Evaluation of DNA extraction methods for freshwater eukaryotic microalgae

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Abstract

The use of molecular methods to investigate microalgal communities of natural and engineered freshwater resources is in its infancy, with the majority of previous studies carried out by microscopy. Inefficient or differential DNA extraction of microalgal community members can lead to bias in downstream community analysis. Three commercially available DNA extraction kits have been tested on a range of pure culture freshwater algal species with diverse cell walls and mixed algal cultures taken from eutrophic waste stabilization ponds (WSP). DNA yield and quality were evaluated, along with DNA suitability for amplification of 18S rRNA gene fragments by polymerase chain reaction (PCR). QiaGenDNeasy® Blood and Tissue kit (QBT), was found to give the highest DNA yields and quality. Denaturant Gradient Gel Electrophoresis (DGGE) was used to assess the diversity of communities from which DNA was extracted. No significant differences were found among kits when assessing diversity. QBT is recommended for use with WSP samples, a conclusion confirmed by further testing on communities from two tropical WSP systems. The fixation of microalgal samples with ethanol prior to DNA extraction was found to reduce yields as well as diversity and is not recommended.

Keywords:
- Waste stabilization ponds
- Microalgae
- Molecular techniques
- DNA extraction
- DGGE
- Community analysis

1. Introduction

Scientific and commercial interest in microalgal communities has increased dramatically in the past five years with the realisation that such communities can be a globally valuable and sustainable source of biomass for the production of biofuels, fertilisers and animal feed. The coupling of microalgal production with wastewater treatment is important for the economic viability of such biomass production (Christenson and Sims, 2011).

Waste stabilization ponds (WSPs) are already used worldwide for low cost wastewater treatment in which microalgae play a crucial role in the assimilation of nitrogen compounds and production of oxygen for bacterial heterotrophic degradation of organic carbon (Camargo Valero and Mara, 2007; Ferrara and Avci, 1982; DiGiano, 1982; Senzia et al., 2002) to prevent eutrophication in receiving waters.

Despite the critical role of microalgae in the removal of nitrogen from wastewater, little is known about the composition of these communities. Microalgae are the key catalysts that control many of the processes in WSPs. An understanding of their ecology (i.e. the rules that govern their behaviour) could transform the design and operation of WSPs from its current simple empirical form to a more mature and predictive engineering practice (Curtis et al., 2003). Furthermore, the success of algal harvesting methods and treatment efficiency are also highly dependent on the algal community present (Christenson and Sims, 2011).
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. They have been applied extensively to study bacterial communities in diverse environments (van Elsas and Boersma, 2011; Sogin et al., 2006; Truu et al., 2009), to assess community dynamics in marine ecosystems (Larsen et al., 2001; Potvin and Lovejoy, 2009; Stoeck et al., 2007), and to study harmful algal blooms (Galluzzi et al., 2005; Vila et al., 2005; Tengs et al., 2001; Connell, 2002). 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 16S rRNA gene (See et al., 2005; Lefranc 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), Yu and Mohn (2001) and Camargo Valero et al. (2009) focused on bacterial populations, while Furtado et al. (2009) isolated and cultured cyanobacteria, before using 16S rDNA gene sequencing to assess their identity. The only study thus far to have assessed eukaryotic microalgae in wastewater treatment plants (Ghosh and Love, 2011) 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.

The DNA extraction method used on a sample can have a major impact on downstream community analysis. Eukaryotic microalgae have a large range of cell wall structures, which creates 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.

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 Blood and Tissue (QBT) kit, Qiagen 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 Galluzzi 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) and the levels of inhibitory substances common in WSP, such as humic acids (Amir et al., 2006), which have the potential to inhibit downstream processes such as PCR (Wilson, 1997).

In the current study we investigated the application of the three commercially available kits outlined above for the extraction of DNA from freshwater eukaryotic algae with a range of cell wall types, 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, and the diversity of dominant species in natural mixed cultures using denaturant gradient gel electrophoresis (DGGE). In addition, we investigated the effect of ethanol fixation on DNA extraction and subsequent PCR, 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 (Wilson, 1997; Marin et al., 2001; Godhe et al., 2002; Ahokas and Erkkila, 1993).

2. Materials and methods

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 100 ml and 12 samples of 250 ml were collected and frozen at −20 °C on return to the laboratory. Another six 250 ml samples were collected. These samples were fixed with 250 ml 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 served the industrial district of the city, with a mixed influent, approximately 50% from industrial sources and 50% from domestic sources. The other system was fed purely domestic wastewater. 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.

2.2. Sample preparation

Samples were defrosted and then centrifuged at 3392 × g (4200 rpm) for 2 h or 7690 × g, 10 min, 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.

2.3. Eukaryotic algal cultures

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

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

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, twice with 100 ml of sample (containing approximately 1.64 x 10^6 cells) and twice with 250 ml of sample (approximately 4.1 x 10^6 cells). The kits were Qiagen DNeasy® Plant Mini kit (QPM), Qiagen DNeasy® Blood and Tissue kit (QBT), and MoBioUltraClean™ 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 50 μl of elution buffer (10 mM 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 100 μl of Buffer AE, (10 mM Tris Cl, 0.5 mM EDTA at pH 9.0) were collected, giving a total of 200 μl.

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 5 h at 56 °C. RNase A and liquid nitrogen were not used.

Two elutions of DNA in 200 μl of Buffer AE, (10 mM Tris Cl, 0.5 mM EDTA at pH 9.0) were collected, giving a total of 400 μl. The extracted DNA elutions were frozen at −20 °C until PCR was carried out.

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 ratio at 260 nm/280 nm) was also assessed using the Nano Drop.

2.6. Amplification of 18S rRNA gene fragments

Amplification of 18S ribosomal RNA gene fragments was carried out in duplicate by PCR using primers Euk1A and Euk516r that target members of the Eukarya domain (Diez et al., 2001) (Table 2). A GC clamp was added to the 5’-end of Euk516r for subsequent DGGE analysis. The final PCR product was approximately 560 base pairs. PCR was carried out using PCR MegamixBlue® (Microzone, UK), 1 μl of each of the primers and 1 μl of DNA extract, using a BioRad C1000™ thermal cycler. The initial denaturing step was 130 s at 94 °C.

<table>
<thead>
<tr>
<th>Algal species and strain number</th>
<th>Class-order</th>
<th>Origin</th>
<th>Culture medium</th>
<th>Characteristic cell features</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydomonas reinhardti</em></td>
<td>Chlorophyceae- Volvocales</td>
<td>Edgewood Park, Connecticut, USA</td>
<td>3N-BBM + V Agar slope</td>
<td>Layered glycoprotein cell wall</td>
</tr>
<tr>
<td>CCAP-11/45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>Chlorophyceae- Volvocales</td>
<td>Molkerteich, Elsnigk, Sachsen-Anhalt, Germany</td>
<td>3N-BBM + V Agar slope</td>
<td>Glucose and mananose or glucosamine cell walls</td>
</tr>
<tr>
<td>CCAP-211/80</td>
<td>Chlorophyceae- Volvocales</td>
<td>Priest Pot, Cumbria, England</td>
<td>3N-BBM + V Liquid media</td>
<td>Globular colonies of 16–32 cells</td>
</tr>
<tr>
<td><em>Pandorina morum</em></td>
<td>Chlorophyceae- Volvocales</td>
<td>Priest Pot, Cumbria, England</td>
<td>EG/JM Liquid media</td>
<td>Cell walls contain decay resistant algaenan</td>
</tr>
<tr>
<td>CCAP-60/2</td>
<td>Chlorophyceae- Chlorococcales</td>
<td>Oyster pond, Martha’s vineyard, Massachusetts, USA</td>
<td>F2 liquid medium with air bubbling</td>
<td>Silica cell wall</td>
</tr>
<tr>
<td><em>Scenedesmus quadricauda</em></td>
<td>Chlorophyceae-Chlorococcales</td>
<td>Edgewood Park, Connecticut, USA</td>
<td>3N-BBM + V Agar slope</td>
<td>Glucose and mananose or glucosamine cell walls</td>
</tr>
<tr>
<td>CCAP-276/21</td>
<td>Bacillariophyceae, Naviculales</td>
<td>Molkerteich, Elsnigk, Sachsen-Anhalt, Germany</td>
<td>3N-BBM + V Liquid media</td>
<td>Globular colonies of 16–32 cells</td>
</tr>
<tr>
<td><em>Navicula pelliculosa</em></td>
<td></td>
<td></td>
<td>EG/JM Liquid media</td>
<td>Cell walls contain decay resistant algaenan</td>
</tr>
<tr>
<td>CCAP-1050/9</td>
<td></td>
<td></td>
<td>F2 liquid medium with air bubbling</td>
<td>Silica cell wall</td>
</tr>
</tbody>
</table>

3N-BBM + V- Bold-Basal medium with 3-fold Nitrogen and vitamins modified; EG- Euglena gracilis media; JM- Jaworski’s medium; F2- medium.
followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 45 s and extension at 72 °C for 130 s. The last cycle extension was held for an extra 6 min, followed by holding at 4 °C. Agarose gel electrophoresis was carried out on 1% agarose gels with a positive and negative control, and a PCR marker (Hyperladder II, Bioline, UK) to determine if DNA of the correct fragment size had been amplified. Ethidium bromide was used to stain DNA for visualisation under ultra violet illumination, using a Dual Intensity Transilluminator (Genetic Research Instrumentation Ltd, UK).

2.7. Diversity analysis

The diversity of the predominant members of the eukaryotic communities was evaluated using DGGE. DGGE was carried out using a BioRad system and a Power Pac 3000. Electrophoresis was run in 0.75 mm thick, 6% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) with a linear gradient of denaturing agents from 15% to 40% (where 100% denaturing agent is defined as 7 mol L-1 urea and 40% deionized formamide). The gradient was optimised to allow for maximum band separation. Electrophoresis was carried out at a total of 900 V hours (a constant 200 V for 4.5 h). Gels were stained using SybrGold for half an hour and visualised with a UV transilluminator with the program Quantity One (BioRad).

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 tested for normality and conformed. Quantity data was tested for normality and conformed. Quality 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 (Equation (1)) 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).

\[ H' = -\sum p_i \ln p_i \]  

(1)

Where \( H' \) = Species Diversity Index

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

3. Results

3.1. Total DNA yield

The quantity of DNA extracted by the three kits and the presence of contaminants are shown on Fig. 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 were used in all further statistical analyses.

All extractions yielded DNA, though this varied greatly in both quality and quantity, with DNA yields from 323 ng for pure culture Scenedesmus quadricauda using the UC kit to 18100 ng 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 Fig. 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 250 ml did not yield significantly more DNA than samples of 100 ml. 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 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.

3.2. 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 280 nm 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Saccharomyces cerevisiae positions</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euk1A</td>
<td>CTGGTTGATCCTGGCCAG</td>
<td>4 to 20</td>
<td>Eukarya</td>
</tr>
<tr>
<td>Euk516r-GC</td>
<td>ACCAGACTGGCCCTCC-</td>
<td>563 to 548</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-CGCGCGGGGGGGGGCGGGGGGGGACGGGGGGG*</td>
<td>a GC-clamp underlined.</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 – Nucleotide sequences of the two primers used to amplify the microalgal DNA.
ranged from 1.0 to 3.4, with 39% of samples tested achieving a value of 1.8 or greater (Table 3). 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 4). 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 230 nm. 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 contamination.

Fig. 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 Nano Drop 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.

Table 3 – 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. a

<table>
<thead>
<tr>
<th>Sample</th>
<th>260/230 ratio</th>
<th>260/280 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UC</td>
<td>QPM</td>
</tr>
<tr>
<td>C. vulgaris</td>
<td>0.08 (0.014)</td>
<td>0.17 (0.014)</td>
</tr>
<tr>
<td>C. reinhardtii</td>
<td>0.21 (0.035)</td>
<td>0.24 (0.042)</td>
</tr>
<tr>
<td>P. morum</td>
<td>0.13 (0)</td>
<td>0.21 (0.028)</td>
</tr>
<tr>
<td>S. quadricauda</td>
<td>0.23 (0.014)</td>
<td>0.18 (0.021)</td>
</tr>
<tr>
<td>N. pelliculosa</td>
<td>0.19 (0.007)</td>
<td>0.2 (0.007)</td>
</tr>
<tr>
<td>Mixed WSP sample, 250 ml</td>
<td>0.36 (0.06)</td>
<td>0.405 (0.04)</td>
</tr>
<tr>
<td>Mixed WSP sample, 250 ml</td>
<td>0.185 (0.01)</td>
<td>0.46 (0.01)</td>
</tr>
<tr>
<td>Mixed WSP sample, 100 ml</td>
<td>0.32 (0.13)</td>
<td>0.37 (0.04)</td>
</tr>
<tr>
<td>Mixed WSP sample, 100 ml</td>
<td>0.75 (0.03)</td>
<td>0.255 (0.01)</td>
</tr>
<tr>
<td>Mixed WSP sample, fixed</td>
<td>0.28 (0.01)</td>
<td>0.675 (0.01)</td>
</tr>
<tr>
<td>Mixed WSP sample, fixed</td>
<td>0.135 (0.04)</td>
<td>0.68 (0)</td>
</tr>
</tbody>
</table>

a 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.
contaminants that absorb light at 230 nm, such as carbohydrates, guanidine thiocyanate, phenols and humic acids (Sambrook et al., 2001). In all samples, 260/230 ratios were below two, with a range of 0.08–1.25. 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.

### 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 *Chlorella vulgaris*, *Pandorina morum* and the diatom *N. pelliculosa* (Fig. 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 (Fig. 3).

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 (Fig. 4). DNA from the QPM kit failed to yield any PCR product in both of the fixed samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean quantity of DNA (ng/elution)</th>
<th>Mean 260/280†</th>
<th>Mean 260/230†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Domestic</td>
<td>Industrial</td>
<td>Domestic</td>
</tr>
<tr>
<td>Anaerobic pond</td>
<td>24020 (10222)</td>
<td>41162 (27008)</td>
<td>1.9 (0.08)</td>
</tr>
<tr>
<td>Facultative pond</td>
<td>14011 (2461)</td>
<td>16423 (2930)</td>
<td>2 (0.02)</td>
</tr>
<tr>
<td>Maturation pond 1</td>
<td>22072 (3195)</td>
<td>16705 (1667)</td>
<td>2 (0.06)</td>
</tr>
<tr>
<td>Maturation pond 2</td>
<td>20445 (6062)</td>
<td>11477 (2287)</td>
<td>2 (0.04)</td>
</tr>
<tr>
<td>Maturation pond 3</td>
<td>7796 (2724)</td>
<td>2 (0.16)</td>
<td>0.77 (0.14)</td>
</tr>
</tbody>
</table>

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

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

Fig. 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.

Fig. 3 — Agarose gel showing PCR products from WSP samples, comparing the success of three DNA extraction kits, QPM, QBT and UC, and a negative control.
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.

3.4. 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 (Fig. 5) 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 5). There was no significant difference in the Shannon–Wiener index ($P = 0.82$) or band richness ($P = 0.18$) for DNA extracted from 250 ml to 100 ml of sample. The Shannon–Wiener index was similar for all of the non-fixed samples, although it was highest for the 100 ml WSP sample when the QBT kit was used.

The DGGE banding pattern obtained from fixed samples is shown in lanes 7–9 of Fig. 5. The 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.

4. 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 100 ml 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. We 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. 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.

![Fig. 4](image-url) Agarose gel showing PCR performed using DNA extracted from ethanol-fixed cells using kits QPM, QBT and UC.

![Fig. 5](image-url) A DGGE gel of 18S rRNA products. Lanes 1–6 are non-fixed WSP samples; 1–250 ml sample QPM, 2–250 ml sample QBT, 3–250 ml sample UC, 4–100 ml sample QPM, 5–100 ml sample QBT, 6–100 ml 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.
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.

5. Conclusions

In this study the effectiveness of a range of commercially available kits for extracting DNA from freshwater algal communities, particularly those in Waste Stabilization Pond systems 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:

- The Qiagen Blood and Tissue kit is recommended for use on samples from Waste Stabilization Ponds. It is 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 or uneven DNA extraction in downstream community analyses. A 100 ml sample or approximately $1.64 \times 10^6$ 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.

- Community analysis of microalgae in Waste Stabilization Ponds and other eutrophic systems, would allow for better understanding of how such systems function, with the potential for process optimisation.

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<table>
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<th>Sample</th>
<th>Shannon Wiener diversity index</th>
<th>Number of bands</th>
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<tr>
<td></td>
<td>QPM</td>
<td>QBT</td>
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<tr>
<td>WSP 250 ml</td>
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<td>WSP 100 ml</td>
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<td>Fixed WSP</td>
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assistance. Also Dr Gary Caldwell and Dr Jonathan Rand, Department of Marine Science and Technology, Newcastle University for their advice and supplying some of the algae for culture. Special thanks to the Larchfield Community, Teesside, and CAGECE, Fortaleza, Brazil for giving us access to their WSP systems.

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