Microbial Community Organisation and Functioning Under Ocean Acidification Conditions

Presented by

Lindsay K. Newbold

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

Research carried out at:

The Centre for Ecology and Hydrology

and

School of Civil Engineering and Geosciences,
Newcastle University

July 2014
Abstract

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). There is significant interest therefore in how pH changes will affect the oceans’ biota and integral processes. This thesis investigates microbial community organisation and functioning in response to predicted end of century CO$_2$ concentrations using an elevated CO$_2$ (~750ppm), large volume (11,000 L) contained seawater mesocosm. This thesis utilises RNA stable isotope probing (SIP) technologies, in conjunction with quantitative reverse transcriptase PCR (RT-qPCR), to investigate the response of microbial communities to elevated CO$_2$. This thesis finds little evidence of changes occurring in bacterial abundance or community composition with elevated CO$_2$, under both phytoplankton pre-bloom/bloom and post-bloom conditions. It is proposed that they represent a community resistant to the changes imposed. In contrast, significant differences were observed between treatments for a number of key eukaryote community members. These findings were investigated in the context of functional change, using the uptake of two key substrates (bicarbonate and glucose) as analogues for photosynthesis and respiration respectively. Unlike community abundance, distinct changes in carbon assimilation were detected in dominant members of the picoplankton. In conclusion the data presented suggest that although current microbial communities hold the capacity to respond to elevated CO$_2$, future responses will likely be taxa specific and controlled by wider community dynamics.
This thesis is dedicated to Isabelle and Michael Newbold,

love Mum.
Acknowledgements

The work presented in this thesis was made possible by the generous funding of both Natural Environmental Research Council (NERC) grant number NE/C507937/1 as part of the post genomics and proteomics programme, and the Centre for Ecology and Hydrology (CEH) internal science budget. I’d also like to thank all of the members of the 2006 Bergen Mesocosm experiment for their invaluable help in data acquisition and advice, many of whom have been acknowledged within the thesis chapters themselves.

I’m incredibly lucky to have had the pleasure of working with amazing supervisors to whom I’ll always be eternally grateful. Andy, Chris and Ian I thank you for your wisdom and advice over the years, without which I’d never have been able to get to this point. I’ll also be forever indebted to Anna for being so generous with her time, knowledge, patience, skills and friendship.

I also really appreciate the brilliant lab colleagues I’ve had over the past 7 years, especially Steve, Rob, Bruce, Dan and Leah for helping to always bring fun to even the most mundane tasks.

I’d like to thank my wonderful family who’ve helped to keep me sane: Mum and Dad thank you for bringing me up with a love for the environment and for always giving me everything you could; Gill for endless hours of support and childcare; Issy and Mikey many thanks for the sleepless nights, early mornings and always making me smile no matter what. Lastly, my incredible husband David for always being there for me with love, support, tea and chocolate (when required) I’m truly blessed to have you all in my life.
# Table of Contents

1 **Introduction: Ocean Acidification and Picoplankton Communities** 1

1.1 The Marine Ecosystem 1

1.2 The Oceans Biogeochemistry and Biological Processes 1

1.2.1 *The carbon cycle* 2

1.2.2 *The marine food web* 3

1.3 Oceanic Microorganisms: The Picoplankton 5

1.3.1 *Capturing marine diversity* 5

1.3.2 *Eukaryotic diversity within picoplankton* 7

1.3.3 *Key lineages of planktonic picoeukaryotes* 8

1.3.4 *Marine bacterial diversity* 11

1.3.5 *Key bacterioplankton lineages* 12

1.4 Climate Change and Ocean Acidification 15

1.4.1 *Climate change* 15

1.4.2 *Ocean acidification* 16

1.4.3 *The effect of ocean acidification upon organisms and processes* 19

1.5 Studying Ocean Acidification and Microbes 21

1.5.1 *Community phylogeny* 21

1.5.2 *Phylogeny and function* 23

1.5.3 *Stable isotope probing (SIP)* 24

1.5.4 *Quantitative PCR (qPCR)* 26

1.5.5 *The Bergen Mesocosm 2006* 27

1.6 Aims and Objectives 29

1.6.1 *Aims and hypotheses* 29

1.7 References 31

2 **Study Site and Experimental Parameters** 62

2.1 Study Site 62

2.2 Experimental Parameters and Time-line 64

3 **Marine Bacterial Communities are Resistant to Elevated Carbon Dioxide Levels** 67

3.1 Summary 68

3.2 Introduction 69

3.3 Results and Discussion 71

3.3.1 *pH and abundance* 71

3.3.2 *Temporal turnover in acidified bacterial communities* 73

3.3.3 *Conclusions* 79

3.4 Experimental Procedures 80

3.4.1 *Experimental set-up and sampling regime* 80

3.4.2 *Enumeration of bacterial cells using flow cytometry* 81

3.4.3 *T-RFLP analysis* 81

3.4.4 *Statistical analyses of data* 82

3.5 Acknowledgements 84
4 The Response of Marine Picoplankton to Ocean Acidification

4.1 Summary
4.2 Introduction
4.3 Results and Discussion
  4.3.1 pH change and nutrient depletion
  4.3.2 Bacterial abundance and acidification
  4.3.3 Eukaryotic abundance and acidification
  4.3.4 Sequence abundance and richness
  4.3.5 Bacterial community response to OA
  4.3.6 Picoeukaryote community response to OA
  4.3.7 Trophic interactions
4.4 Experimental Procedures
  4.4.1 Experimental set-up and sampling regime
  4.4.2 Enumeration of planktonic cells via flow-cytometric analysis
  4.4.3 Nucleic acid extraction, PCR and T-RFLP analysis
  4.4.4 Clone library construction and library sequencing
  4.4.5 Sequence processing and analyses
  4.4.6 Operational taxonomic unit identification
  4.4.7 Statistical analysis
  4.4.8 Curation
4.5 Acknowledgements
4.6 References
4.7 Supplementary Information

5 Active Bicarbonate and Glucose Picoplankton Communities Under Elevated CO₂

5.1 Summary
5.2 Introduction
5.3 Results and Discussion
  5.3.1 Baseline community analysis
  5.3.2 Microcosm and mesocosm community composition
  5.3.3 Dominant bacterial community response to elevated CO₂
  5.3.4 Dominant picoeukaryotic community response to elevated CO₂
  5.3.5 Conclusion
5.4 Experimental Procedures
  5.4.1 Experimental design
  5.4.2 Nucleic acid extraction
  5.4.3 RNA stable isotope probing (SIP)
  5.4.4 Terminal restriction fragment length polymorphism (T-RFLP)
  5.4.5 Statistical analysis
6 The Mamiellales: Strategies for Nutrient Acquisition under Elevated CO₂

6.1 Summary
6.2 Introduction
6.3 Results and Discussion
  6.3.1 Primer design, optimization and experimental validation
  6.3.2 Mamiellales abundance in mesocosms over time
  6.3.3 RT-qPCR validation
  6.3.4 Response of bicarbonate assimilation to elevated CO₂
  6.3.5 Response of glucose assimilation to elevated CO₂
  6.3.6 Conclusion
6.4 Experimental Procedures
  6.4.1 Experimental design
  6.4.2 Nucleic acid extraction
  6.4.3 RNA stable isotope probing (SIP)
  6.4.4 Positive control selection and transcription
  6.4.5 Primer design and PCR optimisation
  6.4.6 (RT)qPCR
  6.4.7 Statistical analysis
6.5 Acknowledgements
6.6 References

7 General Discussion and Conclusions

7.1 Summary of Findings
7.2 How Will Microbes Respond to Predicted Future Levels of Elevated CO₂?
  7.2.1 Prokaryotes
  7.2.2 Picoeukaryotes
7.3 Methodological Considerations and Limitations
  7.3.1 The ability to link phylogeny and function
  7.3.2 The 2006 Bergen mesocosm experiment
  7.3.3 Community fingerprint and diversity techniques
  7.3.4 Stable isotope probing
  7.3.5 (RT) qPCR
7.4 The Future
7.5 References

8 Author Contributions
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>The biological pump</td>
<td>2</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>The pelagic food web</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Distribution of plankton in differing size classes</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>The phylogenetic position of major marine bacterial taxa</td>
<td>12</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>The chemical processes involved in ocean acidification</td>
<td>17</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Graphical outline of a SIP experiment</td>
<td>26</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>The raft housing the mesocosm enclosures</td>
<td>63</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Experimental timeline</td>
<td>66</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Taxa time relationships (TTR’s) for bacterial mesocosm communities</td>
<td>72</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>The distance decay of bacterial similarity</td>
<td>75</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Mean daily nutrient and pH values</td>
<td>100</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Mean daily FACs counts for elevated and ambient Mesocosms</td>
<td>101</td>
</tr>
<tr>
<td>Figure 4.3a</td>
<td>70% majority rules consensus tree of dominant prokaryotic OTU’s</td>
<td>105</td>
</tr>
<tr>
<td>Figure 4.3b</td>
<td>70% majority rules consensus tree of picoeukaryotic OTU’s</td>
<td>106</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Change in Picoplankton community over time as assessed by T-RFLP</td>
<td>109</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Mean daily nutrient and pH values</td>
<td>140</td>
</tr>
<tr>
<td>Figure 5.2a</td>
<td>Changes in T-RFLP abundance during mesocosm and microcosm incubations for dominant prokaryotes</td>
<td>143</td>
</tr>
<tr>
<td>Figure 5.2b</td>
<td>Changes in T-RFLP abundance during mesocosm and microcosm incubations for dominant picoeukaryotes</td>
<td>144</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Ordination plot of community correspondence analysis in glucose and bicarbonate incubations for prokaryote and eukaryote T-RFLP profiles</td>
<td>148</td>
</tr>
<tr>
<td>Figure 6.1</td>
<td>Primer specificity for qPCR assays</td>
<td>168</td>
</tr>
<tr>
<td>Figure 6.2</td>
<td>Mean abundance of Mamiellales signatures</td>
<td>169</td>
</tr>
<tr>
<td>Figure 6.3</td>
<td>Changes in RT-qPCR abundance during SIP microcosm incubations</td>
<td>171</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Taxonomic placement of key picoeukaryotes</td>
<td>9</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>Current tropospheric greenhouse gas concentration</td>
<td>15</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Comparison of regression slopes between all mesocosms for taxa time and distance decay relationships</td>
<td>76</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Summary statistics for partial mantel tests</td>
<td>78</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>CCA analyses for the determination of percentage variation in bacterial mesocosm communities</td>
<td>79</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>CCA analyses for the determination of percentage variation in prokaryotic and eukaryotic communities</td>
<td>149</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Identity and abundance of Mamiellales OTU’s</td>
<td>167</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>S3.7.1</td>
<td>Baseline physical and chemical characteristics</td>
<td>91</td>
</tr>
<tr>
<td>S3.7.2</td>
<td>Changes in mean pCO₂ concentration, pH and bacterial abundance</td>
<td>92</td>
</tr>
<tr>
<td>S3.7.3</td>
<td>Rank abundance plots over time</td>
<td>93</td>
</tr>
<tr>
<td>S4.7.1</td>
<td>Comparison of group abundance using ANOVA</td>
<td>127</td>
</tr>
<tr>
<td>S4.7.2a</td>
<td>Bacterial sequence diversity table</td>
<td>128</td>
</tr>
<tr>
<td>S4.7.2b</td>
<td>Picoeukaryote sequence diversity table</td>
<td>129</td>
</tr>
<tr>
<td>S4.7.3</td>
<td>Accession numbers for sequences used in phylogenetic analysis</td>
<td>132</td>
</tr>
<tr>
<td>S4.7.4</td>
<td>Additional experimental procedures</td>
<td>133</td>
</tr>
<tr>
<td>S5.7.1</td>
<td>Summary of T-RFLP analysis</td>
<td>161</td>
</tr>
<tr>
<td>S5.7.2</td>
<td>Ordination plot of community correspondence analysis (CCA) in prokaryote and eukaryote T-RFLP profiles</td>
<td>162</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction: Ocean Acidification and Picoplanktonic Communities

1.1 The Marine Ecosystem

Water covers more than two thirds of the Earth’s surface and supports all life on Earth. Key to the Earth’s aquatic resources is the marine ecosystem which provides an invaluable component of global geochemical cycles and provides socioeconomic functions, such as food production for millions of people. Marine ecosystem services have been valued at $49.7 \times 10^{12}$ per year, a value far surpassing terrestrial ecosystems (Costanza et al., 2014) yet, over a third (41%) of the world's oceans are severely impacted by anthropogenic activities, the most dominant factor being climate change (Halpern et al., 2008). Changes in marine biodiversity can be directly linked to habitat destruction, pollution, exploitation and indirectly through climate change and linked perturbations in oceanic geochemistry (Jackson et al., 2001; Pandolfi et al., 2003; Worm et al., 2005; Worm et al., 2009). Convincing evidence from terrestrial and marine studies suggest that a diverse biota is essential to ecosystem service sustainability (Griffiths et al., 2001; Sala and Knowlton, 2006; Worm et al., 2006; Butler et al., 2007; Palumbi et al., 2008), therefore both conservation and restoration of marine communities should be a priority. In this chapter I aim to introduce the importance of microorganisms to marine processes and the potential ramifications of climate change, in particular elevated CO$_2$.

1.2 The Ocean’s Biogeochemistry and Biological Processes

The study of marine ecosystems investigates the role of ocean, estuarine, lagoon, coral reef, deep-sea and sea floor communities upon the Earth. Marine ecosystems are integral to the Earth’s biosphere and play a vital role in the cycling of both essential - e.g. oxygen (O), carbon (C), hydrogen (H), nitrogen (N), calcium (Ca), phosphorous (P) and potassium (K) - and trace elements - e.g. iron (Fe) and zinc (Zn) (Gehlen et al., 2011).
1.2.1 *The carbon cycle*

Dissolved oceanic inorganic carbon is estimated to equal around 38400 Gt, a value 50 times higher than that found in the atmosphere, essentially allowing the oceans to drive atmospheric carbon concentration through photosynthetic activities undertaken by phytoplankton (Falkowski *et al.*, 2000). Plankton can be defined as “the small marine or freshwater photosynthetic organisms (phytoplankton) and animals (zooplankton) drifting with the surrounding water” (Lawrence, 2000). This definition should be extended to include marine bacteria (bacterioplankton) and viruses (virioplankton). Despite accounting for only 0.2% of global primary producer biomass, planktonic microorganisms contribute the majority of the oceans primary production, which accounts for half of global primary production (Field *et al.*, 1998). This autotrophically fixed carbon is accessed/released by consumers (inc. heterotrophic eukaryotes and prokaryotes), respiration or decomposition. The oceans and atmosphere...

**Figure 1.1**: Simplified diagram of the biological pump. The biological pump can be split into the soft tissue carbon pump and the carbonate pump. The soft-tissue pump refers to the process by which autotrophically fixed carbon is exported to depth through gravitational settling of particles. Here it is respired through microbiological breakdown, incorporated into sediments or remineralized. In contrast, the calcium carbonate pump is driven by the use of CaCO₃ (calcium carbonate) and subsequent precipitation by marine organisms. Dissolution of CaCO₃ is driven by levels of saturation state (e.g. undersaturation, leads to increased dissolution to compensate), consequentially leading to CO₂ export. Figure drawn from processes described in Weinbauer *et al.* (2011).
interact across large timescales ranging from hours (daily biological production) to millennia (marine sediment interactions). Crucial to this interaction is that surface waters are substantially depleted in dissolved inorganic carbon, when compared to the deep ocean. As a consequence, compensation processes transferring carbon from near surface waters to depth are required, in order to retain this downward carbon gradient, (Gehlen et al., 2011). These pumps are the solubility pump (referring to the physio-chemical processes governing CO$_2$ uptake and transport), carbonate pump (driven by CaCO$_3$ precipitation, settling, solubility and sedimentation) and the soft tissue carbon pump (photosynthetically produced organic carbon, export and remineralisation). The latter two are referred to collectively as the biological pump (see figure 1.1 and for reviews see Raven and Falkowski, 1999; Gehlen al., 2011).

1.2.2 The marine food web

Original marine food webs were considered simple and based upon metazoans (such as fish), grazing on phytoplankton and zooplankton, and zooplankton were considered to graze phytoplankton (Azam, 1998). However, they did not account for the role of bacteria within the oceans. Pioneering studies in the late 1970’s and early 1980’s demonstrated the integral role of microorganisms in marine food webs and biogeochemistry (Pomeroy, 1974; Azam et al., 1983). Microbes are integral in the utilisation of dissolved organic matter (DOM) released from phytoplankton and zooplankton. Subsequent grazing and decay of these bacteria reprocesses this carbon back to the food web - termed the Microbial loop (Azam, 1998; Pomeroy et al., 2007). In surface marine waters 20-40% of bacterial mortality can be attributed viruses, suggesting viral induced mortality is nearly equal to that of grazing (Suttle, 1994, Fuhrman and Noble, 1995). Viruses play an integral role in the marine food web through infection, lysis and the subsequent release of nutrients and DOM, which in turn is accessed by prokaryotes and protists (reviewed in Rohwer, 2009, Zhang, 2011, Weitz and Wilhelm, 2012). This viral mediated oceanic cycling is referred to as the ‘viral loop/shunt’ (Suttle, 2007). DOM can coalesce to form transparent expolymeric particles (TEP) which in turn are accessible to prokaryotes, protists and zooplankton. It’s also important to consider that grazing is an important
pressure in recycling nutrients, by both bacteria and protists. This information is summarised in figure 1.2 (adapted from Weinbauer et al., 2011).

![Figure 1.2: Simplified diagram of the pelagic food web. Four major pathways are illustrated, the classical food web (green), the microbial loop (red), the viral shunt (purple) and the abiotic loop (blue). Nutrient pathways are shown (pink). This figure and legend are adapted from Weinbauer et al. (2011).](image)

Over half of autotrophically fixed oceanic CO₂ is reprocessed or turned over by heterotrophic bacteria and archaea through processes such as the microbial loop and biological pump (Azam, 1998; Jiao et al., 2010). Therefore it can be asserted that microbes are essential to the oceans, and indeed, the Earth’s biogeochemical processes (Falkowski et al., 1998; Falkowski et al., 2008). Before introducing how these biogeochemical processes and mediators may be affected by ocean acidification (OA), it is important to recognise the immense biodiversity present in the ocean.
1.3 Oceanic Microorganisms: The Picoplankton

1.3.1 Capturing marine diversity

Marine diversity ranges from the largest animal on Earth (the blue whale) to the smallest microbes, and hold some of the most diverse (such as coral reefs) and inhospitable ecosystems (such as deep hypersaline anoxic basins) on Earth.

Although microorganisms are integral to all of these ecosystems, this study concentrates on the free-living microbial plankton (more specifically the picoplankton). Since marine planktonic organisms are small and scattered throughout the water column, this has meant that methodological approaches (such as filtration through varying filter sizes) are required to study them.

Therefore, oceanic microorganisms are often defined according to cell diameter, see figure 1.3, adapted from Sieburth et al. (1978) and Sherr and Sherr (2008). Although by no means exclusive, examples of size groupings are as follows: mesoplankton (0.2-20mm), includes small metazoans (such as copepods); microplankton (20-200μm), large protists and most phytoplankton; nanoplankton (2.0-20μm), would include small eukaryotic protists, ciliates and flagellates (thought to be highly important as grazers of picoplankon); picoplankton (0.2-2.0μm), bacteria, archaea and very small eukaryotes and lastly femtoplankton (0.02-0.2μm) would include the virol plankton. It should be noted that such classifications are arbitrary and many taxonomic groupings span size classes: however, sized based approaches have enabled the isolation and comparison of specific groups when studying life history and food web interactions (Worden and Not, 2008). Furthermore, it is also important to note here that trophic strategy is equally as widespread, with autotrophy, heterotrophy and mixotrophy evident in many of the size classes (Zubkov and Tarran, 2008; Zubkov, 2009).

Historically picoplankton was thought to contain only prokaryotic organisms. Here we use the term prokaryote to refer to bacteria and archaea, although this is not taxonomically correct; archaea are thought to be more closely related to eukaryotes, yet the term prokaryotes is still used in a non-phylogenetic context.
Figure 1.3: Distribution of planktonic taxonomic and trophic compartments, in differing size classes. Filled boxes represent heterotrophs and open represent autotrophs. Adapted from Sieburth et al. (1978) and Sherr and Sherr (2008).

Like terrestrial ecosystems, studies of marine microbial diversity were traditionally limited to cultivable organisms. However, only a small fraction of microbial cells can be isolated (the ‘great plate count anomaly’, Staley and Konopka, 1985). 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). Such studies have allowed us to glimpse “the uncultured microbial majority” (Rappe and Giovannoni, 2003) and, with the growing application of high throughput sequencing technologies the understanding of this previously untapped diversity is likely to increase exponentially. However, linking phylogenetic diversity to individual functional roles within a community is problematic at best. Although phylogenetic association to cultured representatives can hint at function, a number of

<table>
<thead>
<tr>
<th>Plankton</th>
<th>Femto- (0.02-0.2 µm)</th>
<th>Pico- (0.2-2.0 µm)</th>
<th>Nano- (2.0-20 µm)</th>
<th>Micro- (20-200 µm)</th>
<th>Meso- (0.2-20 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virioplankton</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterioplankton</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytoplankton</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protozooplankton</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
molecular techniques (outlined in section 1.5) have (and are) being developed to begin to address these questions.

Unlike larger multicellular organisms, members of the picoplankton show little morphological differences. Additionally, their small size and inability to culture the vast majority makes them difficult to name and identify by traditional taxonomic techniques. Traditional taxonomic definitions rarely apply to such organisms, most molecular microbiologists favouring a species concept based upon molecular similarity to delineate taxonomic groups, or operational taxonomic units (OTUs) (Blaxter et al., 2005; Staley, 2006). More recently, the concept of ‘ecotype’ has emerged wherein organisms can be “genetically very similar, but physiologically distinct” (Rocap et al., 2002). For example, the marine bacterial group SAR 11 has distinct sub-clades which are thought to have adapted to specific temporal and depth ranges (Vergin et al., 2013). Additionally, cultured members of the marine picoeukaryote genus Mamiellales are thought to show high and low light adapted strains (Rodríguez et al., 2005). However, this has been shown to be more complex in the environment, where similar strains are found in specific niches driven by temperature and nutrient availability (Demir-Hilton et al., 2011).

Picoplanktonic diversity is taxonomically vast, trophically and functionally complex. Although all three domains of life (Woese and Fox, 1977) are common in the ocean, this thesis concentrates on the picoeukaryotes and bacteria as they numerically dominated the study system.

1.3.2 Eukaryotic diversity within picoplankton

In addition to dramatic changes in both taxonomic resolution and tree structure, the widespread application of molecular techniques has lead to a seemingly endless plethora of newly discovered members of the eukaryotic tree of life. Many are identified solely by molecular signatures, further compounding the problems faced by modern eukaryotic taxonomists (Epstein and López-Garcia, 2008). Much of this newly discovered diversity has been found as a result of a better understanding of ‘pico’ sized eukaryotes. As early as 1951 typical picoeukaryotes such as Micromonas pusilla were described as “abundant and
can only have escaped description earlier because of its minute size” (Knight-Jones and Walne, 1951) yet, the true diversity of picoeukaryotes has only been revealed by studying the molecular diversity of environmental 18S small subunit ribosomal RNA (18S SSU rRNA) ribotypes. Seminal studies found vast numbers of novel 18S signatures from a ‘pico’ sized filtered water in a range of environments (Diez et al., 2001; Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001). The inclusion of these novel groups has meant that eukaryotic microbiology is in a period of change. Traditional taxonomic classifications based largely upon light microscopic observations, are constantly being revised and rewritten with the inclusion of molecular phylogenetic data. Traditional demarkations such as kingdom Protista (Haeckel, 1866), have been completely overturned in favour of the emerging super group concept wherein 5 or more taxonomic super-groups are proposed (Cavaller-Smith, 1993; Baldauf et al., 2000; Simpson and Roger, 2002; Adl et al., 2005; Keeling et al., 2005). Even so, this itself is under constant reconsideration and amendment, with the potential inclusion of ‘mega-groups’ (see table 1.1) (Burki et al., 2007; Burki et al., 2008; Roger and Simpson, 2009; Adl et al., 2012).

1.3.3 Key lineages of planktonic picoeukaryotes

Originally Stramenopiles, Alveolata and Rhizaria were thought to belong to two separate supergroups: Chromalveolata (Alveolata and Stramenopiles) and Rhizaria, however phylogenetic support for this grouping is low (Adl et al., 2005; Keeling et al., 2005; Parfrey et al., 2006). Not to be confused with the prefix SAR given to novel bacterioplankton 16S rRNA phylotypes from the Sargasso Sea (Giovannoni et al., 1990), the grouping of Stramenopiles, Alveolata and Rhizaria (SAR) was first proposed by Burki et al. (2007). Based upon strong phylogenetic evidence, this supergroup cluster contains a large diversity of planktonic eukaryotes, and is supported by modern taxonomic revisions (Adl et al., 2012). One of the first observations from environmental PCR studies was the prevalence within these libraries of previously unknown marine Stramenopiles (MAST) phylotypes (Massana et al., 2004). Massana et al. (2004) found 12 distinct clusters of Novel Stramenopiles which formed monophyletic groups. These MAST taxa were spread across the Stramenopile
linage forming sister groups to both, phototrophic and heterotrophic/mixotrophic lineages. Further, Lin et al. (2012) were able to clearly demonstrate the ingestion of a fluorescently labelled cyanobacterium (*Synechococcus*) by MAST-4 cells supporting the idea that at least one MAST lineage is able to graze bacteria.

Adl and colleagues (2005) split the Alveolata into three highly abundant and important groups; the Apicomplexa, Ciliophora and Dinozoa. Until the application of molecular phylogenetic techniques, the phylum/infrakingdom Alveolata was not recognised, yet it now forms a well supported monophyletic group of primarily single celled organisms which are notable for not only a wide phylogenetic diversity, but also for the adoption of a diverse range of trophic strategies: including phototrophy, phagotrophy and intracellular parasitism (Cavalier-Smith, 1993; Baldauf et al., 2000; Simpson and Roger, 2002; Keeling et al., 2005; Burki et al., 2007; Gould et al., 2008). Alveolates can

<table>
<thead>
<tr>
<th>Mega-group</th>
<th>Super-group</th>
<th>First Rank</th>
<th>Second Rank</th>
<th>Third Rank</th>
<th>Notable Members</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archaeoplastida</td>
<td>Chloroplastida</td>
<td>Rhizaria</td>
<td>Stramenopiles</td>
<td>Sar</td>
<td>Haptophyta</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Archaeplastida</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pelagophyceae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Uncultured Marine Stramenopiles (MAST)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ciliophora</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protalveolata</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dinoflagellata</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Apicomplexa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: Summary of the taxonomic placement of some key eukaryote taxa focusing on picoeukaryotes. Taxonomy follows that of - Adl et al. (2012) and Worden and Not (2008), with the addition of notable members and their linked references Marin and Melkonian (2010) ⁰, del Campo et al. (2013) ¹, Massana et al. (2004); Massana et al. (2006) ² and Grosillier et al. (2006)*. The inclusion of mega-group Diaphoretickes and super-group SAR (Stramenopiles, Alveolata and Rhizaria) follows recent revisions of eukaryotic taxonomy (Burki et al., 2007; Burki et al., 2008; Adl et al., 2012).
be characterised by the presence of “alveoli” - a series of flattened sacs underneath the plasma membrane (Wolters, 1991) and Alveolin proteins (Gould et al., 2008). Like the Stramenopiles, environmental sequencing projects have shown (amongst many novel phylotypes) two key novel pico-sized alveolate groups (NAI and NAII). The position of the novel alveolate groups is contentious, NAI was originally believed to be a distinct sister group to the dinoflagellates but is now (like NAII) thought to cluster within the dinoflagellate order Syndiniales, a taxa containing parasitic members such as Amoebophyra (Groisillier et al., 2006; Guillou et al., 2008). This would hint that at least some of these novel organisms may be parasitic.

First presented by Adl and colleagues (2005) Archaeplastida encompasses the Glaucocephyta, Rhodophyceae (red algae), and Chloroplastida (green algae and plants). Some of the most abundant and ecologically important photosynthetic picoeukaryotes fall within the Chloroplastida class Mamiellophycceae (Marin and Melkonian, 2010). Largely picoeukaryotic the Mamiellales contains some of the smallest known free-living eukaryotes such as Ostreococcus tauri, Micromonas pusilla and Bathycoccus prasinus, found globally and highly abundant in coastal areas. Because this group can be distinguished by their small size (1-2µm diameter), genome (13-22 Mbp) and reduced cellular organisation (one mitochondrion and one chloroplast), they’re often used as a model for the most simplified functional eukaryotic cell (Moreau et al., 2012). Genomes published for Ostreococcus (Derelle et al., 2006; Palenik et al., 2007), Micromonas (Worden et al., 2009) and Bathycoccus (Moreau et al., 2012), suggest that they are able to use the C4 photosynthetic pathway (a method which is believed to be more costly but more efficient than C3 fixation) and gives indirect evidence for sexual reproduction amongst this lineage (Piganeau et al., 2011). Micromonas alone has been found to account for around 45% of picoeukaryotes in the English Channel, clearly demonstrating the importance of this group (Not et al., 2004).

The only described ‘pico’ sized Haptophytes are that of Imantonia rotunda and Phaeocystis cordata (Reynolds, 1974; Zingone et al., 1999; Worden and Not, 2008). However the Haptophytes should be mentioned here due to their
prevalence in bloom forming communities. Of around 300 known haptophyte species in the oceans, around 200 are coccolithophores (Jordan and Chamberlain, 1997). Coccolithophores can be characterised by calcium carbonate shell like structures known as ‘coccoliths’. These structures are thought to be both protective and to serve as a carbon storage mechanism (Sikes et al., 1980). The well known coccolithophorid Emiliania huxleyi, form blooms so large that they’re visible from space (Jordan and Chamberlain, 1997). These organisms are particularly important to carbon cycling as autotrophic carbon sinks and sources of carbon (through decay and sinking, serving as a mechanism of depositing calcium carbonate to oceanic sediments).

1.3.4 Marine bacterial diversity

Like their eukaryotic counterparts, the scope of the diversity of marine prokaryotes has only recently been revealed and, as such, has also undergone major revisions in recent years. Indeed during the 1990’s the inclusion of 16S rRNA data within studies led to a complete revision of not only the diversity of bacterial life but also their functional role in the environment. Because a universal bacterial species concept is contentious, common practice classifies bacterial taxa using molecular similarity cut-offs or operational taxonomic units (OTU’s) (Stackebrandt and Goebel, 1994; Rossello-Mora and Amann, 2001; Staley, 2006). This approach usually treats bacteria of >97% 16S small subunit ribosomal RNA (16S SSU rRNA) sequence homology to be synonymous with “species” level similarity (Stackebrandt and Goebel, 1994). Although arbitrary, the OTU approach is highly useful in quantifying bacterial diversity (Koeppel and Wu, 2013). Using a 97% 16S SSU rRNA sequence similarity cut off the total number of bacterial taxa in the ocean has been estimated to be between 10^6 - 10^9 (Pedrós-Alió, 2006). Bacterial oceanic diversity can be characterised into 8 broad phylogenetic groups: the Proteobacteria, Cyanobacteria, Lentisphaerae, Bacteroidetes, Actinobacteria, Fibrobacter, Planctobacteria and Chloroflexi (see figure 1.4 adapted from Giovannoni and Stingl, 2005). Many of these groups contain members which have been found to be both globally important and numerous. For example, one of the most abundant bacterial ribotypes detected in seawater DNA is that of the SAR11 group (or
Pelagibacteraceae). However, until the application of environmental sequencing studies, it had been unknown to marine microbiologists (Morris et al., 2002a).

### 1.3.5 Key bacterioplankton lineages

One of the foremost bacterial groups is that of the phylum Proteobacteria (Stackebrandt et al., 1988). Proteobacteria can be further broken down into six classes: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria and Zetaproteobacteria (Euzéby, 1997; Emerson et al., 2007; Parte, 2014). Members of all the Proteobacterial classes can be found in marine systems, yet here I concentrate on the Alpha and Gamma classes (Guiry and Guiry, 2013; WoRMS Editorial Board, 2013).

![Phylogenetic tree of major bacterial taxa](image)

**Figure 1.4:** Schematic illustration of the phylogenetic position of major marine bacterial taxa. Figure adapted from Giovannoni and Stingl (2005). Taxa relevant to this study have been included.

The early 1990’s saw a period of landmark papers in environmental marine biodiversity investigating marine planktonic communities (Giovannoni et al., 1990; Britschgi and Giovannoni, 1991; Schmidt et al., 1991). Such studies demonstrated the abundance of Alphaproteobacteria within marine communities. Since their discovery SAR11 bacteria have been shown to
dominate bacterioplankton communities across geographic and depth gradients, and encompass a diverse range of phyla and ecotypes (Morris et al., 2002a; DeLong et al., 2006; Mary et al., 2006; Vergin et al., 2013). SAR11 forms a monophyletic family within the alphaproteobacterial order *Rickettsiales* and is believed to share common ancestry with the eukaryotic mitochondrion (Thrash et al., 2011). SAR11 group organisms metabolize dissolved organic carbon to generate energy via proteorhodopsin or by respiration (Giovannoni et al., 2005a; Giovannoni et al., 2005b). The first cultured isolate from this taxa has been named *Candidatus Pelagibacter ubique* (Rappe et al., 2002). Studies upon *P. ubique* have shown this group are not only one of the smallest known free living bacteria but also have an equally reduced genome size to match (Giovannoni et al., 2005b; Joint, 2008; Grote et al., 2012). Unlike its fellow alphaproteobacterium SAR11, many members of the marine family Rhodobacteraceae are readily found using both traditional marine culture and culture- independent techniques (González and Moran, 1997). This group is both highly diverse and abundant, and contains many significant genera such as the *Rhodobacter*, *Roseobacter*, *Silicobacter* and *Sulfotobacter*. The Rhodobacteraceae are a highly abundant and diverse group with equally diverse biogeochemical characteristics, which have been often associated with algal blooms (Selje et al., 2004; Buchan et al., 2005; Rink et al., 2007; Brinkhoff et al., 2008; Newton et al., 2010). In their recent study of *Roseobacter* genomes Newton and colleagues (2010) were able to identify genetic pathways related to a range of trophic strategies and biochemical utilisation (carbon, phosphorus, sulphur, nitrogen and iron) all of which would be advantageous during a phytoplankton bloom and ensuing nutrient release.

Another important class of marine proteobacteria is that of the *Gammaproteobacteria*. Deep branch phylogeny within this class is difficult to resolve using 16S rRNA gene phylogenies alone (Williams et al., 2010). This is further hampered by the inclusion of novel environmental sequences. The term SAR86 refers to a group of *Gammaproteobacteria* 16S rRNA gene ribotypes first detected in surface marine communities, and subsequently found to be present globally (Britschgi and Giovannoni, 1991; Schmidt et al., 1991;
SAR86 is found to contain 3 main sub-groups (I, II, and III), and, as yet has no cultured representatives (Suzuki et al., 2001a; Sabehi et al., 2004). Studies using SAR86 bacterial artificial chromosomes (BACs) have enabled researchers to ascertain that SAR86 organisms are likely to be aerobic heterotrophs, with the potential for ATP production via proteorhodopsin. Proteorhodopsin was first discovered in a BAC containing a SAR86 18S SSU rRNA, and is now thought to be present in at least 50% of marine bacteria (Béjà et al., 2000; Campbell, et al., 2007). Like SAR11, SAR86 exhibits a streamlined genome (Sabehi et al., 2004; Dupont et al., 2012). Further, Dupont and colleagues (2012) suggested that SAR86 organisms are specialized in lipid and polysaccharide degradation, and hence occupy a niche distinct from other globally distributed proteobacteria such as SAR11.

Members of the Bacteroidetes phylum constitute not only one of the most abundant marine heterotrophic bacterial groups but, also one of the most functionally valuable. Bacteriodetes are believed to have a role as ‘particle specialists’ and are common members of phytoplankton bloom associated bacterial assemblages, where the ability to degrade complex bio molecules is advantageous (Riemann et al., 2000; Kirchman, 2002; Fandino et al., 2005). A recent analysis of Bacteroidetes genomes found adhesion and glycosyl transferase genes typical to an attached lifestyle, and confirmed this group has a key role in polymer degradation through the presence of a high number of glycoside hydrolase and peptidase encoding genes (Fernandez-Gomez et al., 2013). Further, to this strains grown in light and dark conditions provide evidence that at least one member of this group is able to utilise proteorhodopsin to capture and harvest light energy to benefit its growth and survival (Gomez-Consarnau et al., 2007; Gómez-Consarnau et al., 2010).

Finally it is important to mention phylum Cyanobacteria. Phototrophic cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* have been shown to contribute up to 80% of marine oligotrophic primary production (Goericke and Welschmeyer, 1993; Li, 1995; Liu et al., 1999; Rocap et al., 2002). Discovered in 1979 and 1988 respectively, they are likely to be the most
abundant photosynthetic organisms on Earth (Waterbury et al., 1979; Chisholm et al., 1988; Partensky et al., 1999). Considering the wide taxonomic, trophic and functional diversity present within marine picoplankton it is important to consider how they are likely to respond to global climate change, and the ecological impact of such changes.

1.4 Climate Change and Ocean Acidification

1.4.1 Climate change

In 2013 the intergovernmental panel on climate change (IPCC) reported that “Warming of the climate system is unequivocal, and since the 1950s, many of the observed changes are unprecedented over decades to millennia. The atmosphere and ocean have warmed, the amounts of snow and ice have diminished, sea level has risen and the concentrations of greenhouse gases have increased” (IPCC, 2013). Solar energy passes through the atmosphere and is absorbed by the Earth’s surface, warming it up. The greenhouse effect is the process by which reflected thermal energy is absorbed by the atmosphere and its greenhouse gases then, redirected back to the Earth, heating it further. Without this natural process the average temperature of the Earth’s surface would be below the freezing point of water, thereby limiting life. However, any

<table>
<thead>
<tr>
<th>Gas</th>
<th>Pre-1750 trophospheric concentration</th>
<th>Recent trophospheric concentration</th>
<th>Percentage increase since 1750</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Dioxide (CO₂)</td>
<td>280 parts per million (ppm)</td>
<td>400 (ppm)</td>
<td>40.2%</td>
</tr>
<tr>
<td>Methane (CH₄)</td>
<td>700 parts per billion (ppb)</td>
<td>1874/1758 (ppb)</td>
<td>167.7/151.1%</td>
</tr>
<tr>
<td>Nitrous Oxide (N₂O)</td>
<td>270 (ppb)</td>
<td>324/323 (ppb)</td>
<td>20/19.6%</td>
</tr>
</tbody>
</table>

Table 1.2: Comparison of average global pre-industrial and current trophospheric greenhouse gas concentration for 3 major greenhouse gases. Data taken from CDIAC (Blasing, 2013). As per IPCC (2001) convention, anthropogenic contributions prior to 1750 are taken to be negligible. Current values represent recorded annual mean (2012 for CO₂ and 2011 for CH₄ and N₂O).
increases in greenhouse gases would lead to an increased reflected heat thereby increasing surface temperature further, a process known as global warming (IPCC, 2007). Since industrialisation the atmospheric concentration of greenhouse gases such as carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O) have risen dramatically, due to anthropogenic activities such as burning fossil fuels and changes in land use (e.g. deforestation and the intensification of farming). A comparison of some current trophospheric greenhouse gas concentration shows that values for CO₂, CH₄ and N₂O represent a rise of approximately 40, 159 and 20% respectively, since 1750 (see table 1.2).

Influential papers presenting ice core data suggest that present levels are far higher than any in the past 800,000 years (Petit et al., 1999; Siegenthaler et al., 2005; Spahni et al., 2005; Luthi et al., 2008), and data from ancient foraminiferan shells suggests that CO₂ levels were last higher than this around 20 million years ago (Pearson and Palmer, 2000). The intergovernmental panel on climate change (IPCC) fourth assessment report on climate change, used the IPCC special report on emissions scenarios (SRES) to present future climate predictions; - these models suggested that by 2100 atmospheric CO₂ concentration could range between 541 and 970 ppm (IPCC, 2000; IPCC, 2007; IPCC, 2013). In 2013, readings taking for CO₂ at the Mauna Lao research station passed 400ppm, a symbolic benchmark which is likely to represent the norm within a few years (BBC, 2013). If the upward trend in CO₂ and other greenhouse gas emissions continues as predicted, there are likely to be global consequences for both biotic and abiotic processes. However there is another consequence of elevated CO₂ the scale of which has been overlooked until recently, that of ocean acidification (OA).

1.4.2 Ocean acidification

Since industrialisation, global CO₂ emissions have increased and as a result a greater understanding of the relationship between rising atmospheric CO₂, ocean biogeochemistry and the populations therein, is essential (Caldeira and Wickett, 2003; Cicerone et al., 2004; Feely et al., 2004; Orr et al., 2005).
Figure 1.5 outlines the process first coined ‘ocean acidification’ (OA) by Caldeira and Wickett (2003) in which CO$_2$ released into the atmosphere dissolves in seawater and reacts to form carbonic acid (H$_2$CO$_3$), the dissociation of which forms hydrogen and bicarbonate ions. When hydrogen ions are in excess they react with carbonate ions to form more bicarbonate ions. As a result there is a net increase in dissolved carbon dioxide, carbonic acid, bicarbonate ions and hydrogen ions, alongside a decrease in bio-available carbonate ions, overall resulting in a net decrease (acidification) of oceanic pH (see Joint et al. 2011 for review).

The ocean’s buffering capacity is only able to neutralize some additional CO$_2$
therefore, 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).

The concept that oceanic pH can effect organisms is not new. In the first half of the 20$^{th}$ century, a number of early publications were able to highlight the potential effect of changes in hydrogen ion concentration to organisms. Early investigations were able to establish a negative effect upon egg development of a sea urchin (edible sea urchin) and fish species (European plaice) (Moore et al., 1906; Whitley, 1906). Gail (1919) demonstrated a specific pH range (pH 7.4 - 8.6) where Fucus (a brown algae) spore germination was optimal. Although much of this early research has since been re-evaluated, it is important to note that even over a century ago the importance of pH balance in marine systems was conceived. Reviews of this early data can be found elsewhere (Rubey, 1951; Gattuso and Hansson, 2011). Modern ocean acidification research was established by Revelle and Suess (1957). In their seminal paper they were the first to link the uptake of anthropogenically derived CO$_2$ to a decrease in the oceans buffering capacity - the oceans ability to absorb atmospheric CO$_2$. In combination with the observation that changes in carbonate ions (CO$_3^{2-}$), which decrease with elevated pCO$_2$, lead to changes community calcification rates, this has evolved into the modern concept of ocean acidification (Broecker and Takahashi, 1966). Subsequent studies have demonstrated that oceanic pH has changed in response to elevated CO$_2$ using time series data (Bates, 2001; Santana-Casiano et al., 2007; Dore et al., 2009). Many reviews, policy documents and recommendations have since been written in order to try and understand and potentially minimise the effect of OA (Cicerone et al., 2004; Raven et al., 2005; Henderson, 2006; Field et al., 2011; IGBP IOC SCOR, 2013; IPCC, 2013). Yet, all highlight the need for a greater understanding in how projected 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).
1.4.3 The effect of ocean acidification upon organisms and processes

As outlined in section 1.4.2, ocean acidification is occurring and, as such, the associated changes in pH and CO$_2$ are likely to have both a positive and negative effect on the growth of oceanic microorganisms (Riebesell, 2004). The most publicised negative effect of OA comes as a result of a decrease in bio-available carbonate ions limiting calcifying organisms’ ability to make protective shells or skeletons, with organisms such as coral and molluscs appearing particularly vulnerable (Gattuso et al., 1998; Riebesell et al., 2000; Michaelidis et al., 2005; Shirayama and Thornton, 2005; Gazeau et al., 2007; Kuffner et al., 2008). Calcium carbonate occurs in two polymorphic forms (calcite and aragonite) both of which can be accessed. However, there is a critical concentration of carbonate saturation within seawater below which calcium carbonate (CaCO$_3$) will start to dissolve. As aragonite dissolves more readily than calcite, organisms utilising aragonite may respond more rapidly to OA induced changes (Orr et al., 2005). However it is important to note that calcification responses will likely be species specific and that no general trend should be applied for all (Langer et al., 2006). Fine and Tchernov (2007) found that complete recovery was possible in a coral species previously exposed to pH 7.4, clearly demonstrating that ecosystem recovery/maintenance is possible. It is also important to consider that much OA research has focused upon calcifying organisms and the effect upon non-calcifiers may not be as apparent. The early reproductive and juvenile stages of many organisms are sensitive to OA (Kurihara et al., 2004; Kurihara and Shirayama, 2004; Kurihara, 2008; Ceballos-Osuna et al., 2013). Melzner et al. (2009) suggest that metazoan species which tolerate predicted future CO$_2$ concentrations have high metabolic rates and levels of mobility/activity. Such organisms naturally experience varying levels of oxygen consumption and subsequent CO$_2$ excretion during respiration, and therefore may be better able to withstand changes in external acid-base chemistry. However, in contrast intracellular elevated pCO$_2$ levels may lead to shifts in an organisms energy budgets which would likely affect growth, survival, and physiology in general (for reviews see Pörtner et al., 2004; Pörtner et al., 2011).
Secondly, elevated CO$_2$ concentration has 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, the latter *Prochlorococcus* showed only a minimal response. In picoeukaryotes within the *Mamiellales*, *Micromonas*-like *rcbL* (ribulose bisphosphate carboxylase/oxygenase, RubisCO) phylotypes were significantly in higher number, in high CO$_2$ mesocosms, whereas *Bathycoccus*-like *rcbL* phylotypes were not (Meakin and Wyman, 2011). An emerging perception from these and other studies is that organisms which don’t contain effective carbon concentration mechanisms (CCM’s) - which have evolved to support photosynthesis in low concentration CO$_2$ - may be more competitive under elevated CO$_2$ (Engel et al., 2008; Egge et al., 2009; Reinfelder, 2011).

Finally, since ecosystems and community composition are strongly determined by their environment (Martiny et al., 2006), if biogeochemical factors within this environment change then communities are likely to respond. Although many studies have looked at organismal level responses, as yet little work has focused upon microbial community responses to ocean acidification. The application of basic ecological principles has proven to be a powerful tool in explaining the community distribution and abundance patterns of macro-organisms in response to environmental change, yet these ideas have only been applied to microbiology recently (Prosser et al., 2007). An important aspect of community analysis in an environmentally disturbed system (such as CO$_2$ perturbation) is the accurate evaluation of biological integrity and recovery following such an event (Ager et al., 2010) - how will a community respond to change and will it recover? When discussing ocean acidification Joint and colleagues (2011) proposed that ‘marine microbes possess the flexibility to accommodate pH change and there will be no catastrophic changes in marine
biogeochemical processes that are driven by phytoplankton, bacteria and archaea', although Joint does highlight that calcifying and photosynthetic organisms may pose an exception to this. As discussed in the experimental chapters of this thesis, this view is not supported by all (Grossart et al., 2006; Liu et al., 2010; Lidbury et al., 2012), but does serve as a good working hypothesis to investigate microbial community response.

1.5 Studying Ocean Acidification and Microbes

1.5.1 Community phylogeny

As mentioned previously, the majority of environmental microbes are unculturable and, as such, have been identified exclusively upon their phylogenetic signature (Staley and Konopka, 1985; Rappe and Giovannoni, 2003). The biggest breakthrough in microbial ecology during the last century was the now widespread application of molecular techniques to this field. In their benchmark paper Woese and Fox (1977) used differences in conserved regions of the 16S ribosomal gene, to split life into 3 separate domains, and the concept of a phylogenetic marker was born. In combination with the polymerase chain reaction (PCR) and chain terminating sequencing reactions (Sanger sequencing), specific target regions of genetic sequence, e.g. 16S/18S rRNA could be studied (Sanger et al., 1977; Saiki et al., 1985). Clone libraries could be generated from mixed total environmental DNA, by firstly amplifying the desired genetic region (usually 16S or 18S SSU rRNA) and, after insertion of single amplicons into a cloning vector, cultured and sequenced (Olsen et al., 1986; Pace et al., 1986). This method has proven to be a powerful tool in understanding the unknown environmental diversity, and as outlined in section 1.3 has led to the discovery of many new organisms. However, it is important to note that this approach has its limitations. Firstly, clone libraries only represent the number of sequences you have produced, not the total community. One millilitre of seawater in the open ocean contains about $5 \times 10^5$ prokaryote cells (Whitman et al., 1998). Ashelford et al. (2006) defined a large clone library as over 100 sequences, clearly this represented a minute fraction of the total community. Secondly, bias exists in each step of the process including;
preferential amplification of certain gene sequences (Reysenbach et al., 1992), interference from flanking genes (Hansen et al., 1998), template concentration (Chandler et al., 1997), restricted community coverage using ‘universal’ primers (Polz and Cavanaugh, 1998), chimeric sequence formation (Ashelford et al., 2005), polymerase error rate (Tindall and Kunkel, 1988), preferential cloning of small sized PCR amplicons (Huber et al., 2009), a disparity in copy number of genes, accurate and meaningful sequence alignment and phylogenetic tree composition (Page and Holmes, 1998). Many of these have been minimised by improved molecular techniques (Taylor et al., 2007) and developments in bioinformatics. The development of sequence and phylogenetic analysis packages such as; Staden (Staden, 1996; Staden et al., 2000), Phred (Ewing and Green, 1998; Ewing et al., 1998), Phrap (Green, 2008) and PAUP (Swofford, 2002), have enabled the accurate analysis of large numbers of sequences. Such processes have been further aided by the use of chimeric sequence identification and evolutionary model checking programs such as chimera check (Huber et al., 2004), primrose (Ashelford et al., 2006) and Model test (Posada and Crandall, 1998; Posada, 2006). In addition the use of the freely accessible and accurately identified sequence repositories Silva (Pruesse et al., 2007) and Greengenes (DeSantis et al., 2006a; DeSantis et al., 2006b) and the development of comprehensive open source bioinformatics packages e.g MOTHUR (Schloss et al., 2004; Schloss and Handelsman, 2005; Schloss and Handelsman, 2006; Schloss et al., 2009) provide an invaluable resource to the modern microbial ecologist. Yet, the emerging use of next-generation sequencing (NGS) technologies likely holds the biggest advancement in this area. Because, NGS technologies produce millions of sequence reads it is possible to investigate environmental microbial populations at a sequencing depth which was previously impossible (Liu et al., 2012; Egge et al., 2013; Taib et al., 2013). Even so, current sequence read length cannot reach that of traditional Sanger sequencing and clone library analysis is still a powerful tool in investigating environmental sequence diversity.

Because clone libraries can never represent the total community diversity present in a sample and can be prohibited by time and cost, other rapid PCR based community fingerprint techniques have been developed. These include:
temperature/denaturing gradient gel electrophoresis (T/DGGE) (Muyzer, 1999), single strand confirmation polymorphism (SSCP) (Schwieger and Tebbe, 1998), length heterogeneity PCR (LH-PCR) (Suzuki et al., 1998) which has subsequently been superseded by automated ribosomal intergenic spacer analysis (ARISA) (Fisher and Triplett, 1999) and terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997). These techniques have been demonstrated to show similar findings (Smalla et al., 2007). However, terminal restriction fragment length polymorphism (T-RFLP) is often favoured over other community fingerprint techniques because of its relative simplicity, high reproducibility between runs, rapid generation of ‘fingerprints’ from a large number of samples, and quantitative value (Schütte et al., 2008). Molecular fingerprint techniques are reliant on PCR amplification of the target genomic region and will be subject to bias, but it is commonly accepted that comparative inter sample variation is unaffected as they apply in equal measure to all samples (Blackwood et al., 2003).

Although fingerprint techniques are often criticised because of a lack of resolution, they have proven to be an indispensable tool in modern environmental microbiology for the detection of changes in the structure and composition of microbial communities (Ramette, 2009).

1.5.2 Phylogeny and function

It is important to consider not only the structure and composition of a community but also the function it performs. Whether community composition itself will change or not in an experimental system, observed functional changes may occur. Herein lies a problem, as discussed in section 1.3, environmental microbial diversity is not only vast, but severely undercultured (Staley and Konopka, 1985). Therefore, one of the central problems faced by modern microbial ecology is not only the phylogenetic characterisation of such communities, but also the ability to link diversity to their function. Consequently the development of additional molecular approaches has been necessary to address this predicament.
One approach, metagenomics, isolates and inserts total environmental DNA into artificial chromosomes or vectors. In a pioneering study on the metagenome of the Sargasso Sea, it was possible to identify 1800 genomic species, of which 148 were novel, additionally these species contained over 1.2 million genes new to science (Venter et al., 2004). The data produced has been further investigated to include picoeukaryotic sequences, giving a glimpse into the functional genes within this elusive group (Piganeau et al., 2008). The metagenomic approach has been applied to investigate soil (Liles et al., 2003) and wastewater (Strous et al., 2006) and recently extended to look at RNA transcripts or metatranscriptomes. In one such study, marine microbial populations observed during a phytoplankon bloom, studied in a mesocosm CO₂ manipulation study, were noted to have high levels of novelty within their transcriptome (Gilbert et al., 2008). An alternative to looking at a transcriptome would be to measure quantitative gene expression through the use of microarrays (Sebat et al., 2003) or quantitative PCR (qPCR) (see section 1.5.4) (Zhu et al., 2005; Hou et al., 2010; Hunt et al., 2013). Finally, stable isotope probing (SIP) has been used to demonstrate bacterial populations actively metabolising C1 compounds during a phytoplankton bloom (Neufeld et al., 2008) and is further discussed in section 1.5.3.

Each of the techniques listed above have advantages and disadvantages as discussed elsewhere (Manefield et al., 2002b; Griffiths et al., 2004; Handelsman, 2004; Allen and Banfield, 2005; Dumont and Murrell, 2005; Handelsman, 2005; Hofmann et al., 2005; Whiteley et al., 2006; Chen and Murrell, 2010; Mock and Kirkham, 2012), however in combination with modern high throughput sequencing technologies these techniques are emerging as a powerful tool in understanding the ‘microbial black box’ (Tiedje et al., 1999). Below specific techniques used in this study are introduced more comprehensively.

1.5.3 **Stable isotope probing (SIP)**

Stable isotope probing looks at the level of stable isotope integration into cellular biomarkers and therefore, can be used to determine organisms which
are actively utilising a specific labelled substrate. The first to use stable isotopes as markers of microbial function identified the organisms responsible for sulphate reduction coupled to acetate and methane oxidation in sediments, by examining $^{13}$C enriched polar lipid derived fatty acid (PLFA) (Boschker et al., 1998). However the use of PLFA analysis was superseded by approaches which look at the integration of labelled substrate into DNA (DNA-SIP) (Radajewski et al., 2000), and further developed to look at direct integration into the ribosomal RNA molecule (RNA-SIP) (Manefield et al., 2002a; Manefield et al., 2002b; Whiteley et al., 2006). Generally, PLFA-SIP provides the highest sensitivity, yet has fewer potential downstream applications than either DNA or RNA-SIP. DNA-SIP enables a researcher to retrieve actively labelled genomic DNA from an environment and therefore can be used to detect a large range of potential markers. However to obtain labelling sufficient for detection, DNA-SIP requires DNA replication and is therefore limited by cell division (Neufeld et al., 2007a). In contrast, by directly studying labelled rRNA, it is possible to study phylogenetically linkable uptake of a substrate independently of cell replication (Whiteley et al., 2007). Consequently, RNA-SIP is more sensitive than DNA-SIP, although downstream applications are usually, but not always limited to ribosomal RNA analysis (Huang et al., 2009). When combined with community fingerprint techniques, SIP allows for the identification of specific community members which are actively metabolizing a given substrate under the defined experimental parameters. Figure 1.6 summarises a typical nucleic acid SIP experiment, full methodology and reviews available elsewhere (Dumont and Murrell, 2005; Neufeld et al., 2007a; Neufeld et al., 2007b; Whiteley et al., 2007).

Primary studies using DNA SIP were able to demonstrate the presence of active bacterial methylotrophs within forest soils (Radajewski et al., 2000; Radajewski et al., 2002), and methanotrophs in peat soils (Morris et al., 2002b). When extended to include the analysis of RNA, it was possible to identify a novel organism belonging to the bacteria genus *Thauera* key to the degradation of phenol in a bioreactor community (Manefield et al., 2002a). Since these early studies SIP has been used to demonstrate the assimilation of labelled stable isotopes in compounds including carbon dioxide (Griffiths et al., 2004), acetate
(Longnecker and Kujawinski, 2013) and ammonium (Gerbl et al., 2014). Additionally, SIP experiments have been performed in pure cultures (Lueders et al., 2004a) to diverse communities such as those found in soil (Rangel-Castro, 2005; Cébron et al., 2007) and marine environments (Neufeld et al., 2008). These studies, clearly demonstrate that - in conjunction with community fingerprint techniques such as DGGE or T-RFLP - SIP successfully overcomes the ‘microbial black box’ and allows the identification of metabolically active members within a given microbial community.

![Figure 1.6: Graphical outline of a nucleic acid stable isotope probing (SIP) experiment. Environmental community is incubated with desired stable isotope substrate or control and either DNA or RNA extracted. Extract separated by ultracentrifugation, across either a Caesium chloride (CsCl) or Caesium Trifluoroacetate (CsTFA) density gradient. Nucleic acids (NAs) separate based upon molecular weight, those which have integrated heavier stable isotope (e.g $^{13}$C) will be denser than control samples. Gradients can be fractionated, and NAs precipitated for down stream community characterisation applications such as qPCR, molecular fingerprint techniques or metagenomics.](image)

Alternatively, when combined with quantitative PCR techniques (qPCR), SIP is able to accurately quantify the amount of a specific gene which is actively metabolising within a study (Lueders et al., 2004b; He et al., 2012; Sharp et al., 2012).

1.5.4 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) and Quantitative Reverse Transcriptase PCR (qRT-PCR) are considered to be “the method of choice” for the sensitive quantification of the production of a specific nucleic acid (NA) transcript (Bar et
First applied in order to quantify DNA (viral) endpoint PCR transcripts, qPCR has come into popular use when combined with ‘real time’ measurement of PCR product formation (Abbott et al., 1988; Higuchi et al., 1993; Chiang et al., 1996; Gibson et al., 1996; Heid et al., 1996; Bar et al., 2012). Current technologies follow the production of PCR product or cDNA transcript (qRT-PCR) over time by measuring fluorescence. A number of technologies have been developed however, the most commonly applied in microbiological studies are the detection of fluorogenic probes such as TaqMan (Livak et al., 1995; Heid et al., 1996) or a dsDNA binding dye such SYBR green (Wittwer et al., 1997; Ponchel et al., 2003). Although less specific than TaqMan probes, the SYBR green approach is often favoured in environmental microbiology because it monitors the amplification of any double stranded sequence, and is comparatively cheaper than TaqMan. The number of gene copies (or NA quantity if using an accurately quantified standard) of a target taxonomic group can be determined by the number of PCR cycles required to cross a fluorescence detection threshold (quantification cycle, or Cq) (Bustin et al., 2009). qPCR assays have been successfully employed in marine prokaryotes (Suzuki et al., 2000; Suzuki et al., 2001b) and later eukaryotes (Zhu et al., 2005). For example, Zhu and colleagues (2005) developed a qPCR assay to look at specific groups of picoeukaryotes, through which they were able to directly assess the prevalence of order Mamiellales in the Mediterranean Sea. Furthermore, Lueders et al. (2004b) combined both DNA and RNA-SIP with qPCR and RT-qPCR, to track community dynamics in rice field methanotrophs over time, providing evidence of $^{13}$C uptake in prokaryotic methylotrophs and possible indirect uptake in fungi and protozoa. Studies such as Lueders and colleagues (2004b) and Zhu and colleagues (2005) clearly demonstrate the potential to use qPCR in conjunction with SIP to investigate active picoeukaryote communities.

1.5.5 The Bergen mesocosm

As outlined in section 1.4.3, much of the primary work investigating the effect of ocean acidification upon planktonic organisms has been carried out upon single organisms and small scale incubations of mixed populations, for example:
assemblages (Tortell et al., 2002), coccoliths (Riebesell et al., 2000), coral (Gattuso et al., 1998) and their algae (Kuffner et al., 2008), copepods (Kurihara et al., 2004), cyanobacteria (Barcelos e Ramos et al., 2007) gastropods and echinoderms (Kurihara and Shirayama, 2004; Shirayama and Thornton, 2005).Although this approach does allow greater repetition and manipulation than in-situ studies, it may be prone to “bottle effects” (Zobell and Anderson, 1936) and therefore simplify community level interactions. An alternative to this would be an in-situ mesoscale approach, as in Thingstad et al. (2005a) and Boyd et al. (2007), yet the opportunity to manipulate on this scale is rare and statistically limited because of the inability to replicate. An alternative approach is that of a mesocosm study. Mesocosm studies allow direct manipulation and repetition in a large scale naturalistic setting. Mesocosm studies have been successfully employed in the study of seawater acidification in a series of three experiments in 2001, 2003 and 2005 (Riebesell et al., 2008). Named the Pelagic Ecosystem CO₂ Enrichment studies (PeECE I-III), these studies set out to: test the validity of laboratory based studies, examine CO₂ sensitivity transfer from the organism to community and assess the impacts of these findings upon both biogeochemical processes and air sea gas exchange (Riebesell et al., 2008).

In their 2005 policy document, Raven and colleagues (2005) recommended that there was a need for large scale (mesocosm) experiments to further investigate the impact of ocean acidification upon “sensitive organisms, functional groups and ecosystems”. Additionally, they highlighted the current lack of knowledge about the potential effects of OA upon microorganisms. As a result the Bergen Mesocosm Experiment 2006 (a multi consortia initiative funded through the UK Natural Environment Research Council’s ‘Aquatic Microbial Metagenomes and Biogeochemical cycles’ grant) was conceived. The overarching aim of the 2006 Bergen mesocosm experiment was to determine the impact of pH change on key carbon and nitrogen metabolic pathways in marine microbial communities. Participants in the experiment included national research institutes (Plymouth Marine Laboratory and the Centre for Ecology and Hydrology), and a number of universities (inc. Warwick, Newcastle, Stirling, Cardiff and Liverpool). Individual groups investigated the effect of elevated CO₂ upon nitrogen fixation, viral host interactions, methylotrophs, and transcriptomic response. One of the key roles
of CEH within this experiment was to investigate this aim in the context of active $^{13}$C-bicarbonate integration by phototrophs and to follow the transfer of label into the heterotrophic community, through the application of RNA-SIP. As part of the CEH Oxford/Wallingford research team, I utilised the experimental samples and data collected during this experiment, to investigate picoplanktonic diversity as well as carbon utilisation. The aims of this thesis are outlined in section 1.6.

1.6 Aims and Objectives

1.6.1 Aims

Because only a small fraction of the oceans microbial diversity has been cultured and formally described one of the key challenges faced is the ability to link phylogenetic diversity to the functional diversity – “who is there and what are they doing?” (Dubilier, 2007).

**Aim 1: How does bacterial community structure respond to elevated $CO_2$?**

This aim is addressed in chapter 3. The application of basic ecological principles has proven to be a powerful tool in explaining the community distribution and abundance patterns of macro-organisms in response to environmental change, yet these ideas have only been applied to microbiology recently (Prosser et al., 2007). Using a community fingerprint technique (T-RFLP) and the definitions for community disturbance outlined in Martiny and colleagues (2006) this chapter addresses the null hypothesis proposed by Joint and colleagues (2011) that ‘marine microbes possess the flexibility to accommodate pH change and there will be no catastrophic changes in marine biogeochemical processes that are driven by phytoplankton, bacteria and archaea’. This chapter is based upon a first author accepted for publication in Environmental Microbiology Reports.
**Aim 2: How do members of the picoplankton (0.2-2.0 µm) respond to elevated CO₂?**

This is addressed in chapter 4. Although chapter 3 investigates bacterial community structure it is important to understand the taxonomic constituents of this community. Gattuso and colleagues (2011) highlight the need for work integrating community responses to OA and individual responses to elevated CO₂. Chapter 4 utilises flow cytometry, SSU rRNA gene sequencing and T-RFLP to investigate both the phylogenetic diversity and fine resolution dynamics of the dominant members of the picoplankton (both bacterial and eukaryote) to elevated CO₂. This chapter has been formed from a published, first author paper (Newbold et al., 2012).

**Aim 3: Do functional microbial communities respond to OA?**

Microbes are key to oceanic processes through their roles in photosynthesis, grazing and the microbial loop, and as such it is important to consider how OA will affect the function of such communities. One of the most challenging tasks faced by microbial ecologists is to link these functions to uncultured members of a community. Chapter 5 (with some crossover in chapter 6) utilises RNA-SIP to investigate the direct microbial community uptake of CO₂ and glucose within the resident prokaryote and eukaryote communities.

**Aim 4: Is it possible to detect functional shifts in key picoeukaryotes response to elevated CO₂?**

Elevated CO₂ concentration has been linked to higher levels of primary production (Hein and Sand-Jensen, 1997; Schippers et al., 2004). Consequently, there is an emerging perception that phytoplankton may experience a shift in favour of smaller non calcifying organisms, which put less effort into costly carbon concentration mechanisms (CCM’s) (Paulino et al., 2008; Newbold et al., 2012; Brussaard et al., 2013). In the final experimental chapter of this thesis, RNA-SIP and qPCR are used in conjunction to investigate the effect of elevated CO₂ upon the function of the dominant picoeukaryotes *Micromonas* and *Bathycoccus*. 
1.7 References


Knight-Jones, E. W. and Walne, P. R. (1951) 'Chromulina pusilla Butcher, a Dominant Member of the Ultraplankton', *Nature*, 167(4246), pp. 445-446.


Changing Seawater Carbonate Chemistry', *Geochemistry, Geophysics, Geosystems*, 7(9), p. Q09006.


Marin, B. and Melkonian, M. (2010) 'Molecular Phylogeny and Classification of the Mamiellophyceae class. nov (Chlorophyta) based on Sequence


Chapter 2. Study Site and Experimental Parameters

The experimental chapters of this thesis are written in the style of research papers for the publications Environmental Microbiology and Environmental Microbiology Reports, and hence the methods applied are described in each chapter. However, to aid clarity a brief description of the sample site and an experimental time-line follows.

2.1 The Study Site

Situated around 25km from Bergen, Norway, the Marine Biological Research Station, Esplend houses the Large-Scale Mesocosm Facility of the University of Bergen. Aside from multidisciplinary laboratories the facility houses a raft moored in the Raunefjorden, 60.3°N, 5.2 °E with the ability to hold up to 12 Mesocosm enclosures (figure 2.1).

Figure 2.1: The raft housing the mesocosm enclosures
2.2 Experimental Parameters and Time-line

During May 2006 six experimental mesocosm enclosures (3.5m depth, 2m diameter, holding ~1100L) were constructed from polyethylene and suspended 0.5m above the surface water level. Mesocosm supporting structures were anchored to an experimental raft housed in Raunefjorden, Norway 60.3ºN, 5.2 ºE, and 200m from shore. Prior to the commencement of sampling mesocosm enclosures were filled with nutrient deplete unfiltered native fjord water on 2nd of May. In order to minimise contamination from atmospheric conditions enclosures were covered with reinforced lids constructed from high UV transmitting polyethylene. On May 6th a phytoplankton bloom was stimulated through the addition of phosphate and nitrate in all enclosures (concentrations at experimental commencement: 1 µmol l⁻¹ phosphate; 17 µmol l⁻¹ nitrate). Note that mesocosms 2 and 5 used ¹⁵N nitrate as opposed to ¹⁴N in other enclosures. Mesocosm enclosures were exposed to two initial CO₂ treatments high/elevated (mesocosm enclosures 1-3) and ambient (mesocosm enclosures 4-6). High/elevated enclosures were sparged with ambient air enriched to 750 ppmV CO₂ (g) from 4-6th May, until the pH of the seawater within the enclosures had declined to ~7.8 (range 7.81–7.82). Ambient mesocosm enclosures were treated identically, but with ambient air. Subsequently blooming phytoplankton growth reduced CO₂ concentrations in the high CO₂ mesocosms, therefore mesocosm enclosures 1 and 2 were re-acidified 10 days after mesocosm establishment, and ambient condition enclosures 5 and 6 again sparged with air. In order to assess the consequences on the community if the experiment had continued without resparging, the remaining 2 mesocosm bags (3 and 4) were left unsparged. Experimental samples were taken for 18 days beginning the 6th May.

In conjunction with the main mesocosm study, CEH Oxford/Wallingford set up a series of stable isotope microcosm incubations at three key time points in the study. These time points corresponded to an early nutrient replete phase (SIP 1, 7th May), phytoplankton bloom peak (SIP 2, 13th May) and final nutrient deplete phase (SIP 3, 20th May). Microcosm incubations were filled from 4L water sampled directly from all mesocosm bags to fill 5L Nalgene bottles.
containing either $^{12}\text{C}$ or $^{13}\text{C}$ glucose (50mg/L) or sodium bicarbonate (0.15g/L). These microcosms were submerged under surface fjord water and incubated \textit{in situ}. During each incubation daily pH and cell abundance was measured. Plankton was collected from 1L of microcosm water onto 0.2 µm Durapore membranes for a period of 5 days.

A summary timeline of key events within the 2006 BME is found in figure 2.2.
Figure 2.2: Timeline of experimental manipulations within the 2006 Bergen mesocosm experiment.
Chapter 3: Marine Bacterial Communities are Resistant to Elevated Carbon Dioxide Levels

Running title: Marine Bacterial Communities and Elevated CO₂

Lindsay K. Newbold¹,²†, Anna E. Oliver¹†, Andrew S. Whiteley³, and Christopher J. van der Gast¹*

¹NERC Centre for Ecology and Hydrology, Wallingford, OX10 8BB, UK

²School of Civil Engineering and Geosciences, University of Newcastle upon Tyne, Newcastle, NE1 7RU, UK.

³School of Earth and Environment, University of Western Australia, Crawley, WA 6009, Australia

*For correspondence E-mail cjvdg@ceh.ac.uk; Tel. (+44) 1491 692647; Fax (+44) 1491 692424.

† Contributed equally to this work.

Accepted for publication in Environmental Microbiology Reports, © 2014 Society for Applied Microbiology and John Wiley & Sons Ltd. Edits in format and references were made in keeping with the thesis structure however all other wording remains consistent with the submitted publication. Figures have been placed within the text and online supplementary data included listed as supplementary figures S3.7.1 - 3.7.4. The role of individual authors has been outlined on page 207.

Keywords: Ocean acidification; Taxa-time relationships; Distance-decay relationships; taxa turnover; Bacterial Resistance
3.1 Summary

It is well established that the release of anthropogenic derived CO$_2$ into the atmosphere will be mainly absorbed by the oceans, with a concomitant drop in pH; a process termed ocean acidification. As such, there is considerable interest in how changes in increased CO$_2$ and lower pH will affect marine biota, such as bacteria, which play central roles in oceanic biogeochemical processes. Set within an ecological framework, we investigated the direct effects of elevated CO$_2$, contrasted with ambient conditions, on the resistance of marine bacterial communities in a replicated temporal seawater mesocosm experiment. The results of the study strongly indicate that marine bacterial communities are highly resistant to the elevated CO$_2$ and lower pH conditions imposed, as demonstrated from measures of turnover using taxa-time relationships and distance-decay-relationships. In addition, no significant differences in community abundance, structure or composition were observed. Our results suggest that the bacterial fraction of microbial plankton holds enough flexibility and evolutionary capacity to withstand predicted future changes from elevated CO$_2$ and subsequent ocean acidification.
3.2 Introduction

It is well established that most anthropogenically derived carbon dioxide that is released into the atmosphere, as a result of burning fossil fuels and cement production over the past 200 years, will eventually be absorbed by the oceans (Caldeira and Wickett, 2003; Raven et al., 2005). This process of absorption of atmospheric carbon dioxide (pCO$_2$) is changing the chemistry of the oceans and in particular is decreasing pH, making seawater more acidic (Caldeira and Wickett, 2003; Raven et al., 2005; Joint et al., 2011). Joint and colleagues (2011) succinctly described the chemical absorption process; stating that as anthropogenic CO$_2$ increases in the atmosphere, it dissolves in the surface ocean, aqueous CO$_2$ then reacts with water to form a weak acid (carbonic acid, H$_2$CO$_3$), the dissociation of which forms hydrogen (H$^+$) and bicarbonate ions (HCO$_3^-$). The increase in the concentration of hydrogen ions then results in an inevitable drop in oceanic pH: a process which is commonly 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; Raven et al., 2005). The present average surface ocean pH is approximately 8.1, being 0.1 units lower than pre-industrial revolution levels (Caldeira and Wickett, 2003). Atmospheric CO$_2$ is predicted to reach between 550 and 1000 µatm by the year 2100, with a concurrent decline in surface ocean pH of between 0.2 and 0.5 units, for which there is no known analogue from the past 300 million years (Wolf-Gladrow et al., 1999; Nakicenovic et al., 2000).

There is significant interest in how changes in pCO$_2$ levels and subsequent ocean acidification will affect the oceans biota and integral processes (Orr et al., 2005; Fabry et al., 2008; Guinotte and Fabry, 2008; Doney et al., 2009; Kerr, 2010; Sabine and Tanhua, 2010). The marine ecosystem contributes over 90% of the Earth’s biosphere and marine microbes play an essential role in marine biogeochemical cycles central to the biological chemistry of the Earth with around 50% of global primary production attributed to phytoplanktonic bacteria, and protists (Field et al. 1998). Further to this, over half of autotrophically fixed
oceanic CO$_2$ is reprocessed or turned over by heterotrophic bacteria and archaea through processes such as the microbial loop and carbon pump (Azam, 1998; Jiao et al., 2010). An increasing number of studies have reshaped our understanding of the extent and importance of marine bacterial diversity e.g. (Giovannoni et al., 1990; Britschgi and Giovannoni, 1991; Schmidt et al., 1991; Fuhrman et al., 1992; Fuhrman et al., 1993; Rappe et al., 2000), with more recent additional insights into the functional and phylogenetic diversity of the Earth’s oceans, reinforcing the perceived importance of marine microbial communities to the biogeochemical cycles present globally (e.g. Kannan et al., 2007; Rusch et al., 2007; Yooseph et al., 2007).

The application of basic ecological principles has proven to be a powerful tool in explaining the community distribution and abundance patterns of macro-organisms in response to environmental change, yet these ideas have only been applied to microbiology recently (Prosser et al., 2007). An important aspect of community analysis in an environmentally disturbed system (such as CO$_2$ perturbation) is the accurate evaluation of biological integrity and recovery following such an event (Ager et al., 2010) - how will a community respond to change and will it recover? 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 did, likely leading to no loss of function (Grossart et al., 2006; Allgaier et al., 2008). Most recently the European project on ocean acidification (EPOCA) found free living bacterial community structure was not majorly affected by degree of ocean acidification, but by variations in productivity and nutrient availability (Roy et al., 2013; Sperling et al., 2013; Zhang et al., 2013). This highlights not only the often conflicting results found in such studies but also the difficulty in distinguishing direct effects upon bacteria from indirect effects relating to phytoplankton assemblages.

When discussing ocean acidification Joint and colleagues (2011) proposed the null hypothesis that ‘marine microbes possess the flexibility to accommodate pH change and there will be no catastrophic changes in marine biogeochemical
processes that are driven by phytoplankton, bacteria and archaea—a view supported by some studies (Allgaier et al., 2008; Newbold et al., 2012; Roy et al., 2013; Sperling et al., 2013), but not all (Grossart et al., 2006; Liu, 2010; Lidbury et al., 2012). In our previous work we demonstrated that in 5 out of 6 key bacterial groups no significant response to CO₂ perturbation was observed, yet this work reflected only a small proportion of the total community and therefore an in depth study of the direct changes in total bacterial community response is warranted (Newbold et al., 2012).

Here, we test null hypothesis of Joint and colleagues (2011), focusing on direct bacterial community responses to elevated CO₂ in a replicated temporal seawater mesocosm experiment. Specifically, using culture independent methods, we examined bacterial community turnover, composition, structure, and abundance under elevated CO₂ and ambient conditions.

3.3 Results and Discussion

3.3.1 pH and abundance

Seawater samples were collected daily over an 18 day study period from six mesocosms each with a working volume of ~11,000 L. Three mesocosms were enriched with carbon dioxide (elevated CO₂), while the remaining three were used as control (ambient condition) mesocosms. A consequence of increased dissolved carbon dioxide in seawater will be a decrease in pH and subsequent ocean acidification (Joint et al., 2011). This was the case in the experimental mesocosms where an inverse relationship was observed between pH and pCO₂, being autocorrelated as expected (pH = a – b log pCO₂ [r² = 0.99; F₁,₁₀₀ = 2560.2; P < 0.0001]). Measurement and analyses of the physical and chemical parameters within the mesocosms revealed that only pCO₂, pH and total inorganic dissolved (TID) carbon were significantly different between treatments (figure S3.7.1); where pCO₂ and TID carbon were significantly higher and, conversely, pH was significantly lower in mesocosms under elevated CO₂ conditions compared to the ambient control mesocosms (figure S3.7.1; figure S3.7.2a and b).
The mean bacterial abundance within the elevated CO$_2$ mesocosms was $4.5 \times 10^6 \pm 1.03 \times 10^6$ cells ml$^{-1}$ and was not significantly different (ANOVA: $F_{1,4} = 2.05; P = 0.23$) from the mean abundance within the mesocosms under ambient conditions; $5.74 \times 10^6 \pm 9.79 \times 10^5$ cells ml$^{-1}$. The temporal patterns of mean abundance were not significantly different between the elevated CO$_2$ and ambient conditions.

**Figure 3.1**: The taxa-time relationships (TTRs) for bacterial communities in mesocosms under elevated CO$_2$ and ambient conditions. Given are the TTR for each mesocosm (A to F) and the mean TTR by treatment. Error bars represent the standard deviation of the mean ($n = 3$). Also given are the taxa-time power law equation $S = cT^w$: (1) $r^2 = 0.94$, $F_{1,15} = 253.2$; (2) $r^2 = 0.70$, $F_{1,15} = 34.4$; (3) $r^2 = 0.76$, $F_{1,15} = 46.4$; (4) $r^2 = 0.94$, $F_{1,15} = 230.5$; (5) $r^2 = 0.96$, $F_{1,15} = 391.4$; (6) $r^2 = 0.84$, $F_{1,15} = 79.6$; (Elevated CO$_2$ mean) $r^2 = 0.89$, $F_{1,15} = 117.7$; and (Ambient mean) $r^2 = 0.98$, $F_{1,15} = 748.7$. All regression coefficients were significant ($P < 0.0001$).
bacterial abundance mirrored each other until day 14, thereafter mean cell counts under elevated CO$_2$ conditions declined, albeit with high variance, in contrast to mean cell counts under ambient conditions (figure S3.7.2c).

To determine whether the mean abundance distributions over time were significantly different we applied the two-sample Kolmogorov-Smirnov distribution fitting test, which indicated that there was no statistical difference in the bacterial abundance dynamics between treatments ($D = 0.353; P = 0.245$). Furthermore, no significant relationships were observed between bacterial cell counts and pCO$_2$ concentrations or pH in any of the mesocosms ($P > 0.05$ in all cases). This finding is in line with other studies where bacterial abundance was largely unaffected by CO$_2$ perturbation (Grossart et al., 2006; Allgaier et al., 2008; Liu, 2010; Krause et al., 2012; Newbold et al., 2012; Lindh et al., 2013).

### 3.3.2 Temporal turnover in acidified bacterial communities

The bacterial communities within each mesocosm, over the 18 day study period, were analysed by 16S rRNA terminal restriction fragment length polymorphism (T-RFLP). In this study, TRF peak richness and intensity were used to infer the richness and relative abundance of bacterial taxa within each mesocosm. Taxa-time relationships (TTR) were used to investigate the effect of elevated CO$_2$ levels on bacterial diversity (figure 3.1); specifically, to assess temporal taxa turnover of bacterial taxa across the two treatments. The TTR describes how the observed taxa richness of a community in a habitat of fixed size increases with the length of time over which the community is monitored (van der Gast et al., 2008). The TTR was modelled with the power law equation, $S = cT^w$. Where $S$ is the cumulative number of observed taxa over time $T$, $c$ is the intercept and $w$ is the temporal scaling exponent and therefore increasing values of $w$ can be taken as greater rates of taxa turnover. The mean $w$-value within the elevated CO$_2$ mesocosms was $0.145 \pm 0.018$, whereas $w$ was significantly higher (ANOVA: $F_{1,4} = 63.21; P < 0.001$) within the ambient mesocosms, $w = 0.240 \pm 0.011$ (figure 3.1). As slopes, the values of $w$ for each mesocosm between treatments, using the t-distribution method (Fowler et al., 1998), were found to be significantly different (table 3.1a); that is the rate of
turnover within the elevated CO₂ mesocosms produced a significant decrease in cumulative taxa richness and therefore taxa turnover, when compared to the ambient mesocosms.

In addition to the TTR analyses, distance-decay relationships were employed to measure bacterial community turnover rates within the mesocosms (figure 3.2). The distance-decay relationship essentially allows an analysis of how similarity in community composition between sites changes with the geographic distance separating those sites (van der Gast et al., 2011). For the current study, geographical distance was substituted for temporal distance (days) and the rate of decay in community similarity through time was assessed and compared amongst the experimental mesocosms. The distance-decay relationship was modelled with the power law equation, 

\[ S_{\text{SOR}} = cD^d \]

where \( S_{\text{SOR}} \) is the pair-wise similarity between any two samples using the Sørensen index, \( c \) is a constant, \( D \) is temporal distance between pair-wise samples and \( d \) is the rate of decay in similarity or community turnover rate. The mean rate of decay within the elevated CO₂ mesocosms was \( d = -0.030 \pm 0.007 \), however, \( d \) was significantly higher (ANOVA: \( F_{1,4} = 36.07; P < 0.004 \)) within the ambient mesocosms; \( d = -0.167 \pm 0.039 \) (figure 3.2). Using the \( t \)-distribution method, the slopes for each mesocosm distance-decay relationship when compared between treatments were found to be significantly different (table 3.1b). This indicated that the rate of decay, and therefore turnover, was significantly dampened within the elevated CO₂ mesocosms, selecting for a more conserved community composition through time when compared to the more dynamic communities within the ambient mesocosms. Although the distance-decay relationships significantly differed by the overarching treatment, the temporal scaling of bacterial taxa within the mesocosms was driven by time (temporal distance) and not day-to-day differences in pH or pCO₂ concentrations (table 3.2).
Figure 3.2: The distance-decay of bacterial community similarity (Sørensen index ($S_s$)) in mesocosms under elevated CO$_2$ and ambient conditions over time. Given are the distance-decay relationships (DDR) for each mesocosm (1 to 6) and the mean DDR by treatment. Error bars represent the standard deviation of the mean ($n = 3$). Given are the distance-decay power law equation $S_s = cD^d$: (1) $r^2 = 0.05$, $F_{1,134} = 6.5$; (2) $r^2 = 0.02$, $F_{1,134} = 6.4$; (3) $r^2 = 0.09$, $F_{1,134} = 13.9$; (4) $r^2 = 0.28$, $F_{1,134} = 52.8$; (5) $r^2 = 0.40$, $F_{1,134} = 90.5$; (6) $r^2 = 0.43$, $F_{1,134} = 101.6$; (elevated CO$_2$ mean) $r^2 = 0.16$, $F_{1,134} = 24.6$; and (ambient mean) $r^2 = 0.56$, $F_{1,134} = 168.8$. All regression coefficients were significant ($P < 0.05$). Partial Mantel summary statistics are listed in Table 3.2.
<table>
<thead>
<tr>
<th>A Treatment</th>
<th>Elevated CO₂</th>
<th>Ambient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesocosm</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Elevated CO₂</td>
<td>1</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.18</td>
</tr>
<tr>
<td>Ambient CO₂</td>
<td>4</td>
<td>4.48</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.94</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B Treatment</th>
<th>Elevated CO₂</th>
<th>Ambient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesocosm</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Elevated CO₂</td>
<td>1</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.02*</td>
</tr>
<tr>
<td>Ambient CO₂</td>
<td>4</td>
<td>5.34</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.74</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.53</td>
</tr>
</tbody>
</table>

**Table 3.1:** Comparison of power regression slopes between all mesocosms for (A) taxa-time relationships (TTR) and (B) distance-decay relationships. In each case, the t-distribution method test statistic (t) is given in the lower triangle and significance (P) is given in the upper triangle for each comparison. For the taxa-time relationships the degrees of freedom (df) = 1,30, and for the distance-decay-relationships, df = 1, 268. Asterisks denote those slopes that were significantly different at the P < 0.05 level.
As expected, Mantel tests demonstrated pCO₂ and pH were significantly autocorrelated in all mesocosms: Mantel statistic \( r = (\text{Mesocosm 1}) 0.949, (2) 0.966, (3) 0.966, (4) 0.950, (5) 0.943, \) and \( (6) 0.942 \) \( (P < 0.0001 \) in all cases). In addition, Mantel and partial Mantel tests revealed that other environmental variables (including, temperature, salinity, nitrate, phosphate, etc.) did not have significant relationships with similarity in any of the experimental mesocosms. Based on a direct ordination approach, the bacterial community composition was significantly influenced by time, the best explanatory variable in all mesocosms, and phosphate and/or nitrate (table 3.3). In agreement with the Mantel based tests, pH or pCO₂ did not significantly explain any of the variance in the mesocosms communities.

Bacterial taxa abundance distributions for mesocosms under different treatments were plotted as rank-abundance curves to examine differences in evenness and dominance over the course of the study and specifically to determine what impact elevated CO₂ levels had on community structure (figure S3.7.3). It is generally accepted that a reduction of taxa richness will occur in an ecological community as a consequence of an environmental perturbation (Magurran and Phillip, 2001). In addition, the loss of species is accompanied by a change in community structure (Ager et al., 2010). Whereby, unperturbed species-rich assemblages are typically evenly distributed and following a perturbation are replaced by species-poor assemblages with high dominance (Magurran and Phillip, 2001; Ager et al., 2010). To more clearly visualise changes in community structure, the mean slope values \( (b) \) from the rank-abundance plots were used as a descriptive statistic of evenness and plotted over time for each treatment (figure S3.7.3). When the mean slope values were compared \( (\text{CO}_2 \ b = -0.077 \pm 0.026, \) and \( \text{Ambient} \ b = -0.080 \pm 0.014) \) no significant differences in community structure were observed by treatment \( (\text{ANOVA}: F_{1,4} = 0.51; P = 0.514). \)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mesocosm</th>
<th>$r(\text{SD.C})$</th>
<th>$P$</th>
<th>$r(\text{SC.D})$</th>
<th>$P$</th>
<th>$r(\text{SD.p})$</th>
<th>$P$</th>
<th>$r(\text{Sp.D})$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated $CO_2$</td>
<td>1</td>
<td>-0.293</td>
<td>0.001*</td>
<td>0.226</td>
<td>0.995</td>
<td>-0.279</td>
<td>&lt;0.0001*</td>
<td>0.217</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-0.199</td>
<td>0.002*</td>
<td>-0.007</td>
<td>0.473</td>
<td>-0.200</td>
<td>0.001*</td>
<td>0.003</td>
<td>0.515</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-0.325</td>
<td>0.001*</td>
<td>0.115</td>
<td>0.089</td>
<td>-0.333</td>
<td>&lt;0.0001*</td>
<td>0.160</td>
<td>0.968</td>
</tr>
<tr>
<td>Ambient $CO_2$</td>
<td>4</td>
<td>-0.472</td>
<td>&lt;0.0001*</td>
<td>0.331</td>
<td>0.999</td>
<td>-0.643</td>
<td>&lt;0.0001*</td>
<td>0.241</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-0.421</td>
<td>&lt;0.0001*</td>
<td>0.275</td>
<td>0.998</td>
<td>-0.538</td>
<td>&lt;0.0001*</td>
<td>-0.052</td>
<td>0.273</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-0.510</td>
<td>&lt;0.0001*</td>
<td>0.074</td>
<td>0.202</td>
<td>-0.769</td>
<td>&lt;0.0001*</td>
<td>0.247</td>
<td>0.998</td>
</tr>
</tbody>
</table>

**Table 3.2:** Summary statistics for partial Mantel tests. The partial Mantel statistic $r(AB.C)$ estimates the correlation between two proximity matrices, $A$ and $B$, whilst controlling for the effects of $C$. Given are bacterial community similarity $S$ (Sørensen index) and also $C$ and $p$ which are differences in pCO$_2$ and pH, respectively. Also given is $P$ to ascertain whether the partial Mantel regression coefficients were significantly different from zero following 9,999 permutations. $P$-values significant after Bonferroni correction for multiple comparisons ($0.05/18 = 0.003$) are denoted with asterisks.
Allison and Martiny (2008) defined resistance as ‘the degree to which microbial composition remains unchanged in the face of a disturbance’ and resilience as ‘the rate at which microbial composition returns to its original composition after being disturbed’ regardless of the system studied. The EPOCA studies of Roy and colleagues (2013); Sperling and colleagues (2013) and Zhang and colleagues (2013) suggested that variations in nutrients and productivity were the dominant drivers of free living bacterial community change, not increased CO$_2$. In contrast, we found evidence that species turnover was significantly dampened within the elevated CO$_2$ mesocosms, selecting for a more conserved community composition through time, giving clear evidence that the bacteria constituted a community resistant to CO$_2$ perturbation. Further to this, distance decay measures demonstrated that community composition changes little with CO$_2$ perturbation, indicating that the elevated CO$_2$ likely had no direct effect upon the mesocosm community. Others have demonstrated that microbial communities are ‘resistant’ to perturbation (Klamer et al., 2002; Chung et al., 2005; Horz et al., 2005; Kasurinen et al., 2005; Gruter et al., 2006; Bowen et al., 2011). However before generalising it’s important to consider that bacterial communities don’t all respond in the same way (Bissett et al., 2013).

3.3.3 Conclusions

Our findings suggested that the bacterioplankton communities studied were resistant to short term catastrophic pCO$_2$ perturbation. This study corroborates...
the emerging perception that bacteria are able to withstand much environmental change (Liu, 2010; Joint et al., 2011). We cannot however rule out the effect of OA upon the long term resilience of communities. For example Newbold and colleagues (2012) found significant differences in key members of the picoeukaryote community assemblage, a finding also evident in the study of Brussaard and colleagues (2013). Any changes in the pelagic food web are likely to have an effect upon the bacterioplankton as much of bacterial community structure is determined by ‘top down’ pressures (Bell et al., 2010; Martinez-Garcia et al., 2012). To our knowledge recovery has not been measured in a similar mesocosm experiment greater than 30 days (the EPOCA arctic campaign 2010). The changes imposed in our study are meant to simulate conditions faced in 100 years’ time, 100 years represents millions of bacterial generations and therefore the scope for evolutionary adaption is huge. This study highlights the need for long term naturalistic studies, which would examine the effects of ocean acidification upon bacterioplankton in a biologically relevant setting and time scale.

3.4 Experimental procedures

3.4.1 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). We present the data for 3 elevated CO$_2$ (experimental) and 3 ambient CO$_2$ control mesocosms (2 m diameter, 3.5 m deep, ~11,000L). Experimental mesocosm enclosures were gently sparged with CO$_2$ (750 µatm) for 2 days 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 (initial concentrations: 1 µmol l$^{-1}$ phosphate; 17 µmol l$^{-1}$ nitrate). Blooming phytoplankton growth reduced CO$_2$ concentrations in the elevated CO$_2$ mesocosms, therefore 2 of the experimental mesocosm enclosures were re-acidified 11 days after mesocosm establishment (16/5/2006), and 2 ambient condition enclosures again sparged with air (the
remaining 2 mesocosm bags left unsparged). To isolate picoplankton daily samples of ~2 L of water were pre-filtered through Whatman GF/A filters to remove large eukaryote cells and filtrate collected onto 0.2 μm Durapore membranes. These were stored at -80 °C prior to molecular analysis. Note that samples for molecular analysis were not taken on day 12 of the study. Physical and chemical parameters of the water samples (including; atmospheric carbon dioxide (pCO₂), pH, temperature, and salinity) were taken and analysed as described previously (Hopkins et al., 2010), and the summary measurements are presented in figure S3.7.1.

3.4.2 Enumeration of bacterial cells using flow cytometry

Daily flow cytometric counts of absolute concentrations of bacterioplankton were performed using a Becton Dickinson FACSsortTM flow cytometer equipped with an air-cooled blue light laser at 488nm according to the protocols of (Gasol et al., 1999; Zubkov et al., 2001; Tarran et al., 2006; Zubkov et al., 2008).

3.4.3 Terminal restriction fragment length polymorphism (T-RFLP)

Full experimental procedures have been described previously (Newbold et al., 2012). In summary, total nucleic acids were extracted as previously described (Huang et al., 2009). Approximately 20-30 ng of purified template was used per 50 µL PCR reaction. A ~500 bp region of the 16S small subunit ribosomal RNA gene (SSU rRNA) was amplified using fluorescently labelled forward primer (6FAM) 27F and 536R reverse primer (Suzuki et al., 1998). Amplification conditions were as follows; 2 minute pre-denaturation phase at 94 °C followed by 30 cycles of 94 °C for 1 minute, 52 °C for 1 minute, and 72 °C for 3 minutes and a final extension phase of 10 minutes at 72 °C. 20 µL of gel purified PCR product was digested for 4 hours at 37 °C in a 30 µL total reaction volume using 20 units restriction enzyme Mspl (Promega, UK) and buffers. Digestion product (0.5 µL) was combined with 0.5 µL denatured LIZ600 size standard (Applied Biosystems) and 9 µL Hi-Di formamide (Applied Biosystems), and run on an Applied Biosystems 3730 DNA sequencer. The sizes of restriction fragments were calculated and binned using Genemarker™ (Softgenetics) and restriction fragments crossed correlated to specific cloned sequences (see Newbold et al,
2012). Bin widths were checked and manually adjusted to encompass all detected peaks. To differentiate signal from background, a Fluorescence Unit (FU) threshold of 40 units was used for a presence/absence binary matrix. All peaks were manually checked for inclusion in analysis. Relative abundance measures were calculated by dividing individual peak height by total peak height spanning all valid peaks within the analysis. Resultant data were analysed for community richness, composition, and structure.

### 3.4.4 Statistical analyses of data

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). The two-sample Kolmogorov-Smirnov test is used to compare empirical distribution fitting tests from a sample with a known distribution. It can be used, as was the case for the current study, for comparing two empirical distributions (Nikiforov, 1994). The test was performed using the XLSTAT program (version 2012; Addinsoft, France) and applied as previously described (Newbold et al., 2012).

Taxa-time relationships (TTR) were used as one method to visualise and statistically compare differences in marine bacterial temporal scaling between elevated CO$_2$ and ambient mesocosms as previously described (van der Gast et al., 2008). In addition to the TTR, we employed a second method, the distance-decay relationship (DDR), to also examine differences in marine bacterial beta diversity. The DDR describes how similarity in taxa composition between two communities varies with the geographical distance that separates them (Green et al., 2004). In addition, it also allows us to go on to determine how patterns of beta diversity are influenced by environmental factors (Green et al., 2004). In the current study, the DDR has been modified from the power law described previously (van der Gast et al., 2011), to incorporate temporal distance in place of geographic distance. The Sørensen index of community similarity and subsequent average linkage clustering of community profiles were performed using PAST (Paleontological Statistics program, version 2.16), available from the University of Oslo website link.
Two complementary approaches, direct ordination and Mantel test (Tuomisto and Ruokolainen, 2006), were used to relate variability in the distribution of bacteria to environmental factors (pCO₂, temperature, salinity, nitrate, phosphate, particulate organic nitrogen, particulate organic carbon, and total inorganic carbon) and temporal distance (days). For the direct ordination approach, temporal distance and environmental variables that significantly explained variation in bacterial communities were determined with forward selection (999 Monte Carlo permutations; α < 0.05) and used in canonical correspondence analysis (Peros-Neto et al., 2006). Partial canonical correspondence analysis was performed when both time and environmental variables were significant. Analyses were performed in the ECOMII software package (version 2.1.3.137; Pisces Conservation Ltd., Lymington, UK). For the Mantel approach (Mantel, 1967; Green et al., 2004; van der Gast et al., 2011), bacterial similarity matrices for each mesocosm, using raw presence/absence T-RF data, were calculated using the Sørensen index of similarity. Similarity matrices for environmental factors were generated by calculating the absolute difference of values between each pair wise time point. Lower tailed partial Mantel tests were conducted in the XLSTAT program.

Rank-abundance plots were used to determine differences in bacterial community structure (Ager et al., 2010). For each sample the relative abundance of each taxon (TRF) was standardized to percent values before construction of the rank-abundance plots. The rank-abundance plots were visualized by plotting the taxa rank order on the x-axis against relative abundance (log₁⁰ transformed) on the y-axis. For each plot a linear regression model was fitted, represented by the equation, log₁⁰ y = a + bx, where a is the intercept and b is the slope of the plot. The slope (b) was subsequently used as a descriptive statistic for changes in community structure as previously described (Ager et al., 2010).
3.5 Acknowledgments

The authors would like to thank all of the members of the 2006 Bergen Mesocosm Experiment for their invaluable help and data acquisition, especially, Ian Joint, Dorothee Bakker and Isabelle Mary. This work was funded by the UK Natural Environment Research Council (NERC) Grant Number NE/C507937/1 as part of the post-genomics and proteomics programme.

3.6 References


Dynamics Influenced by Elevated CO$_2$ Levels', *Biogeosciences*, 10(2), pp. 719-731.


### 3.7 Supplementary Information

#### Figure S3.7.1: Baseline physical and chemical characteristics for the elevated CO$_2$ and ambient mesocosms. For each parameter within each mesocosm the mean and standard deviation (SD) over 18 days is given. Given is the mean for each parameter ($n=3$) and SD for each treatment. Also given are ANOVA test results, $F$-ratio (including degrees of freedom) and significance ($P$), for each parameter compared under both treatments. Asterisks denote those relationships that were significantly different between treatments at the $P < 0.05$ level. a. Atmospheric CO$_2$ (µatm). b. Temperature in °C. c. Salinity in practical salinity units (PSU). d. Measured in µmol nitrate or phosphate L$^{-1}$. e. Particulate organic (PO) nitrogen or carbon (µg N or C L$^{-1}$). f. Total inorganic dissolved carbon in µmol kg$^{-1}$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Elevated CO$_2$</th>
<th>Ambient</th>
<th>$F_{1,4}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>Mean</td>
</tr>
<tr>
<td>$p$CO$_2^{a}$</td>
<td>566.1 ± 07.2</td>
<td>625.2 ± 155.7</td>
<td>454.4 ± 151.2</td>
<td>548.6 ± 86.7</td>
</tr>
<tr>
<td>$p$H</td>
<td>7.88 ± 0.08</td>
<td>7.85 ± 0.11</td>
<td>7.98 ± 0.12</td>
<td>7.90 ± 0.07</td>
</tr>
<tr>
<td>Temperature$^{b}$</td>
<td>9.70 ± 0.66</td>
<td>9.67 ± 0.65</td>
<td>9.65 ± 0.64</td>
<td>9.67 ± 0.03</td>
</tr>
<tr>
<td>Salinity$^{c}$</td>
<td>31.44 ± 0.08</td>
<td>31.44 ± 0.07</td>
<td>31.48 ± 0.07</td>
<td>31.45 ± 0.07</td>
</tr>
<tr>
<td>Nitrate$^{d}$</td>
<td>6.23 ± 4.67</td>
<td>5.26 ± 4.89</td>
<td>7.14 ± 5.50</td>
<td>6.21 ± 0.94</td>
</tr>
<tr>
<td>Phosphate$^{d}$</td>
<td>0.46 ± 0.25</td>
<td>0.45 ± 0.31</td>
<td>0.52 ± 0.35</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>PO Nitrogen$^{e}$</td>
<td>101 ± 37.6</td>
<td>83 ± 35.4</td>
<td>71 ± 36.8</td>
<td>85 ± 15.1</td>
</tr>
<tr>
<td>PO Carbon$^{f}$</td>
<td>628 ± 208.2</td>
<td>532 ± 196.6</td>
<td>498 ± 183.7</td>
<td>552.7 ± 67.4</td>
</tr>
<tr>
<td>TID Carbon$^{f}$</td>
<td>2092.4 ± 27.1</td>
<td>2103.0 ± 40.9</td>
<td>2052.7 ± 46.6</td>
<td>2082.7 ± 28.5</td>
</tr>
</tbody>
</table>
Figure S3.7.2: Changes in mean (A) pCO$_2$ concentration, (B) pH, and (C) bacterial abundance in mesocosms under elevated CO$_2$ and ambient conditions. Error bars represent the standard deviation of the mean ($n = 3$). Figure adapted from data first presented in Hopkins et al. (2010).
Figure S3.7.3: Changes in bacterial community structure in elevated CO$_2$ and ambient mesocosms visualised using rank-abundance plots over time (days). Given are replicate plots and the mean slope values plus the standard deviation of the mean ($n = 3$) for each time point within each treatment. All regression coefficients were significant ($P < 0.05$).
Chapter 4: The Response of Marine Picoplankton to Ocean Acidification

Lindsay K. Newbold$^{1,2}$†, Anna E. Oliver$^1$†, Tim Booth$^1$, Bela Tiwari$^1$, Todd DeSantis$^3$, Michael Maguire$^2$, Gary Andersen$^3$, Christopher J. van der Gast$^1$ and Andrew S. Whiteley$^1$*

1 Centre for Ecology and Hydrology, Wallingford, Benson Lane, Crowmarsh Gifford, Wallingford, OX10 8BB, U.K

2 Civil Engineering and Geosciences, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, U.K

3 Earth Sciences Division, Lawrence Berkeley National Laboratory, Cyclotron Lane, Berkley, CA 94720, USA.

*For correspondence E-mail aswhi@ceh.ac.uk;

† Contributed equally to this work.

Running title: Response of Picoplankton to Ocean Acidification

Published in Environmental Microbiology (2012) Volume 14, pages 2293-2307, © 2012 Society for Applied Microbiology and Blackwell Publishing Ltd. Edits in format, references and the term ‘High’ CO$_2$ replaced with elevated have been made in keeping with the thesis structure however all other wording remains consistent with the accepted publication. Figures have been placed within the text and online supplementary data included with the original publication listed as supplementary figures S4.7.1 - 4.7.4. The role of individual authors has been outlined on page 207.

Keywords: Picoplankton, Ocean Acidification, Community Structure, Mesocosm.
4.1 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 an ‘elevated 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.
4.2 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 CO₂, 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, CO₂ released into the atmosphere dissolves in seawater and reacts to form carbonic acid (H₂CO₃), the dissociation of which forms hydrogen and bicarbonate ions (H⁺ and HCO₃⁻). 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 CO₂ (Sabine et al., 2004). A decrease in seawater pH and carbonate saturation is set to continue as long as excess CO₂ 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 CO₂ 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 CO₂ 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 CO₂ by increasing both cell volume and primary production (Iglesias-Rodriguez et al., 2008) and the cyanobacteria Trichodesmium responded by increasing CO₂ fixation by 15-128% and N₂ 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 CO₂ 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 rbcL (ribulose bisphosphate carboxylase/oxygenase) sequences were significantly higher in elevated CO₂ mesocosms, whereas numbers of Bathycoccus-like rbcL 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 CO₂ 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 CO₂ 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 and colleagues (2008) found in a high CO₂ 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 $\mu$m 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.
4.3 Results and Discussion

4.3.1 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, CO₂ 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 CO₂ levels were significantly higher (ANOVA: \( r^2 = 0.99; F_{1,34} = 145.1; P < 0.0001 \)) in the elevated CO₂ 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 CO₂ into the mesocosms induced a change in pH from ~8.1 to ~7.8 (figure 4.1). Dissolved nitrate and phosphate were both utilised during the phytoplankton bloom which in turn caused an increase in pH in both the elevated CO₂ 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 CO₂ 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.

4.3.2 Bacterial abundance and acidification

In general, averaging all abundances for each mesocosm over the experiment indicated both experimental (5.11 x 10⁶ ± 2.75 x 10⁶ cells per millilitre) and control mesocosms (6.13 x 10⁶ ± 2.31 x 10⁶ cells per millilitre), 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 (figure 4.2). 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).
Figure 4.1: Mean daily nutrient and pH values for elevated ‘High’ CO₂ 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 and colleagues (2010).
total cell count increases, which peaked on day 6 (~4 x 10^6 and ~0.9 x 10^6 cells per millilitre, for HNA and LNA respectively). Subsequently, both HNA and LNA bacteria exhibited a decrease in number between days 7-10 (figure 4.2).

**Figure 4.2:** Mean daily FACS counts for elevated CO\textsubscript{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.
corresponding to the initiation of the phytoplankton bloom.

During the second (post-bloom, nutrient deplete) phase total bacterial numbers rose rapidly under both regimes (figure 4.2), peaking at day 14 for elevated CO$_2$ (~1 x 10$^7$ cells per millilitre) and day 15 for ambient (~1 x 10$^7$ cells per millilitre). In the elevated 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 x 10$^6$). Ambient cell counts remained comparatively high ~8.1 x 10$^6$. The pattern for total bacterial abundance was mirrored by both the HNA and LNA bacterial operational groupings (figure 4.2). 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 (figure 4.2) from 2.5 x 10$^3$ cells per millilitre (day 1) to 2.5 x 10$^4$ cells per millilitre under elevated CO$_2$ (day 16) and 3.5 x 10$^4$ cells per millilitre 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.

### 4.3.3 Eukaryote abundance and acidification

The mean cell abundances of key eukaryote groups were compared between treatments (figure 4.2), with the exception of the coccolithophores there were no significant differences between treatments (supplementary figure S4.7.1). Despite high variance in cell counts, the mean coccolithophore cell abundances
were significantly lower (ANOVA: $F_{1,34} = 6.15; P = 0.018$) under elevated CO$_2$ conditions ($411 \pm 337.5$ cells per millilitre) when compared to ambient conditions ($942.6 \pm 844.2$ cells per millilitre). 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 and colleagues (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 and colleagues 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 (figure 4.2). Small picoeukaryotes established an initial bloom faster under elevated CO$_2$ conditions when compared to ambient pH conditions. In the elevated CO$_2$ treatment small picoeukaryotes achieved a twofold increase in cell concentration by day 6 ($4.6 \times 10^4$ cells per millilitre) followed by a considerable reduction to $1.2 \times 10^4$ cells per millilitre (day 10). The small picoeukaryote bloom in the ambient treatment took longer to establish but was more prolonged reaching a maximum of $5.1 \times 10^4$ cells per millilitre on day 10. In phase two of the experiment, small picoeukaryotes decreased in abundance (or remained low in the elevated 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 (figure 4.2).

An increase in abundance in elevated CO$_2$ conditions is consistent with Paulino and colleagues (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.

4.3.4 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 of 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 4.3a 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 Rhodobacterales (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 supplementary figure S7.4.2a and b.
Figure 4.3a: 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 = Bacteroidetes. For more specific taxonomic grouping refer to key.
Figure 4.3b: 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.
All major picoeukaryotic lineages typically retrieved from a coastal pelagic marine community were also represented (figure 4.3b). 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).

4.3.5 Bacterial community response to OA

The majority of T-RF fragment lengths were linked to specific sequences within our clone library (supplementary figure 4.7.2a). For simplification, the dynamics of the 6 most abundant bacterial and picoeukaryote peaks were plotted over time (figure 4.4) 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 bp, 435 bp and 436 bp), 2 to Bacteriodetes (peaks 86 bp and 88 bp) and one to the Gammaproteobacteria (peak 136 bp). No significant responses to treatment were detected in the majority of bacteria examined: Rhodobacterales 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 ($D = 0.294, P = 0.387$). Rhodobacterales 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 selected 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 Rhodobacterales 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 that 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.

4.3.6 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 bp) 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 elevated CO$_2$ mesocosms (0.90 in elevated 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 (figure 4.4).

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 elevated 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 and Wyman (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 (figure 4.4). 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 bp (GIA 222) and the Chrysophyceae organisms at peak 231 bp (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 bp) Chrysophyte 227 (peak 227 bp) and Group II Alveolate 373 (GIIA, peak 373 bp) (figure 4.4).

4.3.7 Trophic interactions

Allgaier and colleagues (2008) noted that heterotrophic bacterial dynamics were closely correlated to phytoplankton development and, hence, responded to changes in CO₂. Further, Tank and colleagues (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 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 CO₂ upon the world’s marine ecosystems.
4.4 Experimental Procedures

4.4.1 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 CO₂ experimental and 2 ambient CO₂ control mesocosms (2 m diameter, 3.5 m depth). Experimental mesocosm enclosures were filled with unfiltered native fjord water and gently sparged with CO₂ (750 µatm) for 2 days (4\textsuperscript{th}-6\textsuperscript{th} 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 6\textsuperscript{th} of May (initial concentrations: 1 µmol l\(^{-1}\) phosphate; 17 µmol l\(^{-1}\) nitrate). Blooming phytoplankton growth reduced CO₂ concentrations in the elevated CO₂ 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 initiated on the 6\textsuperscript{th} May (day 1) and filters stored at -80ºC prior to molecular analysis.

4.4.2 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).
4.4.3 *Nucleic acid extraction, PCR and T-RFLP analysis*

Total nucleic acids were extracted using the protocol outlined in Huang and colleagues (2009). Approximately 20-30ng of purified template was used per PCR. For T-RFLP analysis, a 500 bp 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 bp 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 94ºC for 2 minutes followed by 30 thermal cycles of 94ºC for 1 min, 52ºC for 1 min and 72ºC for 3 min. Near full length SSU rRNA amplification consisted of Initial pre-denaturation at 94ºC for 2 minutes followed by 30 thermal cycles of 94ºC for 1 min, 60ºC (16S) or 55ºC (18S) for 2 min and 72ºC for 3 min, all PCR reactions employed a final extension phase of 10 min at 72ºC.

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 37ºC 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 additional experimental procedures in supplementary figure S4.7.4 for full description.

4.4.4 *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, ~480 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 20\textsuperscript{th} of May 2006) from both elevated and ambient CO\textsubscript{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 (7\textsuperscript{th}, 13\textsuperscript{th}, 19\textsuperscript{th} and 22\textsuperscript{nd} May 2006) from both elevated and ambient CO\textsubscript{2} mesocosms (8 libraries, 768 in total). Clones were sequenced using vector primers M13F and R and internal primer 3F (Giribet \textit{et al.}, 1996). Sequencing services were provided by Source BioScience LifeSciences (http://www.lifesciences.sourcebioscience.com/).

\subsection*{4.4.5 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 \textit{et al.}, 1998) and assembled using Phrap (Green, 2008) with default settings. Screening for chimeras was performed using Mallard (16S) (Ashelford \textit{et al.}, 2006) and Bellerophon (18S) (Huber \textit{et al.}, 2004). Any sequences which were of short length, low quality or deemed chimeric were removed from analysis.

\subsection*{4.4.6 OTU identification}

Sequences were aligned using the NAST alignment tool (DeSantis \textit{et al.}, 2006) (16S) or SINA (SILVA INcremental Aligner) web aligner (Pruesse \textit{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 Reversible 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 \textit{et al.}, 2004; Schloss and Handelsman, 2005; Schloss and Handelsman, 2006; Schloss \textit{et al.}, 2009). MOTHUR was again used to designate representative sequences for each OTU in the combined elevated and ambient CO\textsubscript{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 (figure S4.7.3). 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).

### 4.4.7 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).

### 4.4.8 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 supplementary figure S4.7.4 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).

4.5 Acknowledgements

The authors would like to thank all of the members of the 2006 Bergen Mesocosm experiment for their invaluable help in data acquisition and advice, especially Ian Joint, Dorothee Bakker, Rob Griffiths, Isabelle Mary and Dawn Field. Prokaryotic work was funded by the Natural Environmental Research Council (NERC) grant number NE/C507937/1 as part of the post genomics and proteomics programme, and Picoeukaryotic work funded by the Centre for Ecology and Hydrology (CEH) internal science budget.

4.6 References


Ratios in Marine *Synechococcus* and *Prochlorococcus* (Cyanobacteria)*, Journal of Phycology, 43(3), pp. 485-496.


Marker Gene Sequence (MIMARKS) and Minimum Information About any (x) Sequence (MIxS) Specifications’, *Nature Biotechnology*, 29(5), pp. 415-420.


### 4.7 Supplimentary Information

<table>
<thead>
<tr>
<th>Eukaryotic Groups</th>
<th>Treatment</th>
<th>Mean</th>
<th>SD</th>
<th>$F_{1,34}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Small picoeukaryote</strong></td>
<td>Elevated</td>
<td>24068</td>
<td>13154</td>
<td>1.29</td>
<td>0.264</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>29724</td>
<td>16539</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Large Picoeukaryotes</strong></td>
<td>Elevated</td>
<td>2906</td>
<td>2188</td>
<td>4.01</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>6051</td>
<td>6293</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nanoeukaryotes</strong></td>
<td>Elevated</td>
<td>1031</td>
<td>891</td>
<td>0.73</td>
<td>0.399</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>1331</td>
<td>1197</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coccolithophores</strong></td>
<td>Elevated</td>
<td>411</td>
<td>337.5</td>
<td>6.15</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>942.6</td>
<td>844.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cryptophytes</strong></td>
<td>Elevated</td>
<td>89</td>
<td>92.6</td>
<td>2.12</td>
<td>0.155</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>150</td>
<td>151.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure S4.7.1:** Comparison of abundances from key eukaryotic group abundances using ANOVA tests. Given for each group and treatment are the mean abundance, the the standard deviation of the mean (SD), and the ANOVA tests results, including $F$-ratio and significance ($P$).
<table>
<thead>
<tr>
<th>OTU ID</th>
<th>T-RFLP cut site</th>
<th>Phylogenetic ID</th>
<th>Reference sequence</th>
<th>Elevated CO₂</th>
<th>Ambient CO₂</th>
<th>Total Number of sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>435-437</td>
<td><em>Roseobacteria LE17</em></td>
<td>160102C08</td>
<td>371</td>
<td>213</td>
<td>584</td>
</tr>
<tr>
<td>38</td>
<td>520</td>
<td>Bacteroidetes</td>
<td>160402G06</td>
<td>104</td>
<td>235</td>
<td>339</td>
</tr>
<tr>
<td>7</td>
<td>145</td>
<td><em>Candidatus Pelagiobacter</em> (SAR11)</td>
<td>160105C06</td>
<td>134</td>
<td>126</td>
<td>260</td>
</tr>
<tr>
<td>8</td>
<td>136</td>
<td>SAR86-II</td>
<td>160102H02</td>
<td>133</td>
<td>117</td>
<td>250</td>
</tr>
<tr>
<td>1</td>
<td>435-437</td>
<td>Rhodobacteriales</td>
<td>160102D06</td>
<td>57</td>
<td>67</td>
<td>124</td>
</tr>
<tr>
<td>5</td>
<td>86-88</td>
<td>Bacteroidetes</td>
<td>160102F11</td>
<td>75</td>
<td>32</td>
<td>107</td>
</tr>
<tr>
<td>4</td>
<td>488</td>
<td>SAR86-III</td>
<td>160402D04</td>
<td>32</td>
<td>31</td>
<td>63</td>
</tr>
<tr>
<td>81</td>
<td>435-437</td>
<td>Rhodobacteriales</td>
<td>160202B10</td>
<td>17</td>
<td>39</td>
<td>56</td>
</tr>
<tr>
<td>25</td>
<td>136</td>
<td>SAR86-II</td>
<td>160105H11</td>
<td>15</td>
<td>31</td>
<td>46</td>
</tr>
<tr>
<td>21</td>
<td>142</td>
<td>Gammaproteobacteria</td>
<td>160700F10</td>
<td>14</td>
<td>17</td>
<td>31</td>
</tr>
<tr>
<td>31</td>
<td>495</td>
<td>Betaproteobacteria</td>
<td>160304C10</td>
<td>19</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>86-88</td>
<td>Flavobacteria</td>
<td>160200A02</td>
<td>10</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>17</td>
<td>435-437</td>
<td><em>Roseobacteria LE17</em></td>
<td>160205F11</td>
<td>12</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>492</td>
<td>Betaproteobacteria</td>
<td>160304C09</td>
<td>15</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>92</td>
<td>440</td>
<td><em>Roseobacteria LE17</em></td>
<td>160202F03</td>
<td>12</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>59</td>
<td>492</td>
<td>Bacteroidetes</td>
<td>160904G12</td>
<td>4</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>133</td>
<td>486</td>
<td>Gammaproteobacteria</td>
<td>161003F04</td>
<td>4</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>57</td>
<td>435-437</td>
<td>Rhodobacteriales</td>
<td>160602D05</td>
<td>8</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>110</td>
<td>483</td>
<td>Chloroplast OM5</td>
<td>160703A05</td>
<td>4</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>76</td>
<td>440</td>
<td>Alphaproteobacteria</td>
<td>160603C12</td>
<td>4</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>35</td>
<td>435-437</td>
<td><em>Roseobacteria LE17</em></td>
<td>160105G12</td>
<td>8</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>231</td>
<td>435-437</td>
<td>Alphaproteobacteria</td>
<td>160805H04</td>
<td>2</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>52</td>
<td>90</td>
<td>Flavobacteria</td>
<td>160600D10</td>
<td>5</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>18</td>
<td>496</td>
<td>SAR92</td>
<td>160602B07</td>
<td>2</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>65</td>
<td>435-437</td>
<td><em>Roseobacteria LE17</em></td>
<td>160901B05</td>
<td>5</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>204</td>
<td>488</td>
<td>Gammaproteobacteria</td>
<td>160700G11</td>
<td>5</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>286</td>
<td>86-88</td>
<td>Bacteroidetes</td>
<td>160505H10</td>
<td>6</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>15</td>
<td>486</td>
<td>SAR92</td>
<td>160803D06</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>96</td>
<td>435-437</td>
<td>Rhodobacteriales</td>
<td>160205G05</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>102</td>
<td>435-437</td>
<td>Rhodobacteriales</td>
<td>160805G12</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>215</td>
<td>145</td>
<td><em>Candidatus Pelagiobacter</em> (SAR11)</td>
<td>160803B09</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

**Figure S4.7.2a:** 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>
<thead>
<tr>
<th>OTU ID</th>
<th>T-RFLP Cut Site</th>
<th>Phylogenetic ID</th>
<th>Reference sequence</th>
<th>Elevated CO₂</th>
<th>Ambient CO₂</th>
<th>Total Number of sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>265</td>
<td>Bathycoccus</td>
<td>1801A06</td>
<td>80</td>
<td>64</td>
<td>144</td>
</tr>
<tr>
<td>2</td>
<td>265</td>
<td>Micromonas</td>
<td>1804A07</td>
<td>63</td>
<td>12</td>
<td>75</td>
</tr>
<tr>
<td>8</td>
<td>222</td>
<td>Novel Alveolate Group I</td>
<td>1801C02</td>
<td>14</td>
<td>41</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>222</td>
<td>Novel Alveolate Group I</td>
<td>1801A08</td>
<td>16</td>
<td>33</td>
<td>49</td>
</tr>
<tr>
<td>13</td>
<td>221</td>
<td>Chrysophyceae</td>
<td>1804B06</td>
<td>20</td>
<td>22</td>
<td>42</td>
</tr>
<tr>
<td>30</td>
<td>222</td>
<td>Novel Alveolate Group I</td>
<td>1804C03</td>
<td>10</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>15</td>
<td>227</td>
<td>Chrysophyceae</td>
<td>1802A11</td>
<td>17</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>45</td>
<td>373</td>
<td>Novel Alveolate Group II</td>
<td>1815B03</td>
<td>3</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>1</td>
<td>222</td>
<td>Novel Alveolate Group I</td>
<td>1801A01</td>
<td>4</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>27</td>
<td>370</td>
<td>Novel Stramenopiles Group 2</td>
<td>1802G08</td>
<td>9</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>14</td>
<td>222</td>
<td>Chrysophyceae</td>
<td>1806D12</td>
<td>2</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>46</td>
<td>373</td>
<td>Novel Alveolate Group II</td>
<td>1815C01</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>599</td>
<td>Novel Stramenopiles Group 12</td>
<td>1802C04</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>26</td>
<td>290</td>
<td>Novel Stramenopiles Group 12</td>
<td>1802G05</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>21</td>
<td>227</td>
<td>Chrysophyceae</td>
<td>1804A05</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>39</td>
<td>221</td>
<td>Trebouxioiphyceae</td>
<td>1807B08</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>364</td>
<td>Ostreococcus</td>
<td>1815D05</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>227</td>
<td>Chrysophyceae</td>
<td>1802B08</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>44</td>
<td>132</td>
<td>Chrysophyceae</td>
<td>1815A02</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>275</td>
<td>Fungi</td>
<td>1802F03</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>56</td>
<td>265</td>
<td>Dictyochophyceae</td>
<td>1816F05</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>18</td>
<td>380</td>
<td>Rhizaria</td>
<td>1804C10</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>#</td>
<td>ID</td>
<td>Species</td>
<td>Code</td>
<td>M/F</td>
<td>K</td>
<td>L</td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>---------------------------------------------</td>
<td>----------</td>
<td>-----</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>3</td>
<td>376</td>
<td>Novel Alveolate group II</td>
<td>1801A05</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>383</td>
<td>MAST 7 (Marine Novel Stramenopiles Group 7)</td>
<td>1801F10</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>53</td>
<td>376</td>
<td>Ciliophora</td>
<td>1815H04</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>228</td>
<td>Chrysophyceae</td>
<td>1804H06</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>33</td>
<td>275</td>
<td>Ascomycota</td>
<td>1804D06</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>265</td>
<td>Micromonas</td>
<td>1801C01</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>57</td>
<td>350</td>
<td>Ciliophora</td>
<td>1816G03</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>232</td>
<td>Chrysophyceae</td>
<td>1807D03</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>42</td>
<td>231</td>
<td>Ciliophora</td>
<td>1809B06</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>227</td>
<td>Chrysophyceae</td>
<td>1815G07</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>376</td>
<td>Chrysochromulina</td>
<td>1804C04</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>29</td>
<td>285</td>
<td>Rhizaria</td>
<td>1804A10</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>55</td>
<td>275</td>
<td>Ascomycota</td>
<td>1816C11</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>275</td>
<td>Ascomycota</td>
<td>1816G09</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>119</td>
<td>Novel Alveolate Group I</td>
<td>1815F07</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>265</td>
<td>Micromonas</td>
<td>1801F12</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>43</td>
<td>233</td>
<td>MAST 7 (Marine Novel Stramenopiles Group 7)</td>
<td>1809G01</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>279</td>
<td>Imantonia</td>
<td>1806A03</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>223</td>
<td>Metazoa (Echinodermata)</td>
<td>1801B10</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>41</td>
<td>279</td>
<td>Dictyochophyceae</td>
<td>1807E08</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>274</td>
<td>Dictyochophyceae</td>
<td>1815D01</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>213</td>
<td>Cryptomonadales</td>
<td>1816B02</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>365</td>
<td>Ciliophora</td>
<td>1801G01</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>244</td>
<td>Ciliophora</td>
<td>1802B01</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>370</td>
<td>Ciliophora</td>
<td>1804G10</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>38</td>
<td>352</td>
<td>Ciliophora</td>
<td>1806H01</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>376</td>
<td>Ciliophora</td>
<td>1815C12</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>227</td>
<td>Chrysophyceae</td>
<td>1801E06</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure S4.7.2b: 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.
<table>
<thead>
<tr>
<th>Bacterial reference sequences</th>
<th>Picoeukaryote reference sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>AACY01089405 X90641 EF661583</td>
<td>AB193568 AF525860 EF172974 AF109326 AJ514867 DQ244028</td>
</tr>
<tr>
<td>AF359531 DQ372840 AY167339</td>
<td>AY626995 AF525865 EF526977 AF174376 AJ867028 DQ303924</td>
</tr>
<tr>
<td>AF354611 DQ372845 M96746</td>
<td>L81939 AF525868 EF527002 AB183583 AJ867029 DQ310276</td>
</tr>
<tr>
<td>AF353208 DQ372849 DQ167249</td>
<td>AY339145 AF525871 EF527020 AB183605 AM050344 DQ310333</td>
</tr>
<tr>
<td>AF353226 DQ372850 AY744399</td>
<td>AY339149 AF525876 EF527104 AB183618 AM050345 DQ367046</td>
</tr>
<tr>
<td>AF354612 DQ372852 U20797</td>
<td>DQ060805 AF525879 EF527105 AB275040 AY033487 DQ367048</td>
</tr>
<tr>
<td>AY548988 U65908</td>
<td>AY339150 AF525880 EF527106 AB275055 AM114819 DQ293858</td>
</tr>
<tr>
<td>CP000084 AM279169 AF001653</td>
<td>AY339151 AF525881 EF527106 AB275055 AM231737 DQ675152</td>
</tr>
<tr>
<td>DQ234199 AM279197 AF001650</td>
<td>AY339152 AF525882 EF527106 AB290575 AM412525 DQ675156</td>
</tr>
<tr>
<td>AY458647 AM279200 EF182722</td>
<td>Q629387 AY129048 EF527126 AB275058 AM231737 DQ675156</td>
</tr>
<tr>
<td>AF268217 AM279204 AY936189</td>
<td>DQ242509 AY129061 EU247836 AB425943 AY665020 DQ834370</td>
</tr>
<tr>
<td>AF279106 AM279179 CP000435</td>
<td>DQ504335 AY129063 EU304548 AF123927 AY665021 DQ977726</td>
</tr>
<tr>
<td>AF268236 AM279161 M34115</td>
<td>EU162635 AY129064 U14387 AY143943 AY665044 DQ980478</td>
</tr>
<tr>
<td>DQ015813 AF241654 AF245632</td>
<td>DQ278833 AY129067 U73222 AY184167 AY665057 DQ986131</td>
</tr>
<tr>
<td>DQ015817 AF173974</td>
<td>DQ642266 AY129068 U73230 AY257316 AY665094 EFO23353</td>
</tr>
<tr>
<td>DQ015775 AF173975</td>
<td>AY646226 DQ369015 X71140 AY290083 AY665101 EFO23502</td>
</tr>
<tr>
<td>AY794084 NR_027580</td>
<td>AY648222 DQ369016 AF290540 AY363186 AY821968 EFO23594</td>
</tr>
<tr>
<td>AY697879 AY654757</td>
<td>EF532930 AY143572 AY246274 AY372575 AY919815 EFO23894</td>
</tr>
<tr>
<td>AY794144 U70693</td>
<td>AAC020214703 AY143573 AM491021 AY372575 AY919816 EFO43285</td>
</tr>
<tr>
<td>AY794064 U70678</td>
<td>AAX00102636 AY381207 AY472554 AY411268 AY954993 EFO65124</td>
</tr>
<tr>
<td>AATR01000002 U70679</td>
<td>AB058312 AY425313 AY425553 AY707277 AY965868 EFO65125</td>
</tr>
<tr>
<td>AY102027 U70704</td>
<td>AB058311 AY425314 AY472553 AY707284 CR954212 EFO65134</td>
</tr>
<tr>
<td>AY102028 U70715</td>
<td>AB080302 AY425319 AF695161 AY131691 DQ116021 EFO72839</td>
</tr>
<tr>
<td>AY771771 U70696</td>
<td>AB058360 AY590482 AY829996 AY251929 DQ116022 EFO72972</td>
</tr>
<tr>
<td>EF016464 U70717</td>
<td>AB183613 AY626163 AJ402354 AY251930 DQ121425 EFO72972</td>
</tr>
<tr>
<td>AY772092 DQ489286</td>
<td>AY06264 AY642694 AY129036 AY329635 DQ145112 EFO72972</td>
</tr>
<tr>
<td>AB193724 AF353235</td>
<td>AY109323 AY665019 AY525856 AJ495816 DQ243996 EFO72972</td>
</tr>
</tbody>
</table>

Figure S4.7.3: Accession numbers for additional sequences downloaded from GenBank, used in 16S and 18S phylogenetic analysis.
Figure S4.7.4: Additional 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°C 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 CO₂ 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 elevated and ambient CO2 mesocosms. Clones were sequenced using vector primers M13F and R and internal primer 3F (Giribet et al., 1996). Sequencing services were provided by Source BioScience LifeSciences (http://www.lifesciences.sourcebioscience.com/).

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).

References


Chapter 5. Active Bicarbonate and Glucose Picoplankton Communities under Elevated CO$_2$

Lindsay K. Newbold$^1$, 2, Anna E. Oliver $^1$, Christopher J. van der Gast$^1$ and Andrew S. Whiteley $^{1,3}$

$^1$Centre for Ecology and Hydrology, Benson lane, Crowmarsh Gifford, Wallingford, OX10 8BB. UK.

$^2$Civil Engineering and Geosciences, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK.

$^3$School of Earth and Environment, University of Western Australia, Crawley, Australia.

Corresponding author: Andrew S. Whiteley

As yet this chapter is unsubmitted, in keeping with the other chapters it has been written in the style of *Environmental Microbiology Reports*. Figures have been placed within the text and online supplementary data included listed as supplementary figures S5.7.1 - 5.7.4. The role of individual authors has been outlined on page 207.

Keywords: Picoplankton, Ocean Acidification, RNA Stable Isotope Probing, Anthropogenic CO$_2$, Mesocosm.
5.1 Summary

The link between industrialisation and increases in atmospheric CO₂ is irrefutable, and consequently there is significant interest in how related changes in pH will affect the oceans' microbiota and core biogeochemical processes. A growing body of evidence suggests that while microbial abundance will be minimally effected, there may be specific functional responses to elevated CO₂. This study investigates the affect of elevated CO₂ upon carbon assimilation in heterotrophic and phototrophic picoplankton using RNA-SIP. Whilst many taxa appeared unaffected - being significantly associated with temporal dynamics - specific carbon assimilation responses within dominant picoplankton taxa were observed. This indicates that such populations will functionally respond to predicted future CO₂ concentration, yet this response will likely be constrained by nutrient availability.
5.2 Introduction

Evidence for the link between industrialisation and increases in atmospheric CO₂ is undeniable (Petit et al., 1999; Siegenthaler et al., 2005; Lüthi et al., 2008), as is the evidence that excess CO₂ will lead to decreases in oceanic pH a process commonly known as ocean acidification (OA) (Caldeira and Wickett, 2003; Cicerone et al., 2004; Feely et al., 2004; Orr et al., 2005). When dissolved in the oceans, atmospheric CO₂ forms a weak acid (carbonic acid), which can alter seawater chemistry. The most predominant effect is upon the oceans' carbonate chemistry. Increases in carbonic acid leads to increases in bicarbonate ions and dissolved inorganic carbon, but decreases in pH and carbonate ions, which in turn alters the saturation state of key carbonate minerals such as aragonite, calcite and magnesium calcite (Gattuso and Hansson, 2011). Changes in oceanic chemistry are likely to affect the ocean either directly through pH effects or indirectly through impacted marine ecosystems (Gehlen et al., 2011). Ocean acidification appears to influence shell composition (Gattuso et al., 1998; Riebesell et al., 2000; Shirayama and Thornton, 2005; Kuffner et al., 2008) reproductive strategy (Kurihara et al., 2004; Kurihara and Shirayama, 2004; Kurihara, 2008; Ross et al., 2011) and trophic organization (Harvey et al., 2013). Furthermore, there is an emerging perception that phytoplankton may experience a shift in favour of smaller non calcifying organisms, which put less effort into costly carbon concentration mechanisms (CCM’s) (Paulino et al., 2008; Newbold et al., 2012; Brussaard et al., 2013). In contrast to eukaryotes, previous studies suggest that prokaryotic community organisation appears largely unaffected and even resistant to OA (Newbold et al., 2012; Newbold et al., 2014). Recently the European project on ocean acidification (EPOCA) found free living bacterial community structure was not majorly affected by degree of ocean acidification, but by variations in productivity and nutrient availability (Roy et al., 2013; Sperling et al., 2013; Zhang et al., 2013). This makes a direct in depth study of carbon flow within such communities crucial - how will microbial community function respond to elevated CO₂?
Herein lies a problem, the majority of picoplanktonic organisms are unculturable and as such have been identified exclusively upon their molecular signature, meaning their functional roles are largely unknown. The application of stable isotope probing (SIP) - whereby a given community is incubated with a substrate containing a naturally rare stable isotope such as $^{13}\text{C}$, causing metabolically active community members to integrate this ‘heavy labelled’ compound into their nucleic acids which can then be recovered by density gradient ultracentrifugation - in conjunction with community fingerprint techniques such as terminal restriction fragment length polymorphism (T-RFLP) has successfully been applied to allow the identification of metabolically active members within a given microbial community (Manefield et al., 2002a; Morris et al., 2002; Radajewski et al., 2003; Griffiths et al., 2004; Lueders et al., 2004; Rangel-Castro, 2005). Initial approaches investigated the integration of labelled substrate into DNA (DNA-SIP) (Radajewski et al., 2003). SIP was then further developed to look at direct integration into the ribosomal RNA molecule (rRNA-SIP) allowing for the identification of specific community members actively metabolizing a given substrate (Manefield et al., 2002a; Manefield et al., 2002b; Whiteley et al., 2006).

In our previous studies of a large volume mesocosm experiment we found that bacterial communities were seemingly resistant to predicted 2100 elevated CO$_2$ concentrations (~750ppm), but that photosynthetic picoeukaryotes were able to rapidly exploit additional CO$_2$ (Newbold et al., 2012; Newbold et al., 2014). In this study we utilize RNA-SIP to investigate the direct community uptake of sodium bicarbonate (by photosynthesisers) and glucose (by heterotrophs), and apply these results to investigate active carbon flow within the resident prokaryote and eukaryote communities.
5.3 Results and Discussion

5.3.1 Baseline community analysis

The 2006 Bergen Mesocosm experiment (BME) community diversity has been outlined in our previous studies and here has been used as a baseline for non-incubated samples and as a resource for community member identification (Newbold et al., 2012; Newbold et al., 2014). The 2006 BME was split into phases 1 (nutrient replete) and 2 (nutrient deplete) based upon nutrient availability (figure 5.1 reproduced from Newbold et al., 2012), we continue to use this division in the current study. Unfiltered water from these baseline mesocosm bags was used to fill experimental microcosms containing either $^{12}$C-control or $^{13}$C-labelled glucose or sodium bicarbonate (see Experimental procedures for details). After 48 hours the microbial community was collected.

![Figure 5.1: Mean daily nutrient and pH values for elevated '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 denotes the separation between phases one and two. Nutrient data collected by I. Joint, pH data first presented in Hopkins and colleagues (2010). Figure adapted from Newbold et al. 2012](image-url)
by filtration and total RNA extracted. Density gradient ultracentrifugation was used to recover ‘heavy’ $^{13}$C labelled and ‘light’ $^{12}$C unlabelled RNA. The heaviest or most significantly labelled RNA molecules were used to investigate microbial community activity, under the elevated CO$_2$ experimental mesocosm regime imposed.

Refractive index (RI) of blank gradients demonstrated a steady decrease from the heavy to light fractions ($r^2 = 0.9221$). Further, differences in both RNA concentration and RT-PCR product formation were observed from the heavy to light fractions, in both experimental $^{13}$C and control $^{12}$C incubations, $^{13}$C incubations consistently showed a higher RNA and PCR product concentration in heavy fractions which peaked in fraction 5. We assume that the observed changes between heavy and light fractions were due to differing levels of heavy or light substrate assimilation into ribosomal RNA (rRNA), which would be consistent with similar studies (Manefield et al., 2002a; Frias-Lopez et al., 2009). These results suggest that assimilation of $^{13}$C into RNA has occurred within our incubations and are therefore a suitable descriptor of active communities.

### 5.3.2 Microcosm and mesocosm community composition

In our previous study we were able to extensively sample small subunit ribosomal RNA (SSU rRNA) phylotypes from both prokaryote and picoeukaryote communities, using large full length sequence clone libraries (2871, 16S SSU rRNA sequences and 570, 18S SSU rRNA sequences) in conjunction with T-RFLP analysis (Newbold et al., 2012). In this study we utilise this information to identify and compare it to microcosm RNA-SIP incubation community T-RFLP profiles for prokaryotes and eukaryotes. Figure 5.2 and supplementary figure S5.7.1 presents a summary of this information. Bacterial microcosm T-RFLP profiles were similar to those of the mesocosms, with no new terminal restriction fragments (TRF’s) observed. The 6 dominant bacterial TRF’s identified in our previous study remain highly prevalent in this study. It should be noted however that some lower abundance fragments do appear to show preference to bicarbonate or glucose based upon a system of ranking. For
example, TRF peak 483 correlates to Chloroplast/Cyanobacterial full length 16S rRNA sequences, so increased prevalence in SIP incubations targeting photosynthesisers (sodium bicarbonate) is unsurprising (rank 6 in bicarbonate, 53 in glucose and 13 in the mesocosm samples). In contrast TRF peak 200 was in low abundance in both the bicarbonate and mesocosm incubations (ranks 33 and 47) but the most prevalent TRF in the glucose incubations. Sequences correlating to TRF peak 200 were not detected in our extensive clone library so taxonomic affiliation wasn’t possible.

Unlike bacterial communities there were apparent differences between the overall mesocosm and microcosm 18S rRNA gene T-RFLP profiles including a greater 18S diversity (double that of the mesocosm study) and changes in T-RF relative abundance. Although it may represent assimilation of labelled substrate, the difference likely lies in the methodological approach. In our previous study all samples employed a pre-filtration stage in order to remove larger community members and focus upon pico-sized organisms. Due to time constraints we were unable to perform this on SIP microcosm samples. Consequently, the total eukaryote community (including nano and micro plankton), not just picoeukaryotes were studied and the 18S rRNA clone library from our previous study would not be fully representative.

In order to investigate if any significant community level differences were observed between SIP incubations, T-RFLP profiles from fraction 5 of prokaryote and eukaryote communities were tested using canonical correspondence analysis (CCA) against a range of variables: pH, incubation type (ambient or elevated CO₂), substrate (glucose or bicarbonate), time (day in mesocosm experiment, samples corresponded to days 4, 10 and 17) and isotope label (¹²C or ¹³C) (table 5.1 and supplementary figure S5.7.2). None of the variables were found to be significantly collinear (ECOM II software package, Pisces Conservation ltd), however pH and incubation type showed some level of collinarity ($r^2>0.74$ and VIF>4.3 in all analyses). As pH and incubation type were closely linked, pH was removed from analysis. Further, forward selection indicated the most important variables in prokaryotic
5.2a

Proportion of community profile in mesocosm incubations

- **High CO₂**: Bacteriodes (86) and Gammaproteobacteria (136)
- **Ambient CO₂**: Bacteriodes (88) and Gammaproteobacteria (136)
- **Elevated CO₂**: SAR11 (145) and Rhodobacteriales (435)
- **Ambient CO₂**: SAR11 (145) and Rhodobacteriales (436)

**13C - 14C proportional contribution to community profile**

**Time (day)**
Figure 5.2: Changes in TRFLP abundance during mesocosm and stable isotope microcosm incubations, for dominant prokaryotes (5.2a) and picoeukaryotes (5.2b) over time. Changes for individuals within in mesocosm incubations (primary vertical axis) assessed by relative contribution to total T-RFLP profile in elevated (closed circles) and ambient (open circles). Relative uptake of $^{13}$C substrate assessed by peaks relative contribution to total $^{12}$C T-RFLP profile subtracted from relative contribution to total $^{13}$C T-RFLP profile. Positive values for T-RF peaks in bicarbonate (dark grey bars) and glucose (light grey bars) incubations had higher proportional values in $^{13}$C community profile compared to equivalent $^{12}$C incubation (fraction 5) and were therefore assumed to be actively metabolising substrate.
community profile were time, substrate, incubation type and isotope (all \(P=0.01\)) (table 5.1). Together these variables accounted for 81.91% of total community variance. These results indicated that prokaryote communities were most affected by time followed by substrate, \(\text{CO}_2\) treatment and isotope. This finding is in line with others where prokaryote community composition appeared to be derived from nutrient availability and larger community change over time, not acidification (Roy et al., 2013; Sperling et al., 2013; Zhang et al., 2013). Further, the preferences indicated by shifts in TRF abundance would support the view that nutrient availability during blooms creates a succession of separate niches which individual bacterial groups are able to exploit (Teeling et al., 2012).

CCA analysis of eukaryotic communities indicated that 82.56% variance could be explained by the environmental variables; time, substrate and isotope, (\(P=0.01\) for all). Although time is likely the dominant explanatory factor in this study, incubation type although significant (\(P=0.027\)) explained less variation than the other variables. This is perhaps unexpected when you consider the evidence presented in both our previous study, and those of the EPOCA campaign. In these elevated \(\text{CO}_2\) appeared to favour smaller members of the eukaryote community (Newbold et al., 2012; Brussaard et al., 2013; Schulz et al., 2013). The difference again can be explained by the lack of pre-filtration and therefore, in the presence of larger organisms with multiple copies of the 18S rRNA gene. Such organisms would form a higher proportion of 18S rRNA template and consequently be preferentially amplified over the picoeukaryotic community members.

### 5.3.3 Dominant bacterial community response to elevated \(\text{CO}_2\)

CCA analysis of the complete dataset suggested that substrate type was a key explanatory variable in this study, and therefore individuals abundance in glucose and bicarbonate incubations were likely different. Since one of the aims of this study was to investigate OA and picoplankton response to glucose and bicarbonate assimilation, the direct effect of elevated \(\text{CO}_2\) upon the six dominant prokaryote and picoeukaryote TRF’s identified in our previous study
were investigated in depth (although it is acknowledged that other community members may also contribute to these peaks). Dominant picoplankton TRF peaks were identified within SIP microcosm communities (figure 5.2a and b) and compared to other T-RF’s of known trophic function and experimental variables using canonical correspondence analysis, CCA (figure 5.3). The top three significant explanatory variables were time, substrate and isotope in each substrate incubation type (see table 5.1). Figure 5.2a suggests that dominant prokaryotes were assimilating carbon from both bicarbonate and glucose, as represented by those samples that had higher proportional values of $^{13}$C compared to equivalent $^{12}$C samples (fraction 5). Rhodobacterales (T-RF peaks 435 and 436) appear to be actively assimilating $^{13}$C to a higher degree in elevated CO$_2$ SIP incubations, and $^{13}$C glucose appears to be more readily accessed than bicarbonate. This finding is further evidenced by the association of TRF peaks 435 and 436 to elevated CO$_2$ in CCA analysis of glucose incubation (figure 5.3). Rhodobacterales are often considered ecological generalists, and as such are highly diverse and adaptable occupying a wide range of ecological niches (Moran et al., 2004; Polz et al., 2006; Moran et al., 2007; Newton et al., 2010). Wang and colleagues (1993) presented evidence that two Rhodobacterales strains - without Ribulose bisphosphate carboxylase-oxygenase (RubisCO) - were able to grow on media where thiosulphate or sulphide acted as electron donors, and CO$_2$ was the only available carbon source. Additionally, this group contains some of the first described aerobic anoxygenic phototrophs (AAnP’s) whereby they are able to fix CO$_2$ without the production of oxygen (Moran and Miller, 2007; Swingley et al., 2007). Finally, at least one Rhodobacterales species *Roseobacter denitrificans* has been shown to process glucose- through the Entner-Doudoroff pathway (Tang et al., 2009). Therefore, a positive functional response within this group is not entirely surprising.

SAR11 TRF peak 145 $^{13}$C assimilation changed temporally in both glucose and bicarbonate incubations, showing higher assimilation during phase 1 (SIP 1, day 4) in elevated CO$_2$, and assimilating more carbon during phase 2 (SIP3, day 17) in ambient incubations (figure 5a). A recent DNA-SIP study found that bicarbonate assimilation was widespread in marine bacterial communities which
included Rhodobacterales and SAR11 signatures (DeLorenzo et al., 2012). However, CCA analysis indicated SAR11 showed minimal associations with experimental variables, instead associating most strongly with time. Bacteroidetes TRF peaks 86 and 88 showed higher assimilation of $^{13}$C in ambient microcosm incubations (figure 5.2a). However, like SAR11 CCA analysis indicated a closer association with experimental day in both glucose and bicarbonate incubations (figure 5.3). This was mirrored in the Gammaproteobacteria (TRF 136) (figure 5.2a and 5.3), where again time was the greatest explanatory factor.

Although these findings should be taken with caution as actual $^{13}$C integration into RNA was not measured (i.e. by isotope ratio mass spectrometry, IRMS), both the T-RFLP and CCA analysis suggested that although community abundance does not significantly alter (Newbold et al., 2012), a populations function (as in the Rhodobacterales) may respond to elevated CO$_{2}$.

5.3.4 Dominant picoeukaryotic community response to elevated CO$_{2}$

Like the bacterial populations there are observable differences between $^{13}$C and $^{12}$C microcosm incubations, in both elevated and ambient CO$_{2}$ incubations. Figure 5.2b suggests that chrysophycean TRF peak’s 221, 227 and 231 actively assimilate $^{13}$C bicarbonate and therefore likely act autotrophically, a position further supported by the CCA analysis (figure 5.3). This is not surprising considering one of the classifying features of chrysophyceae is the presence of chloroplasts (Adl et al., 2012). Interestingly, Mamiellales peak 265 shows a mixed response to elevated CO$_{2}$ within this analysis. During Phase 1 of the experiment (SIP1, day 4) there is evidence for a stimulatory effect upon photosynthesis in elevated CO$_{2}$ (figure 5.2b). This supports, our previous findings and those of others which suggested that Mamiellales organisms increased abundance in elevated CO$_{2}$, during the nutrient replete phase 1 of this experiment (Meakin and Wyman, 2011; Newbold et al., 2012). Increased relative abundance in the day 4 elevated CO$_{2}$ bicarbonate, would support these findings and indicate that they are assimilating autotrophically derived $^{13}$C during this phase of the experiment. However, as the experiment progressed
active glucose assimilation was observed in both the elevated (day 10) and in the ambient $^{13}$C glucose incubations (days 10 and 17). Mamiellales TRF peak 265 shows a close association to time in bicarbonate incubation CCA analysis.

**Figure 5.3:** Ordination plot of canonical correspondence analysis (CCA) in glucose and bicarbonate, prokaryote and eukaryote T-RFLP profiles. The ordination is obtained through CCA analysis of percentage contribution of individual TRF’s combined with explanatory environmental variables. Only time, isotope and substrate have been included as significant determined by forward selection through permutation tests in ECOM II, software package. Dominant picoplankton TRF’s (closed circles) identified in Newbold *et al.* (2012) are shown in comparison to other community members with known trophic preference (see supplementary figure S4.7.2 for identifications). Percentage values on axes represent percentage of total variation explained.
Table 5.1: Canonical Correspondence Analyses (CCA) for determination of percentage variation in prokaryotic and eukaryotic communities. Values represent CCA analysis for TRF’s from complete dataset (Bicarbonate and Glucose incubations combined), and separate communities from Bicarbonate and Glucose incubations. Forward selection of the most significant variables to include in analysis (represented by*) - $^a$time (day in mesocosm experiment), $^b$substrate (glucose or bicarbonate), $^c$incubation type (ambient or elevated CO$_2$), $^d$pH and $^e$Isotope label ($^{12}$C or $^{13}$C) - was performed in ECOMM II software package based upon 999 iterations. Note although pH was significantly associated with data in some conditions it was removed from analyses due to a high level of colinearity with incubation type.
However, in glucose it is highly associated with $^{13}$C, separating from the chrysophyceae peaks and associating more closely to the heterotrophic fungal TRF peak 275 (figure 5.3). The ability to access both $^{13}$C bicarbonate and glucose may be indicative of an ability of Mamiellales to act mixotrophically. Others have suggested that at least one Mamiellales species, *Micromonas* is able to act in this way (Gonzalez *et al.*, 1993; Sanders and Gast, 2012). It is important to note that the T-RFLP is taxonomically inexact and doesn’t enable the separation of individual species within the Mamiellales, therefore a more quantitative approach (i.e qPCR) would be desirable. Finally figures 5.2b and 5.3 indicate little carbon assimilation of either substrate was observed in the alveolate TRF peaks 222 and 373 during SIP 1 or 2 (phase 1, days 4 and 10), however there was some evidence of $^{13}$C bicarbonate assimilation in TRF peak 373 during the final stage of the experiment (SIP3, day 17), which would correspond to observed increases in abundance during the mesocosm experiment. This is further reflected by an association with autotrophic chrysophyceae peaks in bicarbonate CCA analysis (figure 5.3).

### 5.3.5 Conclusion

These findings corroborate our previous studies where elevated CO$_2$ did not significantly affect community abundance profile. Further, this experiment would suggest that overall community function (in terms of carbon acquisition) did not majorly alter with the changes imposed, but responded more to temporal succession within the blooming community. This can be explained by changes in nutrient availability during blooms creating a succession of separate niches which individual groups are able to exploit (Teeling *et al.*, 2012). There were some exceptions such as Rhodobacterales and Mamiellales which did respond to elevated CO$_2$ and therefore the ability of such organisms to adapt and take functional advantage of excess CO$_2$ may favour them in the future. It was possible to detect differences in response to elevated CO$_2$ in individual community contribution for dominant Rhodobacterales and Mamiellales T-RFLP peaks. If these findings hold true, then they would suggest that planktonic communities are either resistant or able to functionally respond to elevated CO$_2$ by increased photosynthesis and bacterial assimilation of released dissolved
organic carbon. This would, to some extent negate the impact of OA and would support the view of Joint and colleagues (2011) that ‘marine microbes possess the flexibility to accommodate pH change and there will be no catastrophic changes in marine biogeochemical processes that are driven by phytoplankton, bacteria and archaea’.

5.4 Experimental Procedures

5.4.1 Experimental design

The complete experimental design of the May 2006 Bergen mesocosm experiment has been outlined previously (Gilbert et al., 2008; Hopkins et al., 2010; Meakin and Wyman, 2011; Newbold et al., 2012). Here we present the data from a microcosm experiment, run in parallel to the main mesocosm study.

Experimental mesocosm enclosures were filled with unfiltered native fjord water and gently sparged with CO$_2$ (750 µatm) for 2 days (4–6$^{th}$ 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 6$^{th}$ May (initial concentrations: 1 mmol l$^{-1}$ phosphate; 17 mmol l$^{-1}$ nitrate). Blooming phytoplankton growth reduced CO$_2$ concentrations in the elevated-CO$_2$ mesocosms; therefore, mesocosm enclosures were re-acidified after sampling on the 10th day post mesocosm establishment (15/5/2006), and ambient-condition enclosures again sparged with air.

Microcosm incubations used 4L water sampled from mesocosm bags 1 (elevated CO$_2$) and 6 (ambient CO$_2$) to fill 5L Nalgene bottles containing either fully labelled $^{12}$C or $^{13}$C glucose (50mg/L) or sodium bicarbonate (0.15g/L). Microcosms were submerged in surface fjord water and incubated in situ. Following microcosm establishment pH was measured, cells were enumerated and plankton collected from 1L of microcosm water onto 0.2 µm Durapore membranes daily, for a period of 5 days. All membranes were immediately stored at -80 prior to molecular analysis. This process was repeated at 3 key
time points, corresponding to pre-phytoplankton bloom (day 4, 7th), peak phytoplankton bloom (day 10, 13th) and post phytoplankton bloom (day 17, 20th). Samples obtained 2 days after microcosm establishment were later found to show optimal isotope integration therefore only data corresponding to these days has been presented.

5.4.2 Nucleic acid extraction

Total nucleic acids were extracted following the CTAB bead beating protocol outlined by Huang and colleagues (2009). RNA-SIP template of total nucleic acid extract was treated with DNase using DNA-free™ kit, Ambion®. DNase treated RNA was quantified on Qubit® 1.0 Fluorometer with Qubit® RNA HS assay (Invitrogen) using the manufacturers protocol.

5.4.3 RNA Stable isotope probing (RNA-SIP)

RNA SIP protocol followed that outlined by Whiteley and colleagues (2007). Briefly, between 400-500ng RNA was loaded onto a caesium trifluoroacetate (CSTFA) gradient (~2.0 g/ml) and centrifuged in a TLA120.2 rotor on an Optima TLX ultracentrifuge at 64,000 rpm (150,000 x g) for 48 h at 20°C. Gradients were fractionated using peristaltic pump at a flow rate of 0.2 ml min⁻¹. RNAs were isolated from gradient fractions by precipitation with 1 volume of isopropyl alcohol and 1µl glycogen. Fractions were resuspended in 10 µl molecular grade Tris EDTA pH 7.4 and quantified on Qubit® 1.0 Fluorometer with Qubit® RNA HS assay. Prokaryote and eukaryote RNA was reverse transcribed separately using 2µl (1ng) purified RNA template, 1µl (10mM) dNTP's and 1µl (10mM) reverse primers 536R, 16S or Euk570R, 18S (Suzuki et al., 1998; Baldwin et al., 2005) and 1µl SuperScript® II Reverse transcriptase, Invitrogen, following manufacturers protocol including the recommended addition of RNase OUT™, Invitrogen. Additionally as a measure of gradient formation blank gradients were fractionated and refractive index measured at 18°C.
5.4.4 Terminal restriction fragment length polymorphism (T-RFLP)

Full experimental procedures have been described previously (Newbold et al., 2012). Briefly, a 500 b.p. region of the 16S small subunit ribosomal RNA gene (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 gene amplified using primers (6Fam) EukF and Euk570R (Baldwin et al., 2005). Amplification employed a 2 minute pre-denaturation phase at 94 °C followed by 30 cycles of 94 °C for 1 minute, 52 °C for 1 minute, and 72 °C for 3 minutes and a final extension phase of 10 minutes at 72 °C. 20 µL of gel purified PCR product was digested for 4 hours at 37 °C in a 30 µL total reaction volume using 20 units restriction enzyme Mspl (Promega, UK) and buffers. Digestion product (0.5 µL) was combined with 0.5 µL denatured LIZ600 size standard (Applied Biosystems) and 9 µL Hi-Di formamide (Applied Biosystems), and run on an Applied Biosystems 3730 DNA sequencer. The sizes of restriction fragments were calculated and binned using Genemarker™ (Softgenetics) and where possible restriction fragments crossed correlated to specific cloned sequences (see Newbold et al., 2012). Bin widths were checked and manually adjusted to encompass all detected peaks. To differentiate signal from background, a Fluorescence Unit (FU) threshold of 40 units was used for a presence/absence binary matrix. All peaks previously included in mesocosm analysis and additional peaks were manually checked for inclusion in analysis. Relative abundance measures were calculated by dividing individual peak height by total peak height spanning all valid peaks within the analysis. Resultant data was compared to recorded metadata in order to determine if any factors contributed to differences in community composition and abundance.

5.4.5 Statistical analysis

Ecological datasets can be distinguished from other datasets by uneven distribution of individuals (not all species will occur at all sites), and non-linear relationships between species distribution and environmental variables which can often be binary (presence/absence) in nature, therefore multivariate analyses such as canonical correspondence analysis (CCA) can be used to
overcome this problem (ter Braak and Verdonschot, 1995). Here we applied CCA within the in ECOM II software package (Pisces Conservation Ltd) for variable selection and XLSTAT Advance Data Analysis (ADA) module (Addinsoft) to investigate terminal restriction fragment proportional abundance in relation to binary variables; incubation type (ambient or elevated CO₂), substrate (glucose or bicarbonate), and isotope label (¹²C or ¹³C) and continuous parameters time (day in mesocosm experiment) and pH (of microcosm water).

5.5 Acknowledgements

The authors would like to thank all of the members of the 2006 Bergen Mesocosm experiment for their invaluable help in data acquisition and advice, especially Rob Griffiths, Isabelle Mary and Michael Mcguire. Prokaryotic work was funded by the Natural Environmental Research Council (NERC) grant number NE/C507937/1 as part of the post genomics and proteomics programme, and Picoeukaryotic work funded by the Centre for Ecology and Hydrology (CEH) internal science budget.

5.6 References


Newbold, L. K., Oliver, A. E., Whiteley, A. S. and van der Gast, C. J. (2014) 'Marine Bacterial Communities are Resistant to Elevated Carbon Dioxide Levels', Environmental Microbiology Reports. In press.


Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R. M., Lindsay, K., 157


Wang, X., Modak, H. and Tabita, F. (1993) 'Photolithoautotrophic Growth and Control of CO$_2$ fixation in Rhodobacter sphaeroides and Rhodospirillum rubrum


### 5.7 Supplimentary Information

#### Prokaryotes

<table>
<thead>
<tr>
<th>Total Number of TRF’s In Mesocosm</th>
<th>57</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of TRF’s In SIP Microcosms</td>
<td>57</td>
</tr>
<tr>
<td>Mesocosm (in rank order 1-10)</td>
<td>145 436 86 435 88 136 488 486 437 92</td>
</tr>
<tr>
<td>Glucose (in rank order 1-10)</td>
<td>200 522 436 88 171 440 476 448 278 86</td>
</tr>
<tr>
<td>Bicarbonate (in rank order 1-10)</td>
<td>88 436 435 145 486 483 136 86 138 482</td>
</tr>
</tbody>
</table>

#### Eukaryotes

<table>
<thead>
<tr>
<th>Total Number of TRF’s In Mesocosm</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of TRF’s In SIP Microcosms</td>
<td>110</td>
</tr>
<tr>
<td>Source incubation type</td>
<td></td>
</tr>
<tr>
<td>Mesocosm (TRF’s in rank order 1-10)</td>
<td>265 222 221 227 373 231 383 228 360 376</td>
</tr>
<tr>
<td>Glucose (TRF’s in rank order 1-10)</td>
<td>378 275 268 267 265 281 83 370 73 367</td>
</tr>
<tr>
<td>Bicarbonate (TRF’s in rank order 1-10)</td>
<td>268 279 267 380 479 169 370 179 265 220</td>
</tr>
</tbody>
</table>

**Figure S5.7.1:** Summary of T-RFLP analysis. All fraction 5 samples were reverse transcribed and T-RFLP analysis performed. Resultant T-RFLP peaks were ranked on the basis of overall total contribution to community profile, the most abundant peak given rank 1. The top 10 TRF peaks for both glucose and bicarbonate incubations, were compared to ranking in mesocosm incubation, in order to establish any changes in peak prevalence between incubation types.
**Figure S5.7.2:** Ordination plot of canonical correspondence analysis (CCA) in prokaryote and eukaryote T-RFLP profiles. The ordination is obtained through CCA analysis of percentage contribution of individual TRF’s combined with explanatory environmental variables. Only the three most important environmental variables have been included as determined by forward selection through permutation tests in ECOM II, software package. Dominant picoplankton TRF’s (closed circles) identified in Newbold *et al.* (2012) are shown in comparison to other community members with known trophic preference (see figure S4.7.2 for identifications). Percentages on axes represent the percentage of total variance explained by axis.
Chapter 6. The Mamiellales: Strategies for Nutrient Acquisition Under Elevated CO₂

Lindsay K. Newbold¹,², Andrew S. Whiteley³, Christopher J. van der Gast¹ and Anna E. Oliver¹

¹Centre for Ecology and Hydrology, Benson lane, Crowmarsh Gifford, Wallingford, OX10 8BB. UK.

²Civil Engineering and Geosciences, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK.

³School of Earth and Environment, University of Western Australia, Crawley, Australia.

Corresponding author: Anna E. Oliver

As yet this chapter is unsubmitted, in keeping with the other chapters it has been written in the style of Environmental Microbiology Reports. Figures have been placed within the text. The role of individual authors has been outlined on page 207.

Keywords: Mamiellales, Ocean Acidification, RT-qPCR, RNA Stable Isotope Probing, Anthropogenic CO₂, Mesocosm.
6.1 Summary

The link between anthropogenic derived increases in atmospheric CO$_2$ and oceanic pH is firmly established. As a result there is significant interest in how such changes will affect oceanic organisms and biogeochemical processes. A key observation of other marine CO$_2$ manipulation studies is that primary production will be enhanced, favouring small non-calcifying autotrophs, which will likely have consequences for marine carbon availability. This study uses a highly abundant picoeukaryote taxon (Mamiellales) as a model of such populations. We developed a qPCR assay in conjunction with RNA-SIP to investigate carbon assimilation (response to elevated CO$_2$) in individual Mamiellales phylotypes. Phylotype specific carbon assimilation responses within the Mamiellales were observed, indicating that Mamiellales populations - although likely to be constrained by nutrient availability - may be able to exploit future oceanic CO$_2$ concentration.
6.2 Introduction

The link between industrialisation and increases in atmospheric CO$_2$ is irrefutable (Petit et al., 1999; Siegenthaler et al., 2005; Luthi et al., 2008, IPCC., 2013), as is the evidence that excess CO$_2$ will lead to decreases in oceanic pH, a process known as ocean acidification (OA) (Caldeira and Wickett, 2003; Cicerone et al., 2004; Feely et al., 2004; Orr et al., 2005). Perhaps the most publicised effect of OA is a reduction of bioavailable carbonate ions and consequently, reduced calcification in organisms such as corals, molluscs and coccoliths (Gattuso et al., 1998; Riebesell et al., 2000; Michaelidis et al., 2005). Conversely, elevated CO$_2$ concentration has been linked to higher levels of primary production (Hein and Sand-Jensen, 1997; Schippers et al., 2004).

Planktonic microorganisms account for only 0.2% of global primary producer biomass; yet contribute the majority of the oceans' primary production, which in turn accounts for half of global primary production (Field et al., 1998). The ‘pico’ sized 0.2-2.0$\mu$m phytoplankton can be broken down into the photosynthetic prokaryotes - cyanobacterial linages such as Prochlorococcus and Synechococcus - and photosynthetic unicellular eukaryotes. Although contributing numerically less than their prokaryotic counterparts, picoeukaryotes hold a major role in net primary production and therefore act as CO$_2$ sinks (Raven, 1998; Worden et al., 2004; Jardillier et al., 2010; Grob et al., 2011). Previous work has suggested that amongst the picoeukaryotes Mamiellophyceae (order Mamiellales) is highly important in coastal ecosystems, especially polar waters (Not et al., 2005; Worden and Not, 2008; Massana, 2011). The first to propose class Mamiellophyceae, Marin and Melkonian (2010), describe this class as comprising “not only the smallest eukaryotes known, but also arguably some of the ecologically most successful picoeukaryotes in the ocean”.

165
Members of the Mamiellales were some of the first picoeukaryotes described and are readily culturable (Knight-Jones and Walne, 1951). Even so, many environmental strains have been identified exclusively upon their molecular signature, and therefore the functional role of such organisms is unclear. Genomes published for strains of Ostreococcus (Derelle et al., 2006; Palenik et al., 2007), Micromonas (Worden et al., 2009) and Bathycoccus (Moreau et al., 2012) have given huge insight into this order, including their photosynthetic pathway (C4), small genome size, and adaptations for growth in oligotrophic environments (Piganeau et al., 2011). However, genomic studies on a large scale are not only cost prohibitive but also require experimental evidence to investigate and understand such processes. The application of stable isotope probing (SIP) has successfully been applied to identify metabolically active members within given microbial communities (Manefield et al., 2002; Morris et al., 2002; Radajewski et al., 2003; Griffiths et al., 2004; Lueders et al., 2004; Rangel-Castro, 2005). Quantitative PCR (qPCR) and Reverse Transcriptase quantitative PCR (RT-qPCR) have been widely applied in microbial ecology to quantify abundance and expression of taxonomic markers (Smith and Osborn, 2009). By directly studying stable isotope labelled ribosomal RNA (rRNA), in conjunction with RT-qPCR it is possible to measure uptake of a substrate independent of cell replication within targeted organisms.

One of the emerging perceptions from our previous studies, and the work of others, is that members of the Mamiellales were favoured within the elevated CO₂ Bergen mesocosms (Meakin and Wyman, 2011; Newbold et al., 2012). Previously, we found a positive relationship between elevated CO₂ treatment and proportional community contribution of the Mamiellales under nutrient replete conditions (Newbold et al., 2012). Further, in a follow up study, we were able to determine a putative link between elevated CO₂ and carbon assimilation, with possible evidence of mixotrophy within the Mamiellales (Newbold et al., 2014). However, both studies were unable to link changes in specific Mamiellales genera due to limited taxonomic resolution in the methods.
employed. In response, a qPCR assay in conjunction with RNA-SIP was developed to investigate the abundance and functional response of individual Mamiellales phylotypes to elevated CO$_2$ over the duration of the 2006 Bergen mesocosm experiment.

6.3 Results and Discussion

6.3.1 Primer design, optimization and experimental validation

The work presented in this study used samples generated during both the main mesocosm study and a stable isotope probing experiment as outlined in our previous studies (Newbold et al., 2012; Newbold et al., 2014). Here, we successfully developed a qPCR assay in conjunction with rRNA SIP to assess the ability to directly equate differences in function between elevated and ambient CO$_2$ treatments.

<table>
<thead>
<tr>
<th>OTU ID</th>
<th>Phyllogenetic ID</th>
<th>Reference sequence</th>
<th>Elevated CO$_2$</th>
<th>Ambient CO$_2$</th>
<th>Total Number of sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Bathycoccus</td>
<td>1801A06</td>
<td>80</td>
<td>64</td>
<td>144</td>
</tr>
<tr>
<td>2</td>
<td>Micromonas</td>
<td>1804A07</td>
<td>63</td>
<td>12</td>
<td>75</td>
</tr>
<tr>
<td>7</td>
<td>Micromonas</td>
<td>1801C01</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>Micromonas</td>
<td>1801F12</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>Ostreococcus</td>
<td>1815D05</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 6.1: Identity and abundance of Mamiellales OTUs in picoeukaryotic clone libraries. A distance matrix of sequences was used to determine OTUs (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.

An alignment of a total of 144 Bathycoccus-like and 78 Micromonas-like sequences was generated from the clone libraries reported in Newbold et al (2012). These data are summarised in table 6.1. Around 65% of Mamiellales signatures detected during this study were attributed to Bathycoccus-like OTUs.
which were roughly spread equally between elevated and ambient CO$_2$ libraries. In contrast, of the 78 *Micromonas* signatures 85% were detected in elevated CO$_2$ libraries. This alignment was used to design genus specific primers suitable for quantitative PCR (qPCR) targeting the Newbold *et al* (2012) OTUs.

Primer specificity was determined by the sequences within the Newbold *et al* (2012) library and the Silva SSU r 117 database, using prime check tool (Klindworth *et al.*, 2013). Both primer sets were found to have no matches outside of Mamiellophyceae. Furthermore, when tested by standard PCR no cross amplification occurred between standards and non-target controls (*Micromonas* for *Bathycoccus* and vice versa). When tested by qPCR, non targets had quantification cycle (C$_q$) values of greater than 28 (comparable to water) in all but the highest concentrations (see figure 6.1). Subsequent melt curve analysis identified a single peak for both primers. Finally, dilution series of

![Figure 6.1](image-url): Primer specificity for *Bathycoccus* and *Micromonas* qPCR assays. *Bathycoccus* primer set 570F-BATHY03R and *Micromonas* primer set 570F-MICROR, were used to amplify a dilution series of *Bathycoccus*-like 18S standard (closed triangles), *Micromonas*-like standard (open triangle) and water (grey triangle). *Micromonas* was used as non-specific control for *Bathycoccus* assay and vice versa, and water used as negative control.
log copies target against (C_q) value reported efficiency values of 100.4 and 102.37%, and $r^2$ values of 0.999 and 0.990, for *Bathycoccus* and *Micromonas* assays respectively. Reliable qPCR assays should have efficiency values between 90-110%, and $r^2$ values >0.990 (>0.98 for RT-qPCR) (Taylor et al., 2010). It can therefore be concluded that the primer sets presented in this study were highly specific and could be confidently used to quantify resident *Bathycoccus* and *Micromonas* sequences.

### 6.3.2 Mamiellales abundance in mesocosms over time

qPCR was used to track the concentration of *Bathycoccus* and *Micromonas* phylotypes over the course of the Bergen mesocosm experiment (figure 6.2).

![Figure 6.2](image)

**Figure 6.2**: Mean abundance of *Mamiellales* 18S signatures (ng/µg) over duration of the Mesocosm experiment. Elevated (M1) represented by closed circles and Ambient enclosure (M6) represented by open circles. Error bars represent the standard deviation from the mean of triplicate qPCR reactions.
Bathycoccus appeared to be in a concentration of at least two orders of magnitude higher than Micromonas throughout the experiment regardless of treatment. Mamiellales signatures appeared to change over the complete course of the mesocosm experiment however, despite some minor observed treatment preferences (ambient for Bathycoccus, elevated for Micromonas), no significant effect of treatment was found using the Kolmogorov–Smirnov distribution fitting test, \((Bathycoccus \ p=0.25; \ Micromonas \ p=0.078)\). This was contrary to our previous study which indicated that Mamiellales favoured elevated CO\(_2\) (Newbold \textit{et al.}, 2012). Further, Meakin and Wyman (2011) tracked copy number of Bathycoccus and Micromonas RubisCO (\textit{rbcL}) genes over the first 8 days of this experiment. They found that Micromonas significantly favoured elevated CO\(_2\), whereas no treatment effect was observable for Bathycoccus. Although this study observed a higher concentration of Micromonas signatures in elevated CO\(_2\) between days 2-7, no significant difference over the duration of the experiment was seen. Differing methodologies between the studies is likely an explanatory factor. Newbold and colleagues (2012) used T-RFLP and, as such, were not able to separate Bathycoccus and Micromonas signatures. Meakin and Wyman (2011) on the other hand, did use qPCR but looked at chloroplast RubisCO (\textit{rbcL}) genes not 18S SSU rRNA. Additionally, Meakin and Wyman (2011) normalized their qPCR results to a set volume of filtered seawater. Although this approach allowed the calculation of copies per L, others have found that varying inhibitor concentrations in extractions can effect qPCR quantification (Lloyd \textit{et al.}, 2010). To overcome this problem, this study normalized to µg template.

### 6.3.3 RT-qPCR validation

The validity of using RNA stable isotope probing to detect changes in the assimilation of sodium bicarbonate and glucose was established in our previous study (Newbold \textit{et al.}, 2014).
However, one of the challenges highlighted was a lack of phylogenetic resolution, resulting from no pre-filtration and the use of terminal restriction fragment length polymorphism (T-RFLP). The qPCR assay discussed in section 6.3.1 was extended to allow for quantification of rRNA (RT-qPCR). Positive control RNA standards were generated via T7 transcription from plasmids containing target 18S rRNA sequence, selected by OTU affiliation and

Figure 6.3: Changes in RT-qPCR abundance during stable isotope microcosm incubations for *Bathycoccus* and Micromonas. Relative uptake Elevated M1 CO$_2$ incubations (closed circles) compared to ambient M6 incubations (open circles). Relative uptake of $^{13}$C substrate assessed by concentration of target RNA in $^{12}$C incubations subtracted from target concentration in $^{13}$C incubations. Positive values in bicarbonate and glucose incubations are taken to have higher activity in $^{13}$C incubations compared to equivalent $^{12}$C incubation (fraction 5) and were therefore assumed to be actively metabolising substrate. Error bars represent standard deviation from triplicate RT-qPCR reactions. Positive error bars corresponded to $^{13}$C incubation values, negative to $^{12}$C. Vertical line denotes the separation of phases 1 and 2.
sequence insert orientation. These, standards were subsequently used to optimize annealing temperature in one step RT-qPCR reactions, (verified by both melt curve analysis and the inclusion of non-target controls). Accurately quantified standard curves were used to determine PCR reaction efficiency, and subsequently quantify concentration of target *Mamiellales* 18S template in RNA-SIP samples. Standard curves from RT-qPCR of *Bathycoccus* and *Micromonas* gave efficiency values of 101.4 and 108.88, and $R^2$ values of 0.991 and 0.987.

### 6.3.4 Response of bicarbonate assimilation to elevated CO$_2$

Figure 6.3 presents substrate specific assimilation of $^{13}$C across the three time points studied. During the first SIP incubation, levels of *Bathycoccus* bicarbonate assimilation in $^{13}$C incubations did not exceed those of natural $^{12}$C under either regime. In contrast, higher assimilation was observed for *Micromonas* phylotypes. During SIP 2 (end of phase 1, day 10) bicarbonate assimilation was highest in *Bathycoccus* under ambient conditions, whereas *Micromonas* assimilation was roughly equal in both treatments. Finally, bicarbonate assimilation did not surpass that of the background $^{12}$C incubations in the final SIP incubation for either phylotype. The findings of SIP 1 would support Meakin and Wyman (2011), who found higher levels of photosynthetic *Micromonas*-like *rbcL* gene signatures in elevated CO$_2$ during the early stages of the 2006 BME. This SIP experiment would suggest a higher level of photosynthetic carbon assimilation in *Micromonas* under elevated CO$_2$, compared to *Bathycoccus* during the initial days of the nutrient replete phase (see Newbold *et al.*, 2012 figure 4.1 for nutrient data). Interestingly, *Bathycoccus* photosynthetic assimilation was highest in SIP 2 ambient treatment (end of phase 1, day 10). The flow cytometry data presented in Newbold *et al.* 2012 (figure 4.2) suggested that in the ambient treatment, small picoeukaryotes bloomed later (day 10) than in elevated CO$_2$ mesocosms (day 8). Both *Bathycoccus* and *Micromonas* would fall in the small picoeukaryote size grouping (Gómez-Pereira *et al.*, 2013). Therefore, it is most likely that the SIP 2
microcosm’s ambient community represented this bloom peak and hence this is reflected by high levels of *Bathycoccus* bicarbonate assimilation. In contrast, elevated CO$_2$ incubations, having already bloomed, showed comparatively little *Bathycoccus* bicarbonate assimilation. It should be noted however that *Micromonas* assimilation was equal in both treatments at this time point and may therefore represent a group specific response. During the final nutrient deplete phase (SIP3, day 17), assimilation of bicarbonate did not exceed that of the background $^{12}$C incubations in either *Bathycoccus* or *Micromonas*. Newbold and colleagues (2012) observed a secondary bloom in picoeukaryote cell count during this phase and, through T-RFLP analysis, established that within the picoeukaryote community, dominant species abundance shifted favouring groups such as alveolates and chrysophytes. Phytoplankton bloom communities are thought to provide a series of ecological niches based upon nutrient availability, which individuals are able to exploit (Teeling *et al.*, 2012). The observed reductions in *Mamiellales* activity in the second phase of the experiment, despite an observed picoeukaryote bloom community, are like to represent niche separation; whereby conditions favoured other bicarbonate assimilating picoeukaryotes.

### 6.3.5 *Response of glucose assimilation to elevated CO$_2$*

When using RNA-SIP to look at community level functional responses to OA, we previously found evidence for glucose assimilation within the *Mamiellales* (Newbold *et al.*, 2014). This study found more specifically that $^{13}$C glucose was actively assimilated in elevated CO$_2$ during the nutrient replete phase (SIP 1), but minimally assimilated during the remaining phases (figure 6.3). Further, levels of glucose assimilation appeared to exceed that of bicarbonate assimilation during this phase - a response which held for both *Bathycoccus* and *Micromonas*. The presence of chloroplasts and photosynthetic genes within the *Mamiellales* clearly demonstrates a photosynthetic lifestyle (Derelle *et al.*, 2006; Palenik *et al.*, 2007; Worden *et al.*, 2009; Piganeau *et al.*, 2011; Moreau
et al., 2012). Yet, there is a growing body of evidence that picoeukaryotes can act mixotrophically (Zubkov and Tarran, 2008; Hartmann et al., 2013). More specifically there is evidence of mixotrophy within the Mamiellales. Gonzalez and colleagues (1993) determined that cultured *Micromonas* showed high levels of lysozyme activity when incubated with bacteria and suggested that this was due to the ingestion of bacteria. More recently, high levels of mixotrophy have been observed in Artic picoeukaryote populations which were dominated by *Micromonas*-like cells (Sanders and Gast, 2012). The data presented in this study would certainly suggest that dissolved glucose is accessible to both *Bathycoccus* and *Micromonas*. Mixotrophy is often considered an ecological advantage in nutrient deplete (oligotrophic) waters, however, this experiment observed little glucose assimilation in the nutrient deplete phase of the study (SIP 3, day 17) (Hartmann et al., 2012). This indicated that within the confines of this experiment, glucose assimilation was only an advantage to *Bathycoccus* and *Micromonas* during nutrient replete conditions.

Although these data might suggest that glucose assimilation is favoured over that of bicarbonate, these findings should be taken with caution and examined in the context of the broader community. During the first phase of the experiment added nutrients and elevated CO$_2$ will have favoured all autotrophs, most of which have a larger cell mass than the Mamiellales. Therefore, labelled bicarbonate would have been readily assimilated by all autotrophs and consequentially been less bio-available than labelled glucose. Because Mamiellales were rapidly multiplying (as demonstrated by flow cytometry and T-RFLP in our previous study) and presumably assimilating nutrients from every available source, glucose assimilation may have been observed to be artificially higher than bicarbonate. Studies on pure cultures of *Bathycoccus* and *Micromonas* strains would be able to determine if this were the case.
6.3.6 Conclusion

This experiment demonstrates the validity of using both stable isotope probing and qPCR to trace and quantify individuals, at genus level, from a larger mixed community. Phylotype specific carbon assimilation within the Mamiellales in response to elevated CO$_2$ was observed. Further, the presented data suggested that glucose assimilation is present in the Mamiellales but only in elevated nutrient replete CO$_2$ conditions. These findings would support the view that Mamiellales populations, like others, are able to exploit predicted future CO$_2$ concentrations. Even so, nutrient availability will likely provide unique niches which separate an organism’s specific response.

6.4 Experimental Procedures

6.4.1 Experimental design

The complete experimental design of the May 2006 Bergen mesocosm experiment has been outlined previously (Gilbert et al., 2008; Hopkins et al., 2010; Meakin and Wyman, 2011; Newbold et al., 2012). This study presented the data from a microcosm experiment, run in parallel to the main Mesocosm experiment. Experimental mesocosm enclosures were filled with unfiltered native fjord water and gently sparged with CO$_2$ (750 µatm) for 2 days (4–6$^{th}$ 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 6$^{th}$ May (initial concentrations: 1 mmol l$^{-1}$ phosphate; 17 mmol l$^{-1}$ nitrate). Blooming phytoplankton growth reduced CO$_2$ concentrations in the elevated-CO$_2$ mesocosms; therefore after day 10 sampling, mesocosm enclosures were re-acidified (15/5/2006), and ambient-condition enclosures again sparged with air.
As described in Newbold and colleagues (2014) microcosm incubations used 4L water sampled from mesocosm bags 1 (elevated CO$_2$) and 6 (ambient CO$_2$) to fill 5L Nalgene bottles containing either $^{12}$C or $^{13}$C glucose (50mg/L) or sodium bicarbonate (0.15g/L). Microcosms were submerged under surface fjord water and incubated *in situ*. Following microcosm establishment, daily pH was measured and plankton collected from 1L of microcosm water onto 0.2 µm Durapore membranes for a period of 5 days. All membranes were immediately stored at -80 prior to molecular analysis. This process was repeated at 3 key time points, corresponding to pre-phytoplankton bloom (day 4, 7$^{th}$), peak phytoplankton bloom (day 10, 13$^{th}$) and post phytoplankton bloom (day 17, 20$^{th}$). Samples obtained 2 days after microcosm establishment were later found to show optimal isotope integration (via T-RFLP) therefore, only data corresponding to these days has been presented.

### 6.4.2 Nucleic acid extraction

Total nucleic acids were extracted following the CTAB bead beating protocol outlined by Huang and colleagues (2009). RNA-SIP template of total nucleic acid extract was treated with DNase using DNA-free™ kit, Ambion®. DNase treated RNA were quantified on Qubit® 1.0 Fluorometer with Qubit® RNA BR assay.

### 6.4.3 RNA Stable Isotope Probing (RNA-SIP)

RNA-SIP protocol followed that outlined by Whiteley and colleagues (2007). Briefly, between 400-500ng RNA was loaded onto a caesium trifluoroacetate gradient (2.0 g/ml) and centrifuged at 64,000 rpm for 48 h at 20°C on Beckman TLX bench top ultra-centrifuge (TLA120.2 rotor). Gradients were fractionated using a Beckman fraction recovery system and peristaltic pump at a flow rate of 0.2 ml min$^{-1}$. RNAs were isolated from gradient fractions by precipitation with 1 volume of isopropyl alcohol and 1µl glycogen. Fractions were resuspended in
10 µl molecular grade Tris EDTA pH 7.4 and quantified on a Qubit® 1.0 Fluorometer with Qubit® RNA HS assay.

6.4.4 Positive control selection and transcription

This study relied upon accurately quantified DNA and RNA template as positive controls. Newbold and colleagues (2012) identified 5 Mamiellales near full length 18S SSU rRNA gene sequence phylotypes (referred to here as operational taxonomic units, OTUs), of these two dominated; Micromonas-like OTU2 and Bathycoccus- like OTU4. Representative clones (and contained pCR4-TOPO vectors) from OTUs 2 and 4 were selected to act as standards and templates for RNA transcription. Vector inserts contained antisense sequences from Micromonas-like OTU2 clone 1804A07, accession number FR874290 and Bathycoccus-like clone OTU4 1801A12, accession number FR874275. Plasmids were extracted using the QIAprep spin miniprep kit (Qiagen). Linearised plasmid DNA was obtained by digesting 20µl plasmid extract with SpeI (4µl SpeI 10 units/ µl, Promega, 5 µl 10X buffer, 0.2 µl 100 X BSA and 7.1 µl molecular grade water) for 4 hrs at 37°C, followed by heat inactivation at 65°C for 20 mins. Linearised DNA was quantified on Qubit® 1.0 Fluorometer with Qubit® dsDNA BR Assay, and used as DNA standard for qPCR. Between 0.5-2 µg of linearised plasmid DNA was used as a template for RNA transcripts using the HiScribe™ T7 In Vitro Transcription Kit and manufacturers protocol (New England Biolabs inc). Transcripts were concentrated using ethanol precipitation and verified by gel electrophoresis. All residual DNA was removed from RNA standards, using DNA-free™ kit, Ambion®. Standards were quantified on Qubit® 1.0 Fluorometer with Qubit® RNA BR Assay (RNA) Qubit® dsDNA BR Assay (DNA). Template rRNA copy was calculated using the formula:

\[
molecules/ \mu l = a/(plasmid \text{ length} \times 660) \times 6.022 \times 10^{23}
\]
Where \( a \) is the plasmid DNA concentration (g/µl), plasmid length including insert (5731bp for *Bathycoccus*, 5733bp for *Micromonas*), 660 is the average molecular mass of one bp, and 6.022 X10\(^{23}\) is the molar constant (Zhu *et al.*, 2005).

### 6.4.5 Primer design and PCR optimisation

The environmental 18S SSU rRNA sequence data presented in Newbold *et al.*, (2012) was used as a reference dataset to develop genus specific qPCR assays corresponding to a 167bp region of *Bathycoccus* and 150bp region of *Micromonas*- like phylotypes. Target *Bathycoccus* and *Micromonas* 18S SSU rRNA genes were amplified using the universal forward primer 570F- 5’ GTAATTCCAGCTCCAATAGC 3’ (Baldwin *et al.*, 2005), and gene specific reverse primers BATHY03r-5’ACCACGATGACTCCATGCTCA3’ (Zhu *et al.*, 2005) and MICRO- 5’CCAGACCGTTAAGCCAGAGCAC3’.

### 6.4.6 (RT-)qPCR

Quantitative PCR (qPCR) reactions were performed in a final reaction volume of 20µl, consisting of 9 µl EXPRESS SYBR® greenER™ qPCR supermix (invitrogen), 200nM forward and 200nM reverse primers and 10ng template DNA. RT-qPCR was likewise made to final reaction volume of 20µl, with the addition of 10 µl EXPRESS SYBR® greenER™ qPCR supermix (Invitrogen), 200nM forward and 200nM reverse primers, 0.5µl Express one step Superscript® (Invitrogen) and 10ng purified RNA template. All reactions were set up in sterile conditions and performed in twin.tec PCR plates, sealed with masterclear real-time PCR film, on Mastercycler® ep realeplex 4S (all Eppendorf). Thermal cycling conditions consisted of 50°C for 5 minutes, 95°C for 2 minutes, 40 cycles of: 95°C for 15 seconds, 60°C (Bathycoccus)/ 65°C (Micromonas) for 1 minute and final melting curve analysis of 60°C–95°C. All reactions were performed in triplicate as per MIQE guidelines, with suitable dilution series of standards, non-target controls and water (Bustin *et al.*, 2009).
Standard curves of positive template standards were used to determine reaction efficiency in the Agilent Genomic tools calculator (Agilent Technologies, 2013).

Using the formula:  \[ \text{Efficiency} = -1 + 10^{(-1/\text{slope})} \]

### 6.4.7 Statistical analysis

In order to test the similarity of distribution, shape and position of data generated, from the qPCR data, the two-sample Kolmogorov-Smirnov test was utilised. This analysis employs 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 in this study, for comparing two empirical distributions (Nikiforov, 1994). Here this test was applied in XLSTAT software (version 2013.6.04, Addinsoft).

### 6.5 Acknowledgements

The authors would like to thank all of the members of the 2006 Bergen Mesocosm experiment for their invaluable help in data acquisition and advice, especially Steve Summers. This work was funded by the Centre for Ecology and Hydrology (CEH) internal science budget.

### 6.5 References


Knight-Jones, E. W. and Walne, P. R. (1951) 'Chromulina pusilla' Butcher, a Dominant Member of the Ultraplankton', *Nature*, 167(4246), pp. 445-446.


420,000 Years from the Vostok Ice Core, Antarctica', Nature, 399(6735), pp. 429-436.


Chapter 7: General Discussion and Conclusions

7.1 Summary of Findings

This project examined the consequences of elevated CO$_2$ (linked to ocean acidification, OA) on marine microbial communities within the confines of a large volume mesocosm experiment, and through the application of molecular techniques. The broad aims of this thesis were to examine the consequences of elevated CO$_2$ on marine picoplankton community structure, diversity, phylogeny and function (outlined in section 1.6.1). In response the following conclusions have been drawn:

1) The majority of community abundance and functional changes observed within this study can be explained by changes in temporal dynamics, not CO$_2$.

2) Bacterial cell abundance is largely unaffected by elevated CO$_2$.

3) Picoeukaryote cell abundance is significantly higher in elevated CO$_2$.

4) Bacterial community composition is resistant to elevated CO$_2$.

5) Some picoeukaryote populations respond to elevated CO$_2$, but this is likely to be determined by nutrient availability and changes in the wider planktonic food web.

6) Microbial populations hold the capacity to either resist or functionally respond to elevated CO$_2$.

7) Dominant members of picoplanktonic communities either show minimal functional responses to elevated CO$_2$ or respond positively by increasing autotrophic and/or heterotrophic carbon assimilation.

7.2 How Will Microbes Respond to Predicted Future Levels of Elevated CO$_2$?

The work presented in this thesis indicates that presently non-calcifying marine microbes hold enough taxonomic and functional diversity to accommodate
predicted future levels of pH. Therefore, it is unlikely that catastrophic changes in the marine biogeochemical processes driven by picoplankton will occur (Joint et al., 2011). In the experiments presented here, and elsewhere, the majority of microbial community variance was explained by temporal dynamics (Liu et al., 2010; Brussaard et al., 2013; Roy et al., 2013; Sperling et al., 2013; Zhang et al., 2013). Yet, it is important to consider that this thesis did detect some individual responses to elevated CO₂.

### 7.2.1 Prokaryotes

Overall bacterial community diversity within this study was typical of similar environments and studies (Giovannoni et al., 1990; Britschgi and Giovannoni, 1991; Fuhrman et al., 1993; Rappe et al., 1997; Suzuki et al., 1998; Morris et al., 2002; Rusch et al., 2007; Fuhrman, 2009). The six most dominant prokaryotes within both 16S SSU rRNA clone libraries and T-FRLP analysis belonged to four taxa all highly abundant in marine ecosystems; Rhodobacteriales, Bacteroidetes, Candidatus Pelagiobacter (SAR11) and Gammaproteobacteria. Chapter 3 looked at the consequences of predicted year 2100 CO₂ concentrations upon bacterial community turnover and found that not only was community composition conserved over time but that community turnover was dampened with elevated CO₂. These data therefore implied that bacterial communities were resistant to the experimental regime imposed. It is however important to consider that although bacterial communities appear resistant to CO₂ perturbation, the time scale of this experiment would not represent a true OA community. One hundred years represents millions of bacterial generations and therefore the scope for adaption or ‘resilience’ cannot truly be measured in the 18 days represented by the 2006 Bergen mesocosm experiment. Chapter 4 found that there were no significant differences in bacterioplankton cell count between elevated and ambient mesocosms. Further, although dynamic population changes were observed in 5 of 6 key bacterial populations, no significant differences in abundance could be detected (as
assessed by T-RFLP). This finding is in line with other mesocosm studies which report no or minimal responses in bacterial abundance to elevated CO$_2$ (Rochelle-Newall et al., 2004; Grossart et al., 2006; Allgaier et al., 2008; Paulino et al., 2008; Brussaard et al., 2013).

In contrast to bacterial abundance the evidence from the stable isotope probing (SIP) experiment presented in chapter 5, suggested that bacterial populations may functionally respond to future CO$_2$ concentrations. Two of the dominant terminal restriction fragments (TRF’s) identified as belonging to the Rhodobacteriales assimilated a higher proportion of labelled $^{13}$C glucose and sodium bicarbonate in elevated CO$_2$. This result was contrasted by the Bacteroidetes TRF’s which showed higher assimilation in ambient incubations. Grossart et al. (2006) demonstrated that total prokaryotic protein production was enhanced by elevated CO$_2$ in a similar mesocosm study. However this finding was not replicated more recently, where bacterial production significantly decreased with increasing CO$_2$ (Motegi et al., 2013) thereby demonstrating the requirement for further work into bacterial functional response to elevated CO$_2$.

The work of this thesis and other recent studies would suggest that on the whole bacterial response to OA will likely be driven by indirect changes in overall community dynamics rather than directly by degree of acidification (Roy et al., 2013; Sperling et al., 2013). However, there are a number of considerations that should be taken into account:

1) The work presented within this thesis represents only free living bacterioplankton and does not take particle-attached bacteria into consideration. Engel and colleagues (2008) found that free living bacterial diversity was affected by elevated CO$_2$, whereas particle-attached bacterial diversity was independent of CO$_2$ treatment, and strongly coupled to phytoplankton bloom development. However, in a more recent study the authors found that both free-living and particle attached bacterial communities
were strongly associated to phytoplankton bloom development and temperature, not CO$_2$ (Sperling et al., 2013). Additionally, the composition of bacterial populations closely associated to corals has been shown to shift from mutualistic to pathogenic in response to reduced pH, clearly suggesting that not all bacterial populations respond in the same way (Vega Thurber et al., 2009).

2) The work of this thesis focuses on the dominant members of the bacterial community and, as such, ignores much of the ‘rare’ portion of the community. Studies have suggested that rare organisms may represent a ‘microbial seed bank’ – whereby organisms of low abundance might shift to high abundance in response to environmental change (Sogin et al., 2006; Caporaso et al., 2012; Gibbons et al., 2013). Although the methods employed in this study were likely to capture little of this diversity, there was evidence in the SIP incubations of a rare 16S SSU rRNA OTU becoming highly abundant in glucose incubations, which was undetected in the 16S mesocosm library (chapter 5).

3) Viral interactions are a known driver of prokaryote mortality and therefore a key factor in nutrient release (Suttle, 2007). No measure of viral lysis rates or abundance were investigated in this study, however others have found that viral response to OA will likely be minimal (Rochelle-Newall et al., 2004; Larsen et al., 2008) or host dependant (Traving et al., 2014). Therefore, further work investigating the role of picoplankton-virus interactions would be prudent.

4) This study concentrated on marine bacteria and picoeukaryotes excluding the third domain of life, archaea. Like the other domains archaea exhibit a vast marine diversity and play integral roles in nitrogen (and other) biogeochemical cycles (Francis et al., 2007). When universal primers were used to detect archaea present in our system they proved difficult to detect. Furthermore, sequence data from the clone libraries which were produced showed a very low diversity when compared to bacteria and picoeukaryotes (unpublished data) and as a consequence research was focused elsewhere. A recent study
however, has suggested that whereas ammonia oxidizing bacteria (AOB) communities responded to acidification by increased abundance in *Nitrosomonas*, ammonia oxidizing archaea (AOA) showed no significant shifts in community structure, suggesting that archaea may be less sensitive to reduced pH (Bowen et al., 2013). It would be interesting to investigate whether this finding holds for all archaeal communities.

### 7.2.2 Picoeukaryotes

The picoeukaryote diversity revealed in this study, like the bacterial community, matched that found in similar environments (Diez et al., 2001; Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001; Massana et al., 2004; Romari and Vaulot, 2004; Piganeau et al., 2008; Not et al., 2009; Massana et al., 2011). All major picoeukaryotic lineages typically retrieved from a coastal pelagic marine community were represented, with organisms from the Mamiellales, Chrysophyceae, Ciliophora and Alveolata dominating. Chapter 4 determined that the cell abundance of small picoeukaryotes was significantly higher in elevated CO$_2$. When T-RFLP was used to examine changes in the abundance of dominant TRF’s (identified by a large 18S SSU rRNA clone library), half were significantly different between elevated and ambient CO$_2$ mesocosm incubations. TRF’s identified as members of the novel alveolates group I (NAI) and Chrysophyeace, had a significantly higher abundance in ambient mesocosm incubations. In contrast, a TRF peak identified as Mamiellales appeared to be favoured elevated CO$_2$, contributing a significantly higher proportion of the total picoeukaryote community. From this it was concluded that the Mamiellales organisms were able to autotrophically exploit elevated concentrations of CO$_2$. When examined in a functional context (using RNA stable isotope probing, RNA-SIP) distinct differences in the level of glucose and bicarbonate assimilation were observed between CO$_2$ treatments (chapter 5).
Interestingly, the Mamiellales appeared to behave mixotrophically, but only in nutrient replete conditions. Using qPCR and RT-qPCR it was possible to detect phylotype specific carbon assimilation responses to elevated CO₂ within the dominant Mamiellales signatures *Bathycoccus* and *Micromonas* (chapter 6). *Micromonas* appeared to actively assimilate more bicarbonate with elevated CO₂, but only in nutrient replete conditions. *Bathycoccus* bicarbonate assimilation on the other-hand, was highest in bloom peak ambient mesocosms. Further, both *Bathycoccus* and *Micromonas* showed evidence of assimilating glucose, but again only in nutrient replete conditions.

The findings presented in this thesis relating to picoeukaryotes would suggest elevated CO₂ will have an effect upon autotrophic carbon assimilation. However, the exact response is likely to be taxon specific and constrained by nutrient availability. This work adds to the emerging perception that OA will favour small non calcifying autotrophs which put less resources into costly carbon concentrating mechanisms (Paulino *et al.*, 2008; Meakin and Wyman, 2011; Brussaard *et al.*, 2013). The enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO) utilizes dissolved CO₂ in the carbon fixation step of marine photosynthesis. However, RubisCO has a low substrate affinity and therefore requires a mechanism for concentrating CO₂ – carbon concentration mechanism (CCM) (Rost *et al.*, 2008). Organisms which have efficient CCM’s have appeared to be less affected by elevated CO₂ than those lacking efficient CCM’s (Engel *et al.*, 2008). Furthermore, organisms with a large surface to volume ratio, like *Micromonas*, have been shown to capitalize elevated dissolved CO₂ by increased diffusion (Brussaard *et al.*, 2013). It follows that the potential to access alternative carbon sources (act mixotrophically), could serve as a competitive advantage over strict autotrophs (Zubkov, 2009; Sanders and Gast, 2012; Hartmann *et al.*, 2013). If these findings hold true then a shift in phytoplanktonic community composition would have implications for the structure and function of pelagic food webs.
Brussaard and colleagues (2013) found that organisms which prospered with elevated CO$_2$ were more prone to viral lysis, and suggested that this would shift bioaccumulation in living organisms into the dissolved organic carbon pool. Consequently, there would be a reduction in transfer to higher predators and an increase in the importance of the microbial food web. This thesis did find evidence for elevated functional responses in both the Mamiellales and bacterial group Rhodobacteriales which would support this view, however there are additional factors which need also need to be considered:

1) As previously discussed no measure of viral activity was taken into account. Additionally, this thesis focused on picoeukaryotes and therefore the effect of OA upon key grazers of picoplankton such as heterotrophic nanoflagellates (HNF) was only briefly considered in chapter 4. Brussaard and colleagues (2013) found evidence for increased abundance of the nano size class in elevated CO$_2$, yet our study found no significant differences in their cell abundance.

2) The work presented here did not measure actual photosynthetic rate, only changes in microbial abundance and the relative assimilation of bicarbonate into rRNA. Hopkins and colleagues (2010) reported a significant reduction of chlorophyll $a$ in elevated CO$_2$, in the same Bergen mesocosm study, suggesting that overall photosynthesis may have been inhibited, not increased, by elevated CO$_2$.

### 7.3 Methodological Considerations and Limitations

#### 7.3.1 The ability to link phylogeny and function- “who is there and what are they doing” (Dubilier, 2007)

As established in the introduction to this thesis, one of the central challenges of microbial ecology is the linking of phylogeny to function in unculturabable microbes. This thesis applied a number of culture independent techniques to
both establish the diversity present, and to link this diversity to functional
responses to ocean acidification. The methods applied however highlighted a
number of considerations and limitations which need to be explored. These are
outlined in sections (7.3.2 - 7.3.5) below.

7.3.2 The 2006 Bergen mesocosm experiment (BME)

All of the samples used within this study were the result of the 2006 Bergen
mesocosm experiment. Therefore, there are a number of aspects relating to
overall experimental design which should be discussed as they will undoubtedly
have shaped the results presented in this thesis.

One of the strengths of a mesocosm experiment is the ability to make large
scale manipulations in a semi-natural setting, however this means that the
number of replicates is cost prohibited. At the outset of the 2006 BME it was
decided to have three experimental (elevated CO$_2$) and three control (ambient
CO$_2$) mesocosm enclosures. However, as the experiment progressed it became
apparent that the phytoplankton bloom utilized elevated concentrations of
dissolved CO$_2$ and consequently, pH returned to that in line with ambient
conditions (Joint et al., 2011). In response a consortium wide decision was
made to re-acidify two of the experimental enclosures (in order to investigate
communities in elevated CO$_2$ conditions), leaving the remaining experimental
enclosure to fulfil the original experimental design. Although the experiment still
had validity, due to the large volumes investigated, the data produced lost some
of its statistical power. This produced a knock on effect to the parallel SIP
incubations where experimental replication was lost.

It is also important to consider the length of study. In both the bacterial and
eukaryotic communities the majority of variation could be explained by dynamic
temporal changes. Although it was possible to establish that bacterial
communities were resistant to elevated CO$_2$ (chapter 3), the 18 day duration of
the BME 2006 meant resilience (community recovery) couldn’t be accurately measured.

Temporal patterns in the community were also shaped by the addition of nutrients which led to a phytoplankton bloom, and its subsequent decay. The addition of nutrients prior to sampling meant that the communities studied were the result of both nutrient addition and elevated CO$_2$, not in response solely to elevated CO$_2$. A recent mesocosm consortia studied the effect of elevated CO$_2$ on microbial communities prior and post nutrient addition to account for this factor (Schulz et al., 2013). Schulz and colleagues (2013) found distinct changes in plankton community structure when nutrients were added, although higher abundances of picoeukaryotes were noted in elevated CO$_2$ in pre and post-nutrient addition.

It is also important to consider that climate change will work upon a number of environmental parameters including ocean warming, expanding hypoxic regions and changes in salinity (Gattuso et al., 2011). Fu and colleagues (2007) found a synergistic effect upon the photosynthetic rates of the cyanobacterium *Synechococcus*, when looking at elevated CO$_2$ and temperature. Further, Lindh and colleagues (2013) established that temperature was the dominant driver of bacterial community composition, not pH. However, when elevated temperature and CO$_2$ were combined distinct shifts in community composition were seen. These studies clearly demonstrate need to look at all potential climate change factors, not just changes in pH.

Finally, the mechanism by which the mesocosm pH was adjusted may have influenced the results. The BME 2006 adjusted pH by sparging experimental mesocosms with CO$_2$ enriched air. This method was favoured over direct pH adjustment (through the addition of an acid) as it best mimics future OA scenarios - where $p$CO$_2$ increases and pH decreases (Riebesell et al., 2010). Furthermore, it doesn’t change trace metal availability (Shi et al., 2009).
is evidence however that sparging can reduce the growth rate of planktonic species (Shi et al., 2009). Unfortunately there are no reliable alternatives therefore future studies need to keep sparging to a minimum.

In order to account for all of these factors it would be desirable that future experiments increase the number of experimental replicates, number of environmental parameters (including temperature, nutrient and pH gradients) and are performed over a longer duration. These, and numerous other desirable qualities for mesocosm experiments have been outlined elsewhere (Rost et al., 2008; Riebesell et al., 2010).

**7.3.3 Culture independent community fingerprint and diversity techniques**

Many of the drawbacks associated with community fingerprint and diversity techniques were outlined in the introduction to this thesis (1.5.1). The work presented here tried to overcome most bias by improved methodology in extraction, amplification and sequencing. One of the biggest criticisms of culture independent techniques is a lack of taxonomic resolution. This thesis sequenced a library consisting of a total of around 3000 16S and 18S full length rRNA sequences, a value far higher than most similar studies (Ashelford et al., 2006). This depth is now dwarfed by that of equivalent next generation sequence libraries, which have retrieved greater than 10,000,000 reads (Caporaso et al., 2012; Roy et al., 2013). Even so, the read length of such libraries was much shorter (<200 bp) allowing less phylogenetic resolution than the sequences presented here. Next generation sequencing technologies are however evolving at a rapid pace, with 600bp reads being readily achieved at a relatively low cost. As such, they are likely to soon eclipse traditional methods, making in-depth microbial community diversity studies both technologically and financially viable.
7.3.4 RNA stable isotope probing (RNA-SIP)

Like other elements of this study, the data produced by the SIP experiment are likely to reflect a number of methodological choices. Past SIP studies have successfully demonstrated organisms responsible for phenol degradation (Manefield et al., 2002), methane oxidation (Cébron et al., 2007) methanol and methylamine assimilation (Neufeld et al., 2007) and ammonia oxidation (Pratscher et al., 2011) - to name but a few. These substrates were chosen carefully to target relatively select groups of taxa responsible for specific functional roles within the wider community. Here glucose and sodium bicarbonate, substrates accessible to a wide range of the microbial community, were used as tracers for heterotrophy and phototrophy. For example, many bacterial groups contain glycolytic pathways and therefore added glucose was likely readily utilised (Fothergill-Gilmore and Michels, 1993; Canback et al., 2002; Pollack et al., 2013). Furthermore, a recent DNA-SIP study established that bacterial oceanic bicarbonate assimilation is ubiquitous, with bacterial populations employing a number of trophic pathways to access carbon (DeLorenzo et al., 2012). Mixotrophy has been found to be common in both prokaryote and eukaryote marine populations (Zubkov and Tarran, 2008; Zubkov, 2009; Hartmann et al., 2012; Hartmann et al., 2013), and consequently it is difficult to separate carbon assimilatory responses of strict phototrophy or heterotrophy. It is not surprising therefore that only minimal detectable changes were observed between $^{13}$C and background $^{12}$C.

The use of a more sensitive molecular technique, RT-qPCR was able to overcome this issue within the Mamiellales. However, the determination of carbon assimilation mechanisms – in the case of the Mamiellales through direct diffusion or indirectly through phagocytosis of $^{13}$C labelled bacteria or lysed bacterial cellular biomatter - would require an alternative approach. Frias-Lopez and colleagues (2009) added $^{13}$C labelled bacteria to seawater and were able to successfully determine the breadth of eukaryotic mixotrophs within their system.
Further, a recent study, using a combination of flow cell sorting and fluorescence *in situ* hybridization, was able to successfully detect plastidic picoeukaryote cells which had internalised bacterial cells, giving clear evidence for mixotrophy in this size class (Hartmann *et al*., 2013). It would be interesting to apply such approaches to determine if *Mamiellales* are able to graze bacteria directly.

A further factor which appreciably influenced the SIP results was a lack of pre-filtration to remove larger eukaryotes and particulate matter. As discussed in chapter 5, a lack of pre-filtration meant that the SIP rRNA template encompassed the complete community, not just members of the picoplankton. As a result mesocosm clone libraries were not fully representative and the assignment of T-RF peak identity limited. This was a particular problem in the eukaryote T-RFLP analysis where the presence of larger organisms, with multi-copy rRNA genes were likely to have swamped the signal of lower abundance picoeukaryote community members. These may have been actively assimilating $^{13}$C, but were below the detection threshold of T-RFLP. Although the development of a qPCR assay did counteract this issue by specifically targeting *Mamiellales* signatures, it would be advisable that future SIP studies on the functional effects of OA on picoplankton employ a pre-filtration step.

### 7.3.5 (RT) qPCR

This thesis showed the successful development of a (RT) qPCR assay to quantify individual taxa within the Mamiellales, thus minimising the effect of a lack of pre-filtration within the eukaryote community. It would be interesting to extend this assay to include other community members (e.g. the other dominant picoplankton members). However, the quantities of RNA recovered from fractionation were low. Statistically valid (RT) qPCR data requires a high level of repetition and consequently assay number in this study was limited to a few organisms (Bustin *et al*., 2009). This limitation could be overcome through the
use of a multiplex taqMan probe assay - where multiple probes can be run at the same time - requiring less total template. The taqMan approach has been successfully applied to detect members of the *Roseobacter*, SAR11, SAR86, *Synechococcus* and *Cytophaga* taxa, from a mixed environmental community (Suzuki et al., 2001), and also has the advantage of minimizing the effect of non-specific PCR amplification (Smith and Osborn, 2009).

A final consideration is that much of the functional work presented within this thesis concentrated on the assimilation and subsequent integration of carbon compounds into rRNA. Although this gave a measure of phylogenetically active populations, the presented data did not provide any further functional (transcriptomic) information. An attempt was made to produce and sequence mRNA libraries, however, they were dominated by rRNA, despite the use of various ribodepletion methods (A. Oliver, unpublished data). An exciting technology, far too new and cost prohibitive at the time of study, is that of transcriptomics, or next generation RNA sequencing. The first environmental transcriptomes generated were from soils (Leininger et al., 2006; Urich et al., 2008) and later marine plankton (Frias-Lopez et al., 2008). However, the relative mRNA content compared to rRNA of these first studies was low. Even so, the application of such technologies allowed the examination of mRNA in far greater depth than previously available, with the added benefit of a lower template requirement. In combination with the growing development of more successful mRNA enrichment methods, the study of environmental transcriptomes becomes one of the most interesting avenues of future research (Gilbert et al., 2008; Sorek and Cossart, 2010; Cho et al., 2013).

### 7.4 The Future

So “will ocean acidification affect marine microbes?” (Joint et al., 2011). On the whole it appears that the majority of marine picoplankton will be resistant to changes imposed through OA, and as such micro-organismal diversity already
holds the genetic and functional capacity to respond to change. However, the sensitivity of individual organisms varies and, as such, may influence vital oceanic processes such as carbon availability. Future work should target long term holistic studies which look at communities as both diverse and functional entities. Further, studies should focus on the synergistic interactions of proposed climate change models and how multiple factors affect communities.

7.5 References


Hartmann, M., Zubkov, M. V., Scanlan, D. J. and Lepère, C. (2013) *In situ* Interactions Between Photosynthetic Picoeukaryotes and Bacterioplankton in


Pratscher, J., Dumont, M. G. and Conrad, R. (2011) 'Ammonia Oxidation Coupled to CO$_2$ fixation by Archaea and Bacteria in an Agricultural Soil', 203


Author Contributions

Some elements of this thesis, such as overall experimental design and daily nutrient data were part of a research consortium. Consequently, the individual consortia members responsible have been acknowledged within each chapter. The majority of data presented in this thesis was the result of a research group based at CEH. The role of individual authors in each experimental chapter of this thesis are as follows:-

Chapter 3: Marine Bacterial Communities are Resistant to Elevated Carbon Dioxide Levels

Samples collected by L. Newbold and A. Whiteley. Samples processed and analysed by L. Newbold under the supervision of A. Oliver. Statistical analysis was carried out under the supervision of C. van der Gast. Paper was written by L. Newbold with edits by A. Oliver, A. Whiteley and C. van der Gast.

Chapter 4: The Response of Marine Picoplankton to Ocean Acidification

Samples collected by L. Newbold, A. Whiteley and M. Maguire. Statistical analysis was carried out under the supervision of C. van der Gast. Samples processed and analysed by L. Newbold under the supervision of A. Oliver. All phylogenetics performed by L. Newbold. Bioinformatics advice and analysis was provided by T. Booth, B. Tiwari, T. DeSantis and G. Andersen. Paper was written by L. Newbold with edits by A. Oliver, A. Whiteley and C. van der Gast.

Chapter 5: Active Bicarbonate and Glucose Picoplankton Communities Under Elevated CO₂

Samples collected by L. Newbold and A. Whiteley. Samples processed and analysed by L. Newbold under the supervision of A. Oliver. Statistical analysis was carried out by L. Newbold under the supervision of C. van der Gast. Paper was written by L. Newbold with edits by A. Oliver and C. van der Gast.

Chapter 6: The Mamiellales: Strategies for Nutrient Acquisition under Elevated CO₂

Samples collected by L. Newbold and A. Whiteley. Samples processed and analysed by L. Newbold. Assay development L. Newbold. Paper was written by L. Newbold with edits by A. Oliver.