Microbial Community Organisation and Functioning Under Ocean Acidification Conditions

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Presented by

Lindsay K. Newbold

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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.

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Table of Contents

List of Figures

List of Tables

Supplementary Information

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 x10^{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

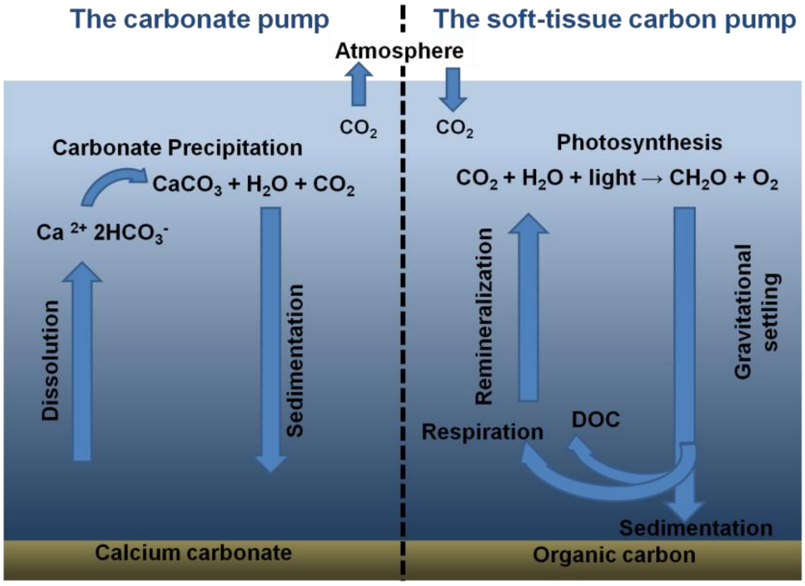

Figure 1.1: Simplified diagram of the biological pump. The biological pump can be split into the soft tissue carbon pump and the carbonate pump. The soft-tissue pump refers to the process by which autotrophically fixed carbon is exported to depth through gravitational settling of particles. Here it is respired through microbiological breakdown, incorporated into sediments or remineralized. In contrast, the calcium carbonate pump is driven by the use of $CaCO_3$ (calcium carbonate) and subsequent precipitation by marine organisms. Dissolution of $CaCO_3$ is driven by levels of saturation state (e.g. undersaturation, leads to increased dissolution to compensate), consequentially leading to CO_2 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 mortaility can be attributed viruses, suggesting viral induced mortailty 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).

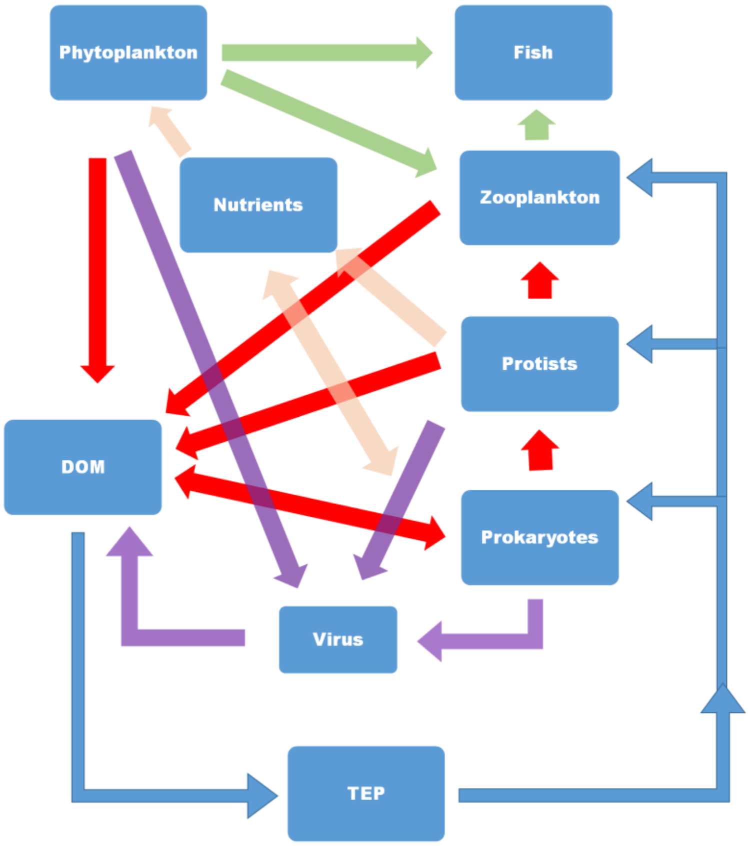

Figure 1.2: Simplified diagram of the pelagic food web. Four major pathways are illustrated, the classical food web (green), the microbial loop (red), the viral shunt (purple) and the abiotic loop (blue). Nutrient pathways are shown (pink). This figure and legend are adapted from Weinbauer *et al.* (2011).

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 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 virioplankton. 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

(Whitman, 2009). However, 'pico' sized eukaryotes are routinely detected by cultivation, epiflourescence microscopy and flow cytometry (Knight-Jones and Walne, 1951; Johnson and Sieburth, 1982; Olson *et al.*, 1985).

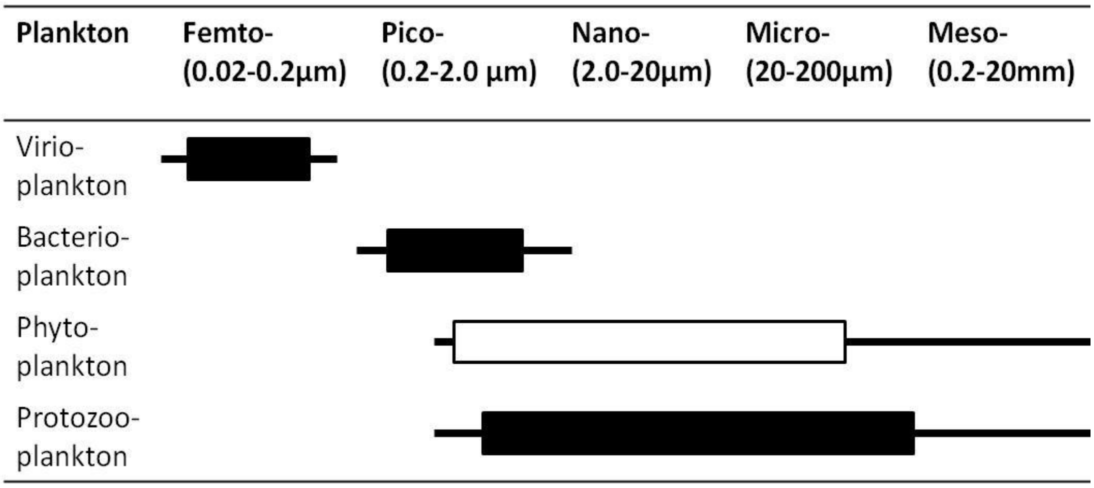

Figure 1.3: Distribution of planktonic taxonomic and trophic compartments, in differing size classes. Filled boxes represent heterotrophs and open represent autotrophs. Adapted from Sieburth *et al.* (1978) and Sherr and Sherr (2008).

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 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-García, 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 (Cavalier-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 singled 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

Mega-group	Super - group	First Rank	Second Rank	Third Rank	Notable Members
Diaphoretickes		**Haptophyta**	Prymnesiophyceae	Prymnesiales	*Imantonia*
	Archaeplastida	**Chloroplastida**	Chlorophyta	Mamiellophyceae◊	*Micromonas* *Bathycoccus* *Ostreococcus*
	Sar +	**Rhizaria**	Cercozoa	Chlorarachniophyta	*Minorisa minuta* †
		Stramenopiles	Pelagophyceae	Pelagomonadales	*Aureococcus* *Pelagococcus*
			Uncultured Marine Stramenopiles (MAST)‡		MAST 1 MAST 4 MAST 6 MAST 7
		Alveolata	Ciliophora	Intramacronucleata	*Prorodon* *Paramecium*
			Protalveolata	Syndiniales	*Amoebophrya* Novel Alveolates group 2 (NA II) *
				Novel Alveolates group 1 (NAI)*	
			Dinoflagellata	Dinophyceae	*Pfiesteria*
			Apicomplexa	Aconoidasida	*Plasmodium*
				Conoidasida	*Cryptosporidium*

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) ◊, del Campo *et al.* (2013) †,Massana *et al.* (2004); Massana *et al.* (2006) ‡ and Groisillier *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 Glaucophyta, Rhodophyceae (red algae), and Chloroplastida (green algae and plants). Some of the most abundant and ecologically important photosynthetic picoeukaryotes fall within the Chloroplastida class Mamiellophyceae (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 prasinos*, 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 *Phaecocystis 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).

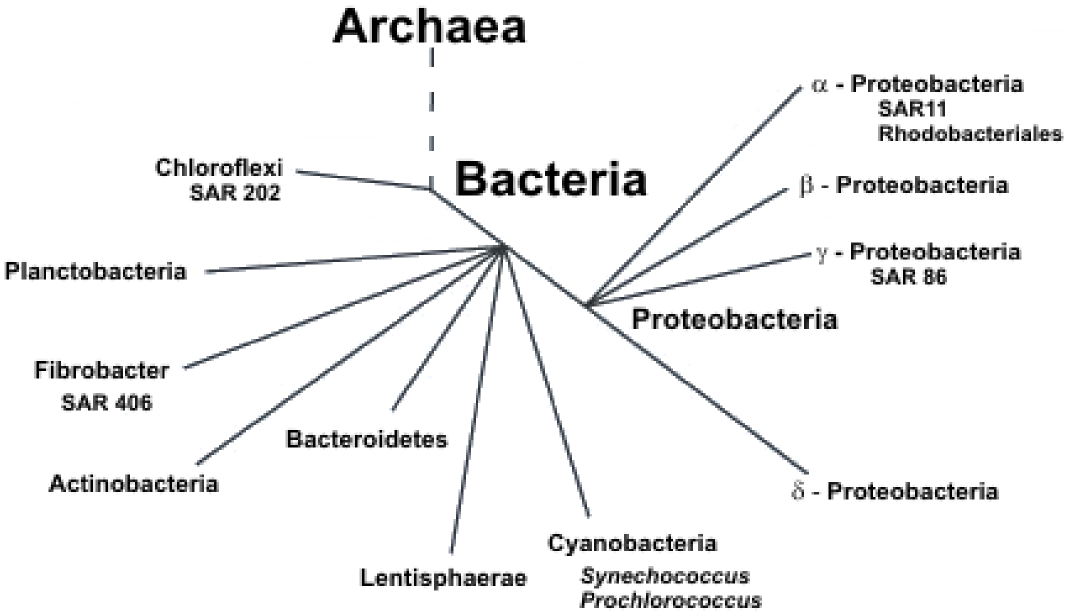

Figure 1.4: Schematic illustration of the phylogenetic position of major marine bacterial taxa. Figure adapted from Giovannoni and Stingl (2005). Taxa relevant to this study have been included.

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 *Sulfitobacter*. 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;

Gonzalez *et al.*, 2000; Malmstrom *et al.*, 2007). 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 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

Gas	**Pre-1750 trophospheric concentration**	**Recent trophospheric concentration**	**Percentage increase since 1750**
Carbon Dioxide (CO_2)	280 parts per million (ppm)	400 (ppm)	40.2%
Methane (CH_4)	700 parts per billion (ppb)	1874/1758 (ppb)	167.7/151.1%
Nitrous Oxide (N_2O)	270 (ppb)	324/323 (ppb)	20/19.6%

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 CO_2 and 2011 for CH_4 and N_2O).

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 (CO_2), methane (CH_4) and nitrous oxide (N_2O) 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 CO_2, CH_4 and N_2O 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 CO_2 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 CO_2 concentration could range between 541 and 970 ppm (IPCC, 2000; IPCC, 2007; IPCC, 2013). In 2013, readings taking for CO_2 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 CO_2 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 CO_2 the scale of which has been overlooked until recently, that of ocean acidification (OA).

1.4.2 *Ocean acidification*

Since industrialisation, global CO_2 emissions have increased and as a result a greater understanding of the relationship between rising atmospheric CO_2, 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_2CO_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

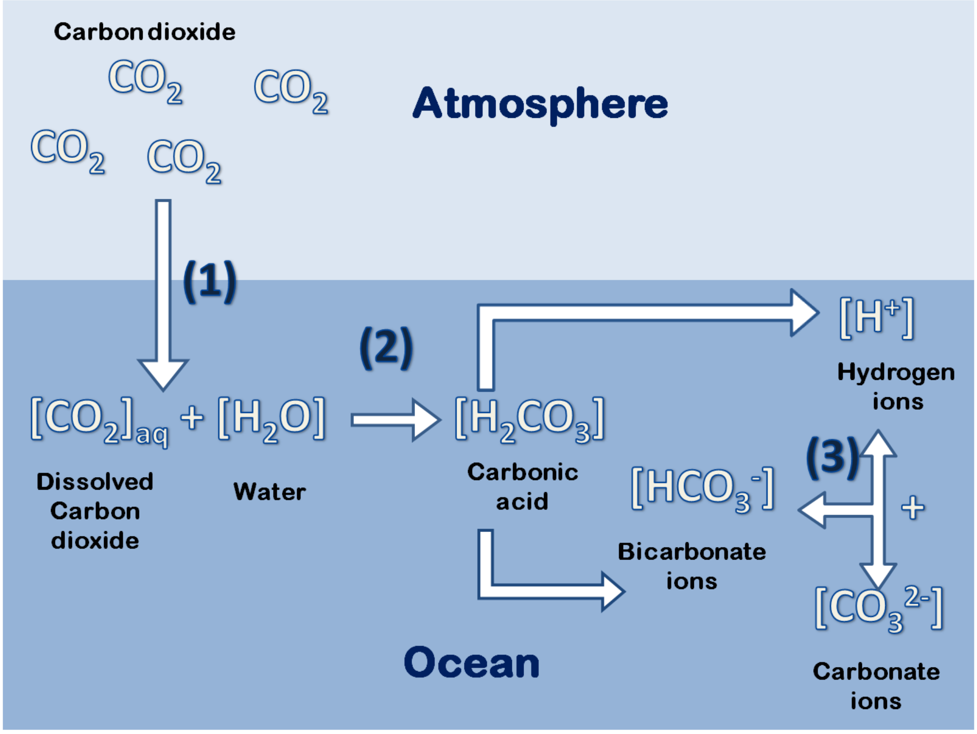

Figure 1.5: Summary of the chemical processes involved in ocean acidification. Atmospheric carbon dioxide (CO_2) dissolves in seawater (1). Dissolved CO_2 reacts with water (H_2O) to form carbonic acid (H_2CO_3), a weak acid (2). H_2CO_3 dissociates to bicarbonate ions (HCO_3^-) and hydrogen ions (H^+) an excess of which combine with carbonate ions (CO_3^{2-}) to form more bicarbonate ions (HCO_3^-)(3). As a result there is a net increase in dissolved carbon dioxide, carbonic acid, bicarbonate ions and hydrogen ions, but a decrease in bio-available carbonate ions. Since pH is determined by the negative log of the activity of hydrogen ions, increases in seawater hydrogen ion concentration will lead to a decrease (or acidification) of oceanic pH. Chemistry taken from Joint *et al.* (2011).

(Sabine *et al.*, 2004) 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 20th 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 juvenille 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 sebsequent CO_2 excreation 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 5×10^5 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 CO_2 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.

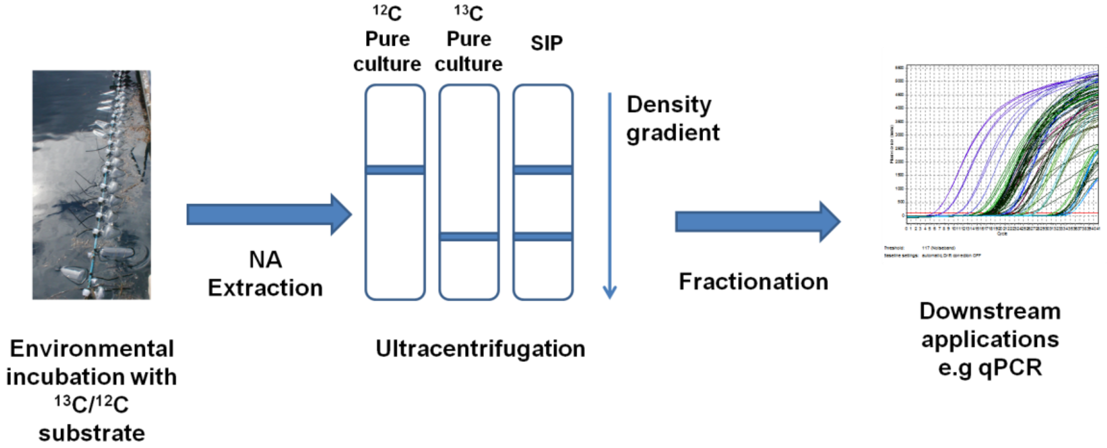

Figure 1.6: Graphical outline of a nucleic acid stable isotope probing (SIP) experiment. Environmental community is incubated with desired stable isotope substrate or control and either DNA or RNA extracted. Extract separated by ultracentrifugation, across either a Caesium chloride (CsCl) or Caesium 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.

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* *al.*, 2012). 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 C_q) (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 CO_2 Enrichment studies (PeECE I-III), these studies set out to: test the validity of laboratory based studies, examine CO_2 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. Particpiants 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 CO_2 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 (0.2-2.0µm) respond to elevated CO_2?

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 CO_2. 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 CO_2. 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 CO_2 and glucose within the resident prokaryote and eukaryote communities.

Aim 4: Is it possible to detect functional shifts in key picoeukaryotes response to elevated CO_2?

Elevated CO_2 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 CO_2 upon the function of the dominant picoeukaryotes *Micromonas* and *Bathycoccus*.

1.7 References

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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, 60.3ºN, 5.2 ºE 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 ^{15}N nitrate as opposed to ^{14}N in other enclosures. Mesocosm enclosures were exposed to two initial CO_2 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 $CO_{2\ (g)}$ 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 CO_2 concentrations in the high CO_2 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}C or ^{13}C glucose (50mg/L) or sodium bicarbonate (0.15g/L). These microcosms were submerged under surface fjord water and incubated *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.

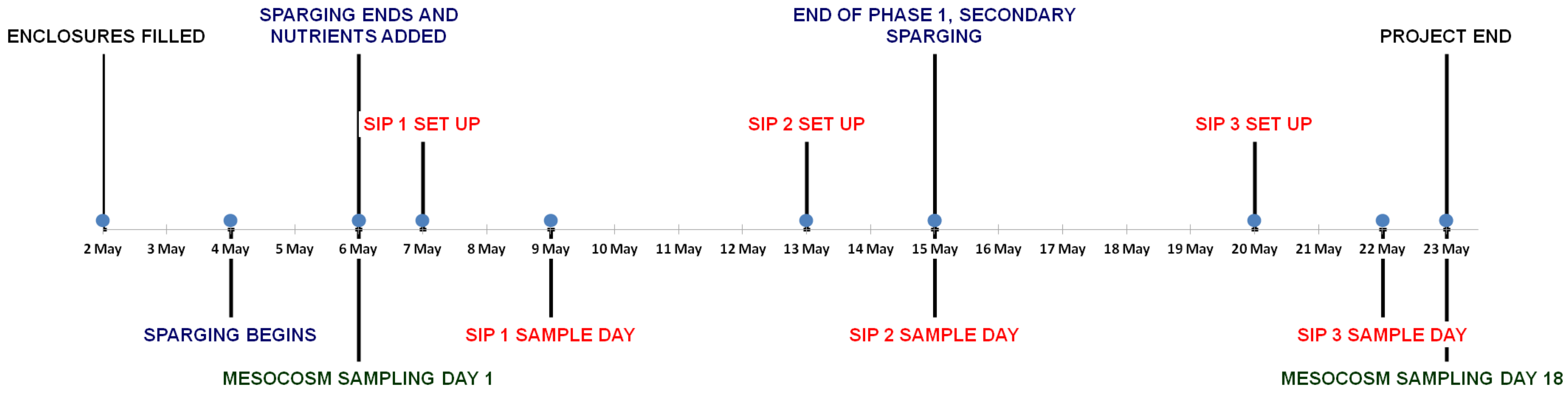

Figure 2.2: Timeline of experimental manipulations within the 2006 Bergen mesocosm experiment.

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

Running title: Marine Bacterial Communities and Elevated CO_2

Lindsay K. Newbold[1,2†], Anna E. Oliver[1†], Andrew S. Whiteley[3], and Christopher J. van der Gast[1*]

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

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

[3]School 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 consistant with the submitted publication. Figures have been placed within the text and online supplimentary data included listed as supplimentary 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_2CO_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 CO_2 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 CO_2 in a replicated temporal seawater mesocosm experiment. Specifically, using culture independent methods, we examined bacterial community turnover, composition, structure, and abundance under elevated CO_2 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 CO_2), 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 pCO_2, being autocorrelated as expected ($\mathrm{pH} = a - b \log pCO_2$ [$r^2 = 0.99$; $F_{1,100} = 2560.2$; $P < 0.0001$]). Measurement and analyses of the physical and chemical parameters within the mesocosms revealed that only pCO_2, pH and total inorganic dissolved (TID) carbon were significantly different between treatments (figure S3.7.1); where pCO_2 and TID carbon were significantly higher and, conversely, pH was significantly lower in mesocosms under elevated CO_2 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 x 10^6 ± 1.03 x 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 x 10^6 ± 9.79 x 10^5 cells ml^{-1}. The temporal patterns of mean

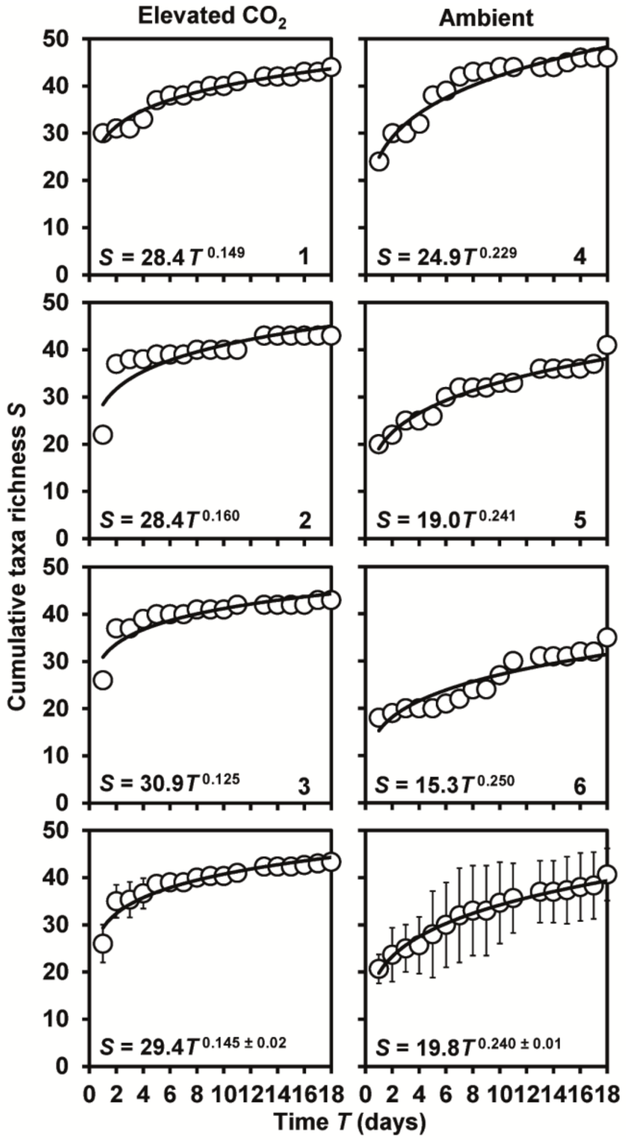

Figure 3.1: The taxa-time relationships (TTRs) for bacterial communities in mesocosms under elevated CO_2 and ambient conditions. Given are the TTR for each mesocosm (A to F) and the mean TTR by treatment. Error bars represent the standard deviation of the mean (n = 3). Also given are the taxa-time power 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 ± 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 CO_2 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, $S_{\mathrm{SOR}} = cD^d$, where S_{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 CO_2 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 CO_2 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 pCO_2 concentrations (table 3.2).

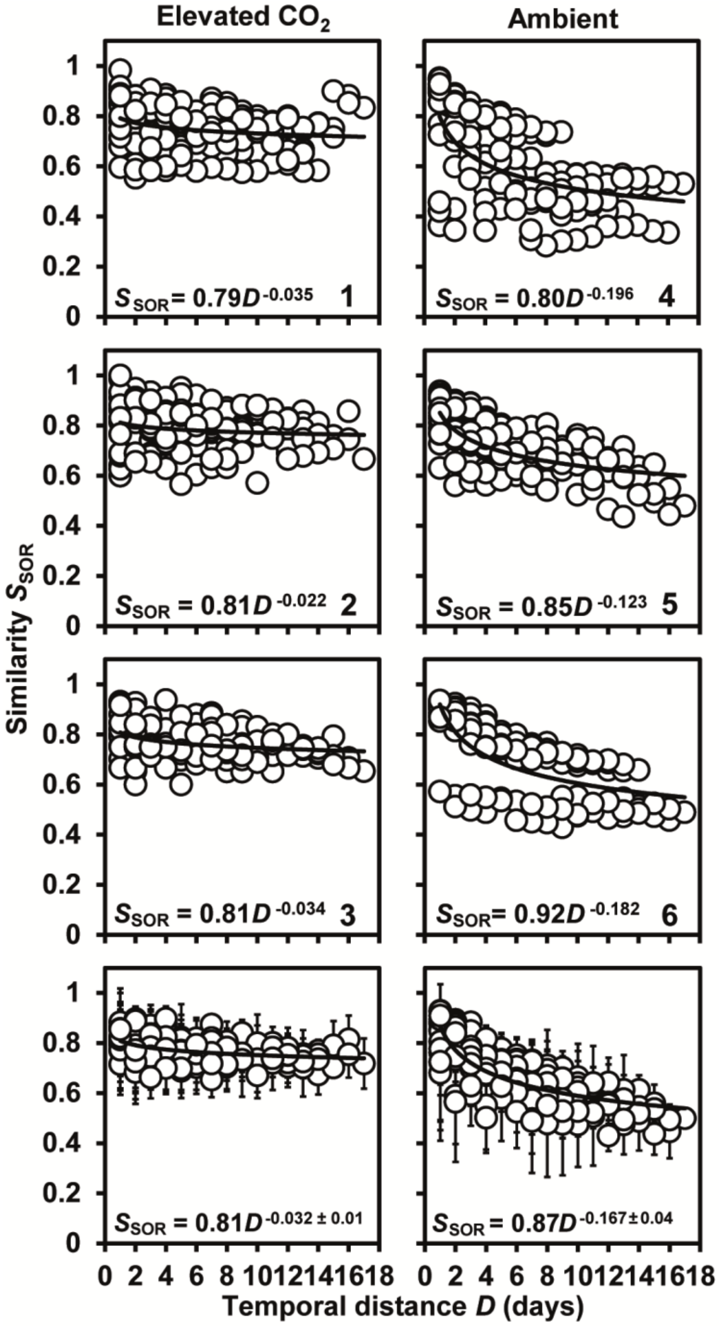

Figure 3.2: The distance-decay of bacterial community similarity (Sørensen index (S_S)) in mesocosms under elevated CO_2 and ambient conditions over time. Given are the distance-decay relationships (DDR) for each mesocosm (1 to 6) and the mean DDR by treatment. Error bars represent the standard deviation 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.

A

Treatment		**Elevated CO$_2$**			**Ambient**		
	Mesocosm	**1**	**2**	**3**	**4**	**5**	**6**
Elevated CO$_2$	*1*		0.72	0.25	<0.0001*	<0.0001*	0.002*
	2	0.36		0.30	0.03*	0.01*	0.03*
	3	1.18	1.06		0.0002*	<0.0001*	<0.0001*
Ambient CO$_2$	*4*	4.48	2.22	4.37		0.55	0.51
	5	5.94	2.71	5.25	0.60		0.76
	6	3.41	2.31	3.73	0.67	0.31	1

B

Treatment		**Elevated CO$_2$**			**Ambient**		
	Mesocosm	**1**	**2**	**3**	**4**	**5**	**6**
Elevated CO$_2$	*1*		0.49	0.98	<0.00001*	<0.00001*	<0.00001*
	2	0.69		0.43	<0.00001*	<0.00001*	<0.00001*
	3	0.02*	0.80		<0.00001*	<0.00001*	<0.00001*
Ambient CO$_2$	*4*	5.34	5.86	5.67		0.02*	0.67
	5	4.74	5.65	5.61	2.41		0.01
	6	6.53	7.30	7.29	0.43	2.63	

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 pCO_2 and pH were significantly autocorrelated in all mesocosms: Mantel statistic r = (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 pCO_2 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 CO_2 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 (CO_2 $b = -0.077 \pm 0.026$, and Ambient $b = -0.080 \pm 0.014$) no significant differences in community structure were observed by treatment (ANOVA: $F_{1,4} = 0.51$; $P = 0.514$).

Treatment	**Mesocosm**	***r*(*SD.C*)**	***P***	***r*(*SC.D*)**	***P***	***r*(*SD.p*)**	***P***	***r*(*Sp.D*)**	***P***
Elevated CO_2	*1*	-0.293	0.001*	0.226	0.995	-0.279	<0.0001*	0.217	0.994
	2	-0.199	0.002*	-0.007	0.473	-0.200	0.001*	0.003	0.515
	3	-0.325	0.001*	0.115	0.089	-0.333	<0.0001*	0.160	0.968
Ambient CO_2	*4*	-0.472	<0.0001*	0.331	0.999	-0.643	<0.0001*	0.241	0.997
	5	-0.421	<0.0001*	0.275	0.998	-0.538	<0.0001*	-0.052	0.273
	6	-0.510	<0.0001*	0.074	0.202	-0.769	<0.0001*	0.247	0.998

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).

	Elevated CO_2			**Ambient CO_2**		
Mesocosm	**1**	**2**	**3**	**4**	**5**	**6**
Time	34.71	30.32	23.48	34.00	49.51	40.54
Phosphate	20.76	17.41	19.92	22.39	30.89	24.02
Nitrate	16.71	-	-	17.70	-	19.47
Undetermined	27.82	52.26	56.60	25.91	19.61	15.97

Table 3.3: Canonical correspondence analyses for determination of percent variation in bacterial communities in mesocosms under elevated CO_2 or ambient conditions by environmental variables and time.

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 ºC 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 (pCO_2), 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 FACSortTM 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 ^{o}C followed by 30 cycles of 94 ^{o}C for 1 minute, 52 ^{o}C for 1 minute, and 72 ^{o}C for 3 minutes and a final extension phase of 10 minutes at 72 ^{o}C. 20 µL of gel purified PCR product was digested for 4 hours at 37 ^{o}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^{TM} (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 (http://folk.uio.no/ohammer/past) run by Øyvind Hammer. The *t*-distribution method was used to compare the regression line slopes generated from the taxa-time and distance-decay relationship analyses as described previously (Fowler *et al.*, 1998).

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 (pCO_2, 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; $\alpha < 0.05$) 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 ($\log^{10}$ transformed) on the *y*-axis. For each plot a linear regression model was fitted, represented by the equation, $\log^{10} y = a + bx$, 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).

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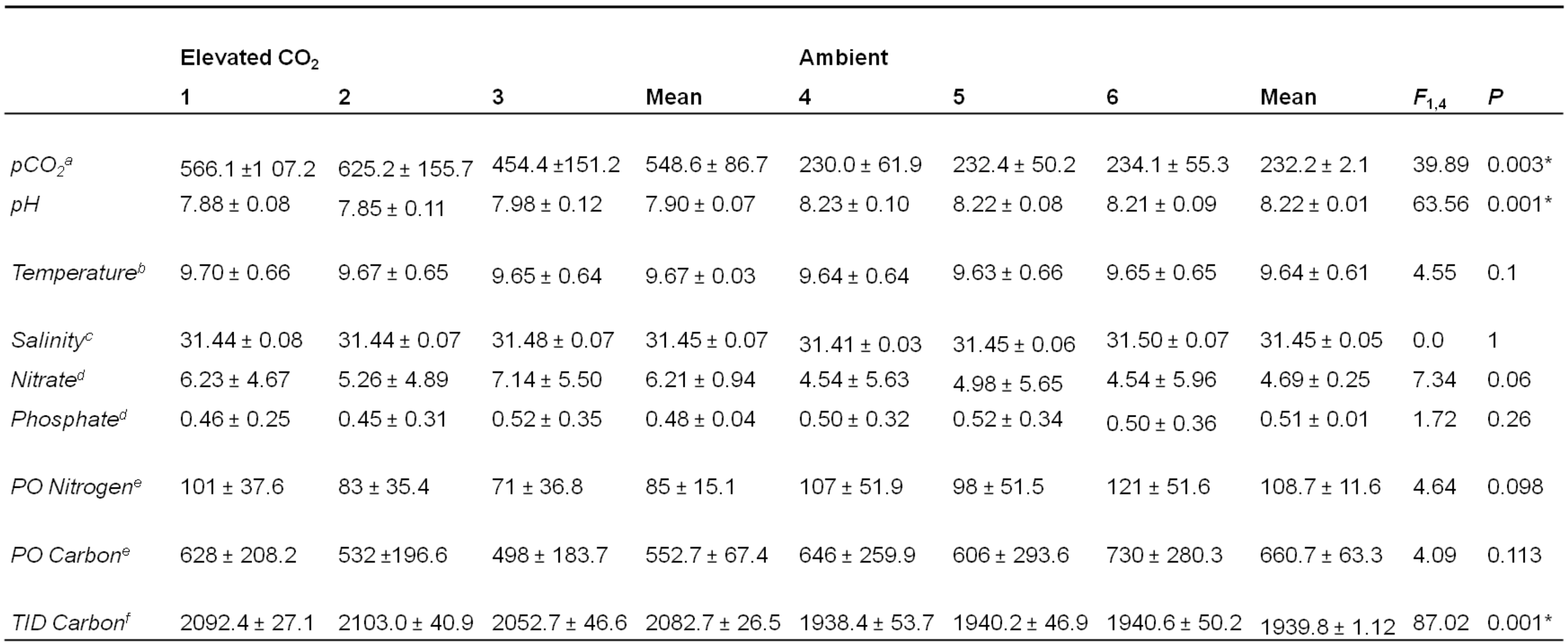
3.7 Supplementary Information



	Elevated CO_2				Ambient					
	1	2	3	Mean	4	5	6	Mean	$F_{1,4}$	*P*
pCO_2[a]	566.1 ±1 07.2	625.2 ± 155.7	454.4 ±151.2	548.6 ± 86.7	230.0 ± 61.9	232.4 ± 50.2	234.1 ± 55.3	232.2 ± 2.1	39.89	0.003*
pH	7.88 ± 0.08	7.85 ± 0.11	7.98 ± 0.12	7.90 ± 0.07	8.23 ± 0.10	8.22 ± 0.08	8.21 ± 0.09	8.22 ± 0.01	63.56	0.001*
Temperature[b]	9.70 ± 0.66	9.67 ± 0.65	9.65 ± 0.64	9.67 ± 0.03	9.64 ± 0.64	9.63 ± 0.66	9.65 ± 0.65	9.64 ± 0.61	4.55	0.1
Salinity[c]	31.44 ± 0.08	31.44 ± 0.07	31.48 ± 0.07	31.45 ± 0.07	31.41 ± 0.03	31.45 ± 0.06	31.50 ± 0.07	31.45 ± 0.05	0.0	1
Nitrate[d]	6.23 ± 4.67	5.26 ± 4.89	7.14 ± 5.50	6.21 ± 0.94	4.54 ± 5.63	4.98 ± 5.65	4.54 ± 5.96	4.69 ± 0.25	7.34	0.06
Phosphate[d]	0.46 ± 0.25	0.45 ± 0.31	0.52 ± 0.35	0.48 ± 0.04	0.50 ± 0.32	0.52 ± 0.34	0.50 ± 0.36	0.51 ± 0.01	1.72	0.26
PO Nitrogen[e]	101 ± 37.6	83 ± 35.4	71 ± 36.8	85 ± 15.1	107 ± 51.9	98 ± 51.5	121 ± 51.6	108.7 ± 11.6	4.64	0.098
PO Carbon[e]	628 ± 208.2	532 ±196.6	498 ± 183.7	552.7 ± 67.4	646 ± 259.9	606 ± 293.6	730 ± 280.3	660.7 ± 63.3	4.09	0.113
TID Carbon[f]	2092.4 ± 27.1	2103.0 ± 40.9	2052.7 ± 46.6	2082.7 ± 26.5	1938.4 ± 53.7	1940.2 ± 46.9	1940.6 ± 50.2	1939.8 ± 1.12	87.02	0.001*

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 ^{o}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}.

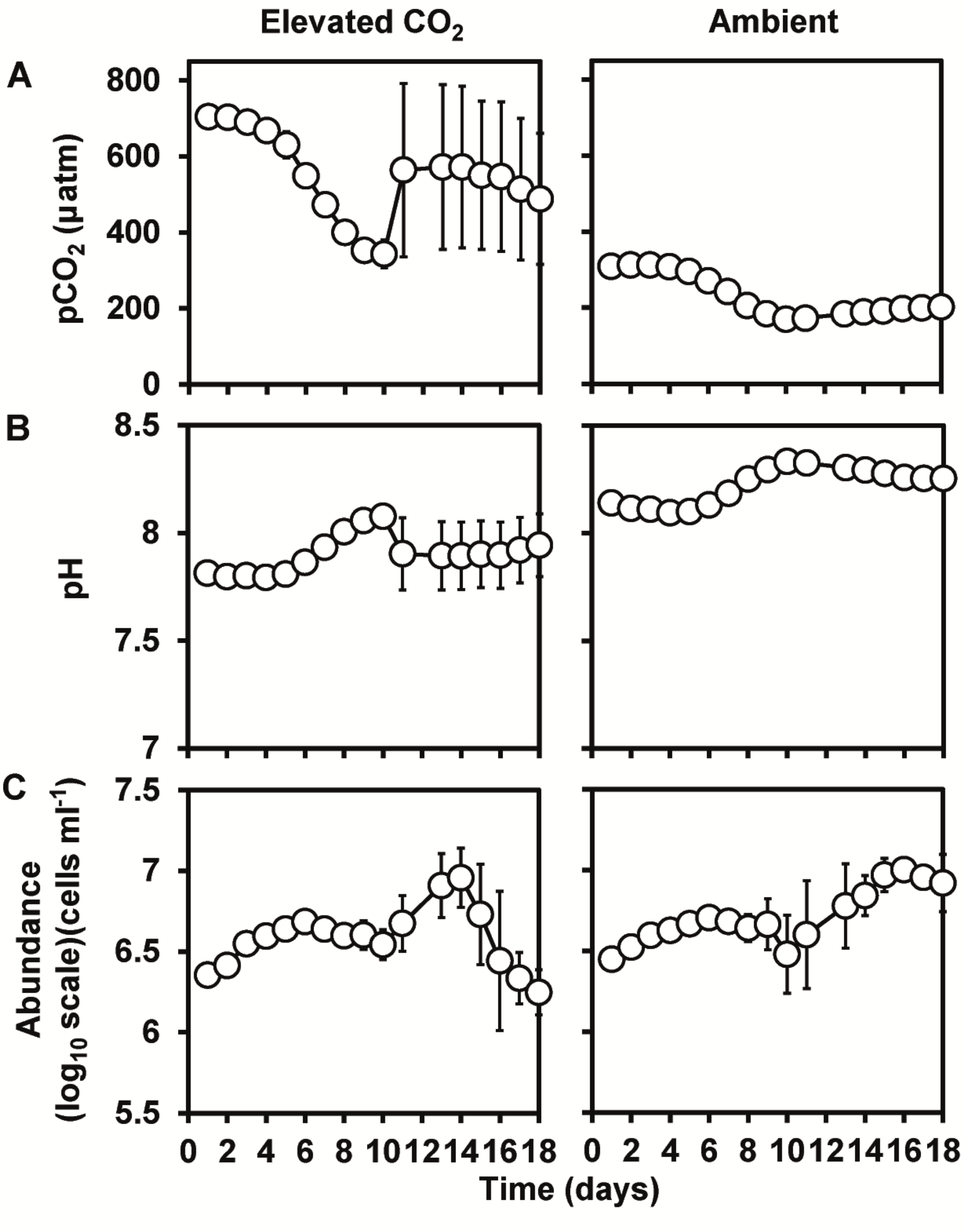


Figure S3.7.2: Changes in mean (A) pCO_2 concentration, (B) pH, and (C) bacterial abundance in mesocosms under elevated CO_2 and ambient conditions. Error bars represent the standard deviation of the mean ($n = 3$). Figure adapted from data first presented in Hopkins *et al.* (2010).

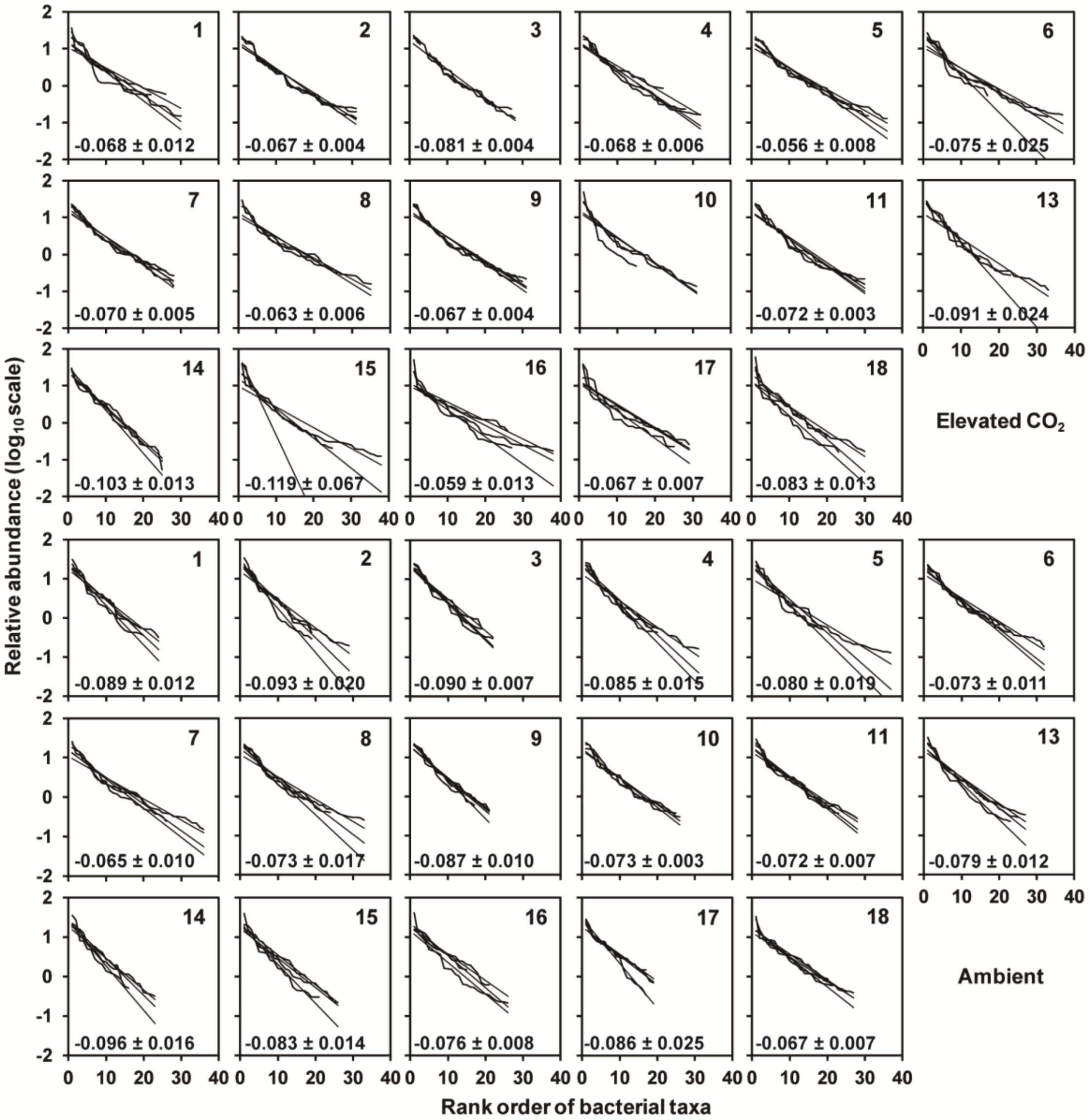


Figure S3.7.3: Changes in bacterial community structure in elevated CO$_2$ and ambient mesocosms visualised using rank-abundance plots over time (days). Given are replicate plots and the mean slope values plus the standard deviation of the mean (n = 3) for each time point within each treatment. All regression 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 consistant with the accepted publication. Figures have been placed within the text and online supplimentary data included with the original publication listed as supplimentary 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 CO_2, ocean biogeochemistry and the populations therein, has been raised (Caldeira and Wickett, 2003; Cicerone *et al*., 2004; Feely *et al*., 2004; Orr *et al*., 2005). Specifically, CO_2 released into the atmosphere dissolves in seawater and reacts to form carbonic acid (H_2CO_3), the dissociation of which forms hydrogen and bicarbonate ions (H^+ and HCO_3^-). An increase in the concentration of hydrogen ions results in a drop in oceanic pH, a process termed 'ocean acidification' (OA), since the ocean's buffering capacity is only able to neutralize some of this additional CO_2 (Sabine *et al*., 2004). A decrease in seawater pH and carbonate saturation is set to continue as long as excess CO_2 enters the atmosphere (Brewer *et al*., 1997; Feely *et al*., 2004). Currently, a pH change in the region of 0.3-0.4 units is predicted by the end of the century (Caldeira *et al*., 2007; Feely *et al*., 2008). Consequently, there is significant interest in how these pH changes will affect the oceans biota and integral processes (Fabry *et al*., 2008; Guinotte and Fabry, 2008; Doney *et al*., 2009; Kerr, 2010; Sabine and Tanhua, 2010).

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

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

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

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

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

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, CO_2 and pH were significantly autocorrelated throughout the experiment (Regression analysis: $r^2 = 0.99$; $F_{1,34} = 45963.7$; $P < 0.0001$) and the mean CO_2 levels were significantly higher (ANOVA: $r^2 = 0.99$; $F_{1,34} = 145.1$; $P < 0.0001$) in the elevated CO_2 mesocosms (638.9 ± 125.9 µatm) when compared to the ambient mesocosms (246.3 ± 57.4 µatm). During phase one of the experiment the introduction of CO_2 into the mesocosms induced a change in pH from ~8.1 to ~7.8 (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 CO_2 and ambient condition mesocosms, rising to ~pH 8 and ~pH 8.3 respectively and in line with an uptake of excess carbon dioxide during photosynthesis. In the second phase of the experiment, following the second CO_2 amendment on day 10, the pH in acidified mesocosms was maintained between ~pH 7.8 and ~pH 7.9. Dissolved nitrate and phosphate fluctuated in overall concentration but remained relatively low in comparison to pre-bloom levels.

4.3.2 *Bacterial abundance and acidification*

In general, averaging all abundances for each mesocosm over the experiment indicated both experimental ($5.11 \times 10^6 \pm 2.75 \times 10^6$ cells per millilitre) and control mesocosms ($6.13 \times 10^6 \pm 2.31 \times 10^6$ 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).

Marked growth was shown by both the HNA and LNA bacteria, in parallel with

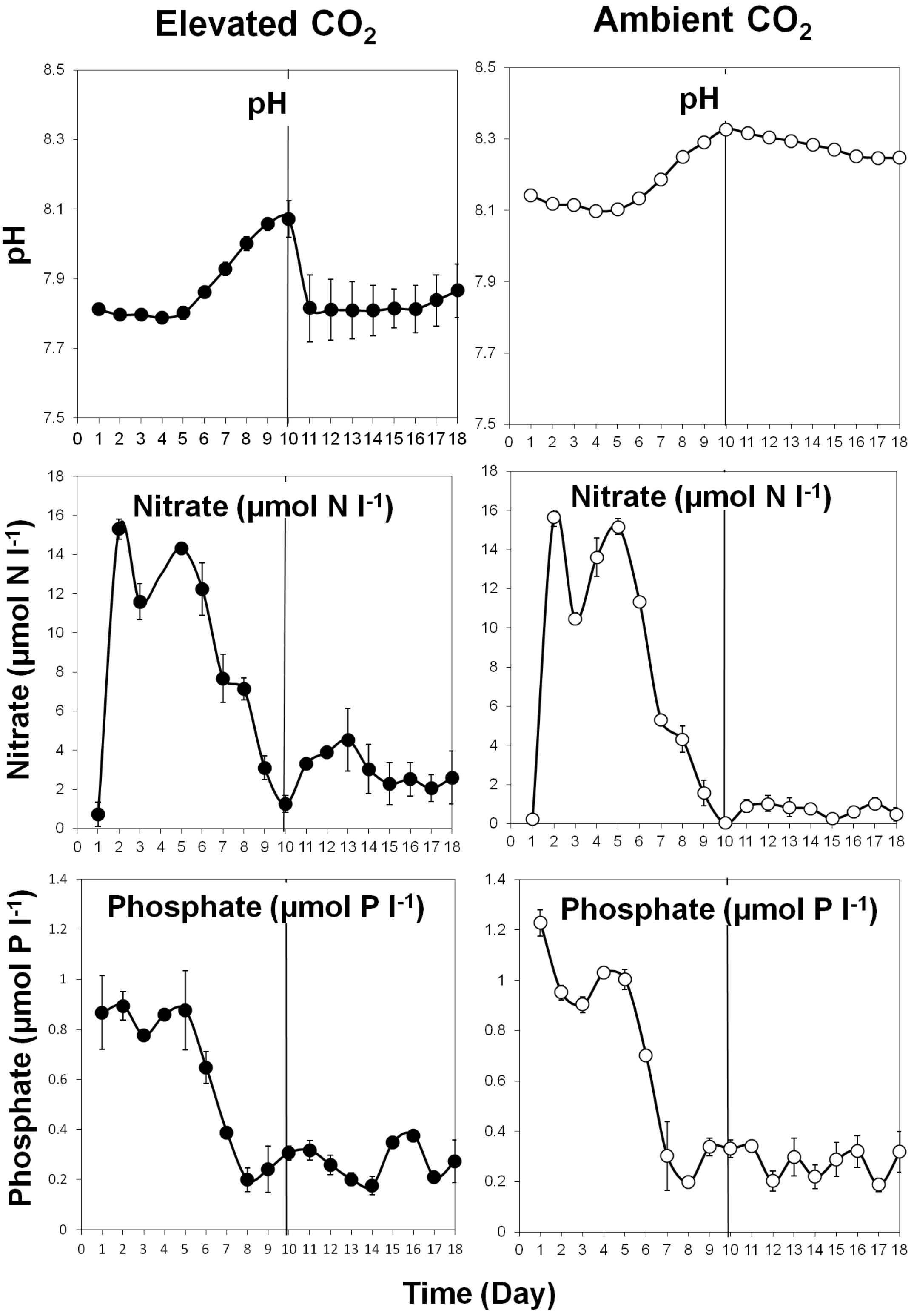


Figure 4.1: Mean daily nutrient and pH values for elevated 'High' CO_2 mesocosms (closed circle) and ambient mesocosms (open circle). Error bars represent standard deviation from the mean of the 2 replicate mesocosms. Solid vertical bar separates phases one and two. Nutrient data collected by I. Joint 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),

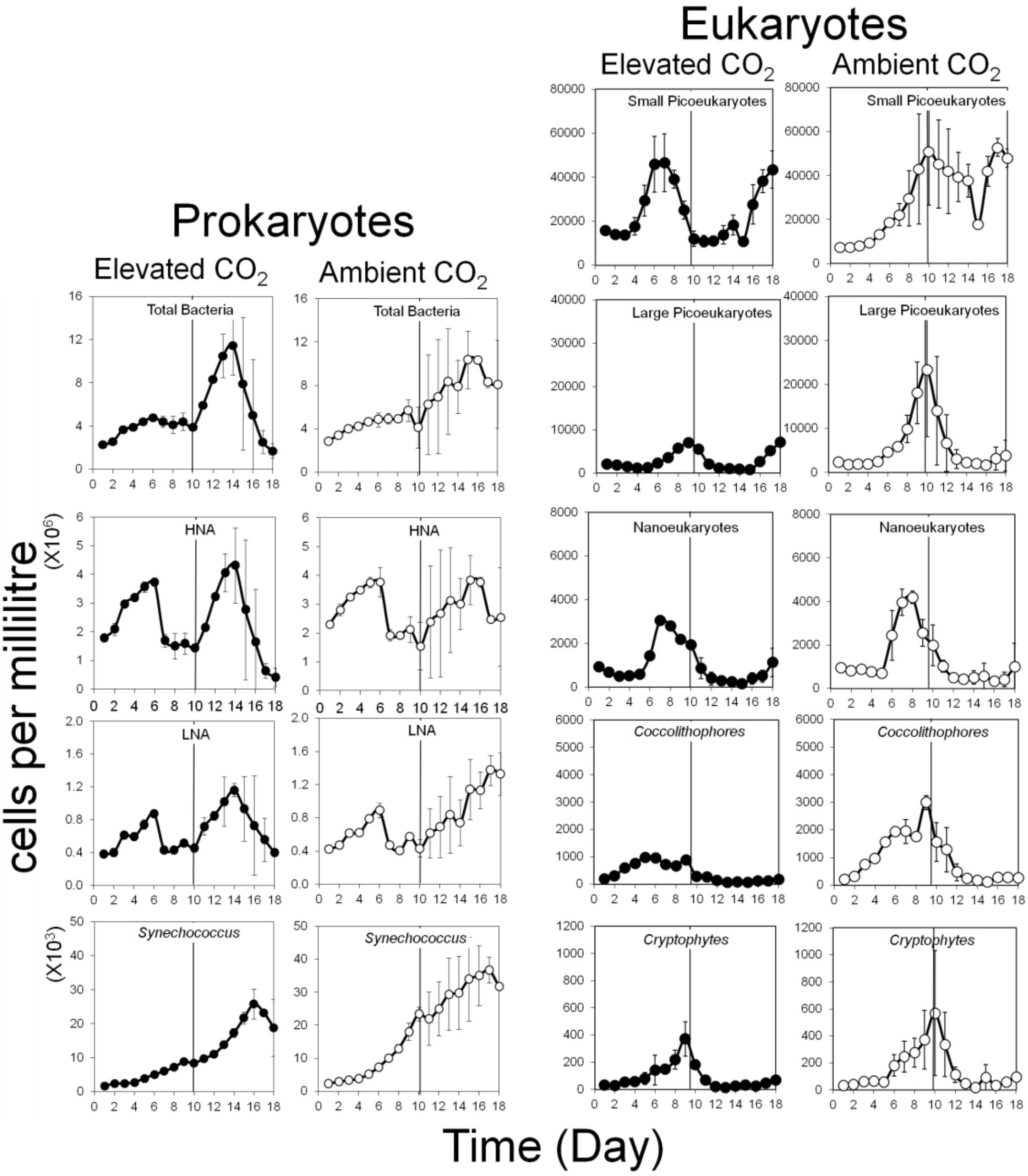


Figure 4.2: Mean daily FACS counts for elevated CO_2 mesocosms (closed circle) and ambient mesocosms (open circle). Prokaryotic groupings include total bacteria, High Nucleic Acid content bacteria (HNA), Low Nucleic Acid content bacteria (LNA) and *Synechococcus*. Eukaryotic groupings include small picoeukaryotes, 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 (~$1x10^7$ cells per millilitre) and day 15 for ambient (~1×10^7 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 (~1.7×10^6). Ambient cell counts remained comparatively high ~$8.1x10^6$. 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 2.5×10^3 cells per millilitre (day 1) to 2.5×10^4 cells per millilitre under elevated CO_2 (day 16) and 3.5×10^4 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 ± 337.5 cells per millilitre) when compared to ambient conditions (942.6 ± 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 (4.6×10^4 cells per millilitre) followed by a considerable reduction to 1.2×10^4 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 5.1×10^4 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.

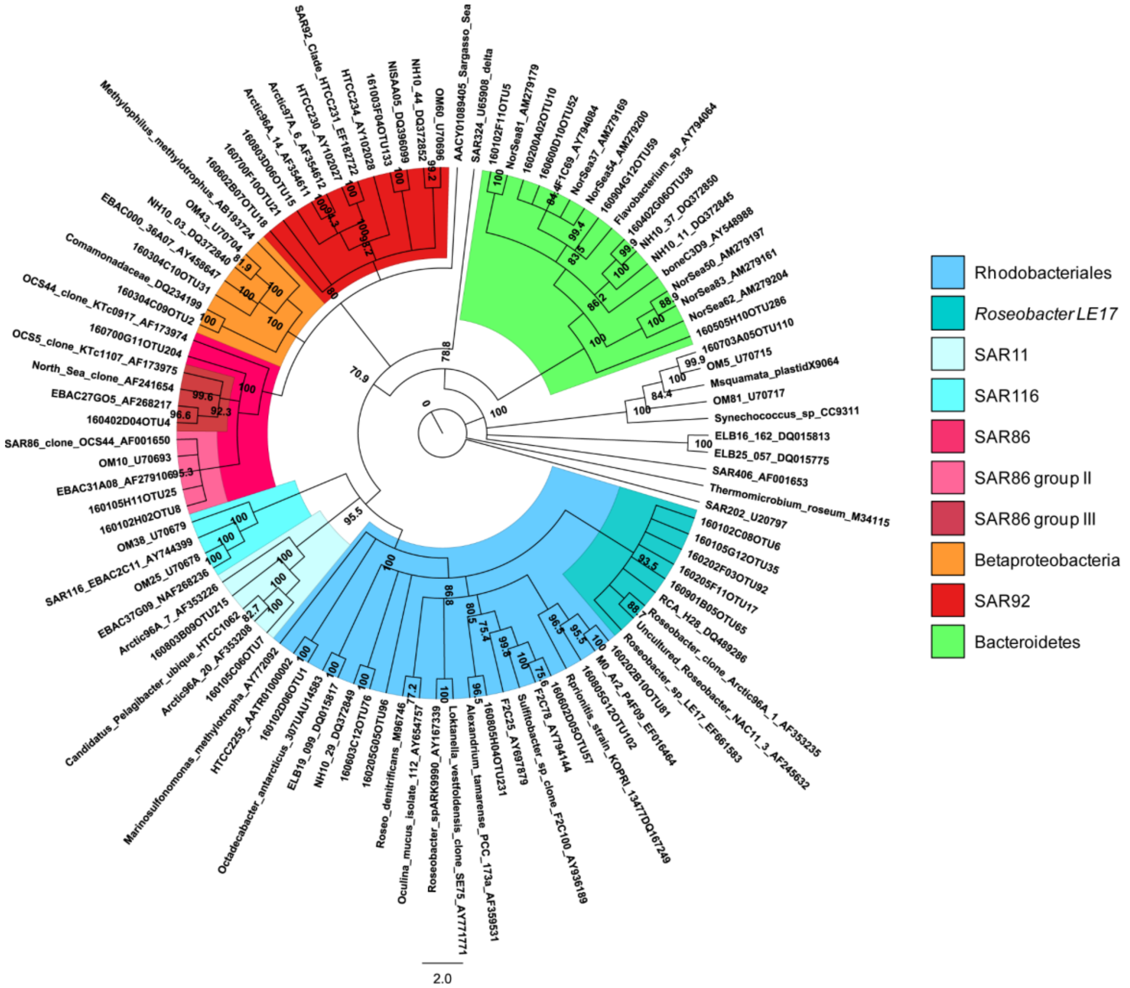


Figure 4.3a: A 70 % Majority Rule Consensus Tree of dominant prokaryotic OTUs as inferred by Neighbour Joining distance criterion under GTR+I+G model. Bootstrap support from 1000 replicates are shown at nodes. Phyla/sub-phyla are highlighted as follows: Blue = Alphaproteobacteria, Red = Gammaproteobacteria, Orange = Betaproteobacteria and Green = Bacteriodetes. For more specific taxonomic grouping refer to key.

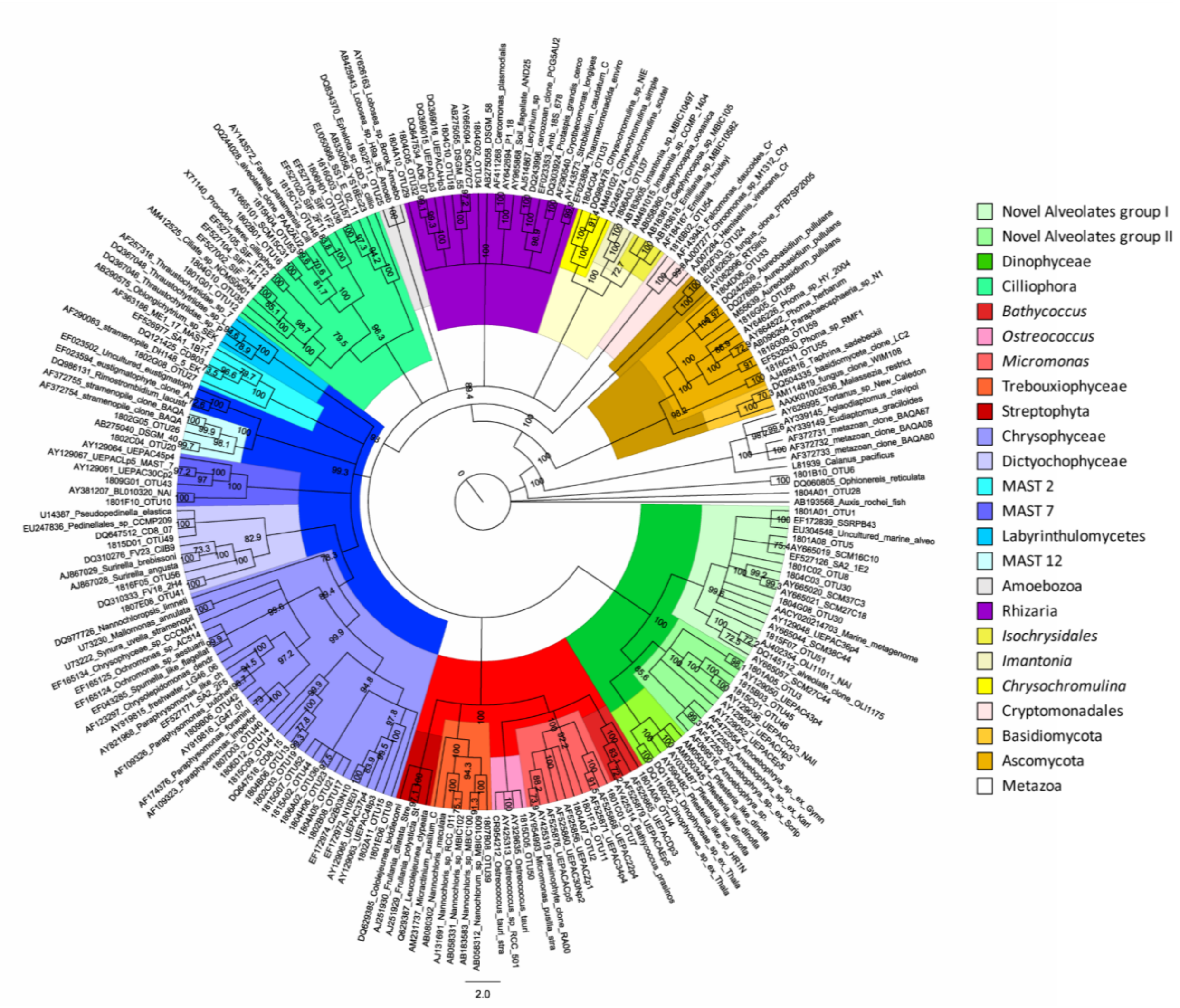


Figure 4.3b: A 70 % Majority Rule Consensus Tree of picoeukaryotic OTUs as inferred by Neighbour Joining distance criterion under GTR+I+G model. Bootstrap support from 1000 replicates are shown at nodes. Phyla/sub-phyla are highlighted as follows: Blue = Stramenopiles, Red = Archaeplastida, Purple = 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 costal 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.

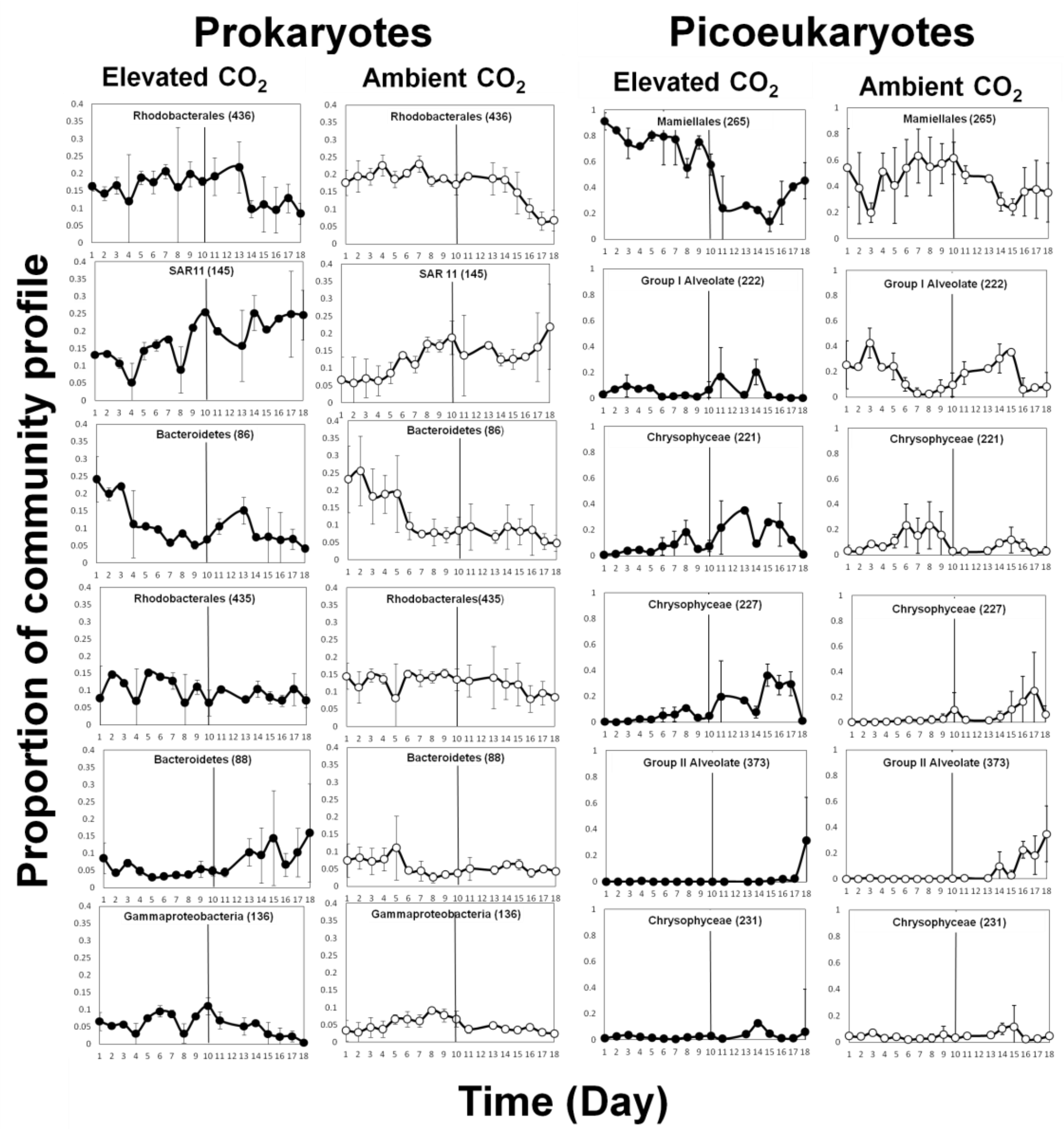


Figure 4.4: Picoplankton community change over time as assessed by T-RFLP. Community T-RFLP profiles were generated for all samples. Mean values for the 6 most abundant bacterial and picoeukaryotic T-RFs are shown for ambient (open circle) and elevated CO_2 mesocosms (closed circle). Solid vertical bar separates phases one and two.

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 CO_2. 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 CO_2 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 CO_2 experimental and 2 ambient CO_2 control mesocosms (2 m diameter, 3.5 m depth). Experimental mesocosm enclosures were filled with unfiltered native fjord water and gently sparged with CO_2 (750 µatm) for 2 days (4th-6th May) until a pH~7.8 was established. To control for sparging effects, ambient condition mesocosm enclosures were sparged with air. In order to simulate natural conditions more closely, a phytoplankton bloom was induced through the addition of nitrate and phosphate in all mesocosms on the 6th of May (initial concentrations: 1 µmol l^{-1} phosphate; 17 µmol l^{-1} nitrate). Blooming phytoplankton growth reduced CO_2 concentrations in the elevated CO_2 mesocosms, therefore mesocosm enclosures were re-acidified 10 days after mesocosm establishment (15/5/2006), and ambient condition enclosures again sparged with air. To isolate picoplankton daily samples of ~2 L of water were pre-filtered through Whatman GF/A (1-6 µm nominal pore size) filters to remove large eukaryote cells and filtrate collected onto 0.2 µm Durapore membranes. Sampling was initiated on the 6th 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 20th of May 2006) from both elevated and ambient CO_2 mesocosms (10 libraries, 4800 clones in total). Clones were sequenced using vector primers M13F and R at the NERC Biomolecular Analysis Facility-Edinburgh (NBAF-E). For the 18S SSU rRNA gene ninety six clones were randomly picked from each of 4 libraries corresponding to days 2, 8, 14 and 17 (7th, 13th, 19th and 22nd May 2006) from both elevated and ambient CO_2 mesocosms (8 libraries, 768 in total). Clones were sequenced using vector primers M13F and R and internal primer 3F (Giribet *et al*., 1996). Sequencing services were provided by Source BioScience LifeSciences (http://www.lifesciences.sourcebioscience.com/).

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

4.4.6 *OTU identification*

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

16S OTUs with 10 or more associated sequences and all 18S OTUs were realigned (as above) to include a selection of published sequences (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).

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4.7 Supplimentary Information

Eukaryotic Groups	**CO_2 Treatment**	**Mean**	**SD**	**$F_{1,34}$**	***P***
Small picoeukaryote	Elevated	24068	13154	1.29	0.264
	Ambient	29724	16539		
Large Picoeukaryotes	Elevated	2906	2188	4.01	0.053
	Ambient	6051	6293		
Nanoeukaryotes	Elevated	1031	891	0.73	0.399
	Ambient	1331	1197		
Coccolithophores	Elevated	411	337.5	6.15	0.018
	Ambient	942.6	844.2		
Cryptophytes	Elevated	89	92.6	2.12	0.155
	Ambient	150	151.8		

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 deviaition of the mean (SD), and the ANOVA tests results, including *F*-ratio and significance (*P*).

OTU ID	T-RFLP cut site	Phylogenetic ID	Reference sequence	Elevated CO_2	Ambient CO_2	Total Number of sequences
6	435-437	*Roseobacteria LE17*	160102C08	371	213	584
38	520	Bacteroidetes	160402G06	104	235	339
7	145	*Candidatus Pelagiobacter* (SAR11)	160105C06	134	126	260
8	136	SAR86-II	160102H02	133	117	250
1	435-437	Rhodobacteriales	160102D06	57	67	124
5	86-88	Bacteroidetes	160102F11	75	32	107
4	488	SAR86-III	160402D04	32	31	63
81	435-437	Rhodobacteriales	160202B10	17	39	56
25	136	SAR86-II	160105H11	15	31	46
21	142	Gammaproteobacteria	160700F10	14	17	31
31	495	Betaproteobacteria	160304C10	19	6	25
10	86-88	Flavobacteria	160200A02	10	12	22
17	435-437	*Roseobacteria LE17*	160205F11	12	8	20
2	492	Betaproteobacteria	160304C09	15	4	19
92	440	*Roseobacteria LE17*	160202F03	12	6	18
59	492	Bacteroidetes	160904G12	4	13	17
133	486	Gammaproteobacteria	161003F04	4	13	17
57	435-437	Rhodobacteriales	160602D05	8	7	15
110	483	Chloroplast OM5	160703A05	4	11	15
76	440	Alphaproteobacteria	160603C12	4	10	14
35	435-437	*Roseobacteria LE17*	160105G12	8	5	13
231	435-437	Alphaproteobacteria	160805H04	2	11	13
52	90	Flavobacteria	160600D10	5	7	12
18	496	SAR92	160602B07	2	9	11
65	435-437	*Roseobacteria LE17*	160901B05	5	6	11
204	488	Gammaproteobacteria	160700G11	5	6	11
286	86-88	Bacteroidetes	160505H10	6	5	11
15	486	SAR92	160803D06	3	7	10
96	435-437	Rhodobacteriales	160205G05	2	8	10
102	435-437	Rhodobacteriales	160805G12	3	7	10
215	145	*Candidatus Pelagiobacter* (SAR11)	160803B09	5	5	10

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.

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

3	376	*Novel Alveolate group II*	1801A05	1	2	3
10	383	*MAST 7 (Marine Novel Stramenopiles Group 7)*	1801F10	2	1	3
53	376	*Ciliophora*	1815H04	3		3
23	228	*Chrysophyceae*	1804H06	2	1	3
33	275	*Ascomycota*	1804D06	3	0	3
7	265	*Micromonas*	1801C01	2	0	2
57	350	*Ciliophora*	1816G03		2	2
40	232	*Chrysophyceae*	1807D03	1	1	2
42	231	*Chrysophyceae*	1809B06		2	2
52	227	*Chrysophyceae*	1815G07	2		2
31	376	*Chrysochromulina*	1804C04	2	0	2
29	285	*Rhizaria*	1804A10	2	0	2
55	275	*Ascomycota*	1816C11		2	2
59	275	*Ascomycota*	1816G09		2	2
51	119	*Novel Alveolate Group I*	1815F07	1		1
11	265	*Micromonas*	1801F12	1	0	1
43	233	*MAST 7 (Marine Novel Stramenopiles Group 7)*	1809G01		1	1
37	279	*Imantonia*	1806A03		1	1
6	223	*Metazoa (Echinodermata)*	1801B10	1	0	1
41	279	*Dictyochophyceae*	1807E08		1	1
49	274	*Dictyochophyceae*	1815D01	1		1
54	213	*Cryptomonadales*	1816B02		1	1
12	365	*Ciliophora*	1801G01	1	0	1
16	244	*Ciliophora*	1802B01	1	0	1
35	370	*Ciliophora*	1804G10	1	0	1
38	352	*Ciliophora*	1806H01		1	1
48	376	*Ciliophora*	1815C12	1		1
9	227	*Chrysophyceae*	1801E06	1	0	1

19	221	*Chrysophyceae*	1802C03	1	0	1
36	221	*Chrysophyceae*	1806A02		1	1
47	221	*Chrysophyceae*	1815C09	1		1
28	249	*Metazoa (Chordata)*	1804A01	1	0	1
32	188	*Cercozoa/Rhizaria*	1804C05	1	0	1
34	385	*Cercozoa/Rhizaria*	1804G02	1	0	1
58	275	*Ascomycota*	1816G05		1	1
25	218	*Amoebozoa*	1802F11	1	0	1

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.

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

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 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) and analysed on an Applied Biosystems 3730 DNA sequencer. Fragments were calculated and binned using Genemarker (Softgenetics). Briefly, bin widths were checked and manually adjusted to encompass all detected peaks. To differentiate signal from background Fluorescence Unit (FU) a threshold of 40 units was used to determine which T-RF's to include and subsequently a cut off of 20 FU's was used for a presence/absence binary matrix. All peaks were manually checked for inclusion in analysis. When required, relative abundance measures were calculated by dividing individual peak height by total peak height spanning all valid peaks within the analysis. In order to investigate community structure these data were ranked based upon total abundance, then change in the 6 most dominant peaks plotted over time. When required, e.g. to putatively identify T-RF fragments, the cut site position was determined by running unaligned non-chimeric sequences, trimmed to short amplicon primer region through T-RFLPmap (Field and Griffiths, 2008). The fragment length of specific clones was then cross correlated to this data to determine the identity of specific T-RF's in relation to clone sequences generated from the mesocosms.

Sequence assembly and quality assessment

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

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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 supplimentary data included listed as supplimentary 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 CO_2 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 CO_2. This study investigates the affect of elevated CO_2 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 CO_2 concentration, yet this response will likely be constrained by nutrient availability.

5.2 Introduction

Evidence for the link between industrialisation and increases in atmospheric CO_2 is undeniable (Petit *et al.*, 1999; Siegenthaler *et al.*, 2005; Luthi *et al.*, 2008), as is the evidence that excess CO_2 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 CO_2 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 CO_2?

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}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

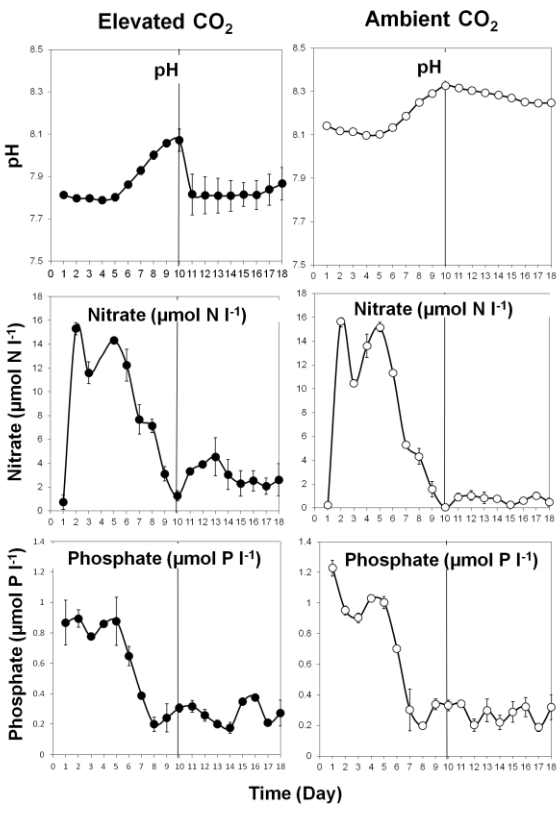


Figure 5.1: Mean daily nutrient and pH values for elevated ‘High’ CO_2 mesocosms (closed circle) and ambient mesocosms (open circle). Error bars represent standard deviation from the mean of the 2 replicate mesocosms. Solid vertical bar denotes the separation between phases one and two. Nutrient data collected by I. Joint , pH data first presented in Hopkins and colleagues (2010). Figure adapted from Newbold *et al*. 2012

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.

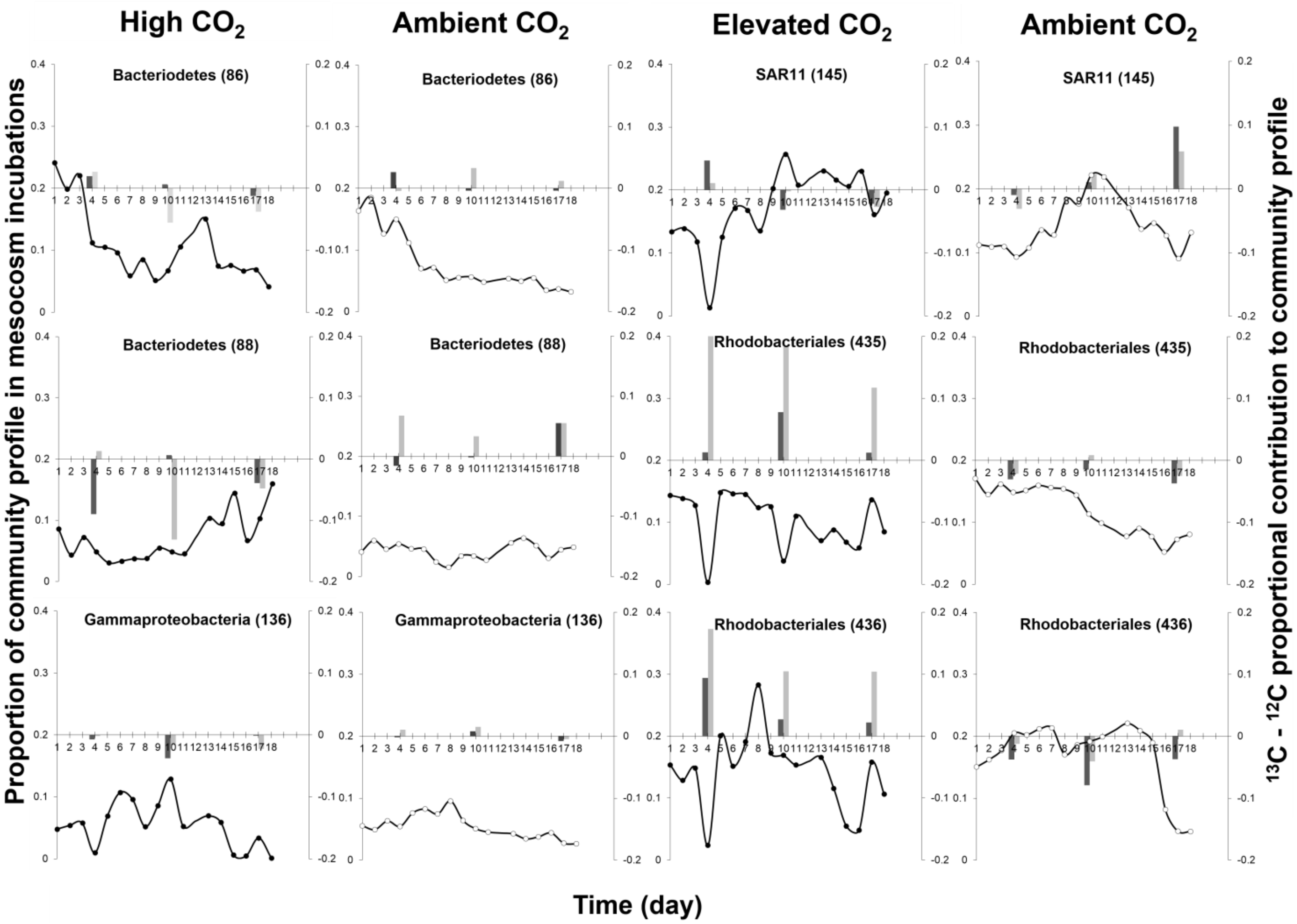
5.3.2 *Microcosm and mesocosm community composition*

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 CO_2), substrate (glucose or bicarbonate), time (day in mesocosm experiment, samples corresponded to days 4, 10 and 17) and isotope label (^{12}C or ^{13}C) (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


High CO$_2$
Ambient CO$_2$
Elevated CO$_2$
Ambient CO$_2$
Bacteriodetes (86)
Bacteriodetes (86)
SAR11 (145)
SAR11 (145)
Bacteriodetes (88)
Bacteriodetes (88)
Rhodobacteriales (435)
Rhodobacteriales (435)
Gammaproteobacteria (136)
Gammaproteobacteria (136)
Rhodobacteriales (436)
Rhodobacteriales (436)
Proportion of community profile in mesocosm incubations
^{13}C - ^{12}C proportional contribution to community profile
Time (day)
0
0.1
0.2
0.3
0.4
-0.2
-0.1
0
0.1
0.2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

5.2b

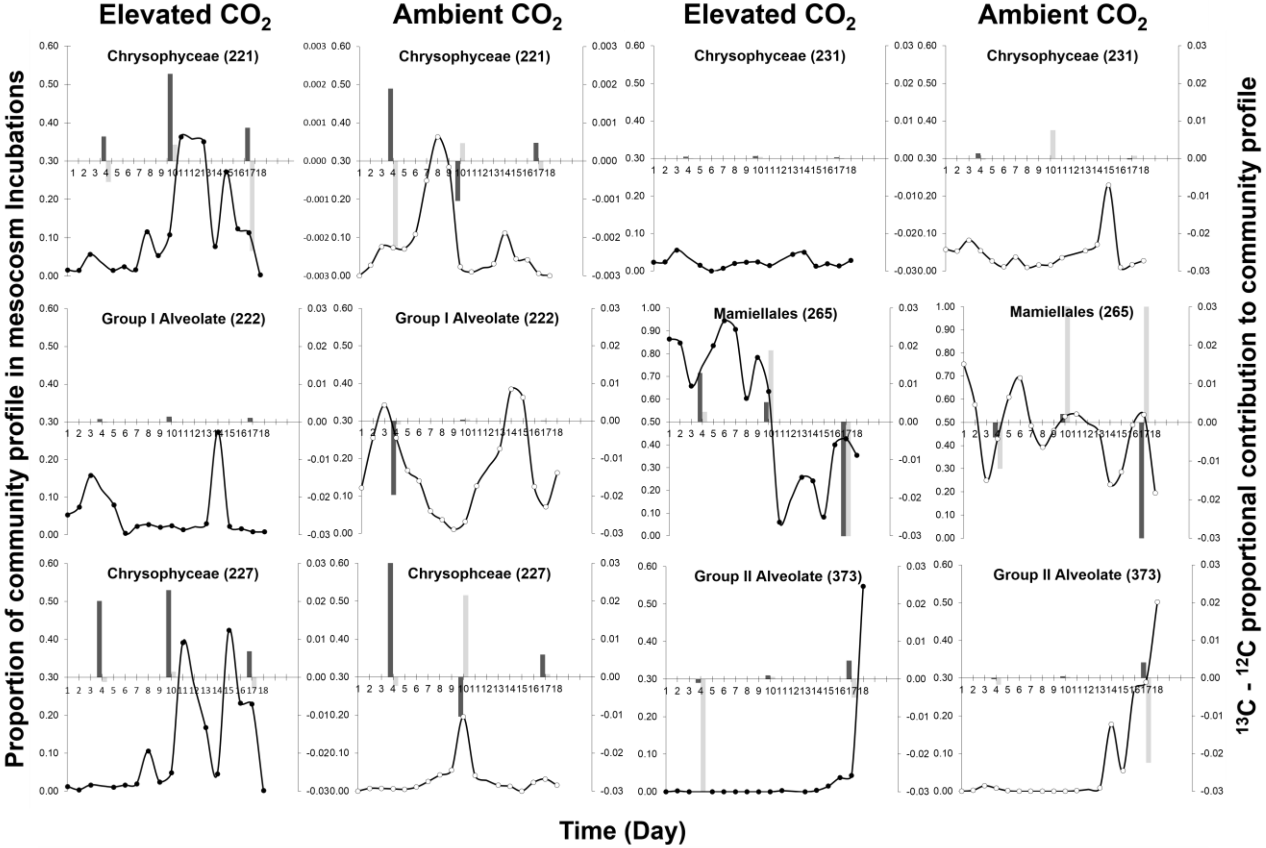


Figure 5.2: Changes in TRFLP abundance during mesocosm and stable isotope microcosm incubations, for dominant prokaryotes (5.2a) and picoeukaryotes (5.2b) over time. Changes for individuals within in mesocosm incubations (primary vertical axis) assessed by relative contribution to total T-RFLP profile 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, 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 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 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 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 Rhodobacteriales 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 chrysophyceae 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.

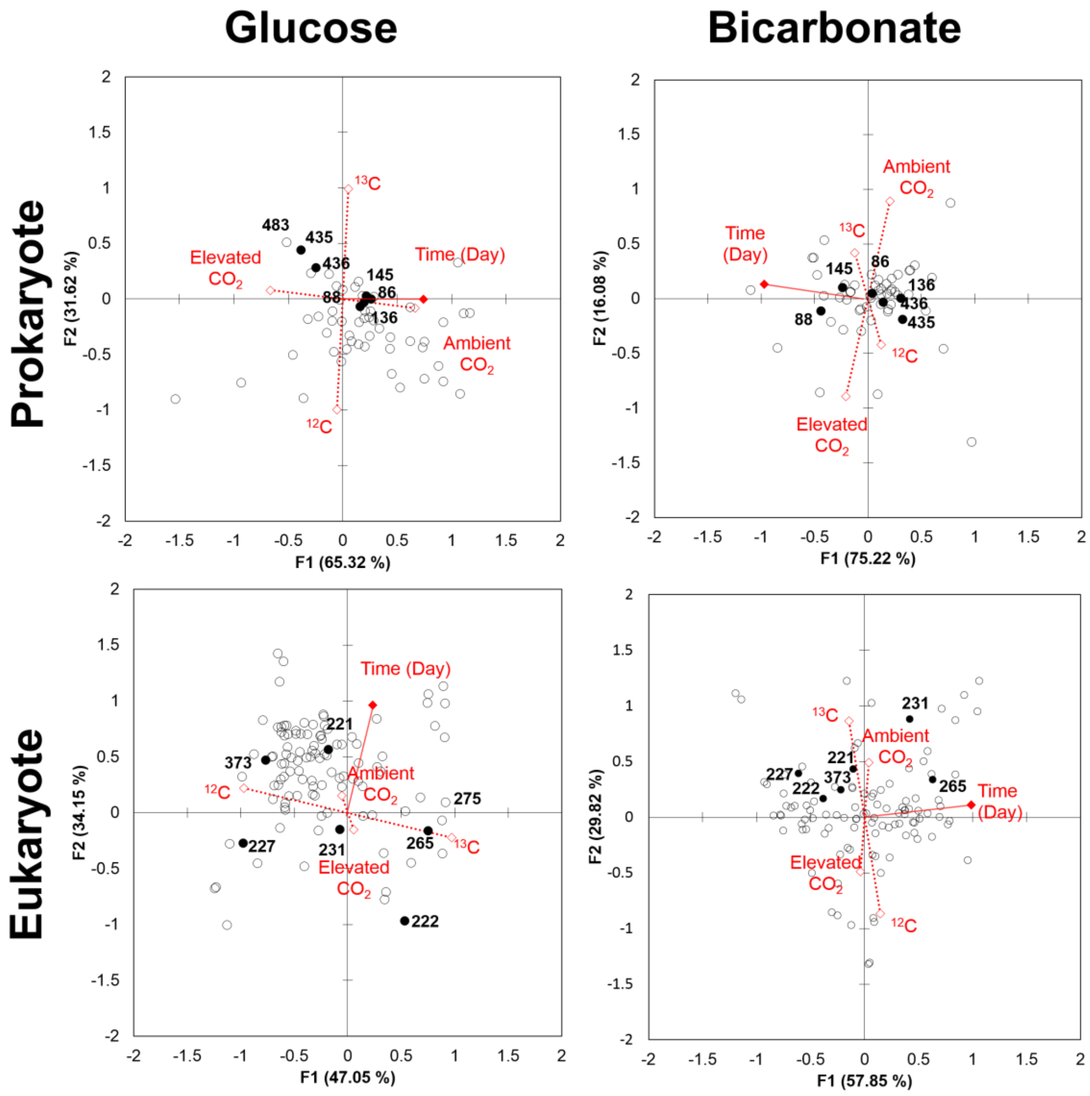


Figure 5.3: Ordination plot of canonical correspondence analysis (CCA) in glucose and bicarbonate, prokaryote and eukaryote T-RFLP profiles. The ordination is obtained through CCA analysis of percentage contribution of individual TRF's combined with explanatory environmental variables. Only time, isotope 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.

Prokaryote	***Total***		***Bicarbonate***		***Glucose***	
Environmental variable	Variance (%)	Probability (*P*)	Variance (%)	Probability (*P*)	Variance (%)	Probability (*P*)
Time^a	9.377	0.001*	33.450	0.001*	12.854	0.001*
Substrate^b	8.085	0.001*				
Incubation Type ^{c}	4.757	0.001*	8.167	0.001*	10.589	0.001*
pH^d	4.131	0.002	7.567	0.001	7.774	0.001
Isotope^e	4.118	0.001*	5.156	0.002*	10.863	0.001*

Eukaryote	***Total***		***Bicarbonate***		***Glucose***	
Environmental variable	Variance (%)	Probability (*P*)	Variance (%)	Probability (*P*)	Variance (%)	Probability (*P*)
Time^a	4.421	0.001*	19.611	0.001*	10.179	0.001*
Substrate^b	7.005	0.001*				
Incubation Type^c	2.483	0.027	5.710	0.051*	5.668	0.006*
pH^d	2.574	0.018	3.766	0.353	5.310	0.025
Isotope^e	4.484	0.001*	9.072	0.004*	13.603	0.001*

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 colinnearity 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 (4–6th May) until a pH~ 7.8 was established. To control for sparging effects, ambient-condition mesocosm enclosures were sparged with air. In order to simulate natural conditions more closely, a phytoplankton bloom was induced through the addition of nitrate and phosphate in all mesocosms on 6th 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 BR 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 Flourometer 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 18ºC.

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). Amplfication employed a 2 minute pre-denaturation phase at 94 ^{o}C followed by 30 cycles of 94 ^{o}C for 1 minute, 52 ^{o}C for 1 minute, and 72 ^{o}C for 3 minutes and a final extension phase of 10 minutes at 72 ^{o}C. 20 µL of gel purified PCR product was digested for 4 hours at 37 ^{o}C in a 30 µL total reaction volume using 20 units restriction enzyme *MspI* (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^{TM} (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 CO_2), substrate (glucose or bicarbonate), and isotope label (^{12}C or ^{13}C) and continuous parameters time (day in mesocosm experiment) and pH (of microcosm water).

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5.7 Supplimentary Information

Prokaryotes										
Total Number of TRF's In Mesocosm	57									
Total Number of TRF's In SIP Microcosms	57									
Mesocosm (in rank order 1-10)	145	436	86	435	88	136	488	486	437	92
Glucose (in rank order 1-10)	200	522	436	88	171	440	476	448	278	86
Bicarbonate (in rank order 1-10)	88	436	435	145	486	483	136	86	138	482
Eukaryotes										
Total Number of TRF's In Mesocosm	38									
Total Number of TRF's In SIP Microcosms	110									
Source incubation type										
Mesocosm (TRF’s in rank order 1-10)	265	222	221	227	373	231	383	228	360	376
Glucose (TRF’s in rank order 1-10)	378	275	268	267	265	281	83	370	73	367
Bicarbonate (TRF’s in rank order 1-10)	268	279	267	380	479	169	370	179	265	220

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.

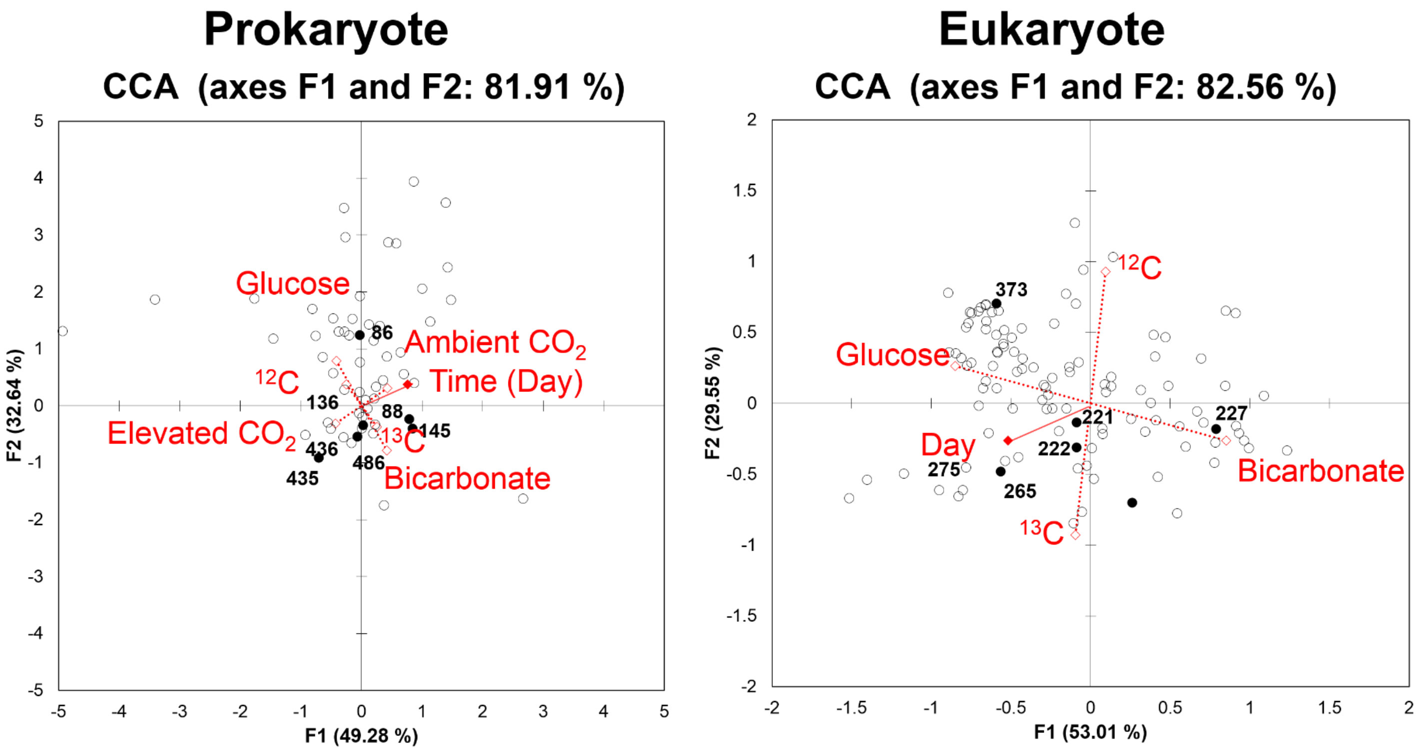

Figure S5.7.2: Ordination plot of canonical correspondence analysis (CCA) in prokaryote and eukaryote T-RFLP profiles. The ordination is obtained through CCA analysis of percentage contribution of individual TRF's combined with explanatory environmental variables. Only the three most important environmental 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 CO_2

Lindsay K. Newbold[1, 2], Andrew S. Whiteley[3], Christopher J. van der Gast[1] and Anna E. Oliver[1]

[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: 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 CO_2, 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 0.2-2.0μm phytoplankton can be broken down into the photosynthetic prokaryotes - cyanobacterial linages such as *Prochlorococcus* and S*ynechococcus* - 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".

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 photosynthetetic pathway (C4), small genome size, and adaptions 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 CO_2 Bergen mesocosms (Meakin and Wyman, 2011; Newbold *et al.*, 2012). Previously, we found a positive relationship between elevated CO_2 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 CO_2 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.

OTU ID	Phylogenetic ID	Reference sequence	Elevated CO_2	Ambient CO_2	Total Number of sequences
4	*Bathycoccus*	1801A06	80	64	144
2	*Micromonas*	1804A07	63	12	75
7	*Micromonas*	1801C01	2	0	2
11	*Micromonas*	1801F12	1	0	1
50	*Ostreococcus*	1815D05	5	0	5

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 SSUr 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

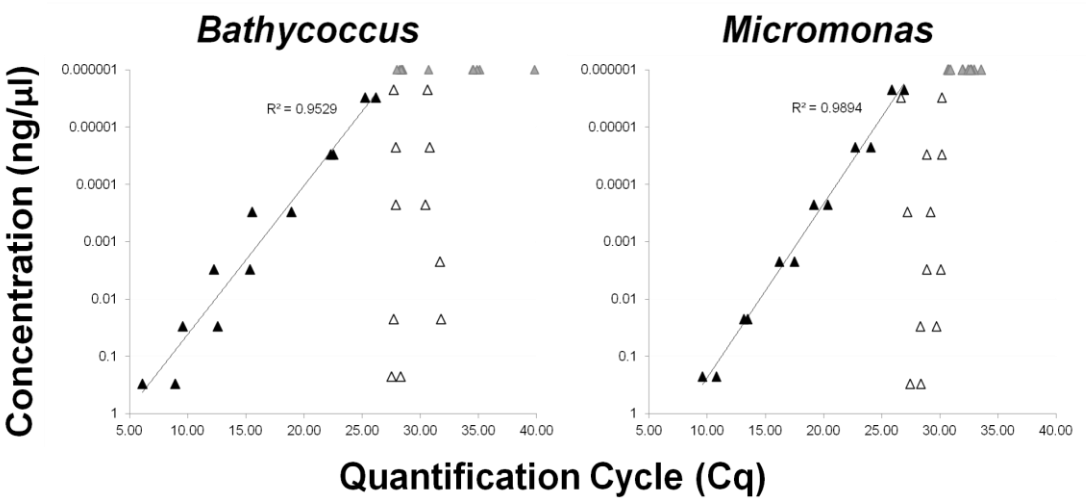

Figure 6.1: Primer specificity for *Bathycoccus* and *Micromonas* qPCR assays. *Bathycoccus* primer set 570F-BATHY03R and *Micromonas* primer set 570F-MICROR, were used to amplify a dilution series of *Bathycoccus-like* 18S standard (closed triangles), *Micromonas-like* standard (open triangle) and water (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).

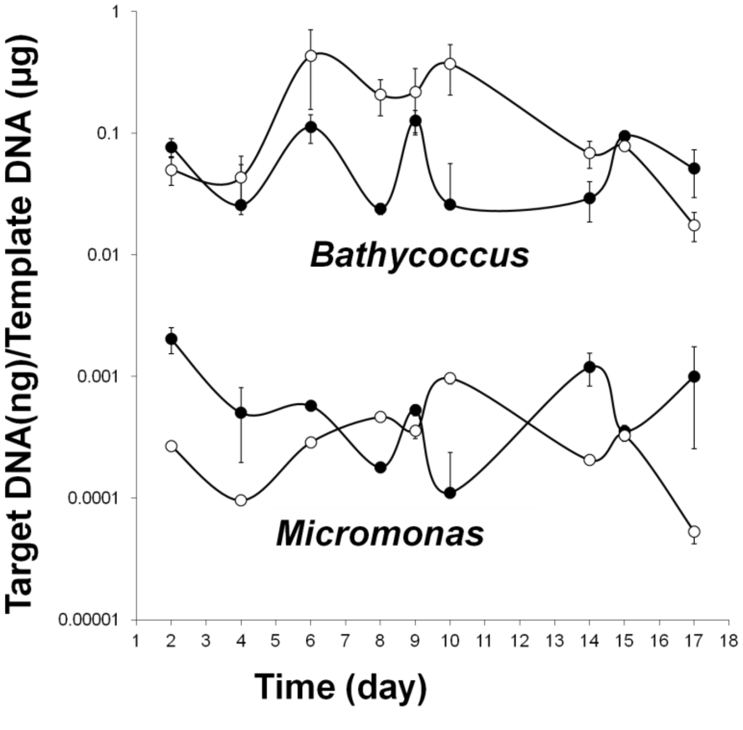

Figure 6.2: Mean abundance of *Mamiellales* 18S signatures (ng/µg) over duration of the Mesocosm experiment. Elevated (M1) represented by closed circles and Ambient enclosure (M6) represented by open circles. Error bars represent the standard deviation from the mean of triplicate qPCR reactions.

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 *et al.*, 2012). Further, Meakin and Wyman (2011) tracked copy number of *Bathycoccus* and *Micromonas* RubisCO (*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 (*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 *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 *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

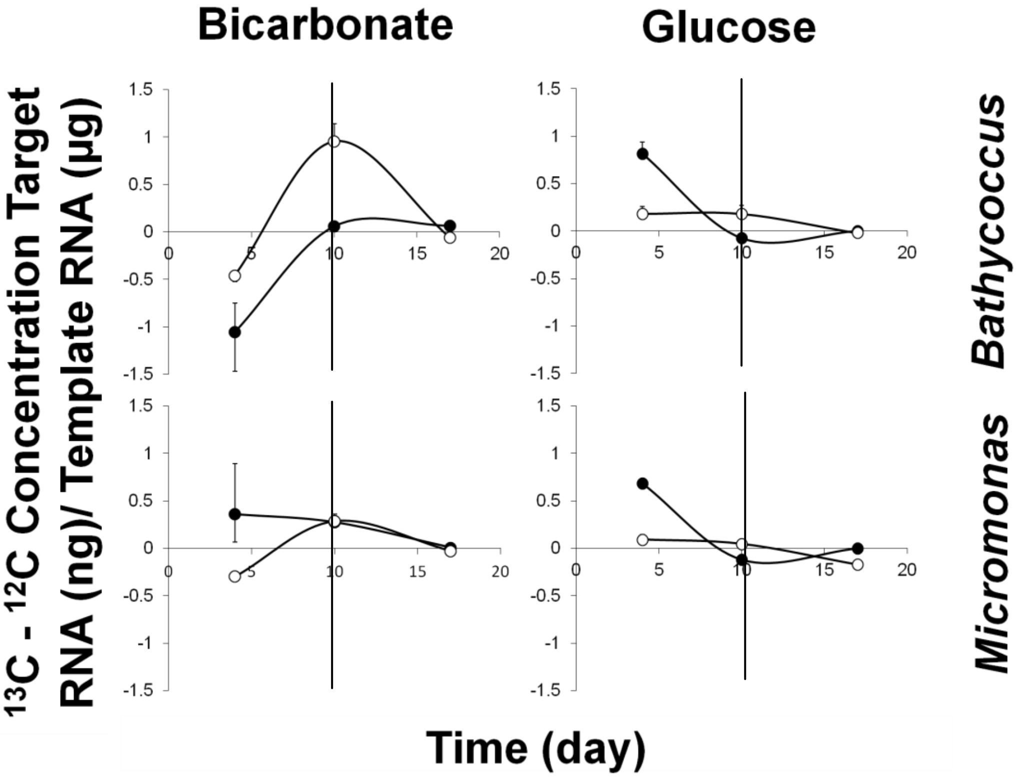

Figure 6.3: Changes in RT-qPCR abundance during stable isotope microcosm incubations for *Bathycoccus* and Micromonas. Relative uptake Elevated M1 CO_2 incubations (closed circles) compared to ambient M6 incubations (open circles). Relative uptake of ^{13}C substrate assessed by concentration of target 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 (4–6th May) until a pH~ 7.8 was established. To control for sparging effects, ambient-condition mesocosm enclosures were sparged with air. In order to simulate natural conditions more closely, a phytoplankton bloom was induced through the addition of nitrate and phosphate in all mesocosms on 6th 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, 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 (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^{O}C, followed by heat inactivation at 65 ^{O}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 Flourometer with Qubit® RNA BR Assay (RNA) Qubit® dsDNA BR Assay (DNA). Template rRNA copy was calculated using the formula:

$$\text{molecules/ µl} = a/(\text{plasmid length} \times 660) \times 6.022 \times 10^{23}$$

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'ACCACGATGACTCCATGTCTCA3' (Zhu *et al.*, 2005) and MICROR- 5'CCAGACCGTTAAGCCCAGAGCAC3'.

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 realplex 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: Efficiency = $-1+10^{(-1/slope)}$

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

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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 CO_2.

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 CO_2 concentrations upon bacterial community turnover and found that not only was community composition conserved over time but that community turnover was dampened with elevated CO_2. 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 CO_2 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 CO_2 within the dominant Mamiellales signatures *Bathycoccus and Micromonas* (chapter 6). *Micromonas* appeared to actively assimilate more bicarbonate with elevated $CO_{2,}$ 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 CO_2 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 CO_2 in the carbon fixation step of marine photosynthesis. However, RubisCO has a low substrate affinity and therefore requires a mechanism for concentrating CO_2 – carbon concentration mechanism (CCM) (Rost *et al.*, 2008). Organisms which have efficient CCM's have appeared to be less affected by elevated CO_2 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 CO_2 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 unculturable 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 pCO_2 increases and pH decreases (Riebesell *et al.*, 2010). Furthermore, it doesn't change trace metal availability (Shi *et al.*, 2009). There 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 *taq*Man probe assay - where multiple probes can be run at the same time - requiring less total template. The *taq*Man 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 phylogentically 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

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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 CO_2

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 CO_2

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.