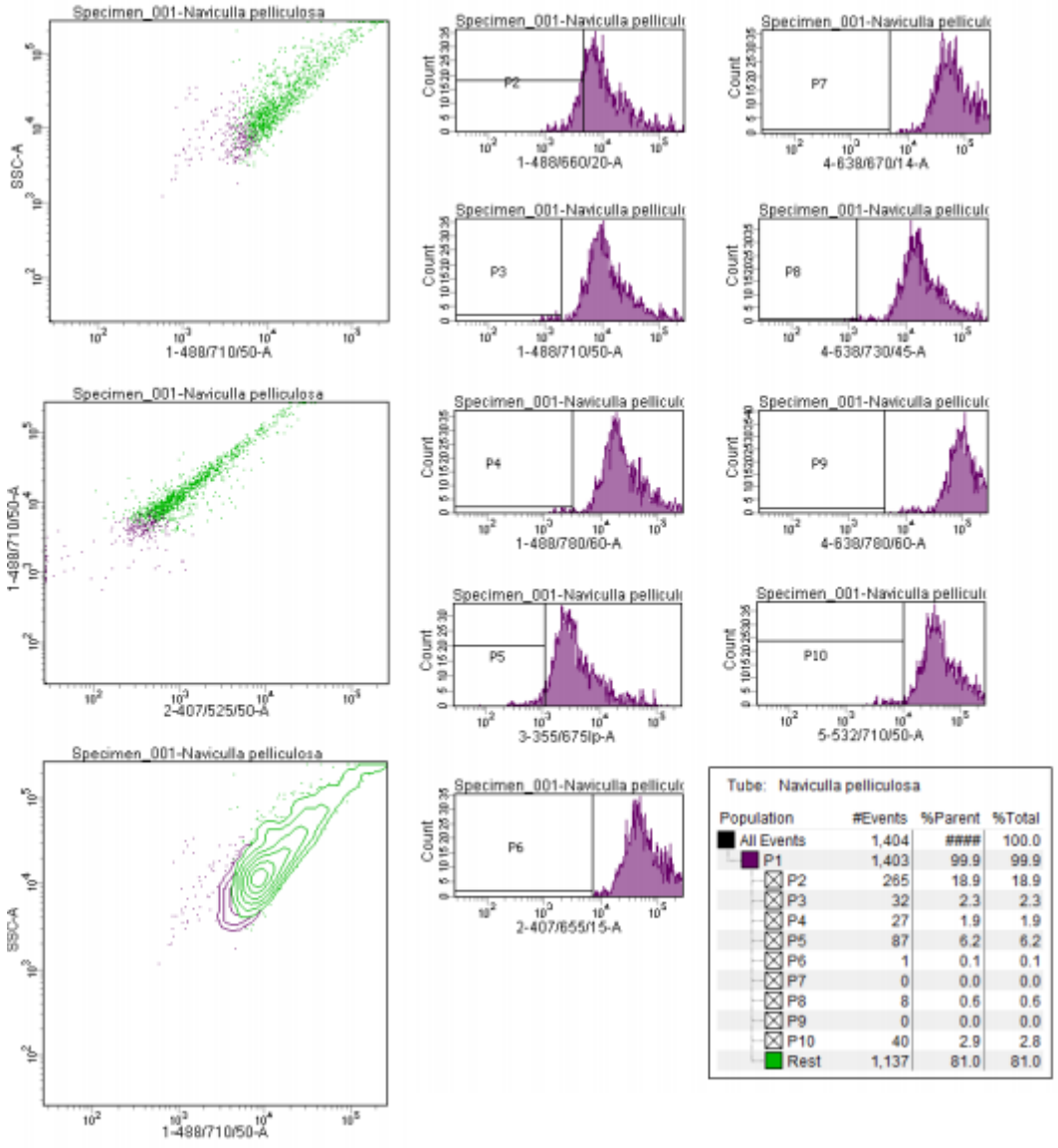
Cultures were assessed using a range of lasers and detectors to determine, which would be the most appropriate for setting up photosynthetic versus non-photosynthetic gating.

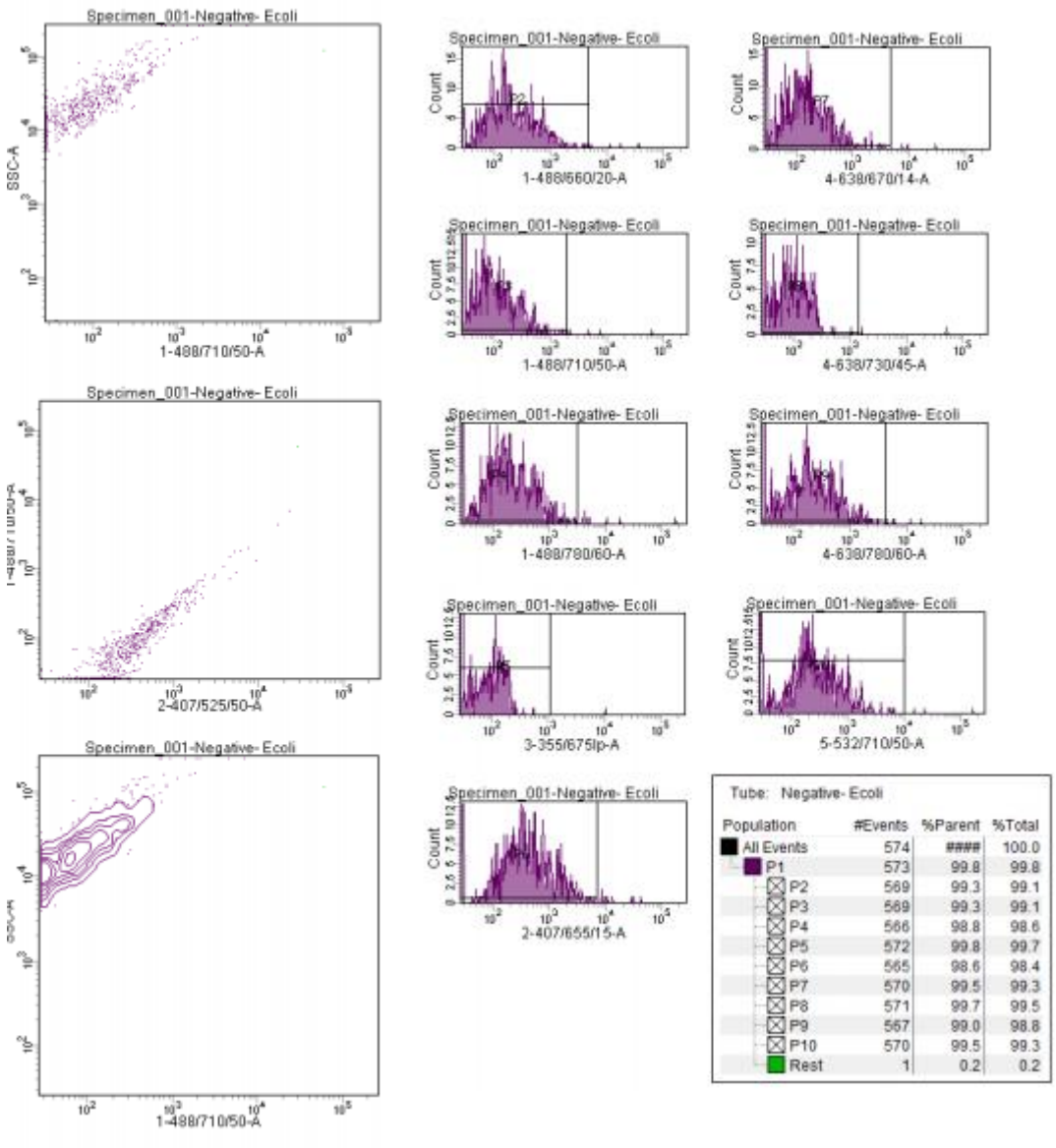


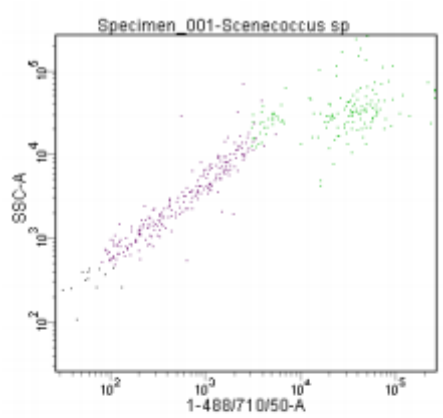


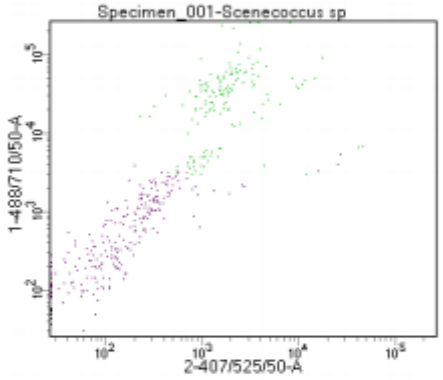
Tube: Chlorella vulgaris

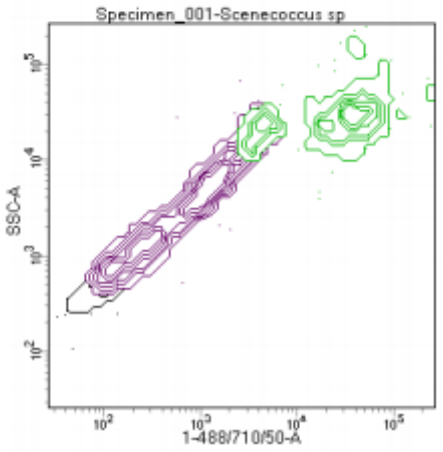
Population	#Events	%Parent	%Total
All Events	5,000	####	100.0
P1	5,000	100.0	100.0
P2	338	6.8	6.8
P3	127	2.5	2.5
P4	106	2.1	2.1
P5	422	8.4	8.4
P6	301	6.0	6.0
P7	87	1.7	1.7
P8	138	2.8	2.8
P9	79	1.6	1.6
P10	333	6.7	6.7
Rest	4,577	91.5	91.5

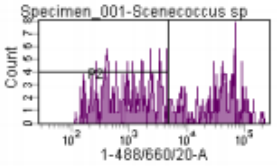

Specimen_001-Naviculla pelliculosa
SSC-A
1-488/710/50-A
P2
1-488/660/20-A
P7
4-638/670/14-A
P3
1-488/710/50-A
P8
4-638/730/45-A
2-407/525/50-A
P4
1-488/780/60-A
P9
4-638/780/60-A
P5
3-355/675lp-A
P10
5-532/710/50-A
P6
2-407/655/15-A
Tube: Naviculla pelliculosa
Population #Events %Parent %Total
All Events 1,404 #### 100.0
P1 1,403 99.9 99.9
P2 265 18.9 18.9
P3 32 2.3 2.3
P4 27 1.9 1.9
P5 87 6.2 6.2
P6 1 0.1 0.1
P7 0 0.0 0.0
P8 8 0.6 0.6
P9 0 0.0 0.0
P10 40 2.9 2.8
Rest 1,137 81.0 81.0

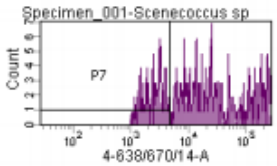

Specimen_001-Negative- Ecoli
SSC-A
1-488/710/50-A
Specimen_001-Negative- Ecoli
2-407/525/50-A
Specimen_001-Negative- Ecoli
1-488/710/50-A
Specimen_001-Negative- Ecoli
Count
1-488/660/20-A
Specimen_001-Negative- Ecoli
Count
4-638/670/14-A
Specimen_001-Negative- Ecoli
Count
1-488/710/50-A
Specimen_001-Negative- Ecoli
Count
4-638/730/45-A
Specimen_001-Negative- Ecoli
Count
1-488/780/60-A
Specimen_001-Negative- Ecoli
Count
4-638/780/60-A
Specimen_001-Negative- Ecoli
Count
3-355/675lp-A
Specimen_001-Negative- Ecoli
Count
5-532/710/50-A
Specimen_001-Negative- Ecoli
Count
2-407/655/15-A
Tube: Negative- Ecoli
Population #Events %Parent %Total
All Events 574 #### 100.0
P1 573 99.8 99.8
P2 569 99.3 99.1
P3 569 99.3 99.1
P4 566 98.8 98.6
P5 572 99.8 99.7
P6 565 98.6 98.4
P7 570 99.5 99.3
P8 571 99.7 99.5
P9 567 99.0 98.8
P10 570 99.5 99.3
Rest 1 0.2 0.2

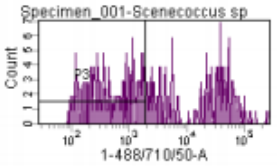


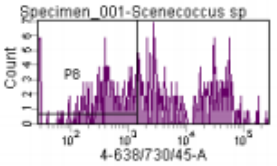


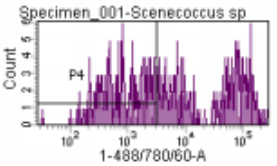


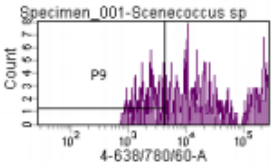


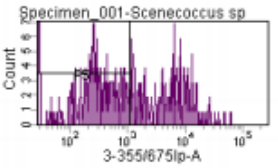


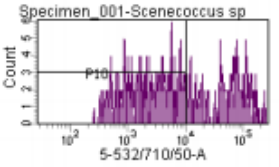


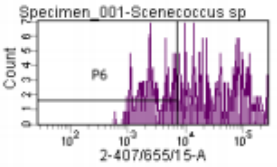






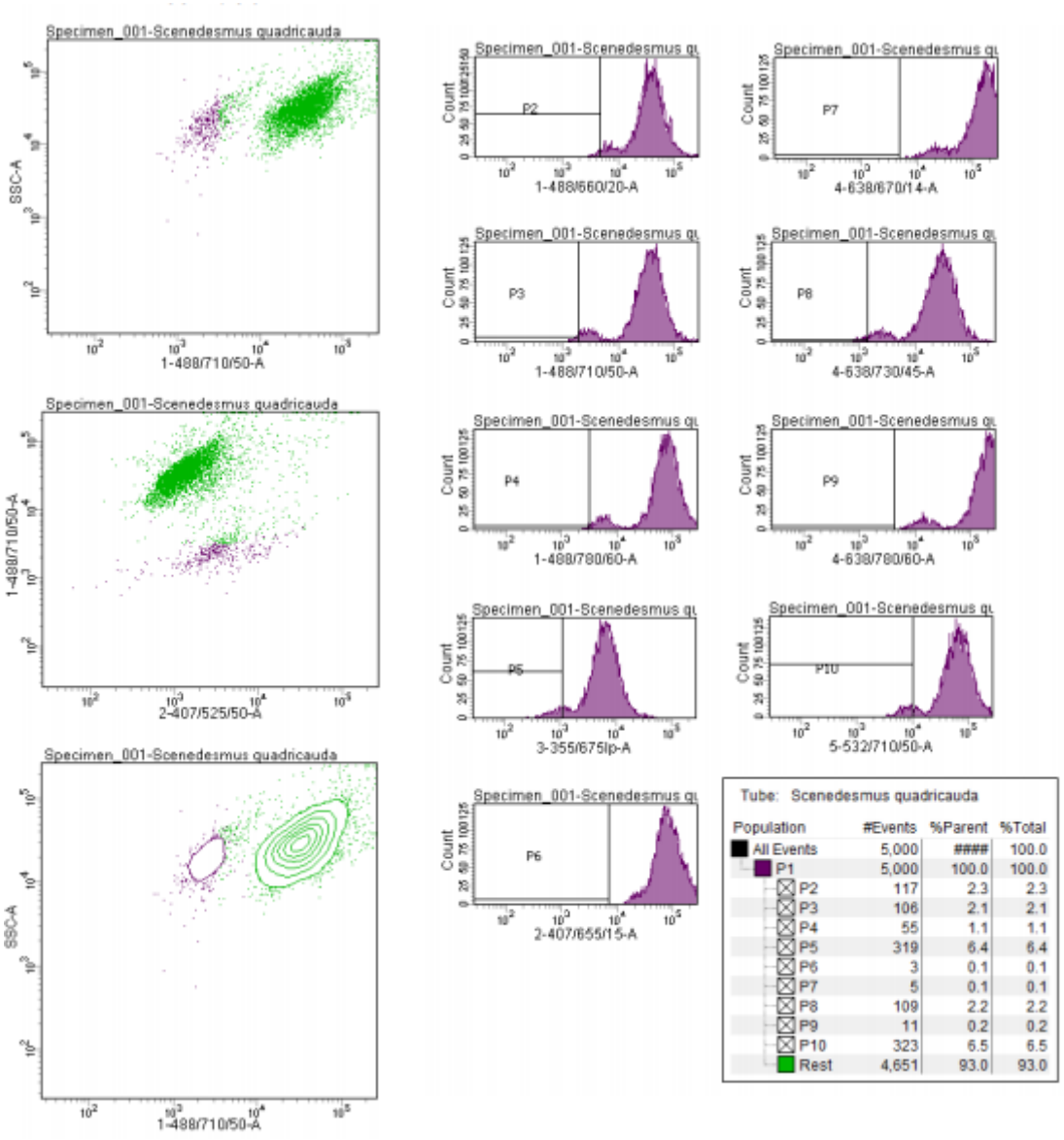






Tube: Scenecoccus sp

Population	#Events	%Parent	%Total
All Events	385	####	100.0
P1	371	96.4	96.4
P2	216	58.2	56.1
P3	188	50.7	48.8
P4	173	46.6	44.9
P5	214	57.7	55.6
P6	134	36.1	34.8
P7	93	25.1	24.2
P8	154	41.5	40.0
P9	96	25.9	24.9
P10	216	58.2	56.1
Rest	151	40.7	39.2


Specimen_001-Scenedesmus quadricauda
SSC-A
1-488/710/50-A
Specimen_001-Scenedesmus quadricauda
1-488/710/50-A
2-407/525/50-A
Specimen_001-Scenedesmus quadricauda
SSC-A
1-488/710/50-A
Specimen_001-Scenedesmus qu
Count
P2
1-488/660/20-A
Specimen_001-Scenedesmus qu
Count
P7
4-638/670/14-A
Specimen_001-Scenedesmus qu
Count
P3
1-488/710/50-A
Specimen_001-Scenedesmus qu
Count
P8
4-638/730/45-A
Specimen_001-Scenedesmus qu
Count
P4
1-488/780/60-A
Specimen_001-Scenedesmus qu
Count
P9
4-638/780/60-A
Specimen_001-Scenedesmus qu
Count
P5
3-355/675lp-A
Specimen_001-Scenedesmus qu
Count
P10
5-532/710/50-A
Specimen_001-Scenedesmus qu
Count
P6
2-407/655/15-A
Tube: Scenedesmus quadricauda
Population #Events %Parent %Total
All Events 5,000 #### 100.0
P1 5,000 100.0 100.0
P2 117 2.3 2.3
P3 106 2.1 2.1
P4 55 1.1 1.1
P5 319 6.4 6.4
P6 3 0.1 0.1
P7 5 0.1 0.1
P8 109 2.2 2.2
P9 11 0.2 0.2
P10 323 6.5 6.5
Rest 4,651 93.0 93.0

Appendix 5 Summary of the similarities for the dominant excised eukaryotic bands from Chapter 6

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class
1	*Paramecium multimicronucleatum* (HE662762) [*]	*100*	Industrial water	Alveolata	Oligohymenophora
2	*Tetrahymena pigmentosa* (M26358) (Sogin *et al.*, 1986b)	*100*	Genomic DNA	Alveolata	Oligohymenophora
	Tetrahymena patula (M98017) (Sogin *et al.*, 1986b)	100	Genomic DNA	Alveolata	Oligohymenophora
	Tetrahymena nanneyi (M98016) (Sogin *et al.*, 1986b)	100	Genomic DNA	Alveolata	Oligohymenophora
	Tetrahymena hyperangularis (M98014) (Sogin *et al.*, 1986b)	100	Genomic DNA	Alveolata	Oligohymenophora
3	*Tubulinida sp.* (HQ687486) *(Dykova et al., 2011)*	99	Liver, genomic DNA	Amoebozoa	Tubulinea
4	*Tokophyra lemnarum* (AY332720) (Snoeyenbos-West *et al*., 2004)	99	Genomic DNA	Alveolata	Phyllopharyngea
5	*Lepadella patella* (AY218117) (Giribet *et al*., 2004)	99	Genomic DNA	Rotifera	Eurotatoria
6	*Uncultured eukaryote* (GU970424) (Valster *et al.*, 2010)	92	Bionmass from rapid sand		
7	*Tubulinida sp.* (HQ687486) *(Dykova et al., 2011)*	98	Liver, genomic DNA	Amoebozoa	Tubulinea
8	*Oxytrichia longa* (AF164125) [*]	99	Complete sequence, genomic DNA	Alveolata	Spirotrichea
9	*Oxytrichia longa* (AF164125) [*]	99	Complete sequence, genomic DNA	Alveolata	Spirotrichea
10	Uncultured ciliate clone (EU143872) (Chen *et al.*, 2008b)	99	Freshwater lake	Alveolata	
11	Uncultured alveolata (GQ844635) (Chen *et al*., 2008b)	97	Freshwater lake	Alveolata	
12	Uncultured freshwater eukaryote (AB721051) [*]	99	Water purification plant		
13	*Cyclidium glaucoma (AJ749839) (Finlay et al., 2006)*	100	Culture collection	Alveolata	Oligohymenophora
14	*Schroedenella apiculata (AB037098)(Hegewald and Hanagata, 2000)*	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Scenedesmus obtusus (AB037091) (Hegewald and Hanagata, 2000)	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Coelastropsis costata (AB037083) (Hegewald and Hanagata, 2000)	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Scenedesmus ovalternus (X81966) (Kessler et al., 1997)	99	Genomic DNA	Chlorophyta	Chlorophyceae
15	*Scenedesmus obliquus (FR86573) [*]*	100	Genomic DNA, culture collection	Chlorophyta	Chlorophyceae
	Scenedesmus pectinatus (FR865730) [*]	100	Genomic DNA, culture collection	Chlorophyta	Chlorophyceae
	Scenedesmus deserticola (AY510463) (Lewis and Flechtner, 2004)	100	Soil isolate	Chlorophyta	Chlorophyceae
	Scenedesmus littoralis (Hanagata, 2001)	100	Genomic DNA	Chlorophyta	Chlorophyceae
	Scenedesmus rubescens (Kessler et al., 1997)	100	Genomic DNA	Chlorophyta	Chlorophyceae

16	*Tetranesphris brasiliensis* (HM565929) (Krienitz *et al.*, 2011a)	100	Genetic DNA	Chlorophyta	Chlorophyceae
17	*Paramecium tetraurelia* (X03772) (Sogin and Elwood, 1986)	100	Genomic DNA	Alveolata	Oligohymenophora
18	*Pyrobotrys stellata* (Nakada *et al.*, 2010)	98	Genomic DNA	Chlorophyta	Chlorophyceae
19	*Chlorella sorokiniana* (AB731602) (Hoshina *et al.*, 2013)	99	Lake, Culture collection	Chlorophyta	Trebouxiophyceae
	Chlorella vulgaris (GQ122369) [*]	99	Culture collection	Chlorophyta	Trebouxiophyceae
	Meyerella planktonica (AY543042) (Fawley *et al.*, 2005)	99	Lake, USA	Chlorophyta	Trebouxiophyceae
20	*Ochromonas vasocystis* (EF165111) [*]	99	Genomic DNA	Stramenopiles	Chrysophyceae
21	*Uncultured chytridiomycota* (GQ995419) (Freeman *et al.*, 2009)	97	Soil	Fungi	Chytridiomycota
22	*Paramecium multimicronucleatum* (HE662762)	99	Industrial water	Alveolata	Oligohymenophora
23	*Desmodesmus intermedius* (FR865703) (FR865700) [*]	99	Culture collection	Chlorophyta	Chlorophyceae
24	*Gaertneriomyces spectabile* (FJ827661) (Wakefield *et al.*, 2010)	97	*Genomic DNA*	Fungi	Chytridiomycete
25	*Kirchneriella obesa* (HM483513) (Krienitz *et al.*, 2011b)	99	Genomic DNA	Chlorophyta	Chlorophyceae
26	*Kirchneriella obesa* (HM483513) (Krienitz *et al.*, 2011b)	99	Genomic DNA	Chlorophyta	Chlorophyceae
27	*Chlorella sorokiniana* (EF030563) (Summerer *et al.*, 2008)	99	Genomic DNA	Chlorophyta	Trebouxiophyceae
	Micractinium pusillium (FM205836) (Luo *et al*., 2010)	99	Culture collection	Chlorophyta	Trebouxiophyceae
28	Uncultured ciliate clone (JF720678) (Chen *et al*., 2008b)	99	Freshwater lake	*Alveolata*	
29	*Opisthonecta minima* (EF417834) (Williams and Clamp, 2007)	100	Genomic DNA	Alveolata	Oligohymenophora
30	*Chlorella sorokiniana* (AB731602) (Hoshina and Fujiwara, 2013)	99	Culture collection	Chlorophyta	Trebouxiophyceae
	Chlorella vulgaris (AB080308) (Yamamoto *et al*., 2003)	99	Genomic DNA	Chlorophyta	Trebouxiophyceae

Appendix 6 Summary of the similarities for the dominant excised cyanobacterial bands, from Chapter 6.

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class	RDP classifier
1	Uncultured Cyanobacteria bacterium (CU920275) (Riviere *et al.*, 2009)	*98*	Anaerobic wastewater sludge digester	Cyanobacteria		Bacteria, Firmicutes, Clostridia
2	Uncultured bacterium clone (JQ072402) [*]	97	Brewery wastewater clarifier outfall	Bacteria		Bacteria, Proteobacteria,
3	Uncultured organism clone (JN528545) (Harris *et al.*, 2013)	93	Microbial mat in hypersaline evaporation pond	Bacteria		Bacteria, Proteobacteria,
4	Uncultured *Attheya* clone (GQ183295) (Allen *et al.*, 2010)	98	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	Cyanobacteria/Chloroplast[75%] Cyanobacteria[44%] Family VIII
	Uncultured *Thalassiosira* (GQ183223) (Allen *et al.*, 2010)	*98*	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	
5	Uncultured *Attheya* clone (GQ183295) (Allen *et al.*, 2010)	96	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	Cyanobacteria/Chloroplast[58%] Chloroplast[32%] Chloroplast[32%] Bangiophyceae[27%]
	Uncultured *Thalassiosira* (GQ183223) (Allen *et al.*, 2010)	*96*	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	
6	Uncultured *Attheya* clone (GQ183295) (Allen *et al.*, 2010)	97	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	Cyanobacteria/Chloroplast[64%] Chloroplast[38%] Chloroplast[38%] Bangiophyceae[36%]
	Uncultured *Thalassiosira* (GQ183223) (Allen *et al.*, 2010)	*97*	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	
7	Uncultured *Attheya* clone (GQ183295) (Allen *et al.*, 2010)	98	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	Cyanobacteria/Chloroplast[75%] Cyanobacteria[44%] Family VIII[20%]
	Uncultured *Thalassiosira* (GQ183223) (Allen *et al.*, 2010)	*98*	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	
8	Uncultured *Attheya* clone (GQ183295) (Allen *et al.*, 2010)	98	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	Cyanobacteria/Chloroplast[58%] Chloroplast[44%] Chloroplast[44%] Bangiophyceae[33%]
	Uncultured *Thalassiosira* (GQ183223) (Allen *et al.*, 2010)	*98*	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	
9	Uncultured *Attheya* clone (GQ183295) (Allen *et al.*, 2010)	98	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	Cyanobacteria/Chloroplast[58%] Chloroplast[44%] Chloroplast[44%] Bangiophyceae[33%]
	Uncultured *Thalassiosira* (GQ183223) (Allen *et al.*, 2010)	*98*	Aquatic sediment from constructed wetland	Stramenopiles	Cosinodiscophyceae	

10	Uncultured *Fischerella sp.* Clone (KC211807) (Coman et al., 2013)	97	Hot spring microbial mat	Cyanobacteria		Cyanobacteria/Chloroplast[27%] Cyanobacteria[18%] Family X[4%] GpX[4%]
11	Uncultured bacterium clone (GU636277) (Jeong *et al.*, 2011)	97	River water			Cyanobacteria/Chloroplast[25%] Chloroplast[20%] Chloroplast[20%] Bangiophyceae[13%]
12	*Oscillatoria sp.* (JN399097) [*]	98	Genomic DNA	Cyanobacteria	Oscillatoriophycideae	Cyanobacteria/Chloroplast[32%] Cyanobacteria[26%] Family X[5%] GpX[5%]
13	*Utricularia gibba* chloroplast (KC997777) (Ibarra-Laclette, 2013, in press) One of many 100% matches to plant chloroplasts	100	Complete genome	Plant	Streptophyta	Cyanobacteria/Chloroplast[100%] Chloroplast[100%] Chloroplast[100%] Streptophyta[100%]
14	Uncultured bacterium (HQ905766) [*]	99	Phyllosphere of tomatoes			Cyanobacteria/Chloroplast[61%] Chloroplast[46%] Chloroplast[46%] Streptophyta[44%]
15	*Oscillatoria sp.* (JN399097) [*]	98	Genomic DNA	Cyanobacteria	Oscillatoriophycideae	Cyanobacteria/Chloroplast[33%] Cyanobacteria[26%] Family X[5%] GpX[5%]
16	*Chlorella variabilis* plastid (HQ914635) [*]	99	Complete genome	Chlorophyta	Trebouxiophyceae	Cyanobacteria/Chloroplast[100%] Chloroplast[99%] Chloroplast[99%] Chlorophyta[93%]
	Chlorella pyrenoidosa plastid (AJ387756) [*]	99	Genomic DNA	Chlorophyta	Trebouxiophyceae	
	Chlorella sorokiniana plastid (X65689) [*]	99	*Genomic DNA*	Chlorophyta	Trebouxiophyceae	
17	*Oscilatoriales* cyanobacterium (HQ912983) [*]	100	*Lake*	Cyanobacteria	Oscillatoriophycideae	Cyanobacteria/Chloroplast[90%] Cyanobacteria[88%] Family XIII[61%] GpXIII[61%]
	Planktothrix pseudogardhii (FM177501) [*]	100	Lake	Cyanobacteria	Oscillatoriophycideae	
	Planktothrix mougeotii (FJ184392) (Lin *et al.*, 2010)	100	Genomic DNA	Cyanobacteria	Oscillatoriophycideae	

Appendix 7 Summary of the similarities for the dominant excised eukaryotic bands, Chapter 7, Case Study 1

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class
1	*Opisthonecta minima* (EF417834) (Williams and Clamp, 2007)	99	Genomic DNA	*Alveolata*	*Oligohymenophorea*
2	*Parachlorella kessleri* (JQ797561) [*]	99	Fresh water lake	*Chlorophyta*	*Trebouxiophyceae*
3	*Chlorella sorokiniana* (AB731602) (Hoshina *et al.*, 2013)	99	Genomic DNA	*Chlorophyta*	*Trebouxiophyceae*
4	*Chlorella sorokiniana* (AB731602) (Hoshina *et al.*, 2013)	99	Genomic DNA	*Chlorophyta*	*Trebouxiophyceae*
5	*Brachionus calyciflorus* (GQ503607) [*]	100	Freshwater Lake, Russia	*Rotifera*	*Monogononta*
6	Uncultures eukaryote clone (JX069052) (Thomas *et al.*, 2012)	98	Southern Alberta River, environmental sample		
7	*Tubulinida* species (HQ687486) (Dykova *et al.*, 2011)	99	Genomic DNA	*Amaebozoa*	*Tubulinea*
8	*Parachlorella kessleri* (JQ797561) [*]	99	Fresh water lake	*Chlorophyta*	*Trebouxiophyceae*
9	*Paramecium multimicronucleatum* (HE662762) [*]	100	Industrial water	*Alveolata*	*Oligohymenophorea*

Appendix 8 Summary of the similarities for the dominant excised cyanobacterial bands, Chapter 7, Case study 1

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class	RDP classifier
1	Unclassified *Streptophyta* (JQ701246) [*]	*94*	Water from experimental oligotrophic mesocosm	Viridiplantae	Streptophyta	Cyanobacteria/ chloroplast
	Chlorella species (HF536585) [*]	94	Sediment sample	Chlorophyta	Trebouxiophyceae	
	Uncultured *cyanobacterium* (JX463374) [*]	94	Coral disease mat	Cyanobacterium		
2	Uncultured *bacterium* (GU638170) (Jeong *et al.*, 2011)	96	River water			Cyanobacteria/ chloroplast
3	Unclassified *Streptophyta* (JQ701246) [*]	*99*	Water from experimental oligotrophic mesocosm	Viridiplantae	Streptophyta	Cyanobacteria/ chloroplast
	Chlorella species (HF536585) [*]	99	Sediment sample	Chlorophyta	Trebouxiophyceae	
	Uncultured cyanobacterium (JX463374) [*]	99	Coral disease mat	Cyanobacterium		
4	Unclassified *Streptophyta* (JQ701246)	*99*	Water from experimental oligotrophic	Viridiplantae	Streptophyta	Cyanobacteria/

	[*]		mesocosm			chloroplast
	Chlorella species (HF536585) [*]	99	Sediment sample	Chlorophyta	Trebouxiophyceae	
	Uncultured cyanobacterium (JX463374) [*]	99	Coral disease mat	Cyanobacterium		
5	*Planktothrix rubescens* (HF678515) [*]	99	CCAP- Lake Zurich	Cyanobacterium	Oscillatoriophyta	
6	Uncultured *bacterium* clone OF3 (JN941845) [*]	100	Fresh water lake surface	Bacteria		Cyanobacteria/ chloroplast
	Planktothrix pseudagardhii (JQ894510) [*]	100	Hydrocarbon contaminated sediment	Cyanobacterium	Oscillatoriophyta	
	Planktothrix mougeotii (FJ184392) (Lin *et al.*, 2010)	100	Genomic DNA	Cyanobacterium	Oscillatoriophyta	
	Oscillatoria species (GQ351575) [*]	100	Genomic DNA	Cyanobacterium	Oscillatoriophyta	
7	Uncultured *bacterium* clone OF3 (JN941845) [*]	98	Fresh water lake surface	Bacteria		
	Planktothrix pseudagardhii (JQ894510) [*]	98	Hydrocarbon contaminated sediment	Cyanobacterium	Oscillatoriophyta	
	Planktothrix mougeotii (FJ184392) (Lin *et al.*, 2010)	98	Genomic DNA	Cyanobacterium	Oscillatoriophyta	
	Oscillatoria species (GQ351575) [*]	98	Genomic DNA	Cyanobacterium	Oscillatoriophyta	
8	Uncultured *bacterium* (GU636558) (Jeong *et al.*, 2011)	97	River water	Bacterium		Cyanobacteria/ chloroplast
9	Unclassified *Streptophyta* (JQ701246) [*]	99	Water from experimental oligotrophic mesocosm	Viridiplantae	Streptophyta	Cyanobacteria/ chloroplast
	Chlorella variabilis plastid (HQ914635) [*]	99	Complete genome	Chlorophyta	Trebouxiophyceae	
10	Unclassified *Streptophyta* (JQ701246) [*]	99	Water from experimental oligotrophic mesocosm	Viridiplantae	Streptophyta	Cyanobacteria/ chloroplast
	Chlorella variabilis plastid (HQ914635) [*]	99	*Complete genome*	Chlorophyta	Trebouxiophyceae	
11	Unclassified *Streptophyta* (JQ701246) [*]	99	*Water from experimental oligotrophic mesocosm*	*Viridiplantae*	*Streptophyta*	*Cyanobacteria/ chloroplast*
	Chlorella sorokiniana plastid (JN865974) [*]	99	Fresh water	Chlorophyta	Trebouxiophyceae	
	Chlorella variabilis (JN865973) [*]	99	Freshwater	Chlorophyta	Trebouxiophyceae	

12	*Planktothrix rubescens* (HF678515) [*]	99	CCAP- Lake Zurich	*Cyanobacterium*	*Oscillatoriophyta*	*Cyanobacteria*
	Planktothrix agardhii (HF678485) [*]	99	CCAP- Lake Zurich	*Cyanobacterium*	*Oscillatoriophyta*	
13	*Arthrospira platensis* (KC536648) [*]	99	Genomic DNA	*Cyanobacterium*	*Oscillatoriophyta*	*Cyanobacteria*
	Arthrospira maxima (GQ206141) [*]	99	Genomic DNA, lake	*Cyanobacterium*	*Oscillatoriophyta*	
14	*Arthrospira platensis* (KC536648) [*]	100	Genomic DNA	*Cyanobacterium*	*Oscillatoriophyta*	*Cyanobacteria*
	Arthrospira maxima (JX827162) [*]	100	Genomic DNA	*Cyanobacterium*	*Oscillatoriophyta*	
	Lyngbya hieronymusli (JN854140) [*]	100	Genomic DNA, lake	*Cyanobacterium*	*Oscillatoriophyta*	
	Planktothrix cryptovaginata (JN854139) [*]	100	Genomic DNA, lake	*Cyanobacterium*	*Oscillatoriophyta*	
15	*Pedinomonas sp.* plastid (HE610169) (Marin, 2012)	99	Plastid DNA	*Chlorophyta*	*Pedinophyceae*	*Chloroplast, chlorophyta*
16	Uncultured marine microorganism (EU183683) (Ceotto *et al*., 2008)	99	Marine, Hawaii			*Cyanobacteria/ chloroplast*
17	*Planktothrix rubescens* (HF678515) [*]	96	CCAP- Lake Zurich	*Cyanobacterium*	*Oscillatoriophyta*	*Cyanobacteria*
	Planktothrix agardhii (HF678485) [*]	96	CCAP- Lake Zurich	*Cyanobacterium*	*Oscillatoriophyta*	

Appendix 9 Summary of the similarities for the dominant excised eukaryotic bands, Chapter 7, Case study 2 (using ncbi- ncleuotide BLAST).

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class
1	Uncultured alveolate clone EU162627	83	Freshwater lake	Alveolata	
2	Uncultured alveolate clone EU162627	84	Freshwater lake	Alveolata	
3	*Paramecium tetraurelia* EF502045	99	Genomic DNA	Alveolata	Oligohymenophorea
4	*Apiaceae* environmental EF024041	92	Aspen rhizosphere		
5	*Kirchneriella obesa* HM483513	98	Genomic DNA	Chlorophyta	Chlorophyceae
6	*Kirchneriella obesa* HM483513	100	Genomic DNA	Chlorophyta	Chlorophyceae
7	*Kirchneriella obesa* HM483513	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Kirchneriella dianae HM483512	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Ankistrodesmus bibraianus Y16938	99	Genomic DNA	Chlorophyta	Chlorophyceae
8	*Kirchneriella obesa* HM483513	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Kirchneriella dianae HM483512	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Ankistrodesmus bibraianus Y16938	99	Genomic DNA	Chlorophyta	Chlorophyceae
9	*Kirchneriella obesa* HM483513	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Kirchneriella dianae HM483512	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Ankistrodesmus bibraianus Y16938	99	Genomic DNA	Chlorophyta	Chlorophyceae
10	Uncultured alveolate clone EU162627	84	Freshwater lake	Alveolata	
11	*Paramecium tetraurelia* EF502045	100	Genomic DNA	Alveolata	Oligohymenophorea
12	*Uronema nigricans* JF973324	100	Genomic DNA	Alveolata	Oligohymenophorea
13	*Pyrobotrys stellata* AB542920	100	Genomic DNA	Chlorophyta	Chlorophyceae
14	*Kirchneriella obesa* HM483513	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Kirchneriella dianae HM483512	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Ankistrodesmus bibraianus Y16938	99	Genomic DNA	Chlorophyta	Chlorophyceae
15	*Kirchneriella obesa* HM483513	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Kirchneriella dianae HM483512	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Ankistrodesmus bibraianus Y16938	99	Genomic DNA	Chlorophyta	Chlorophyceae
16	*Kirchneriella obesa* HM483513	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Kirchneriella dianae HM483512	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Ankistrodesmus bibraianus Y16938	99	Genomic DNA	Chlorophyta	Chlorophyceae
17	*Kirchneriella obesa* HM483513	100	Genomic DNA	Chlorophyta	Chlorophyceae

18	Uncultured alveolate clone EU162627	84	Freshwater lake	Alveolata	
19	*Kirchneriella obesa* HM483513	99	Genomic DNA	Chlorophyta	Chlorophyceae
	Tetranephris brasiliensis HM565927	98	Genomic DNA	Chlorophyta	Chlorophyceae
20	*Pyrobotrys stellata* AB542920	100	Genomic DNA	Chlorophyta	Chlorophyceae
	Chlamydomonas applanata AB701512	99	Genomic DNA, culture collection	Chlorophyta	Chlorophyceae

Appendix 10 Summary of the similarities for the dominant excised cyanobacterial bands, Chapter 7, Case study 2 (using ncbi-nucleotide database BLAST).

Band	Closest relative (accession no.)	Similarity %	Source	Affiliation	Class	RDP classifier
1	*Chroococcidiopsis thermalis* NR_102464	100	Genomic DNA-culture collection	Cyanobacteria	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[99%] Cyanobacteria[97%] Family I[93%] GpI[93%]
2	*Telosma cordata* plastid KF539853	100	Plastid DNA	Plant	Viridiplantae	Bacteria[100%] Cyanobacteria/Chloroplast[100%] Chloroplast[100%] Chloroplast[100%] Streptophyta[100%]
	Large number of other plastid entries	100				
3	*Stockwellia quadrifida* chloroplast, KC180807	100	Plastid DNA	Plant	Viridiplantae	Bacteria[100%] Cyanobacteria/Chloroplast[100%] Chloroplast[100%] Chloroplast[100%] Streptophyta[100%]
	Large number of other plastid entries	100				
4	*Symploca sp*. AB863135	100	Genomic DNA	Cyanobacteria	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[97%] Cyanobacteria[94%] Family I[94%] GpI[94%]
	Lyngbya cf. majuscula AB863125	100	Genomic DNA	Cyanobacteria	Cyanophyceae	
	Oculatella sp. KC311928	100	Genomic DNA	Cyanobacteria	Cyanophyceae	
	Pseudophormidium sp. KC311926	100	Genomic DNA	Cyanobacteria	Cyanophyceae	
	Pseudanabaenaceae cyanobacterium KC311922	100	Soil	Cyanobacteria	Cyanophyceae	
	Phormidesmis sp. KC311917	100	Soil	Cyanobacteria	Cyanophyceae	
	Large number of other cyanobacterial entries	100				
5	Uncultured bacterium clone GU624265	99	Pig faeces	Bacteria		Bacteria[99%] "Proteobacteria"[30%] Deltaproteobacteria[22%]

						Desulfuromonadales[11%] Geobacteraceae[9%] Geopsychrobacter[8%]
6	*Planktothrix rubescens* CCAP 1460/9 HF678515	100	Culture collection	Cyanobacteria	Cyanophyceae	Bacteria[98%] Cyanobacteria/Chloroplast[75%] Cyanobacteria[69%] Family XII[40%] GpXII[40%]
	Planktothrix rubescens CCAP 1460/18 HF678490	100	Culture collection	Cyanobacteria	Cyanophyceae	
	Planktothrix agardhii CCAP 1460/13 HF678485	100	Culture collection, freshwater lake	Cyanobacteria	Cyanophyceae	
7	*Planktothrix rubescens* CCAP 1460/9 HF678515	100	Culture collection	Cyanobacteria	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[64%] Cyanobacteria[60%] Family XII[38%] GpXII[38%]
	Planktothrix rubescens CCAP 1460/18 HF678490	100	Culture collection	Cyanobacteria	Cyanophyceae	
	Planktothrix agardhii CCAP 1460/13 HF678485	100	Culture collection, freshwater lake	Cyanobacteria	Cyanophyceae	
8	Uncultured bacterium clone FJ879949	97	Rat faeces			Bacteria[100%] Firmicutes[34%] Clostridia[30%] Clostridiales[25%] Eubacteriaceae[10%] Alkalibacter[9%]
9	*Stockwellia quadrifida* chloroplast, KC180807	100	Chloroplast DNA	Plant	Viridiplantae	Bacteria[100%] Cyanobacteria/Chloroplast[100%] Chloroplast[100%] Chloroplast[100%] Streptophyta[100%]
	Halospirulina sp. JX912466	100	Irrigated crop	Cyanobacteria	Cyanophyceae	
10	Uncultured bacterium clone JX225104	89	Subsurface aquifer sediment	Bacteria		Bacteria[90%] "Aquificae"[14%] Aquificae[14%] Aquificales[14%]
11	*Chroococcidiopsis thermalis* PCC 7203 NR_102464	100	Culture collection, soil	Cyanobacteria	Cyanophyceae	Bacteria[100%] Cyanobacteria/Chloroplast[99%] Cyanobacteria[97%] Family I[93%] GpI[93%]
	Chroococcidiopsis thermalis CCAP 1423/1 JX316763	100	Culture collection, roman baths	Cyanobacteria	Cyanophyceae	
	Chroococcidiopsis cubana SAG 39.79 JF810080	100	Culture collection, soil	Cyanobacteria	Cyanophyceae	
12	*Asclepias syriaca* chloroplast KF386166	100	Plastid DNA	Plant	Viridiplantae	Bacteria[100%] Cyanobacteria/Chloroplast[99%] Chloroplast[97%] Chloroplast[97%] Streptophyta[97%]
	Large number of other plastid entries	100				
13	Telosma cordata plastid KF539853	100	Plastid DNA	Plant	Viridiplantae	Bacteria[100%] Cyanobacteria/Chloroplast[72%] Chloroplast[57%] Chloroplast[57%]
	Large number of other plastid entries	100				

Appendix 11 Chemical and physical data for the samples from the Ginebra facultative ponds, conventional and baffled, Chapter 7, Case study 2.

Sample	BOD	COD (mg/l)	PO_4 (mg/l)	NH_4 (mg/l)	TKN (mg/l)	N-NO3 (mg/l)	pH	Dissolved oxygen (mg/l)	Temperature (°C)	Light intensity
C1s-18	9.84	293	2.565	17.05	21.85	1.13	7.89	4.15	26.5	0
C1d-18		333	2.766	19.69	24.58	7.58	6.99	1.38	27.2	0
C1s-6	4.68	393	3.013	21.5	29.7	4.6	7.1	0.38	23.2	3.64
C1d-6	7.8	303	3.3	24.5	30.8	1.8	7.07	0.36	23.5	0.006
C1s-9	24.24	408	3.005	20.32	26.53	4.36	7.31	0.96	26.5	680
C1d-9		558	3.206	19.47	26.53	3.35	6.98	0.35	24.3	3.46
C1s-12	8.28	358	2.848	16.37	28.11	3.18	8.54	20.55	31.8	627
C1d-12	8.88	503	2.894	18.63	28.93	3.01	6.96	2.3	28.5	7.33
B1s-18	5.52	238	2.931	18.18	26.30	1.47	8.87	9.52	27.3	0
B1d-18	3.24	343	2.664	24.86	27.66	1.35	6.97	1.04	26.4	0
B1s-6	0.828	153	3.161	22	33.6	2.83	7.19	0.46	23.4	2.74
B1d-6	5.76	483	3.062	21.6	27.8	4.5	7.09	0.42	22.9	0
B1s-9	28.2	613	3.095	24.27	33.84	4.59	7.76	4.68	27.7	784
B1d-9	18.72	253	3.029	23.14	29.73	1.93	7.18	0.57	26.8	1.56
B1s-12	5.4	523	3.518	21.45	31.05	2.13	8.75	22.2	32.5	798
B1d-12	19.08	493	2.959	20.75	29.92	1.86	6.98	1.42	26.8	0.236

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