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The Response of Marine Picoplankton to Ocean Acidification

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Summary

Since industrialisation global CO_2 emissions have increased, and as a consequence oceanic pH is predicted to drop by 0.3-0.4 units before the end of the century - a process coined 'ocean acidification' (OA). Consequently, there is significant interest in how pH changes will affect the oceans' biota and integral processes. We investigated marine picoplankton (0.2-2 µm diameter) community response to predicted end of century CO_2 concentrations, via a 'high CO_2' (~750 ppm) large volume (11,000 L) contained seawater mesocosm approach. We found little evidence of changes occurring in bacterial abundance or community composition due to elevated CO_2 under both phytoplankton pre-bloom/bloom and post-bloom conditions. In contrast, significant differences were observed between treatments for a number of key picoeukaryote community members. These data suggested a key outcome of ocean acidification is a more rapid exploitation of elevated CO_2 levels by photosynthetic picoeukaryotes. Thus, our study indicates the needs for a more thorough understanding of picoeukaryote mediated carbon flow within ocean acidification experiments, both in relation to picoplankton carbon sources, sinks and transfer to higher trophic levels.

Introduction

The marine ecosystem accounts for over 90% of the Earth's biosphere and its microbes play an essential role in marine biogeochemical cycles central to the biological chemistry of the Earth (Falkowski et al., 2008; Worden and Not, 2008). Picoplankton communities (prokaryotes and eukaryotes of 0.2-2.0 μm cell diameter) are known to function as phototrophs, heterotrophs and potentially mixotrophs (Groisillier et al., 2006; Zubkov and Tarran, 2008; Zubkov, 2009). Although picoeukaryotic abundance can be lower than that of their prokaryotic counterparts, their large cell volume means that they often contribute a higher proportion of total picoplanktonic biomass in marine ecosystems (Moran, 2007).

In the last decade, the putative importance of the relationship between rising atmospheric CO_2, ocean biogeochemistry and the populations therein, has been raised (Caldeira and Wickett, 2003; Cicerone et al., 2004; Feely et al., 2004; Orr et al., 2005). Specifically, CO_2 released into the atmosphere dissolves in seawater and reacts to form carbonic acid (H_2CO_3), the dissociation of which forms hydrogen and bicarbonate ions (H^+ and HCO_3^-). An increase in the concentration of hydrogen ions results in a drop in oceanic pH, a process termed 'Ocean Acidification' (OA), since the ocean's buffering capacity is only able to neutralize some of this additional CO_2 (Sabine et al., 2004). A decrease in seawater pH and carbonate saturation is set to continue as long as excess CO_2 enters the atmosphere (Brewer et al., 1997; Feely et al., 2004). Currently, a pH change in the region of 0.3-0.4 units is predicted by the end of the century (Caldeira et al., 2007; Feely et al., 2008). Consequently, there is significant interest in how these pH changes will affect the oceans biota and integral processes (Fabry et al., 2008; Guinotte and Fabry, 2008; Doney et al., 2009; Kerr, 2010; Sabine and Tanhua, 2010).

Changes in pH and CO_2 are likely to have both positive and negative effects upon the growth of oceanic organisms. Aside from the direct physiological and metabolic cost of a change in pH upon organisms (see Pörtner et al 2004 for a summary), a decrease in bio-available carbonate ions results in difficulties for organisms which utilise calcium carbonate to make protective shells or skeletons (Gattuso et al., 1998; Riebesell et al., 2000; Shirayama and Thornton, 2005; Gazeau et al., 2007; Kuffner et al., 2008).

In contrast, increased CO_2 concentration has also been linked to higher levels of primary production (Hein and Sand-Jensen, 1997; Schippers et al., 2004). For example, the marine coccolithophore *Emiliania huxleyi* responded to increased CO_2 by increasing both cell volume and primary production (Iglesias-Rodriguez et al., 2008) and the cyanobacteria *Trichodesmium* responded by increasing CO_2 fixation by 15-128% and N_2 fixation by 35-100% (Hutchins et al., 2007). However, not all organisms will respond in the same way, even within the same family. Fu and colleagues (2007) examined two marine cyanobacteria, *Synechococcus* and *Prochlorococcus,* the former showed a fourfold increase in photosynthesis, when incubated in increased CO_2 and temperature conditions, yet the latter *Prochlorococcus* showed only a minimal response. This variation in response isn't limited to prokaryotes, in the picoeukaryote order Mamiellales*,* numbers of *Micromonas*-like *rcbL* (ribulose bisphosphate carboxylase/ oxygenase) sequences were significantly higher in high CO_2 mesocosms, whereas numbers of *Bathycoccus*- like *rcbL* sequences were evenly spread across treatments (Meakin and Wyman, 2011).

Previous mesocosm studies investigating community response to OA suggested that the total abundance of bacteria did not significantly differ between CO_2 perturbation treatments although changes in free living bacterial community composition can be linked OA, however this likely leads to no loss of function (Grossart et al., 2006; Allgaier et al., 2008). Initially autotrophic picoeukaryotes were also thought not to be significantly affected by elevated CO_2 environments (Engel et al., 2005), yet a recent mesocosm experiment has suggested that this is not likely to be the case (Paulino et al., 2008). Paulino et al (2008) found in a high CO_2 post bloom community that a marked increase in picoeukaryote concentration was observed. This was linked to an ability to out compete larger community members in times of nutrient depletion (Thingstad et al., 2005). In these studies bacterial population dynamics were closely tied to that of the eukaryotic population, and the interaction between autotrophs, heterotrophs and their grazers is key to understanding the response of picoplankton to OA.

Although these experiments set out a basis to understand the effects of ocean acidification upon marine microbiota, the techniques and approaches previously used targeted broad phylogenetic

levels and have often lead to conflicting results (Joint et al., 2011). Environmental DNA sequencing projects have reshaped our understanding of the extent and importance of marine microbial diversity, both prokaryotic (Giovannoni et al., 1990; Britschgi and Giovannoni, 1991; Schmidt et al., 1991; Fuhrman et al., 1992; Fuhrman et al., 1993; Rappe et al., 2000; Rusch et al., 2007) and picoeukaryotic (Diez et al., 2001; Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001; Romari and Vaulot, 2004; Piganeau et al., 2008; Not et al., 2009). Consequently, the application of more sensitive community fingerprinting techniques to investigate the response to CO_2 changes in the total picoplanktonic community and the interaction between its constituent members is necessary.

In response to the questions raised during the seminal mesocosm studies previously outlined (see Riebesell et al., 2008), the 2006 Bergen Mesocosm experiment aimed to investigate the effect of OA upon bacterial populations. During this experiment it has already been observed that trace gas concentrations were affected by elevated CO_2 and that a large level of novelty within the transcriptome of the microbial population was present (Gilbert et al., 2008; Hopkins et al., 2010). Meakin and Wyman (2011) clearly demonstrated that two closely related prasinophytes differed in response to treatment. In this study we extend these studies by investigating community diversity and dynamics in response to elevated CO_2 concentration (~750 ppm, equivalent to year 2100 predictions). Specifically, we investigated the fine resolution dynamics within key marine microbial picoplankton communities (prokaryotes and eukaryotes of 0.2-2.0 μm cell diameter) subjected to increased atmospheric CO_2 during phytoplankton bloom and post bloom conditions, in a large (11,000 L) contained seawater mesocosm experiment.

Results and discussion

pH change and nutrient depletion.

In order to simplify discussion of this study it was decided to split the study into two phases: phase one, a pre-bloom/bloom, nutrient replete phase (days 1-10) and phase two, a post-bloom, nutrient deplete phase (days 11-18). Chlorophyll a data has previously been presented and supports the delimitation of these phases (Hopkins et al., 2010). As expected, CO_2 and pH were significantly autocorrelated throughout the experiment (Regression analysis: r^2 = 0.99; $F_{1,34}$ = 45963.7; P <

0.0001) and the mean CO_2 levels were significantly higher (ANOVA: r^2 = 0.99; $F_{1,34}$ = 145.1; P < 0.0001) in the high CO_2 mesocosms (638.9 ± 125.9 µatm) when compared to the ambient mesocosms (246.3 ± 57.4 µatm). During phase one of the experiment the introduction of CO_2 into the mesocosms induced a change in pH from ~8.1 to ~7.8 (Fig.1). Dissolved nitrate and phosphate were both utilised during the phytoplankton bloom which in turn caused an increase in pH in both the high CO_2 and ambient condition mesocosms, rising to ~pH 8 and ~pH 8.3 respectively and in line with an uptake of excess carbon dioxide during photosynthesis. In the second phase of the experiment, following the second CO_2 amendment on day 10, the pH in acidified mesocosms was maintained between ~pH 7.8 and ~pH 7.9. Dissolved nitrate and phosphate fluctuated in overall concentration but remained relatively low in comparison to pre-bloom levels.

Bacterial abundance and acidification.

In general, averaging all abundances for each mesocosm over the experiment indicated both experimental ($5.11 \times 10^6 \pm 2.75 \times 10^6$ cells/ml) and control mesocosms ($6.13 \times 10^6 \pm 2.31 \times 10^6$ cells/ml), did not significantly differ with treatment (ANOVA: $F_{1,34}$ = 1.44; P = 0.238). Total bacterial numbers slowly increased during the beginning of the first (nutrient replete) phase of the experiment, irrespective of treatment (Fig.2). Since total cell count can be affected by its constituent subpopulations, we further resolved into the High and Low Nucleic Acid groupings (herein HNA and LNA). Marked growth was shown by both the the HNA and LNA bacteria, in parallel with total cell count increases, which peaked on day 6 (~4×10^6 and ~0.9×10^6 cells/ml, for HNA and LNA respectively). Subsequently, both HNA and LNA bacteria exhibited a decrease in number between days 7-10 (Fig.2), corresponding to the initiation of the phytoplankton bloom.

During the second (post-bloom, nutrient deplete) phase total bacterial numbers rose rapidly under both regimes (Fig.2), peaking at day 14 for high CO_2,(~1×10^7 cells/ml) and day 15 for ambient (~1×10^7 cells/ml). In the high CO_2 treatment this was followed by a rapid drop in cell numbers, comparable to those observed at the initiation of the experiment (~1.7×10^6). Ambient cell counts remained comparatively high ~8.1×10^6. The pattern for total bacterial abundance was mirrored by both the HNA and LNA bacterial operational groupings (Fig.2). To determine if any of these observations were significant the two-sample Kolmogorov-Smirnov distribution fitting test was applied, and indicated that

there was no statistical difference in the bacterial abundance dynamics between treatments for both the major bacterial nucleic acid types (D = 0.278, P = 0.425).

The phototrophic bacteria *Synechococcus* gradually increased over the duration of the experiment (Fig.2) from 2.5 x10^3 cells/ml (day 1) to 2.5 x 10^4 cells/ml under elevated CO_2 (day 16) and 3.5x10^4 cells/ml under ambient conditions (day 17). Similarly to other bacteria, *Synechococcus* did not respond in terms of abundance or dynamics to the experimental treatment, reflected by the fact that no significant difference was found in the bacterial cell count distributions for the two treatments (D = 0.0389, P = 0.098).

Previous studies have also indicated that increased acidification has no significant influence on the abundance of total bacteria (Rochelle-Newall et al., 2004; Grossart et al., 2006; Allgaier et al., 2008), and thus, these broad data confirm previous observations.

Eukaryote abundance and acidification.

The mean cell abundances of key eukaryote groups were compared between treatments (Fig. 2), with the exception of the coccolithophores there were no significant differences between treatments (Table S1). Despite high variance in cell counts, the mean coccolithophore cell abundances were significantly lower (ANOVA: $F_{1,34}$ = 6.15; P = 0.018) under high CO_2 conditions (411 ± 337.5 cells/ml) when compared to ambient conditions (942.6 ± 844.2 cells/ml). More specifically, when analysing the pattern of evolution of cell counts over time, only the small picoeukaryotes significantly differed (Kolmogorov-Smirnov test statistic, D = 0.500, P = 0.021) between treatments, suggesting that the temporal distribution of only these organisms responded to the experimental regime imposed. Hopkins et al (2010) found that the abundances of large picoeukaryotes, cryptophytes, and coccolithophores were suppressed in high CO_2 conditions at localised time points, yet we found no significant evidence for this in the evolution of cell count distributions over time; large picoeukaryotes (D = 0.444, P = 0.056), nanoeukaryotes (D = 0.278, P = 0.503), coccolithophores (D = 0.389, P = 0.132) and cryptophytes (D = 0.389, P = 0.132). Our study would suggest the differences observed by Hopkins et al are likely to be temporary and that the community is able to adjust in the relatively short time period studied.

Cell abundance data, derived from flow cytometry suggested that small picoeukaryotes also numerically dominated the eukaryotic organisms examined during this study (Fig.2). Small picoeukaryotes established an initial bloom faster under high CO_2 conditions when compared to ambient pH conditions. In the high CO_2 treatment small picoeukaryotes achieved a twofold increase in cell concentration by day 6 (4.6×10^4 cells/ml) followed by a considerable reduction to 1.2×10^4 cells/ml (day 10). The small picoeukaryote bloom in the ambient treatment took longer to establish but was more prolonged reaching a maximum of 5.1×10^4 cells/ml on day 10. In phase two of the experiment, small picoeukaryotes decreased in abundance (or remained low in the high CO_2 treatment) until day 16 when a secondary bloom initiated and numbers rapidly increased to levels comparable to those observed at the peak of phase one (Fig.2).

An increase in abundance in high CO_2 conditions is consistent with Paulino et al's (2008) work however, in their study differences in small picoeukaryote abundance were most pronounced under nutrient depletion towards the end of their experiment. Here, we observed differences in abundance throughout.

Sequence abundance and richness.

In order to map bacterial community structure effects by acidification, we first sought to confirm that the populations within the mesocosms were representative of marine communities, and not simply random assemblages due to 'bottle effects' (Zobell and Anderson, 1936).

Provisional identification attributed bacterial sequences to a broad range of phylogenetic groups typical to marine samples including the Proteobacteria, Bacteriodetes, Cyanobacteria and Actinobacteria. Of these, a total number of 469 bacterial OTUs were identified at the 97% similarity level. Figure 3a represents bacterial OTUs containing 10 or more sequences. Tree topology supported high taxonomic ranking with abundant OTUs falling within well supported clusters. The highest number of bacterial SSU rRNA sequences could be attributed to the Rhodobacteriales (861 sequences) and within it the most abundant OTU (OTU 6, 584 sequences) was closely affiliated with other cultured marine *Roseobacter* sequences (93.5% bootstrap support). Additionally, we found

significant numbers of sequences relating to SAR11 (OTU 7, 260 sequences). Although not as prevalent as the Alphaproteobacteria, a sizeable number of Gammaproteobacterial sequences were also detected within our clone libraries, most closely related to environmental sequences belonging to SAR86 groups II (OTUs 8 and 25 totalling, 296 sequences), III (OTU 4, 63 sequences) and SAR92 (OTUs 18 and 15 totalling 21 sequences). Finally, sequences associated with members of the phylum Bacteriodetes were commonly detected, the most abundant OTU (38) containing 339 sequences. A complete list of picoplankton OTU identity is given in Table S3.

All major picoeukaryotic lineages typically retrieved from a costal pelagic marine community were also represented (Figure 3b). Tree topology supported high level taxonomic identity. Archaeplastida and Stramenopiles formed well supported monophyletic groups, and within these the sequences clustered within well supported sub-groups. The Alveolata were paraphyletic with respect to the Ciliophora when using a 70% support value, yet support within contained groups was high. The highest sequence OTU diversity (at 98% identity) was found within the Chrysophyceae (14), Ciliophora (7) and group I Alveolates (5). However, the most abundant OTUs corresponded to the photosynthetic Mamiellales organisms *Bathycoccus* (OTU 4) and *Micromonas* (OTU 2); together contributing 38% of the entire sequences detected within the 18S clone libraries (219). Members of the Novel Alveolates group I (NAI) contributed over 25% of sequences and Chrysophyceae, 17%.

As such, we confirmed that the large volume mesocosms utilised here contained communities similar to those found within other marine environments both at local and global scales. (Zubkov et al., 2002; Worden, 2006; Allgaier et al., 2008).

Bacterial community response to OA.

The majority of T-RF fragment lengths were linked to specific sequences within our clone library (Fig. S1). For simplification, the dynamics of the 6 most abundant bacterial and picoeukaryote peaks were plotted over time (Fig.4) and tested for significance using the two-sample Kolmogorov-Smirnov distribution fitting test. Of the most abundant bacterial T-RFs 3 were attributed to the Alphaproteobacteria (peaks 145 b.p., 435 b.p. and 436 b.p.) 2 to Bacteriodetes (peaks 86 b.p. and 88 b.p.) and one to the Gammaproteobacteria (peak 136 b.p.). No significant responses to treatment

were detected in the majority of bacteria examined; Rhodobacteriales 436 (D = 0.353, P = 0.190), SAR11 145 (D = 0.412, P = 0.081), Bacteriodetes 86 (D = 0.176, P = 0.930) Bacteriodetes 88 (D = 0.294, P = 0.387) Gammaproteobacteria 136 (D = 0.294, P = 0.387). Rhodobacteriales 435 showed a significant difference between treatments (D = 0.471, P = 0.031) which was attributed to fluctuations in the first phase of the experiment.

Various studies have demonstrated that the distinct dissolved organic carbon (DOC) compounds released by algae during the course of a phytoplankton bloom and post bloom conditions select for specific bacterial sub-communities or populations (Riemann et al., 2000; Schäfer et al., 2001; Grossart et al., 2005). In this study there were indications that bacterial populations can be linked to phase of experiment. A high prevalence of organisms such as the Rhodobacteriales at the beginning of the study, and increased levels of SAR 11 at the end of the study are likely to be indicative of their ideal nutrient concentrations during these phases (see Hopkins et al, 2010 and BMED for further nutrient data).

Therefore, in combination with the cell abundance data we have demonstrated that bacterioplankton communities undergo dynamic changes during phytoplankton bloom and post-bloom conditions, but on the whole do not significantly differ with acidification. With respect to this, we surmised that if short term acidification effects did not alter microbial community dynamics significantly, then longer term (e.g. 100 year) effects will more than likely have minimal effects due to the time allowed for physiological adaptation to the prevailing changes. Our assumption has validity when considering the diversity of habitats and pH gradients to which bacteria are already exposed. Many bacterial populations already experience pH as low or even lower than those projected for the end of the century and continue to function (Joint et al., 2011). When looking at population changes with depth (which constitutes a natural pH gradient) at the ALOHA sampling station many key organisms were detected across the depth gradient (DeLong et al., 2006) suggesting some natural populations are already able to withstand a range of pH. For individual taxa, recent studies have highlighted the some common marine species, e.g. *Vibrio* spp. are able to regulate internal pH (Labare et al., 2010), the prevalence of such compensation mechanisms and the ability to withstand changes in future pH

would confer a large advantage under elevated CO_2 concentrations (equivalent to year 2100 predictions) and warrants further investigation in key marine taxa.

Picoeukaryote community response to OA.

Unlike the bacterioplankton, significant differences in picoeukaryote community composition were observed between treatments, but this varied between group studied and phase of the experiment.

Picoeukaryotes belonging to the Mamiellales (*Micromonas* and *Bathycoccus* with a peak at 265 b.p.) together formed around 38 % of the total sequences detected and were found to significantly differ between treatments when using T-RFLP (Kolmogorov-Smirnov test statistic, D = 0.47, P = 0.04). More specifically, in early phase one elevated CO_2 promoted Mamiellales 265 causing it to form a higher proportion of the total community profile within the high CO_2 mesocosms (0.90 in high CO_2 compared to 0.50 in ambient CO_2 day 1). This difference became less pronounced as phase one progressed (days 2-10). In the post-bloom mesocosms (phase two) abundance decreased (days 11-14) and then recovered for the remainder of the study (Fig.4).

Although the application of T-RFLP is unable to differentiate between *Micromonas* and *Bathycoccus* phylotypes, a higher percentage of *Micromonas* sequences were detected in the high CO_2 treatment (85 %) than in ambient treatment CO_2 (15 %), whereas *Bathycoccus* sequences were evenly distributed (55 % and 45 % respectively). Corroborating these data, Meakin et al (2011) found *Micromonas* like *rcbL* phylotypes were significantly higher in elevated CO_2 than those of *Bathycoccus* during the first phase of the same mesocosm experiment. Further, they postulated that these differences were due an inefficient operation of carbon concentration mechanisms (CCM) within *Micromonas*, which would be favoured under future predicted increases in CO_2 concentration (Engel et al., 2008).

In the post-bloom, nutrient deplete, phase two, changes in T-RFLP abundance was seen for all of the abundant T-RFs (Fig.4). The contribution of Mamiellales 265 to the T-RFLP profile was similar to the levels observed in the ambient treatment suggesting that under phosphate and nitrate limitation the positive effect of CO_2 amendment is counteracted. The proportional reduction of Mamiellales 265

corresponded to an increase in the contribution of the other dominant community members. Both Group I Alveolate at peak 222 b.p. (GIA 222) and the Chrysophyceae organisms at peak 231 b.p. (Chrysophyte 231) significantly favoured ambient conditions, (both D = 0.53, P = 0.02). It is likely that heterotrophic organisms would be favoured in the second phase of the experiment and increased abundance of lineages such as the Alveolates and Chrysophyceae, both of which are thought to contain heterotrophic or mixotrophic organisms (Jones, 2000; Moreira and Lopez-Garcia, 2002; Andersen, 2004), supports a switch to heterotrophy/bacterivory after the phytoplankton bloom. No significant differences between treatments were found in the distribution of Chrysophyte 221 (peak 221 b.p.) Chrysophyte 227 (peak 227 b.p.) and Group II Alveolate 373 (GIIA, peak 373 b.p.) (Fig.4).

Trophic interactions.

Allgaier and colleagues (2008) noted that heterotrophic bacterial dynamics were closely correlated to phytoplankton development and, hence, responded to changes in CO_2. Further, (Tank et al., 2009) suggested that in their study cascading trophic interactions were a key driver of bacterial response to pH perturbation. During the first phase of the experiment added phosphate and nitrate was utilised by the phytoplankton bloom (inc. picoeukaryotes) thereby depleting the dissolved N and P concentration. Bacterivory would serve as an important mechanism to overcome this limitation during the nutrient deplete phase two. Although undoubtedly heterotrophic nanoflagellates were likely key grazers within the community, there is evidence that mixotrophy is high in oligotrophic waters (Unrein et al, 2007). In their study Zubkov and Tarran, 2008 noted that plastid containing eukaryotes < 5 µm (which were numerically dominated by picoeukaryotes) can graze a significant proportion of marine bacteria (40-95 %) suggesting that mixotrophy is common in nutrient deplete waters. Indeed further to this, there is evidence that *Micromonas* is able to act mixotrophically (Gonzalez et al., 1993). We hypothesize that autotrophy and potentially mixotrophy within in the picoeukaryote population may explain some of the patterns observed in this experiment (fast bloom promotion, population maintenance and bacterial abundance cycling). Further, with potential switching of carbon processing pathways (sources and sinks for carbon), and their increased abundance under acidification, we suggest that key future research areas within ocean acidification studies should examine picoeukaryote mediated carbon

flow, its magnitude and effects upon higher trophic levels if we are to fully understand the effects of increased atmospheric CO_2 upon the world's marine ecosystems.

Experimental procedures

Experimental set up and sampling regime.

The complete experimental set up has been outlined previously (Gilbert et al., 2008; Hopkins et al., 2010; Meakin and Wyman, 2011). Here, we present the data for 2 elevated CO_2 experimental and 2 ambient CO_2 control mesocosms (2 m diameter, 3.5 m deep). Experimental mesocosm enclosures were filled with unfiltered native fjord water and gently sparged with CO_2 (750 µatm) for 2 days (4th-6th May) until a pH ~ 7.8 was established. To control for sparging effects ambient condition mesocosm enclosures were sparged with air. In order to simulate natural conditions more closely, a phytoplankton bloom was induced through the addition of nitrate and phosphate in all mesocosms on the 6th of May (initial concentrations: 1 µmol l^{-1} phosphate; 17 µmol l^{-1} nitrate). Blooming phytoplankton growth reduced CO_2 concentrations in the high CO_2 mesocosms, therefore mesocosm enclosures were re-acidified 10 days after mesocosm establishment (15/5/2006), and ambient condition enclosures again sparged with air. To isolate picoplankton daily samples of ~2 L of water were pre-filtered through Whatman GF/A (1-6 µm nominal pore size) filters to remove large eukaryote cells and filtrate collected onto 0.2 µm Durapore membranes. Sampling was initated on the 6th May (day 1) and filters stored at -80 ºC prior to molecular analysis.

Enumeration of planktonic cells via flow cytometric analysis.

Daily flow cytometric counts of absolute concentrations of major bacterial and eukaryotic groups were performed using a Becton Dickinson FACSort flow cytometer equipped with an air-cooled blue light laser at 488nm according to previously documented protocols (Gasol et al., 1999; Zubkov et al., 2001; Acinas et al., 2004; Tarran et al., 2006; Zubkov and Burkill, 2006; Zubkov et al., 2008).

Nucleic acid extraction, PCR and T-RFLP analysis.

Total nucleic acids were extracted using the protocol outlined in Huang *et al*. (2009). Approximately 20-30ng of purified template was used per PCR. For Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis, a 500 b.p. region of the 16S small subunit ribosomal RNA (SSU rRNA) was amplified using labelled primers (6FAM)27F and 536R (Suzuki et al., 1998), and a 600 b.p. region of 18S SSU rRNA amplified using primers (6Fam)EukF and Euk570R(Baldwin et al., 2005). For the construction of clone libraries, near full length fragments of the 16S and 18S SSU

rRNA genes were amplified using 27F-1492R(16S) and EukF-EukR(18S) (DeLong, 1992). For short SSU rRNA amplification thermal cycling conditions were as follows: Initial pre-denaturation at 94 ºC for 2 minutes followed by 30 thermal cycles of 94 ºC for 1 min, 52 ºC for 1 min and 72 ºC for 3 min. Near full length SSU rRNA amplification consisted of Initial pre-denaturation at 94 ºC for 2 minutes followed by 30 thermal cycles of 94 ºC for 1 min, 60 ºC (16S) or 55 ºC (18S) for 2 min and 72 ºC for 3 min, all PCR reactions employed a final extension phase of 10 min at 72 ºC.

T-RFLP PCR products were gel purified using a QIAquick Gel Extraction Kit (QIAGEN) and 20 µl of product was digested for 4 hours at 37 ºC in a 30 µl total reaction volume using 20 units restriction enzyme MspI (Promega). Digestion products (0.5 µl) were combined with denatured 0.5 µl LIZ600 size standard (Applied Biosystems) and 9 µl of Hi-Di formamide (Applied Biosystems), analysed on an Applied Biosystems 3730 DNA sequencer and the sizes of restriction fragments were calculated. Binning analysis was performed using Genemarker (Softgenetics) and restriction fragment cross correlated to specific cloned sequences. (See online experimental procedures for full description).

Clone library construction and library sequencing.

Near full length SSU rRNA PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, California). For the 16S SSU rRNA gene, ~ four hundred and eighty clones were randomly picked from each of 5 libraries corresponding to sample days 2, 8, 9, 14 and 15 (7th, 13th, 14th, 19th and the 20th of May 2006) from both high and ambient CO_2 mesocosms (10 libraries, 4800 clones in total). Clones were sequenced using vector primers M13F and R at the NERC Biomolecular Analysis Facility-Edinburgh (NBAF-E). For the 18S SSU rRNA gene ninety six clones were randomly picked from each of 4 libraries corresponding to days 2, 8, 14 and 17 (7th, 13th, 19th and 22nd May 2006) from both high and ambient CO_2 mesocosms (8 libraries, 768 in total). Clones were sequenced using vector primers M13F and R and internal primer 3F (Giribet et al., 1996). Sequencing services were provided by Geneservice (www.geneservice.co.uk).

Sequence processing and analysis

Sequence processing was carried out within the Staden pregap4 and gap4 framework (Staden, 1996), base-called using Phred (Ewing and Green, 1998; Ewing et al., 1998) and assembled using

Phrap (Green, 2008) with default settings. Screening for chimeras was performed using Mallard (16S) (Ashelford et al., 2006) and Bellerophon (18S) (Huber et al., 2004). Any sequences which we were of short length, low quality or deemed chimeric were removed from analysis.

OTU identification.

Sequences were aligned using the NAST alignment tool (DeSantis et al., 2006) (16S) or SINA (SILVA INcremental Aligner) web aligner(Pruesse et al., 2007)(18S). Modeltest (Posada and Crandall, 1998) was used to determine optimal likelihood settings to calculate a distance matrix in PAUP4b8 (Swofford, 2002) using the general Time Reversable model (GTR)(Tavaré, 1986) and a gamma distribution. The resultant matrix was used to calculate the number of operational taxonomic units (OTUs) using DOTUR and MOTHUR (Schloss et al., 2004; Schloss and Handelsman, 2005; Schloss and Handelsman, 2006; Schloss et al., 2009). MOTHUR was again used to designate representative sequences for each OTU in the combined high and ambient CO_2 libraries using a 97 % (16S) and 98 % (18S) similarity cut-off, which roughly corresponds to a species/genus level (Stackebrandt and Goebel, 1994; Romari and Vaulot, 2004).

16S OTUs with 10 or more associated sequences and all 18S OTUs were realigned (as above) to include a selection of published sequences (Table S2). The 16S alignment contained 99 sequences and 1799 nucleotide positions and the 18S alignment contained 218 sequences and 2399 nucleotide positions after duplicate sequences and common gaps were removed.

For each dataset PAUP4b8 (Swofford, 2002) was used to generate a Neighbour Joining (NJ) tree using the likelihood criterion, and bootstrap support values for 1000 replicates. The resultant tree was used to determine the taxonomic affiliation of each OTU, identity was given via inclusion in the nearest supported cluster (at 70 % bootstrap value or higher).

Statistical analyses.

One-way ANOVA tests, regression analysis, coefficients of determination (r^2), residuals and significance (P) were calculated using Minitab software (version 14.20, Minitab, University Park, PA, USA). In order to test the similarity of distribution, shape and position of data generated, from the

FACS count and the T-RFLP community distribution data, the two-sample Kolmogorov-Smirnov test was utilised. This analysis employed distribution fitting tests for comparing an empirical distribution determined from a sample with a known distribution. It can also be used, as was the case for the current study, for comparing two empirical distributions (Nikiforov, 1994).

Curation.

Samples, extractions and clone libraries were bar-coded and curated using Handlebar (Booth et al., 2007). Biogeochemical data including key nutrients, chlorophyll concentration, temperature, salinity, sequence information, T-RFLP traces and additional metadata is available at http://nebc.nerc.ac.uk/bergendb, The Bergen Mesocosm Experiment Database (BMED). Hosting of BMED was performed on the NEBC Bio-Linux scientific computing platform (Field et al., 2006) (See online material and methods for full description). A total of 2871 (16S SSU rRNA) and 573 (18S SSU rRNA) non-chimeric gene sequences with the associated MIMARKS (Yilmaz et al., 2011a; Yilmaz et al., 2011b) compliant metadata have been deposited at EBI using Webin under the accession numbers FR683104 - FR685974 (16S) FR874265 - FR874837 (18S).

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Figure legends

Figure 1. Mean daily nutrient and pH values for high CO_2 mesocosms (closed circle) and ambient mesocosms (open circle). Error bars represent standard deviation from the mean of the 2 replicate mesocosms. Solid vertical bar separates phases one and two. Nutrient data collected by I. Joint and pH data first presented in Hopkins et al (2010).

Figure 2. Mean daily FACS counts for high CO_2 mesocosms (closed circle) and ambient mesocosms (open circle). Prokaryotic groupings include total bacteria, High Nucleic Acid content bacteria (HNA), Low Nucleic Acid content bacteria (LNA) and *Synechococcus*. Eukaryotic groupings include small picoeukaryotes, large picoeukaryotes, nanoeukaryotes, Coccolithophores and Cryptophytes. Error bars represent standard deviation from the mean of the 2 replicate mesocosms. Solid vertical bar separates phases one and two.

Figure 3a: A 70 % Majority Rule Consensus Tree of dominant prokaryotic OTUs as inferred by Neighbour Joining distance criterion under GTR+I+G model. Bootstrap support from 1000 replicates are shown at nodes. Phyla/sub-phyla are highlighted as follows: Blue = Alphaproteobacteria, Red = Gammaproteobacteria, Orange = Betaproteobacteria and Green = Bacteriodetes. For more specific taxonomic grouping refer to key.

Figure 3b: A 70 % Majority Rule Consensus Tree of picoeukaryotic OTUs as inferred by Neighbour Joining distance criterion under GTR+I+G model. Bootstrap support from 1000 replicates are shown at nodes. Phyla/sub-phyla are highlighted as follows: Blue = Stramenopiles, Red = Archaeplastida,

Purple = Rhizaria (inc Cercozoa), Yellow = Prymnesiophyceae, Orange = Fungi and Green = Alveolata. For more specific taxonomic grouping refer to key.

Figure 4. Picoplankton community change over time as assessed by T-RFLP. Community T-RFLP profiles were generated for all samples. Mean values for the 6 most abundant bacterial and picoeukaryotic T-RFs are shown for ambient (open circle) and high CO_2 mesocosms (closed circle). Solid vertical bar separates phases one and two.

Table S1 Comparison of abundances from key eukaryotic group abundances using ANOVA tests. Given for each group and treatment are the mean abundance, the the standard deviaition of the mean (SD), and the ANOVA tests results, including *F*-ratio and significance (*P*).

Table S2: Accession numbers for additional sequences downloaded from GenBank, used in 16S and 18S phylogenetic analysis.

Table S3a: Identity and abundance of OTU's from prokaryotic clone libraries. A distance matrix of sequences was used to determine OTU's (similarity level 97 % for bacteria) and their abundance across the total experiment using MOTHUR. Taxonomic identity of reference sequences was determined by phylogenetic placement. The number of sequences detected in clone libraries pooled by treatment is given.

Table S3b: Identity and abundance of OTU's from picoeukaryotic clone libraries. A distance matrix of sequences was used to determine OTU's (similarity level 98 % for picoeukaryotes) and their abundance across the total experiment using MOTHUR. Taxonomic identity of reference sequences was determined by phylogenetic placement. The number of sequences detected in clone libraries pooled by treatment is given.

Online experimental procedures

T-RFLP

T-RFLP PCR products were gel purified using a QIAquick Gel Extraction Kit (QIAGEN) and 20 µl of product was digested for 4 hours at 37 ºC in a 30 µl total reaction volume using 20 units restriction enzyme MspI (Promega). Digestion products (0.5 µl) were combined with denatured 0.5 µl LIZ600 size standard (Applied Biosystems) and 9 µl of Hi-Di formamide (Applied Biosystems) and analysed on an Applied Biosystems 3730 DNA sequencer. Fragments were calculated and binned using Genemarker (Softgenetics). Briefly, bin widths were checked and manually adjusted to encompass all detected peaks. To differentiate signal from background Fluorescence Unit (FU) a threshold of 40 units was used to determine which T-RF's to include and subsequently a cut off of 20 FU's was used for a presence/absence binary matrix. All peaks were manually checked for inclusion in analysis. When required, relative abundance measures were calculated by dividing individual peak height by total peak height spanning all valid peaks within the analysis. In order to investigate community structure these data were ranked based upon total abundance, then change in the 6 most dominant peaks plotted over time. When required, e.g. to putatively identify T-RF fragments, the cut site position was determined by running unaligned non-chimeric sequences, trimmed to short amplicon primer region through T-RFLPmap (Field and Griffiths 2008). The fragment length of specific clones was then cross correlated to this data to determine the identity of specific T-RF's in relation to clone sequences generated from the mesocosms.

Sequence assembly and quality assessment

Near full length SSU rRNA PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, California). For the 16S SSU rRNA gene, five hundred clones were randomly picked from each of 5 libraries corresponding to sample days 2, 8, 9, 14 and 15 (7th, 13th, 14th, 19th and the 20th of May 2006) from both high and ambient CO_2 mesocosms. Clones were sequenced using vector primers M13F and R at the NERC Biomolecular Analysis Facility-Edinburgh (NBAF-E). For the 18S SSU rRNA gene ninety six clones were randomly picked from each of 4 libraries corresponding to days 2, 8, 14 and 17 (7th, 13th, 19th and 22nd May 2006) from both high and ambient CO_2 mesocosms. Clones were sequenced using vector primers M13F and R and internal

primer 3F (Giribet et al 1996). Sequencing services were provided by Geneservice (www.geneservice.co.uk)

SSU rRNA sequence processing was carried out within the Staden pregap4 and gap4 framework (Staden 1996), using a custom script (FRProcessing_triplets.pl), and accompanying parameter settings file (pregap4params_v2.txt). Script and parameter file can be downloaded from: http://nebc.nerc.ac.uk/tools/code-corner/scripts/sequence-processing#-frprocessing_pairs_v2-pl. Processing was run on the Bio-Linux platform (Field et al 2006) on a Dell Optiplex 755 32 bit system with 4Gb RAM. Briefly, sequences were base-called using Phred (Ewing and Green 1998, Ewing et al 1998) with the trim-alt option with cut off specified at 0.025. Vector clipping was done with the pregap4 sequencing vector clip module and reads were assembled using Phrap (Green 2008) with default settings. Assembly logs were screened for low quality sequences or assembly problems. Final cleaned, assembled consensus sequences were exported via the Staden gap4 program. Screening for chimeras was performed using Mallard (16S) (Ashelford et al 2006) and Bellerophon (18S) (Huber et al 2004).

Online experimental procedure references

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1 ***Table S3b:***

OTU ID	T-RFLP Cut Site	Phylogenetic ID	Reference sequence	High CO_2	Ambient CO_2	Total Number of sequences
4	265	*Bathycoccus*	1801A06	80	64	144
2	265	*Micromonas*	1804A07	63	12	75
8	222	Novel Alveolate Group I	1801C02	14	41	55
5	222	Novel Alveolate Group I	1801A08	16	33	49
13	221	Chrysophyceae	1804B06	20	22	42
30	222	Novel Alveolate Group I	1804C03	10	15	25
15	227	Chrysophyceae	1802A11	17	2	19
45	373	Novel Alveolate group II	1815B03	3	14	17
1	222	Novel Alveolate Group I	1801A01	4	12	16
27	370	MAST 2 (Marine Novel Stramenopiles Group 2)	1802G08	9	1	10
14	222	Chrysophyceae	1806D12	2	5	7
46	373	Novel Alveolate group II	1815C01	2	4	6
20	599	MAST 12 (Marine Novel Stramenopiles Group 12)	1802C04	6	0	6
26	290	MAST 12 (Marine Novel Stramenopiles Group 12)	1802G05	6	0	6
21	227	Chrysophyceae	1804A05	4	2	6
39	221	Trebouxiophyceae	1807B08		5	5
50	364	*Ostreococcus*	1815D05	5		5
17	227	Chrysophyceae	1802B08	5	0	5
44	132	Chrysophyceae	1815A02	5	0	5
24	275	Fungi	1802F03	3	1	4
56	265	Dictyochophyceae	1816F05		4	4
18	380	Rhizaria	1804C10	4	0	4
3	376	Novel Alveolate group II	1801A05	1	2	3
10	383	MAST 7 (Marine Novel Stramenopiles Group 7)	1801F10	2	1	3
53	376	Ciliophora	1815H04	3		3
23	228	Chrysophyceae	1804H06	2	1	3
33	275	Ascomycota	1804D06	3	0	3

7	265	*Micromonas*	1801C01	2	0	2
57	350	Ciliophora	1816G03		2	2
40	232	Chrysophyceae	1807D03	1	1	2
42	231	Chrysophyceae	1809B06		2	2
52	227	Chrysophyceae	1815G07	2		2
31	376	*Chrysochromulina*	1804C04	2	0	2
29	285	Rhizaria	1804A10	2	0	2
55	275	Ascomycota	1816C11		2	2
59	275	Ascomycota	1816G09		2	2
51	119	Novel Alveolate Group I	1815F07	1		1
11	265	*Micromonas*	1801F12	1	0	1
43	233	MAST 7 (Marine Novel Stramenopiles Group 7)	1809G01		1	1
37	279	Imantonia	1806A03		1	1
6	223	Metazoa (Echinodermata)	1801B10	1	0	1
41	279	Dictyochophyceae	1807E08		1	1
49	274	Dictyochophyceae	1815D01	1		1
54	213	Cryptomonadales	1816B02		1	1
12	365	Ciliophora	1801G01	1	0	1
16	244	Ciliophora	1802B01	1	0	1
35	370	Ciliophora	1804G10	1	0	1
38	352	Ciliophora	1806H01		1	1
48	376	Ciliophora	1815C12	1		1
9	227	Chrysophyceae	1801E06	1	0	1
19	221	Chrysophyceae	1802C03	1	0	1
36	221	Chrysophyceae	1806A02		1	1
47	221	Chrysophyceae	1815C09	1		1
28	249	Metazoa (Chordata)	1804A01	1	0	1
32	188	Cercozoa/Rhizaria	1804C05	1	0	1
34	385	Cercozoa/Rhizaria	1804G02	1	0	1
58	275	Ascomycota	1816G05		1	1
25	218	Amoebozoa	1802F11	1	0	1

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Table S3a:

OTU ID	T-RFLP cut site	Phylogenetic ID	Reference sequence	High CO_2	Ambient CO_2	Total Number of sequences
6	435-7	*Roseobacteria LE17*	160102C08	371	213	584
38	520	Bacteroidetes	160402G06	104	235	339
7	145	Consistiales (SAR11)	160105C06	134	126	260
8	136	SAR86-II	160102H02	133	117	250
1	435-7	Rhodobacteriales	160102D06	57	67	124
5	86-88	Bacteroidetes	160102F11	75	32	107
4	488	SAR86-III	160402D04	32	31	63
81	435-7	Rhodobacteriales	160202B10	17	39	56
25	136	SAR86-II	160105H11	15	31	46
21	142	Gammaproteobacteria	160700F10	14	17	31
31	495	Betaproteobacteria	160304C10	19	6	25
10	86-88	Flavobacteria	160200A02	10	12	22
17	435-7	*Roseobacteria LE17*	160205F11	12	8	20
2	492	Betaproteobacteria	160304C09	15	4	19
92	440	*Roseobacteria LE17*	160202F03	12	6	18
59	492	Bacteroidetes	160904G12	4	13	17
133	486	Gammaproteobacteria	161003F04	4	13	17
57	435-7	Rhodobacteriales	160602D05	8	7	15
110	483	Chloroplast OM5	160703A05	4	11	15
76	440	Alphaproteobacteria	160603C12	4	10	14
35	435-7	*Roseobacteria LE17*	160105G12	8	5	13
231	435-7	Alphaproteobacteria	160805H04	2	11	13
52	90	Bacteroidetes	160600D10	5	7	12
18	496	SAR92	160602B07	2	9	11
65	435-7	*Roseobacteria LE17*	160901B05	5	6	11
204	488	Gammaproteobacteria	160700G11	5	6	11
286	86-88	Bacteroidetes	160505H10	6	5	11
15	486	SAR92	160803D06	3	7	10
96	435-7	Rhodobacteriales	160205G05	2	8	10
102	435-7	Rhodobacteriales	160805G12	3	7	10
215	145	Consistiales (SAR11)	160803B09	5	5	10

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Table S2: *Accession numbers for additional sequences, used in phylogenetic analysis*

Bacterial reference sequences			Picoeukaryote reference sequences					
AACY01089405	X90641	EF661583	AB193568	AF525860	EF172974	AF109326	AJ514867	DQ244028
AF359531	DQ372840	AY167339	AY626995	AF525865	EF526977	AF174376	AJ867028	DQ303924
AF354611	DQ372845	M96746	L81939	AF525868	EF527002	AB183583	AJ867029	DQ310276
AF353208	DQ372849	DQ167249	AY339145	AF525871	EF527020	AB183605	AM050344	DQ310333
AF353226	DQ372850	AY744399	AY339149	AF525876	EF527104	AB183618	AM050345	DQ367046
AF354612	DQ372852	U20797	DQ060805	AF525879	EF527105	AB275040	AY033487	DQ367048
AY548988	DQ396099	U65908	AF372731	AY129037	EF527106	AB275055	AM114819	DQ629385
CP000084	AM279169	AF001653	AF372732	AY129048	EF527126	AB275058	AM231737	DQ647512
DQ234199	AM279197	AF001650	AF372733	AY129050	EF527171	AB290575	AM412525	DQ647516
AY458647	AM279200	EF182722	Q629387	AY129052	EU050966	AB330056	AM491015	DQ647534
AF268217	AM279204	AY936189	DQ242509	AY129061	EU247836	AB425943	AY665020	DQ834370
AF279106	AM279179	CP000435	DQ504335	AY129063	EU304548	AF123297	AY665021	DQ977726
AF268236	AM279161	M34115	EU162635	AY129064	U14387	AF143943	AY665044	DQ980478
DQ015813	AF241654	AF245632	M55639	AY129065	U73222	AF184167	AY665057	DQ986131
DQ015817	AF173974		DQ278883	AY129067	U73230	AF257316	AY665094	EF023353
DQ015775	AF173975		AY646226	DQ369015	X71140	AF290083	AY665101	EF023502
AY794084	NR_027580		AY864822	DQ369016	AF290540	AF363186	AY821968	EF023594
AY697879	AY654757		EF532930	AY143572	AJ246274	AF372754	AY919815	EF023894
AY794144	U70693		AACY020214703	AY143573	AM491021	AF372755	AY919816	EF043285
AY794064	U70678		AAXK01002636	AY381207	AF472554	AF411268	AY954993	EF165124
AATR01000002	U70679		AB058312	AY425313	AF472553	AJ007277	AY965868	EF165125
AY102027	U70704		AB058331	AY425314	AF47255	AJ007284	CR954212	EF165134
AY102028	U70715		AB080302	AY425319	AF069516	AJ131691	DQ116021	EF172839
AY771771	U70696		AB058360	AY590482	AY082996	AJ251929	DQ116022	EF172972
EF016464	U70717		AB183613	AY626163	AJ402354	AJ251930	DQ121425	
AY772092	DQ489286		AB096264	AY642694	AY129036	AY329635	DQ145112	
AB193724	AF353235		AF109323	AY665019	AF525856	AJ495816	DQ243996	