Protist and Cyanobacterial Contributions to Particle Flux in Oligotrophic Ocean Regions

by

Jessica Amacher

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Graduate Supervisory Committee:

Susanne Neuer, Chair
Ferran Garcia-Pichel
Michael Lomas
Martin Wojciechowski
Valerie Stout

ARIZONA STATE UNIVERSITY

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ABSTRACT

The oceans play an essential role in global biogeochemical cycles and in regulating climate. The biological carbon pump, the photosynthetic fixation of carbon dioxide by phytoplankton and subsequent sequestration of organic carbon into deep water, combined with the physical carbon pump, make the oceans the only long-term net sink for anthropogenic carbon dioxide. A full understanding of the workings of the biological carbon pump requires a knowledge of the role of different taxonomic groups of phytoplankton (protists and cyanobacteria) to organic carbon export. However, this has been difficult due to the degraded nature of particles sinking into particle traps, the main tools employed by oceanographers to collect sinking particulate matter in the ocean. In this study DNA-based molecular methods, including denaturing gradient gel electrophoresis, cloning and sequencing, and taxon-specific quantitative PCR, allowed for the first time for the identification of which protists and cyanobacteria contributed to the material collected by the traps in relation to their presence in the euphotic zone. I conducted this study at two time-series stations in the subtropical North Atlantic Ocean, one north of the Canary Islands, and one located south of Bermuda. The Bermuda study allowed me to investigate seasonal and interannual changes in the contribution of the plankton community to particle flux. I could also show that small unarmored taxa, including representatives of prasinophytes and cyanobacteria, constituted a significant fraction of sequences recovered from sediment trap material. Prasinophyte sequences alone could account for up to 13% of the clone library sequences of trap material during

bloom periods. These observations contradict a long-standing paradigm in biological oceanography that only large taxa with mineral shells are capable of sinking while smaller, unarmored cells are recycled in the euphotic zone through the microbial loop. Climate change and a subsequent warming of the surface ocean may lead to a shift in the protist community toward smaller cell size in the future, but in light of these findings these changes may not necessarily lead to a reduction in the strength of the biological carbon pump.

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Introduction

Marine phytoplankton and the biological carbon pump

The biological carbon pump, the photosynthetic fixation of CO_2 by phytoplankton in the euphotic zone and the subsequent sequestration of organic carbon into deep water, is responsible for a gross global flux of about 16 petagrams of carbon per year from the euphotic zone to the ocean interior (Falkowski et al. 1998). The particulate matter that contributes to the sinking particle flux consists either of aggregated phytoplankton cells, amorphous detritus, or fecal pellets produced by grazers feeding on the phytoplankton in the euphotic zone. Once the particles sink out of the surface mixed layer, they begin their descent through the deep ocean where much of the organic matter is respired and remineralized in its upper 500 m. In combination with the physical carbon pump, the oceans have been a major regulator of climate and have been the only true net sink for anthropogenic CO_2 over the past 200 years (Sabine et al., 2004). Although much of the fixed carbon presumably remains in the upper water column, where it is recycled within the microbial loop (Sherr and Sherr, 1994), particles sinking to depths of at least 500 m are effectively removed from exchange with the atmosphere for tens to hundreds of years (Ono et al., 2001). A portion of this carbon (approximately 1%) continues to the ocean floor and is incorporated into the sediment, out of possible contact with the atmosphere on geological time scales.

Marine phytoplankton are the unicellular primary producers responsible for most of the photosynthesis, and thus carbon export, in the ocean. Phytoplankton include both eukaryotic (protists) and prokaryotic (cyanobacterial) members. They make up the base of marine food webs and play a central role in energy flow and elemental cycling. Through the actions of the biological carbon pump they play a major role in the removal of anthropogenic carbon dioxide from the atmosphere.

The most common cyanobacteria in the ocean are the marine clade of *Synecococcus* and *Prochlorococcus* (DuRand et al., 2001). Protists are a polyphyletic group made up of phototrophic (phytoplankton), heterotrophic (microzooplankton), and mixotrophic unicellular organisms. Taxonomic groupings, particularly for protist taxa, have been disputed since genetic information obtained through molecular studies have begun to be incorporated into their systematics. Adl et al. (2005) describes the ranking of eukaryotes into super-groups and further into first rank and second rank categories. These groupings are based not on the traditional taxonomy using shared characteristics, but rather on molecular data grouping sequences into clades. Using this classification system these authors list the protist super-group Chromalveolata, which includes Alveolates (ciliates, dinoflagellates and marine alveolates), Stramenopiles (diatoms, and novel marine stramenopiles, marine stramenopiles), Haptophyta (Prymnesiophyceae, coccolithophorids) and Cryptophyceae. Archaeplastida, another protist super-group includes the Chloroplastida (Chlorophyta, Prasinophytae), Glaucophyta and Rhodophyceae (red algae). Next,

the Excavata include the Euglenozoa group. Lastly, Rhizaria are microzooplankton and include foraminifera, radiolaria and cercozoa as defined in Adl et al. (2005). More recently, Baldauf (2008) published an updated eukaryotic phylogenetic tree with similar groupings constructed with both molecular and ultrastructural data (Figure 1) in attempt to bring together traditional and molecular approaches.

Several novel lineages of as yet uncultured marine organisms have been discovered through 18S rRNA gene sequencing and classified by phylogenetic analysis (Guillou et al., 2008; ie. Massana and Pedrós-Alió, 2008; Not et al., 2009). Two major groups of these newly classified clades are the marine alveolates and marine stramenopiles. Marine alveolates are members of the Alveolata related to dinoflagellates and are thought to correspond to the Syndiniales, an order consisting of marine parasitoid dinoflagellates (Massana and Pedrós-Alió, 2008; Siano et al., 2011). Five distinct lineages have been classified and categorized as marine alveolates groups I-V (Guillou et al., 2008). Members of this group are abundant in clone library surveys throughout the oceans (Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001; Not et al., 2009). Group II, the Amoebophryidae, is the most diverse but has only one cultured and formally described genus, *Amoebophyra*, a parasite of dinoflagellates (Guillou et al., 2008; Siano et al., 2011). Marine stramenopiles (MAST), members of the Stramenopile group, are thought to be small heterotrophic flagellates widely distributed in marine communities (Massana et al., 2004; Massana et al., 2006). This group consists of twelve distinct phylogenetic lineages (Massana et al., 2004). Recently,

the species *Solenicola setigera*, a small colonial parasite or epiphyte to the diatom *Leptocylindrus mediterraneus* belonging to the MAST 3 clade, has become the first cultured member of this group (Gómez et al., 2011).

Biogeochemists traditionally though that large taxa with heavy mineral tests, such as diatoms, have traditionally been thought to dominate particle flux (Boyd and Newton, 1999; Michaels and Silver, 1988; Sarthou et al., 2005). However, the idea that small phytoplankton may also be important, perhaps even more important than large cells, in particle export has recently begun to emerge (Table 1). Packaging of small cells into large aggregates or the rapidly-sinking fecal pellets of grazers (Legendre and Le Fèvre, 1995; Olli and Heiskanen, 1999; Richardson and Jackson, 2007; Waite et al., 2000) allows them to sink through the water column at a faster rate than could be predicted by physics on the basis of their size (Jackson, 2001). In a modeling study Richardson and Jackson (2007) hypothesize that picoplankton contribute to export production, the portion of primary production that sinks out of the euphotic zone, at a rate proportional to their primary production. Brew et al. (2009) found aggregation to be an important mechanism controlling export, and also revealed a significant correlation between particle flux and picoplankton abundance. Lomas and Moran (2011) found that nanoeukaryotes as well as cyanobacteria contribute significantly to particulate organic carbon (POC) flux. In subtropical gyres, which are dominated by picoplankton (Steinberg et al., 2001) small phytoplankton may be as important to carbon flux as diatoms and other large-celled taxa.

Collecting sinking particulate matter

Cells or cell detritus in the ocean are collected with particle traps of various designs, which are deployed or moored at different water depth intervals and time periods. Most cells sinking into these traps are packaged into aggregates or fecal pellets of grazers. In the following chapters we report on data gathered using two styles of drifting particle traps. The ESTOC particle trap design of Neuer et al. (2007) consists of an array of three traps deployed at 200, 300 and 500 m. Sample bottles in this design are filled with a 40 psu salt solution and 2% formalin, and are attached to the bottom of a larger cylinder filled with filtered seawater (Fig. 2). At BATS the particle interceptor traps (PITs, Fig. 2) were designed as described in the JGOFS protocols and consist of cylinders filled with brine solution. These are equipped with a drain valve under the base, which holds a 90 mm membrane filter for the collection of trap material (http://bats.bios.edu/bats_methods.html). Arrays were deployed at 150, 200 and 300 m depth. In each of these arrays sinking particles are intercepted by the cylinders and sink into the brine solution, then after a given time period the traps are recovered and the sampling bottles removed and filtered onto a GF/F filter (ESTOC) or directly filtered through the drain valve onto a polycarbonate filter (BATS).

Identifying organisms in sediment trap material

Identification and enumeration of organisms contained in particle trap material is difficult due to the packaging into fecal pellets and amorphous aggregates and to degradation of pigments. Various techniques have been used to

analyze the composition of sedimenting material. Organic and inorganic carbon, particulate organic nitrogen, and opal are routinely measured using elemental analyzers (Fischer et al., 1996). However, in order to identify specific taxa contributing to sinking particle flux other methods must be employed. Identifying the hard parts of taxa with mineral tests can easily be accomplished through microscopy. Silica frustules of diatoms do not dissolve readily (Lewin, 1961), but stay intact in traps and can be visualized by light microscopy, identified by shape and enumerated (Abrantes et al., 2002; Painter et al., 2010; Salter et al., 2007; Smythe-Wright et al., 2010). Coccolithophorids can be identified and quantified using scanning electron microscopy (SEM) counts of their coccoliths, and related to a flux from their abundance in particle traps (Sprengel et al., 2000; Sprengel et al., 2002). Silica (SiO_2) flux, a regular measurement taken at BATS, can also be measured as a proxy for the flux from diatoms (Krause et al., 2009; Michaels and Silver, 1988; Sampei et al., 2011). These methods measure silica and calcium carbonate flux, and assume that they indicate the concurrent downward flux of cellular material. However, it is also possible that the organic material of the respective organisms is recycled within the mixed layer, with only the hard parts sinking into the trap material (Benitez-Nelson et al., 2007; Dugdale et al., 1995). This would lead to overestimates of the contribution of these organisms to organic carbon flux. Additionally, in oligotrophic gyre regions, where pico- and nanoplankton without any mineral hard parts dominate the community, these methods may only account for a small portion of the total organic particle flux that will result in a distorted view of the organismal contribution to flux. In

another approach, Wakeham et al. (2009) measured the concentrations of organic biomarkers (amino acids, lipids, and pigments) in particle traps to determine the contribution of diatoms, coccolithophorids, and small phytoplankton to overall flux. However, this does not provide detailed taxonomic information. Lipid biomarkers were used by Salter et al. (2010) to determine the contributions of diatoms and coccolithophorids to deep ocean particle flux, and Bourguet et al. (2009) analyzed these same organic biomarkers in order to determine the composition of the bacterial community in the NW Mediterranean and its response to environmental changes. Under-water video profiling has also been used to determine organic flux (Stemmann et al., 2002), but this approach also does not allow for identification of specific taxa. In the previous study and that of Lomas and Moran (2011), who collected samples using both Niskin bottles and in-situ pumps to collect size-fractionated particles, high performance liquid chromatography (HPLC) was used to identify diagnostic pigments present in the water column. This method was used in order to determine which taxa may be sinking below the euphotic zone. However, HPLC may be hindered by the degradation of pigments during their descent through the water column.

DNA-based molecular methods have become an alternative approach to obtain detailed taxonomic information of plankton communities in the water column (Countway et al., 2007; Díez et al., 2001a; b; Moon-van der Staay et al., 2001; Not et al., 2007). Techniques such as denaturing gradient gel electrophoresis (DGGE) fingerprinting, cloning and sequencing, and quantitative PCR provide a way to identify and distinguish between taxa regardless of their

size, appearance or pigmentation. Recently it has been found that DNA can be extracted from particle trap material and individual taxa can be identified using clone libraries of the 18S rRNA gene (Amacher et al., 2009). Being a relatively stable molecule, DNA can potentially be collected and extracted even from fecal pellets (Martin et al., 2006; Nejstgaard et al., 2003; Troedsson et al., 2009) and is more resistant to breakdown than other tracer compounds, such as pigments, proteins or lipids (Nejstgaard et al., 2008). Additionally, Martin et al. (2006) found that prey species contained in fecal pellets reflected the actual predator's diet, suggesting that no differential degradation of DNA occurred. These DNA-based molecular methods allow us to identify taxa in particle trap material and to distinguish between small organisms in a way that is not otherwise possible, and allow the comparison between the planktonic community in the water column with plankton sinking into particle traps below the euphotic zone.

Study area (BATS and ESTOC)

The studies presented in the subsequent chapters were carried out at the European Station for Time-Series in the Ocean, Canary Islands (ESTOC) and at the Bermuda Atlantic Time-Series (BATS) station. These stations are located in the eastern (ESTOC) and western (BATS) subtropical North Atlantic gyre. ESTOC is located in the Canary Current approximately 100 km north of the Canary Islands and BATS in the Sargasso Sea, approximately 90 km southeast of Bermuda. Monthly sampling at ESTOC began in 1994, and BATS has been

sampled monthly since 1988, with sampling at the nearby Hydrostation S since 1954 (Schroeder and Stommel, 1969).

The Sargasso Sea stays stratified during most of the year (Steinberg et al., 2001) during which time primary production is thought to be nitrogen limited (Lipschultz, 2001). In late winter (between January and March) a breakup of the seasonal thermocline, driven by increased wind stress and changes in heat flux, results in deep convective mixing (maximum mixed layer depth of 100 – 350 m, 202 m annual average (Cianca et al., 2007) increasing the availability of nutrients in the euphotic zone (average depth 100 m), causing a sharp increase in abundance (a bloom) of phytoplankton as seen in elevated chlorophyll *a* levels (Helmke et al., 2010). The proximity of BATS to the Gulf Stream results in mesoscale variability in hydrography that can provide nutrient inputs throughout the rest of the year (Cianca et al., 2007). Lomas et al. (2009a; b) recently studied the impact of late-winter storms on the phytoplankton community near BATS, finding that the intermittent passage of winter storms can temporarily destabilize the water column, leading to pulses of nitrate and causing small blooms that involve a rapid succession of phytoplankters from diatoms to prymnesiophytes and finally cyanobacteria. By contrast, continuous winter storm activity can cause continuous mixing. This results in increased phytoplankton biomass but without the rapid succession of the community. The input of nutrients into the upper water column can cause blooms of phytoplankton and lead to a temporary rise in the strength of the biological carbon pump.

At ESTOC the maximum mixed layer depth is 100-160 m, with seasonal stratification breaking up in late winter due to cooling and increased surface mixing as a result of winter storms (Cianca et al., 2007). Euphotic zone depth is similar to that in Bermuda, averaging 93 m in spring and 126 m in winter (Zielinski et al., 2002). Neuer et al. (2007) observed a clear seasonality of chlorophyll, primary production, and POC flux with highest levels during the winter bloom period and smaller intermittent peaks occurring in summer and fall caused by the strong summer Trade Winds. Mesoscale eddy activity is lower than at BATS (Cianca et al., 2007). The ESTOC station, located approximately 500 km from the coast of NW Africa, is influenced seasonally by episodic dust deposition blowing offshore from the Saharan Desert, a major input of the trace element, iron. While this input of iron does not increase primary production or export production, it results in increased downward flux of lithogenic matter and may influence the timing of particle flux (Neuer et al., 2004).

Most of what is known about the phytoplankton community in the euphotic zone at BATS comes from microscopy counts, flow cytometry, and HPLC/pigment data. This oligotrophic area is mainly dominated by picoplankton, with significant seasonal and interannual variability in phytoplankton community structure (Caron et al., 1999; DuRand et al., 2001; Steinberg et al., 2001). The most numerically abundant phytoplankton members are the cyanobacteria *Synechococcus*, whose peak abundance occurs during the spring bloom, and *Prochlorococcus*, which is the most abundant taxon during the summer and fall (DuRand et al., 2001). The eukaryotic phytoplankton community is dominated by

nanoplankton (DuRand et al., 2001; Lomas and Moran, 2011). Prymnesiophytes and pelagophytes are the most abundant eukaryotes (Steinberg et al., 2001). The coccolithophorid *Emiliania huxleyi*, a prymnesiophyte, dominates surface waters during the winter/spring period (DuRand et al., 2001; Haidar and Thierstein, 2001; Lomas and Bates, 2004). Dinoflagellates and prasinophytes are less abundant as compared with the previously mentioned groups (Lessard and Murrell, 1996; Lomas and Bates, 2004; Steinberg et al., 2001). Diatom blooms are rare, with biogenic particulate silica concentration and production rates among the lowest in the oceans, but they are generally present during bloom periods (Krause et al., 2009; Steinberg et al., 2001). A study using ^{30}Si tracers at BATS found that diatoms may contribute up to 90% of POC export during the winter/spring bloom and up to 30% annually in the Sargasso Sea (Nelson and Brzezinski, 1997).

Much less is known about the phytoplankton community at ESTOC due to the lack of time-series records on pigments, flow cytometry, or microscopy. Coccolithophorids, including *Emiliana huxleyei, Florisphaera profunda*, and *Gephyrocapsa ericsonii* are the numerically most abundant group of the nanoplankton determined by microscopy (Abrantes et al., 2002; Sprengel et al., 2000). Small picoplankton, including *Synechococcus*, account for 2% - 28% of the autotrophic biomass, whereas nanoplankton, including coccolithophorids, account for 65%, and taxa greater than 10 µm, including diatoms, make up only 7% of the autotrophic biomass (Neuer et al., 2007).

Particulate downward flux can be measured using particle traps, which have been deployed monthly at BATS since 1988 and at ESTOC since 1996. Helmke et al. (2010) found average daily particulate organic carbon (POC) flux from particle traps at BATS from 150 m, 200 m and 300 m to be 29.1, 22.0 and 16.3 mg m^{-2} d^{-1} respectively. POC flux at ESTOC was almost an order of magnitude smaller with average daily POC flux from 200 m, 300 m, and 500 m of 4.5, 3.0, and 2.4 mg m^{-2} d^{-1}. POC flux at BATS was highest in winter and spring, low in summer, and at the lowest levels in fall. At ESTOC flux values were generally higher in late winter/spring, followed by summer, and lowest in fall. The comparatively lower export production at ESTOC may be associated with smaller new nutrient imports, weaker eddy-activity, and lower nitrogen fixation rates (Cianca et al., 2007; Helmke et al., 2010; McGillicuddy Jr. et al., 2003; Neuer et al., 2002).

Future Trends

As global atmospheric temperatures increase with rising atmospheric CO_2 levels, the ocean becomes warmer and more stratified, resulting in shallower mixed layers with less nutrient input from deeper waters (Bopp et al., 2005; Falkowski and Oliver, 2007). This will likely lower productivity and the strength of the biological carbon pump. Under these changing conditions, marine communities will also likely undergo shifts in abundance and composition, an effect that may influence the efficiency of the biological carbon pump in unpredictable ways. A corollary of the associated increase in dissolved CO_2 is the

acidification of seawater and a decrease in the saturation state of calcium carbonates (calcite and aragonite). This can lead to decreased rates of calcification of coccolithophorids and foraminifera (Hays et al., 2005). Taucher and Oschlies (2011) note that net primary production (NPP) in future oceans may also be directly dependent on temperature, with elevated temperatures reducing NPP. Their model and several others (Bopp et al., 2001; Cox et al., 2000; Fung et al., 2005) project a decrease in export production. Falkowski and Oliver (2007) state that in highly stratified, low nutrient conditions smaller cells will be selected for, leading to an increase of small sized phytoplankton and thus, if small size indeed means low export, a decreased downward export scenario for future oceans.

However, these projections have been made based on the assumption that the contribution of small protists and cyanobacteria to export flux is negligible, or in the case of some models impossible. The recent findings that small phytoplankton can and do contribute to POC flux through packaging into aggregates and fecal pellets suggests that a shift to smaller cells may not necessarily lead to a decline in export flux in oligotrophic ocean regions. Figure 3 shows the new concept of the biological carbon pump, which includes sinking of both large and small sized plankton via aggregate and fecal pellet formation. Incorporating these new data into ecosystem models that consider the roles of functional groups (Hood et al., 2006; Le Quéré et al., 2005) will allow for more accurate predictions of future changes in plankton communities and their role in biogeochemical cycles.

Summary of findings

The primary goal of this dissertation is to investigate the contributions of specific taxonomic groups of phytoplankton to particle flux in the subtropical North Atlantic gyre using DNA-based molecular techniques. This dissertation is divided into four chapters, each following the format of a scientific journal article.

Chapter 1 describes a proof of concept study using samples collected in March 2005 at ESTOC. The aim was to determine whether the extraction of DNA and subsequent cloning and sequencing of the 18S rRNA gene could be applied to particle trap material as a means of identifying specific taxa and comparing water column plankton communities to trap material. Results revealed a surprisingly high diversity of cells contained in particle trap material, including sequences matching to taxa in the pico- and nano- size range. Additionally, a significant difference between taxa found in the water column and trap libraries was detected. Most notably, sequences closely matching to diatoms were common in the water column but were only found in very low numbers in the trap samples, while sequences matching to smaller sized taxa were found as a higher proportion of trap libraries. This study showed not only that DNA-based molecular techniques can be used to determine relative taxon-specific contributions to particle flux, but that these methods have the potential to reveal new information relating to the flux of particles in the ocean. This paper has been published in the December 2009 issue of Deep Sea Research Part I.

Chapter 2 investigates the potential strengths and weaknesses of DNA-based molecular methods. Here we examine the biases inherent in two techniques:

denaturing gradient gel electrophoresis (DGGE), a technique allowing for the simultaneous analysis of multiple samples on a single gel, providing a community 'fingerprint'; and clone libraries, which separate environmental sequences through the insertion of sequences into a plasmid and transformation into an *E. coli* cell. Cultured organisms were amplified singly and in combinations of predetermined cell numbers and biovolumes using "universal" eukaryotic primers for the 18S rRNA gene. We found that while each culture could be amplified by PCR and detected in DGGE and clone libraries when extracted alone, certain organisms, including *E. huxleyi* and other haptophytes, diatoms, and prasinophytes, were biased against when co-extracted with other organisms. These findings do not negate the usefulness of these methods in environmental studies, but serve as a guide to the interpretation of results and emphasize the importance of using a combination of molecular and DNA-independent methods for the accurate representation of marine community structure. Additionally, these methods, when used in comparative studies such as those that follow in the next chapters, will still allow for comparisons to be made between samples treated in the same way because the inherent bias will affect the samples equally. A shortened version (excluding the DGGE component) of this paper has been accepted by the Journal of Plankton Research.

Chapter 3 presents results of a two year time-series at the BATS station from May 2008-April 2010. This study investigates the plankton and cyanobacterial communities present in the upper water column and their contributions to downward particle flux. This is accomplished with DGGEs,

which allow us to not only obtain a fingerprint of the community, but to determine richness of phylotypes for each sample in the time series. Additionally, taxonomic information can be determined by excising and sequencing dominant bands. For the second half of the time series, actual DNA concentrations were quantified through the co-extraction and quantification by qPCR of an exogenous plasmid. This allowed us to determine the DNA concentration in the water column and for the first time to calculate the flux of DNA into the 150 m particle traps. Seasonal as well as an interesting interannual variation can be seen in the richness of phylotypes detected in both eukaryotic and cyanobacterial DGGEs. The first and second years of the time series were quite different with much deeper mixing in the winter of 2010 than in 2009. In the second year of sampling, the eukaryotic richness in the water column samples increased while cyanobacterial richness decreased. Sequencing of dominant bands revealed pico- and nanoplankton as well as cyanobacterial sequences, including *Synechococcus* and *Prochlorococcus*, in trap samples. These results show for the first time directly that these small organisms contribute to POC flux.

Chapter 4 expands upon the previous chapter with an in depth analysis of selected samples from the time series at BATS. Eukaryotic clone libraries of the 18S rRNA gene were constructed for samples from the 2009 and 2010 late winter bloom as well as sampling points during which interesting episodic events occurred. Samples for cloning and sequencing were chosen based on hydrography data, POC flux, and DGGE results. Results of this study show that small taxa, including members of the prasinophyte, prymnesiophyte, marine alveolate and

MAST groups, could be recovered from the 150 m particle trap roughly in proportion to their abundance in the water column, with prasinophyte sequences enriched in the traps in some months during the winter phytoplankton bloom. Sequences matching to dinoflagellates were the most abundant in water column and trap libraries and were also recovered in traps proportionally to their relative abundance in the water column. Diatoms were a minor contributor to trap libraries, reflecting a low abundance in the water column, but were present in the water column at the deep chlorophyll maximum in February 2009 and March 2010, and in the traps in December 2008, July 2009, and March 2010. Additionally, quantitative PCR using Mamiellales-specific primers was conducted for samples from the winter bloom periods. These results show for the first time quantitatively the relative contribution of these picoplanktonic organisms to particulate organic carbon flux.

In combination, these works show that DNA-based molecular methods, including DGGE, cloning and sequencing, and qPCR, allow for an analysis of the protist contribution to particle flux in a way that would not be possible before. These results clearly show that small pico- and nanoplankton are contributors to POC flux into the deep ocean.

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Table Captions.

Table 1. Selected studies supporting the sinking of small phytoplankton through aggregation and fecal pellet formation.

Figure Captions.

Figure 1. Consensus phylogenetic tree of major eukaryotic groups based on molecular and ultrastructural data. The two proposed roots for the tree are indicated by the black dashed lines. From Baldauf (2008).

Figure 2. Particle trap arrays used at ESTOC (Neuer et al., 2007, left) and BATS (http://bats.bios.edu/bats_methods.html, right).

Figure 3. The biological carbon pump. Modified from U.S. Joint Global Ocean Flux Study (JGOFS, http://www1.whoi.edu).

Tables.

Table 1.

Source	Method	Location	Findings
Lomas and Moran, 2011	Size-fractionated pumps, HPLC	BATS, Sargasso Sea	Nanoeukaryotes and cyanobacteria contribute to POC export
Legendre and Le Fèvre, 1995	Review		Small plankton cells are readily exported when incorporated into large particles, in fecal pellets, or by symbiosis with larger cells
Olli and Heiskanen, 1999	Inverted light, epifluorescence microscopy	Baltic Sea	Aggregates heavily colonized by picoeukaryotes and cyanobacteria, significant contribution of these taxa to sedimentation rates
Richardson and Jackson, 2007	Model		Contribution of picoplankton to export is proportional to their total net primary production
Waite et al., 2000	Epifluorescence, light microscopy	Southern Ocean	Significant sedimentation of picoplankton embedded in aggregates, found in heterotrophic flagellates, fecal pellets and organic matrices
Jackson, 2001	Model		Small plankton can be incorporated into large aggregates, causing them to sink faster and thus contribute to downward particle flux
Brew et al., 2009	Size-fractionated pumps, flow cytometry	BATS, Sargasso Sea	Particle flux increases as picoplankton abundance increases
Amacher et al., 2009	18S rRNA gene clone libraries	ESTOC, Eastern subtropical North Atlantic	Sequences corresponding to small organisms found in particle traps
Martin et al., 2006	18S rRNA gene DGGEs	Laboratory	DNA from krill gut and fecal pellets can be amplified and prey identified on DGGE, contents reflect food source
Nejstgaard et al., 2003	PCR with species-specific 18S rRNA gene primers	Laboratory	Copepods prey species was detected in extracts of whole animals and of their fecal pellets
Troedsson et al., 2009	Quantitative PCR of 18S rRNA gen	Laboratory	DNA can be amplified from gut contents and detected using qPCR
Pfannkuche and Lochte, 1993	HPLC, epifluorescence microscopy	Northeast Atlantic	Cyanobacteria found in fecal pellets and gut contents of salps, and in sediment trap material and detritus
Turley and Mackie, 1995	Epifluorescence microscopy	Northeast Atlantic	Heterotrophic bacteria and cyanobacteria transported to the deep sea attached to, or incorporated in rapidly sinking particles
Jansen and Bathmann, 2007	Epifluorescence microscopy	North Sea	Intact cells found in copepod fecal pellets
Wilson and Steinberg, 2010	Epifluorescence, light microscopy	Subtropical Pacific (Hawaii Ocean Time-series), subarctic Pacific	Zooplankton can ingest picoplankton and cyanobacteria when packaged into aggregates. Whole cells are visible in guts

Figures.

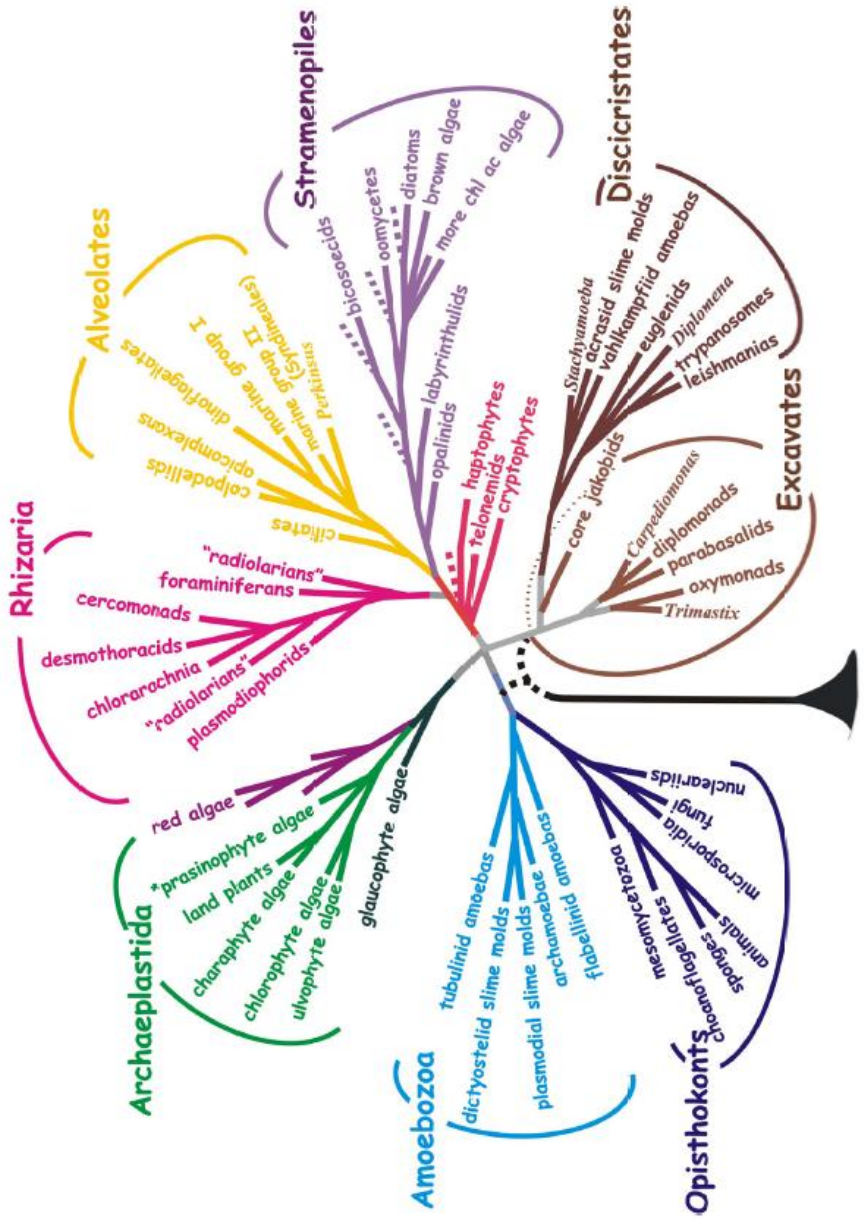

Figure 1.

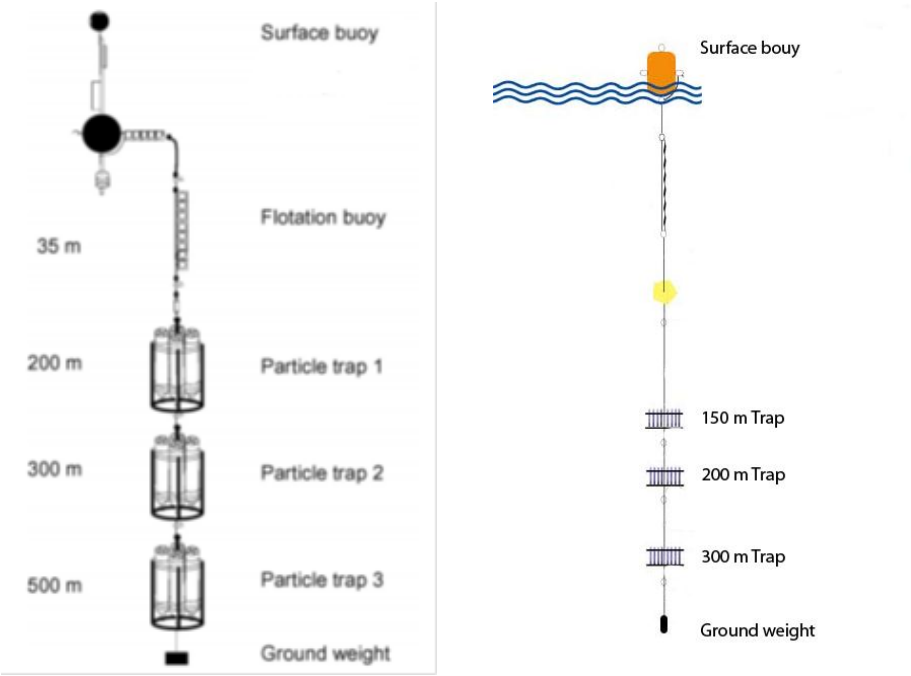

Figure 2.

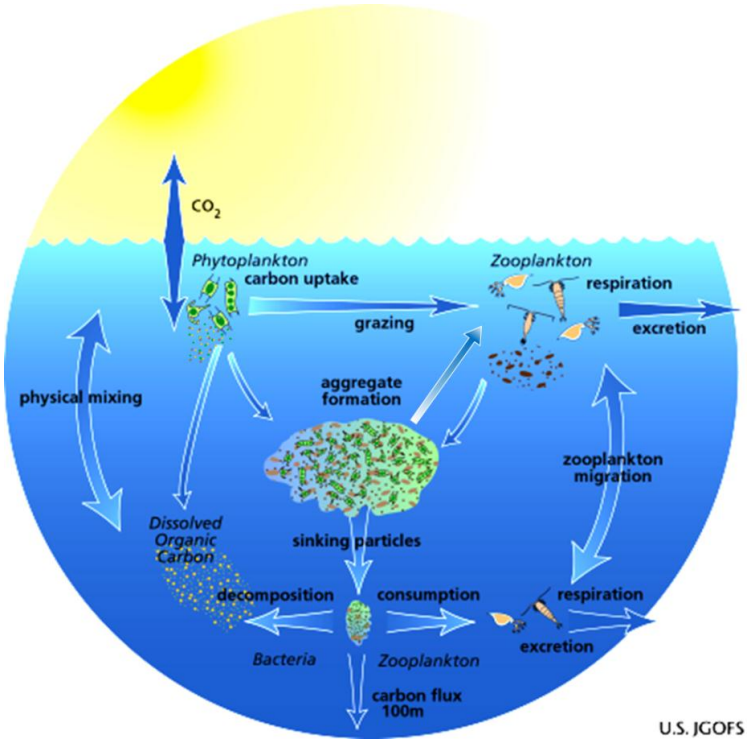

Figure 3.

CHAPTER 1

MOLECULAR APPROACH TO DETERMINE CONTRIBUTIONS OF THE PROTIST COMMUNITY TO PARTICLE FLUX

Jessica Amacher[1], Susanne Neuer[1*], Ramon Massana[2], Ian Anderson[1]

[1]Arizona State University, School of Life Sciences, Tempe, AZ 85287

[2]Institut de Ciències del Mar, CSIC, Passeig Marítim de la Barceloneta, 37-49. E-08003 Barcelona (Spain)

ABSTRACT

The importance of key taxonomic groups of eukaryotic phytoplankton as contributors to downward particle flux was evaluated near the European time-series station (ESTOC) in the eastern subtropical North Atlantic in March 2005. For the first time, molecular (cloning and sequencing) techniques were used to compare plankton communities from various depths in the euphotic zone with clone libraries from surface-tethered particle traps. Analyses of 18S rDNA clone libraries revealed compositional differences between the phytoplankton assemblages in the photic zone and those retrieved from shallow particle traps below, suggesting that not all phytoplankton contribute equally to particle flux. Contrary to expectations, our study also showed that it is not diatoms, despite their high abundance in the water column, but rather small phytoplankton taxa that dominated sequences recovered from trap material. We provide here first observational evidence that large taxa with mineral tests may not necessarily contribute more to export production than do smaller taxa even if the former are abundant in the water column.

1. Introduction

The ocean is a major regulator of climate and has been the only true net sink for anthropogenic CO_2 over the past 200 years (Sabine et al., 2004). Marine phytoplankton are responsible for a portion of this drawdown of carbon by means of the biological carbon pump: the photosynthetic fixation of CO_2 and subsequent sequestration of mainly particulate organic carbon into deep water (Falkowski et al., 1998). Although much of the fixed carbon presumably remains in the upper water column, where it is recycled within the microbial loop (Sherr and Sherr, 1994), particles sinking to depths of at least 500 m are effectively removed from exchange with the atmosphere for tens to hundreds of years (Ono et al., 2001).

Large, heavy phytoplankton with mineral tests, such as diatoms, have been thought to contribute more to carbon export than smaller organisms because of faster sinking rates (Boyd and Newton, 1999; Michaels and Silver, 1988; Sarthou et al., 2005). However, packaging of pico- (<2 µm) and nanoplankton (2-10 µm) may allow these small cells to sink faster than they would alone and thus may contribute as much to particle flux as larger organisms. In a recent modeling study, Richardson and Jackson (2007) suggested that these important primary producers may form fast-sinking aggregates or become incorporated into fecal pellets, allowing them to contribute to export production at a rate proportional to their primary production.

Traditionally, marine planktonic community composition has been determined using microscopic techniques, pigment analysis, and flow cytometry.

But these techniques are insufficient to distinguish between many small protist taxa, nor are they able to identify cells packaged into aggregates or contained in the fecal pellets, which is mainly the material collected by particle traps. Molecular techniques have recently become useful tools for community analysis and have shed new light on the diversity of the marine eukaryotic phytoplankton assemblages in the ocean (Countway et al., 2007; Díez et al., 2001; Moon-van der Staay et al., 2001; Not et al., 2007). However, these techniques have not previously been used to analyze particles collected by traps.

Here we apply cloning and sequencing of environmental eukaryotic (18S) ribosomal DNA from an oligotrophic subtropical gyre region near the European Station for Time-Series in the Ocean, Canary Islands (ESTOC). This technique allows us to "visualize" the compositional make-up of invisible components of the particulate material sinking out of the euphotic zone, enabling a comparison with the communities in the water column from which they are derived and an assessment of specific contributions to overall downward flux.

2. Methods

2.1. Field sampling

Surface-tethered particle traps were deployed and water-column samples collected in March 2005 near the European time-series station, ESTOC, located approximately 100 km north of the Canary Islands in the eastern subtropical North Atlantic (Fig. 1) on a cruise with FS Poseidon (POS 320). The design of

the particle traps was according to Neuer et al. (2007). Briefly, a surface buoy with an ARGOS transmitter, flash and a Radar reflector was connected to an array of three traps at 200, 300 and 500 m depth, each with four sampling cups. The main buoyancy was located at approximately 30 m to avoid the influence of wind on the traps. Each sampling cup was filled with sterile filtered seawater with added trace-metal free NaCl for a final salinity of 40. Three bottles contained buffered formaldehyde (final concentration 2%), while the fourth remained unfixed for use in molecular analysis.

Two series of traps were deployed for this study, one for 26 hours and another for 52 hours. We chose to construct clone libraries from the shallowest (200 m) and deepest (500 m) traps. However, the clone libraries from the set of traps deployed for 52 hours were uninformative, as the library at 200 m was dominated by copepod sequences (total of 66 metazoan, 4 alveolate and 2 diatom sequences) and at 500 m with radiolarian sequences (106 radiolarian, 2 diatom, 4 alveolate, and 1 each ciliate, cercozoan, crythecomonas and euglenozoa sequences), and thus are not shown here. We conclude that the deployment time was too long and the unfixed samples were compromised by too many zooplankton grazers that had entered the traps alive. Based on these observations, we recommend limiting the deployment time to 24 h when working with un-fixed traps, especially if molecular techniques are employed to characterize the composition of the community in the traps.

The first trap was deployed on 9 March 2005 (drifting from 29˚ 12.44' N, 015˚ 55.96' W to 29˚ 16.6' N, 015˚ 59.15' W). At deployment and recovery of the trap, using sampling bottles attached to a rosette, water samples were collected at five depths in the upper 200 m (approximately the base of the winter mixed layer) for measurement of chlorophyll concentration, microscopy and molecular analyses. Salinity, temperature and in vivo chlorophyll fluorescence were determined in the upper 500 m of the water column with a CTD (Conductivity, Temperature, Depth probe, Neil Brown General Oceanics Rosette with 21 10 L Niskin bottles) and a fluorometer. Because the main buoyancy of the traps was located at approximately 30 m depth, below the wind induced Ekman layer, the deployment and recovery stations should represent approximately the same water body and plankton communities and should thus be comparable.

2.2. Trap samples

Samples from the unfixed sampling cup for use in DNA extraction (one at each depth) were split into two 100-mL samples, filtered onboard onto two glass fiber (GF/F) filters and immediately added to a cryotube containing TESC buffer (100 mM Tris-HCl [pH 8], 100 mM EDTA, 1.5 M NaCl, 1% [wt/vol] hexadecylmethylammonium bromide, (Garcia-Pichel et al., 2001) stored in liquid nitrogen onboard and during transit, then at -80 °C at Arizona State University until extraction of the DNA. We chose to use unfixed material for molecular analysis to optimize extraction yield. While DNA can be extracted and amplified after formalin fixation, extraction yields are lower (results not shown) and PCR

(polymerase chain reaction) amplification may be reduced by as much as 79% compared to unfixed material (Fiallo et al., 1992).

To quantify flux of particulate matter into traps, total weight and particulate organic carbon (POC) and particulate organic nitrogen (PON) content were determined from two of the 2% buffered formalin fixed sampling cups after return to Arizona State University. First, zooplankton that had entered the traps alive (so-called swimmers) were carefully removed under a Nikon SMZ-U dissecting scope. The swimmer-free sample was then gently mixed, equal volumes were poured into separate bottles, and each sample was filtered onto a Poretics (0.2 μm pore size) polycarbonate filter. Particulates were rinsed with sterile Milli-Q (15-18 MΩ) water and washed from the filter into a pre-weighed ultra-clean silver capsule (Elemental Microanalysis Limited), dried and weighed to determine the dry weight of the sample. The material in the silver capsules was then acidified with 1-2 drops of concentrated hydrochloric acid to remove inorganic carbon, and POC and PON were quantified using elemental analysis (Costech Elemental Analyzer). Flux was recorded as weight of organic carbon per unit area (m^2) and time (day) using appropriate conversion factors (Neuer et al., 2007)

2.3. Water samples

Chlorophyll-a concentration was determined by filtering 200-250 mL of seawater in duplicate onto GF/F filters in the ship's laboratory. Filters were kept frozen and stored until extraction in 10 mL of 90% acetone for 24 hours at 4 ˚C.

The concentration of the extracted chlorophyll was determined by fluorescence on a TD-700 fluorometer (Turner Designs) as in Welschmeyer (1994).

Samples for epifluorescence microscopy were collected from the water column from the same samples used for molecular analysis (5 depths between 10 and 200 m) at the time of deployment of the traps and the time of their recovery. These samples were not collected from the traps because epifluorescence microscopy is difficult to use for counting cells contained in aggregates and fecal pellets, which make up most of the material collected in particle traps and only intact cells or mineral tests are identifiable. Samples for epifluorescence microscopy were fixed with gluteraldehyde for a few seconds (final concentration ca. 0.1 M), stained with DAPI (4',6-diamidino-2-phenylindole, a fluorescent stain that binds to DNA) (final concentration ca. 0.03 M), then filtered onto black Poretics (0.2 µm pore size) polycarbonate filters in the ship's laboratory. Filters were mounted on a microscope slide using immersion oil and frozen at -40 °C until microscopic analysis in the home laboratory. Cells were counted in size classes under blue (photosynthetic pigments) and UV (DAPI stained nucleic acids) excitation as in Neuer et al. (1994) using a Zeiss Axioscope. Abundance was calculated as cells mL^{-1} using appropriate conversion factors. The accuracy of the counts can be estimated using a Poisson distribution as in Lund et al. (1958). At least 30 cells were counted in either one or more stripes across the whole filter or in 5 fields (or partial fields) distributed across the filter. When 30 cells are counted, 95% confidence limits (CI) predict an actual range of cell numbers

between 21 and 43 cells (corresponding to ±30-43% of cells counted) assuming normal distribution across the filter. In this study up to 250 cells (e.g., *Synechoccous*, small phototrophs) were counted, which would give a 95% confidence range of 220-284 cells (corresponding to ±12-14% of cells counted). Note that this statistic reflects the counting error per se and the within sample variance (Lund 1958) but we have no repeat samples from the same Niskin bottle or from a replicate hydrocast to evaluate other sources of error.

Euphotic-zone samples for molecular analysis were prefiltered through a 100 μm mesh filter; 2 L of each water sample was filtered onto GF/F filters and preserved in the same manner as the trap samples (described above). Molecular analysis was conducted for euphotic zone samples from the trap deployment station only. We then compared the community at the deployment with that at the recovery station using epifluorescence microscopy (Fig. 2).

2.4. Molecular Analysis

Extractions were carried out using the Phenol:Chloroform:Isoamyl alcohol (PCI, Sigma) method as described in Countway et al. (2005) Frozen, preserved filters were thawed and alternately bead-beated and heated at 70 °C three times. NaCl and cetyltrimethyl ammonium bromide (CTAB) were then added to make a final concentration of 0.7 M NaCl and 0.01% CTAB. The mixture was then heated at 70 °C for an additional 10 minutes before addition of an equal volume of (25:24:1) PCI followed by vortexing and centrifuging. The supernatant was removed to a new tube and this procedure was repeated once with PCI and twice

with (24:1) Chloroform:isoamyl alcohol. Samples were then precipitated overnight at -20 °C in 95% ethanol with 0.1 x volume of 10.5 M ammonium acetate. The next day tubes were centrifuged before decanting liquid, rinsing with 70% ethanol and drying in an oven for 1-2 hours. Finally, 30 µL of water was added to each tube. DNA concentration was measured for each sample using a NanoDrop ND-1000 Spectrophotometer. Approximately 10-50 ng of DNA extract was used as template for PCR using primers EukA (AACCTGGTTGATCCTGCCAGT) and EukB (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al., 1988) as in Diez et al. (2001). These primers amplify nearly full length (approximately 1800 bp) 18S rDNA from most eukaryotes. Each reaction contained 5 µL of 10X Takara Ex Taq Buffer, 4 µL of dNTP's (200 µM each), 0.25 µL of each primer (0.3 µM), 39.75 µL of water, and 0.25 µL of Takara Ex Taq polymerase along with the template. The thermal PCR protocol consisted of an initial denaturation at 94 °C for 3 minutes; 30 cycles of 94 °C for 45 seconds, 55 °C for 1 minute, 72 °C for 3 minutes; then a final extension at 72 °C for 5 minutes. PCR products were then quantified on an agarose gel and purified using the QIAquick PCR purification kit (Qiagen).

Two libraries were constructed from the trap (200 and 500 m) and three from the water column (10, 55 and 150 m). The Topo TA cloning kit (Invitrogen) was used to construct clone libraries from PCR products. 138 to 190 clones from each library were reamplified by PCR to check for the successful uptake of the

inserted fragment. Positive clones were then purified and sequenced using the Euk528F (GCGGTAATTCCAGCTCCAA) primer (Elwood et al., 1985) with the Applied Biosystems 3730 sequencer. After removing metazoan sequences and unreadable sequences we were left with a range of 41 to 94 sequences per library (Table 1).

Sequences from each library were categorized into broad taxonomic "supergroups" (Adl et al., 2005) (Table 1) using their closest match on the NCBI (National Center for Biotechnology Information) database. Sequences from all libraries were entered into the DNA Baser program (version 2.11.0.933, http://www.dnabaser.com) and grouped in operational taxonomic units (OTU) using an identity threshold of 95%. For statistical analyses and determination of OTU, a range between 95% to 99% similarity is a standard used in most of the literature. Countway et al. (2005, 2007) uses 95%. Not et al. (2007) compared thresholds of 80% to 99% similarity, for most taxa there was little difference between 95% and 99% similarity. Since our study was intended to assess proportional relationships between taxa in the euphotic zone compared with the traps, we chose a threshold of 95%. The numbers of sequences in each OTU per library were then imported into PRIMER v.6 (Clarke and Gorley, 2006) for cluster analysis.

Relative abundances of each OTU were arranged in a matrix (5 libraries by 99 OTU's) and square root transformed to downweight the effect of abundant OTU's on similarities. Using PRIMER v.6, Bray-Curtis coefficients of

similarities (*S*), (Bray and Curtis, 1957) were calculated and analyzed using cluster analysis and non metric multidimensional scaling (MDS), a technique used to find similarities and dissimilarities between datasets. A WPGMA (weighted pair group method with averaging) diagram was constructed and a similarity profile (SIMPROF) test (Clarke and Gorley, 2006) run to establish significance of dendrogram nodes.

3. Results

3.1. Chlorophyll and temperature profiles

Samples were collected during the end of the winter bloom, and surface chlorophyll levels at the deployment station (1.28 mg m^{-3}, Fig. 2A) were on the high end of the range for this oligotrophic region (Neuer et al., 2007) and are somewhat higher than what is shown by MODIS satellite imagery (Fig. 1). Note, however, that this image is an average of the surface chlorophyll of the entire month of March 2005. The mixed layer depth was approximately 200 m, with the deep chlorophyll maximum (DCM, 1.54 mg m^{-3}) at around 150 m (Fig. 2A). The temperature through the mixed layer was relatively uniform at around 18 °C down to approximately 150 m. The recovery station (Fig. 2C) displayed a similar profile. However, chlorophyll-a levels had declined to 1.06 mg m^{-3} at the surface and to 1.04 mg m^{-3} at the DCM, and a second shallower chlorophyll peak (1.50 mg m^{-3}) was observed at 25 m.

3.2. Microscopy

At the deployment station (Fig. 2B) phototrophic flagellates (nearly all between 1-5 μm) were most abundant at 55 and 85 m (5.5 x 10^3 cells mL^{-1}, 5.6 x 10^3 cells mL^{-1}). A 10-m sample was not available for the deployment station. At the recovery station (Fig. 2D) phototrophic flagellates were much higher at 10 and 35 m (3 x 10^4 cells mL^{-1}, 2.6 x 10^4 cells mL^{-1}) and decreased from 35 m to 200 m. *Synecococcus* (ca 1.5 μm cell diameter) was also most abundant near the surface (deployment station 9.8 x 10^3 cells mL^{-1} at 55 m, recovery station 2.6 x 10^4 cells mL^{-1} at 10 m). Their numbers declined through the rest of the water column to 8.5 x 10^2 cells mL^{-1} at the 200-m deployment station and 3.9 x 10^2 cells mL^{-1} at the 200-m recovery station. *Prochlorococcus* may have been present also, but the small size of the cells and the rapid bleaching of their pigments made them difficult to accurately enumerate using epifluorescence. Flow cytometry, the standard way to enumerate *Prochlorococcus*, was not performed here. Diatoms at deployment had a large peak in abundance at 150 m, the DCM (5.5 x 10^2 cells mL^{-1}) (97% of cells >20 μm). The recovery station shows a similar pattern but with a peak at the DCM less than half of that at the deployment station (2.5 x 10^2 cells mL^{-1}) (67% of cells >20 μm). The diatom *Asterionellopsis sp.* (approximately 8 μm x 25-30 μm) dominated the community at the DCM at the deployment station with 4.2 x 10^2 cells mL^{-1} (95% CI of 3.6 x 10^2 – 4.8 10^2 cells mL^{-1}) compared to 2.6 x 10^2 cells mL^{-1} (95% CI of 2.2 x 10^2 – 3.1 x 10^2 cells mL^{-1}) at the recovery station. Many sequences from this genus were found in the clone library at the DCM (see Supplemental Table,

http://www.sciencedirect.com/science/article/pii/S0967063709001721).

Phototrophic dinoflagellates (88% of which were <10 μm) were most abundant at 55 m at deployment (1.2 x 10^2 cells mL^{-1}) and at 35 m at recovery (2.0 x 10^2 cells mL^{-1}). Heterotrophic dinoflagellates (85% smaller than 5 μm) had the highest abundance at 85 m at the deployment station (1.9 x 10^2 cells mL^{-1}) and at 10 m at the recovery station (1.3 x 10^3 cells mL^{-1}). Heterotrophic protists other than dinoflagellates (nearly all between 2-6 μm) were most abundant at deployment at 85 m (2.2 x 10^3 cells mL^{-1}) and at recovery declined from 10 m (1.9 x 10^3 cells mL^{-1}) down to 200 m (2.1 x 10^2 cells mL^{-1}).

3.3. Flux

Fluxes of particulate organic carbon (POC) were 14.7 (± 3.4) mg m^{-2} day^{-1} at 200 m, increasing to 21.4 (± 2.2) mg m^{-2} day^{-1} at 300 m and then decreasing to 3.3 (± 1.5) mg m^{-2} day^{-1} at 500 m. Fluxes of particulate organic nitrogen (PON) were 2.1 (± 0.04) mg m^{-2} day^{-1} at 200 m, increasing slightly to 3.8 (± 0.9) mg m^{-2} day^{-1} at 300 m and then dropping to 0.5 (± 0.3) mg m^{-2} day^{-1} at 500 m. These values are 2-3 times higher than those recorded previously in late winter and early spring; POC flux determined in March 1998 was between 2.5 and 6.5 mg m^{-2} day^{-1} and PON flux was between 0.3 and 0.9 mg m^{-2} day^{-1} in the upper 500 m (Neuer et al., 2007). These unusually high fluxes fit with the unusually high chlorophyll-a levels encountered during this cruise.

3.4. Clone Libraries

In this work we present the results of 5 clone libraries of community 18S rDNA genes, 3 of the water column at the deployment station (10, 50 and 150 m) and 2 from the unfixed trap material (200 and 500 m). The majority of sequences from all five clone libraries fell into the Stramenopile (diatoms, marine stramenopiles (MAST)) or Alveolata (dinoflagellates, ciliates, novel alveolate groups I and II) first rank super groups (Adl et al., 2005) (see Supplemental Table http://www.sciencedirect.com/science/article/pii/S0967063709001721). There is an obvious difference between OTU's found in the euphotic zone and those found in trap material (Fig. 3, Table 1). Stramenopile OTU's made up only 10.7% of trap libraries as compared to 35.3% in euphotic zone libraries while Alveolata OTU's were roughly equal in the water column and the trap libraries (Table 1).

At the phylotype/clade-specific level ("Phylogenetic group" in Supplemental Table http://www.sciencedirect.com/science/article/pii/S0967063709001721) we observed that upper water column communities were strongly dominated by sequences corresponding to diatoms, dinoflagellates and marine alveolates, with marine stramenopiles, ciliates, radiolaria, prasinophytes and cryptophytes making up the rest (Fig. 3). At the 150 m DCM, diatoms accounted for nearly three quarters of the analyzed clones (Fig. 3C). This finding was confirmed by epifluorescence microscopy (Fig. 2B). *Asterionellopsis sp.* (cell length ca. 30-40 µm) was the largest contributor to diatom sequences at 150 m but was not a large component in the other samples. Other significant contributors to the three

libraries included sequences traceable to diatom species *Thalassiosira delicatula* and *Minidiscus trioculatus* (see Supplemental Table http://www.sciencedirect.com/science/article/pii/S0967063709001721).

In the 200-m trap (Fig. 3D) about half of the clones could be assigned to radiolarians, and nearly one third to alveolates (including a large percentage of dinoflagellates). Sequences from cryptophytes, stramenopiles, euglenozoa and prasinophytes made up the rest of the library. At 500 m, alveolate sequences accounted for just over half of the clones, but there was a greater diversity overall than in surface samples. Radiolarians were also important in this library. Copepod sequences were significant contributors to several of the libraries. However, metazoan sequences have been left out of this analysis as we focus specifically on the protistan community.

The relative distribution of taxa present in the water column and in the trap material suggests that not every taxonomic group contributes equally to particle flux. The results of the multivariate cluster analysis of Bray-Curtis coefficients indicates higher similarity among the three water-column libraries and among the two trap libraries than between water column and trap libraries. The WPGMA diagram (Fig. 4) shows that the three water-column libraries were determined by the SIMPROF test to be significantly different from the two trap libraries ($p<0.05$).

4. Discussion

Our results have shown clone libraries to be successful in providing information about the protist communities not only from the water column but also from trap material. Although we cannot directly infer the amount of carbon flux contributed by each group, this technique has enabled us to investigate the *relative* contributions of various protist groups to the downward flux of particulate matter.

There may be some degradation of DNA by the time the particles are collected by the traps. But intact and even culturable cells as well as pigments have been found in aggregates and salp feces collected in particle traps and sediments (Fischer et al., 1996; Pfannkuche and Lochte, 1993; Turley and Mackie, 1995) and in fecal pellets (Jansen and Bathmann, 2007; Martin et al., 2006). Furthermore, cells need not be living or even intact in order to obtain DNA sequences that can be used for molecular analysis. 18S rDNA has been used in various studies of copepod diets (Martin et al., 2006; Nejstgaard et al., 2003; Troedsson et al., 2009). These latter studies have shown that digestion and degradation of sinking particles may only be partial and that DNA is a chemically stable molecule and should be more resistant to breakdown than other tracer compounds, such as pigments and proteins (Nejstgaard et al., 2008). Furthermore, in a study by Martin et al. (2006), prey species found in fecal pellets reflected the actual diet, suggesting no differential degradation of DNA. Cells need not be recognizable for DNA analysis, making this technique particularly

useful for particle trap collected material, for which identification by visual inspection is inherently difficult.

A comparison of the clone libraries revealed a clear difference between sequences found in the euphotic zone and those found in trap material (Fig. 3, 4 and Table 1). Interestingly, diatoms contributed up to half of all sequences from the euphotic zone, while only a small percentage of all sequences from trap material originated from diatoms. Conversely, marine alveolate sequences, for example, were found in roughly the same proportions in the euphotic zone as in the trap libraries. The significant difference between the trap and euphotic zone libraries detected by the SIMPROF test confirms the difference between the water-column community and organisms contained in particles collected in the traps. These results may suggest the presence of a "compositional filter" acting on these communities during the process of sinking. This study was completed using data from a single 24 hour trap and corresponding euphotic zone samples and represents only a snapshot in time. We are currently involved in a more intensive study at the Bermuda Time Series Station which will allow us to investigate seasonal changes.

Our results are particularly surprising with respect to the diatoms, thought to dominate particle flux (Sarthou et al., 2005). Diatom sequences made up at least a third of all sequences in each of the libraries from the euphotic zone but only a small proportion of the libraries of either trap sample, and only one sequence belonging to *Asterionellopsis sp.*, the most abundant diatom found in the

water column, was found in the trap samples (see Supplemental Table http://www.sciencedirect.com/science/article/pii/S0967063709001721). Diatoms do not normally make up a significant portion of communities in the oligotrophic North Atlantic gyre (Steinberg et al., 2001). The area surrounding ESTOC is characterized by low phytoplankton biomass, typical of an oligotrophic gyre. These samples however, were taken in mid-March, near the end of the winter bloom, when productivity is highest (Neuer et al., 2007). During this time period, one would expect to have the highest contribution to particle flux coming from these abundant diatoms, yet our data show the opposite trend (Fig. 3). It is possible that the diatoms had not yet begun to sink; however, our microscopy data indicate cell numbers of diatoms had declined slightly between the deployment and the recovery stations. Benitez-Nelson et al. (2007) found that a diatom bloom in a cold-core cyclonic eddy off Hawaii did not result in a large export flux, as would be expected, and that 90% of diatom frustules above the DCM observed by epifluorescence microscopy were empty of organic material. Thus another explanation for the missing diatoms is shallow remineralization of their organic matter. However, visual inspection of our trap material did not show any diatom frustules, which should have arrived in the traps in this case. Thus, several reasons such as shallow respiration/dissolution, advection and an absence of sinking cells could be the reason for the missing diatoms in our trap material.

In contrast, small taxa without mineral tests, such as prasinophytes and uncalcified prymnesiophytes, whose sequences were found at 200 and 500 m,

were found in the sedimenting material (Fig. 3). Particularly interesting is the finding in the trap samples of sequences belonging to the prasinophyte genera *Micromonas*, *Ostreococcus* and *Bathycoccus* (see Supplemental Table http://www.sciencedirect.com/science/article/pii/S0967063709001721), small phytoplanktonic cells (1-2 µm in diameter) that are known to be ubiquitous in temperate and low latitude waters (Vaulot et al., 2008). Of these pico-eukaryotes, only *Ostreococcus* was found in the water column (55m, see Supplemental Table http://www.sciencedirect.com/science/article/pii/S0967063709001721) but all are photosynthetic and must have been present in the euphotic zone. It is possible that the picoeukaryotes were diluted out by clones of the other larger taxa in the euphotic zone and were therefore not found in the clone libraries. It is even more remarkable then that they could be detected in the trap libraries. Numerous past studies have reported that small cells can be incorporated into large aggregates or rapidly-sinking fecal pellets of grazers (Legendre and Le Fèvre, 1995; Olli and Heiskanen, 1999; Richardson and Jackson, 2007), allowing them to sink through the water column at a rate faster than their cell size would allow (Jackson, 2001) .

In related studies of plankton communities in the western North Atlantic subtropical gyre, dinoflagellates and other alveolate groups dominate clone libraries while diatoms are not often found (Countway et al., 2007; Not et al., 2007). This also occurs in other marine systems, such as the Mediterranean Sea (Massana et al., 2004a) and the Indian Ocean (Not et al., 2008). However, samples were not collected during bloom situations, and with the exception of the

Countway et al. (2007) study, were prefiltered through a 3 µm mesh, which would exclude most diatoms. Additionally, each of these studies reports a very large diversity of eukaryotes at every location and depth for which molecular studies have been employed, similar to what has been found here. Similarities and differences between the Countway et al. (2007), Not et al. (2007), and the present study are outlined in Table 2. Neither of the latter studies, however, provided time-series data from any location, in part because of the time-intensive nature of clone library analysis. Note that the number of sequences cloned in each of these libraries differ (Table 2), and a larger clone library has a greater chance to capture rare phylotypes. Abrantes et al. (2002) investigated diatom abundance from water column and traps using light microscopy around ESTOC and found that diatom abundance can more than double in February or March compared to any other month, coinciding with the winter bloom in that area of the subtropical gyre. In both Abrantes et al. (2002) and in our samples, the genera *Chaetoceros, Eucampia, Pleurosigma, Pseudo-nitzschia, Stephanodiscus,* and *Thalassiosira* were found. However, *Pleurosigma, Stephanodiscus,* and *Thalassiosira* were found only in the trap material in the Abrantes et al. (2002) study, but were found in the euphotic zone in this work. *Asterionellopsis sp.,* abundant in the euphotic zone in our study was found by Abrantes et al. (2002) off Cape Ghir.

The proportion of sequences of a particular phylotype in each clone library, when compared across depths, generally follows the same pattern as observed by epifluorescence microscopy. These similarities strengthen our

confidence in the use of molecular data to decipher differences between samples from water column and traps. For example, diatom sequences made up about a third of the 10-m clone library, roughly a quarter of the 55-m and more than half of the 150-m library (Fig. 3A-C). Epifluorescence counts of diatoms at the deployment station show a large increase in diatom abundance from 55 to 150 m (Fig. 2B). *Asterionellopsis sp.* was very abundant at deployment (150 m) both in the clone libraries and in the epifluorescence counts. Using microscopy, cells that could be identified only as "heterotrophic protists" may have been the ubiquitous Marine Stramenopile (MAST) cells (Massana et al., 2004b) or the marine alveolates found in the clone libraries. These groups make up about the same number of sequences in the 55 and 150 m clone libraries and have similar abundance in the epifluorescence samples at those depths. "Phototrophic flagellates" may have included the prasinophyte, chrysophyte, and prymnesiophyte sequences. Phototroph sequence numbers in the clone libraries remain similar from 10 m to 150 m and epifluorescence counts at the deployment station do not decrease much from 55 to 150 m. Epifluorescence counts showed cyanobacteria, mainly *Synecococcus*, to be the numerically most abundant at both stations throughout the water column. In the Sargasso Sea, *Prochlorococcus* and *Synechococcus* represent more than 85% of the total number of picoplankton enumerated (DuRand et al., 2001). Our clone libraries were constructed using eukaryotic primers; cyanobacteria such as *Synechococcus* and *Prochlorococcus* would not be captured. When comparing molecular data to epifluorescence

counts, we do not expect the results to match exactly, but rather to complement each other. The combination of these methods should provide a comprehensive representation of the protist community in this environment.

This is the first study to examine the relative contribution of different taxa to the particles collected in traps using DNA based molecular techniques. Clone libraries have allowed us to investigate the eukaryotic protist community composition in the water column at a greater resolution than is possible using traditional methods. For the trap material, this is, to date, the only taxon-specific method that can be used to identify protists that are otherwise indistinguishable in detritus because of lack of mineral tests. Time-series data using molecular techniques may shed light on the temporal changes of the phytoplankton community and their contribution to particle flux. These data and future studies may help understand regional and seasonal differences in the contribution of protist to particle flux and can improve "green" ecosystem models that incorporate information about functional groups (Hood et al., 2006; Le Quéré et al., 2005) to better predict changes in plankton communities and their role in biogeochemical cycles.

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Table Captions.

Table 1. Number of OTU's and total sequenced clones (in brackets) based on 95% similarity in each first rank Super-group category (Adl et al., 2005) for each depth in the water column and trap clone libraries. Stramenop. = Stramenopiles; Radiolar. = Radiolaria; Cryptoph. = Cryptophyta; Chloroplas. = Chloroplastida; Eugleno. = Euglenozoa; Opisthok. = Opisthokonta.

Table 2. Comparison of this study with clone libraries constructed from water column samples in the Sargasso Sea. Numbers of clones in each library in brackets.

Figure Captions.

Fig. 1. Aqua MODIS (Moderate Resolution Imaging Spectroradiometer aboard the Aqua satellite) false color satellite image (monthly average for March 2005, 4km data) of the northern Canary Islands region, located in the eastern subtropical Atlantic off northwest Africa, showing the location of ESTOC and particle trap (insert). Because of the resolution of the image the deployment and recovery stations are shown as a single point on the map. Colors indicate chlorophyll concentration (sidebar).

Fig. 2. Vertical profiles of temperature, chlorophyll and cell abundance. Temperature, chlorophyll fluorescence and extracted chlorophyll for the (A) deployment and (C) recovery stations. Cell abundance as a function of depth for the deployment station (B) and recovery station (D) as determined by epifluorescence microscopy cell counts. (note logarithmic scale).

Fig. 3. Percent composition of 18S rDNA clone libraries for (A-C) water column and (D, E) trap libraries. Numbers next to group names indicate numbers of cloned sequences belonging to each group. Metazoan sequences have been left out of the dataset in order to compare only protist taxa.

Fig. 4. Comparison of trap and water column library compositions. WPGMA cluster diagram of Bray-Curtis similarities calculated from square root transformed relative OTU abundances from water column and trap libraries. The asterisk indicates significant differences ($P < 0.05$) between the nodes as determined by the SIMPROF test.

Tables.

Table 1.

Sample	Stramenop.	Haptophyta	Radiolar.	Alveolata	Cryptoph.	Cercozoa	Chloroplas.	Eugleno.	Opisthok.	Total
10 m	7 [22]	0	0	12 [24]	1 [1]	0	0	0	0	20 [47]
55 m	9 [16]	0	1 [4]	17 [20]	0	0	1 [1]	0	0	28 [41]
150 m	8 [41]	0	0	11 [16]	0	1 [1]	0	0	0	20 [58]
total	24 [79]	0	1 [4]	40 [60]	1 [1]	1 [1]	1 [1]	0	0	68 [146]
%	35.3 [54.1]	0	1.5 [2.7]	58.8 [41.1]	1.5 [0.7]	1.5 [0.7]	1.5 [0.7]	0	0	100
200 m Trap	3 [3]	0	3 [51]	15 [34]	2 [4]	0	1 [1]	1 [1]	0	25 [94]
500 m Trap	3 [4]	1 [1]	6 [15]	16 [22]	0	0	2 [4]	2 [4]	1 [1]	31 [51]
total	6 [7]	1 [1]	9 [66]	31 [56]	2 [4]	0	3 [5]	3 [5]	1 [1]	56 [145]
%	10.7 [4.8]	1.8 [0.7]	16.1 [45.5]	55.4 [38.6]	3.6 [2.8]	0	5.4 [3.4]	5.4 [3.4]	1.8 [0.7]	100

Table 2.

Organism	Not et al. 2007 [225]	Countway et al. 2007 [918]	This Study Water Column [146]	This Study Traps [145]
Stramenopiles	17	42	79	7
Haptophyta	5	6		1
Radiolaria	46	259	4	66
Alveolata	153	514	60	56
Cryptophyta	1	4	1	4
Cercozoa	1	1	1	
Chloroplastida		7	1	5
Euglenozoa		72		5
Choanomonada	1	1		1
Picobiliphytes	1			
Rhodophyta		3		
Telonema		9		

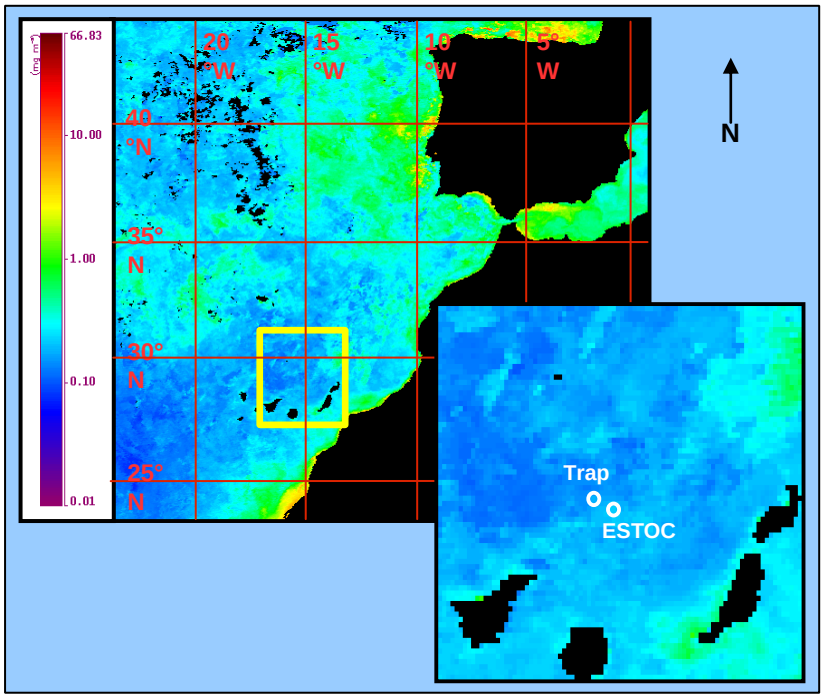
66.83
10.00
1.00
0.10
0.01
20° W
15° W
10° W
5° W
40° N
35° N
30° N
25° N
N
Trap
ESTOC

Fig. 1.

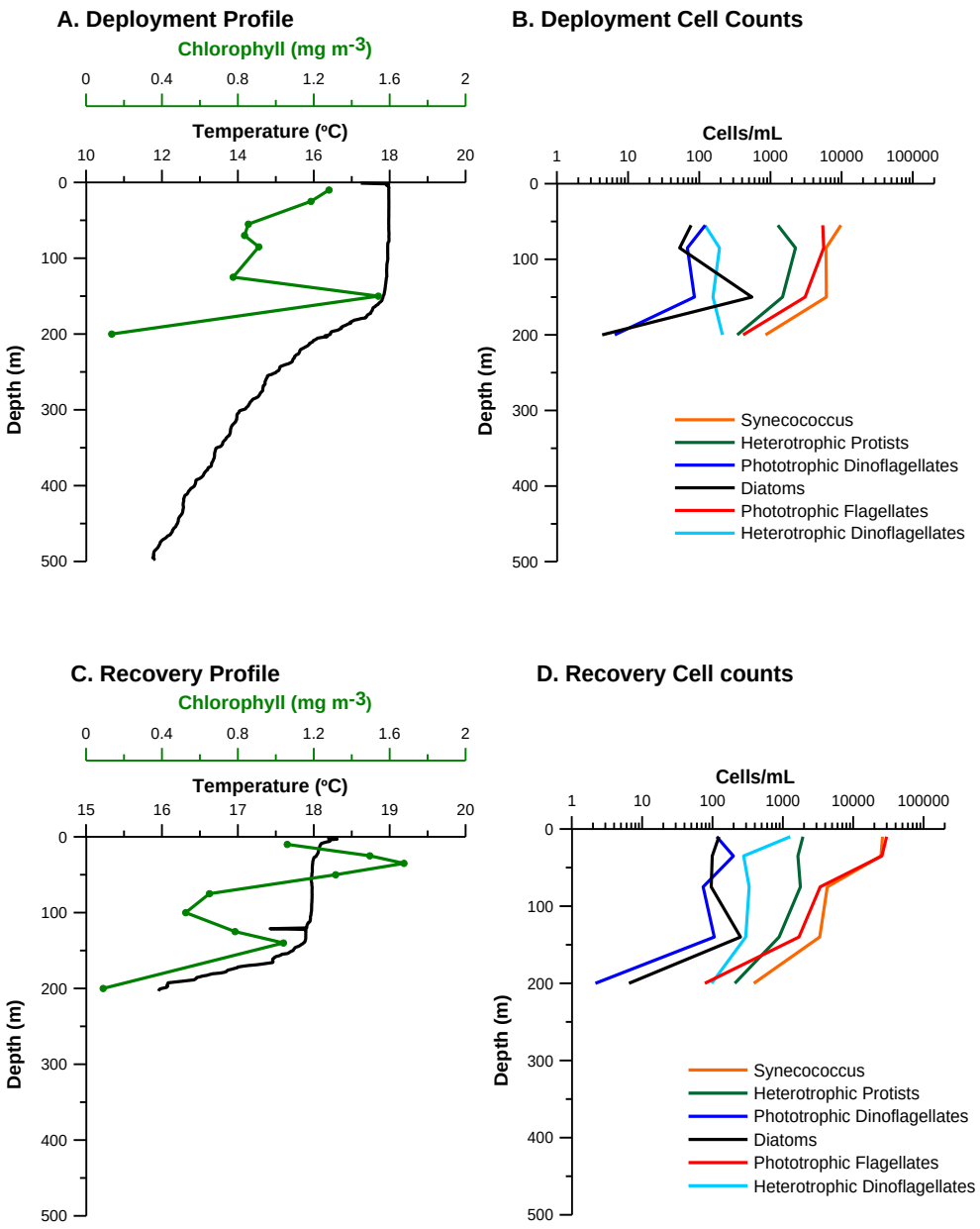
A. Deployment Profile
Chlorophyll (mg m-3)
0
0.4
0.8
1.2
1.6
2
Temperature (ºC)
10
12
14
16
18
20
Depth (m)
0
100
200
300
400
500
B. Deployment Cell Counts
Cells/mL
1
10
100
1000
10000
100000
Synecococcus
Heterotrophic Protists
Phototrophic Dinoflagellates
Diatoms
Phototrophic Flagellates
Heterotrophic Dinoflagellates
C. Recovery Profile
Chlorophyll (mg m-3)
Temperature (ºC)
15
16
17
18
19
20
D. Recovery Cell counts

Fig. 2.

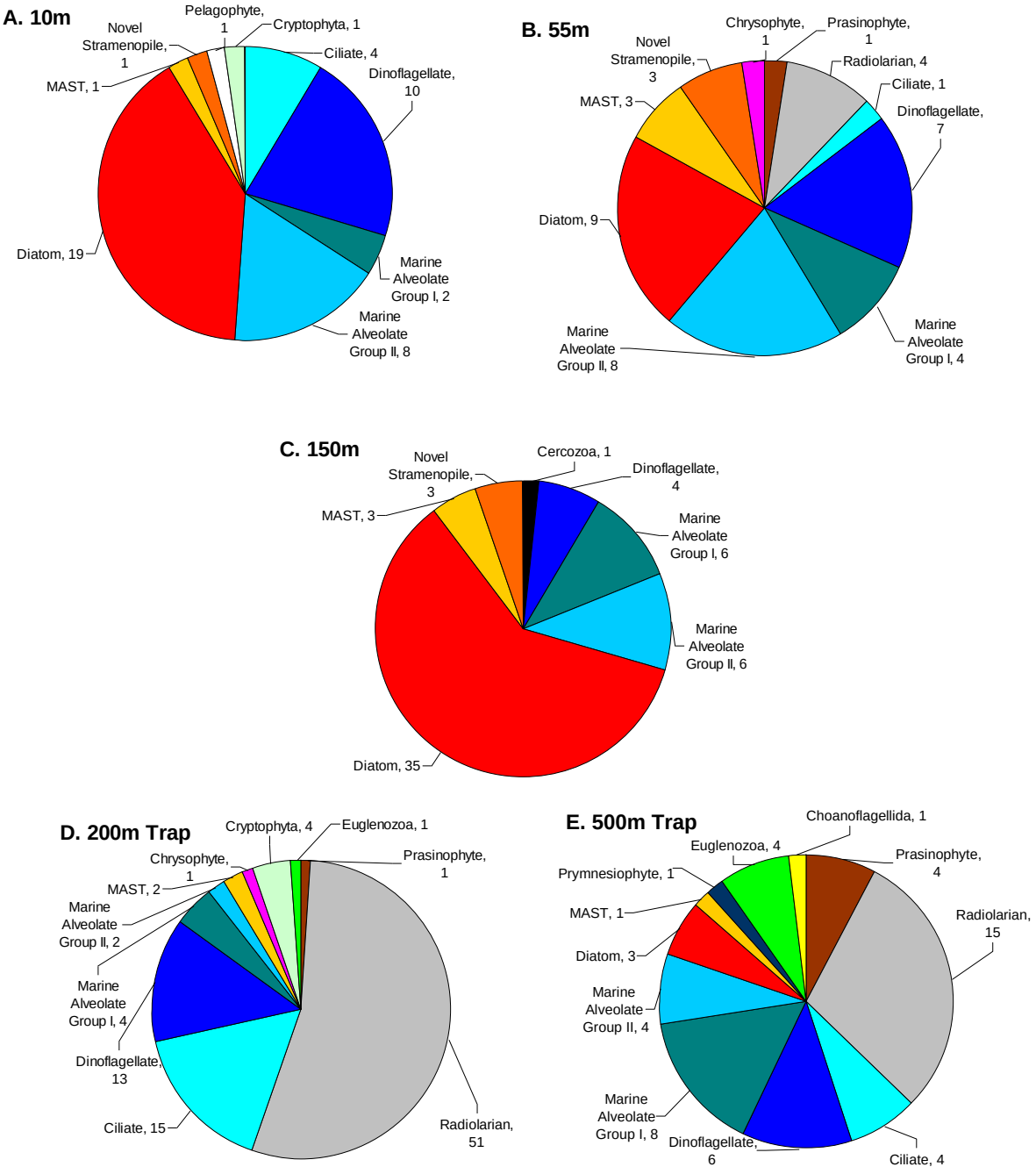
A. 10m
Pelagophyte, 1
Cryptophyta, 1
Novel Stramenopile, 1
Ciliate, 4
MAST, 1
Dinoflagellate, 10
Diatom, 19
Marine Alveolate Group I, 2
Marine Alveolate Group II, 8
B. 55m
Chrysophyte, 1
Prasinophyte, 1
Novel Stramenopile, 3
Radiolarian, 4
Ciliate, 1
MAST, 3
Dinoflagellate, 7
Diatom, 9
Marine Alveolate Group II, 8
Marine Alveolate Group I, 4
C. 150m
Cercozoa, 1
Novel Stramenopile, 3
Dinoflagellate, 4
MAST, 3
Marine Alveolate Group I, 6
Marine Alveolate Group II, 6
Diatom, 35
D. 200m Trap
Cryptophyta, 4
Euglenozoa, 1
Chrysophyte, 1
Prasinophyte, 1
MAST, 2
Marine Alveolate Group II, 2
Marine Alveolate Group I, 4
Dinoflagellate, 13
Ciliate, 15
Radiolarian, 51
E. 500m Trap
Choanoflagellida, 1
Euglenozoa, 4
Prasinophyte, 4
Prymnesiophyte, 1
Radiolarian, 15
MAST, 1
Diatom, 3
Marine Alveolate Group II, 4
Marine Alveolate Group I, 8
Dinoflagellate, 6
Ciliate, 4

Fig. 3.

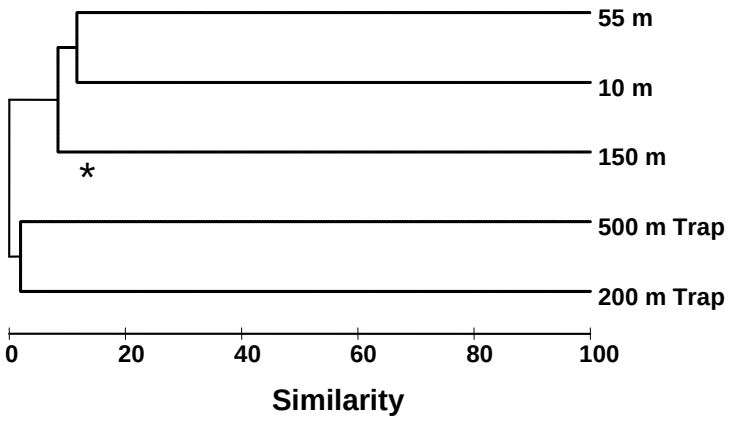

Fig. 4.

CHAPTER 2

BIASES ASSOCIATED WITH DNA-BASED MOLECULAR STUDIES OF MARINE PROTIST DIVERSITY

Jessica Amacher, Charles Baysinger, Susanne Neuer*.

Arizona State University, School of Life Sciences, Tempe, AZ 85287

Abstract

DNA-based molecular methods have been increasingly employed in surveys of marine protist communities. The most commonly applied methods include denaturing gradient gel electrophoresis (DGGE) and clone libraries of 18S rRNA amplicons. These methods have led to discoveries about diversity and community structure of marine eukaryotes that would not have been possible using traditional methods. Despite their benefits over culture-dependent or microscopy-based surveys, these methods are known to have underlying biases. In this study, we investigated the effects of varying population density and of co-occurring organisms on the extent of these biases. To do this, we carried out laboratory experiments of known concentrations of prymnesiophytes, diatoms, dinoflagellates, chlorophytes, and prasinophytes. Among the prymnesiophytes we focused on *Emiliania huxleyi* as a model organism. *E. huxleyi* is a common marine haptophyte of considerable importance among marine primary producers, but is underrepresented in genetic libraries. We found that, while it is possible to amplify and detect *E. huxleyi* using DGGE and clone libraries of 18S rRNA genes, they could not be recovered in clone libraries in a mixture of organisms at the abundance levels of oligotrophic gyre regions (ca. 50 cells mL^{-1}). At temperate North Atlantic bloom concentrations (ca. 4.5×10^3 cells mL^{-1}) *E. huxleyi* could be amplified and detected in clone libraries. In addition, we could identify bias associated with organisms present in the mixture, most clearly seen

when the dinoflagellate *Scrippsiella sp.* was present. These findings highlight the importance of using a variety of molecular and traditional methods to accurately determine community composition and to acknowledge limitations in the interpretation of these methods.

1. Introduction

Culture-independent DNA-based molecular methods have been widely applied to the analysis of eukaryotic microbial community structure in the ocean over the past decade (Countway et al., 2007; Díez et al., 2001a; 2001b; Moon-van der Staay et al., 2001; Not et al., 2007). Environmental surveys of the phytoplankton community can be carried out by amplifying the small subunit ribosomal RNA genes by polymerase chain reaction (PCR) using "universal" primers designed to amplify all cyanobacteria (16S rRNA) or all eukaryotes (18S rRNA). DNA extracted from environmental samples is PCR amplified and then individual 18S rRNA sequences can be separated using clone libraries (Díez et al., 2001a) or denaturing gradient gel electrophoresis (DGGE) (Díez et al., 2001b) and rRNA gene sequences identified. These methods eliminate the problems associated with other techniques aimed at elucidating taxonomic diversity. For example, culturing techniques favor "weed" species that are able to grow well under specific laboratory conditions, cell abundance measurements obtained with microscopy and flow cytometry are usually not able to distinguish between small taxa or those with similar shape and pigmentation, and pigment-based analyses are limited to autotrophic organisms and have considerable taxonomic ambiguity. Molecular environmental surveys have revealed unexpectedly high diversity, several novel lineages of protists, and new insights into marine protist ecology and distribution especially of the pico- and nanoplankton (Caron et al., 2009;

2004) that would not have been possible using traditional methods such as microscopy, flow cytometry, and HPLC.

While clone libraries and DGGE have been invaluable in elucidating the vast diversity of marine protists, inherent biases are known to exist in these methods. Possible biases have been described that are associated with extraction of DNA or PCR amplification steps (Hong et al., 2009; Sánchez et al., 2009; Wintzingerode et al., 1997), primer mismatches (Liu et al., 2009), or with cloning and DGGE (Sekiguchi et al., 2001). Problems associated with PCR bias include inhibition due to higher GC content, and mismatches between the "universal" primers and the 18S rRNA sequences of the diverse communities of eukaryotes in environmental communities. Darling et al. (1996) describes the reduced amplification of foraminifera by PCR due to their unusually long 18S sequences. Additionally, some larger organisms, such as diatoms and dinoflagellates, contain higher copy numbers of 18S rRNA genes than other, typically smaller, organisms like prasinophytes and haptophytes (Not et al., 2009; Zhu et al., 2005). Additionally, comparatively lower cell concentrations may be responsible for the absence of certain taxa in environmental 16S rRNA clone libraries (Chandler et al., 1997). While a single organism amplified on its own may be amplified by universal primers, in a mixture of organisms, as in environmental samples, primer competition may result in preferential amplification and overrepresentation of certain taxa (Farris and Olson, 2007; Potvin and Lovejoy, 2009).

One taxonomic group that is known to be biased against is the haptophytes (or prymnesiophytes) (Liu et al., 2009). This taxonomic group is a part of the Chromalveolata supergroup (Adl et al., 2005) and includes coccolithophorids, calcareous plate-forming phytoplankton found throughout the world's oceans (DuRand et al., 2001; Haidar and Thierstein, 2001; Lomas and Bates, 2004). *Emiliania huxleyi* is the most commonly found species of coccolithophorid in open ocean environments (DuRand et al., 2001; Haidar and Thierstein, 2001; Sprengel et al., 2002). Previous studies have shown that, despite their high abundance as measured by pigment analysis, haptophyte sequences were not found to be abundant using clone library sequence data (Liu et al., 2009; Moon-van der Staay et al., 2001; Not et al., 2008). But haptophytes are not the only taxonomic group which have an associated bias in clone library studies. A study by Potvin and Lovejoy (2009) using artificial and environmental combinations of arctic communities showed that certain taxa (including a haptophyte, a prasinophyte, and certain diatom species) may be biased against in clone libraries. In another study using 18S rRNA clone libraries, Not et al. (2009) found that some groups seem to be biased against, including haptophytes, marine stramenopiles (MAST), and picobiliphytes, and some are biased for, including cryptophytes, radiolaria, and dinoflagellates. The disconnect between the abundance of some taxonomic groups in the oceans and lack of sequences found using molecular methods is a potential problem in any study using molecular

techniques to survey marine eukaryotic microbial community structure, and this problem deserves further investigation.

We aim in this study to not only quantify and compare the bias in DGGE and clone libraries, but also to test the effect of varying cell concentrations, biovolume and co-occurring organisms on the apparent bias using monoalgal and mixed cultures in the laboratory. We have focused in this study on simulating the composition of field populations of the North Atlantic oligotrophic gyre in the region of the Bermuda Atlantic Time-series Study (BATS), as this location is considered a typical oligotrophic open ocean site, and the plankton communities at that station are well known and have been studied over several decades. Our experiments extend upon a recent study by Potvin and Lovejoy (2009) with an analysis of the effect of bias in DGGE in addition to clone libraries. Our experiments are the first to elucidate the compound effect of both low natural abundances of certain groups in the field and bias exerted by other organism groups present in a population, and should aid in the interpretation of molecular surveys of community composition in the field.

2. Methods

2.1. Cultures

Organisms were selected from several of the major taxonomic groups of marine eukaryotic phytoplankton in order to simulate the composition of a natural

open ocean photosynthetic community. Included in this study are the following monoalgal cultures from the Provasoli-Gulliard National Center for Culture of Marine Phytoplankton (CCMP, https://ccmp.bigelow.org/): the haptophytes *Emiliania huxleyi* (CCMP371), *Gephyrocapsa oceanica* (CCMP2054), *Prymnesium parvum* (UTEX LB2797) and *Isochrysis galbana* (CCMP1323); the chlorophyte *Dunaliella tertiolecta* (CCMP1320); prasinophytes *Prasinococcus capsulatus* (CCMP1407) and *Bathycoccus prasinos* (CCMP1898); dinoflagellates *Scrippsiella sp.* (CCMP1073) and *Oxyrrhis marina* (CCMP1788); and the diatom *Thalassiosira weissflogii* (CCMP1051). Each organism was cultured using the specific media recommended for their optimal growth by the CCMP (https://ccmp.bigelow.org/). Cells from each culture were harvested in late exponential phase.

2.2. Cell abundance and Biovolume Calculations

To determine cell abundance, 1 mL of each culture was cytologically fixed with glutaraldehyde (ca. 0.1 M final conc.), stained with 4,6-diamidino-2-phenylindole (DAPI, ca. 2 mM final conc.), and filtered onto a 0.22 μm pore size black polycarbonate filter. Cells were enumerated under an Axio A1 Carl Zeiss Imager using blue-light excitation. Biovolume was determined using cell dimensions measured with a calibrated ocular micrometer under an epifluorescence microscope then determining cell volume using appropriate geometric shapes (Olenina et al., 2006). Biovolume was multiplied by the total

abundance for each organism (Table 1). Samples were filtered within 2-3 hours onto GF/F filters using vacuum filtration. The filters were placed in a 2 mL microcentrifuge tube containing 600 µL of lysis buffer (40 mM EDTA, pH 8, 100 mM Tris-HCl, pH 8, 100 mM NaCl, 1% sodium dodecyl sulfate, and water) and stored at -40˚ C until extraction.

To determine whether each organism could be amplified and detected singly and in a mixture in DGGEs and clone libraries, each organism was filtered separately and as part of two cell combinations. The first combination contained *E. huxleyi, P. parvum, D. tertiolecta, P. capsulatus, Scrippsiella sp.*, and *T. weissflogii* (Combination 1, Fig. 1, Table 1). A second mixture contained the heterotrophic dinoflagellate *O. marina, E. huxleyi , G. oceanica, P. parvum, P. capsulatus, T. weissflogii, B. prasinos, I. galbana* (Combination 2, Fig. 1). *D. tertiolecta* was not added to the mixture *per se*, but was introduced as prey species of the *O. marina*, and was therefore present at low concentrations.

To test the effect of cell abundance and biovolume as well as co-occurring organisms, cells were filtered in three different treatments: “Equal cell number”, “Equal biovolume”, and “Field concentration” (Fig. 1). The filtration volume in each case was adjusted to approximate cell abundance in a 2 L filtration volume of natural Sargasso Sea water (Amacher et al., 2009). Cell numbers for the “Field concentration” treatment were chosen to approximate cell concentrations as in DuRand et al. (2001) for the oligotrophic BATS station; these *in situ*

concentrations are approximately 50 cells mL^{-1} for *E. huxleyi* , and 6000 cells mL^{-1} total for all other eukaryotes. Because diatoms and dinoflagellates were not found in high abundance in the DuRand et al. (2001) study, they were filtered in the same concentration as *E. huxleyi*. For Combination 2, in which the dinoflagellate *Scrippsiella sp.* was replaced by *O. marina* and *G. oceanica*, *P. parvum* and *B. prasinos* were added, the number of coccolithophorids in our study was modified to reflect the average number of *E. huxleyi* found at BATS by Haidar et al. (2001) (10 cells mL^{-1}). We later determined that the region of the 18S rRNA amplified from *E. huxleyi* and *G. oceanica* (each 10 cells mL^{-1}) using the DGGE primers are nearly indistinguishable (99.5% similar), so we will consider these sequences together for the remainder of the experiment. We believe that these numbers more accurately reflect the abundance of *E. huxleyi* present in the oligotrophic open ocean. The abundances of the other organisms were kept equal to that used in Combination 1. Though these "Field concentration" samples were intended to roughly approximate the diversity present in the Sargasso Sea near BATS, the actual diversity is undoubtedly greater and more variable. For the "Equal cell number" treatment cell abundance was calculated as described above and an appropriate volume of each culture was added for a final mixture containing equal numbers of each cell type (Table 1). The "Equal biovolume" treatment was made similarly, but average biovolume per cell was calculated before adjusting the volume filtered for each cell type so that

the total biovolume for each culture was approximately equal (Table 1). These treatments were made to test whether *E. huxleyi* is biased against even when equally abundant in a mixed sample.

2.3. DNA extraction and PCR Amplification

DNA was extracted from the filtered samples using a phenol, chloroform, iso-amyl alcohol (PCI, Sigma) organic extraction as detailed in Countway et al. (2007). DNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer. The DNA extracts from each sample were amplified by PCR for either DGGE or clone libraries. For DGGE, an approximately 500 bp region of the 18S rRNA gene was amplified with the universal eukaryotic primers Euk1A (Sogin and Gunderson, 1987) and Euk516rGC (Amann et al., 1990), set A in Díez et al. (2001b)). Each PCR reaction contained 5 µL of 10x Ex Taq Buffer, 4 µL of a 200 µM dNTP solution, 0.3 µl of each primer (0.05 µM final concentration), 1 µL of 10% bovine serum albumin, and 0.25 µL of Takara Taq polymerase. The PCR for the DGGE analyses consisted of an initial denaturation of 94°C for 130 s, 30 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 130 s, and a final elongation step of 72°C for 290 s.

For clone libraries, a 1.8 kbp pair region of 18S rRNA was amplified with the universal eukaryotic primers EukA and EukB (primer set C in Diez et al. (2001b)). To amplify these samples, 0.25 µl of a 50 µM solution of each primer was used. The PCR protocol consisted of an initial denaturing step of 94°C for

three minutes, 30 cycles of 94°C for 45 s, 55°C for 60 s, 72°C for 3 minutes, and a final elongation step of 72°C for 10 min. All PCR products were run on an agarose gel to check for proper amplification. Phusion™ clone libraries contained 10 µL of 5x Phusion™ HF Buffer, 1 µL of a 200 µM dNTP solution, 0.5 µl each primer (0.08 µM final concentration), 1 µL of 10% bovine serum albumin, and 0.5 µL of Finnzymes Phusion™ polymerase. The PCR protocol consisted of an initial denaturation of 98°C for 30 s, then 30 cycles of 98°C for 10 s, 61°C for 30 s, and 72°C for 30 s, and a final elongation step of 72°C for 450 s.

2.4. DGGE

DGGEs were prepared as in Diez et al. (2001b) with a 30-45% gradient of formamide and urea and run for 16 hours at 100 V. Approximately 300 ng of PCR product was loaded into each well. DGGEs were stained with SybrGold nucleic acid stain (Invitrogen) and visualized on a blue light Invitrogen Safe Imager™. Excised bands were re-amplified and then sequenced using the Euk1A primer.

Three denaturing gradient gels were run for this study. The samples used for DGGE 1 consisted of the "Field concentration" and "Equal cell number" of Combination 1, and the samples used for DGGE 2 consisted of the "Equal biovolume" concentrations of the same combination. The samples used for DGGE 3 were composed of the "Field concentration" and "Equal biovolume" treatments of Combination 2 (Fig. 1, Table 1).

2.5. Clone libraries

Clone libraries were constructed using the TOPO TA cloning kit (Invitrogen). PCR products purified using the EZNA Cycle Pure cleanup kit (Omega) were inserted into the pCR 2.1-TOPO plasmid cloning vector (Invitrogen) and then transformed into chemically competent *E. coli*. Cells were grown at 37 ^{o}C overnight on Luria-Bertani agar plates with 50 mg mL^{-1} kanamycin for plasmid uptake screening and 20 mg mL^{-1} X-Gal for blue/white colony selection. Selected colonies were re-amplified using the set C primers and sequenced using the Euk 528F universal eukaryotic primer (Elwood et al., 1985). For Phusion™ libraries, amplified PCR products were purified as described above, inserted into the pCR 2.1-TOPO plasmid, and then sent to the University of Washington High-Throughput DNA lab (http://www.htseq.org/) for cloning and sequencing.

Using clone libraries, we carried out a quantitative test of any bias against the 18S rRNA genes of any of the organisms in the mixed cultures by amplification using the universal eukaryotic primers EukA and EukB. Making the assumption that the size (biovolume) of an organism is roughly linearly related to the copy number of the genes Zhu et al. (2005), we considered an organism to be biased against if it was underrepresented in a clone library of a mixture of organisms at equal biovolume.

A total of seven clone libraries were constructed for this study. The first and second clone libraries contained DNA from the Combination 1 "Field concentration" and "Equal biovolume" treatments. The third and fourth clone libraries contained DNA from the Combination 2 "Field concentration" and "Equal biovolume" treatments (Fig. 1). The fifth clone library was constructed using DNA from an excised band from DGGE 1. The purpose of this fifth library was to test if the band of a competing sequence covered up that of *E. huxleyi* in a DGGE gel (Sekiguchi et al., 2001)(see results below). Finally, the sixth and seventh libraries were constructed using the high fidelity Phusion™ DNA polymerase (New England Biolabs) to amplify the "Equal Biovolume" treatments for both combinations with the universal eukaryotic primer set and a clone library was constructed for each. These last two libraries were constructed to determine whether any difference in amplification exists between libraries constructed using Phusion™ DNA polymerase- and Taq DNA polymerase-amplified sequences.

2.6. Sequencing

Re-amplified bands and picked colonies were purified using the EZNA Cycle Pure Kit (Omega) and sequenced by the DNA Laboratory at Arizona State University (http://sols.asu.edu/labs/dna_lab/index.php). Sequences returned to us were analyzed by Chromas Lite (http://www.technelysium.com.au/chromas_lite.html) and their closest similarity match determined by BLAST (http://blast.ncbi.nlm.nih.gov/) analysis.

3. Results and Discussion

Using cultured organisms from a range of different taxonomic groups, we analyzed banding patterns of both individual species and combinations of organisms on denaturing gradient gels. One experiment simulated cell concentrations that would be found at the BATS station in the Sargasso Sea (DuRand et al., 2001; Haidar and Thierstein, 2001) to determine whether each organism could be amplified by PCR and visualized by DGGE at concentrations matching those from the field. Then, to determine whether amplification of some rarer species such as *E. huxleyi* would amplify if cell concentrations were equal for all taxa present in the mixture we kept the cell numbers approximately equal to 1×10^3 ml^{-1} for all taxa. DGGE 1 shows results from the "Field concentration" and "Equal cell number" treatments for this combination (Fig. 2). *E. huxleyi* was visible in the gel when amplified separately regardless of the cell concentration, but was not present in either of the combination samples. Note that the *E. huxleyi* band is slightly higher in the gel than the *Scrippsiella sp.* band and the band found in the combination lane, which matched to *Scrippsiella sp.* when sequenced. This result shows that while *E. huxleyi* can be amplified and detected on a denaturing gradient gel at both high and low (oligotrophic gyre) cell concentrations, it may not be detectable using DGGE when other organisms are amplified together with *E. huxleyi*.

In DGGE 1 (Fig. 2A) two bands at the same position on the gel as *E. huxleyi* seemed to overlap with the location of the *Scrippsiella sp.* band. After sequencing, both bands closely matched *Scrippsiella sp.* based on NCBI BLAST similarity scores. This could mean that *E. huxleyi* was not amplified by PCR in the presence of *Scrippsiella sp.* or another organism in the mixture. Alternatively, it could mean that the *Scrippsiella sp.* and *E. huxleyi* bands overlapped in the gel and DNA from each organism was present in the same band, but the *Scrippsiella sp.* band masked the *E. huxleyi* signal. An experiment testing these two alternative hypotheses is described below. If a PCR bias does exist, *Scrippsiella sp.* would be preferentially amplified, masking the *E. huxleyi* signal. Furthermore, it seems that any bias that exists is not limited to *E. huxleyi*. Another haptophyte (*P. parvum*) and a chlorophyte (*P. capsulatus*) were not present in the combination samples in DGGE 1. While it appears that both the "Field concentration" and "Equal cell number" combination lanes contain a band aligned with the *P. parvum* band run singly, the band at the same position in the "Equal cell number" lane was identified as *Scrippsiella sp.* The band in the "Field concentration" lane was identified as *P. parvum.* For *P. capsulatus*, the bands in the "Field concentration" and "Equal cell number" combination lanes at the corresponding position were identified as *Scrippsiella sp.* and *D. tertiolecta,* respectively. This result is unexpected as the bands for those species amplified singly were not in the same location on the gel (Fig. 2A). This effect may be due to heteroduplex formation

between similar single stranded DNA sequences during PCR (Guldberg and Güttler, 1993), or double banding resulting from errors by Taq polymerase during PCR (Janse et al., 2004).

Another potential source of bias might be due to unequal copy numbers of 18S rRNA genes in different species. Zhu et al. (2005) shows a positive correlation between cell length and rRNA copy number in a cell. To reduce this as a source of bias, we calculated the biovolume of each organism (Olenina et al., 2006) and tested mixtures of organisms of equal biovolumes rather than equal cell numbers. DGGE 2 shows results from the "Equal biovolume" treatment (Fig. 2B). *E. huxleyi* was not present in the combination sample, and again a band that could potentially have been *E. huxleyi* was identified as *Scrippsiella sp.* The bands that aligned with *P. capsulatus* and *P. parvum* were identified as *D. tertiolecta* and *Scrippsiella sp*, respectively. While *E. huxleyi* was visible on a DGGE gel when amplified in isolation, it appears that its DNA either did not amplify while in the presence of DNA from other organisms or was simply not visible as a distinct band on the gel. The same seems to be true for *P. capsulatus* and *P. parvum*. This result supports the hypothesis that *E. huxleyi* and other taxa are biased against in a mixture of co-occurring organisms.

To determine whether *E. huxleyi* can be detected when any competing bands are eliminated, an alternative combination of organisms was made containing the dinoflagellate *O. marina* instead of *Scrippsiella sp.* In DGGE 3

(Fig. 3), *E. huxleyi* was found in both the "Field concentration" and "Equal biovolume" combinations. The intensity of the band was higher in the "Equal biovolume" combination, as expected. Interestingly, neither *O. marina* nor *T. weissflogii* sequences were found in either combination, though bands from each were present when run separately on the DGGE gel. In the "Field concentration" combination, all other organisms were visible on the DGGE, while in the "Equal biovolume" combination, *P. capsulatus* was also not detected.

We were also interested in whether the band in DGGE 1 that aligned closely to *E. huxleyi* but was identified as *Scrippsiella sp.* could also have contained *E. huxleyi* DNA. Sekiguchi et al. (2001) found, through 16S rRNA cloning of single excised DGGE bands, that a single band does not necessarily represent only a single bacterial strain. When this band was excised from the DGGE, the sequence, which matched to *Scrippsiella sp.*, did not contain a high amount of background "noise", suggesting there was minimal contamination from any other sequences. However, a clone library of this excised band, constructed using the DGGE primer set, revealed *Scrippsiella sp.* (8 clones), *E. huxleyi* (6 clones), and also *D. tertiolecta* (1 clone). This result indicates that an organism that amplifies preferentially or is more abundant in a sample may mask a less abundant organism, or one that is biased against if both DNA fragments migrate to the same position in a DGGE gel. This presents interesting implications for

diversity and richness estimates calculated using DGGE, highlighting the importance of cautious interpretation of DGGE results.

3.1. Clone Library Analyses

Two "Field concentration" clone libraries were constructed using the two different combinations of organisms. In each clone library, 48 clones were sequenced and matched to the NCBI database (some unreadable sequences were eliminated). This number was considered sufficient based on the small number of species in each mixture (6 in Combination 1 and 8 in Combination 2) and was supported by rarefaction curves (data not shown). In Clone library 1 (Combination 1, Fig. 4), *D. tertiolecta* (24 clones) and *P. capsulatus* (18 clones) dominated the library, while *T. weissflogii* (4 clones) and *Scrippsiella sp.* (1 clone) were found in fewer numbers. No *P. parvum* or *E. huxleyi* sequences were retrieved. In Clone library 3 (Combination 2, Fig. 4), *P. parvum* (16 clones), *P. capsulatus* (13 clones), *T. weissflogii* (9 clones) and *D. tertiolecta* (7 clones) comprised most of the library. One clone each of *I. galbana* and *B. prasinos*, but no *E. huxleyi* or *O. marina* sequences were retrieved. However, *E. huxleyi* sequences were detected in a control reaction with *E. huxleyi* alone at field concentrations. Therefore, while it is possible to detect *E. huxleyi* on its own in clone libraries, we conclude that in open ocean field concentrations, when *E. huxleyi* is present in a mixture of organisms it is either biased against in clone libraries.

Consequently we constructed an "Equal biovolume" clone library to attempt to reduce the competition of more abundant organisms and those with higher copy numbers of 18S rRNA genes. While size may not correlate exactly with copy number, there is a trend of greater rRNA copy number with increased cell size (Zhu et al., 2005). Therefore, in this combination, if each of the organisms had an equal chance to be amplified and cloned, they should be represented equally in the clone libraries. But again, some organisms were represented in much higher numbers than others. In Clone library 2 (Fig. 5A), *D. tertiolecta* (23 clones), *T. weissflogii* (10 clones), and *Scrippsiella sp.* (7 clones) dominated the library, while *P. capsulatus* (3 clones) and *P. parvum* (2 clones) sequences were found in lesser numbers and no *E. huxleyi* / *G. oceanica* sequences were retrieved. Based on the total number of clones, we would expect 8 clones for each organism. In Clone library 4 (Fig. 5B), *B. prasinos* (13 clones), *P. capsulatus* (10 clones), and *T. weissflogii* (9 clones) were the most dominant sequences, while only a single *P. parvum* sequence and no *I. galbana* or *O. marina* sequences were found. This library was, however, the only library in which *E. huxleyi* sequences were retrieved (4 clones). For this number of clones sequenced, we would expect 8 clones for *E. huxleyi* / *G. oceanica and* 4 clones for each other organism. Potvin and Lovejoy (2009) also found an uneven recovery of clones from their artificial community of mixed cultures filtered at equal cell numbers using different primer combinations. They found that the haptophytes

were biased against in most cases, as were the diatoms and a prasinophyte, and there seemed to be a strong bias favoring the dinoflagellate. In our "Equal biovolume" treatment, *E. huxleyi* were several orders of magnitude more abundant than in the Sargasso Sea, closer to bloom concentrations found in the temperate North Atlantic (eg. Balch et al., 1991) indicating that at low field concentrations, it is unlikely to find *E. huxleyi* sequences in clone libraries or DGGEs. Our results show that in the case of *E. huxleyi*, in addition to a bias induced by other organisms, there is also a dilution effect due to its low concentration in an oligotrophic open ocean setting.

Two additional clone libraries were created from the "Equal Biovolume" mixtures of both Combination 1 and 2 using Phusion™ polymerase in order to compare the results of this high fidelity polymerase with our results using Taq polymerase. Phusion™ polymerase has been used in order to alleviate the PCR bias caused by higher GC content sequences (Liu et al., 2009). Instead, our results indicated a more pronounced bias than those constructed with the Taq polymerase. In the Combination 1 library, the majority of sequences matched closely to *Scrippsiella sp.* (71 clones). The remainder of the sequences were identified as *D. tertiolecta* (18 clones), *B. prasinos* (3 clones), and *T. weissflogii* (1 clone). The Combination 2 library (Phusion™ polymerase based) was almost entirely composed of *B. prasinos* (86 clones), with only 1 *D. tertiolecta* and 3 *T. weissflogii* clones. This result was surprising, but there are several possible

explanations. First, since one of the benefits of Phusion™ polymerase is faster and more accurate amplification, sequences with a few mismatches may be less likely to amplify as well as sequences perfectly matched to the primer. Furthermore, the average GC content of haptophytes was reported to be high (≈57%) in the rRNA gene (Liu et al., 2009), but in the 18S rRNA regions amplified using the DGGE and clone library primers, we found only a small difference between the GC content of the haptophytes used in the present study as compared to other groups (Supplemental 1). In this case, a polymerase designed for high GC sequences would not necessarily favor the amplification of these haptophytes.

3.2. Comparison of methods

E. huxleyi was biased against in clone libraries and in Combination 1 DGGEs but was detected in the Combination 2 DGGEs in both the "Field concentration" and "Equal biovolume" mixtures. In Combination 2, *Scrippsiella sp.* was not included, which seemed to eliminate the problem of the overlapping band. *P. parvum* and *I. galbana*, members of the haptophytes, were generally biased against in clone libraries but were present in most DGGE combinations. In the DGGE experiments, only *T. weissflogii* and *O. marina* were not detected in either combination. *O. marina* sequences were not detected in clone libraries either, but *T. weissflogii* was usually well represented. These differences between the results of the clone library and DGGE methods may be due to the different

primer sets used to amplify the DNA or due to some inherent bias specific to the particular method. For example, competing bands in DGGEs or the insertion of environmental DNA into a plasmid in clone libraries may cause different results in one method versus another. An interesting result is that the particular combination of organisms in a sample seem to change what other taxa are represented in a clone library or DGGE. Additionally, biases do not seem to be taxon-specific. One species from a particular taxonomic group may be well represented while another is absent. Thus, while not all biases can be eliminated, DGGE and clone libraries, when used in combination, can lead to a better representation of the actual community composition in environmental studies of marine protists than when these methods are employed in isolation.

3.3. Conclusions

In this study we investigated the bias of DNA-based molecular methods using two different known cultured mixtures containing various eukaryotic phytoplankton groups at either typical oligotrophic field concentrations, equal cell numbers, and equal biovolumes. We found that biases may be dependent upon cell concentration, on the composition of organisms in a mixed sample, and also on the method (DGGE, clone library) used. While it is possible to amplify our target organism, *E. huxleyi*, by PCR and its sequences can be obtained using clone libraries and DGGE, it is biased against when present in a mixture of organisms. This is not only true for samples at field concentrations, in which *E. huxleyi* may

be too dilute to detect when more abundant organisms are present, but also for our "Equal cell number" and "Equal biovolume" treatments in which *E. huxleyi* is an equal or even a greater component of the mixture. Therefore, dilution alone does not seem to be the cause of this differential representation of taxa. Generally the chlorophyte, prasinophytes and diatoms were biased for and the haptophytes were biased against in clone libraries. In DGGE, the diatom, prasinophyte and haptophytes were biased against in most cases, depending on the combination of organisms present. Additionally, we found that *E. huxleyi* may be covered up if another band migrates to the same location on the gel. The dinoflagellates, a group with a high rRNA copy number (Zhu et al., 2005) showed mixed results in both clone libraries and DGGEs with *Scrippsiella sp.* biased for and *O. marina* biased against.

The biases inherent in these molecular methods will skew the results of environmental surveys of marine protists, a problem that need to be acknowledged when interpreting DGGE fingerprints and clone libraries. Comparative studies of community changes between different sites (Not et al., 2008), depths (Countway et al., 2007; Not et al., 2007), or between water column and traps (Amacher et al., 2009) will still be useful because the comparison is based on the same methodology. In the experiments reported here, we showed that DGGE and clone libraries each have inherent limitations and strengths. A combination of these methods, along with DNA-independent tools such as

microscopy, flow cytometry, and high performance liquid chromatography (HPLC), should provide a comprehensive representation of marine eukaryotic community structure.

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Table Captions.

Table 1. Organism combinations, biovolume per cell, cell mL^{-1}, and biovolume mL^{-1} used in each experiment.

Figure Captions.

Fig. 1. The experimental setup used in our study. Experiments 1, 2, and 3 used the mixture of organisms that contained *Scrippsiella sp.* (Combination 1) and correspond to DGGEs 1 and 2 and clone libraries 1 and 2. Experiments 4 and 5 used the mixture of organisms that contained *O. marina* (Combination 2), the results of which are found in DGGE 3 and clone libraries 3 and 4.

Fig. 2. DGGE 1 and 2. A. DGGE 1, Combination 1 "Field concentrations" (Fld) and "Equal cell number" (EC) of either single organism samples or combinations of species (CMB). B. DGGE 2, Combination 1 "Equal biovolume" (EB) of single species and their combination (CMB). DT (*Dunaliella tertiolecta*), PP (*Prymnesium parvum*), TW (*Thalassiosira weissflogii*), PC (*Prasinococcus capsulatus*), EH (*Emiliania huxleyi*), S (*Scrippsiella sp.*).

Fig. 3. DGGE 3, Combination 2 “Equal Biovolume” (EB) and “Field concentration” (Fld). PC (*Prasinococcus capsulatus*), PP (*Prymnesium parvum*), OM (*Oxyrrhis marina*), IG (*Isochrysis galbana*), EH (*Emiliania huxleyi*), BP (*Bathycoccus prasinos*), GO (*Gephyrocapsa oceanica*), TW (*Thalassiosira weissflogii*).

Fig. 4. Number of clones of each genus in “Field concentration”. A. clone library 1 (Combination 1). B. Clone library 3 (Combination 2).

Fig. 5. Number of clones of each genus in equal biovolume libraries. A) Clone library 2 (Combination 1), and B) clone library 4 (Combination 2). Dashed line indicates the expected number of clones.

Tables.

Table 1.

Combination	Supergroup	Class	Organism Name	Biovolume Per Cell (μm^3)	Field Concentration		Equal Cells Concentration		Equal Biovolume Concentration	
					Cell mL^{-1}	Biovolume μm^3 mL^{-1}	Cell mL^{-1}	Biovolume μm^3 mL^{-1}	Cell mL^{-1}	Biovolume μm^3 mL^{-1}
1	Chlorophyta	Chlorophyceae	*D. terrioleca*	201	2.01E+03	4.03E+05	1.05E+03	2.11E+05	2.10E+02	4.21E+04
	Chlorophyta	Prasinophyceae	*P. capsulatus*	65	1.99E+03	1.30E+05	1.04E+03	6.79E+04	6.28E+02	4.11E+04
	Haptophyta	Prymnesiophyceae	*P. parvum*	49	2.00E+03	9.79E+04	1.04E+03	5.08E+04	8.48E+02	4.16E+04
	Haptophyta	Prymnesiophyceae	*E. huxleyi*	14	5.20E+01	7.35E+02	1.04E+03	1.47E+04	2.95E+03	4.17E+04
	Alveolates	Dinophyceae	*Scrippsiella sp.*	2232	5.05E+01	1.13E+05	1.04E+03	2.32E+06	1.86E+01	4.16E+04
	Stramenopiles	Diatom	*T. weissflogii*	423	5.20E+01	2.20E+04	1.05E+03	4.44E+05	9.92E+01	4.20E+04
2	Prasinophyta	Prasinophyceae	*B. prasinos*	4	1.00E+03	4.19E+03			1.51E+04	6.35E+04
	Haptophyta	Prymnesiophyceae	*I. galbana*	49	9.99E+02	4.91E+04			1.29E+03	6.34E+04
	Chlorophyta	Prasinophyceae	*P. capsulatus*	65	9.99E+02	6.54E+04			9.71E+02	6.35E+04
	Haptophyta	Prymnesiophyceae	*P. parvum*	49	1.00E+03	4.91E+04			1.29E+03	6.34E+04
	Haptophyta	Prymnesiophyceae	*E. huxleyi*	14	5.04E+00	7.13E+01			4.50E+03	6.36E+04
	Haptophyta	Prymnesiophyceae	*G. oceanica*	34	5.02E+00	1.68E+02			1.89E+03	6.34E+04
	Alveolates	Dinophyceae	*O. marina*	2356	2.65E+01	6.24E+04			2.71E+01	6.38E+04
	Stramenopiles	Diatom	*T. weissflogii*	423	1.00E+02	4.23E+04			1.49E+02	6.29E+04

Figures.

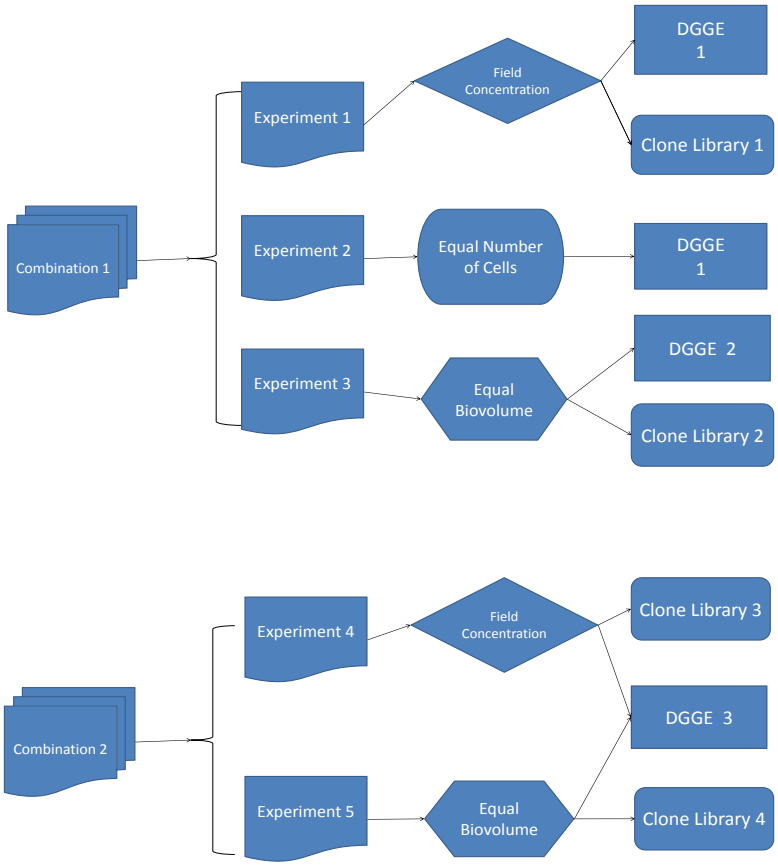

Fig. 1.

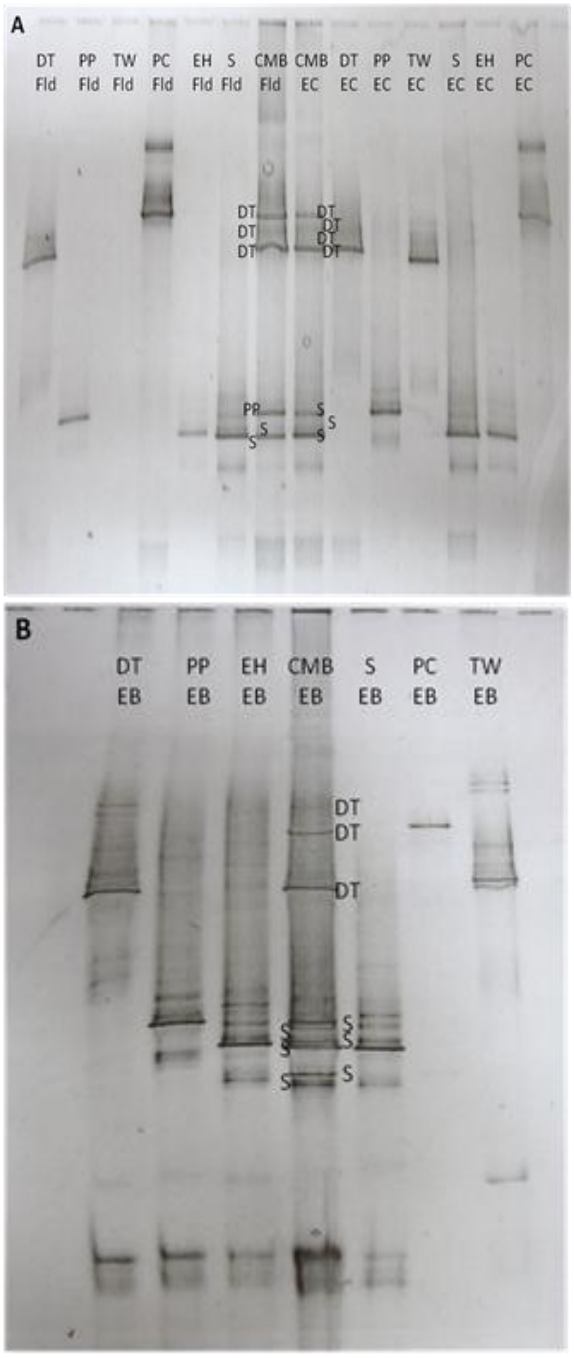
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Fig. 2.

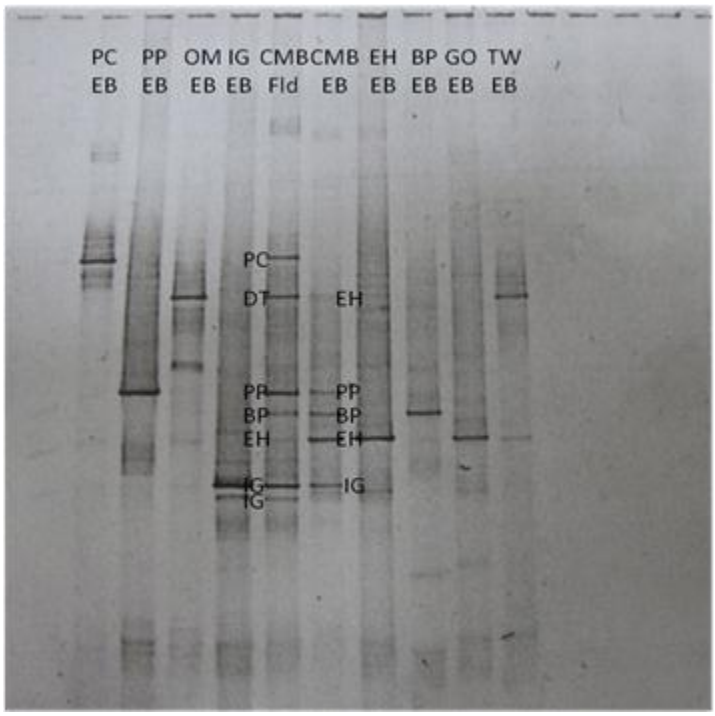
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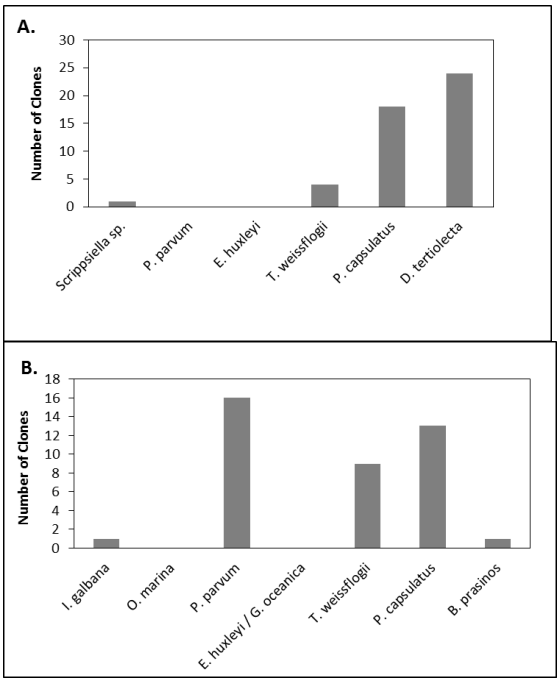
Fig. 3.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Number of Clones
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20
15
10
5
0
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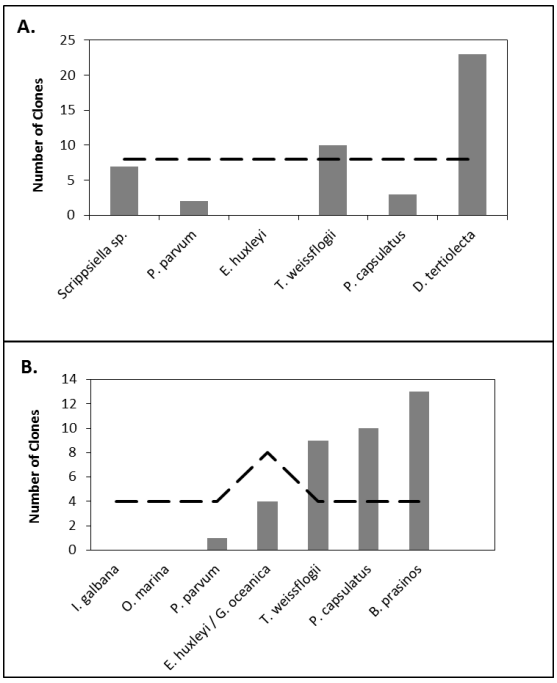
P. parvum
E. huxleyi
T. weissflogii
P. capsulatus
D. tertiolecta
B.
Number of Clones
18
16
14
12
10
8
6
4
2
0
I. galbana
O. marina
P. parvum
E. huxleyi / G. oceanica
T. weissflogii
P. capsulatus
B. prasinos

Fig. 4.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Number of Clones
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E. huxleyi
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P. capsulatus
D. tertiolecta
B.
Number of Clones
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I. galbana
O. marina
P. parvum
E. huxleyi / G. oceanica
T. weissflogii
P. capsulatus
B. prasinos

Fig. 5.

CHAPTER 3

DNA-BASED MOLECULAR FINGERPRINTING OF EUKARYOTIC PROTISTS AND CYANOBACTERIA CONTRIBUTING TO SINKING PARTICLE FLUX AT THE BERMUDA ATLANTIC TIME-SERIES STUDY

Jessica Amacher[1], Susanne Neuer[1], Charles Baysinger[1], Michael Lomas[2]

[1]Arizona State University, School of Life Sciences, Tempe, AZ 85287

[2]Bermuda Institute of Ocean Sciences, St. George's GE01, Bermuda

Abstract

We used denaturing gradient gel electrophoresis (DGGE) to examine the protist and cyanobacterial communities in the water column and in corresponding 150 m particle interceptor traps at the Bermuda Atlantic Time-series Study (BATS) in a two year monthly time-series from May 2008 to April 2010. Our results show that cyanobacteria, including *Prochlorococcus* and *Synechococcus*, as well as small-sized eukaryotic taxa, including the prasinophyte genera *Ostereococcus*, *Micromonas*, and *Bathycoccus*, can contribute to downward particle flux. Dinoflagellates were the most commonly detected taxa in both water column and trap samples throughout the time series. Diatom sequences were found infrequently in the water column, and they were only very rarely in trap material. Ribosomal RNA sequences matching to the cyanobacteria *Prochlorococcus* and *Synechococcus* were consistently found in the water column, and detected in trap samples for each available time point. Both seasonal and interannual variability affected sinking patterns. The two years of this study were quite different hydrographically, with higher storm activity and the passing of a cyclonic eddy in winter 2010, causing unusually deep winter mixing. This was reflected in the water column fingerprints, which were richer in eukaryotes and poorer in cyanobacteria in winter of 2010 compared with that of 2009. Eukaryotic richness increases could be tracted to increased diversity of picoeukaryotes and prasinophyte / prymnesiophytes. The decrease in

cyanobacteria richness was in turn reflected in the trap composition, but the increase in eukaryotes was not, perhaps indicating a disproportionate contribution of certain taxa to sinking particle flux.

1. Introduction

The oceans are important regulators of climate and the only true net sink for anthropogenic carbon dioxide (Sabine et al., 2004). Marine phytoplankton play a major role in the removal of carbon dioxide from the atmosphere and subsequent sequestration into the deep sea, a process known as the biological carbon pump. While much of the organic matter is oxidized via respiration and re-mineralized through the microbial loop (Sherr and Sherr, 1994), particles sinking to depths of 500 m or more are effectively removed from exchange with the atmosphere from tens to hundreds of years (Ono et al., 2001). Approximately 1% of this carbon reaches the sea floor and is incorporated into the sediment, removing it from contact with the atmosphere on geological time scales. Subtropical gyres make up nearly 60% of the world's oceans (Eppley and Peterson, 1979; (Longhurst, 1998) and are responsible for a significant portion of this flux despite their oligotrophic nature (Emerson et al., 2001).

The planktonic community in these oligotrophic gyre regions is dominated by pico- and nanoplankton as well as cyanobacteria for most of the year (DuRand et al., 2001). Contrary to the traditional size-based tenet that only the larger organisms sink, we now have growing evidence that small phytoplankton and even cyanobacteria contribute to the flux out of the upper water column. In a modeling study, Richardson and Jackson (2007) hypothesize that picoplankton contribute to export production at a rate proportional to their primary production.

Brew et al. (2009) suggested that aggregation may be an important mechanism controlling export, and also revealed a significant correlation between particle flux and picoplankton abundance. In a study by Lomas and Moran (2011) high performance liquid chromatography (HPLC)-based pigment analysis was used to identify taxonomic groups collected with Niskin bottles and in-situ pumps from within and below the euphotic zone. Their study found that nanoeukaryotes contribute to particulate organic carbon (POC) flux in proportion to their autotrophic biomass, while cyanobacteria contribute one-tenth of their autotrophic biomass to POC flux. Amacher et al. (2009) showed that in 18S rRNA gene clone libraries at ESTOC (European station for time series in the ocean), small plankton representation in trap samples was roughly in proportion to their abundance in the water column, but also that diatoms, while abundant in the water column were rare in corresponding trap samples. The mechanism behind the downward flux of small cells is thought to be the packaging into aggregates, which can then either sink and/or be grazed and released as fecal pellets, that allows them to sink through the water column at a faster rate than their individual size would predict (Jackson, 2001; Legendre and Le Fèvre, 1995; Olli and Heiskanen, 1999; Richardson and Jackson, 2007). In subtropical gyre regions, which are dominated by pico- and nanoplankton (Steinberg et al. 2001) it is important to consider that small phytoplankton may play a major role in the flux of carbon out of the euphotic zone.

The Bermuda Atlantic Time-series Study (BATS), located in the western subtropical North Atlantic gyre, has been sampled on a monthly basis since 1988. This area stays stratified during most of the year (Steinberg et al., 2001). In late winter a breakup of the thermocline, driven by increased wind stress and changes in heat flux, results in deep convective mixing (Cianca et al., 2007) increasing the availability of nutrients in the euphotic zone (average depth 100 m), causing a phytoplankton bloom as seen by elevated chlorophyll a (Chl-a) levels (Helmke et al., 2010). Most of what is known about the phytoplankton community in the euphotic zone at BATS comes from microscopy, flow cytometry, and HPLC data. This area is dominated by small pico- and nanoplankton during most of the year, with significant seasonal and interannual variability in phytoplankton community structure (Caron et al., 1999; DuRand et al., 2001; Steinberg et al., 2001). *Synechococcus* dominates the cyanobacterial community during the winter/spring bloom, while *Prochlorococcus* dominates during summer and fall (DuRand et al., 2001). The eukaryotic community is dominated by nanoplankton; abundant eukaryotes include prymnesiophytes and pelagophytes, with dinoflagellates and prasinophytes less abundant and diatom blooms occurring rarely, typically during winter/spring bloom periods (DuRand et al., 2001; Krause et al., 2009; Lessard and Murrell, 1996; Lomas and Bates, 2004; Steinberg et al., 2001). Particulate organic carbon (POC) flux is regularly measured at BATS with particle interceptor traps (PITs) at monthly intervals. POC flux at BATS has been found to

be highest in winter and spring, lower in summer, and lowest in fall (Helmke et al., 2010). Identification of taxa in particle traps proves to be more difficult due to the packaging of cells into amorphous aggregates and fecal pellets and the degradation of pigmentation. Organisms with mineral shells, such as diatoms and coccolithophorids, can be identified, but this potentially overestimates the contributions of those phytoplankton taxa to flux, and underestimates that of small and unarmored taxa.

DNA-based molecular methods can now be used to identify eukaryotes and cyanobacterial taxa contained in both water column samples and in trap material. Amacher et al. (2009) used clone libraries of small subunit 18S rRNA genes to identify eukaryotic taxa contained in trap material and to determine the proportional contribution of taxonomic groups to flux. Denaturing gradient gel electrophoresis (DGGE) is another technique frequently used with 16S and 18S rRNA genes to obtain a community "fingerprint" (ie. Countway et al., 2007; Díez et al., 2001a; b; Moon-van der Staay et al., 2001; Not et al., 2007). This technique allows for the simultaneous analysis of multiple samples on a single gel. Differences between samples can quickly be compared and quantified. Additionally, individual bands, representing a particular phylotype, can be excised from the gel, sequenced, and identified, allowing us to determine which organisms contribute to the flux of carbon out of the euphotic zone. While there may be some degradation of DNA in trap material, several studies of copepod

diets (Martin et al., 2006; Nejstgaard et al., 2003; Nejstgaard et al., 2008; Troedsson et al., 2009) have shown that digestion and degradation of DNA passing through zooplankton guts may only be partial. Additionally, Martin et al. (2006) found that prey species contained in fecal pellets reflected the actual diet, suggesting no differential degradation of DNA. The percent of fecal pellets contributing to sinking material is highly variable but in the North Atlantic subtropical gyre, fecal pellets may make up an average of 20-30% of total POC flux at depth (900 m, Fischer et al., 1996; 200 m, Huskin et al., 2004). Intact and even culturable cells have been found in aggregates and fecal pellets of salps (filter feeding planktonic tunicates) collected in particle traps and sediments (Fischer et al., 1996; Pfannkuche and Lochte, 1993; Turley and Mackie, 1995), in fecal pellets (Jansen and Bathmann, 2007; Martin et al., 2006), and in the hindguts of mesozooplankton (Wilson and Steinberg, 2010). In Martin et al. (2006), prey species found in fecal pellets reflected their actual diet, suggesting no differential degradation of DNA. Furthermore, because DNA is a chemically stable molecule more resistant to breakdown than other tracer compounds, such as pigments and proteins, cells need not be living or even intact in order to obtain DNA sequences that can be used for molecular analysis (Nejstgaard et al., 2008). This makes 16S and 18S rRNA clone libraries and DGGE particularly useful techniques in the identification of organisms contributing to flux into particle traps.

In this study we use DGGE to examine eukaryotic protist and cyanobacterial communities in the upper water column and taxa collected in 150 m particle trap material. We hypothesize that pico- and nanoplankton contribute to particle flux and also that there will be seasonal variability in the taxa which contribute most to particle flux. Regular monthly sampling at the BATS station over a two year time period allowed us to observe the sinking patterns of different taxa in response to seasonality as well episodic events. Additionally, we determined the efficiency of DNA recovery in order quantify the DNA present in water column samples and to determine the flux of DNA out of the euphotic zone. These data give us a better understanding of the dynamics of plankton communities and their contribution to flux.

2. Methods

2.1. Field Sampling

Sampling was conducted on a monthly basis (twice monthly during the winter bloom) from May 2008-April 2010 at the Bermuda Atlantic Time-series Study (BATS) station. Samples for DNA analysis were collected in addition to the core parameters as part of the BATS program (including temperature, salinity, nutrients, chlorophyll, primary production, flow cytometry, HPLC, and deployment of surface-tethered particle interceptor traps, http://bats.bios.edu/). In this study we will be including several of these core parameters in our analysis.

From the upper water column, samples were collected in Niskin bottles attached to a rosette with conductivity, temperature, and depth (CTD) sensors from four depths in the upper water column. For molecular analysis, a 2 L sample was prefiltered through a 100 μm mesh (to exclude larger zooplankton) and filtered onto GF/F glass fiber filters, placed in 1 mL lysis buffer (40 mM EDTA, pH 8, 100 mM Tris-HCl, pH 8, 100 mM NaCl, 1% sodium dodecyl sulfate, and water (Countway et al., 2005)), immediately frozen in liquid nitrogen for shipping, and finally stored at -80 °C at ASU until extraction.

In addition to the standard BATS three day particle interceptor traps (PITs) deployed at 150, 200 and 300 m. A fixative of 0.74% formalin (final concentration) is added to each PITS tube, and separate unfixed particle traps were deployed for 24 hours at 150 m for this project. In order to identify specific taxa contributing to particle flux, the 150 m fixed and 24-hour unfixed trap samples were subjected to DNA-based molecular analysis. Particles in each PIT collection tube were drained through a 0.8 μm polycarbonate filter which sits at the base of the tube. The filters were then frozen in liquid nitrogen after retrieval, again thawed in the lab to pick out zooplankton swimmers using a dissecting microscope, placed in 1 mL lysis buffer, frozen again in liquid nitrogen for shipping, and then stored at -80 °C at ASU until extraction. Filters from two tubes of the 24 hour unfixed trap were combined in the first step of extraction for a

higher yield of DNA. Particulate organic carbon flux was determined from the three day fixed traps as part of the BATS core program.

2.2. DNA Extraction

Extractions were carried out using the Phenol: Chloroform: Isoamyl alcohol (PCI, Sigma) method as described in Countway et al. (2005). In this method filters preserved in lysis buffer are alternately bead beaten and heated, and DNA is then extracted with PCI, cleaned with chloroform: isoamyl alcohol, precipitated in ethanol overnight, and then eluted in 60 µL of deionized water for each water column sample and 100 µL for each trap sample (see Amacher et al., 2009, Chapter 1 for further detail). DNA concentrations were quantified with a NanoDrop ND-1000 Spectrophotometer.

2.3. PCR

To amplify eukaryotic 18S rRNA genes approximately 10-50 ng of DNA extract was used as template for PCR using primers Euk1A (Sogin and Gunderson, 1987) and Euk516rGC (Amann et al., 1990) as in Diez et al. (2001b). Each reaction contained 5 µL of 10X Takara Ex Taq Buffer, 4 µL of dNTP's (200 µM each), 1 µL 10% bovine serum albumin (BSA) 0.3 µL of each primer (0.3 µM), 38.15 µL of water, and 0.25 µL of Takara Ex Taq polymerase along with the template. PCR reactions consisted of an initial denaturation at 94 °C for 130 s; 30 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 130 s; and a final extension at 72 °C for 7 minutes.

Cyanobacterial 16S rRNA genes were amplified with the primers Cya359fGC and Cya781r (Nübel et al., 1997). Each reaction contained 5 μL of 10X Takara Ex Taq Buffer, 4 μL of dNTP's (200 μM each), 1 μL 10% bovine serum albumin (BSA) 0.3 μL of each primer (0.3 μM), 38.25 μL of water, and 0.25 μL of Takara Ex Taq polymerase along with the template. PCR reactions consisted of an initial denaturation at 94 °C for 5 minutes; 30 cycles of 94 °C for 1 minute, 60 °C for 1 minute, 72 °C for 1 minute; and a final extension at 72 °C for 9 minutes. Prior to molecular analysis, PCR products were run on an agarose gel to check for proper amplification and approximate DNA concentration.

2.4. Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed for each sample. Through several trials, we established that a gradient of 20-45% denaturant for eukaryotic and 30-70% denaturant for cyanobacterial gels allows for sufficient separation of bands throughout the gel. Approximately 500-700 ng of PCR product was loaded into each well. Gels were run at 100 V for 16 hours, stained with SybrGold and imaged with the BioRad Fluor-S imager.

When possible, two of each band type (bands on the same horizontal plane) were excised from the gel and allowed to elute in 30 μL of sterile MilliQ water overnight at 4 °C, then stored at -40 °C. Bands too close to another band in the lane or not clearly visible were not excised. For sequencing, DNA from each band was reamplified using eukaryotic primers Euk1A and Euk516r and

cyanobacterial primers Cya359f and Cya781r, then purified with the EZNA Cycle-Pure kit (Omega). Bands were sequenced in one direction with the Applied Biosystems 3730 sequencer at Arizona State University. The closest similarity match for each sequence was determined by BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/) analysis to determine broad taxonomic groupings.

Using the Quantity One software package, each band on the gel was selected in order to determine its intensity and position on the gel. Bands on the same horizontal lines between lanes were considered to be the same phylotype. Bray-Curtis coefficients of similarities (Bray and Curtis, 1957) between water column and trap samples within each time point were calculated and analyzed using cluster analysis and non-metric multidimensional scaling (MDS) using the PRIMER v.6 program (Clarke and Gorley, 2006). UPGMA (unweighted paired group method with averaging) diagrams were constructed and similarity profile tests (SIMPROF, (Clarke and Gorley, 2006)) were run to establish significance between samples. Relative contributions of each band as a fraction of the whole sample were determined using the Quantity One software output and used to calculate richness, Shannon diversity, and evenness for each sample.

2.5. Efficiency of DNA recovery

For all samples from July 2009 through April 2010 the exogenous plasmid pGEM-3Z (Promega) was added prior to extraction in order to determine the

efficiency of DNA recovery. This method has been used by Coyne et al. (2005) and Halliday et al. (2010) in studies of harmful algal species and *Enterococcus* in beach sands. These studies have shown the coextraction of the exogenous plasmid to be effective and accurate method for the quantification of DNA in environmental samples. A 500 µL stock of lysis buffer was prepared with 1 µg pGEM plasmid; 36 µL of this stock was added to each water column sample and 60 µL to each trap sample to achieve a final ideal concentration (assuming 100% recovery) of 1.2 ng μL^{-1} in the extract as in Halliday et al. (2010). The actual amount of pGEM in each extract was then quantified by qPCR. The primers M13F and pGEMR (Coyne et al., 2005) amplified a 208 bp region of the plasmid. A standard curve was constructed with known concentrations of pGEM in a dilution series of 10 ng to 10^{-4} ng. 20 µL reactions were prepared in triplicate each with 5 uL of diluted template DNA, 100 ng each of primers M13F and pGEMR, 12.5 uL SYBR Green master mix and 1.9 uL sterile MilliQ water. Quantitative PCR reactions were run on an ABI7900HT thermocycler. The protocol consisted of an initial denaturation at 95 °C for 3 min; 50 cycles of 95 °C for 15 s and 60 °C for 1 min, then a melting cycle beginning at 60 °C and ending at 95 °C with a 2% ramp rate. Duplicate reactions were run for each extract with an additional 1.2 ng of pGEM plasmid to determine qPCR inhibition. This allowed us to calculate the corrected efficiency of recovery as described in Halliday et al. (2010).

This corrected efficiency of pGEM recovery was assumed to represent the approximate recovery of total DNA for each extraction. Therefore, the total amount of DNA in each of the samples at the time of collection could be estimated by dividing the amount of extracted DNA as measured with the NanoDrop ND-1000 Spectrophotometer by the relative efficiency of pGEM recovery. These calculations allowed us to more accurately estimate the DNA concentration present in the water column and the DNA flux determined with the 150 m trap.

3. Results

3.1. Hydrography and chlorophyll distribution

Temperature profiles reveal patterns of summer stratification and winter mixing with stronger stratification in 2009 and deeper mixing into the 18 °C mode water in winter 2010 (Fig 1). This pattern is reflected in the Chl-a distribution (Fig. 2) with higher Chl-a in winter 2010 than 2009 and lower levels in the summer of 2009 as compared to summer 2008. Additionally, several potential mesoscale eddies can be detected in this temperature profile (Fig. 1) and can be observed in altimetry data collected during this time period (http://science.whoi.edu/users/valery/altimetry/).

3.2. Efficiency of DNA recovery and water column DNA distribution

The efficiency of pGEM recovery (Supplemental 1) for water column samples was higher than that of the trap samples, with average recovery of 38.6% and 24.2%, respectively. These are slightly lower efficiencies than samples extracted from beach sands in Halliday et al. (2010), at an average of 55.5% for wet and 66.3% for dry sands. Unfixed traps had a higher average recovery efficiency than fixed traps (31.3% unfixed and 15.3% fixed). However, the range of efficiency was surprisingly large. For water column samples qPCR calculated efficiencies varied between 6.8-100% with the majority falling between 20-40%. Unfixed traps ranged from 4.0-88.4% and fixed traps between 1.5-35.5%.

Figure 3 shows calculated DNA concentrations from the water column samples. The winter bloom had the highest DNA concentrations with a few peaks in fall. This pattern corresponds to the higher Chl-a levels found during the winter bloom (Fig. 2). Concentrations ranged from $2.3x10^{2} – 1.4x10^{4}$ ng L^{-1}. In the April 2010 samples we detected a miniscule level of pGEM recovery in both the water column and trap samples (<0.03%). If it is assumed that the DNA loss is proportional to that of pGEM, dividing the DNA concentration of the extracted product (as measured with the NanoDrop ND-1000 Spectrophotometer) by the percent recocery resulted in unrealistically high DNA concentrations. Therefore, we excluded those data in our calculations.

3.3. Flux

POC export (Fig. 4) at 150 m ranged from 6.9 ±4.1 mg m^{-2} d^{-1} in April 2010 to 105.4 ±9.7 mg m^{-2} d^{-1} in February 2010 (Fig. 4). These carbon flux values are consistent with those previously observed at the BATS (http://bats.bios.edu/, Fig. 5). There is no clear seasonality distinguishable in this dataset, although the highest flux values were observed during the late winter bloom. The second half of the sampling period (May 2009-April 2010) had a slightly higher carbon flux on average. Flux of DNA in the 150 m trap (Fig. 4) ranged from 2.59 – 146 µg DNA m^{-2} d^{-1}. The highest DNA flux values were found in July 2009 and March 2010. DNA concentration in the water column was also high in the water column in March 2010, reflecting the high DNA flux (Fig. 3). However, in July 2009 the DNA concentration in the water column was relatively low. A linear relationship exists between DNA flux and POC flux (Pearson correlation coefficient=0.915, p=0.001) and regression analysis shows that DNA flux increases with increasing POC flux (R^2=0.838, p=0.001). Note that several of the POC and DNA flux values during the 2010 winter bloom do not coincide (Fig. 4). This is because the three day PITs traps, which are used to determine POC flux, are deployed only during the regular monthly cruises, but the 24 hr traps, used for DNA analysis, are collected at each cruise. Additionally, the pGEM plasmid for February 5th, 2010 was not recoverable and for April 21st, 2010 it was unreasonably small (<0.03%), making our calculations for DNA flux impossible. Thus these samples are not included in Fig. 4.

3.4. Community fingerprint and richness

Denaturing gradient gels were run for each time-point in order to visualize the "fingerprint" of banding patterns for the community in either a water column sample or traps. Examples of a typical gels run with eukaryotic and cyanobacterial samples and corresponding dendrograms from the spring bloom (March 24, 2010) are shown in Figures 6 and 7. Dendrograms constructed for eukaryotic DGGEs typically revealed the greatest similarities between the samples closest in depth (Supplemental 2). A significant difference between the trap and water column banding patterns was often detected especially during the second year of the time series (May 2009-April 2010), but in some cases traps grouped together with deeper samples from the water column (May, July, August, and December 2008, February 2009). In other cases one trap was significantly different and the other grouped together with deeper samples (October, November 2008, May, August 2009) or there was no significant difference detected between water column and trap samples (September 2008). For the cyanobacteria, we see a similar pattern. For nearly all samples that we could amplify, trap DNA from fixed and unfixed samples, we found the traps grouping together with a significant difference between the water column and trap samples.

The most intense bands from each gel were cut out, eluted and sequenced to determine the source of the amplified band. Major taxonomic groups found in eukaryotic gels are shown in Table 1. In eukaryotic gels, dinoflagellate sequences

were nearly always present in both water column and traps. Ciliates, stramenopiles, prymnesiophytes, and radiolarians were also common, but were not always detected in the traps. Diatoms were detected in only three sampling dates, March, April, and August 2009. In each of these sample sequences matching to the centric diatom genus *Thalassiosira* were found at each water column depth and in the traps with the exception of April 2009 when there was no available trap sample. Additionally, in March 2009 sequences matching most closely to the pennate diatom genus *Diadesmis* were found in the water column samples but not in the traps. For those groups that were only detected occasionally, there does not seem to be a clear seasonal pattern in the presence of particular taxonomic groups.

In cyanobacterial gels (Table 2), *Synechococcus* and *Prochlorococcus* were detected in every water column sample (with 1 exception in July 2009) and in every available trap sample. As shown in Table 2 we were not able to amplify cyanobacterial DNA from trap samples for several sampling dates from the first year (July-October 2008) or for any of the samples from May 2009-April 2010 (April 2009 trap samples were not available) even after multiple attempts using different concentrations of template DNA, primers, BSA, and Taq polymerase as well as adjusting annealing temperatures and numbers of cycles. After these trials we believe that the cyanobacteria template DNA was below the detection limit for PCR, and perhaps even too low a ratio of cyanobacterial to eukaryotic DNA for

the primers to be able to efficiently attach to the correct binding sites. This hypothesis would fit with the lower cyanobacterial and higher eukaryotic cell concentration found by epifluorescence microscopy in Hansen et al. (in preparation) during the second year of sampling. Despite this reduced trap sample set, in every single trap sample in which we could amplify cyanobacterial DNA, *Synechococcus* and *Prochlorococcus* were always present.

Small subunit rRNA genes (16S) from the plastid DNA genomes of photosynthetic eukaryotes (including diatoms and other stramenopiles, dinoflagellates, prymnesiophytes, prasinophytes, cryptophytes and euglenozoa, see Table 2) could also be amplified using our cyanobacterial primers and detected with DGGE. Diatoms (including sequences matching to plastid DNA of the genera *Eucampia*, *Rhizosolenia*, *Amphora*, *Thalassiosira*, *Cylindrotheca*, *Minutocellus*, and *Cymbella*) were present in several additional time points: in the water column in May, August, and November 2008, and both February 2010 samples, and in the trap in June, and November 2008. Other stramenopile sequences from the Pelagophyte, Dictyophyte, and Raphidophyte groups were detected each month from July 2008-May 2009 but were not found in subsequent months. Several other eukaryotic groups (dinoflagellates, prymnesiophytes, prasinophytes, and cryptophytes) were found more often in the second half of the two-year sampling period. In the results of the cyanobacterial DGGEs, stramenopiles, diatoms, and heterotrophic bacteria were detected in the traps, but

dinoflagellate sequences were found only in the water column. This contradicts the results of the eukaryotic DGGEs, which show dinoflagellate sequences in both the water column and trap libraries in all but a few sampling dates (Table 1) and may be due to differences in the dinoflagellate species that can be amplified with the different primer sets. In these cyanobacterial DGGEs, sequences identified as dinoflagellates included members of the genera *Amylax* and *Dinophysis*. It is possible, however that some may belong to the marine alveolates, largely uncultured members of the Alveolata related to the Syndiniales, a group of marine parasitoid dinoflagellates (Massana and Pedrós-Alió, 2008; Siano et al., 2011).

Estimates of eukaryotic richness as shown by DGGE analysis display a clear increase in the upper water column in summer 2009–winter 2010 as compared with summer 2008–summer 2009 (Fig. 8). However, this increase is not reflected in the trap samples. The increase in the estimated richness based on the water column data seems to coincide with the increase of Chl-a during the 2010 winter bloom and also with the increased DNA concentration in the water column. Interestingly, the opposite pattern is observed for estimates of cyanobacterial richness from DGGEs in the upper water column (Fig. 9) with decreased richness in the second half of this sampling period. There seems to be a general trend of a slightly greater number of eukaryotic plastid phylotypes in the second year, but fewer different strains of *Prochlorococcus*, *Synechococcus* and fewer other cyanobacteria (Fig. 10).

4. Discussion

4.1. Role of pico- and nanoplankton in particle flux

Cyanobacteria, including *Prochlorococcus* and *Synechococcus*, as well as small eukaryotic phytoplankton, including prasinophytes, prymnesiophytes, marine alveolates, and stramenopiles, were seen to contribute toward the particle flux into the 150 m traps during our study period, in agreement with our hypothesis. Although cyanobacterial DNA could not always be amplified from trap material, the presence of both *Synechococcus* and *Prochlorococcus* sequences in each of those available trap samples clearly shows that these very small organisms can sink and thus contribute to carbon flux. This was likely due to the cyanobacterial DNA in the extract being below the detection limit by PCR. We will learn more about the proportional contribution through analysis of clone libraries (Chapter 4).

4.2. Seasonal and interannual variability

The first (May 2008-April 2009) and second (May 2009-April 2010) years of this time series were hydrographically quite different. During the second year the area experienced stronger summer stratification and a much deeper winter mixing event. This resulted in higher Chl-a concentrations in the water column in summer 2008 than in 2009 and higher winter bloom Chl-a levels in 2010 as compared to winter 2009. This fortuitous observation of a strong interannual variability allows us to make comparisons between the plankton communities and

flux. The overall trend in the molecular data clearly shows a greater eukaryotic and lower cyanobacterial richness during the second half of the time series as compared to the first. These data agree with those of Hansen et al. (in preparation), who describes a much higher abundance of picoeukaryotes and lower abundance of *Synechococcus* as measured by flow cytometry and epifluorescence counts. *Prochlorococcus* is more abundant in fall 2009 but less abundant in winter 2010 than in the previous year. The abundance of prymnesiophyte and prasinophytes is also higher during the 2010 winter bloom.

The overall trend in sequences excised and identified from cyanobacterial DGGEs indicates an increase in eukaryotic plastid sequences and fewer strains of cyanobacteria in the second half of the time series (Fig. 10). This decreased richness and abundance of cyanobacteria may be accompanied by a reduced contribution of cyanobacteria to particle flux. Lomas and Moran (2011) found that cyanobacteria contribute only one tenth of their primary production to particle flux measured in their samples. However, those samples were collected using pumps that cannot effectively collect fecal pellets. Richardson and Jackson (2007) suggest that up to 89% of POC export can be through grazing by mesozooplankton, which consume picoplankton through detritus and smaller grazers. Therefore, this may exclude a potentially important mechanism for transport of these small cells to depth. The pattern of increased eukaryotic richness from May 2009-April 2010 is more difficult to interpret because of the

high diversity of these communities. More than half of the band types were not sequenced or resulted in unusable sequences, most likely due to overlapping bands. Those patterns that are discernable do not shed much light on the cause behind this increase in richness. A slight increase in dinoflagellate sequences in summer and fall of 2009, more ciliate sequences in the second half of the time series, and more prasinophyte and radiolarian sequences are found in the second year. However, there are fewer stramenopile, prymnesiophyte, and metazoan sequences in year 2. These trends may be misleading as they represent only the sequenced bands excised from the DGGE and not the full community. Additionally, some taxa, including diatoms, prasinophytes, and prymnesiophytes may be biased against in DGGEs when amplified in combination with other organisms (Chapter 2). The use of other techniques, such as clone libraries, may shed more light on the cause behind the increased richness in the second half of the sampling period.

No obvious seasonality is apparent in POC flux. However, flux was highest during the winter bloom of 2010 with February 2010 the highest in the time series (Fig. 4). During this time phytoplankton abundance was at a high with elevated Chl-a levels (Fig. 2) and DNA concentration in the water column (Fig. 3). DNA flux and POC flux are positively correlated (see above, Fig. 4) showing that increases in POC flux likely originate from plankton, though this does not allow us to distinguish between phototrophic and heterotrophic organisms.

Eukaryotic richness in the water column was high throughout this spring bloom period (Fig. 8), although richness in the trap does not seem to change, suggesting a disproportionate contribution of some taxa to particle flux. Eukaryotic DGGE results show most of the diversity during this time stemming from dinoflagellates, ciliates, and radiolarians, but prasinophyte sequences matching to *Ostreococcus* or *Bathycoccus* were also found in the water column. Dinoflagellate, ciliate, and radiolarian sequences contributed to flux into the trap (data not shown). DGGE analysis of cyanobacterial sequences included eukaryotic plastid sequences from dinoflagellates, diatoms, and the prymnesiophyte *Chrysochromulina* in addition to *Synechococcus* and *Prochlorococcus*. Unfortunately cyanobacterial trap data are not available during this time period, so no conclusions can be made regarding the flux of these taxa.

Prior to the winter bloom in December 2008 a weak positive anomaly indicates a possible anticyclonic eddy, a warm-core rotating water mass on the scale of kilometers, caused a deepening of the thermocline (Fig. 1). Several dinoflagellate, prasinophyte, prymnesiophyte, and ciliate sequences were detected by the eukaryotic DGGE. Interestingly, prasinophyte sequences matching to *Micromonas pusilla*, *Micromonas sp.* and *Bathycoccus prasinos* were detected in both water column and trap samples, again supporting the hypothesis that small phytoplankton can contribute to particle flux. Cyanobacterial DGGE sequences for this month include *Prochlorococcus* and *Synechococcus* in both the water

column and trap samples and *Plectonema sp.*in the water column only. Plastid DNA sequences from the stramenopile *Aureococcus anophagefferens* were found in the water column and trap, and the dinoflagellate *Amylax triacantha* only in the water column. Hansen et al. (in preparation) finds a relatively high abundance of *Prochlorococcus* during this time as well as heterotrophic nanoflagellates and dinoflagellates. Cryptophyte abundance was at the highest in the time-series, although no sequences matching to this group were detected in the DGGE gels. POC flux was fairly high in December (Fig. 4), most likely as an effect of the early winter mixing. These results suggest that small phytoplankton including cyanobacteria may have been substantial contributors to downward flux during this time.

As this bloom progressed we observed sequences matching to dinoflagellates, stramenopiles, prymnesiophytes, and cyanobacteria in the February 2009 traps and the diatom *Thalassiosira weissflogii*, *Prochlorococcus* and *Synechococcus* in the March 2009 trap. Taxa found in the water column but not in the trap in February 2009 included radiolarians and apicomplexa. In March, ciliate, dinoflagellate, prasinophyte, and stramenopile sequences were detected in the water column but were not found in the traps. April 2009 had a diverse community consisting of apicomplexa, cercozoa, chrysophyte, ciliate, diatom, dinoflagellate, radiolarian, stramenopile, and cyanobacterial sequences. Unfortunately, no trap was available for this time period due to weather

conditions. Cell counts from Hansen et al. (in preparation) reveal abundant *Synechococcus*, comparatively low *Prochlorococcus*, low picoeukaryotes, and the most abundant period of cryptophytes. This progression differs from Lomas et al. (2009), who found a succession from diatoms to prymnesiophytes and then to cyanobacteria, however the time scales differ between these studies. Chl-a stays consistent February through March, decreasing in the upper water column in late March and April.

In contrast to the previous winter, a cyclonic cold-core eddy passed through the BATS region in March 2010, causing a shoaling of the 18 °C mode water, which combined with strong winter storms led to mixing of this colder water up to the sea surface. This deep mixing effect resulted in a strengthening of the winter bloom. Chl-a levels were higher than at any point during the time series in March and April 2010. Eukaryotic DGGE results show a diverse community with sequences matching to ciliates, dinoflagellates, prasinophytes, prymnesiophytes and stramenopiles. Taxa contributing to flux into the trap during this time included dinoflagellates *Amoebophrya sp.* (which may indicate a Marine Alveolate) and *Heterocapsa rotundata*, and the chlorophyte *Nanochlorum sp.* Several prymnesiophyte as well as cryptophyte sequences were found during this time in the cyanobacterial DGGE water column samples. Epifluorescence cell counts from the water column indicate the highest abundance of picoeukaryotes in the time series, high cryptophyte, prasinophyte and prymnesiophyte abundance,

and slightly higher *Synechococcus* and diatom abundance (Hansen et al., in preparation). The DNA concentrations in the water column were high during this time point, also indicating a bloom event (Fig. 3). POC flux was high in February and March 2010 and DNA flux reached the highest level in the time series (Fig. 4). This suggests that the deep mixing period in winter, combined with the eddy event led to a substantial phytoplankton bloom resulting in higher flux levels than those seen in the previous winter.

This bloom progressed differently than the previous year. Chl-a was low in December 2009, and higher than in the previous year and a half beginning in February (BATS was not sampled during January because the ship was in the ship-yard), gradually increasing through April. Phylotypes found in the trap material included ciliates, dinoflagellates, streptophyta, and radiolarians in December 2009; ciliates, dinoflagellates, prasinophytes, and radiolarians in February; ciliate, chlorophyte, dinoflagellate, and radiolarian sequences in March; and ciliate, dinoflagellate, prasinophyte and radiolarian sequences in April. Additionally, sequences found in the water column but not in the trap included stramenopiles in December, prasinophytes in February, stramenopile and prymnesiophyte sequences in March and streptophyta in April. Traps could not be amplified for the cyanobacterial DGGEs during this time, but there were a wide variety of eukaryotic plastid sequences in the water column at this time, with dinoflagellate sequences in December; cryptophyte, diatom, dinoflagellate,

prymnesiophyte, and prasinophyte sequences in February; cryptophyte, dinoflagellate, and prymnesiophyte sequences in March; and prymnesiophyte sequences in April. *Prochlorococcus* and *Synechococcus* were present during each time point. Hansen et al. (in preparation) found *Synechococcus* and *Prochlorococcus* to be much less abundant than in the 2008-2009 bloom. In contrast to the more frequent diatom plastid sequences found in the cyanobacterial DGGEs, cell counts of diatoms were lower than in 2009. Picoeukaryote (from flow cytometry counts), and prymnesiophyte/prasinophyte and cryptophyte abundances (from epifluorescence counts) were high during this bloom period (Hansen et al., in preparation), which seems to fit with the sequences detected in these DGGEs. The progression of this bloom mirrors that seen by Lomas et al. (2009) better than the 2008-2009 bloom, with diatom sequences appearing in early February through early March, and prymnesiophytes from late February through April. Both *Prochlorococcus* and *Synechococcus* were present throughout.

4.3. Episodic events

Mesoscale eddies, rotating water masses on the scale of tens of kilometers occur in three types: cyclonic, anticyclonic, and mode water eddies. These features frequently pass through the BATS region (Cianca et al., 2007; McGillicuddy et al., 2007). Mode water and cyclonic eddies can result in a shallowing of the seasonal thermocline and in increased nutrient input, elevated

primary production, and potentially can increase the efficiency of the biological carbon pump. Anticyclonic eddies result in a downwelling of the seasonal thermocline and are not generally associated with a biological response (McGillicuddy et al., 2007; Mouriño-Carballido, 2009). While wind interactions can confound the interpretation of eddy activity (McGillicuddy et al., 2007), satellite altimetry data allows us to track the passage of eddies through the BATS site (http://science.whoi.edu/users/valery/altimetry/). A positive sea level anomaly is associated with an anticyclonic or mode water eddy, a negative anomaly with a cyclonic eddy. Several potential eddies can be detected during our sampling period.

Altimetry data suggest a fairly weak positive anomaly and deepening of the seasonal thermocline (Fig. 1) in May-June 2008, indicating the passage of an anticyclonic eddy through the BATS site. Chl-a at the deep chlorophyll maximum was higher during May 2008 than at any point besides the 2010 winter bloom. In May 2008 we detected a peak in pico- and nanoeukaryote abundance through flow cytometry and a peak in prymnesiophytes and prasinophytes as well as centric and pennate diatoms through epifluorescence counts. Cell abundance of *Synechococcus* was high in the upper water column and *Prochlorococcus* abundance was high closer to the DCM (Hansen et al., in preparation). Richness of the eukaryote DGGEs was particularly low in the upper water column (Fig. 8) while richness in the cyanobacterial DGGEs were at the highest levels in the time

series, particularly in the fixed trap samples (Fig. 9). Eukaryotic DGGEs were dominated by dinoflagellate sequences, with several bands matching to ciliates, radiolarians, and prasinophytes. A variety of dinoflagellate sequences were detected in the traps, including potential members of the marine alveolates. Ciliate sequences were also found in the traps. Cyanobacterial DGGEs mainly showed *Synechococcus* and *Prochlorococcus* sequences in trap material, but sequences matching to the stramenopile *Aureococcus anophagefferens* and the diatoms *Rhizosolenia setigera* and *Amphora coffeaeformis* were also detected. During this time POC flux was comparatively low (Fig. 4).

In October and November 2008 a positive anomaly passed through the area. This possible anticyclonic eddy caused a deepening of the thermocline (Fig. 1). Chl-a levels were not particularly high during this time (Fig. 2). Richness of both the eukaryotic and cyanobacterial DGGEs was quite low as well (Fig. 8, 9). Several dinoflagellate, stramenopile, prasinophyte, prymnesiophyte, ciliate, and radiolarian sequences were detected on these DGGEs. Trap samples from October could not be amplified with the cyanobacterial primers, but the November traps contained sequences matching to the stramenopiles *Aureococcus anophagefferens* and *Dictyochophyte sp.* and the diatom *Rhizosolenia setigera* in addition to several strains of *Synechococcus* and *Prochlorococcus*. Hansen et al. (in preparation) found *Prochlorococcus* and dinoflagellates to be abundant during these months. Additionally, that study revealed a substantial peak in the

abundance of pennate diatoms at 90 m in October, however no sequenced bands matching to diatoms were detected in the DGGEs. The POC flux may have been affected by this event, with slightly higher flux levels in October, but a fairly low flux in November (Fig. 4).

Altimetry data for May and June 2009 and a corresponding deepening of the thermocline (Fig. 1) also suggest the passage of an anticyclonic eddy. Chl-a is fairly low (Fig. 2), POC flux is roughly average (Fig. 4), as is the DNA concentration in the water column as well as DNA flux in June 2009 (Fig. 3, 4). May 2009 had the highest eukaryotic DGGE richness found in the trap samples but the water column richness was not particularly high (Fig. 8). Cyanobacterial DGGEs show a fairly high richness in the upper water column in May 2009, but after this point cyanobacterial richness rapidly decreased for the remainder of the time series (Fig. 9). Eukaryotic DGGEs for these months show mainly dinoflagellate and radiolarian sequences in the water column and trap samples. Cyanobacterial DGGEs show plastid sequences from stramenopiles and the prymnesiophyte *Chrysochromulina* in the water column along with several strains of *Prochlorococcus* and *Synechococcus*, but it is not known whether these contributed to the trap as DNA could not be amplified with the cyanobacterial primers after April 2009. Hansen et al. (in preparation) found slightly elevated *Prochlorococcus* abundance during these months and high nanoeukaryote abundance, but most other groups showed fairly low abundance.

While there is no available altimetry data for July-December 2009, an uplift of the thermocline (Fig. 1) and elevated POC and DNA flux (Fig. 4) in July 2009 may indicate the passage of a cyclonic eddy. Chl-a levels and water column DNA concentration were quite low during this time (Fig. 2, 3). Cell abundance of both *Prochlorococcus* and nanoeukaryotes were decreasing from the levels found in previous months (Hansen et al., in preparation). Eukaryotic DGGEs from May-July 2009 show mainly dinoflagellate and radiolarian sequences in the water column and trap samples. Richness in both cyanobacterial and eukaryotic DGGEs was fairly low with the exception of a large peak in eukaryotic richness at 120 m depth (Fig. 8, 9). Richness in the eukaryotic trap samples was comparatively low (Fig. 8). Several prymnesiophyte and stramenopile sequences were also found in the water column plastid DGGEs during this time, but it is unknown whether these may have contributed to flux due to the absence of amplified trap DNA.

In August 2009, the thermocline depth increased again, indicating a possible anticyclonic eddy. Neither POC, water column DNA concentration, nor DNA flux were particularly high (Fig. 3, 4) which is to be expected for this kind of eddy, and Chl-a levels dropped off at this point, not increasing again until the winter bloom (Fig. 2). Richness in cyanobacterial DGGEs was low, while in the eukaryotic DGGE a high richness was measured at the surface and at 60m depth. Eukaryotic DGGE results show this time period dominated again by dinoflagellates, marine alveolates, ciliates, and radiolarians. Dinoflagellate and

radiolarian sequences were found in the trap as well as the diatom genus *Thalassiosira*. In the cyanobacterial DGGE only *Synechococcus*, *Prochlorococcus* and the dinoflagellate *Dinophysis mitra* were found in the water column. The only groups found abundantly in the water column during this time based on cell counts were *Prochlorococcus*, high prymnesiophyte and prasinophyte abundance at 85 m, and high abundance of heterotrophic nanoflagellates and dinoflagellates (Hansen et al., in preparation).

Analysis of these events seems to indicate that episodic events and perhaps seasonality do have an effect on the composition of plankton contributing to downward particle flux, supporting our second hypothesis. This is evident both by changes in the composition of the community in the water column as well as differences in trap material. Additionally, while dinoflagellates (the most common taxa throughout the time series) and sequences matching to larger taxa were often detected in trap material, a variety of sequences matching to small taxa were also found in the traps.

4.4. Limitations of the method and future efforts

Denaturing gradient gels enable us to analyze a large amount of samples quickly and are useful in making comparisons between samples. Taxonomic resolution from DGGEs is limited due to the relatively short fragment size (approximately 300-500 bp). Additionally, since only the most dominant bands can be excised and sequenced from these gels and overlapping bands cannot be

sequenced, this method does not allow for the identification of the full community. The presence of metazoan sequences in samples amplified with universal eukaryotic primers also presents a problem in that metazoan sequences cannot be completely excluded. In order to achieve a more detailed analysis we choose several samples of interest for cloning and sequencing (Chapter 4). This method allows for a greater taxonomic resolution, taxonomic identification of the community is limited only by library size, and metazoan sequences can easily be removed. Clone libraries are, however, more time intensive and are thus difficult to use exclusively in time-series studies. These methods used in parallel allow us to track major trends throughout a longer time series and also complete a detailed analysis of specific points of interest.

The more we understand about the role of plankton in the biological carbon pump, the better we can predict how the ocean will react to global climate change. Rising global temperatures and CO_2 levels will increase sea surface temperatures, leading to increased stratification with reduced nutrient input to the sea surface (Bopp et al., 2005; Falkowski and Oliver, 2007) and lowered pH of the ocean (Hays et al., 2005). These conditions will have various effects on the plankton community, most likely selecting for small sized phytoplankton, which because of their larger surface to volume ratio are better competitors in highly stratified, nutrient-poor conditions (Falkowski and Oliver, 2007). In several modeling studies, this has been projected to lead to a decrease in export flux

(Bopp et al., 2001; Cox et al., 2000; Fung et al., 2005). However, in light of recent data showing the ability of small phytoplankton to sink through packaging mechanisms, this shift to smaller cells may not necessarily lead to a less efficient biological carbon pump. Modeling studies incorporating these findings will allow for more accurate predictions of the role of ocean biogeochemistry especially in oligotrophic gyres in a changing climate.

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Table Captions.

Table 1. Overview of taxonomic groups represented in bands from eukaryotic denaturing gradient gels. W= water column T= trap. Dinoflag.=Dinoflagellate, Alveol.=Marine Alveolate, Stramen.=Stramenopile, Prymn=Prymnesiophyte, Prasino.=Prasinophyte, Chloro.=Chlorophyte, Radiolar.=Radiolarian, Cercoz.=Cercozoan, Strept.=Streptophyta.

Table 2. Overview of taxonomic groups represented in bands from cyanobacterial denaturing gradient gels. W= water column T= trap. Synecho.=Synechococcus, Prochloro,=Prochlorococcus, Stramen.=Stramenopile, Dinoflag.=Dinoflagellate, Prymn=Prymnesiophyte, Prasino.=Prasinophyte, Verrucomicro.= Verrucomicrobia.

Figure Captions.

Fig. 1. Contour plot of temperature in the upper 150 m from May 2008-April 2010.

Fig. 2. Contour plot of extracted chlorophyll in the upper 150 m from May 2008-April 2010.

Fig. 3. Contour plot of DNA concentration (ng/L) as calculated by quantitative PCR.

Fig. 4. Time series of particulate organic carbon flux at 150 m as measured by particle interceptor traps from May 2008-May 2010 (grey bars) and DNA flux from July 2009-March 2009 as calculated by quantitative PCR (black circles).

Fig. 5. POC flux data from the BATS time series site from 1988-2010.

Fig. 6. Examples of denaturing gradient gels from this study. Eukaryotic (A) and cyanobacterial (B) DGGE gel from March 24, 2010.

Fig. 7. Similarity dendrograms for DGGE gels from Fig. 6. Eukaryotic (A) and cyanobacterial (B) dendrograms from March 24, 2010.

Fig. 8. Time series of eukaryotic richness (DGGE).

Fig. 9. Time series of cyanobacterial richness (DGGE).

Fig. 10. Cyanobacterial DGGE sequence matches. Matched bands corresponding to cyanobacterial sequences (red), eukaryotic plastid sequences (blue) and unknown or unsequenced band types (green) in each cyanobacterial DGGE.

Tables.

Table 1.

Taxa	May-08	Jun-08	Jul-08	Aug-08	Sep-08	Oct-08	Nov-08	Dec-08	Feb-09	Feb-09	Mar-09	Apr-09	May-09	Jun-09	Jul-09	Aug-09	Sep-09	Oct-09	Nov-09	Dec-09	Feb-10	Feb-10	Mar-10	Mar-10	Apr-10
Ciliate.		WT	W	W	WT	WT		W			W	W				W		W	WT	WT	WT	W	W	WT	WT
Dinoflag.	WT	WT	WT	WT	WT	WT	WT	WT	WT	W	W		WT	W	WT	WT	WT	WT	WT	WT	WT	WT	WT	W	WT
Alveol.		W			W		WT			W		W							W						
Diatom											WT	W				WT									
Stramen.					W	WT			WT	WT		W						W	WT	W			W		
Prymn.			T	WT	W			W	WT	W														W	
Prasino.	W							WT			W										W	WT			WT
Chloro.																								WT	
Radiolar.		WT		WT		WT			W			W	WT		WT	T		WT	WT	T	T		W	WT	
Cercoz.			W									W							W						
Strept.																				WT					W

Table 2.

Taxa	May -08	Jun -08	Jul -08	Aug -08	Sep -08	Oct -08	Nov -08	Dec -08	Feb -09	Feb -09	Mar -09	Apr -09	May -09	Jun -09	Jul -09	Aug -09	Sep -09	Oct -09	Nov -09	Dec -09	Feb -10	Feb -10	Mar -10	Mar -10	Apr -10
Synecho.	WT	WT	W	W	W	W	WT	WT	WT	WT	WT	W	W	W		W	W	W	W	W	W	W	W	W	W
Prochloro.	WT	WT	W	W	W	W	WT	WT	WT	W	WT	W	W	W	W	W	W	W	W	W	W	W	W	W	W
Diatom	W	T		W			WT														W	W			
Stramen.		WT	W	W	W	W	WT	WT	W	W	W	W	W												
Dinoflag.								W		W	W	W		W	W	W				W	W	W		W	
Prymn.														W	W		W				W	W	W	W	W
Prasino.																					W	W			
Euglenozoa					W	W																			
Cryptophyte																					W	W		W	
Verrucomicr.		WT																							
Oscillatoria					W		WT	W					W												
Cyanobium					W	W											W	W	W						
*=No Trap			*	*	*	*						*	*	*	*	*	*	*	*	*	*	*	*	*	*

Figures.

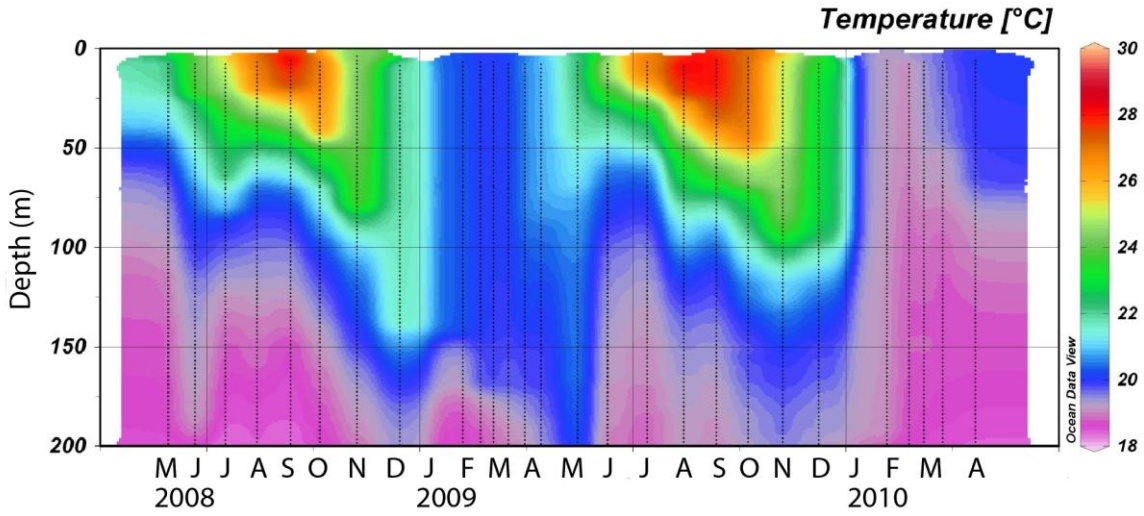


Fig. 1.

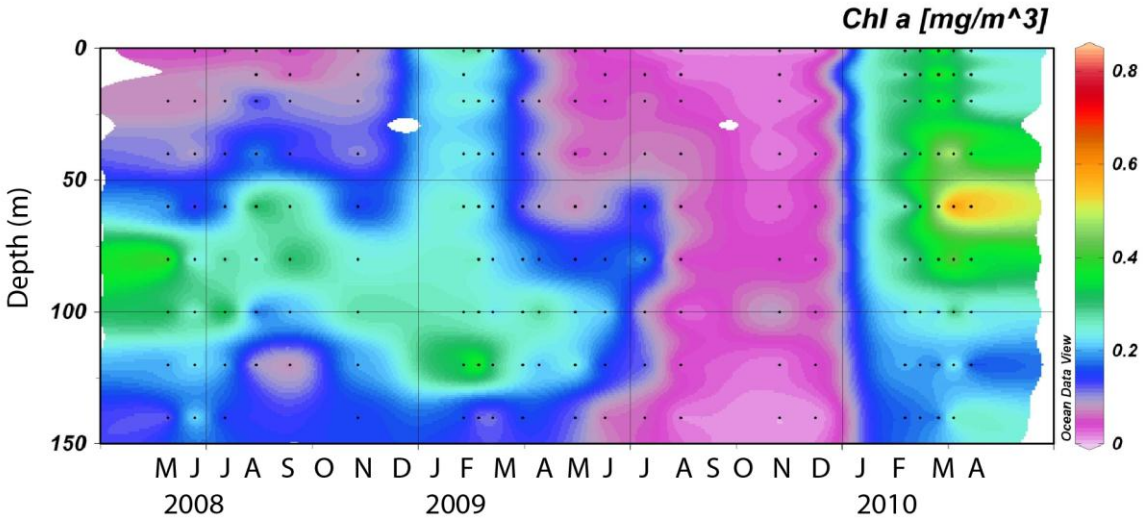


Fig. 2.

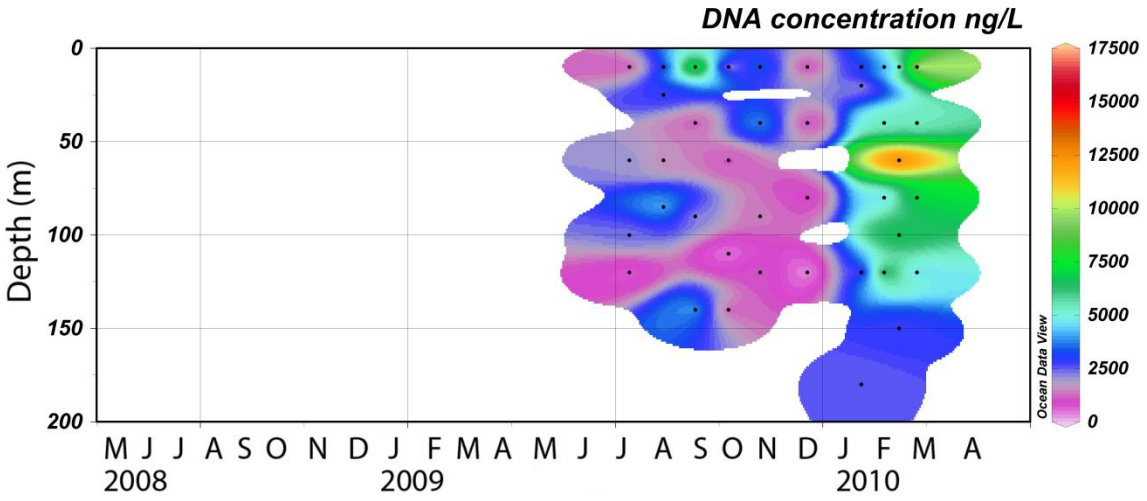

DNA concentration ng/L
Depth (m)
0
50
100
150
200
M J J A S O N D J F M A M J J A S O N D J F M A
2008
2009
2010
17500
15000
12500
10000
7500
5000
2500
0
Ocean Data View

Fig. 3.

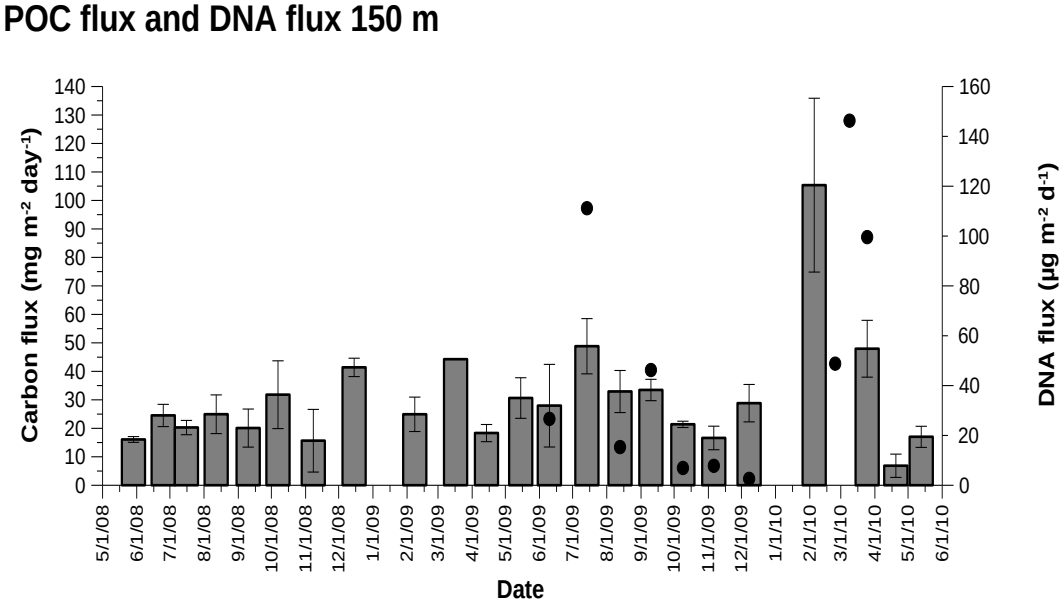

POC flux and DNA flux 150 m
Carbon flux (mg m-2 day-1)
DNA flux (µg m-2 d-1)
Date
5/1/08
6/1/08
7/1/08
8/1/08
9/1/08
10/1/08
11/1/08
12/1/08
1/1/09
2/1/09
3/1/09
4/1/09
5/1/09
6/1/09
7/1/09
8/1/09
9/1/09
10/1/09
11/1/09
12/1/09
1/1/10
2/1/10
3/1/10
4/1/10
5/1/10
6/1/10

Fig. 4.

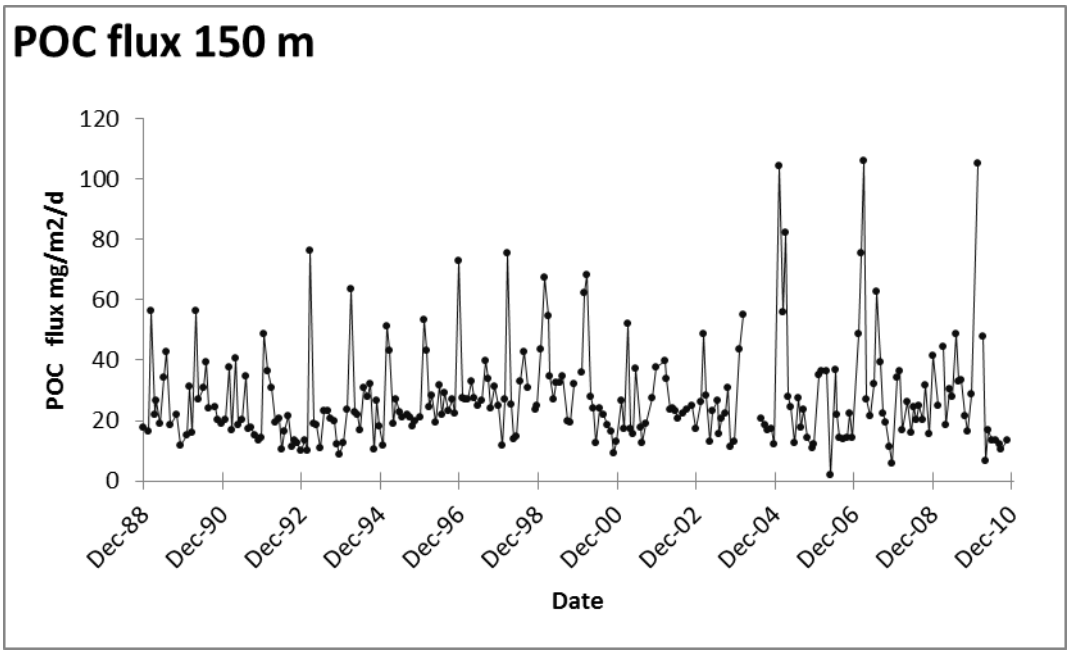

POC flux 150 m
POC flux mg/m2/d
120
100
80
60
40
20
0
Dec-88
Dec-90
Dec-92
Dec-94
Dec-96
Dec-98
Dec-00
Dec-02
Dec-04
Dec-06
Dec-08
Dec-10
Date

Fig. 5.

A. **B.**

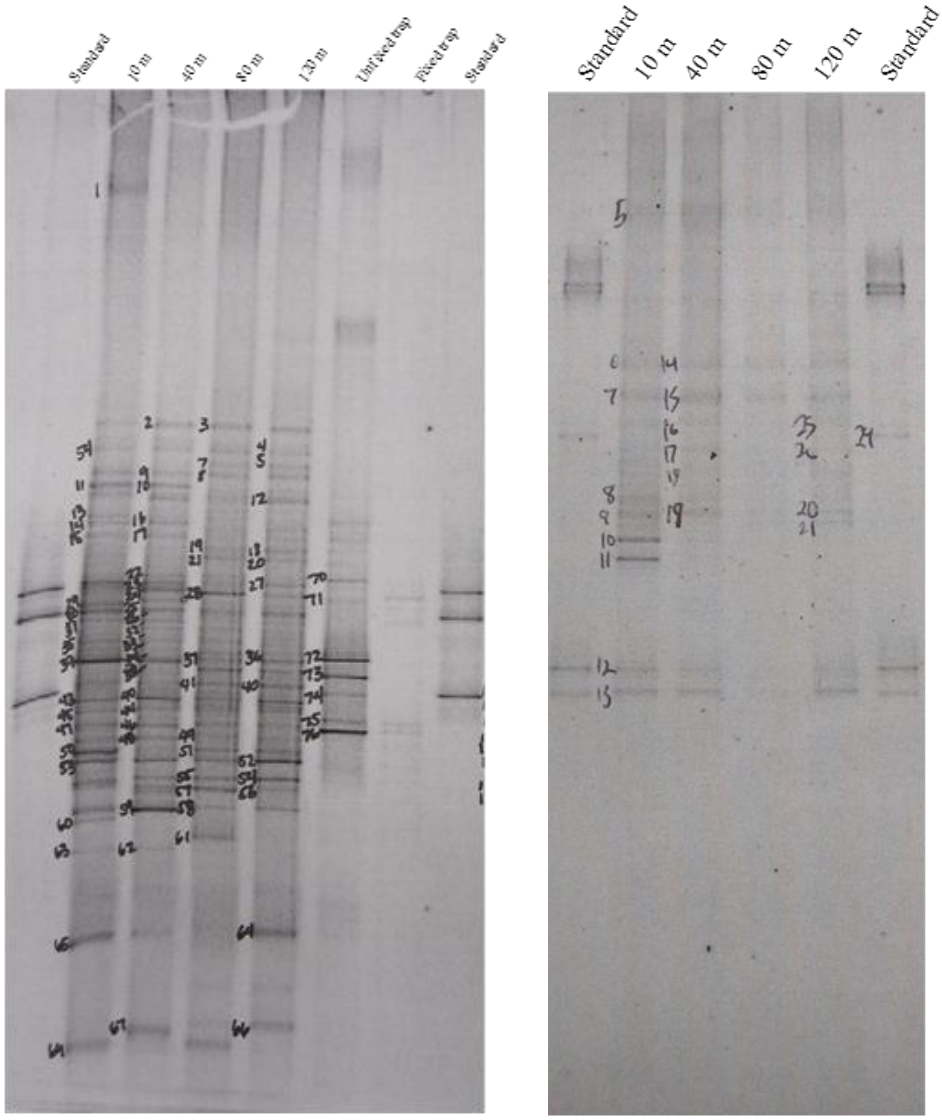
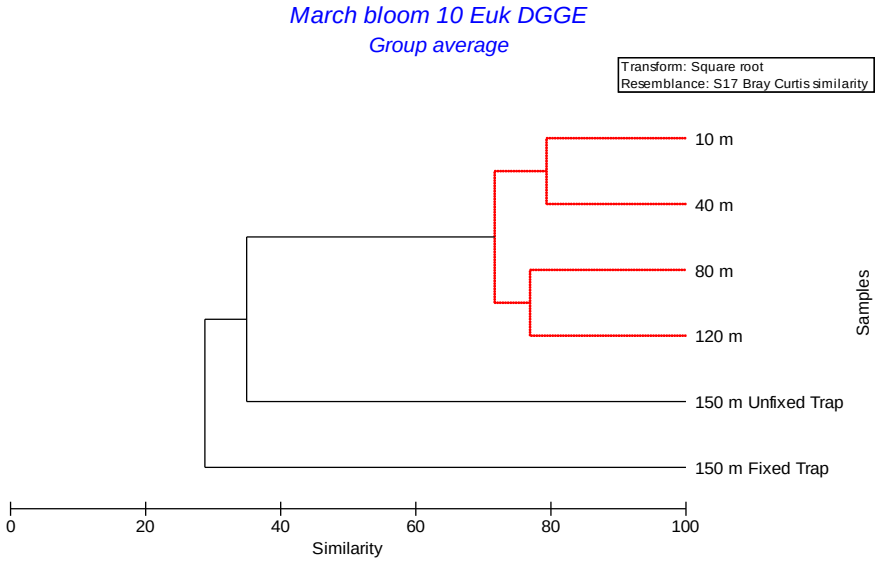

Standard
10 m
40 m
80 m
120 m
Unfixed trap
Fixed trap
Standard
Standard
10 m
40 m
80 m
120 m
Standard

Fig. 6.

A.


March bloom 10 Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
120 m
150 m Unfixed Trap
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

B.

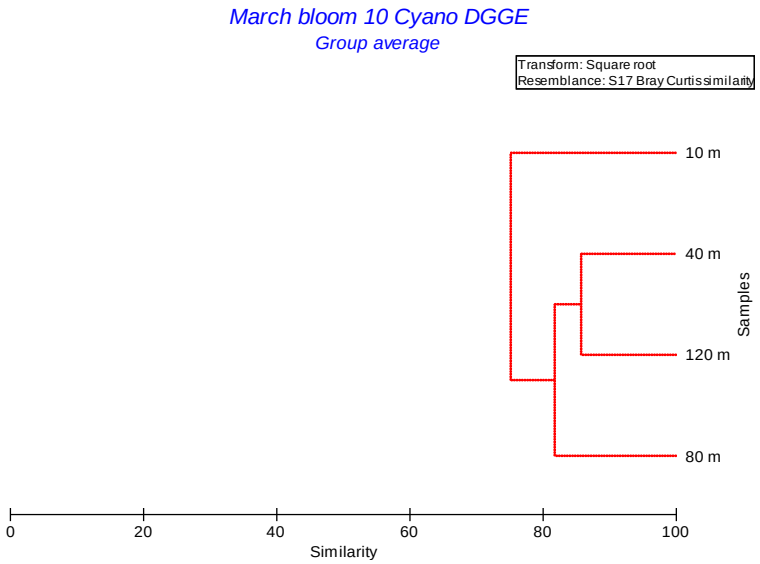

March bloom 10 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
120 m
80 m
Samples
0
20
40
60
80
100
Similarity

Fig. 7.

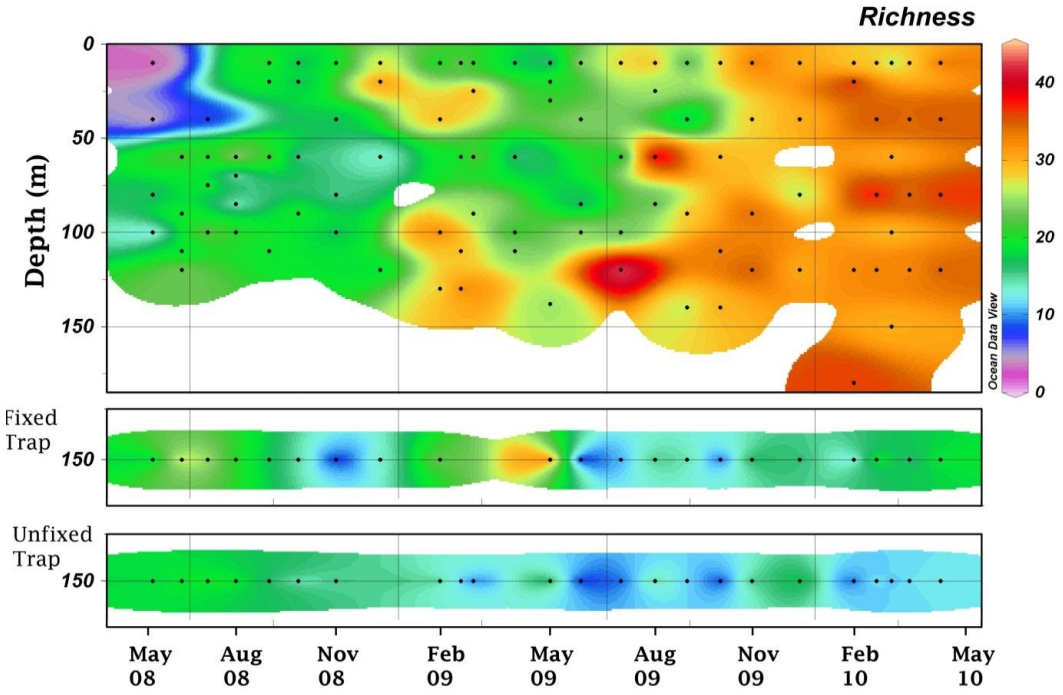

Richness
Depth (m)
0
50
100
150
40
30
20
10
0
Ocean Data View
Fixed Trap
150
Unfixed Trap
150
May 08
Aug 08
Nov 08
Feb 09
May 09
Aug 09
Nov 09
Feb 10
May 10

Fig. 8.

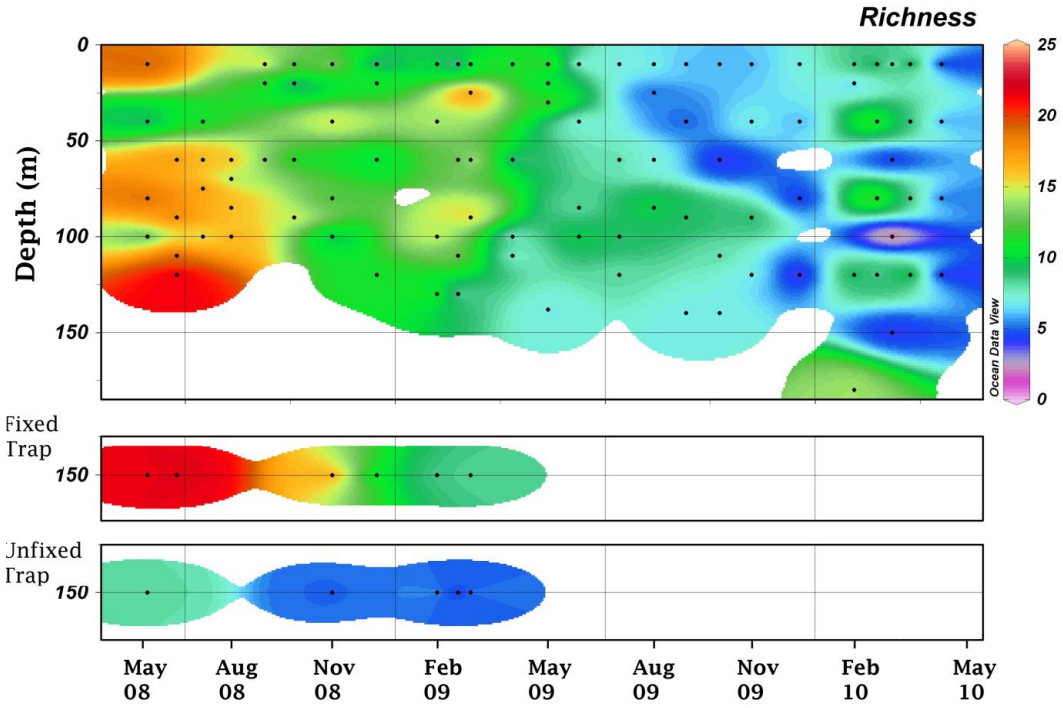

Richness
Depth (m)
0
50
100
150
25
20
15
10
5
0
Ocean Data View
Fixed Trap
150
Unfixed Trap
150
May 08
Aug 08
Nov 08
Feb 09
May 09
Aug 09
Nov 09
Feb 10
May 10

Fig. 9.

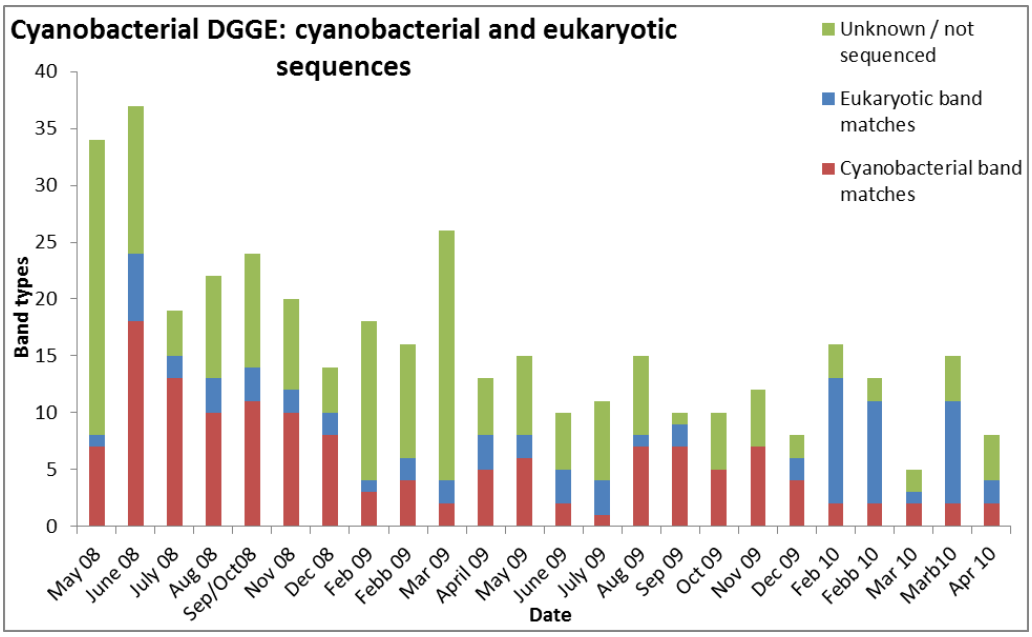

Cyanobacterial DGGE: cyanobacterial and eukaryotic sequences
Unknown / not sequenced
Eukaryotic band matches
Cyanobacterial band matches
Band types
0
5
10
15
20
25
30
35
40
May 08
June 08
July 08
Aug 08
Sep/Oct08
Nov 08
Dec 08
Feb 09
Febb 09
Mar 09
April 09
May 09
June 09
July 09
Aug 09
Sep 09
Oct 09
Nov 09
Dec 09
Feb 10
Febb 10
Mar 10
Marb10
Apr 10
Date

Fig. 10.

CHAPTER 4

THE CONTRIBUTION OF PROTISTS TO PARTICLE FLUX AT THE BERMUDA ATLANTIC TIME-SERIES STUDY

Jessica Amacher[1], Susanne Neuer[1*], Charles Baysinger[1] Michael Lomas[2]

[1]Arizona State University, School of Life Sciences, Tempe, AZ 85287

[2]Bermuda Institute of Ocean Sciences, St. George's GE01, Bermuda

Abstract

The importance of small planktonic organisms in downward particle flux has been postulated in numerous studies, but because cells contained in particle trap material are difficult to identify with traditional methods, few direct observations exist. Clone libraries of small subunit ribosomal RNA (SSU RNA) genes now allow us to determine the relative contributions of different taxa to particle flux. Here we present results from a selection of water column and 150 m particle trap samples from a two year molecular time-series at the Bermuda Atlantic Time-series Study (BATS). Samples were selected from the winter–spring bloom periods in 2009 and 2010, and also from October 2008 and July 2009, which were selected for cloning and sequencing. Temperature, chlorophyll, particulate organic carbon flux, and denaturing gradient gel electrophoresis data were used to identify unusual events in the upper water column. Our results indicate that protist taxa contribute to flux at a rate proportional to their abundance in the water column. Dinoflagellate and marine alveolate SSU RNA sequences made up the greatest fraction of both the water column and trap libraries. Diatom SSU RNA sequences were present in several samples, but were not abundant. Only one time point was available in which diatom sequences were detected in both the water column and trap libraries. In this case their relative abundance in the trap library was found to be roughly proportional to that of the water column libraries. SSU RNA sequences matching to taxonomic groups

known to include small-sized cells, for example the prasinophyte genera *Bathycoccus*, *Micromonas* and *Ostreococcus*, were detected in a greater proportion in trap material than in the corresponding water column samples. In one sample from the late winter bloom, prasinophyte sequences accounted for up to 4.5% of water column libraries and 13% of the trap library. Additionally, group-specific qPCR for Mamiellales provided the first quantitative estimates of the percent contribution of a group of small-sized taxa to POC flux. Our results indicate a flux of Mamiellales ranging from 0.002 to 0.47 mgC m^{-2} d^{-1}, contributing up to 5.7% of total POC flux.

1. Introduction

Marine phytoplankton play an essential role in the global carbon cycle. Through the process of photosynthesis, they convert dissolved carbon dioxide into organic matter, and eventually sink into the deep ocean in the form of aggregates and fecal pellets, a process known as the biological carbon pump. While much of this organic carbon is remineralized through the microbial loop (Sherr and Sherr, 1994), once particles sink below 500 m they are removed from the atmosphere for tens to hundreds of years (Ono et al., 2001; Sherr and Sherr, 1994). This process, along with the physical carbon pump, constitutes the only true net sink for atmospheric carbon dioxide (Sabine et al., 2004). In changing global conditions, temperature increases and rising atmospheric CO_2 levels will result in warmer, more acidic, more stratified oceans with shallower mixed layer depths and decreased nutrient input (Bopp et al., 2005; Falkowski and Oliver, 2007). Plankton communities are likely to undergo changes in these conditions, with decreased nutrient availability selecting for smaller cells (Falkowski and Oliver, 2007).

Larger organisms, particularly those with mineralized tests, have been thought to contribute more to particulate organic carbon flux (POC) with the contribution of smaller cells in the pico- (<2 μm) and nano- (2-20 μm) size classes considered negligible (Boyd and Newton, 1999; Michaels and Silver, 1988; Sarthou et al., 2005). However, there is increasing evidence that small cells

can also contribute to particle flux through packaging into aggregates and rapidly sinking fecal pellets. Richardson and Jackson (2007) hypothesized in a modeling study that picoplankton contribute to export production at a rate proportional to their primary production. In a DNA-based molecular study Amacher et al. (2009) were able to identify sequences closely matching small taxa in clone libraries of trap DNA, and found a roughly proportional contribution of these small taxa in water column and trap libraries. Unexpectedly, diatom sequences were rare in traps despite their high abundance in the water column. Brew et al. (2009) found a significant correlation between picoplankton abundance in the water column and POC flux, with aggregation being an important mechanism contributing to this flux. Recently Lomas and Moran (2011) found that nanoeukaryotes and cyanobacteria are significant contributors to POC flux in particles collected using both Niskin bottles and size-fractionated in-situ pumps. Understanding the role of different taxonomic groups in the biological carbon pump is necessary to predict the consequences of global climate change. This is particularly important in subtropical gyre regions, which are dominated by pico- and nanoplankton (Steinberg et al., 2001) and make up nearly 60% of the world's oceans (Eppley and Peterson, 1979; (Longhurst, 1998).

Particle flux can be quantified with free floating sediment traps below the euphotic zone. However, identifying organisms contained in trap material is difficult due to the packaging of cells into aggregates and fecal pellets and

degradation of pigments, making identification by microscopy, flow cytometry, or high performance liquid chromatography (HPLC) nearly impossible. DNA-based molecular methods allow us to determine more specific taxonomic classifications of organisms contributing to downward flux (Amacher et al., 2009). A relatively stable molecule, DNA is more resistant to breakdown than other tracer compounds, such as pigments, proteins, or lipids (Nejstgaard et al., 2008). Intact and even culturable cells have been found in aggregates and fecal pellets collected in particle traps (Fischer et al., 1996; Pfannkuche and Lochte, 1993; Turley and Mackie, 1995), in fecal pellets (Jansen and Bathmann, 2007; Martin et al., 2006), and in the hindguts of mesozooplankton (Wilson and Steinberg, 2010). Several studies have shown that DNA can be extracted from guts and fecal pellets of zooplankton (Martin et al., 2006; Nejstgaard et al., 2003; Troedsson et al., 2009). Techniques such as denaturing gradient gel electrophoresis (DGGE, see Chapter 3), cloning and sequencing (Amacher et al., 2009, see Chapter 1, this study), and quantitative PCR (this study) can be used to identify organisms contained in trap material and to compare the relative abundance of taxa in the water column with those collected in particle trap material, allowing us to determine which organisms may be important in POC flux.

In this study we use cloning and sequencing as well as quantitative PCR to determine the contributions of protist taxa to POC flux near the Bermuda Atlantic Time-series Study (BATS). This work is part of a two year time-series from May

2008-April 2010. A full time series using DGGE was conducted (Chapter 3) allowing us to observe seasonality and changes in the contributions of different taxa to particle flux over time. Here we will investigate the contribution of the eukaryotic community during the winter bloom periods of 2009 and 2010, as well as during fall (October 2008) and summer (July 2009) sample. The latter dates were selected because temperature and chlorophyll a (chl-a) profiles, POC flux values, and results of DGGEs indicated unusual events in the upper water column. Clone libraries, while more time intensive, provide greater taxonomic resolution and allow for the identification of both rare and abundant taxa compared to DGGEs.

2. Methods

2.1. Field Sampling

Samples for this work were collected as part of a two year time-series from May 2008-April 2010 carried out at the Bermuda Atlantic Time-series Study (BATS) as described in Chapter 3. Samples for DNA analysis were collected from the upper water column in Niskin bottles on a rosette with attached conductivity, temperature, and depth (CTD) sensors. Both fixed (72 hr) and unfixed (24 hr) particle interceptor traps (PITs) were deployed at 150 m in order to collect particles sinking below the mixed layer. Particulate organic carbon flux

was determined from the three day fixed traps as part of the BATS core program. See Chapter 3 for further detail.

2.2. DNA Extraction and PCR

Extractions were carried out using the Phenol:Chloroform:Isoamyl alcohol (PCI, Sigma) method as described in Countway et al. (2005), see Chapter 1 for further detail. Approximately 10-50 ng of DNA extract was used as template for PCR using primers EukA and EukB (Medlin et al., 1988) as in Diez et al. (2001). This primer set amplifies the nearly full (~1800 bp) 18S rRNA gene. Each reaction contained 5 µL of 10X Takara Ex Taq Buffer, 4 µL of dNTP's (200 µM each), 1 µL 10% bovine serum albumin (BSA) 0.3 µL of each primer (0.3 µM), 38.15 µL of water, and 0.25 µL of Takara Ex Taq polymerase along with the template. The thermal cycler program consisted of an initial denaturation at 94 °C for 3 minutes; 30 cycles of 94 °C for 45 s, 55 °C for 1 minute, 72 °C for 3 minutes; and a final extension at 72 °C for 5 minutes. PCR products were run on an agarose gel to check for proper amplification and approximate concentration and then purified with the EZNA Cycle Pure cleanup kit (Omega).

2.3. Clone libraries

Clone libraries were constructed for samples selected based on prior results of DGGE and hydrographic data. For this in depth clone library analysis, we chose to focus on the 2009 and 2010 winter bloom periods as well as some interesting dates from fall 2008 and summer 2009. Samples were chosen after

consideration of hydrographical data and results from DGGEs (see Chapter 3). For each sampling date two depths from the water column (10 m and the deep chlorophyll maximum) and one 150 m trap sample was selected (Table 1). Unfixed traps were selected unless the sample was unavailable or DGGE results indicated that the fixed trap would be more suitable due to occurrence of fungi and metazoans in the unfixed traps. Purified PCR products were inserted into the pCR 2.1-TOPO plasmid (Invitrogen) and sent on ice to the University of Washington High-throughput DNA sequencing lab (http://www.htseq.org/) for cloning and sequencing. Positive clones were sequenced in one direction with the Euk528F primer (Elwood et al., 1985), yielding approximately 700-800 bp sequences. Broad taxonomic groups were determined for each sequence using their closest similarity match from BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/) analysis. Sequences were then grouped into operational taxonomic units (OTUs) with the DNA Baser program (version 2.11.0.933, http://www.dnabaser.com) using an identity threshold of 95%. Relatedness between each library was determined from the relative abundances of OTUs from each library and calculating Bray-Curtis coefficients of similarities (S), (Bray and Curtis, 1957) with PRIMER v. 6. Dendrograms showing the degree of similarity between samples were made for each set of libraries.

2.4. Quantitative PCR

The primers EUK528f (Elwood et al., 1985) and PRAS04r (Zhu et al., 2005), specific for the order Mamiellales, members of the Chloroplastida group (Adl et al., 2005), were utilized for qPCR of selected samples in order to quantify the contributions of some of the smallest organisms (including *Bathycoccus*, *Micromonas*, and *Ostreococcus*) recovered from both water column and trap samples. A standard curve was constructed with known concentrations of *Micromonas pusilla* (CCMP487) DNA in a dilution series of 10^{-4} ng to 10 ng of DNA per reaction. Reactions were prepared in triplicate each with 5 µL of diluted template DNA (1.7-2.5 ng), 300 nM each of primers EUK528f and PRAS04r, 10 µL SYBR Green master mix, and sterile MilliQ water up to 20 µL. Quantitative PCR reactions were run on an ABI7900HT thermocycler. The protocol consisted of an initial denaturation at 94 °C for 2 min; 40 cycles of 94 °C for 15 s and 60 °C for 1 min, then a melting cycle beginning at 60 °C and ending at 94 °C with a 2% ramp rate.

3. Results and Discussion

3.1. Physical conditions and carbon flux

The temperature profile for October 2008 shows a strongly stratified water column, mixed down to approximately 50 m depth (Fig. 1A). Chlorophyll data was not available for this time point. Flux values at 150 m were slightly higher than in previous months (see Chapter 3), sharply decreasing with depth down to

300 m. A marked dip in the seasonal thermocline and a positive anomaly detected by altimetry data (http://science.whoi.edu/users/valery/altimetry/) indicates the passage of an anticyclonic eddy. The elevated flux along with a peak in diatom abundance at 90 m as measured by epifluorescence counts (Hansen et al., in preparation) led us to select this sample for analysis through cloning and sequencing.

The mixed layer depth deepened to 150 m in December 2008, gradually increasing to nearly 175 m depth through late February. No chlorophyll data were available for December 2008 nor March 2009, but chl-a values from February 2009 on were relatively high, increasing from early to late February. The February 27th, 2009 sample also showed a substantial peak in chl-a at 120 m depth. POC flux values reached higher levels than in previous months. These samples were chosen for construction of clone libraries in order to follow the progression of the full winter bloom.

During July 2009 the passage of a cyclonic eddy (see Chapter 3), an event known to have a biological effect (Mouriño-Carballido, 2009), may have caused a bloom situation. This seems to have led to an elevated flux level. In addition, the flux of DNA into the trap was at the second highest level detected in the time series (see Chapter 3, Fig. 1F). However, both chl-a levels and DNA concentration in the water column were relatively low (Fig. 1F, Chapter 3). These features made this an interesting time point for further investigation.

Another cyclonic eddy passed through the area during the 2010 winter bloom, leading to an enhanced winter mixing into the 18 °C mode water and a more pronounced bloom (indicated by the elevated chl-a and POC flux during this time (see Chapter 3, Fig. 1G)) compared to winter 2009. Temperature profiles suggest that the bloom began later than in 2008-2009 (see Chapter 3), leading us to choose March and April for these winter bloom libraries. The highest peak in flux was measured in February 2010, but was not investigated in this study as flux values were not available prior to selecting the 2010 clone libraries.

3.2. Clone libraries

Figure 1 shows pie graphs of each of the 24 clone libraries constructed for this study, grouped by sampling date. A minimum of 96 and up to 192 clones per library were sequenced and subjected to BLAST analysis. Low quality sequences were removed as were those matching to metazoans or fungi as our interest in this study focused on protist species and because multicellular organisms would be highly overrepresented (see Table 2). Some of these libraries had too many fungi or metazoan sequences compared with protist sequences to construct a substantial library (Feb. 7, 2009 trap; Mar. 2009 trap; April 2010 trap, see Table 2).

In each of the libraries constructed for this study more than half of the protist clones matched to sequences from the alveolate group, including ciliates, dinoflagellates, marine alveolate groups I, II and III (MALV, Guillou et al., 2008; Massana and Pedrós-Alió, 2008), and uncategorized Syndiniales taxa.

Dinoflagellate and MALV taxa generally seemed to be present in the trap material roughly in proportion to their presence in the water column. Ciliates often made up a higher proportion of taxa identified in the water column libraries than in the corresponding trap samples (October 2008, early and mid-February, March 2009, March and April 2010, see Fig. 2). Small protists in the prasinophyte (including *Ostreococcus*, *Micromonas*, and *Bathycoccus*) and prymnesiophyte (including *Chrysochromulina*, *Emiliania*, and *Scyphosphaera*) groups made up a smaller proportion of each library, but were represented in both water column and trap samples. In some cases prasinophyte sequences accounted for a larger percentage of the trap library than the water column libraries (Oct. 2008, Feb. 21st, 2009, Mar. 2010, see Fig. 3). Diatom sequences were detected but only represented a small proportion of libraries from only four sampling dates (March, 2010 DCM, trap; July 2009 trap; Feb. 21, 2009 DCM; Dec. 2008 trap).

In October 7th, 2008 (Fig. 1A) a dip in the seasonal thermocline as well as a positive sea level anomaly indicated the passage of an anticyclonic eddy (see Chapter 3). POC flux at 150 m was slightly higher than in previous months (see Chapter 3). Epifluorescence microscopy data (Hansen et al., in preparation) indicated a large peak in pennate diatom abundance at 90 m during this time period. However, no diatom sequences were detected in these libraries, nor were they found in the DGGEs of that sample (see Chapter 3). This may be attributed to a bias against diatoms in clone libraries, as observed for the centric diatom

Attheya and an undetermined pennate diatom in a study by Potvin and Lovejoy (2009). However, we could not confirm this observation, instead finding the diatom species *Thalassiosira weissflogii* to be biased for in clone libraries (see Chapter 2). Additionally, the concentration of these organisms in the water column may factor into the absence of diatom sequences in these libraries. Amacher et al. (2009) found a high abundance of diatoms, including *Asterionellopsis sp.*, *Thalassiosira delicatula*, and *Minidiscus trioculatus*, in clone libraries of the water column from the time-series station, ESTOC (European Station for Time-Series in the Ocean, Canary Islands). However, diatom cell concentration was significantly higher in the Amacher et al. (2009) study (550 cells mL^{-1}), than in this 90 m October 2008 sample (97 cells mL^{-1}). Alveolate sequences made up the majority of the clones in both the water column and trap samples. Sequences matching to members of the potentially parasitic MALV and uncharacterized Syndiniales groups (Siano et al., 2011) were the largest contributors to sequences recovered from trap material on this sampling date. Dinoflagellate sequences made up roughly a third of those found in the trap material. While few prasinophyte sequences were detected, they were present in the trap. DGGE results show ciliate, dinoflagellate, stramenopile, and radiolarian sequences in both water column and trap samples (see Chapter 3), all of which were present in these libraries. No significant difference was detected between water column and trap libraries, indicating that there was an overall proportional

contribution of different taxonomic groups to particle flux at 150 m. This seems to be confirmed by the comparative ratios of ciliates, dinoflagellates, MALV, and MAST sequences from these clone libraries (Fig. 2, 3). A significant difference between the water column and unfixed traps was detected in the DGGE for this month, however. This may simply be accounted for by differences between the methods as the fixed traps are deployed an additional two days allowing for the collection of a greater amount of sinking particles and thus more DNA and likely more protist phylotypes.

December 15th, 2008 (Fig. 1B) was another interesting time point. A significant deepening of the thermocline to about 150 m may indicate early winter mixing and perhaps also an anticyclonic eddy (see Chapter 3). POC flux was average, but slightly higher than in the previous or subsequent sampling dates. In epifluorescence counts, Hansen et al. (in preparation) found the highest levels of cryptophytes during this time period. In the 10 m library, sequences matching to cryptophyte taxa made up about 10% of the 10 m library and two sequences in this group were detected in the trap library. Dinoflagellate sequences made up the largest portion of each library. Several sequences from potentially small-sized taxa were detected in these libraries as well as in the 150 m trap. MALV sequences made up 22% and prasinophytes nearly 3% of the trap library. Marine stramenopiles (MAST, largely uncultured heterotrophic flagellates, Massana et al., 2004; Massana et al., 2006), prymnesiophyte and Biliphyte sequences were

also detected. Dinoflagellate and prasinophyte sequences were present in both the water column and traps in the DGGE while ciliates and prymnesiophytes were present only in the water column samples (see Chapter 3). Additionally, DGGE results show samples in the upper water column significantly different from the 120 m water column and 150 m trap samples. While no significant difference was detected between these clone libraries, the 120 m water column sample did group with the 150 m trap sample.

Although no samples were available for January 2009, elevated chl-a levels and mixing down to 150 m depth in February 7th, 2009 indicate that the winter bloom had begun (Fig. 1C). POC flux levels were roughly average during this time. The size of the trap library was reduced due to the high abundance of fungi sequences, limiting our ability for interpretation. Despite the small number of sequences, this trap library was the only one in which no dinoflagellate sequences were detected. However, MALVs made up 88% of the trap library and only about a third of each of the water column libraries. A single MAST and a stramenopile sequence matching to *Thraustochytriidae sp.* made up the rest of the trap library. DGGE results for this date include dinoflagellates, stramenopiles and prymnesiophyte sequences in both the water column and trap and radiolarian sequences in the water column only (see Chapter 3). These results differ from what is found in the trap clone library, but this may likely be due to the small number of clones sequenced (18 clones sequenced). A significant difference

between the water column and trap libraries was detected in both clone library and in the DGGE. This may indicate a disproportionate contribution of some taxa to particle flux. Indeed, MALV and MAST make up a larger proportion of the trap library than the water column libraries (Fig. 2). Ciliate, dinoflagellate, prasinophyte and prymnesiophyte sequences are detected only in the water column and not in the trap (Fig. 3).

On February 21st, 2009 the upper water column was mixed slightly deeper than on the previous sampling date, and an interesting spike in chl-a could be observed at 120 m (Fig. 1D). Prasinophytes, including sequences matching to the genera *Bathycoccus* and *Ostreococcus*, were enriched in the trap library, making up only 4% of the water column libraries, but 13% of the trap library (Fig. 3). No obvious differences were seen in the 130 m library that would account for the high peak in chlorophyll at 120 m. Dinoflagellates made up a smaller percentage of the trap libraries than their relative abundance in the water column libraries and MALV sequences were present in nearly the same proportion in the trap as in the water column. (Fig. 2). Ciliates and radiolarian sequences each accounted for around 10% of water column libraries; prymnesiophyte and diatoms 1-2% of water column libraries but each was absent from the trap libraries (Fig. 2, 3). In the DGGE of this date only stramenopile sequences were detected in both the water column and the traps, seeming to fit the clone library data fairly well as MAST and other stramenopile sequences made up the majority of the trap library.

Dinoflagellate, MALV and prymnesiophyte sequences were detected in the water column only (see Chapter 3). While the clone library water column samples grouped together and were found to be significantly different from the trap, the DGGE results showed the 120 m and trap samples grouping together, with a significant difference between these and the samples from the upper water column (Fig 1D, Chapter 3). In both February 2009 trap libraries larger organisms, such as dinoflagellates, ciliates, and radiolaria, made up a smaller percentage of, or were absent from, the trap libraries. In the February 7th trap MALV sequences made up nearly 90% of the library (Fig. 1C), and on February 21st stramenopile sequences accounted for half of the trap library (Fig. 1D).

On March 17th, 2009 POC flux was the highest observed during the 2008-2009 bloom period (Fig. 1E). This trap had an overabundance of clones matching to fungi (Table 2). Only two dinoflagellate sequences and one stramenopile sequence were available after removing these, thus making a comparison of water column and trap libraries impossible. Sequences found in the water column indicate a diverse protist community, with ciliate, dinoflagellate, and MALV taxa making up half or more of the libraries. Prasinophyte sequences, including those matching to the genera *Micromonas*, *Bathycoccus*, and *Ostreococcus*, made up about 16% of the libraries. Sequences matching to radiolaria and prymnesiophytes were also significant contributors to these libraries. Ciliate, dinoflagellate, and prasinophyte sequences were detected in the water column samples using DGGE,

which fits well with the clone library results (see Chapter 3). Diatom sequences were detected in both the water column and traps in DGGE samples, but no sequences matching to diatoms were found in the clone libraries, perhaps as a result of sample size or bias favoring diatoms in DGGEs and against diatoms in clone libraries (see Chapter 2). While no significant difference was detected between the clone libraries from this month, a significant difference between the water column and trap samples was detected in the DGGE (Fig. 1E, Chapter 3). This may be a result of the very small sample size for this trap library.

On July 14th, 2009 the second highest flux levels in the time series were detected and thermal profiles suggest the passage of a cyclonic eddy (Fig. 1F). Epifluorescence counts indicate a greater abundance in pennate diatoms, with a small peak at 60 m depth (6.5 cells mL^{-1} (Hansen et al., in preparation), but no diatom sequences were detected in these libraries. Flow cytometry measurements from that study also measured slightly elevated levels of pico- and nanoeukaryotes. Ciliates and dinoflagellates are present in relatively the same proportion, but MALVs make up less of the trap library than in the water column libraries (Fig. 2). MAST make up a larger proportion in the trap than the water column (Fig. 3). Only dinoflagellate and radiolarian sequences were detected in the DGGE for this month, found to be present in both the water column and the trap (see Chapter 3). This result differs from the diversity of organisms detected in the clone libraries during this month, and serves as an example of the greater

resolution of clone libraries in identification of the full plankton community. The trap library was found to be significantly different from the water column samples in both the clone library and DGGEs (Fig. 1F, Chapter 3), again suggesting that conditions at this time led to a disproportionate contribution of some taxa to particle flux.

March 24th, 2010 was at the peak of the winter bloom (Fig. 1G). The cyclonic eddy that passed through during this time seemed to have caused an increase in winter mixing, resulting in elevated chl-a levels as well as the POC flux, which were particularly high during this month. Flow cytometry showed a peak in the abundance of picoeukaryotes, and epifluorescence cell counts indicate high cryptophyte, prasinophyte and/or prymnesiophyte abundance, and slightly elevated diatom abundance at 40 m (21.6 cells mL^{-1} (Hansen et al., in preparation). Cryptophyte sequences closely matching to the genus *Teleaulax* were detected only in the 10 m library. Prasinophyte and prymnesiophyte sequences, including *Micromonas*, *Bathycoccus*, and *Ostreococcus* were detected in the water column libraries, and Prasinophyceae, *Pyramimonas*, and *Pycnococcus* in the trap library. Diatom sequences of the genera *Fragilariopsis*, *Thalassiosira*, and *Skeletonema* were detected at 80 m, accounting for 2.1% of the library, and *Haslea*, *Minidiscus*, *Minutocellus*, and *Cylindrotheca*, making up 3.2% of the 150 m trap clone library. Ciliate sequences, while abundant at 10 m, were relatively less abundant at 80 m and were absent from the 150 m trap library.

MALVs make up a 40-50% of the 80 m and 150 m libraries, but less than 10% of the 10 m library though the average ratios of the water column are close to their ratio in the trap library (Fig. 2). The contribution of dinoflagellate sequences to the trap library is proportional to the water column libraries (Fig. 2). Prasinophytes, MAST, and diatoms made up a greater proportion of the trap library than the water column libraries (Fig. 3). DGGE sequences detected during this month included ciliate, chlorophyte, and radiolarian sequences in both the water column and the traps and dinoflagellates and prasinophytes in the water column only (see Chapter 3), but these results were not representative of the sequences detected in the clone libraries for this date. The 80 m water column and 150 m trap samples were not significantly different from each other, but the 10 m sample was found to be significantly different from both the 80 m water column and the trap libraries. However, the 80 m water column and 150 m trap samples were less than 5% similar to each other. A different result was seen in the DGGE, with the water column samples grouping together and a significant difference between the water column and the traps (see Chapter 3).

By April 21st, 2010 the water column began to stratify and a peak in chl-a had developed at 80 m depth (Fig. 1H). POC flux was greatly reduced from the high levels seen in late March. This trap library was also significantly reduced due to the high numbers of fungi sequences, but enough protist sequences were detected to enable the comparison with water column libraries. As seen in

previous months, ciliate sequences were found abundantly in the water column but only one sequence was found in the trap library. Dinoflagellate and MALV sequences were found in roughly the same proportion in the water column and the trap, although the ratios in the traps were slightly higher than in the water column libraries (Fig. 2). Sequences matching to prasinophytes made up a slightly higher proportion of the trap library (5.7%) than the average found in the water column libraries (4.1%) (Fig. 3). MAST sequences also were enriched in the trap library (Fig. 3). The April 2010 DGGE results show ciliate, dinoflagellate, and prasinophyte sequences in both the water column and the trap and streptophyta in the water column only (see Chapter 3). Streptophyta, members of the Charophyta in the Chloroplastida super group (Adl et al., 2005), were not detected in these libraries, but the rest seems to fit with the clone library results. It is worth noting that in each case the diversity and taxonomic resolution detected with clone libraries provides a considerably improved representation of the community present in the water column and traps during each time point. No significant difference was detected between the water column and trap libraries at this time, though the water column samples grouped together. DGGE results again show a significant difference between the water column and trap samples (see Chapter 3).

Our results show a similar community composition as that found by Countway et al. (2007) in 18S rRNA gene clone libraries of the euphotic zone at oligotrophic stations in the Sargasso Sea in August 2000. Dinoflagellate and

MALV sequences made up over half of each clone library with stramenopiles, ciliates, prymnesiophytes, cryptophytes, chlorophytes and others making up a smaller proportion. Although deep water samples (2500 m) were collected by these authors, no particle trap libraries were constructed for comparison with the present study. Results of 18S rRNA gene clone libraries at ESTOC in March 2005, however, were quite different (Amacher et al., 2009). In that study, diatom sequences, primarily of the genera *Asterionella*, *Thalassiosira*, and *Minidiscus* accounted for up to 60% of sequences in clone libraries of the water column but were not detected in the 200 m trap and made up only 6% of the 500 m trap. Radiolarian sequences made up the majority of the trap libraries, with dinoflagellates, MALVs, and ciliates making up the second largest percentage. Similar to the findings of this study, prasinophyte sequences, including *Bathycoccus*, *Micromonas*, and *Ostreococcus*, were found to be slightly enriched in the traps. The hypothesis postulated by Helmke et al. (2010) that more unballasted cells contribute to BATS sinking flux than at ESTOC seems to be supported in part by the relative abundance of sequences matching to unmineralized taxa in BATS clone libraries, and also by the high relative contribution of radiolarian sequences in the ESTOC libraries in Amacher et al. (2009). However, the abundance of diatom sequences in the water column and absence from trap clone libraries at ESTOC (Amacher et al., 2009) does not

support this hypothesis. Further study, particularly at ESTOC, may shed more light on the cause of the differences between these two sites.

3.3. Mamiellales cell concentration and flux

Clone libraries are useful in determining relative abundance and taxonomic identifications, but do not give us quantitative estimates of the contribution of different groups. They are dependent on 18S rRNA gene copy number, and there may also be inherent biases for and against certain organism groups (see Chapter 2). Quantitative PCR with group-specific primers was used here in addition to clone libraries as a way to quantitatively determine the contribution of a specific taxonomic group to particle flux. The Mamiellales group includes some of the smallest phytoplankton taxa, including *Bathycoccus*, *Micromonas*, and *Ostreococcus*. Taxa from this group were found to be fairly large contributors to both water column and traps in both DGGEs (see Chapter 3) and clone libraries. These conditions make this group ideal for quantitative analysis by qPCR with this data set.

Cell concentrations of taxa from the group Mamiellales estimated by qPCR ranged from 7.90×10^{1} cells mL^{-1} in April 2010 to 2.01×10^{5} cells mL^{-1} in March 2009 (Fig. 4). These estimates were compared with epifluorescence counts of small (1-2 µm) phototrophs (Hansen et al., in preparation) and flow cytometry counts of picoeukaryotes from the BATS data set. For several samples all three estimates were quite comparable. However, the qPCR estimates for March 2009

10 m sample, both February and March 2010 samples, and the April 2010 80 m sample were substantially higher than the epifluorescence or flow cytometry counts. While primer specificity was assessed by Zhu et al. (2005) there may be some level of nonspecific binding of primers to non-target DNA, resulting in higher estimates than their actual abundance in the water column. In addition, there may be an effect of a higher 18S rRNA gene copy numbers of some Mamiellales species. *Micromonas pusilla* was used as a standard for this qPCR and thus cell abundance has been calculated as *Micromonas* cell equivalents, which is not necessarily the same as cells mL^{-1}. According to Zhu et al. (2005) *Bathycoccus* has approximately 8.3 copies per cell, *Micromonas* 4.1 copies per cell, and *Ostreococcus* 2.7 copies per cell. Depending on the composition of these and other Mamiellales taxa in any given sample the qPCR derived estimates may be higher or lower than the actual abundance in the water column. In these clone libraries, the qPCR estimates that are lower than epifluorescence or flow cytometry counts have only *Micromonas* sequences. In most of the samples with higher qPCR estimates *Bathycoccus*, *Ostreococcus*, or other prasinophyte sequences were also detected. Zhu et al. (2005) found similar results, with cell abundance of Mamiellales from samples collected at Blanes Bay in the Mediterranean Sea using qPCR comparable, but generally higher compared with enumeration by FISH (fluorescent in-situ hybridization). Genera-specific qPCR primers (as in Countway and Caron, 2006; Zhu et al., 2005) would help to shed

more light on this incongruity between qPCR and other estimates of cell abundance.

There seems to be a trend of higher cell abundance as measured by qPCR with a higher percent contribution to clone libraries. For example, the highest cell abundance determined by qPCR in the water column was found in March 2009 at 10 m. Prasinophyte sequences made up 21.3% of this library, the highest percentage found in any of these clone libraries. However, the estimates of Mamiellales cells mL^{-1} using this approach are not directly comparable with the clone libraries, because the percent of clones per library indicate the relative contribution of the copy number of DNA from specific taxa, while qPCR results estimate a specific cell abundance in the water column or flux into the trap.

Carbon flux to 150 m by Mamiellales was also estimated using this method. Cell number as estimated by qPCR was converted to pg carbon per cell using the conversion ($pgC\ cell^{-1} = 0.216 \times biovolume^{0.939}$) by Menden-Deuer and Lessard (2000). The diameter of an average *Micromonas* cell (1.5 µm) was used to calculate biovolume according to the geometric equations found in Hillebrand et al. (1999). Flux ranged from 0.002 – 0.469 $mg\ m^{-2}\ d^{-1}$. The relative contribution to total POC flux by this group was usually small (0.004-0.067%) but in December 2008 and April 2010 the contribution to flux was more significant with 1.13% and 5.65%, respectively (Table 3). During these times the percent contribution of prasinophyte sequences to each trap library were also relatively

high, with 2.6% in December 2008 and 5.7% in April 2010. However, the samples with the highest percent contribution of prasinophyte sequences to clone libraries were February 21st, 2009 (13%) and March 24th, 2010 (9.7%). Again, a direct comparison with clone library results cannot be made, but we can make rough comparisons based on the taxa that are present in the libraries. In the March 2010 trap library, several sequences matching to diatom and dinoflagellate taxa were identified. It is possible that the smaller percent contribution of Mamiellales to POC flux was in part due to the higher carbon content of these larger taxa compared with that of the small-sized Mamiellales cells. No POC flux data was available for the February 2009 sample as flux is only determined during the regular monthly cruises when the 3 day PITs are deployed. However, we can speculate that the Mamiellales would most likely make up a large percentage of total POC flux at this point as the majority of the taxa contributing to this library were potentially small-sized as well (up to 93%).

While comparison with epifluorescence and flow cytometry counts suggest that these estimates may be overestimated in some cases, these are the first quantitative estimates of the contribution of a specific taxonomic group of plankton in the pico- and nano- size range to POC flux. There is an enormous potential for the use of this method with primers specific for other taxonomic groups. A greater taxonomic resolution could be achieved through the use of genus or species-specific primers. More direct comparisons could be made

between the contributions of different functional groups of protists and even cyanobacteria to POC flux.

3.4. Conclusions

Contrary to the long held notion that large taxa, such as diatoms and coccolithophorids, dominate particle flux (Boyd and Newton, 1999; Michaels and Silver, 1988; Sarthou et al., 2005), we find that their relative abundance in trap material is in proportion to that found in the water column. This suggests that these taxa do not contribute more to downward flux than small unarmored organisms. Our results also provide strong support for the hypothesis that pico- and nanoplankton can contribute to particle flux at a rate proportional to their primary production in the water column, as hypothesized by Richardson and Jackson (2007). The likely mechanism allowing small plankton to sink out of the water column is through the packaging of cells into aggregates and fecal pellets (Legendre and Le Fèvre, 1995; Olli and Heiskanen, 1999; Richardson and Jackson, 2007; Waite et al., 2000). This study builds on results of studies such as Brew et al. (2009) who found aggregation to be an important mechanism controlling export, and revealed a strong correlation between particle flux and picoplankton abundance; and Lomas and Moran (2011), who found nanoplankton to be significant contributors to POC flux. In oligotrophic gyre regions, where pico- and nanoplankton dominate the plankton community (Steinberg et al., 2001), this mechanism of sinking may play an essential role in flux out of the

water column. The ability of small cells to sink out of the water column will be especially important in warming conditions, which may lead to more strongly stratified oceans with less availability of nutrients, conditions favoring cells with a higher surface to volume ratio (Falkowski and Oliver, 2007). Incorporating the contributions of different taxonomic groups to carbon flux into climate change models will help us to better predict the role the oceans will play in the future.

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Table captions.

Table 1. Sampling dates and depths of samples from which clone libraries were constructed.

Table 2. Non-protist contributions to clone libraries.

Table 3. Carbon flux by Mamiellales quantified by qPCR. Represented as a percentage of total particulate organic carbon flux.

Figure Captions.

Fig. 1. Clone library results and water column profile. For A. October 7, 2008; B. December 15, 2008; C. February 7, 2009; D. February 21, 2009; E. March 17, 2009; F. July 14, 2009; G. March 24, 2010; H. April 21, 2010. (A-H) Top: Pie graphs showing relative contribution of clones for each taxonomic group (see key on right). Metazoan and fungi sequences have been left out of the dataset in order to compare only protist taxa. Bottom left: Dendrogram showing Bray-Curtis similarities between libraries from each sampling date. Black lines indicate significant difference ($p \leq 0.05$). Bottom right: Profile of temperature (solid line), particulate organic carbon flux (dashed line) and extracted chlorophyll a (green).

Fig. 2. Ratios of ciliates (red), dinoflagellates (green), and alveolates (purple) in water column versus trap clone libraries. Sampling dates are denoted by symbols. October 7, 2008 (solid squares), December 15, 2008 (solid circles), February 7, 2009 (diamonds), February 21, 2009 (x), March 17, 2009 (x with vertical line), July 2009 (dash), March 24, 2010 (+), and April 21, 2010 (open circles).

Fig. 3. Ratios of prasinophytes (purple), prymnesiophytes (orange), diatoms (green), and MAST (blue) in water column versus trap clone libraries. Sampling dates are denoted by symbols. October 7, 2008 (solid squares), December 15, 2008 (solid circles), February 7, 2009 (diamonds), February 21, 2009 (x), March 17, 2009 (x with vertical line), July 2009 (dash), March 24, 2010 (+), and April 21, 2010 (open circles).

Fig. 4. Cell concentration in selected samples as measured by quantitative PCR with Mamiellales specific primers (blue bars), epifluorescence microscopy counts (maroon bars), and picoeukaryote flow cytometry counts (green bars).

Tables.

Table 1.

Date	Water Column Depths (m)	Trap
October 7, 2008	10, 90	150m Unfixed
December 15, 2008	10, 120	150m Fixed
February 7th, 2009	10, 130	150m Unfixed
February 21st, 2009	10, 130	150m Unfixed
March 17th, 2009	10, 90	150m Unfixed
July 14, 2009	10, 60	150m Unfixed
March 24, 2010	10, 80	150m Unfixed
April 21, 2010	10, 80	150m Fixed

Table 2.

Date	Library (depth (m))	Cnidaria	Copepods	Fungi	Other	Total sequences	Protist %	Metazoan or fungi %
October 7, 2008	**10**	8	27	-	-	174	80%	20%
	90	1	12	-	2	178	92%	8%
	trap	3	60	60	-	173	29%	71%
December 15, 2008	**10**	8	56	-	1	147	56%	44%
	120	1	27	-	-	94	70%	30%
	trap	33	17	-	2	169	69%	31%
February 7, 2009	**10**	19	35	4	-	101	43%	57%
	130	-	50	7	-	110	48%	52%
	trap	-	-	85	-	103	17%	83%
February 21, 2009	**10**	1	20	-	-	88	76%	24%
	130	-	38	-	-	85	55%	45%
	trap	-	4	73	-	123	37%	63%
March 17, 2009	**10**	1	28	-	3	93	66%	34%
	90	-	22	-	-	70	69%	31%
	trap	-	-	85	-	88	3%	97%
July 14, 2009	**10**	6	50	-	1	182	69%	31%

	60	3	33	-	-	172	79%	21%
	trap	-	1	18	-	167	89%	11%
March 24, 2010	**10**	5	38	-	2	129	65%	35%
	80	8	27	1	3	179	78%	22%
	trap	6	13	6	3	182	85%	15%
April 21, 2010	**10**	1	9	-	-	175	94%	6%
	80	1	31	-	-	176	82%	18%
	trap	1	15	122	1	174	20%	80%

Table 3.

Sample	Trap carbon flux by Mamiellales (mg m^{-2} d^{-1})	150 m POC flux (mg m^{-2} d^{-1})	% of POC flux by Mamiellales
Dec 15, 2008	0.4689	41.39	1.1%
Feb 7, 2009	0.0167	24.92	0.067%
Feb 21, 2009	0.0056	no data	
Mar 17, 2009	0.0122	44.25	0.028%
Feb 3, 2010	0.0093	105.36	0.009%
Feb 23, 2010	0.0075	no data	
Mar 24, 2010	0.0021	47.93	0.004%
Apr 21, 2010	0.3877	6.86	5.6%

Figures.

Figure 1A.

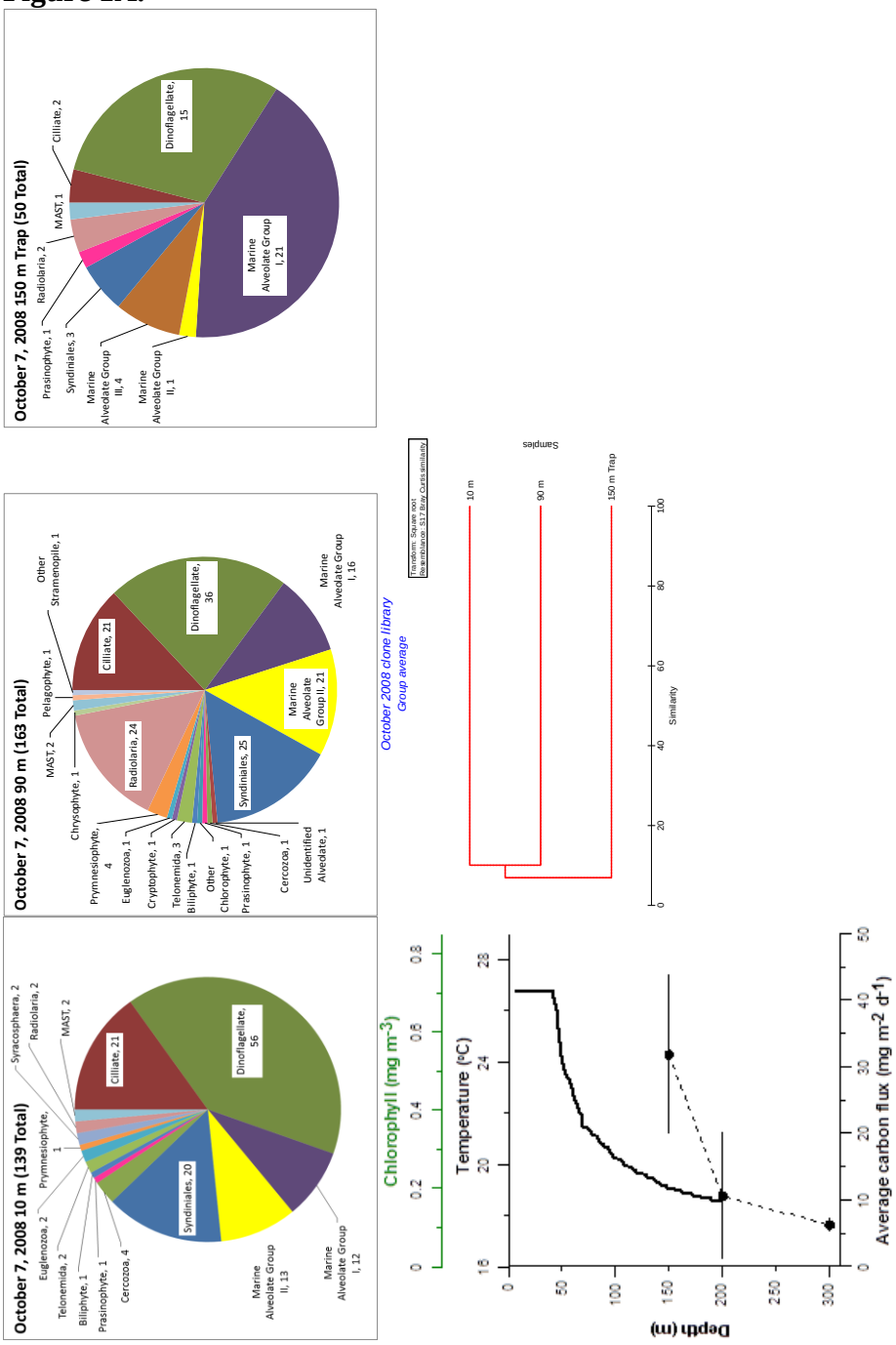


Figure 1B.

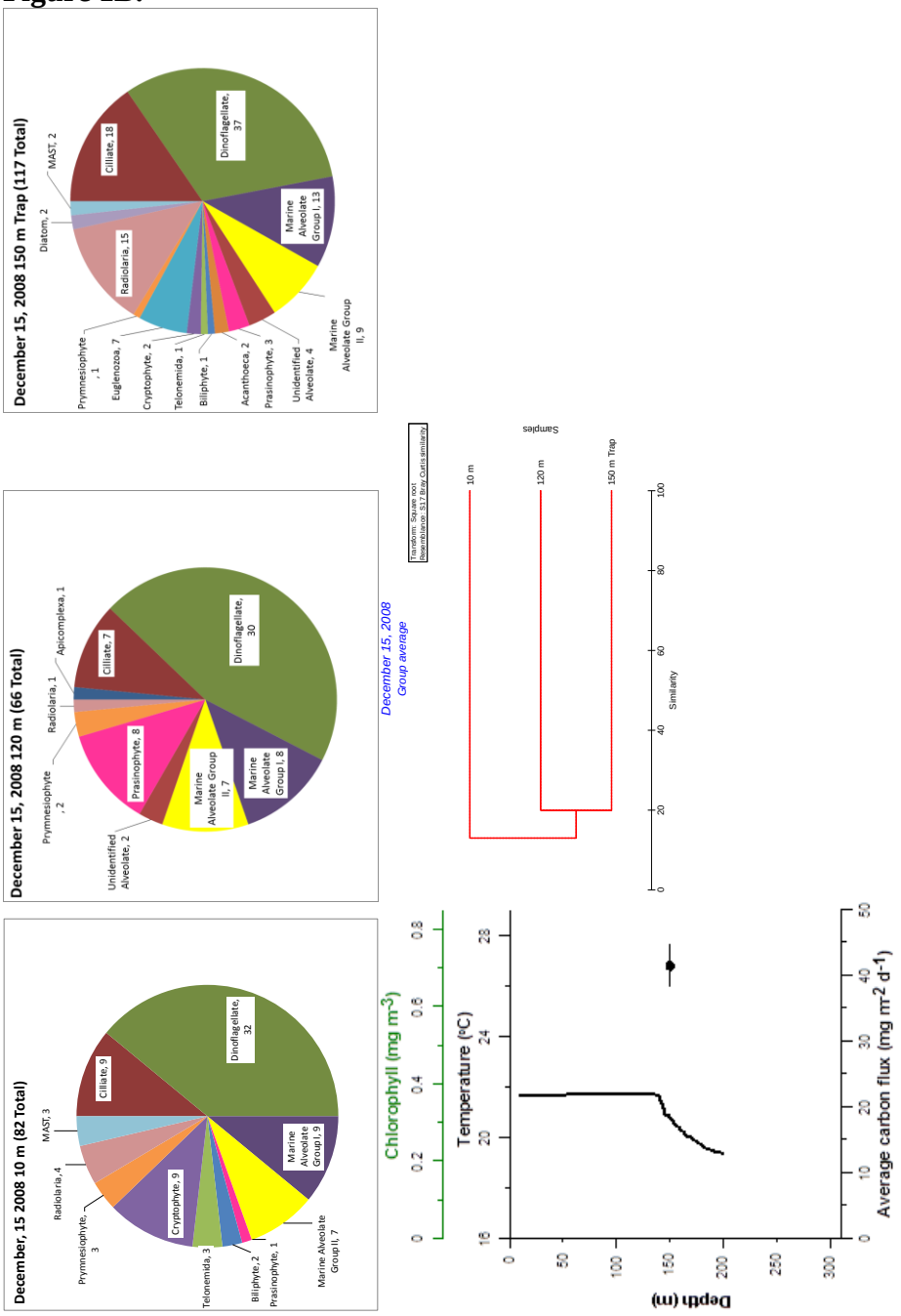

December 15, 2008 150 m Trap (117 Total)
Dinoflagellate, 37
Marine Alveolate Group I, 13
Marine Alveolate Group II, 9
Unidentified Alveolate, 4
Prasinophyte, 3
Acanthoeca, 2
Biliphyte, 1
Telonemida, 1
Cryptophyte, 2
Euglenozoa, 7
Prymnesiophyte, 1
Radiolaria, 15
Diatom, 2
MAST, 2
Ciliate, 18
December 15, 2008 120 m (66 Total)
Dinoflagellate, 30
Marine Alveolate Group I, 8
Marine Alveolate Group II, 7
Prasinophyte, 8
Unidentified Alveolate, 2
Prymnesiophyte, 2
Radiolaria, 1
Apicomplexa, 1
Ciliate, 7
December, 15 2008 10 m (82 Total)
Dinoflagellate, 32
Marine Alveolate Group I, 9
Marine Alveolate Group II, 7
Prasinophyte, 1
Biliphyte, 2
Telonemida, 3
Cryptophyte, 9
Prymnesiophyte, 3
Radiolaria, 4
MAST, 3
Ciliate, 9
December 15, 2008
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Samples
10 m
120 m
150 m Trap
Similarity
0
20
40
60
80
100
Chlorophyll (mg m-3)
0
0.2
0.4
0.6
0.8
Temperature (°C)
16
20
24
28
Depth (m)
0
50
100
150
200
250
300
Average carbon flux (mg m-2 d-1)
0
10
20
30
40
50

Figure 1C.

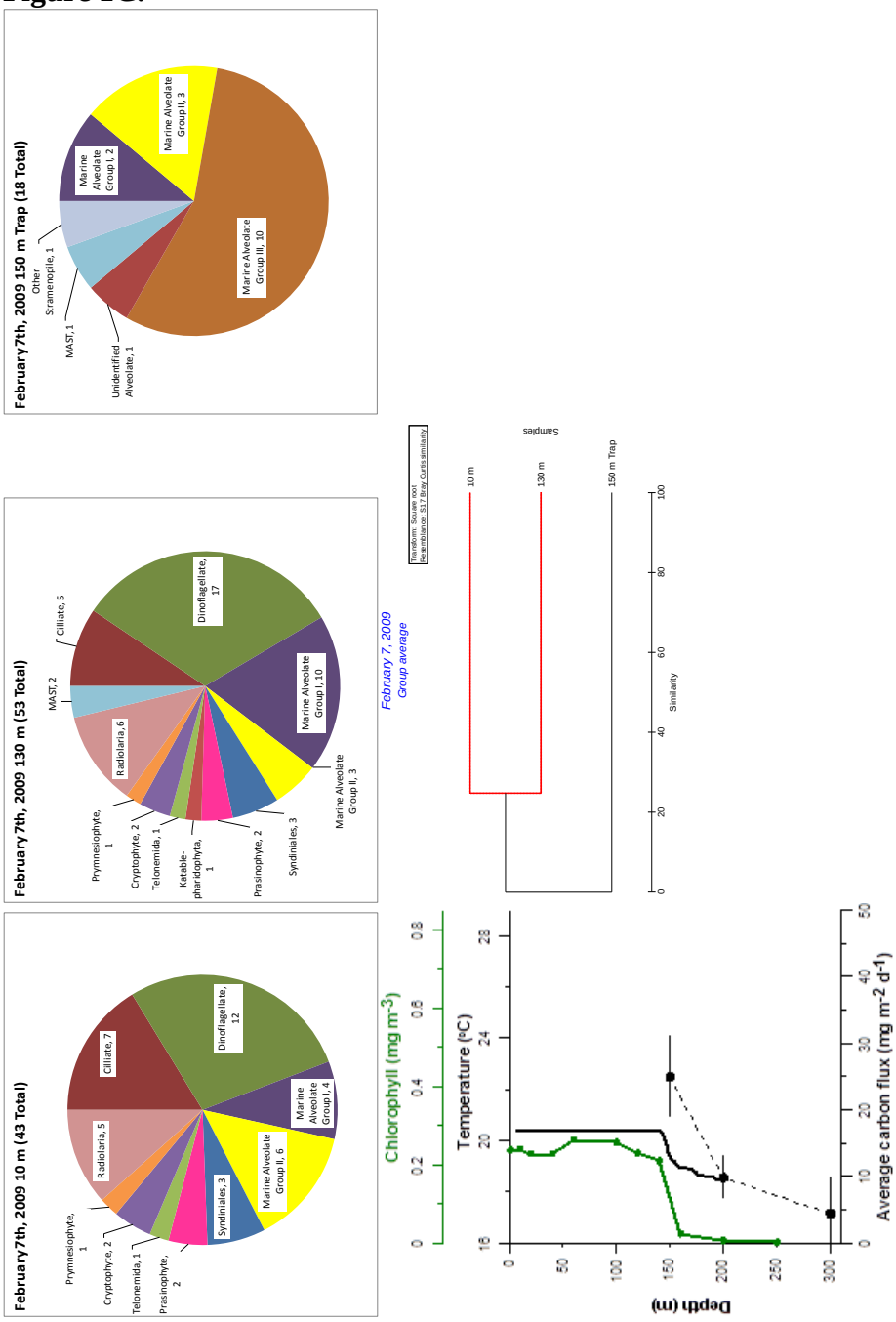

February7th, 2009 150 m Trap (18 Total)
Marine Alveolate Group I, 2
Marine Alveolate Group II, 3
Marine Alveolate Group III, 10
Other Stramenopile, 1
MAST, 1
Unidentified Alveolate, 1
February7th, 2009 130 m (53 Total)
Ciliate, 5
Dinoflagellate, 17
MAST, 2
Marine Alveolate Group I, 10
Radiolaria, 6
Marine Alveolate Group II, 3
Prymnesiophyte, 1
Cryptophyte, 2
Telonemids, 1
Katable-pharidophyta, 1
Prasinophyte, 2
Syndiniales, 3
February7th, 2009 10 m (43 Total)
Dinoflagellate, 12
Ciliate, 7
Marine Alveolate Group I, 4
Radiolaria, 5
Marine Alveolate Group II, 6
Syndiniales, 3
Prymnesiophyte, 1
Cryptophyte, 2
Telonemids, 1
Prasinophyte, 2
February 7, 2009
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Samples
10 m
130 m
150 m Trap
Similarity
0
20
40
60
80
100
Chlorophyll (mg m-3)
0
0.2
0.4
0.6
0.8
Temperature (°C)
16
20
24
28
Depth (m)
0
50
100
150
200
250
300
Average carbon flux (mg m-2 d-1)
0
10
20
30
40
50

Figure 1D.

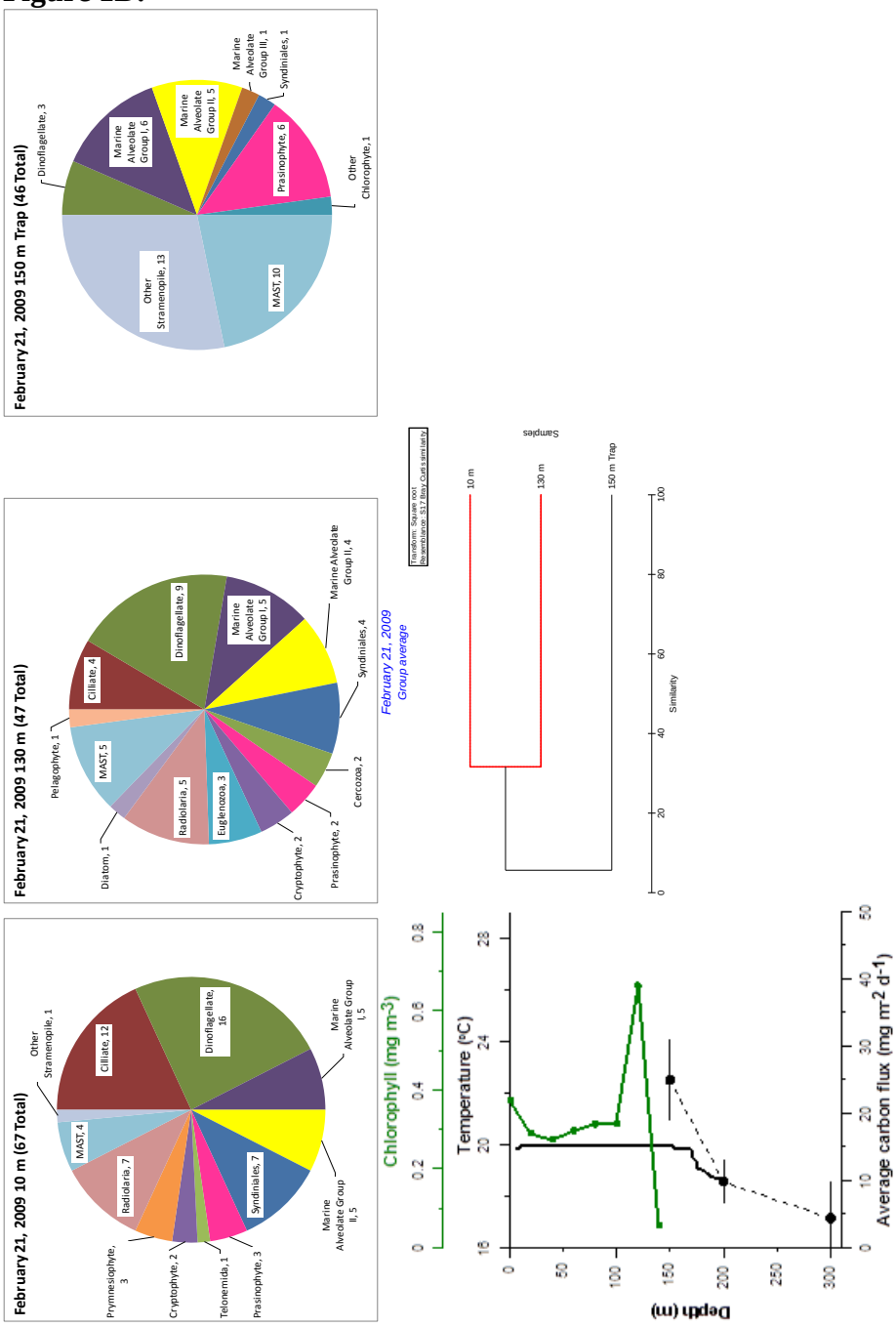

February 21, 2009 150 m Trap (46 Total)
Dinoflagellate, 3
Marine Alveolate Group I, 6
Marine Alveolate Group I, 5
Marine Alveolate Group II, 1
Syndiniales, 1
Prasinophyte, 6
Other Chorophyte, 1
MAST, 10
Other Stramenopile, 13
February 21, 2009 130 m (47 Total)
Pelagophyte, 1
Ciliate, 4
Dinoflagellate, 9
Marine Alveolate Group I, 5
Marine Alveolate Group II, 4
Syndiniales, 4
MAST, 5
Radiolaria, 5
Euglenozoa, 3
Cercozoa, 2
Diatom, 1
Cryptophyte, 2
Prasinophyte, 2
February 21, 2009 10 m (67 Total)
Other Stramenopile, 1
Ciliate, 12
Dinoflagellate, 16
Marine Alveolate Group I, 5
MAST, 4
Radiolaria, 7
Syndiniales, 7
Prymnesiophyte, 3
Cryptophyte, 2
Telonemida, 1
Prasinophyte, 3
Marine Alveolate Group II, 5
February 21, 2009
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Samples
10 m
130 m
150 m Trap
Similarity
0
20
40
60
80
100
Chlorophyll (mg m-3)
0
0.2
0.4
0.6
0.8
Temperature (°C)
16
20
24
28
Depth (m)
0
50
100
150
200
250
300
Average carbon flux (mg m-2 d-1)
0
10
20
30
40
50

Figure 1E.

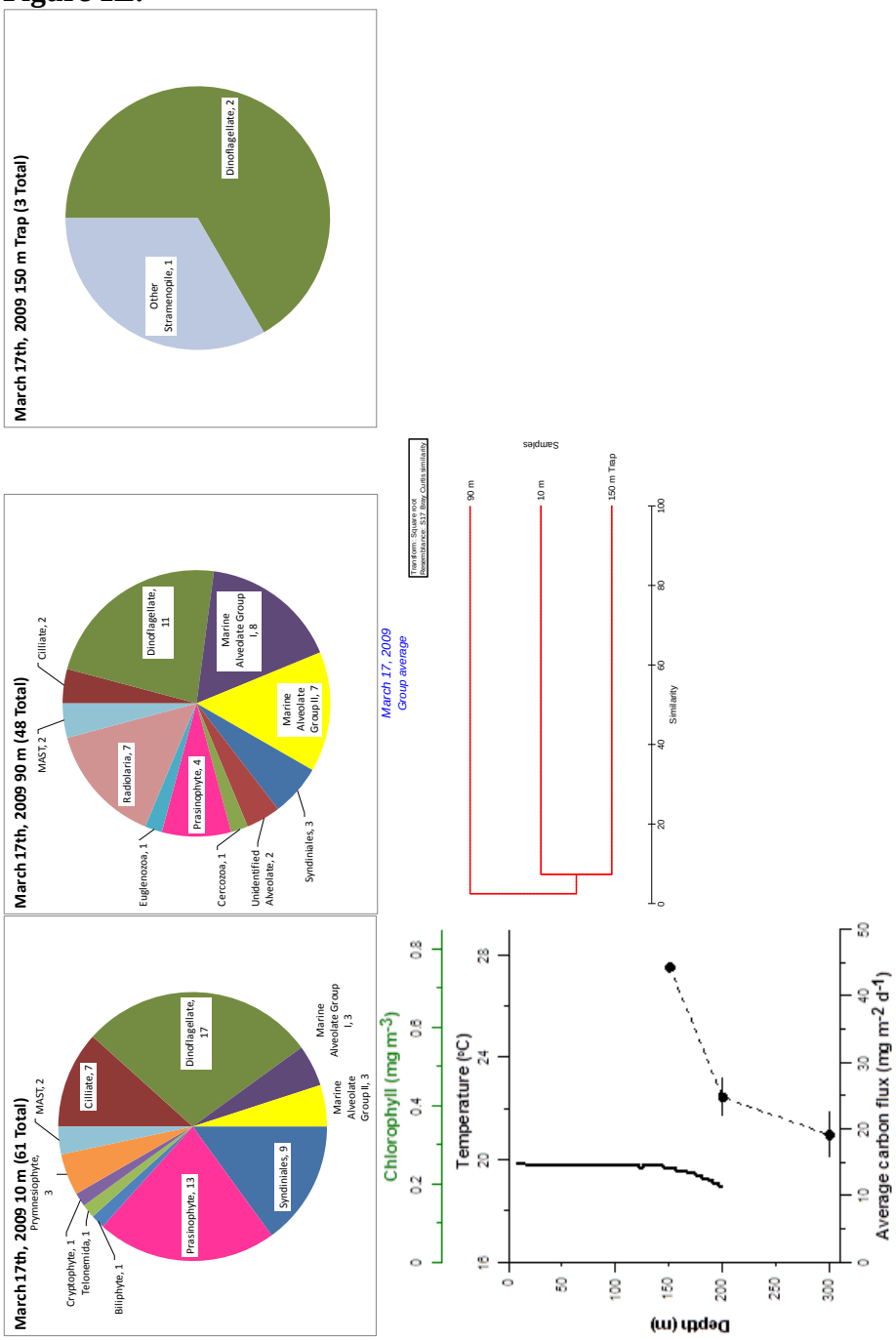

March 17th, 2009 150 m Trap (3 Total)
Dinoflagellate, 2
Other Stramenopile, 1
March 17th, 2009 90 m (48 Total)
Ciliate, 2
MAST, 2
Radiolaria, 7
Euglenozoa, 1
Prasinophyte, 4
Cercozoa, 1
Unidentified Alveolate, 2
Syndiniales, 3
Marine Alveolate Group II, 7
Marine Alveolate Group I, 8
Dinoflagellate, 11
March 17th, 2009 10 m (61 Total)
Prymnesiophyte, 3
MAST, 2
Ciliate, 7
Cryptophyte, 1
Telonemida, 1
Biliphyte, 1
Prasinophyte, 13
Syndiniales, 9
Marine Alveolate Group II, 3
Marine Alveolate Group I, 3
Dinoflagellate, 17
March 17, 2009
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Samples
90 m
10 m
150 m Trap
Similarity
0
20
40
60
80
100
Chlorophyll (mg m-3)
0
0.2
0.4
0.6
0.8
Temperature (°C)
16
20
24
28
Depth (m)
0
50
100
150
200
250
300
Average carbon flux (mg m-2 d-1)
0
10
20
30
40
50

Figure 1F.

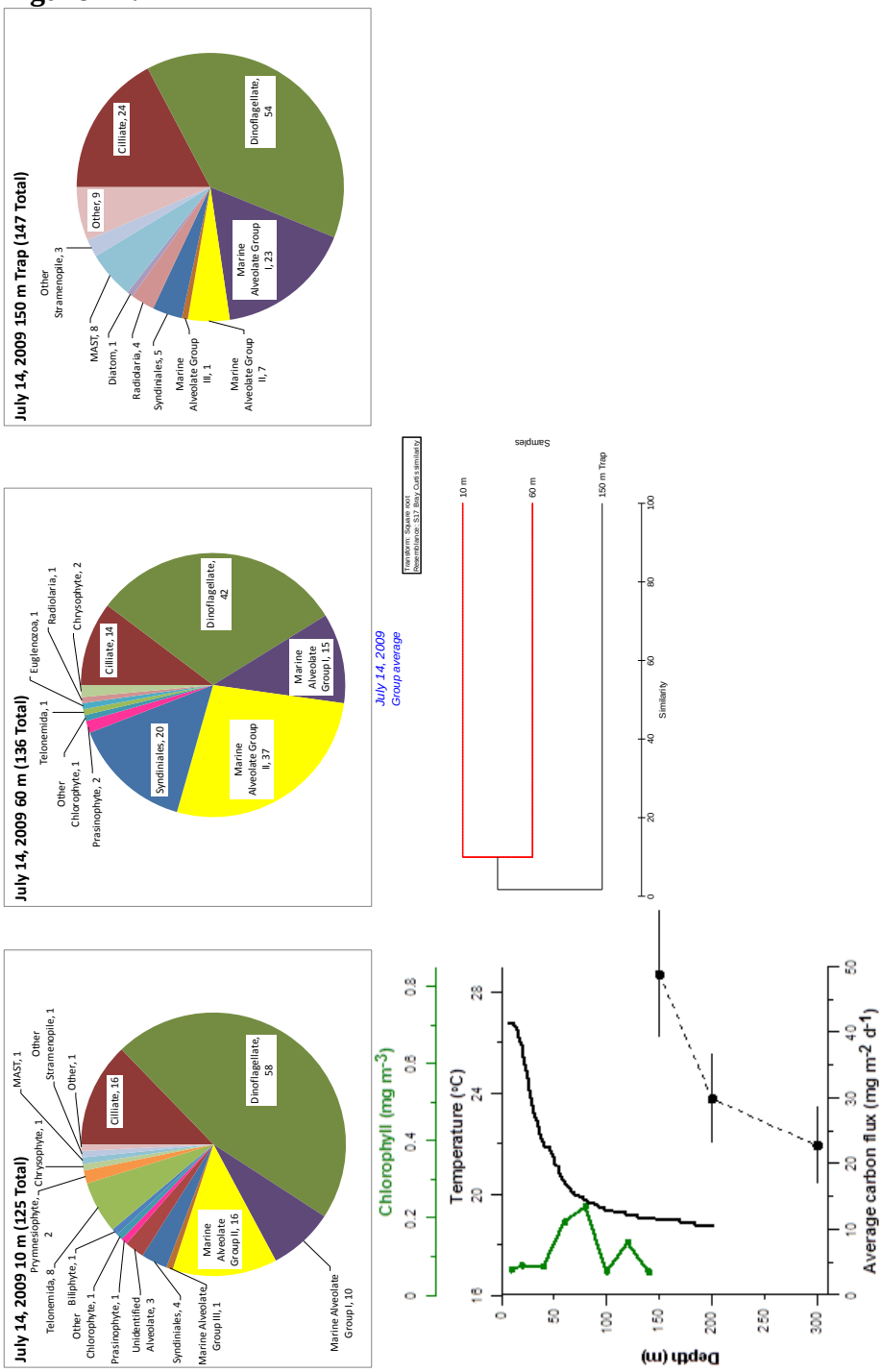

July 14, 2009 150 m Trap (147 Total)
Dinoflagellate, 54
Ciliate, 24
Other, 9
Marine Alveolate Group I, 23
Other Stramenopile, 3
MAST, 8
Diatom, 1
Radiolaria, 4
Syndiniales, 5
Marine Alveolate Group III, 1
Marine Alveolate Group II, 7
July 14, 2009 60 m (136 Total)
Dinoflagellate, 42
Ciliate, 14
Marine Alveolate Group I, 15
Marine Alveolate Group II, 37
Syndiniales, 20
Euglenozoa, 1
Radiolaria, 1
Chrysophyte, 2
Telonemida, 1
Other Chlorophyte, 1
Prasinophyte, 2
July 14, 2009 10 m (125 Total)
Dinoflagellate, 58
Ciliate, 16
Marine Alveolate Group II, 16
Marine Alveolate Group I, 10
MAST, 1
Other Stramenopile, 1
Other, 1
Chrysophyte, 1
Prymnesiophyte, 2
Telonemida, 8
Biliphyte, 1
Other Chlorophyte, 1
Prasinophyte, 1
Unidentified Alveolate, 3
Syndiniales, 4
Marine Alveolate Group III, 1
July 14, 2009
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Samples
10 m
60 m
150 m Trap
Similarity
0
20
40
60
80
100
Chlorophyll (mg m-3)
0
0.2
0.4
0.6
0.8
Temperature (°C)
16
20
24
28
Depth (m)
0
50
100
150
200
250
300
Average carbon flux (mg m-2 d-1)
0
10
20
30
40
50

Figure 1G.

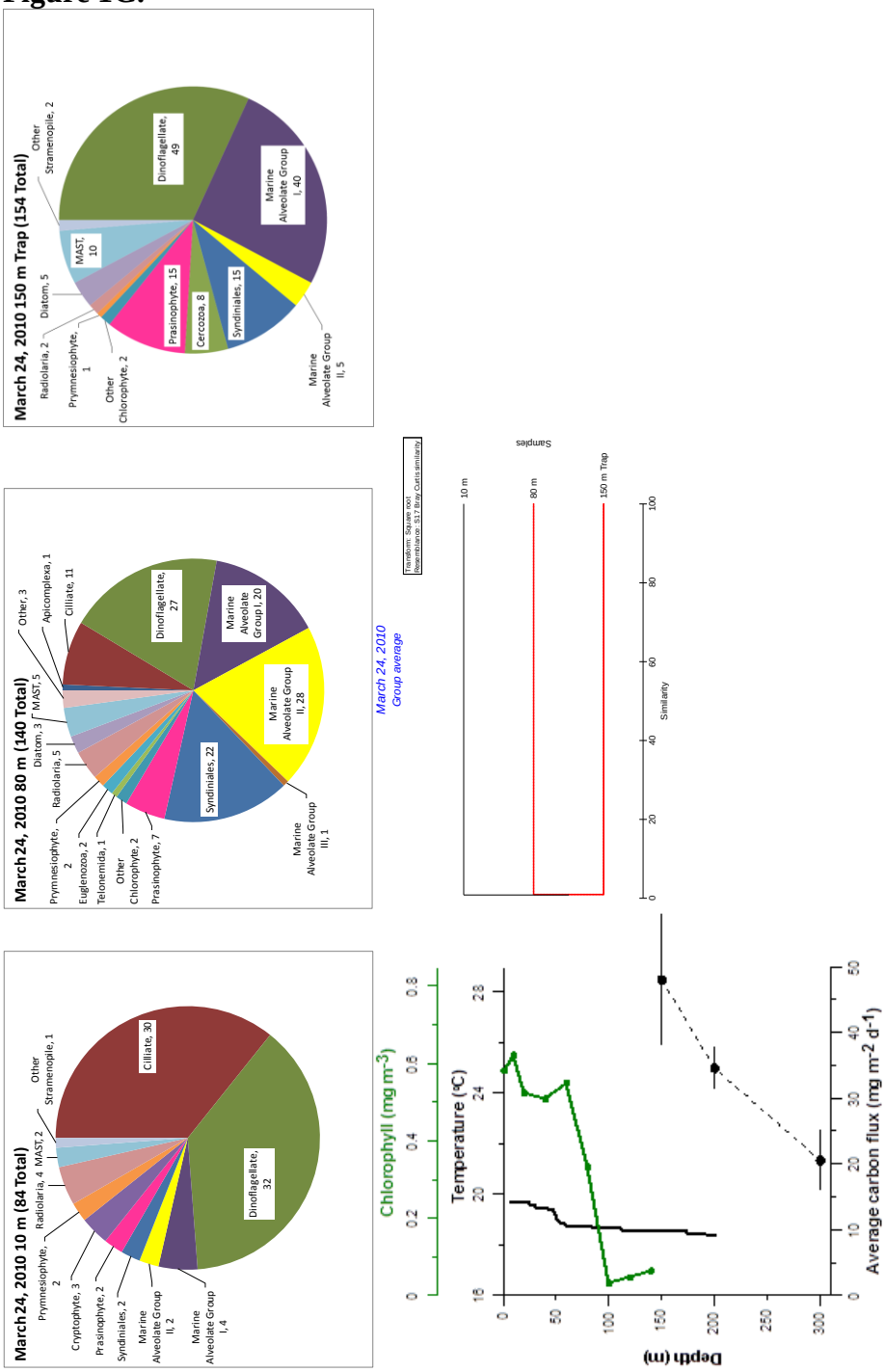

March 24, 2010 150 m Trap (154 Total)
Dinoflagellate, 49
Marine Alveolate Group I, 40
Syndiniales, 15
Cercozoa, 8
Prasinophyte, 15
Other Chlorophyte, 2
Prymnesiophyte, 1
Radiolaria, 2
Diatom, 5
MAST, 10
Other Stramenopile, 2
Marine Alveolate Group II, 5
March 24, 2010 80 m (140 Total)
Dinoflagellate, 27
Marine Alveolate Group I, 20
Marine Alveolate Group II, 28
Syndiniales, 22
Marine Alveolate Group III, 1
Prasinophyte, 7
Other Chlorophyte, 2
Telonemida, 1
Euglenozoa, 2
Prymnesiophyte, 2
Radiolaria, 5
Diatom, 3
MAST, 5
Other, 3
Apicomplexa, 1
Ciliate, 11
March 24, 2010 10 m (84 Total)
Ciliate, 30
Dinoflagellate, 32
Marine Alveolate Group I, 4
Marine Alveolate Group II, 2
Syndiniales, 2
Prasinophyte, 2
Cryptophyte, 3
Prymnesiophyte, 2
Radiolaria, 4
MAST, 2
Other Stramenopile, 1
March 24, 2010
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
80 m
150 m Trap
Samples
Similarity
0
20
40
60
80
100
Chlorophyll (mg m-3)
0
0.2
0.4
0.6
0.8
Temperature (°C)
16
20
24
28
Depth (m)
0
50
100
150
200
250
300
Average carbon flux (mg m-2 d-1)
0
10
20
30
40
50

Figure 1H.

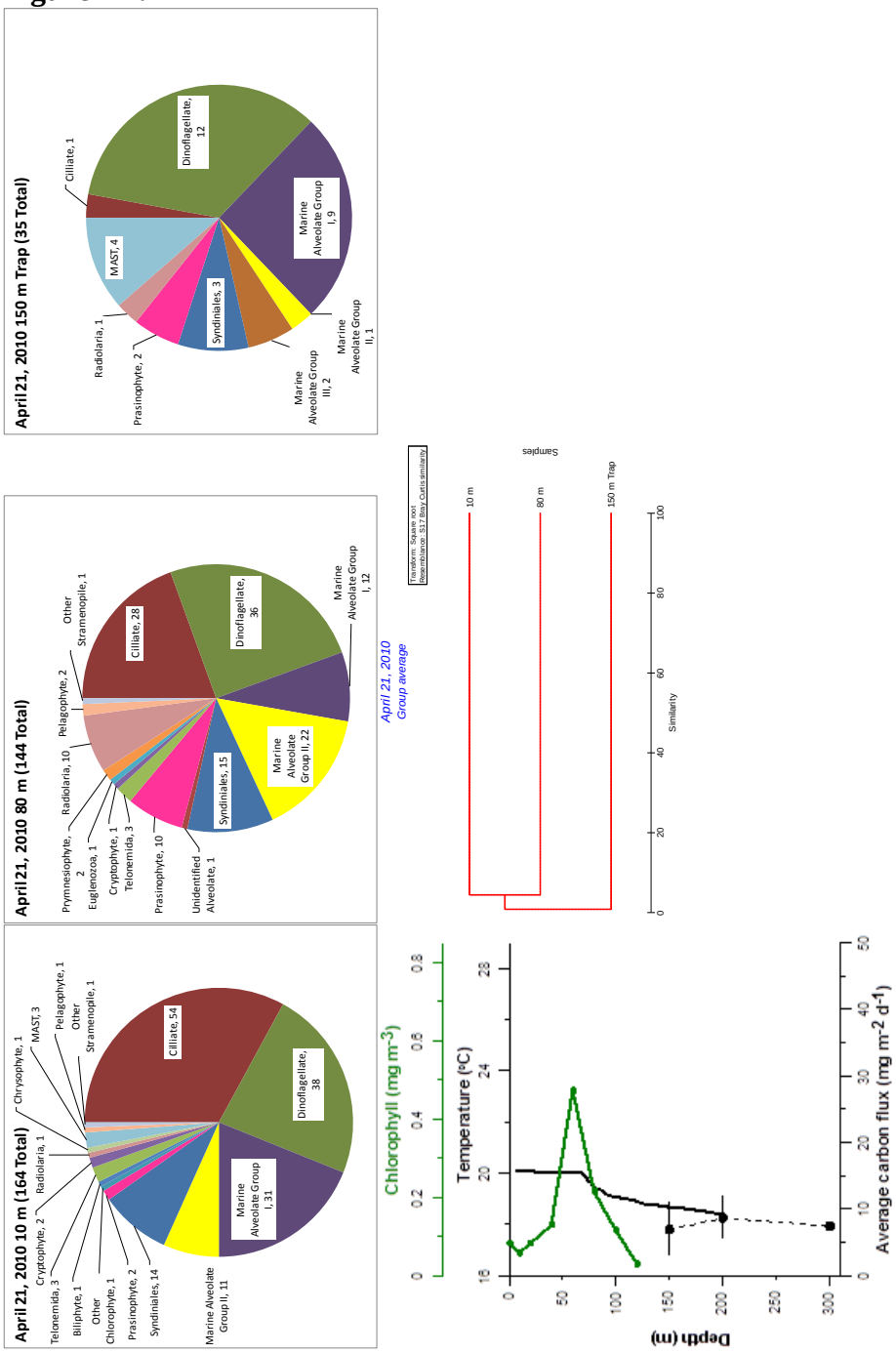


Fig. 1.

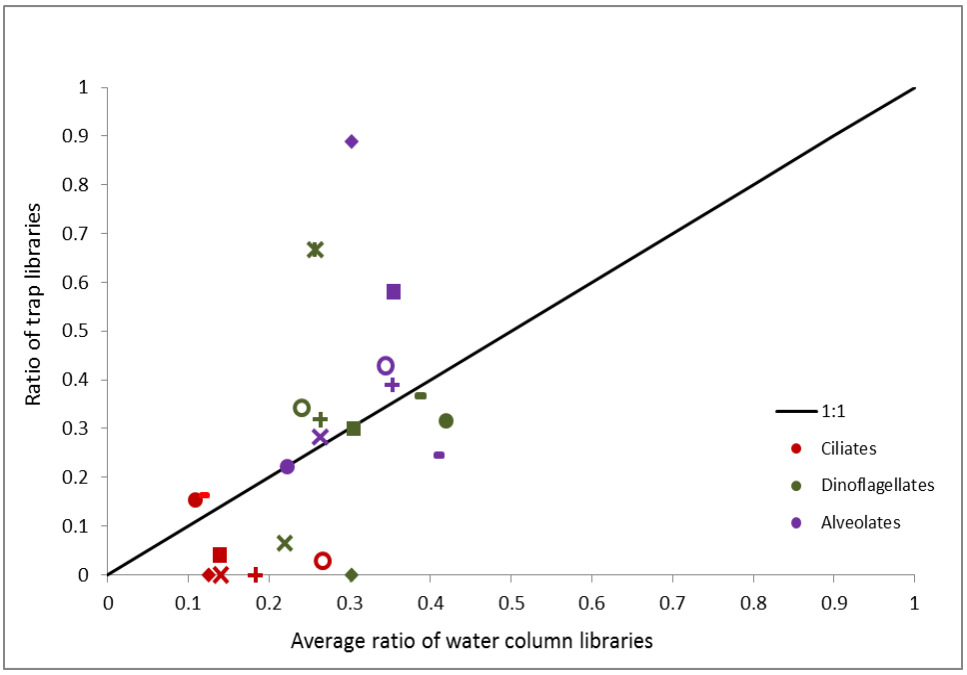

Ratio of trap libraries
Average ratio of water column libraries
1:1
Ciliates
Dinoflagellates
Alveolates

Fig. 2.

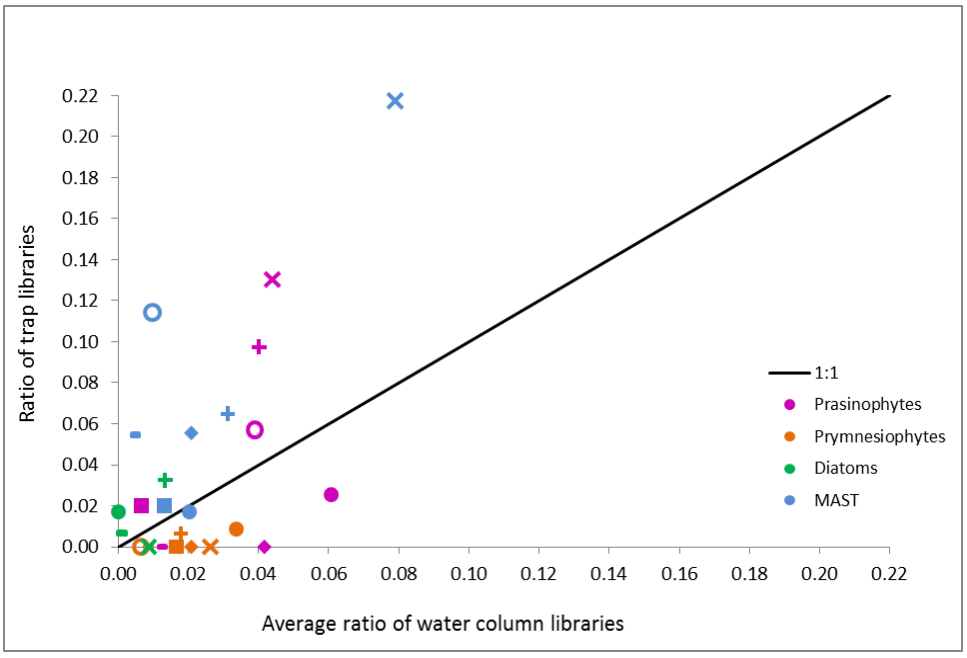

Ratio of trap libraries
Average ratio of water column libraries
1:1
Prasinophytes
Prymnesiophytes
Diatoms
MAST

Fig. 3.

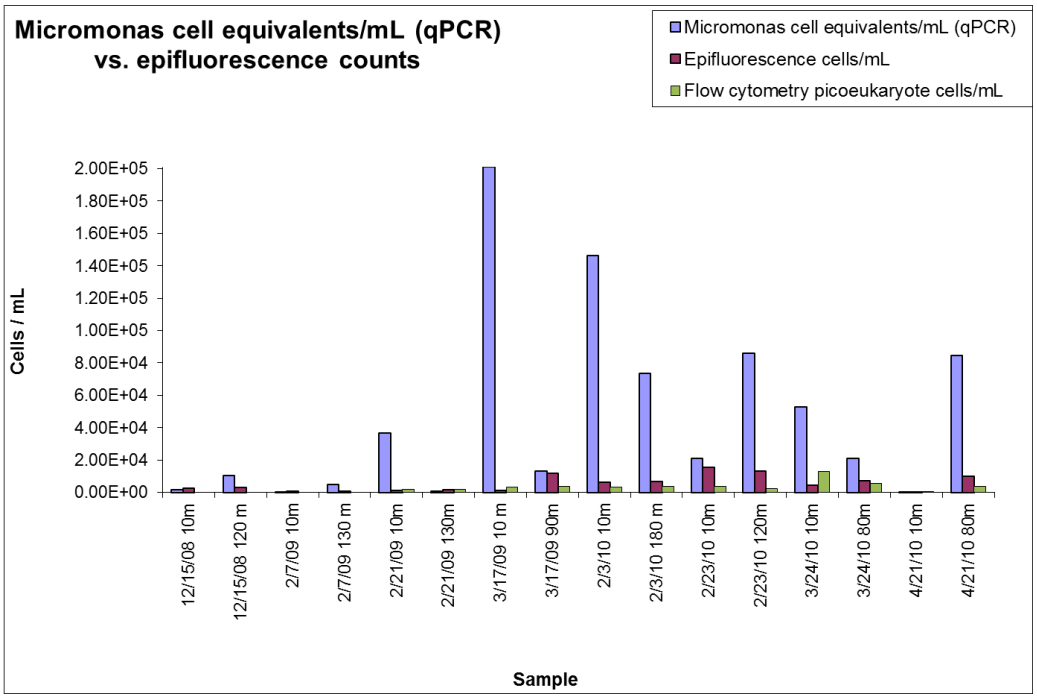

Micromonas cell equivalents/mL (qPCR) vs. epifluorescence counts
Micromonas cell equivalents/mL (qPCR)
Epifluorescence cells/mL
Flow cytometry picoeukaryote cells/mL
Cells / mL
2.00E+05
1.80E+05
1.60E+05
1.40E+05
1.20E+05
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
12/15/08 10m
12/15/08 120 m
2/7/09 10m
2/7/09 130 m
2/21/09 10m
2/21/09 130m
3/17/09 10 m
3/17/09 90m
2/3/10 10m
2/3/10 180 m
2/23/10 10m
2/23/10 120m
3/24/10 10m
3/24/10 80m
4/21/10 10m
4/21/10 80m
Sample

Fig. 4.

APPENDIX A

CHAPTER 2, SUPPLEMENTAL

Organism	DGGE	Clone Library	18S as per NCBI
E. huxleyi	46.4	50.1	49.6
Scrippsiella sp.	46.0	46.1	46.0
D. tertiolecta	46.4	48.7	48.0
P. parvum	46.9	50.4	49.2
P. capsulatus	46.1	47.9	46.9
T. weissflogii	46.7	45.7	45.5
I. galbana	46.3	51.0	50.2
O. marina	43.7	Not detected	44.1
B. prasinos	46.0	46.3	46.0

Supplemental 1. Percent GC content of 18S rRNA gene sequence fragments using the DGGE and clone library primers, and the full 18S rRNA gene sequence of each organism used in this experiment.

APPENDIX B

CHAPTER 3 SUPPLEMENTAL

Supplemental 1.

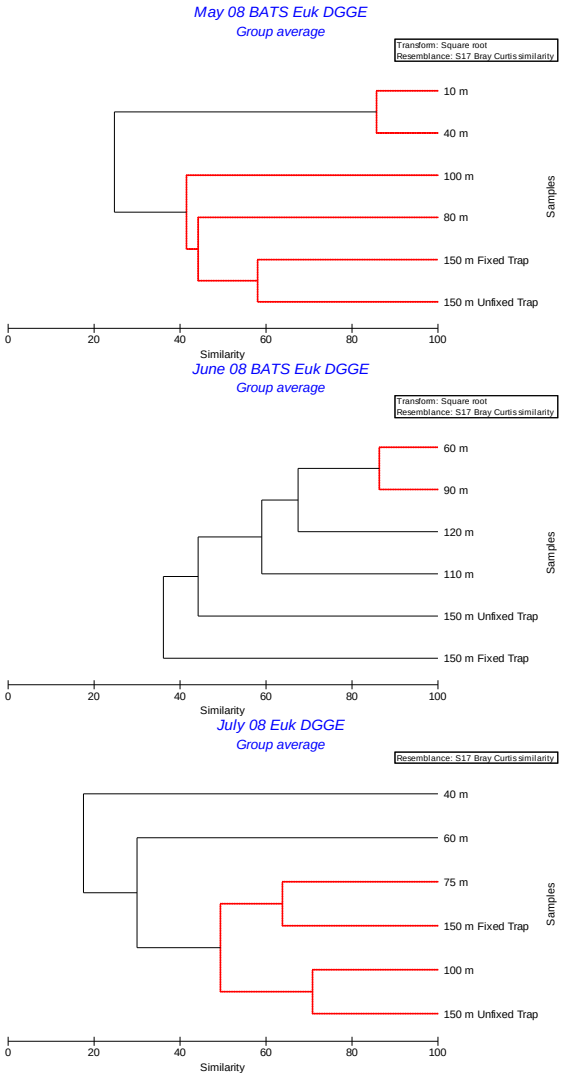
Date	Sample	E/I (Corrected efficiency of recovery)
June 10, 2009	150 m trap fixed	9.7%
	150 m trap unfixed	57.2%
July 14, 2009	10 m	76.0%
	60 m	27.1%
	100 m	27.3%
	120m	62.8%
	150 m trap fixed	1.5%
	150 m trap unfixed	88.4%
August 14, 2009	10 m	22.4%
	25 m	48.6%
	60 m	71.6%
	85 m	19.4%
	150 m trap fixed	25.6%
	150 m trap unfixed	4.0%
September 10, 2009	10 m	11.4%
	40 m	74.4%
	90 m	22.1%
	140 m	27.0%
	150 m trap fixed	35.5%
	150 m trap unfixed	4.8%
October 10, 2009	10 m	48.4%
	60 m	50.2%
	110 m	44.1%
	140 m	36.5%
	150 m trap fixed	14.3%
	150 m trap unfixed	81.3%

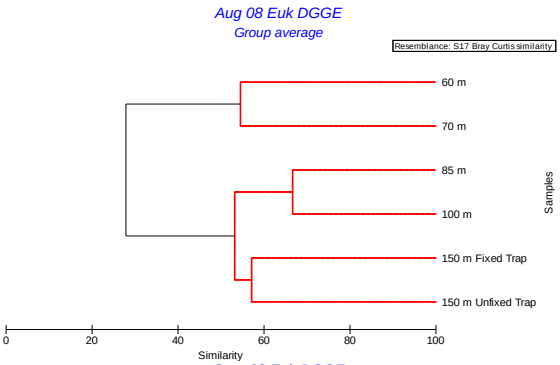
November 6, 2009	10 m	17.2%
	40 m	20.0%
	90 m	57.5%
	120 m	43.2%
	150 m trap fixed	12.3%
	150 m trap unfixed	23.0%
December 8, 2009	10 m	92.83%
	40 m	64.07%
	80 m	121.7%
	120 m	19.46%
	150 m trap fixed	16.93%
	150 m trap unfixed	34.31%
February 3, 2010	10 m	39.07%
	20 m	47.95%
	120 m	57.12%
	180 m	29.12%
	150 m trap fixed	
	150 m trap unfixed	
February 23, 2010	10 m	12.28%
	40 m	23.52%
	80 m	13.05%
	120 m	19.34%
	150 m trap fixed	6.3%
	150 m trap unfixed	4.51%
March 8, 2010	10 m	53.41%
	60 m	17.25%
	100 m	29.76%
	150 m	24.70%
	150 m trap unfixed	7.6%
March 24, 2010	10 m	10.76%
	40 m	30.12%

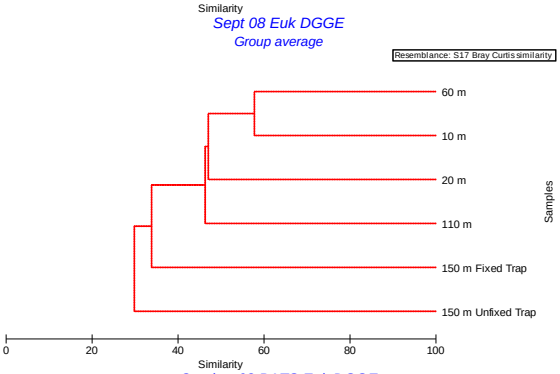
	80 m	6.79%
	120 m	22.97%
	150 m trap fixed	
	150 m trap unfixed	8.4%
April 21, 2010	10 m	0.0%
	40 m	0.00%
	80 m	0.03%
	120 m	0.02%
	150 m trap fixed	0.04%
	150 m trap unfixed	0.01%

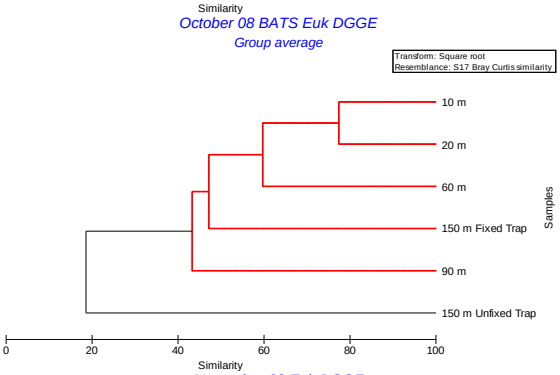
Supplemental 2. Eukaryotic and cyanobacterial UPGMA dendrograms for each month of denaturing gradient gel results. Black lines indicate significant difference ($p < 0.05$) A. Eukaryotic DGGE dendrograms. B. Cyanobacterial DGGE dendrograms.

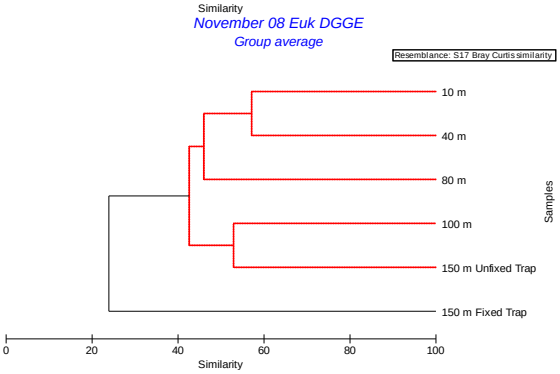
A.

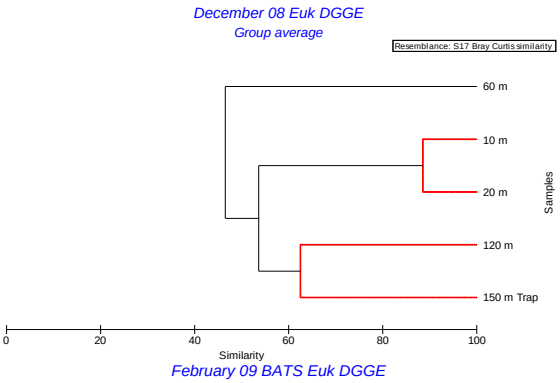


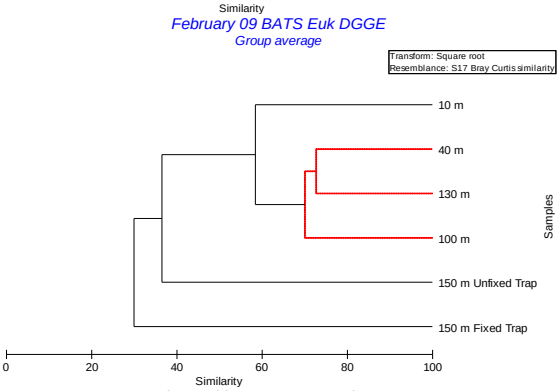

Aug 08 Euk DGGE
Group average
Resemblance: S17 Bray Curtis similarity
60 m
70 m
85 m
100 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

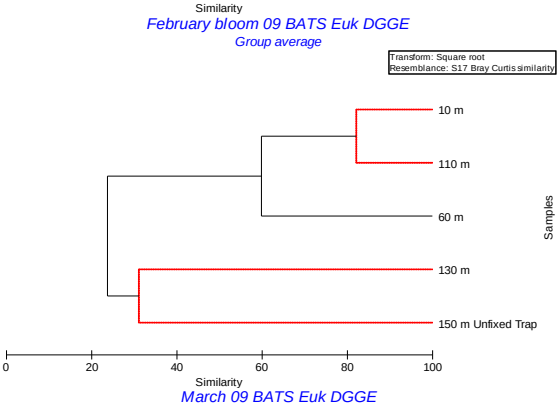

Sept 08 Euk DGGE
Group average
Resemblance: S17 Bray Curtis similarity
60 m
10 m
20 m
110 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

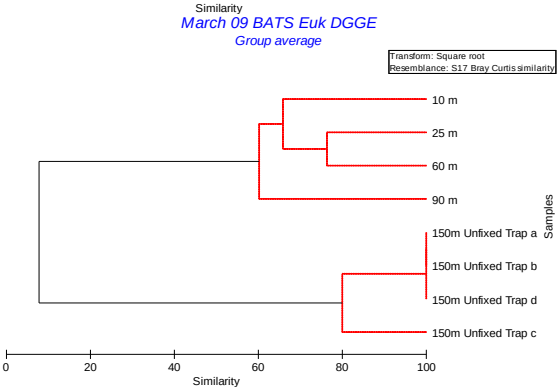

October 08 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
20 m
60 m
150 m Fixed Trap
90 m
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

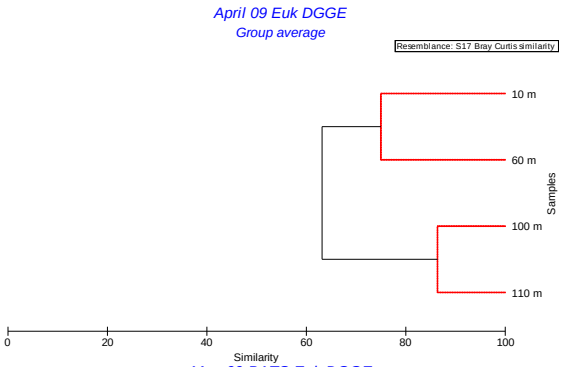

November 08 Euk DGGE
Group average
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
100 m
150 m Unfixed Trap
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

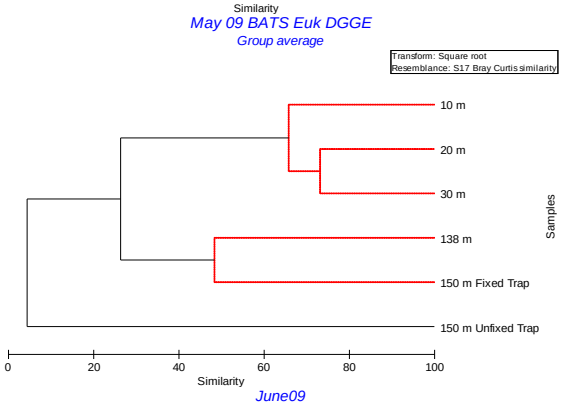

December 08 Euk DGGE
Group average
Resemblance: S17 Bray Curtis similarity
60 m
10 m
20 m
120 m
150 m Trap
Samples
0
20
40
60
80
100
Similarity

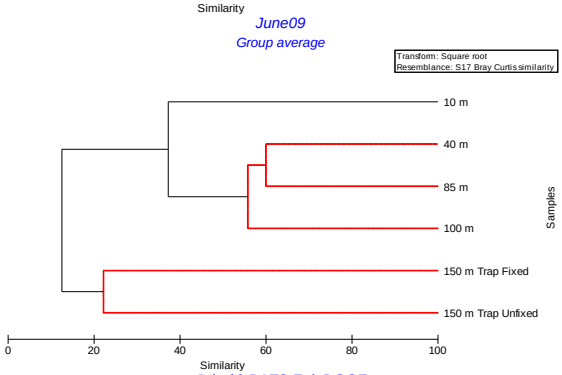

February 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
130 m
100 m
150 m Unfixed Trap
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

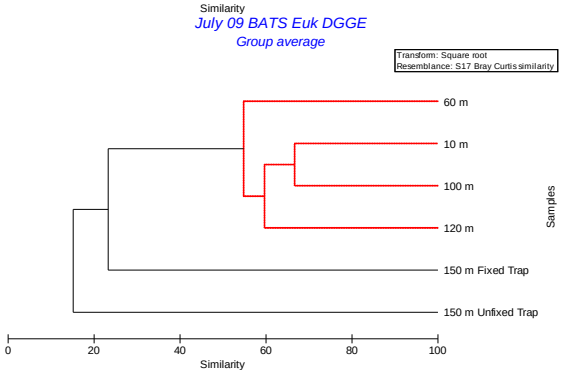

February bloom 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
110 m
60 m
130 m
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

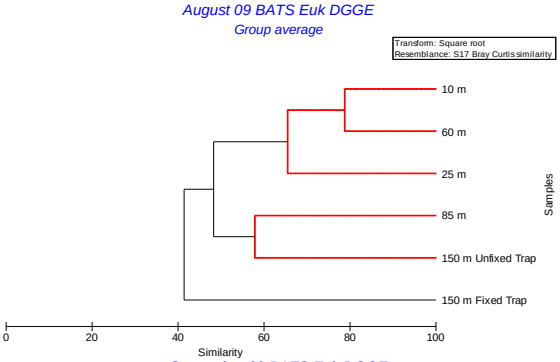

March 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
25 m
60 m
90 m
150m Unfixed Trap a
150m Unfixed Trap b
150m Unfixed Trap d
150m Unfixed Trap c
Samples
0
20
40
60
80
100
Similarity

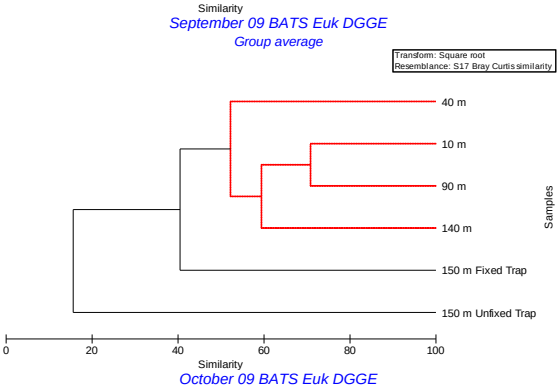

April 09 Euk DGGE
Group average
Resemblance: S17 Bray Curtis similarity
10 m
60 m
100 m
110 m
Samples
0
20
40
60
80
100
Similarity

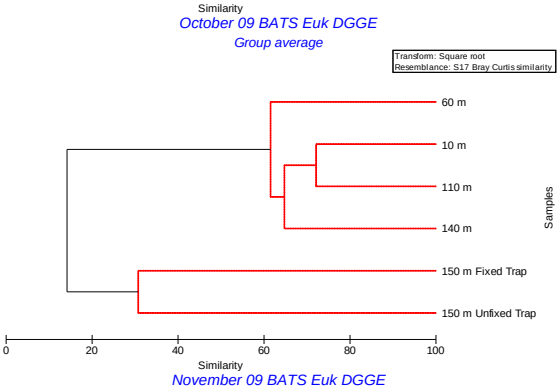

May 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
20 m
30 m
138 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

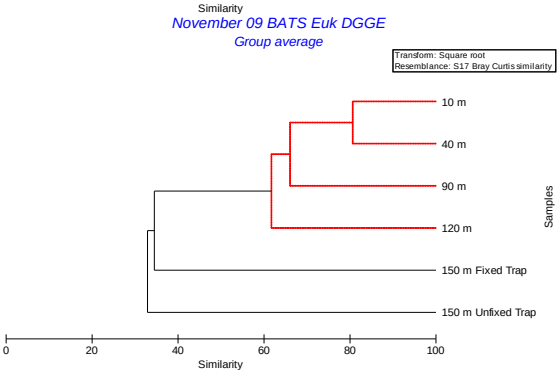

June09
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
85 m
100 m
150 m Trap Fixed
150 m Trap Unfixed
Samples
0
20
40
60
80
100
Similarity

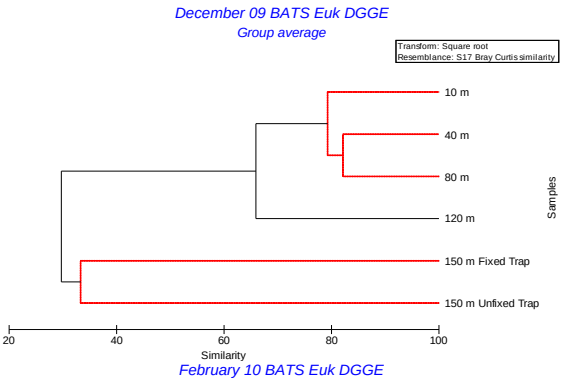

July 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
60 m
10 m
100 m
120 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

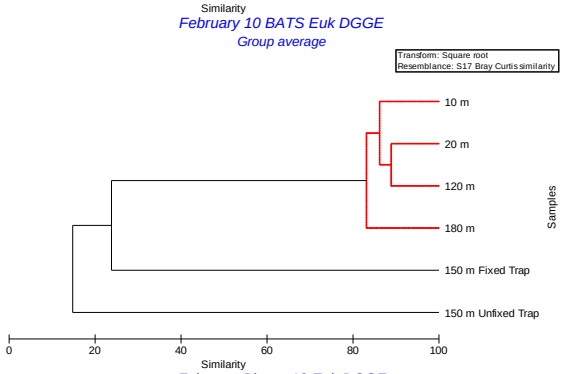

August 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
60 m
25 m
85 m
150 m Unfixed Trap
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

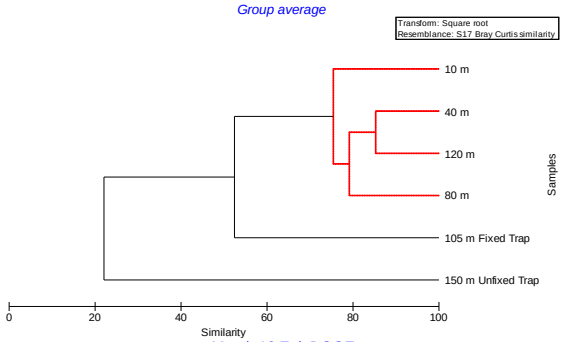

September 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
40 m
10 m
90 m
140 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

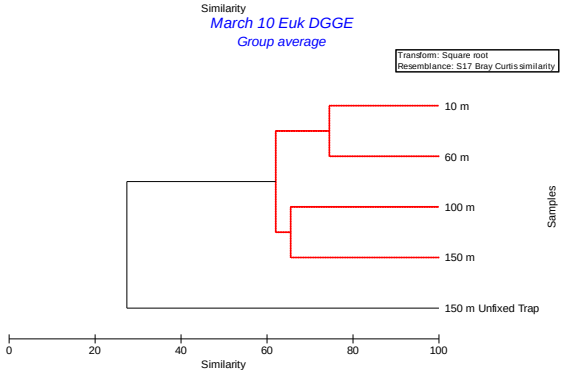

October 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
60 m
10 m
110 m
140 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

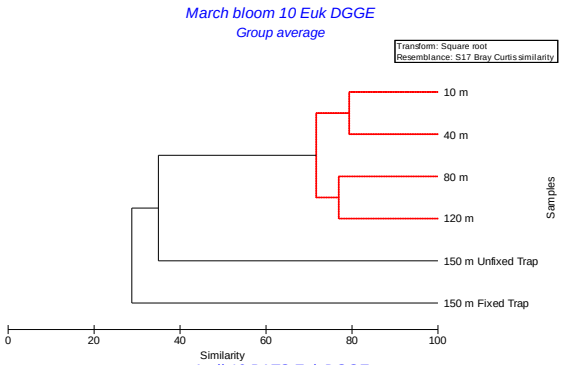

November 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
90 m
120 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

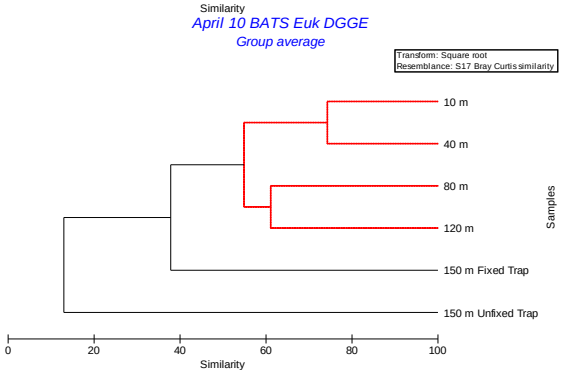

December 09 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
120 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
20
40
60
80
100
Similarity


February 10 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
20 m
120 m
180 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

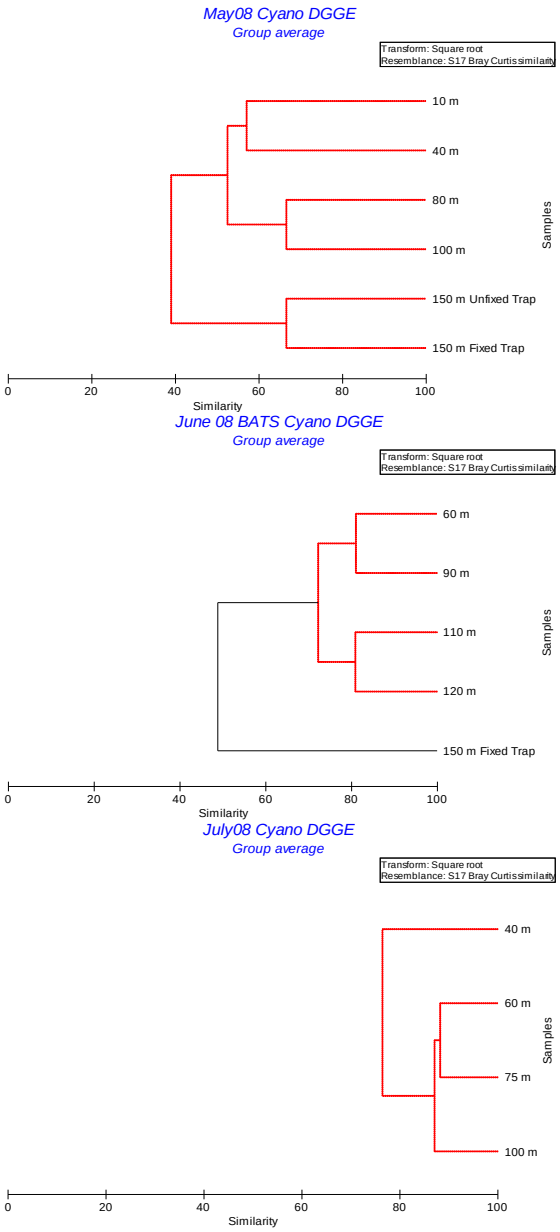

February Bloom 10 Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
120 m
80 m
105 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

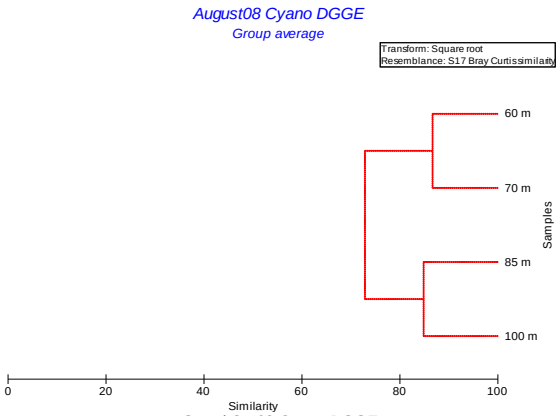

March 10 Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
60 m
100 m
150 m
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

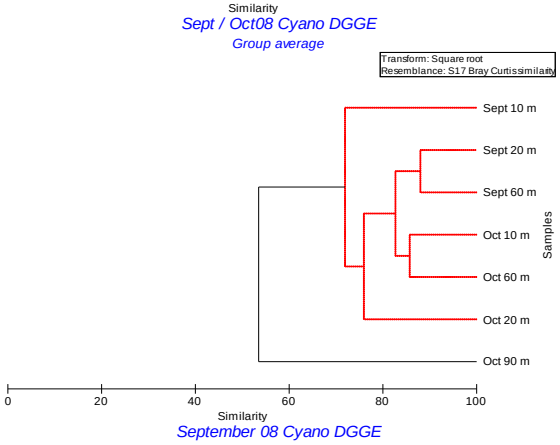

March bloom 10 Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
120 m
150 m Unfixed Trap
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

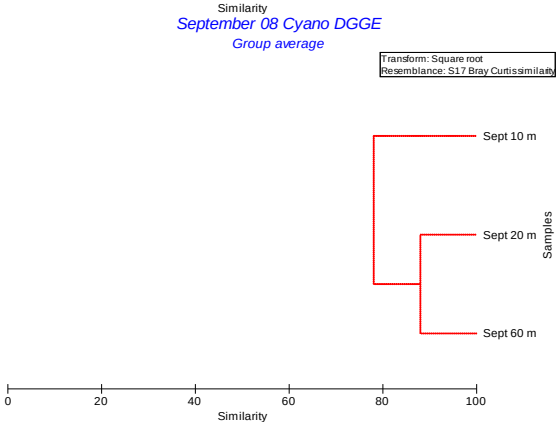

April 10 BATS Euk DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
120 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

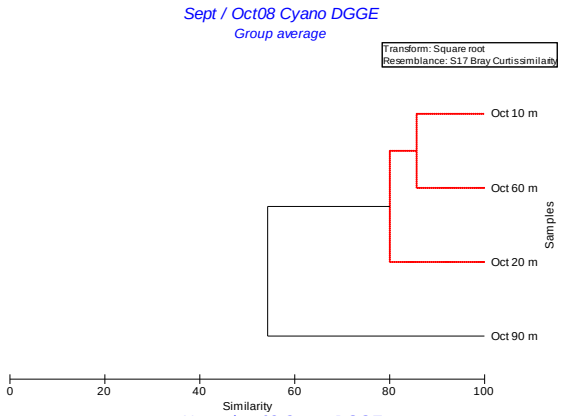
B.

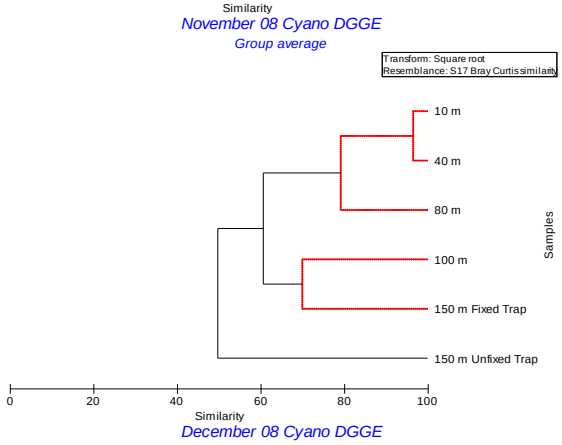

May08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
100 m
150 m Unfixed Trap
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity
June 08 BATS Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
60 m
90 m
110 m
120 m
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity
July08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
40 m
60 m
75 m
100 m
Samples
0
20
40
60
80
100
Similarity

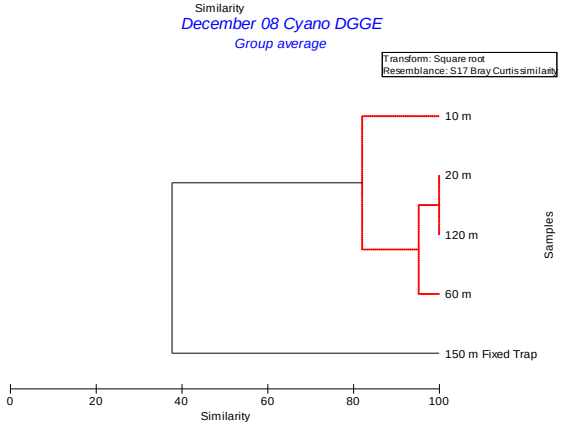

August08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
60 m
70 m
85 m
100 m
Samples
0
20
40
60
80
100
Similarity

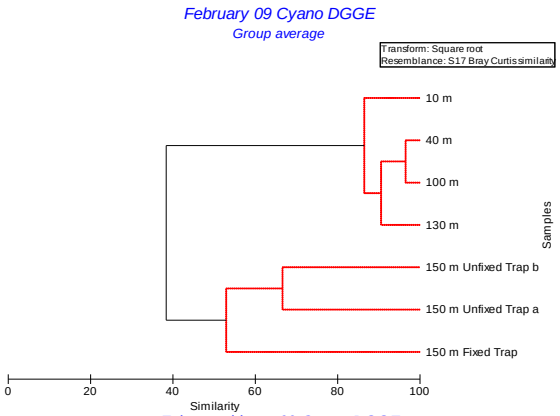

Sept / Oct08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Sept 10 m
Sept 20 m
Sept 60 m
Oct 10 m
Oct 60 m
Oct 20 m
Oct 90 m
Samples
0
20
40
60
80
100
Similarity

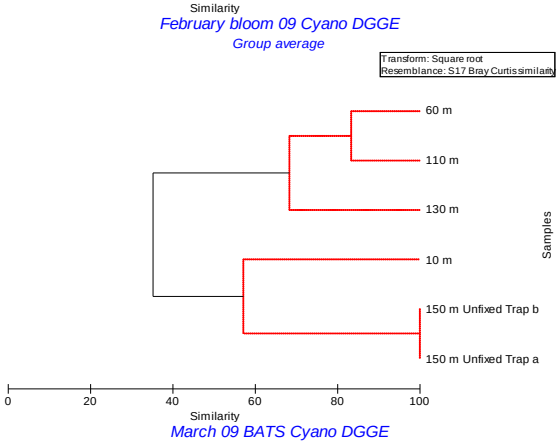

September 08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Sept 10 m
Sept 20 m
Sept 60 m
Samples
0
20
40
60
80
100
Similarity

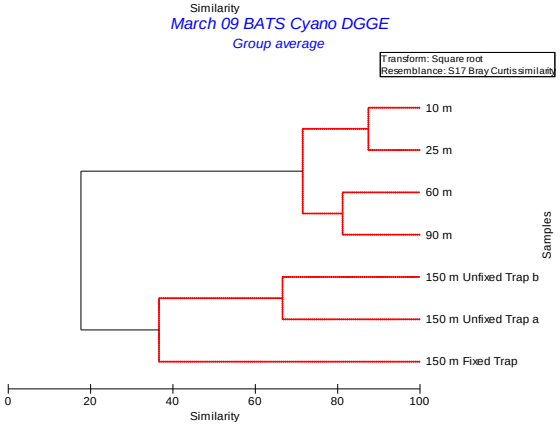

Sept / Oct08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
Oct 10 m
Oct 60 m
Oct 20 m
Oct 90 m
Samples
0
20
40
60
80
100
Similarity

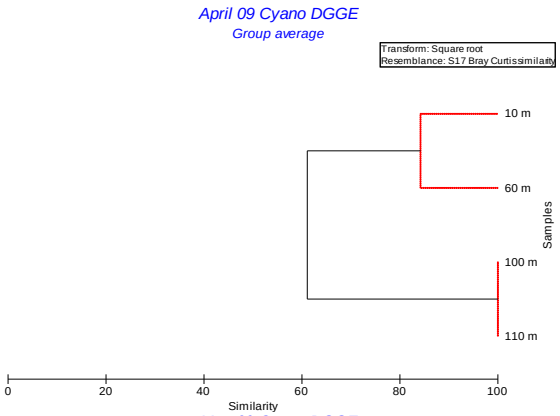

November 08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
100 m
150 m Fixed Trap
150 m Unfixed Trap
Samples
0
20
40
60
80
100
Similarity

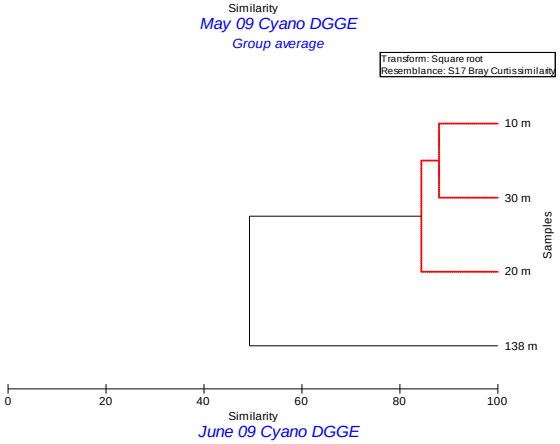

December 08 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
20 m
120 m
60 m
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

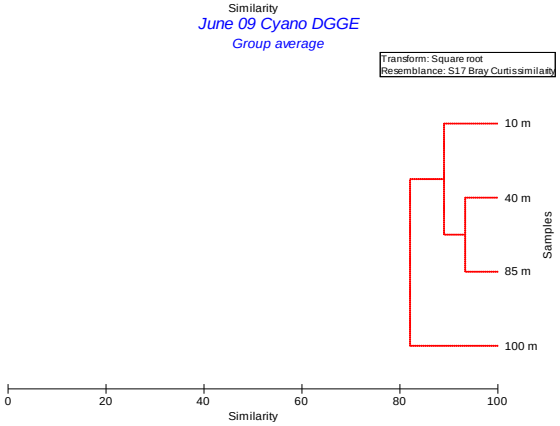

February 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
100 m
130 m
150 m Unfixed Trap b
150 m Unfixed Trap a
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

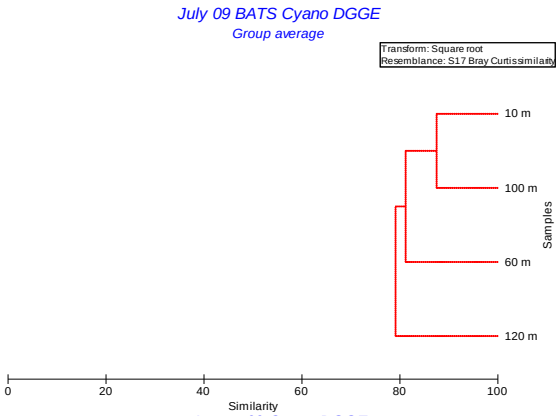

February bloom 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
60 m
110 m
130 m
10 m
150 m Unfixed Trap b
150 m Unfixed Trap a
Samples
0
20
40
60
80
100
Similarity

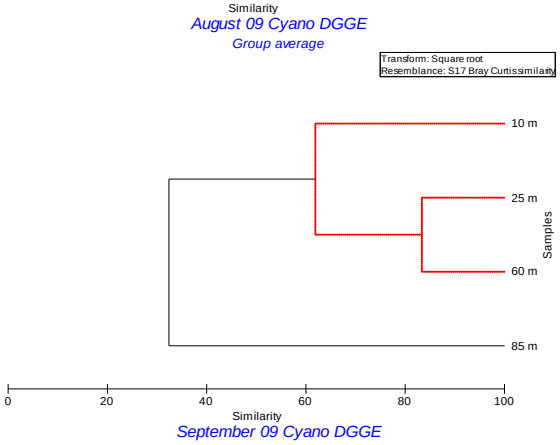

March 09 BATS Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
25 m
60 m
90 m
150 m Unfixed Trap b
150 m Unfixed Trap a
150 m Fixed Trap
Samples
0
20
40
60
80
100
Similarity

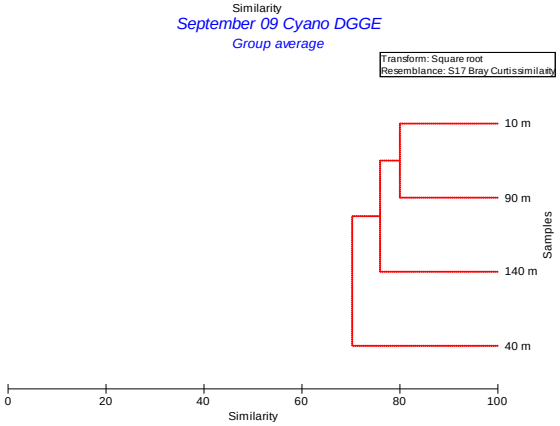

April 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
60 m
100 m
110 m
Samples
0
20
40
60
80
100
Similarity

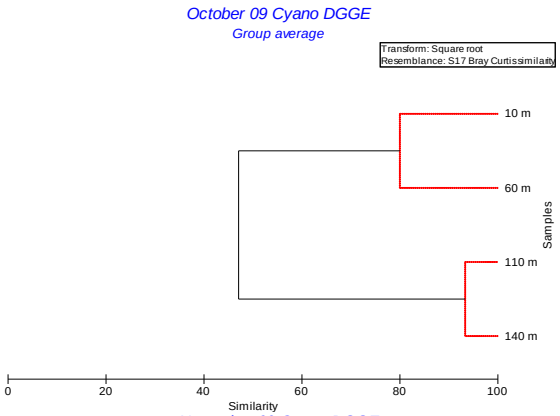

May 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
30 m
20 m
138 m
Samples
0
20
40
60
80
100
Similarity

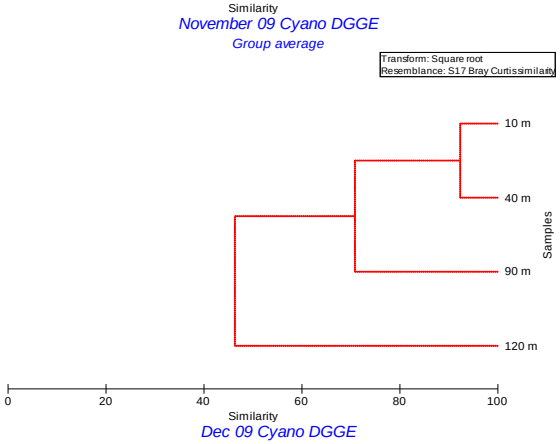

June 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
85 m
100 m
Samples
0
20
40
60
80
100
Similarity

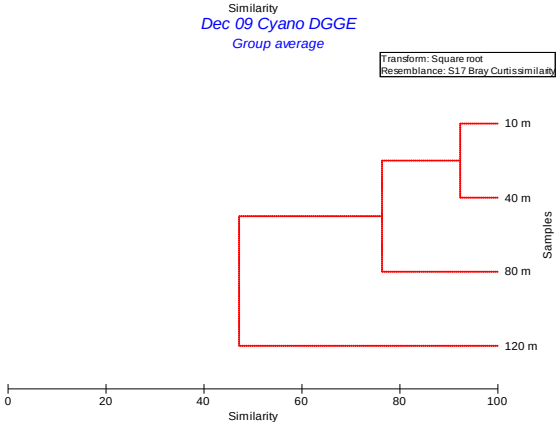

July 09 BATS Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
100 m
60 m
120 m
Samples
0
20
40
60
80
100
Similarity


August 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
25 m
60 m
85 m
Samples
0
20
40
60
80
100
Similarity


September 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
90 m
140 m
40 m
Samples
0
20
40
60
80
100
Similarity

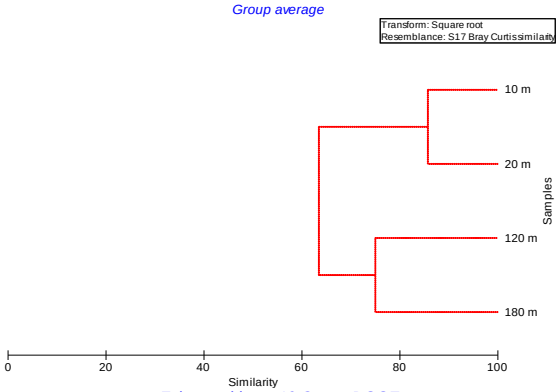

October 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
60 m
110 m
140 m
Samples
0
20
40
60
80
100
Similarity


November 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
90 m
120 m
Samples
0
20
40
60
80
100
Similarity


Dec 09 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
120 m
Samples
0
20
40
60
80
100
Similarity

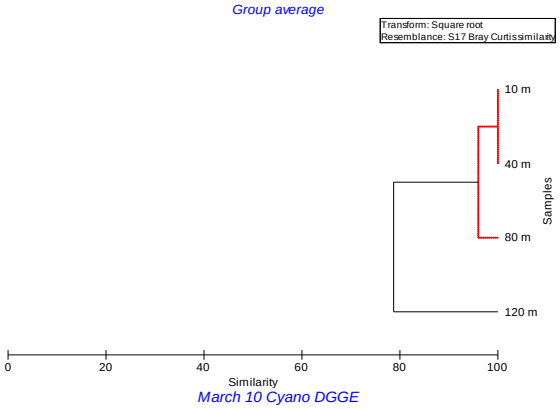
February 2010 BATS Cyano DGGE

Group average



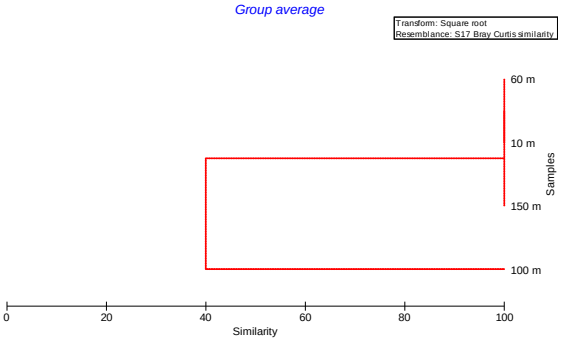
February bloom 10 Cyano DGGE

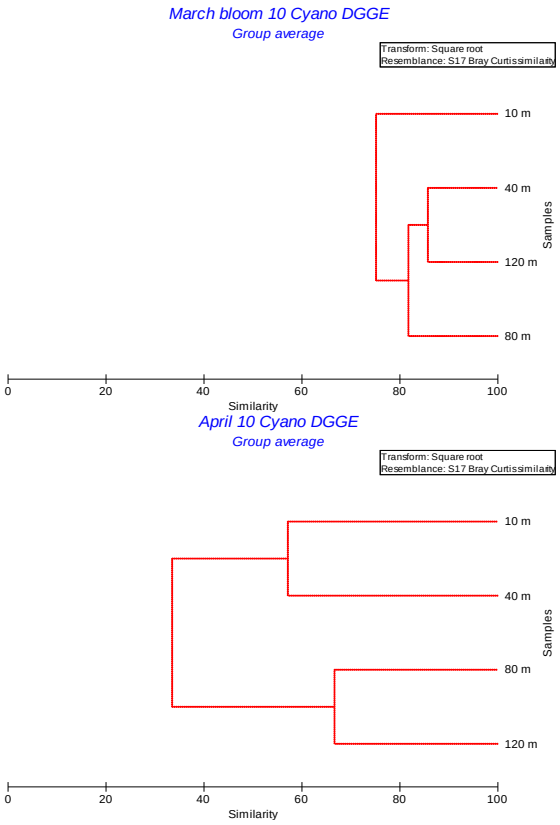
Group average



March 10 Cyano DGGE

Group average




March bloom 10 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
120 m
80 m
Samples
0
20
40
60
80
100
Similarity
April 10 Cyano DGGE
Group average
Transform: Square root
Resemblance: S17 Bray Curtis similarity
10 m
40 m
80 m
120 m
Samples
0
20
40
60
80
100
Similarity

223

223