The combined effect of daylength and CO_2 on coccolithophore physiology

Laura M. J. Bretherton

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School of Biological Sciences

University of Essex

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Summary

Atmospheric CO_2 levels have been increasing at an accelerated rate for the last 250 years, much of which is absorbed by the oceans, resulting in a process called ocean acidification (OA). This phenomenon has the capacity to disrupt many marine biological processes that utilise carbon, in particular photosynthesis and calcification, and as such phytoplankton have been a main topic of OA studies. While research has accelerated over the last decade, establishing general trends still remains confounded by methodological inconsistencies. Coccolithophores, particularly the species *Emiliania huxleyi*, are both ecologically and biogeochemically important phytoplankton; however, one strain (NZEH) has produced highly varied results. Here, we present a multivariate analysis that suggests previous inconsistencies between past studies of NZEH may be driven by variance of the light:dark (L:D) cycle used for growth. Experimental analysis on NZEH showed that under a 14:10h L:D cycle, CO_2 induces significantly slower growth rates and higher PIC and POC $cell^{-1}$, but this effect is dampened under 24h of light. This was widened to encompass more taxa, including more isolates of *E. huxleyi* (PLY70-3, PLY124-3, RCC962), and two other species of coccolithophore; *Gephyrocapsa oceanica* and *Coccolithus pelagicus*. L:D cycle changed the observed OA response, with two main responses divided by biogeographical origin. In tropical taxa, 24h light enhanced the effects of increased photosynthesis, but dampened the decrease in calcification in response to CO_2. For temperate taxa, 24h dampened both the increases in photosynthesis and calcification with CO_2. Evaluation of photobiology reveals that both CO_2 and longer photoperiods induce a "high light" acclimation response, and changes in coccosphere thickness suggest it has a photoprotective role. Finally, results from bioassay experiments on natural phytoplankton populations in the polar regions show that CO_2 response is hard to predict and based on community composition and ambient starting conditions. This work serves to further highlight the importance of environmental variables that moderate the OA response in accurately understanding future biogeochemical cycles. Future models attempting to predict the impact of OA upon marine systems must critically account for interactive role of light availability.

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List of Abbreviations

Ω	Carbonate saturation state
BIOAcid	Biological Impacts of Ocean Acidification Programme
CCM	Carbon concentrating mechanism
DIC	Dissolved inorganic carbon
EP	Extracellular polymers
EPOCA	European Project on Ocean Acidification
FRRf	Fast repetition rate fluorometry
L:D cycle	Light:dark cycle
OA	Ocean acidification
PIC	Particulate inorganic carbon
ppm	Parts per million
POC	Particulate organic carbon
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SA:V	Surface area:volume
TA	Total alkalinity
TEP	Transparent exopolymer particles
UKOARP	UK Ocean Acidification Research Programme

Chapter 1: Introduction

1.1. Marine Phytoplankton

Microscopic phytoplankton are key organisms on planet Earth thriving within all aquatic ecosystems where light is available for growth. Photosynthesis by phytoplankton in the oceans is particularly important contributing around 50 Pg C $year^{-1}$ (Chavez *et al.* 2011) amounting to ~50% to global net primary productivity (Falkowski and Raven 2007). This productivity sequesters CO_2 from the atmosphere and subsequently fuels marine food webs or exports fixed organic carbon from the surface to the deep ocean via sinking. Phytoplankton also play fundamental roles in biogeochemical cycling of other key elements, such as nitrogen (e.g. nitrogen fixation by cyanobacteria) and silicon (the diatoms produce a frustule made of silica), and associated bio-products (so-called secondary metabolites) in the form of trace gases as dimethylsulphide (DMS) that act as cloud condensing nuclei and alter the Earth's albedo (Charlson *et al.* 1987; Hopkins *et al.* 2010).

Under optimal environmental conditions phytoplankton can form huge blooms across vast spatial scales that are visible from space (Fig. 1.1.1). In particular, the seasonal spring blooms that characterise coastal, sub-polar and the temperate North Atlantic waters are major biogeochemical and ecological events. Changes in light (Raitsos *et al.* 2011), nutrient availability, turbulence (Huisman *et al.* 1999), temperature (Wolf and Woods 1988; Raitsos *et al.* 2011) and mixing (Townsend *et al.* 1994) have all been identified as key triggers for the start of these blooms. The exponential increase in phytoplankton abundance is crucial for the survival of zooplankton, such as copepods that time their breeding so their hatchlings have a food supply. In turn these zooplankton are an important food source for higher trophic guilds, such as fish and mammals, i.e. fish stocks available to human subsistence. However, changes to the climate, in particular 'global warming' induced by elevated atmospheric CO_2 (and other heat trapping

aerosols e.g. methane) caused by anthropogenic activity are altering the duration and timing of blooms. Warmer ocean temperatures can also depress phytoplankton blooms by increasing grazing pressure (Oviatt *et al.* 2002); for example a change in just 2°C has been observed to cause copepod maturation to occur 3 weeks earlier, resulting in increased grazing pressure on phytoplankton (Durbin and Durbin 1992).

Given the importance of phytoplankton in cycling key nutrients and fuelling ocean food webs it is critical we better understand how climatic change influences their productivity. In recent years the scientific community has rapidly acknowledged that warmer oceans are not the only consequence of elevated atmospheric CO_2.

Fig. 1.1.1. An extensive phytoplankton bloom of coccolithophores (pale blue plumes) off the Irish coast on 2nd June, 2006. NASA image by Jess Schmaltz, based on MODIS data.

1.2. Ocean Acidification

Since the beginning of the Industrial Revolution, the rate of increase in atmospheric CO_2 concentration has been an order of magnitude larger than the Earth has experienced in millions of years (Doney and Schimel 2007). Current CO_2 levels are about 385ppm, the highest atmospheric CO_2 has been for the last 800,000 years (Luthi *et al.* 2008). This increase is predicted to further accelerate with the planet's growing population and the emerging economies of less-developed countries, with CO_2 levels of between 730 and 1020ppm by the year 2100 (IPCC 2007). Potential consequences of increased CO_2 have received much media attention, especially the predicted changes in the Earth's temperature; however, such attention has (until recently) overshadowed another equally important outcome of increased atmospheric CO_2 concentrations, "ocean acidification" (Caldeira and Wickett 2003).

The world's oceans naturally absorb CO_2, so-called 'buffering', and it has been estimated that current atmospheric levels would be even higher (around 450ppm) if it were not for this key process (Doney *et al.* 2009). However, buffering capability is not limitless and excess CO_2 ultimately results in a downward shift of pH, and hence acidification (Raven *et al.* 2005; Gattuso and Lavigne 2009). Models predict that parts of the Southern Ocean will be severely affected by ocean acidification as early as 2030 (McNeil and Matear 2008), and there have already been incidences of upwelled corrosive water from the deep ocean off the coast of South America (Feely *et al.* 2008).

Interest in how ocean acidification (OA) will affect marine organisms is still in its infancy. In the late 20th century, research conducted into the effects of changes in carbonate chemistry on biology was sporadic, and not in the context of anthropogenic CO_2, e.g. (Borowitzka 1981; Fabry 1990; Amoroso *et al.* 1998) and it was not until the turn of the 21st century when research began to focus on how increased atmospheric CO_2 would affect seawater and in turn marine organisms, e.g. Gattuso *et al*. (1998) and Riebesell *et al*. (2000). By 2005, the

Royal Society had issued a report to better identify mechanisms, processes and pathways by which OA could operate (Raven *et al.* 2005). While this report stimulated more intensive research in OA and its effects on marine organisms, these findings were initially clouded by methodological inconsistencies and a lack of understanding of carbonate chemistry and therefore how best to alter the carbonate system for robust OA investigations (Hurd *et al.* 2009; Shi *et al.* 2009; Riebesell *et al.* 2010). With much of methodological issues now largely resolved (e.g. (Riebesell *et al.* 2010), it is apparent now that the rate at which we develop our understanding of the biological responses to ocean acidification must exceed the rate of any harmful changes in the marine environment.

1.3. Seawater Carbonate Chemistry

CO_2 in the atmosphere naturally dissolves into the oceans and the subsequent carbon chemistry of seawater is governed by the following reaction(s):

$$CO_{2(atmos)} \leftrightarrow CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow 2H^+ + CO_3^{2-} \quad (1)$$

These reactions are all reversible, and exist in near equilibrium (Millero *et al.* 2002). At present, average sea surface pH values are around 8.1, meaning approximately 90% of inorganic carbon exists as bicarbonate, 9% is carbonate and just 1% is CO_2 (Fig. 2). As atmospheric CO_2 rises, more dissolves into seawater and results in more H^+. CO_3^{2-} acts as a buffer, and "mops up" these protons, with the end result being an increase in CO_2/HCO_3^- and thus a decrease in CO_3^{2-}. In fact, the widely accepted prediction of a 0.3 – 0.4 drop in oceanic pH in the next century will equate to an increase of $[H^+]$ by around 150%, with a subsequent 50% drop in carbonate ions (Orr *et al.* 2005) (refer to Fig. 1.3.1).

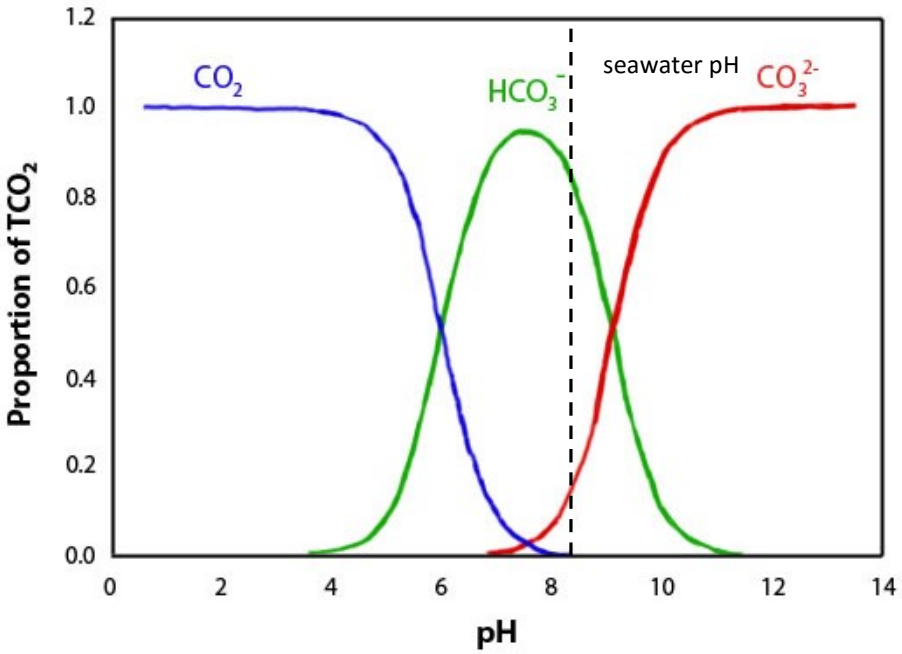

Fig. 1.3.1. The speciation of carbon in seawater at different pH values. The black dashed line indicates current seawater pH.

The concentration of carbonate also affects the saturation state (Ω) of seawater with regards to calcium carbonate ($CaCO_3$). In principle, Ω describes how likely $CaCO_3$ is to precipitate or dissolve, and can be calculated using the following equation:

$$\Omega = [Ca^{2+}][CO_3^{2-}]/K'_{sp} \qquad (2)$$

Here, K'_{sp} is the apparent solubility constant, which varies depending on many factors like temperature, pressure or the form of calcium carbonate (Mucci 1983). Since $[Ca^{2+}]$ does not vary much as it is closely linked to salinity, Ω is governed mostly by the amount of carbonate available in the seawater. Therefore, ocean acidification will affect the saturation of seawater, with potentially major consequences for calcifying organisms, see (Hoegh-Guldberg *et al.* 2007; Ridgwell *et al.* 2009; Beaufort *et al.* 2011); however, this subject is returned to in later sections. $CaCO_3$ is more likely to precipitate when Ω is greater than 1, and more likely to dissolve when it is below 1. Already we have seen the saturation horizon (where Ω = 1), in some areas rising in

depth from its original position before industrial times (Feely *et al.* 2004), and the first undersaturated waters, where $\Omega < 1$, are predicted to occur in high latitude waters by 2030 (McNeil and Matear 2008).

In general, warmer tropical regions are super-saturated with $CaCO_3$ by a factor of 4.2, whereas colder waters by only 1.2 (Takahashi et al. 2014). Several models have predicted that the surface waters of polar regions will be the first to experience under-saturation with regard to $CaCO_3$ (Orr *et al.* 2005; McNeil and Matear 2008; Steinacher *et al.* 2009). Observations from the Arctic basin have already revealed aragonite under-saturation in the surface waters of the Canadian Arctic Archipelago (Chierici and Fransson 2009), the Canada Basin (Yamamoto-Kawai *et al.* 2009), the Siberian Shelf (Anderson *et al.* 2011), and the Hudson Bay System (Azetsu-Scott *et al.* 2014) as a result of both riverine input and sea ice meltwater. In the Southern Ocean, meltwater also has a strong influence on aragonite saturation in the Weddell Sea (Weeber *et al.* 2015), as well as in the Amundsen and Ross Seas (Mattsdotter Björk *et al.* 2014). Further away from the ice shelf, Mattsdotter Björk *et al*. (2014) found that aragonite saturation is more strongly controlled by primary productivity.

Under-saturation of surface and near-surface waters can also occur seasonally in coastal upwelling systems (Feely *et al.* 2008; Alin *et al.* 2012; Harris *et al.* 2013; Ohman *et al.* 2013; Martz *et al.* 2014), and while this has occurred since pre-industrial times (Alin *et al.* 2012), the increase in anthropogenically derived CO_2 in upwelled water is exacerbating the impact it has on surface waters (Feely *et al.* 2008). Upwelling can also cause significant changes in the saturation horizon depth (the depth at which $\Omega=1$). In the California Current System, upwelled water can cause shoaling of the saturation horizon from >200m to <75m (Bednaršek and Ohman 2015). Generally, long-term observations have shown that OA has caused shoaling of the saturation horizon depth, averaging 1 to 2m yr^{-1} between 1991 and 2008 in the Pacific Ocean (with regional variability) (Feely *et al.* 2012).

1.4. Methods in Ocean Acidification

Over the last decade, research into ocean acidification has increasingly intensified, in particular with the formation of many international research groups, such as the EPOCA, BIOAcid and UKOARP. The main goals of such groups are to further improve the methods used in OA studies, and to start considering impacts on more ecosystem scale processes, such as nitrogen and carbon cycling. There has also been a move to study natural assemblages of organisms to observe how the community as a whole responds to OA, but this also requires knowledge of the mechanistic responses exhibited in each individual species (whether positive or negative) via controlled laboratory experiments. In either case, robust approaches to manipulate the carbon chemistry that enable comparison between studies is essential (Ridgwell *et al.* 2009).

There are different methods widely used to alter the carbon chemistry of seawater. The first is using the additions of very small volumes of acid (usually HCl) and/or a base (usually NaOH) to induce the correct change in pH (Riebesell *et al.* 2000; Langer *et al.* 2006; Shi *et al.* 2009). Whilst this does create a change in the carbon speciation, it crucially does not increase the DIC pool. Using this method is also accompanied by a decrease in alkalinity (Iglesias-Rodriguez *et al.* 2008), demonstrated in Fig. 1.4.1. Ocean acidification is not likely to cause any changes in seawater alkalinity, therefore any changes that occur in laboratory cultures are not realistic. To counter both these issues, additional bicarbonate can also be added to the media to increase [DIC] and stabilise the alkalinity (Gattuso *et al.* 2010).

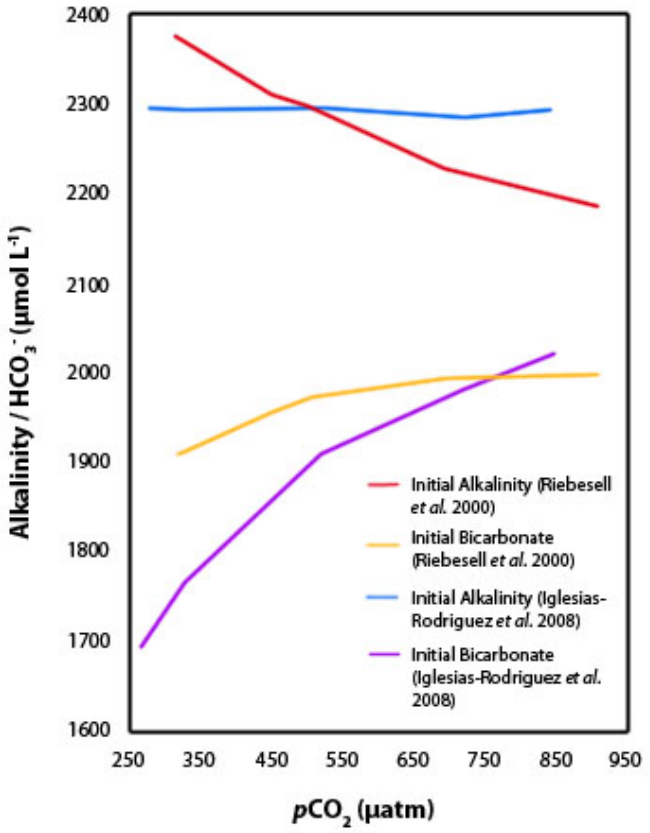

Fig. 1.4.1. Changes in alkalinity and bicarbonate with pCO_2 using acid/base additions (Riebesell *et al.* 2000) and bubbling with CO_2 in air (Iglesias-Rodriguez *et al.* 2008). Bubbling with CO_2 causes an increase in [DIC] but keeps alkalinity fairly stable. After Iglesias-Rodriguez *et al.* (2008).

The other common method of carbon chemistry perturbation is to bubble media with CO_2 gas in air (Fu *et al.* 2007; Iglesias-Rodriguez *et al.* 2008), adjusted to the required concentration either using pre-mixed gases or a gas mixer. The easiest way of doing this is via two tanks of gas, one containing air and the other containing a known concentration of CO_2 (e.g. 10% CO_2). The air is passed through a CO_2 scrubber and is mixed with the 10% CO_2 at a known rate to give the desired concentration of CO_2. This is then bubbled through the media at a constant rate through an air stone to allow the CO_2 to dissolve and change the carbon chemistry.

A gas bubbling/mixing approach offers a much more realistic change to the carbonate system in relation to OA (refer back to Fig. 1.4.1), since it adds additional carbon to increase the DIC pool while maintaining a more constant alkalinity, even at lower pH values (Gattuso *et al.*

2010). As such, gas bubbling is the recommended method in the guidelines for best practises in ocean acidification research, issued by EPOCA. Even so, control with gas mixing assumes a "perfect" system where the carbonate chemistry remains stable. The problem in applying these techniques to laboratory experiments is that biological processes also have the ability to perturb the carbonate system, especially in a closed system. In general, there two processes that act on carbon chemistry; photosynthesis and respiration. Photosynthesis can cause a drift in alkalinity as it involves uptake of nutrients and alters the ratios of minor ions in the medium as well as resulting in a drawdown of CO_2. Respiration, on the other hand, produces CO_2 and changes [DIC] and consequently the pH. It then becomes difficult to understand the observed responses of the cultured organisms as the carbonate system is not at the desired state.

To avoid biological induced drifts cultures can be controlled by self-correcting ("stat") systems, such as pH or pCO_2 stat systems, e.g. (Sciandra *et al.* 2003; Leonardos and Geider 2005; Brading *et al.* 2011). Such a process is in principle straight forward; the parameter of interest is continually monitored and corrected by adding a known amount of CO_2 or CO_2-free air, often by an automated system. While this keeps the carbonate chemistry within a small, defined range of values, the pH-stat approach in particular requires accurate probes and in turn regular calibration. Alternatively, the amount of biological material can be kept low so that the biological drifts are negligible; however, less biological material may ultimately limit the scope of the research that ultimately be conducted.

1.5. Responses of Key Phytoplankton Groups to OA

OA has the potential to moderate two natural processes; photosynthesis (increase in available CO_2 and/or HCO_3^-) and calcification (a decrease in CO_3^{2-} and Ω). Phytoplankton are thought to be C-limited in the modern ocean, mainly due to ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the primary carboxylating enzyme in the dark reactions of photosynthesis. RubisCO is inefficient in its carboxylating role as it has a low affinity for CO_2;

its half-saturation constant is 20-70µmol kg^{-1} CO_2 (Badger *et al.* 1998), but normal concentrations of CO_2 in seawater range from just 10-25µmol kg^{-1} (Riebesell 2004). Such inefficiency in the modern ocean is probably due to the evolutionary history of these organisms, as many groups evolved at a time when atmospheric CO_2 were much higher than they are now (Tortell 2000) but further by RubisCO's double function as an oxygenase, so CO_2 molecules compete with O_2 molecules for the active site. Thus it would be logical to assume that the rates of photosynthesis exhibited by phytoplankton are undersaturated, and the increase in CO_2 concentration would stimulate photosynthesis (e.g. Rost *et al*. 2008); however, many species of phytoplankton exposed to elevated CO_2 to date via controlled laboratory experiments appear to show very little change in photosynthesis rate, indicating saturation despite an inefficient enzyme and scarcity of the substrate, e.g. (Rost *et al.* 2003) (Fig. 5). That said, such a general response is not ubiquitous; for example, Clark and Flynn (2000) examined a range of phytoplankton and found that for some species, RubisCO would still be half-saturated even at 750 ppm CO_2.

Limited photosynthetic responses to elevated CO_2 appears to be driven by employing carbon concentrating mechanisms (CCMs), which help phytoplankton to keep RubisCO saturated with CO_2 despite the low ambient concentration, (see (Giordano *et al.* 2005). A variety of CCMs exists, mainly in the form of carbonic anhydrase either outside or inside the cell and enable phytoplankton to utilise HCO_3^- as a carbon source and speeding up the conversion to CO_2. The amount of energy invested in CCMs varies between groups, and it is therefore likely that responses of photosynthesis will be species-dependent. Together, factors such as RubsiCO specificity and CCM activity, likely ensure that the response of photosynthesis to elevated CO_2 is not ubiquitous across groups or even closely related species (e.g. Brading *et al.* 2011). This response is likely further complicated by the one phytoplankton group that also calcifies: the coccolithophores. Therefore, the following sections will now consider the various growth (photosynthesis and calcification) responses observed to date for the main phytoplankton groups.

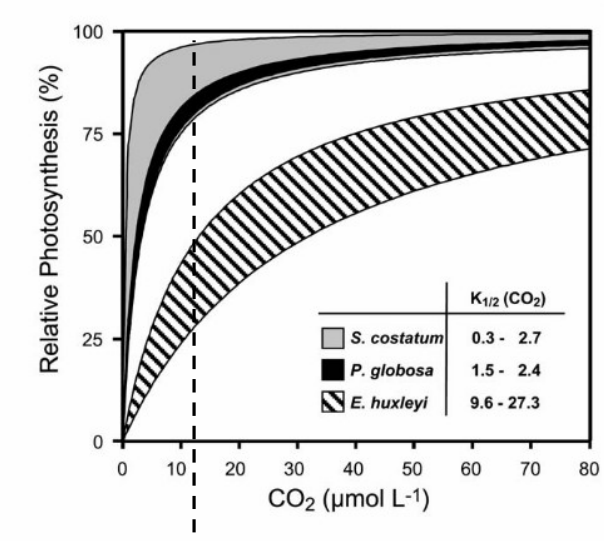

Fig. 1.5.1. Changes in the relative photosynthesis between three different species (*Skeletonema costatum*, *Phaeocystis globosa* and *Emiliania huxleyi*) in response to changes in pCO_2 concentration. The dotted line indicates the current average CO_2 concentration in natural seawater. Results from Rost *et al*. (2003), diagram from Rost and Riebesell (2004).

1.5.1. Coccolithophores

Much of the phytoplankton research to date examining ocean acidification has focussed mainly on the coccolithophores, a globally distributed group of phytoplankton that form ornate shells from calcium carbonate. As both a photosynthesising and calcifying group of phytoplankton, they contribute significantly to the global carbon cycle (Riebesell *et al.* 2009). They calcify prolifically (Tyrrell *et al.* 1999; Shutler *et al.* 2010), and coccolithophore-derived calcite provides ballast material to accelerate the sinking velocities of organic particles, enhancing carbon sequestration in the deep ocean (Klaas and Archer 2002; Ziveri *et al.* 2007; Chow *et al.* 2015). Therefore, understanding the sensitivity of coccolithophores to OA is of vital importance to our ability to predict the impacts of climate change on biogeochemical cycles.

Calcification is dependent on Ω, which in turn is driven by changes in $[CO_3^{2-}]$, and as such coccolithophores are often classed as at-risk organisms with regards to OA. Conversely, coccolithophores are known to possess inefficient CCMs (Rost *et al.* 2003) and rely on simple

diffusion of CO_2 for much of their carbon uptake (Tortell 2000). Therefore, OA could have a negative impact on calcification, but enhance photosynthesis by relieving carbon limitation. While coccolithophores have been studied extensively, their response to CO_2 remains unclear due to the considerable variability and conflicting nature of observations reported in the literature. Even within the same species, results can vary extensively. The most widely tested organism, *Emiliania huxleyi*, has produced a wide range of responses to CO_2, with some studies indicating a negative effect on calcification (Riebesell *et al.* 2000; Zondervan *et al.* 2002; Sciandra *et al.* 2003), some reporting no change (Langer *et al.* 2009; Richier *et al.* 2010), and some even observing an increase in calcification (Iglesias-Rodriguez *et al.* 2008; Fiorini *et al.* 2011a).

Differences in CO_2 response can be attributed to variability in other parameters that are not being controlled in a consistent manner across studies, and specific interactions of CO_2 and other environmental factors have since been investigated. As previously discussed, the method of perturbation has been a debateable topic for some time and held as one of the main reasons differences between earlier work on *E. huxleyi* is not consistent with later studies (see Iglesias-Rodriguez *et al*. 2008 and accompanying comments). There is evidence to suggest that *E. huxleyi* responds in a similar manner to either DIC or TA manipulations (Hoppe *et al.* 2011), but multifactorial experiments have shown that modification of different aspects of the carbonate system does affect specific physiological responses in *E. huxleyi*, suggesting that it is decreasing pH that negatively affects growth, organic carbon production and calcification rates at high CO_2 (Bach *et al.* 2011; Bach *et al.* 2013).

The interaction of CO_2 with other abiotic factors has also been investigated. The effects of temperature and CO_2 are particularly pertinent as these two environmental factors will increase simultaneously as climate change progresses. A few studies have found that POC production increases with PIC decreases with increasing CO_2 and temperature together (Feng *et*

al. 2009; De Bodt *et al.* 2010). Recently, Sett *et al*. (2014) found that increased temperatures alter the OA response of *E. huxleyi* and *Gephyrocapsa oceanica* by shifting their optimum CO_2 ranges towards higher concentrations. CO_2 and irradiance also have some interactive effect, with the OA response of POC production being heavily influenced by light intensity in *E. huxleyi*, while PIC production was unaffected by CO_2 at low irradiances (Zondervan *et al.* 2002). Feng *et al*. (2008) looked at the effect of all three factors (CO_2, temperature and irradiance) and found some interactions. Growth rates in *E. huxleyi* are accelerated by temperature, but only at low irradiances, while photosynthesis is enhanced with both CO_2 and temperature. PIC:POC is strongly negatively affected by irradiance, but CO_2 only appears to have a negative effect with high irradiance. In addition to these interactive environmental factors, some species and strain-specific responses have been observed (Langer *et al.* 2006; Langer *et al.* 2009), and it is likely that, given the high genetic and physiological diversity within *E. huxleyi* (Brand 1982; Iglesias-Rodriguez *et al.* 2006; Smith *et al.* 2012), there may not be a uniform response to OA.

In order to reconcile these sources of variability, several meta-analyses have been performed on available data. Findlay *et al*. (2011) found overall that PIC:POC generally decreases with CO_2, and that most of the variability is driven by differences in P availability and TA. More recently, Meyer and Riebesell (2014) agreed with these findings, also concluding that PIC:POC is negatively affected by OA in both *E. huxleyi* and *G. oceanica*. They also report that there is no indication that carbonate manipulation method affects PIC:POC in coccolithophores, but that it does appear to have an effect on photosynthesis. Both of these meta-analyses conclude that more work is needed to understand how other environmental factors affect the observed OA response in this key group of organisms.

1.5.2. Diatoms

As with coccolithophores, diatoms are another widespread group of phytoplankton that often form large blooms, especially in the polar regions of the globe. Also, they are important biogeochemically since they form shells out of silicate called frustules, and therefore also contribute to the cycling of Si and hence a widely studied phytoplankton group. That said, very little work about the effects of ocean acidification on diatoms exists, especially compared to the coccolithophores.. Some early research suggested that diatoms, especially larger ones, are limited by CO_2 (Riebesell *et al.* 1993); however, subsequent experimental work has confirmed the presence of highly efficient CCMs in many diatoms species that aid the uptake of both CO_2 and HCO_3^- (Burkhardt *et al.* 2001; Rost *et al.* 2003; Trimborn *et al.* 2008; Trimborn *et al.* 2009). This observation is more consistent with more recent work that demonstrates diatom photosynthesis rates do not appear to increase with elevated pCO_2 as the CCMs are effective enough to keep the rate at saturation (Rost *et al.* 2003).

Again, conflicting observations of how different species respond to CO_2 amongst diatoms are probably due variations in the relative effectiveness of CCMs between species. Field experiments on natural populations has demonstrated that the dominant diatom species can change in response to elevated CO_2 in areas such as the equatorial Pacific (Tortell *et al.* 2002) and the Southern Ocean (Tortell *et al.* 2008); specifically, increases in pCO_2 resulted in increased productivity and larger chain-forming diatoms dominating the blooms. Selection for 'larger' cells under higher CO_2 amongst diatoms is a tantalising result since it supports the notion that larger cells may be CO_2 limited as a result of a smaller SA:vol ratio even with a highly efficient CCM and therefore more competitive under higher CO_2 conditions (e.g. Finkel et al. 2010). However, as yet this notion has not been experimentally tested. Furthermore, despite the role of diatoms in Si-cycling, how elevated CO_2 affect silicification and cellular quotas of Si has been largely neglected. Only on study on frustule formation and Si quotas in diatoms in response

to changes in CO_2 was not focusing on ocean acidification, and therefore did not use "meaningful" pCO_2 levels. Milligan *et al*. (2004) observed increased dissolution of the frustule at higher CO_2 concentrations, but the two pCO_2 levels used were 100ppm and 750ppm. Therefore, without a present-day level, it is difficult to interpret these results in the context of the changes that will occur in the coming decades.

1.5.3. Other Phytoplankton Groups

The majority of ocean acidification research has focussed on groups that have key roles in biogeochemical cycling, especially the coccolithophores. Coccolithophores contribute to organic and inorganic mineralisation of carbon, and changes in their physiology could affect carbon export to the deep ocean and have significant net impacts on the sequestration of CO_2. However, such restricted taxonomic focus effectively limits the level of relevance considering how natural communities will respond to ocean acidification, with subsequent impact on food webs and marine ecology. Few studies have focussed on taxa other than coccolithophores or diatoms, but still demonstrate that responses will not be the same across all groups of phytoplankton.

In the oligotrophic waters of the tropics, N_2-fixing cyanobacteria support a large amount of productivity and contribute to the nitrogen cycle, and ultimately the carbon cycle, yet remain largely ignored in ocean acidification research. The majority of cyanobacteria studies have used the filamentous diazotroph *Trichodesmium*, a genus responsible for the majority of marine N_2-fixation. Across the few studies performed to date (Hutchins *et al.* 2007; Levitan *et al.* 2007), a strong increase in nitrogen fixation, photosynthesis and growth rate with increased pCO_2 was generally observed which could have huge impacts on oligotrophic environments. The exact mechanism behind this response is unclear, but further experimentation suggests that changes in CCM activity help to resolve issues in resource allocation between photosynthesis and N_2-fixation (Kranz *et al.* 2009). Interestingly, global nitrification rates are predicted to decrease in

response to ocean acidification, drawing attention yet again to the lack of other species experimented upon. Non-nitrifying cyanobacteria are equally poorly represented, with a single study in 2007 using two species (Fu *et al.* 2007). *Synechococcus* increased productivity and growth under higher CO_2 whilst *Prochlorococcus* showed no response. The difference in response between genus amongst coccolithophores and diatoms (above) and the general dominance throughout much of the world's oceans of *Synechococcus* and *Prochlorococcus* underlines the need to study a wider variety of organisms within this and between groups of phytoplankton. Very few studies to date have looked at dinoflagellates, another group of phytoplankton that form essential ecological roles, such as forming symbioses (e.g. *Symbiodinium*, Brading *et al.* 2011).

1.6. Interactions of OA with other environmental factors

A major gap in most environmental/climatic change studies is understanding how factors work together (interactively) to regulate key rate and biomass properties (e.g. Boyd et al. 2010); most studies focus on how a single variable may influence changes in productivity, biomass yields and allocation of resources (e.g. nutrient stoichiometry), which is not an accurate representation of how environmental change operates. OA is no exception and will inevitably accompany other environmental changes brought about by elevated atmospheric CO_2, such as temperature. However, only a handful of studies have looked at the combined effects of OA with another environmental factors: CO_2 and the other variable(s) of interest can have a synergistic effect, intensifying the response to either one on its own. For example, one strain of *E. huxleyi* was found to reduce calcification rates at higher CO_2, but the effect was exacerbated with the addition of UV-A light, and intensified even more when treated with UV-A and UV-B light (Gao *et al.* 2009). Similarly, increased temperature exacerbates the increased growth and photosynthetic rates in response to both elevated CO_2 for *Synechococcus* (but not for *Prochlororcoccus*) (Fu *et al.* 2007).

With knowledge that differences in methods can be easily reconciled and often ruled out as driving differences between OA studies, and other environmental factors can intensify (or potentially dampen) the OA response, it is likely that differences of 'environment' as well as taxa (above) may explain contrasting results between OA studied across closely related species/groups; standard growth conditions are not used across studies (but perhaps should not be given that many isolates come from very different optimum growth conditions). An overview of the research on OA shows that the light intensities used to grow *E. huxleyi* vary between studies from 50µmol s^{-1} m^{-2} all the way to 500µmol s^{-1} m^{-2} and work by Feng *et al.* (2008) has shown that at higher irradiances, elevated CO_2 has a stronger negative effect on PIC:POC in *E. huxleyi*, and the ratio will remain constant at low irradiances, independent of CO_2 concentration or temperature. Calcification rates have been shown to reduce further in some strains of *E. huxleyi* under N-limitation (Lefebvre *et al.* 2011).

Other variables to consider then might include cell size, as the relationship to CO_2 availability changes as cells become larger. Big cells have a smaller surface-area-to-volume ratio (SA:V) and are thought to be limited by CO_2 (Riebesell *et al.* 1993) as they have less of a capacity to acquire carbon through simple diffusion. As such, there is possibly a greater reliance on CCMs in larger cells than in smaller cells. At higher CO_2, diffusion limitation is relieved, and down-regulation of CCMs could free up resources to be invested elsewhere, such as growth. Field work has supported this theory, with larger colonial diatoms becoming dominant with CO_2 enrichment (Tortell *et al.* 2002; Tortell *et al.* 2008). Mesocosm studies by Paulino *et al*. (2008) found increasing the CO_2 available resulted in no detectable change in the growth of small picophytoplankton, but stimulated the growth of the larger nanophytoplankton. Conversely, in the Canadian Arctic, average cell size is decreasing, with pico- and bacterioplankton having become the dominant groups (Li *et al.* 2009). Rising average temperatures have resulted in more sea ice melting, flushing more freshwater into the Arctic Basin and diluting the available

nutrients, in particular nitrogen. The larger SA:V of smaller cells means they can outcompete larger cells for nutrient uptake and dominate. This should be taken into consideration when comparing the responses of highly diverse groups, such as the diatoms where size can vary greatly between species of the same genus.

Coupled to light and nutrient availability is also turbulence, which is also closely tied to the method used in experimental set up. Bubbling media with CO_2 causes small scale turbulence that can slow down the growth rate of the plankton culture, or damage particularly fragile species (Hurd *et al.* 2009). On top of this, certain groups of phytoplankton favour turbulence, like diatoms, while others are negatively affected by it, like dinoflagellates. In reality, OA will accompany increased stratification and less seasonal mixing (Sarmiento *et al.* 2004), so the turbulence experienced in cultures may not reflect true future conditions. Indeed, for some groups where turbulence is favoured, the method of bubbling in CO_2 may be affording the culture an advantage that will not necessarily be present in the future ocean. The take home message here is that focusing on individual factors helps to understand the mechanisms by which phytoplankton can respond; however, researchers need to consider the factors chosen relative to how the environment is changing as a whole.

Little research has yet to consider the combined interactive effects of growth environment, however, as explained in the section on coccolithophores, a meta-analysis of several *E. huxleyi* studies, an organism that exhibits the widest variation in response to OA (though this is more than likely due to the sheer amount of research conducted on *E. huxleyi* in comparison to other species), used multivariate statistics to determine what are the most important factors driving the OA response (Findlay *et al.* 2011). The factors that most controlled the variations in PIC:POC, aside from CO_2, were P concentration and TA. The multivariate statistics were not applied to other physiological responses, like growth rate, and it is not clear if the same trend would apply to non-calcifiers (i.e. POC:PON). However, this is a first important

step to demonstrate that changes in core growth variables can change the outcome of how we perceive the OA-response.

Despite an important first step by Findlay *et al*. (2011), their results do not help to explain the discrepancies between all studies, in particular those that focus on the *E. huxleyi* strain PLY M219, or "NZEH"; this strain has been something of a "controversial" organism, with Iglesias-Rodriguez *et al.* (2008) reporting no change in PIC:POC with elevated CO_2, a contradiction to earlier studies on *E. huxleyi*. More recent studies on NZEH have found a decrease in PIC:POC in response to higher CO_2 (Shi *et al.* 2009; Hoppe *et al.* 2011). Importantly, the method used to grow this strain was generally the same throughout, with similar light intensities, nutrient regime, temperature employed. However, one important factor that was not consistent across the studies, and not considered in the meta-analysis by Findlay *et al.* (2011), is daylight period: a 12:12, 18:6 and 24:0 light:dark (L:D) cycle for Iglesias-Rodriguez *et al*. (2008), Hoppe *et al.* (2011) and Shi *et al.* (2009), respectively. Daylight period can have significant effects on the physiology of phytoplankton. Many species increase their maximum growth rate with increasing day length (Foy and Gibson 1993; Thompson 1999), though some plateau at very long daylight periods (Nielsen 1992; Foy and Gibson 1993); and importantly the photophysiology of *E. huxleyi* has been shown to alter in response to changes in daylight period, with increasing day length resulting in a lower photosynthetic rate, and less photosynthetic efficiency (Nielsen 1997). It is entirely likely that differences in daylight period may contribute to different responses to OA, as the cultures are in different physiological states. Longer daylengths effectively provide an extended window in which to photosynthesise and in turn synthesise (in)organic constituents; therefore, not only could daylength affect how much (and the timing with which) any one consistent is ultimately produced.

1.7. Thesis Aims and Objectives

The overall aim of this thesis is to determine whether and how the light-dark cycle (L:D) regulates the response to elevated CO_2 in phytoplankton. Experimentation will examine how changes in L:D cycle affect the magnitude and timing of PIC:POC allocation under elevated CO_2 conditions. This work will initially focus on *E. huxleyi* strain NZEH in order to compare different L:D conditions used in past studies. I will experimentally examine the physiology of the NZEH strain under high and low CO_2 under both a 14:10h L:D cycle and continuous light (24h). This experimental analysis will be widened to include a range of taxa, including more *E. huxleyi* isolates, and two other species of coccolithophore to determine i) how "typical" NZEH is in terms of coccolithophore biology, ii) whether or not biogeography has an influence on an organism's response to both CO_2 and L:D cycle. These organisms come from a range of starting conditions, most notably *E. huxleyi* RCC862 and *G. oceanica* originate from the tropics, while the rest of the organisms were isolated from temperate regions. This potentially means there are different capacities to deal with changes in photoperiod across these taxa. Analysis of coccolithophore photobiology, which still remains poorly resolved, will be conducted using fast repetition rate (FRR) fluorometry. FRR fluorometry data can be used to evaluate the photosynthesis response to CO_2 and L:D cycle, and will also look at how the coccosphere affects these changes. Finally, experimental work on natural populations in the polar regions will add better perspective as to whether these laboratory results have real world applications.

Chapter 2: Moderation of the response of *Emiliania huxleyi* strain NZEH to ocean acidification and photoperiod

2.1 Introduction

Atmospheric CO_2 has risen drastically since the Industrial Revolution nearly 250 years ago. Present day levels are ~385ppm but are predicted to reach ~730–1020ppm by 2100 if the current rate of anthropogenic emissions continue (IPCC 2007). The oceans contribute to almost 50% of all productivity on the planet (Behrenfeld *et al.* 2009) and thus act as a strong CO_2 sink, already absorbing around 40% of anthropogenically derived CO_2 (Sabine and Tanhua 2010). CO_2 reacts with water to form carbonic acid but this is offset by a back reaction with carbonate thereby providing "natural buffering". However, this buffering capacity is not limitless, and ultimately excess CO_2 will drive ocean pH down, a process termed ocean acidification (OA) (Caldeira and Wickett 2003; Raven *et al.* 2005). The reduction of carbonate and pH therefore has a strong influence on the seawater dissolved inorganic carbon (DIC) and in turn the organisms that rely on this pool for key processes such as photosynthesis and calcification.

Recent research over the past 5-10 years has focussed on how phytoplankton will be affected by OA (see Rost *et al*. (2008) for a review). Particular attention has been given to the coccolithophores, a globally distributed group of calcifying phytoplankton, and specifically the species *Emiliania huxleyi*, an important bloom-former that exhibits genotypic variation over global spatial scales (Brand 1982). Early studies demonstrated a decline of coccolithophore calcification to elevated CO_2 levels, e.g. (Riebesell *et al.* 2000; Sciandra *et al.* 2003); however, more intensive analyses demonstrated that this calcification response is in fact highly variable both between species (Langer *et al.* 2006) and even between strains of one species, such as *E. huxleyi* (see Ridgewell *et al.* (2009)). Notably, experimental work on *E. huxleyi* strain PLY M219/NZEH has demonstrated a range of physiological responses despite using similar methods

for replicating OA conditions (Iglesias-Rodriguez *et al.* 2008; Shi *et al.* 2009; Hoppe *et al.* 2011; Jones *et al.* 2013; Rouco *et al.* 2013). Differences in other key variables used to grow NZEH, such as nutrient availability and light regime may mediate this OA response (e.g. Findlay *et al.* (2011)); thus identifying such 'mediating factors' that complicate reconciliation amongst studies is key to better understand (and predict) just how OA will impact primary biogeochemical processes of the oceans.

The contradictory results from the same isolate, NZEH (Iglesias-Rodriguez *et al.* 2008; Shi *et al.* 2009; Hoppe *et al.* 2011; Jones *et al.* 2013; Rouco *et al.* 2013) are particularly interesting since they rule out the possibility of intra-specific variability among coccolithophore isolates (e.g. Suggett et al. (2007)), but rather points to differences in the way environmental factors are being controlled across OA experiments. At face value, the main disparity between these studies is the light-dark (L:D) cycle used to grow the organisms, ranging from relatively short (12h) up to constant light (24h). Importantly, light availability in terms of intensity, is known to influence physiological responses to OA (Gao *et al.* 2012), including coccolithophores (McCarthy *et al.* 2012); however, differences in L:D cycle are also well documented to alter algal physiology but have not yet been examined in the context of OA. For example, some phytoplankton species show a decrease in growth rates as daylength increases (Burkhardt *et al.* 1999; Rost *et al.* 2002), while others have shown that their grow rates are independent of L:D cycle (Nielsen 1992; Foy and Gibson 1993; Price *et al.* 1998). More specifically, *E. huxleyi* has a nearly threefold higher chlorophyll a (chl a) specific photosynthetic rate when grown under a 12:12h L:D cycle, compared to continuous light (Nielsen 1997). Perhaps most importantly relative to OA, changes in photoperiod can profoundly alter carbon uptake by phytoplankton. Specifically, *E. huxleyi* cells show a far greater dependency on HCO_3^- when grown under shorter photoperiods, compared to continuous light (Rost *et al.* 2006), perhaps indicating that carbon concentrating mechanisms (CCMs) are down-regulated when light is constant.

In order to evaluate whether L:D cycle is indeed an important variable in regulating the OA response of NZEH in past experiments, I conducted a fully factorial multivariate experiment altering pCO_2 (400 µatm vs 1000 µatm) and photoperiod (14h vs 24h). I specifically tested the hypothesis that under elevated pCO_2, NZEH maintain its growth rate, but with a cost of decreased PIC production rate in continuous light, but reduces growth to maintain PIC production under 14:10h L:D.

My aim was initially examined by sampling at one time point throughout the day, but further experiments evaluated the growth dynamics (sampling after lights are turned on, and just before they turn off) throughout the day for the cells under the different daylength regimes: as such, I was able to further evaluate the robustness of sampling between treatments at a single time point. The results are specifically considered as part of a multifactorial analysis of growth factors on NZEH OA responses along with past results for the strain.

2.2. Methods and Materials

2.2.1. Culturing Conditions

Six replicate cultures of *E. huxleyi* (strain PLY M219, also known as "NZEH") were simultaneously grown in climate-controlled growth cabinets maintained at 17°C with a photon flux density (PFD) of ~300µmol photons m^{-2} s^{-1} in a growth cabinet (Sanyo Gallenkamp, Fitotron PG660, Loughborough UK), and grown in artificial seawater enriched with f/2 medium nutrients (Guillard 1975) and selenium (1.0 x 10^{-4} M solution). Three replicates were grown at ambient pCO_2, and three at a "future" higher pCO_2, thus providing true biological replicates at each CO_2 level; these levels were initially set as 385ppm and 1000ppm (see Section 2.3.2 for details on carbonate chemistry). These six replicates were grown with two different photoperiods; 14h and 24h. During the 14h photoperiod, the lights were switched on at 0700 GMT and turned off at 2100 GMT. Cultures were monitored daily via cell counts (Section 2.3.3)

and were maintained semi-continuously by dilution when necessary to maintain cells in exponential growth. The cultures were grown in steady state growth for 3 weeks (ca. 15 generations) to allow for acclimation to the CO_2 and/or light conditions. During this period, the cultures were sampled every day to take cell counts for the purpose of calculating growth rates, as well as general observations of the state of the cultures. These growth rates were used to calculate the dilution factor for each vessel to keep the cultures in exponential growth. Additionally, the pH, alkalinity and [DIC] of each vessel were measured every day to monitor the state of the carbonate chemistry. All other physiological paramters were measured only on the final day of the experiment (i.e. at the end of the 3 week period. The growth rates and carbonate chemistry parameters presented here are data from the final day of the experiment.

2.2.2. Controlling and monitoring the carbonate system

The carbonate system was adjusted by bubbling CO_2 in air into the culture media via a series of mass flow controllers (MFCs). Both a cylinder of 10% CO_2 (BOC) and an air compressor (Bambi, HT15 oil-free compressor) were each connected to a MFC (Bronkhorst High-Tech, EL-FLOW F-201AV) via nylon tubing. The air was first passed through soda lime (Sigma 23888) to remove any CO_2. Both MFCs were connected to a laptop with software provided by Bronkhorst that both controlled and recorded the flow of gas. From the MFCs, the gas lines were fed into a gas mixer, and through to a set of 4 taps. One tap fed the gas into an infra-red gas analyser (IRGA) (Li-Cor, LI-820 analyser) connected to the laptop to verify the CO_2 concentration, while the other 3 taps ran into one set of triplicate cultures at a rate of 150mL min^{-1}. All air-in and air-out ports on the culture vessels were fitted with 0.2µm hydrophobic air filters (Millipore, MA, USA).

The air-in line to all culture vessels ended in a glass tube with an air stone at the bottom to ensure better diffusion of the gases into the media. All culture vessels sat on a magnetic

stirring plate that turned a small magnetic stir bar to both help the gases mix better, and minimise clumping.

Several parameters of the carbonate chemistry were measured every day to monitor how it was changing. The actual pCO_2, along with other important parameters such as $[HCO_3^-]$, $[CO_3^{2-}]$ and **Ω**, were calculated by putting this data into the CO2SYS software (ver. 14) (Pierrot *et al.* 2006).

Alkalinity was measured by taking 20mL aliquots from each vessel that were gently gravity filtered with a 26mm 0.2µm syringe filter (Minisart filter, Sigma-Aldrich), and used for a volumetric titration using an auto-titrator (Metrohm 870 Karl Fischer Volumetric Titrino). A probe records pH using the total hydrogen scale as small volumes of HCl are added to the sample. Acid is added until the sample reaches the second equilibrium point (EP2) of seawater. This occurs where pH ≈ 4.5, and $[H^+]$ is equal to $[HCO_3^-]$. TA can then be calculated with the following equation:

$$TA\ (\mu mol\ kg^{-1}) = \left[\frac{vol_{acid}\ \cdot\ N_{acid}}{vol_{sample}}\right] \cdot 1000000 \qquad (1)$$

where vol_{acid} is the volume of acid required to complete the titration, N_{acid} is the normality of the HCl used, and vol_{sample} is the volume of sample used for the measurement. Sample pH was also recorded with the titrator, as the probe was constantly calibrated using a standard.

To measure the DIC in the media, 15mL aliquots were gravity-filtered through syringe filters, and the filtrate transferred to glass vials that had been heated in a muffle furnace at 500°C for 3 hours to remove any carbon. These samples were run through a total carbon analyser (Shimadzu TOC-VCSH Total Organic Carbon Analyzer with ASV-I autosampler), which gave a

reading in μmol kg^{-1}. Regular calibration of the instrument using bicarbonate standards was necessary.

It was important to ensure full CO_2 control over the biology and hence I initially evaluated the extent to which cells could divide and grow before significantly altering the carbon chemistry analysis of the seawater carbon chemistry, as part of a preliminary experiment using batch cultures of NZEH (Fig. 2.3.1). The media was not changed over the course of this experiment, and samples were taken for cell counts and to characterise the carbonate chemistry. Alkalinity was chosen as the main indicator of how the media was changing because a) the alkalinity should remain relatively constant; and b) this is the parameter most affected by calcification. As cell density increased, *E. huxleyi* caused a drawdown of alkalinity, as expected. The cell density chosen to grow the cultures at was 150,000 cells ml^{-1}, which is a trade-off between minimising alkalinity drawdown (~200μmol kg^{-1}), and having enough material to be able to take samples from the vessels.

The alkalinity of the media used here is low relative to expected alkalinity of seawater (~2400μmol kg^{-1}). Initially, autoclaving was the method used to sterilise the culture media, but this causes a drive off of CO_2. All subsequent cultures were grown in media that was filter sterilised through a 0.2μm filter (Polycap 36AS, Whatman Filters), and was more realistic in terms of carbonate chemistry. Full characterisation of the carbonate system in the experimental cultures can be found in Table 2.3.1.

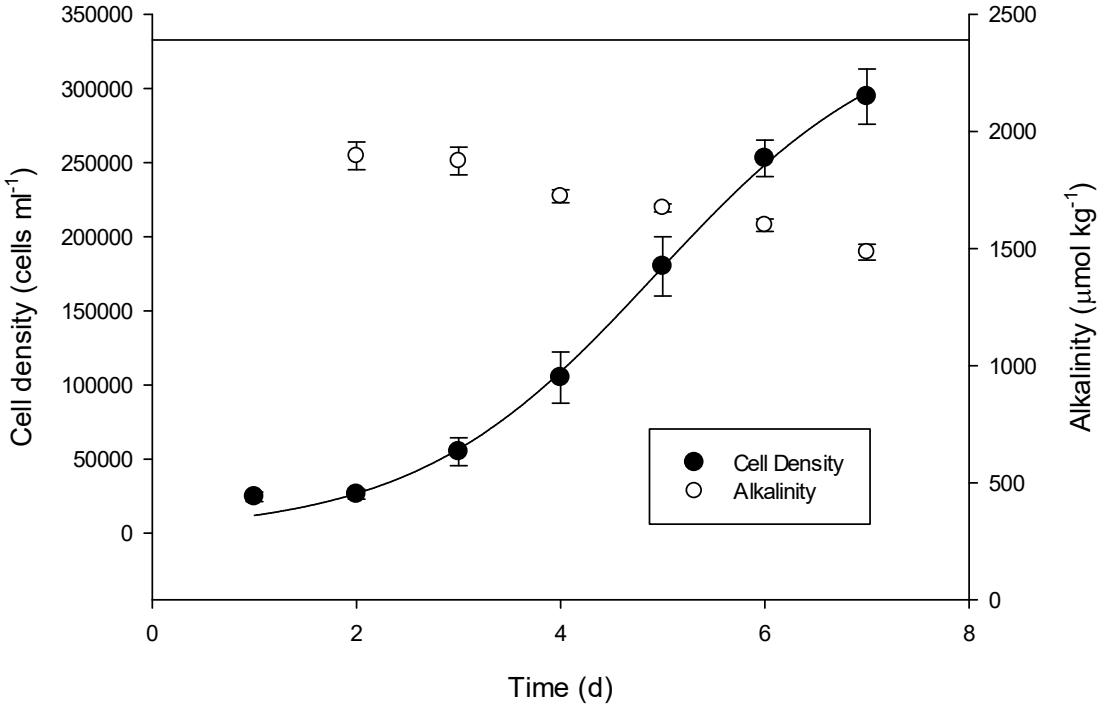

Fig. 2.2.1. Changes in alkalinity with cell density in batch cultures of *E. huxleyi* NZEH used to predict how the carbonate chemistry of artificial media is perturbed by biological activity (error bars are +/- S.D., *n*=3).

Table 2.2.1. Actual carbonate parameters in experimental cultures (+/- S.D., n=3). Alkalinity, pH and DIC are direct measurements, all other parameters are calculated from that. Data used is from 3 days of sampling, after growing ca. 15 generations of *E. huxleyi* NZEH.

	Target pCO_2 (µatm)	Actual pCO_2 (µatm)	Alkalinity (µmol kg^{-1})	pH	DIC (µmol kg^{-1})	[HCO_3^-] (µmol kg^{-1})	[CO_3^{2-}] (µmol kg^{-1})	Ω
14h	400	433.07	2329.78	8.03	2106.11	1927.68	163.12	3.92
		(49.69)	(22.60)	(0.01)	(30.57)	(39.87)	(14.50)	(0.35)
	1000	782.59	1937.66	7.73	1851.02	1749.96	73.39	1.76
		(37.56)	(40.40)	(0.02)	(31.86)	(27.75)	(5.47)	(0.13)
24h	400	454.74	2215.14	8.00	2018.46	1859.45	142.93	3.43
		(43.50)	(41.34)	(0.03)	(48.11)	(50.58)	(6.93)	(0.17)
	1000	1032.23	1921.97	7.64	1865.11	1768.83	59.77	1.92
		(167.34)	(41.71)	(0.03)	(41.14)	(41.00)	(10.31)	(0.25)

2.2.3. Growth rates and cell size

Cells were counted daily by taking a 2mL sample from each culture and measured microscopically using a Neubauer haemocytometer slide (Fisher Scientific, Loughborough, UK). Growth rates (μ) were calculated using the following equation:

$$\mu\,(d^{-1}) = (\ln c_0 - \ln c_1) / \Delta t^{-1} \qquad (2)$$

where c_0 is the initial count (cells mL^{-1}), c_1 is the final count (cells mL^{-1}), and Δt is the time between the two counts (days). Cell size was measured by taking a 15mL sample and running it through a Coulter counter. Coccosphere thickness was calculated by running the sample again after adding 5μL of weak HCl acid (to remove the coccoliths). The difference in diameter was taken as the coccolith thickness.

2.2.4. Chlorophyll a analysis

A volume of 150mL was filtered through 25mm MF300 glass fibre filters (Fisher Scientific, Massachusetts, USA) which were then flash-frozen in liquid nitrogen. Samples were stored at -80°C for later analysis. Pigments were extracted from the cells by grinding the filters each in 5mL of 90% acetone and then refrigerating in the dark for 2 hours. Each was then then centrifuged at 4500rpm for 5 minutes, and the supernatant pipetted into cuvettes. Absorbance was measured at 630nm, 644nm and 750nm using a spectrophotometer (U-3000, Hitachi High Technologies, Wokingham, UK) relative to acetone blanks to correct the readings. Chlorophyll concentrations were finally determined using the equations of Ritchie (2006), and normalised to cell concentration and volume.

2.2.5. PIC and POC Analysis

Two aliquots of 150mL were each filtered down onto ashed (heated in a muffle furnace at 500°C for 3 hours) 25mm MF300 glass fibre filters (Fisher Scientific, Massachusetts, USA) and then placed in a desiccator to dry for 24 hours. Samples were stored in cryotubes to be analysed

at a later date. One of each pair of filters was acidified with ~2M HCl to drive off organic carbon, and further dried for 24 hours. The total carbon on each filter was measured using a carbon analyser (Shimadzu TOC-VCSH Total Organic Carbon Analyzer with ASV-I autosampler) calibrated using a glucose standard. Particulate inorganic carbon (PIC) was measured on the acidified filters, and Particulate Organic Carbon (POC) was calculated by subtracting PIC from the total carbon measured on the non-acidified filters. Both PIC and POC were then normalised to cell concentration and volume.

2.3. Results

2.3.1. Past evidence for L:D cycle influence upon NZEH OA response

Past NZEH studies were initially evaluated to quantify the possible extent of L:D upon the OA response. Data mined from studies with continuous light (L:D 24:0, Shi et al. 2009; Hoppe et al. 2011) clearly exhibited a very different OA response of growth rate, but also cellular PIC and POC content in comparison to data from studies using a L:D cycle (Iglesias-Rodriguez et al. 2008) (Figs. 2.4.1A, B, C). Specifically growth rates decrease with CO_2 for the shorter photoperiod (Fig. 2.4.1A) but were never >0.8 d^{-1}, even in the low CO_2 cultures. Under continuous light, growth rates remained constant despite changes in CO_2, but were consistently higher than those of the shorter L:D cycle cultures (always >1 d^{-1}). Comparing R^2 values confirms that while CO_2 strongly controls growth rate in the L:D cycle cultures ($R^2 = 0.91$), there is little to no CO_2 effect under continuous light ($R^2 = 0.27$).

Similar to the growth rate, both the response of POC and PIC cellular content to OA differed between data from continuous light and L:D 14:10; here, PIC $cell^{-1}$ and POC $cell^{-1}$ increased with CO_2 under the short photoperiod (Fig. 2.3.1), though POC increases by a much more considerable amount (a 3-fold increase, compared to a 1.5-fold increase in PIC).

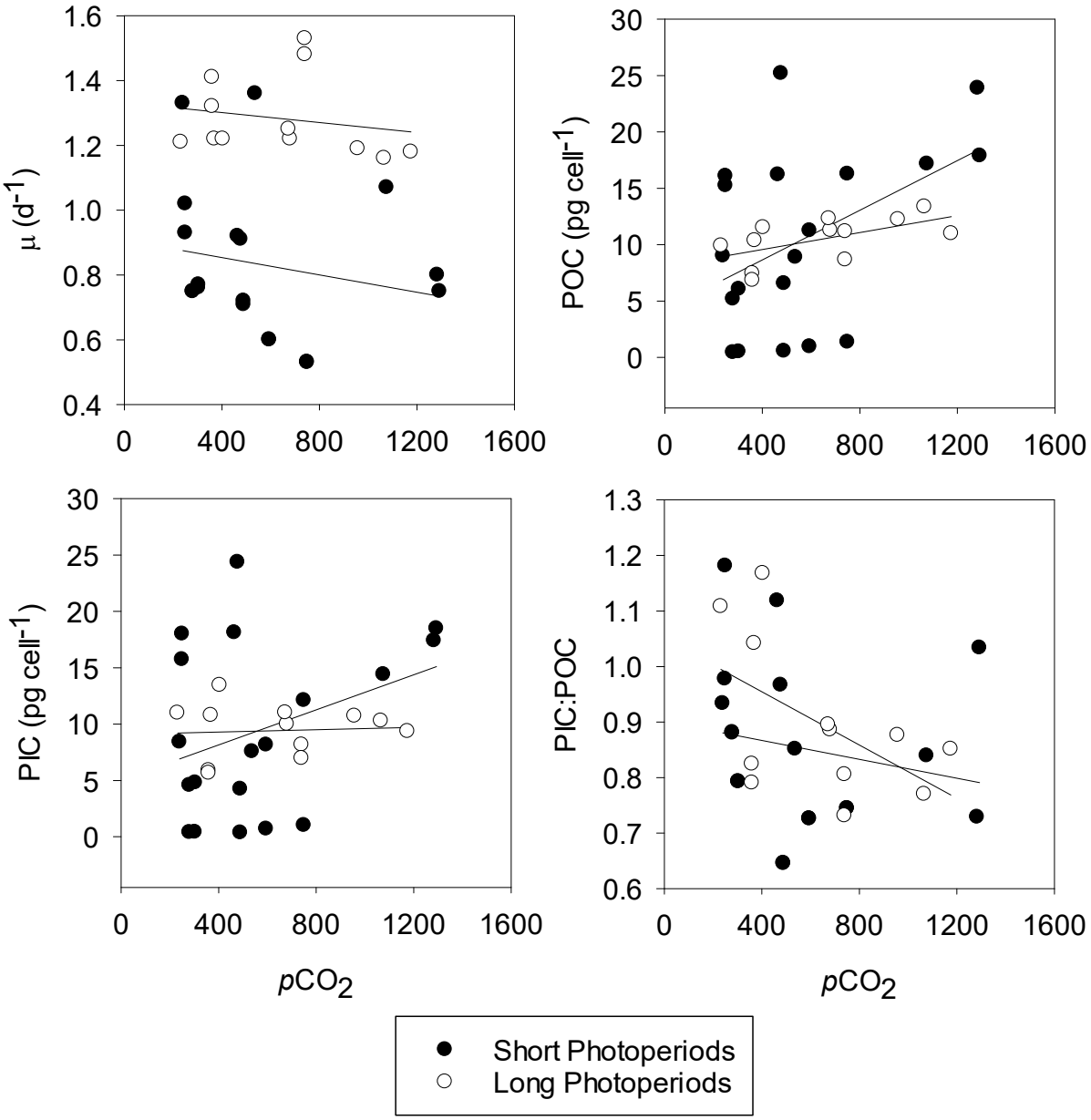

Fig. 2.3.1. Responses of *E. huxleyi* NZEH growth rate (A), POC per cell (B) and PIC per cell (C) to changes in pCO_2. Data are from 3 different studies; Iglesias-Rodriguez *et al*. (2008) (closed circles), Shi *et al*. (2009) and Hoppe *et al*. (2011) (open circles). The closed circles also correspond to a "short" L:D period (12h), versus the open circles where the experiments used a "long" L:D period (18h in Hoppe *et al*., 24h in Shi *et al*.). R^2 values for each data set can be found in Table 2.4.1.

However, the CO_2 effect is lost under continuous light, as indicated by the low R^2 values (0.31 for POC, 0.15 for PIC). Both PIC and POC production are higher at ambient CO_2 conditions when grown under 24h of light, compared to the shorter photoperiod.

Observation of the relationships between growth rate, PIC and POC and pCO_2 in these past studies indicated a major issue in attempting to reconcile data across several studies; specifically the trends observed were particularly biased by Rouco *et al*. (2013) and Jones *et al*. (2013). The values obtained in Jones et al for these parameters are very high relative to the average for NZEH, and Rouco *et al*. (2013) looked at the combined effect of nutrients and pCO_2, further skewing some of the data. Removal of these studies from the analysis revealed clearer trends. An ANCOVA analysis on the results showed that L:D cycle was a significant covariable for growth rate and POC, but only when the two studies mentioned were not included.

The multiple regression analysis indicated that L:D cycle was a key variable in predicting both growth rate and POC, while light intensity proved to be more important when determining PIC production of NZEH. The concentrations of both NO_3 and PO_4 made up the other constituents of the growth rate model ($F_{3,34}$=24.578; p<0.05; R^2=0.675). The beta weights indicate that there is a positive correlation with all variables included in the model, meaning longer L:D cycles induce higher growth rates.

The PIC model shows a strongly negative correlation with light intensity and temperature ($F_{3,34}$=9.318; p<0.05; R^2=0.342). The POC model included the most variables ($F_{3,34}$=17.418; p<0.05; R^2=0.720).

Table 2.3.1. Mulitple regression output indicating variables controlling changes in growth rate, PIC or POC cell^{-1} using all published data on *E. huxleyi* NZEH to date. Environmental variables included were temperature, L:D cycle, light intensity, PO_4, NO_3, salinity, pCO_2 and alkalinity.

	Included variables	Beta weights	*p*
Growth Rate	L:D cycle	0.401	0.007
	PO_4	0.570	0.001
	NO_3	0.241	0.038
PIC	Light intensity	-0.902	0.000
	Temperature	-0.587	0.009
POC	Light intensity	-1.623	0.000
	pCO_2	0.386	0.000
	Temperature	-1.323	0.000
	PO_4	-1.058	0.000
	L:D cycle	1.000	0.000

2.3.2. Experimental test of L:D influence upon the OA response of NZEH

Growth rates at ambient pCO_2 under both light regimes were comparable, at 0.89 d^{-1} (14h) and 0.93 d^{-1} (24h); however, growth rate decreased by ~20% under CO_2 enrichment for L:D 14:10, but changed very little with CO_2 under continuous light. In contrast to the growth rate, POC $cell^{-1}$ and PIC $cell^{-1}$ under ambient pCO_2 were affected by L:D cycle, whereby POC $cell^{-1}$ was ~30% less under continuous light compared to the 14h cultures, but PIC $cell^{-1}$ was ~120% higher in cultures grown under continuous light. In all cases, POC $cell^{-1}$ and PIC $cell^{-1}$ increased with CO_2, but continuous light dampened this response. When grown with the 14h photoperiod, POC increased by ~40%, but when grown in continuous light, this increase was only ~15%. PIC increased by 33% with CO_2 under the 14h light regime, but again this increase was dampened to ~17% when the cultures were grown under continuous light.

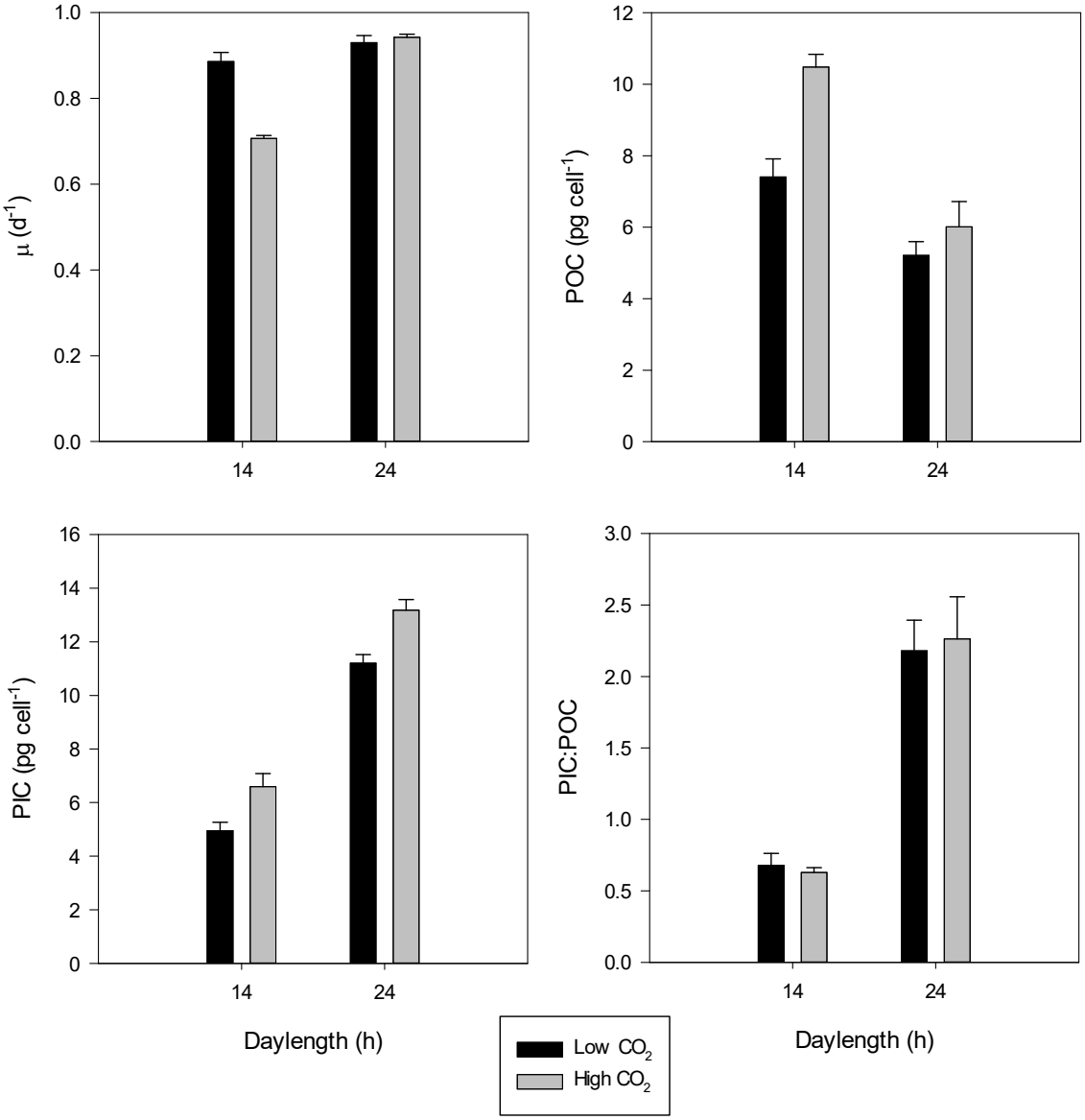

Fig. 2.3.3. The combined effects of photoperiod and pCO_2 on growth rate (A), POC cell^{-1} (B), PIC cell^{-1} (C), and PIC:POC (D) of cultures of *E. huxleyi* NZEH. See Table 2.3.1 for full characterisation of carbonate system in each treatment. Error bars are +/- S.D., n=3. There is a significant combined effect of the two treatments on POC (2-way ANOVA, $p<0.05$).

Table 2.3.2. Summary of 2-way ANOVA examining the influence of L:D cycle (14:10 and 24h)and CO_2 on growth rates, PIC cell^{-1}, POC cell^{-1} and PIC:POC in *E. huxleyi* NZEH. Significant outcomes and interactions are highlighted in bold.

Treatment	df	Growth		PIC		POC		PIC:POC	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
L:D cycle	1	29.76	**<0.001**	22.11	**0.001**	14.49	**0.005**	0.007	0.93
CO_2	1	72.43	**<0.001**	278.08	**<0.001**	42.93	**<0.001**	69.85	**<0.001**
L:D x CO_2	1	40.05	**<0.001**	0.18	0.60	5.02	**0.05**	0.13	0.73

Changes in cell size corresponded to changes in POC cell^{-1}, with cells being ~50% smaller by volume (and therefore containing less POC) in the continuous light treatment. L:D cycle had a much bigger impact on cell size than CO_2 concentration did.

The coccosphere was thicker overall in cultures grown under the shorter photoperiod (0.45 and 0.58µm, versus 0.15 and 0.36µm), despite cells being larger (see Fig. 2.4.4. D). CO_2 always induced thicker coccospheres, with a ~30% increase in 14h cultures, and a ~150% increase in 24h cultures. CO_2 only had a significant effect on coccosphere thickness under 24h of light. It is interesting to note that while cell size conforms to POC cell^{-1}, coccosphere thickness does not relate well to PIC cell^{-1}, suggesting that PIC is possibly over estimated in the 24h cultures.

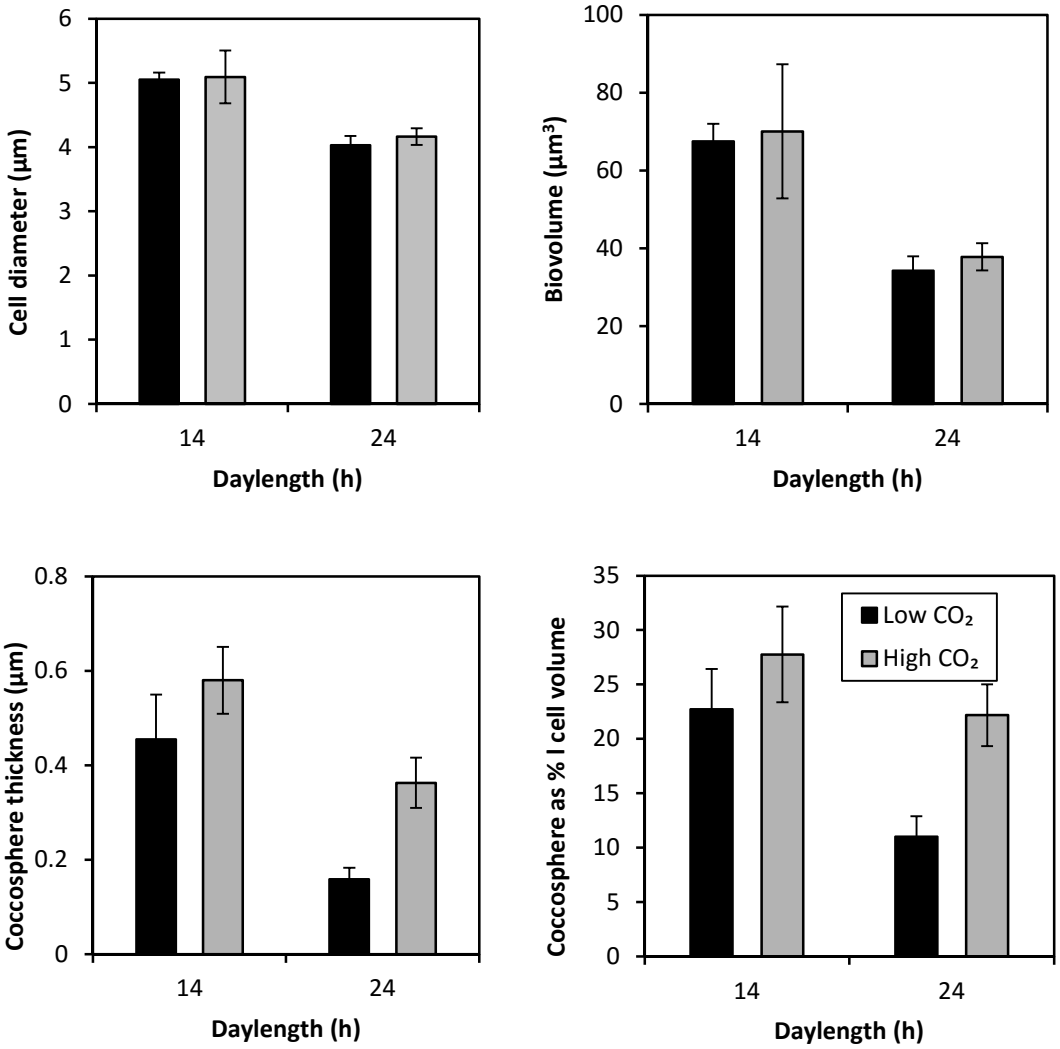

Fig. 2.3.4. The effect of photoperiod and pCO_2 on cell size and structure, which relate to changes in cell diameter and cell volume (not including the coccosphere), coccosphere thickness, and how the coccosphere relates to cell size. Error bars are +/- S.D., n=3.

CO_2 enrichment had a strong positive effect on chlorophyll cell-1 under the 14h photoperiod, yielding a 3.5-fold increase from 0.61pg cell^{-1} to 2.25 pg cell^{-1}. CO_2 induced a much smaller increase under continuous light, ~25%, but chlorophyll levels at ambient CO_2 were much higher than the 14h cultures, being of a similar value to the high CO_2 cultures at 14h, and rose to 3.03pg cell^{-1}. When normalised to cell volume, the differences in chlorophyll content become far more clear. Because the cells are much smaller, chlorophyll per cell volume is much higher in the 24h cultures, 19.96pg cell^{-1} at ambient CO_2, and doubling to 41.0pg cell^{-1} at high CO_2.

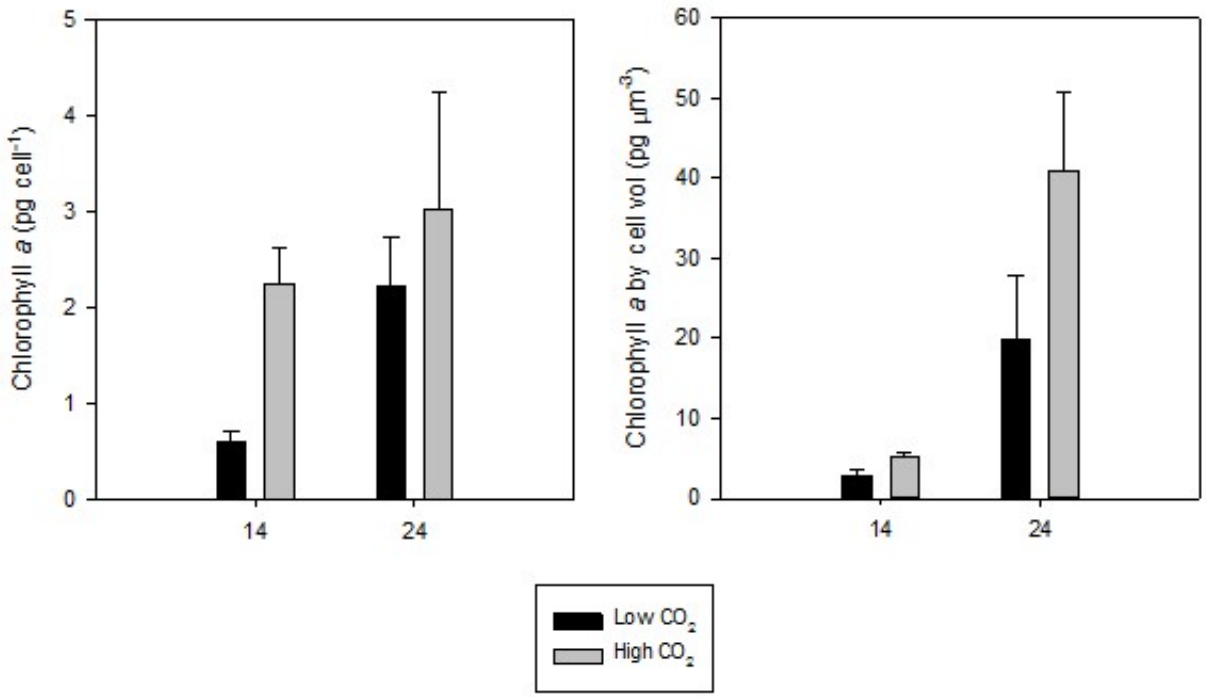

Fig. 2.3.5. The combined effects of photoperiod and pCO_2 on pg chlorophyll a per cell (A) and chlorophyll normalised to cell volume (B) on cultures of *E. huxleyi* NZEH. Black bars are low CO_2 cultures, shaded bars are high CO_2. Error bars are +/- S.D., n=3.

Table 2.3.3. Summary of 2-way ANOVA examining the influence of L:D cycle (14:10 and 24h)and CO_2 on cell volume, coccosphere thickness and chlorophyll cell^{-1} in *E. huxleyi* NZEH. Significant outcomes and interactions are highlighted in bold.

Treatment	df	Cell volume		Coccosphere thickness		Chlorophyll by cell volume	
		F	*p*	*F*	*p*	*F*	*p*
L:D cycle	1	18.57	**0.002**	0.32	0.58	9.32	**0.01**
CO_2	1	45.19	**<0.001**	37.41	**<0.001**	133.74	**<0.001**
L:D x CO_2	1	1.09	0.32	0.008	0.93	6.43	**0.03**

2.4. Discussion

Daylength is an important factor in modulating the response of growth rate to CO_2 in NZEH. The decrease in μ with CO_2 observed in 14h cultures was not present under continuous light (Fig. 2.3.3A). As *E. huxleyi* uses an inefficient CCM (Rost *et al.* 2003), it seems unusual that elevated CO_2 would cause a decrease in growth rate (14h cultures), but could indicate a

reallocation of resources. While cells grown under the shorter photoperiod produce less PIC at high CO_2 than those grown in continuous light (Fig. 2.3.3C), they are also bigger (Figs. 2.3.4A and B) and contain more POC (Fig. 2.3.3B). Comparatively, the cells in the high CO_2 treatment under continuous light grow fast and produce more PIC, but are far smaller.

This implies that perhaps daylength changes the way cells allocate resources under high CO_2 conditions, specifically that shorter photoperiods result in a prioritisation of cell maintenance, while continuous light favours cell division. In nature, *E. huxleyi* forms blooms in stratified, high light conditions (Balch *et al.* 1991; Tyrrell *et al.* 1999). *E. huxleyi* has a lower affinity for inorganic carbon under continuous light, as well as low C fixation rates, but is still able to maintain high growth rates under these conditions (Rost *et al.* 2002; Rost *et al.* 2006). Perhaps prioritising cell division during long photoperiods is an adaptation to enable this species to quickly capitalise on bloom conditions and reach high densities.

Desynchronisation of cell division under continuous light could also affect some of the differences in physiology relating to cell growth. When grown under L:D cycles, the cell cycle is synchronised throughout the culture, meaning all cells divide at a similar time (Jochem and Meyerdierks 1999; Müller *et al.* 2008). The high growth rates achieved under continuous light here (0.92 and 0.93 at low and high CO_2 respectively) also coincide with smaller cell size. However, the low CO_2 cultures grown under 14h had comparable growth rate, but the cells were larger. As cells only divide once they have reached a critical diameter (Müller *et al.* 2008), it is likely that the smaller cell size in continuous light cultures is as a result of desynchronisation.

While daylength does not have an effect on the OA response of PIC $cell^{-1}$, it does modulate its production overall. Cultures grown under the short photoperiod produced between 5 and 6 pg $cell^{-1}$, and this more than doubles to between 11 and 14 pg $cell^{-1}$ under continuous light (Fig. 2.3.3C). This equates to an increase in calcification as the PIC:POC values are very high

(Fig. 2.3.3D), indicating that it is L:D cycle rather than CO_2 that has a more important role in controlling PIC:POC in NZEH. These are interesting results that could again reflect a disruption in the daily cell cycle under continuous light. Calcification is linked to the cell division cycle, and more specifically, only takes place during the G1 (gap 1, assimilation before DNA synthesis) phase (Müller *et al.* 2008). In fact, the coccolith vesicle is not present in *E. huxleyi* cells until after mitosis has occurred (Linschooten *et al.* 1991), so calcification should be limited by high growth rates. This is the opposite of what was observed here, where cells that are growing slower and allocating more resources to cell maintenance (and therefore likely to be in G1 phase) produce less PIC than those growing more rapidly and of smaller size. It has been reported that calcification is slowed during periods of darkness (Balch *et al.* 1992; Paasche and Brubak 1994; Paasche 2001), so perhaps L:D cycle has an impact on the amount of time spent in each phase of the cell cycle. The daily cell cycle is poorly understood in phytoplankton, so it is unclear how exactly it is affected by continuous light.

E. huxleyi typically blooms in highly stratified waters during spring and summer, where cells encounter high surface irradiances. Calcification has been proposed as a means of photoprotection, and that could be why PIC production increases under continuous light. It has previously been demonstrated that *E. huxleyi* is capable of dissipating excess excitation energy from sudden increases in irradiance through elevated rates of calcification (Ramos *et al.* 2012). Additionally, naked strains of *E. huxleyi* show less photoprotective capability (Xu and Gao 2012) and are more easily damaged by UV radiation (Gao *et al.* 2009).

These results could also indicate that a stronger link between calcification and photosynthesis exists than previously thought, as evidenced by the higher PIC:POC values with continuous light. While some studies have shown that photosynthesis is not directly dependent on calcification (Trimborn *et al.* 2007; Leonardos *et al.* 2009), it could still act as a CCM. As already discussed in Chapter 1, photosynthetic rates in most phytoplankton are already near their

maximum, despite modern CO_2 concentrations not being at saturating levels, due to the presence of CCMs (Tortell 2000; Giordano *et al.* 2005). *E. huxleyi* operates an inefficient, low-affinity CCM (Rost *et al.* 2003). Interestingly, *E. huxleyi* also preferentially uses HCO_3^- as an inorganic carbon source when grown under L:D cycles, but almost entirely depends on CO_2 under continuous light (Rost *et al.* 2006). Intracellular calcification is proposed to work as a CCM by providing excess protons to drive the dehydration of HCO_3^- to CO_2 (Nimer and Merrett 1992; Paasche 2001). Therefore, increasing calcification (i.e. PIC cell^{-1}) may allow cells to use HCO_3^- as a carbon source even under continuous light, thus maintaining high growth rates.

Under continuous light, PIC cell^{-1} showed an inverse relationship with coccosphere thickness (see Figs. 2.3.4C and D). Daily observations of the cultures under a light-microscope showed many lose liths in the culture media, indicating that while continuous light increased PIC production, less of it remained attached to the cells. It appears that continuous light may in fact cause over-production of PIC, which may be again related to disruptions in the cell cycle. As already mentioned, periods of darkness result in lower rates of calcification, and as such, it may be that dark intervals are required to stop cells from over-calcifying.

It is interesting to note that the phenomenon of shedding liths has been observed in nature in *E. huxleyi* blooms (Balch *et al.* 1991; Tyrrell *et al.* 1999). The reasons for coccolith shedding are still poorly resolved. Infection from viruses has been shown to induce the loss of coccoliths (Bidle *et al.* 2007; Frada *et al.* 2008), but as this consistently only happened under continuous light, even for other coccolithophores tested (see Chapter 3), it is unlikely that this is the cause here.

Coccolithophores produce many extracellular polysaccharides (EP), including transparent exopolymer particles (TEP). Some of the precursors of TEP have long been known to be important in both the production and attachment of coccoliths to the coccosphere (de Jong *et al.*

1979). TEP production is in fact enhanced by elevated CO_2 (Engel 2002; Engel *et al.* 2004; Borchard and Engel 2012), which could explain why the coccosphere is thicker in high CO_2 cultures even under the shorter photoperiod (Fig. 2.3.4C). However, the effects of light availability on TEP have not been tested. Shedding coccoliths has been found to increase coagulation of both particulate matter and cells (Chow *et al.* 2015), and thus aid in ballasting. It could be that shedding occurs in response to long periods of light as a means of photoprotection, by aggregating cells and sinking them to darker waters. At the same time, elevated temperatures enhance the degradation of aggregates formed from TEP and other polysaccharides (Piontek *et al.* 2009), while ocean acidification conditions reduce its stickiness (Mari 2008) meaning that as climate change progresses, this shedding could become a much less effective means of photoprotection.

Using a Coulter counter to measure the size of the coccosphere is a common method in the literature (see Aloisi 2015). However, the Coulter counter is unable to 'see' the coccolith shield, and as such underestimates the coccosphere size compared to values derived from SEM (Langer *et al.* 2006; Oviedo *et al.* 2014) and light-microscope observations (van Rijssel and Gieskes 2002). Iglesias-Rodriguez *et al*. (2008) also reported that the Coulter counter underestimated coccosphere volume compared to flow cytometry measurements. Aloisi (2015) developed an equation for calculating the coccosphere volume using PIC and POC $cell^{-1}$, which produces values that coincide with coccosphere size determined through SEM and light-microscope measurements, but are always larger than values observed with a Coulter counter. This equation assumes that PIC $cell^{-1}$ is all associated with the coccosphere at the time of measurement, which would overestimate the coccosphere size for the cultures presented here when grown under continuous light. Therefore, more accurate characterisation of the coccosphere could be achieved using SEM micrographs, as in Poulton *et al*. (2011).

Conclusions

The results from experimental analysis showed that L:D cycle was a modulating factor for the OA response of growth rate, but had little impact on the way other aspects of NZEH physiology responded to CO_2. This lines up well with meta-analysis of the literature, where L:D cycle was the most important factor in predicting growth rate only. While it does not explain all the variation in the literature, the results do demonstrate that L:D cycle is a far more important factor in controlling PIC:POC than CO_2. Continuous light appears to create a stronger link between photosynthesis and calcification, potentially owing to the use of calcification as a CCM to access HCO_3^- as a carbon source. It also induces over-production of coccoliths, which could be a by-product of using calcification as a CCM, or caused by disruptions in cell cycle, or changes to TEP production. Continuous light also causes desynchronisation of cells within the culture, leading to smaller mean cell size.

These results can only be applied to NZEH, and do not take into consideration any intra- or inter-specific variation. It is unclear how unique or ubiquitous NZEH is with regard to coccolithophore physiology, and analysis on more strains and species is required to ascertain the importance of L:D cycle as a modulating environmental factor.

Chapter 3: Inter- and intra-specific responses amongst coccolithophores to the interaction of photoperiod and ocean acidification

3.1. Introduction

Ocean acidification (OA) research of coccolithophores has to date focused overwhelmingly on the species *Emiliania huxleyi*, due in part to its ecological and biogeochemical importance in waters of higher latitudes; this focus is perhaps unsurprising given that *E. huxleyi* produces large blooms detectable by satellite imagery, and contributes a large amount to the calcite production in the North Sea (Daniels *et al.* 2012). However, results from OA studies examining the same species are often highly variable as a result of contrasting environmental growth conditions (see Findlay et al. 2011), but is further confounded by the fact that there is high physiological and morphological variation within the *E. huxleyi* species (Brand 1982; Young 1994; Hiramatsu and De Deckker 1996; Wolfe and Steinke 1996). The notion of high functional diversity of the same species examined from laboratory cultures is intriguing since in nature many sub-populations of the same species can occur, which are geographically isolated and significantly genetically different from each other (Brand 1982; Iglesias-Rodriguez *et al.* 2006) thus indicating the potential for highly plastic physiological responses to exist to maintain population viability as environmental conditions change.

A total of five morphotypes have so far been identified in *E. huxleyi* (Young *et al.* 2003), which can exhibit highly variable calcification rates even under ambient CO_2 conditions. Little is known of the relative abundance of these morphotypes within *E. huxleyi* populations, and it is not fully understood if they are the result of a shift in genotype (i.e. distinct sub-populations) or phenotype (i.e. same genetic species, but coccolith formation is altered by environmental factors) (Smith *et al.* 2012). The few studies that have looked at the natural distribution of these morphotypes have found that they correlate to different water bodies (Findlay and Giraudeau

2000; Hagino *et al.* 2005), suggesting strong environmental control selecting for types of specific physiologies.

Even more interesting in the context of this thesis is the observation that the dominant morphotype in a population can change with shifts in seawater carbonate chemistry. Smith *et al*. (2012) found that during the winter period in the Bay of Biscay, the *E. huxleyi* population shifts from predominantly morphotype A to the overcalcified morphotype A, despite the seasonal decrease in **Ω** for that time of year. In contrast, the population returns to A morphotype in the summer. It is unclear why a decrease in $CaCO_3$ saturation causes overcalcification but Smith et al. (2012) suggest that this morphotype is particularly hardy and can endure the winter months, and/or changes in other environmental variables are likely important in triggering this response, but that genetic analysis is required to elucidate whether these morphotypes denote a genetic shift in the community, or are simply another life stage of *E. huxleyi*. To date it is still well established that there is no uniform OA response between different strains of *E. huxleyi* (Langer et al. 2009).

While the importance of *E. huxleyi* is well established, other species of coccolithophores can make significant contributions to the inorganic carbon pool globally. For example, in the south east Pacific, around 30% of suspended calcite can be attributed to not just *E. huxleyi*, but also *Gephyrocapsa spp*. and *Crenalithus spp*. (Beaufort *et al.* 2008); similarly, in the Mediterranean, *Syracosphaera sp.* and *Gephyrocapsa oceanica* can contribute to up to 10% of the coccolithophore population in some areas, while *G. mullerae* comprises nearly 25% (Álvarez *et al.* 2010).

Coccolithophore species composition is strongly driven by environmental changes, and diversity is in fact highest in stratified, warm oligotrophic waters where salinity is relatively high. This is particularly striking in studies such as that by Winter *et al*. (1979), where 52 species

of coccolithophore were recorded in the Gulf of Eilat, despite the average salinity of 41 ppt. More recent work in the south Atlantic shows that coccolithophore populations can vary even over relatively small spatial scales where the environmental conditions are variable. Boekel and Beaufort (2004) found four distinct coccolithophore assemblages in the waters west of South Africa, with each occupying a particular environmental niche. *E. huxleyi*-dominated waters ere characteristic of upwellings, whereas warmer oligotrophic conditions yielded far greater diversity and a higher abundance of many tropical and sub-tropical species such as *Umbilicophaera sibogae*. In deep (>4000m) low nutrient waters, the dominant species became *Calcidiscus leptoporus* and *G. ericsonii*.

Despite the wider diversity of coccolithophore species beyond *E. huxleyi*, surprisingly only 5 other species of coccolithophore have been studied in the context of OA. These experiments have demonstrated species specific responses: *Coccolithus pelagicus*, one of the most heavily calcifying extant species, shows no change in PIC production with CO_2, whereas *C. leptoporus*, another heavy calcifier, has an optimum CO_2 range, with PIC production dropping both above and below current CO_2 levels (Langer *et al.* 2006). *G. oceanica* appears to thrive in high CO_2 conditions, with no change in coccolith size but an increased growth rate, whilst *Coccolithus braarudii* shows malformation and smaller coccoliths (Rickaby *et al.* 2010). *Syracosphaera pulchra* does not show any changes in PIC production or growth rate with CO_2 (Fiorini *et al.* 2011a), but there does appear to be an interactive effect of CO_2 and temperature on this species that becomes far more pronounced in the haploid stage (Fiorini *et al.* 2011b).

As with the relative wealth of OA studies *E. huxleyi*, past studies on other coccolithophore species have rarely used consistent growth conditions thereby confounding robust inter-comparisons. In order to begin to reconcile this limitation, and consider the wider implications of the role of daylength observed in Chapter 2, I therefore expanded my experimental method to include not only other strains of *E. huxleyi*, but other coccolithophore

species. My main aim was to test if the difference in OA response observed in NZEH with L:D cycle also occurred more widely.

3.2. Methods and Materials

3.2.1. Growth and selection of strains

Five different coccolithophores were selected for this experiment; three different isolates of *E. huxleyi* (PML70-3, PML124-3, RCC962), as well as strains of two other species, *Gephyrocapsa oceanica* (RCC1804) and *Coccolithus pelagicus* (PLY182); these various strains were chosen to represent a cross-section of isolation locations, dates, species morphotype, and ultimately of cellular inorganic content (PIC cell^{-1}) under steady state ambient pCO_2 conditions (see Table 3.2.1). Strains *E. huxleyi* 70-3 and 124-3 were recently isolated on cruise D366 (June/July 2011) and provided by Cecilia Balestreri (Marine Biological Association). Strain 70-3 was isolated from the northern North Sea, and is a B morphotype, whilst strain 124-3 was isolated from the Bay of Biscay and is an A morphotype, Together these *E. huxleyi* strains represent genetic variants acclimated to different carbonate chemistry conditions, as well as a "light" and "heavy" calcifier. Strain 962 is a sub-tropical strain that provides more diversity to the temperate-centric research on *E. huxleyi*. *C. pelagicus* is a heavily calcifying coccolithophore commonly found in temperate to sub-polar regions, whereas *G. oceanica* is found throughout temperate and sub-tropical regions (and therefore provides a second representative of coccolithophores from warmer climates).

The 5 coccolithophores were grown at low (400µatm) and high (1000µatm) CO_2, under both a 14:10h L:D cycle and continuous light. All strains were grown as for NZEH (detailed in Section 2.2.1), with the exception of the use of *K* media (Keller *et al.* 1987) in filtered natural seawater (Sargasso Sea) for *E. huxleyi* 962 and *G. oceanica*. Similarly, details for all variables measured and reported here, specifically carbonate chemistry, growth rates, PIC and POC cell^{-1},

chlorophyll and cell size, were determined as in Section 2.2. The temperate isolates (*E. huxleyi* 70-3, 124-3, and *C. pelagicus*) were grown at 16°C, while the two tropical isolates (*E. huxleyi* 962 and *G. oceanica*) were grown at 20°C.

Table 3.2.1. A summary of the 5 coccolithophore cultures grown under different CO_2 and L:D cycles. Ambient PIC $cell^{-1}$ is the mean at 400 µatm CO_2 when grown in a L:D cycle of 14:10h. NZEH data from Chapter 2 is shown for comparison (+/-S.E.).

Species	Strain	Isolation Date	Isolation Location	Ambient PIC (pg $cell^{-1}$)
Emiliania huxleyi	RCC962	29/10/2004	French Polynesia -8.3333, -141.2500	3.24 (1.41)
Emiliania huxleyi	PML70-3	02/07/2011	North Sea 56.2954, 3.3642	2.86 (0.09)
Emiliania huxleyi	PML124-3	25/06/2011	Bay of Biscay 46.1059, -7.1378	8.11 (0.13)
Emiliania huxleyi	NZEH	1992	South Pacific -47.6993, 174.0294	11.20 (0.31)
Gephyrocapsa oceanica	RCC1804	09/12/2008	Sipadan, Malaysia 4.1103, 118.6309	4.73 (1.00)
Coccolithus pelagicus	PLY182	1958	English Channel 50.1833, -4.3000	256.67 (4.17)

3.3. Results

3.4.1. Growth Rate Response

Consistent with my results previously with NZEH (Chapter 2, Fig. 2.3.1), the 14h light regime induced a decrease in growth rate (μ, d_{-1}) for all species tested here except for *C. pelagicus* (which remained unaffected). This effect was strongest on the two sub-tropical strains, *E. huxleyi* 962 and *G. oceanica*, whose growth rates decreased by ca. 50% and 66% percent respectively with increased CO_2. The two temperate isolates of *E. huxleyi* exhibited a much smaller decrease, both growing at ~0.8 d^{-1} under ambient CO_2 conditions and decreasing to 0.7 (strain 70-3) and 0.66 (strain 124-3), though this is not statistically significant in either case. In contrast, continuous light removed any CO_2 effect from growth rate, but there are species specific responses to the change in L:D cycle. In both sub-tropical species, there is a decrease in the ambient CO_2 growth rate compared to 14h, but an increase in the high CO_2 rates. The temperate *E. huxleyi* isolates both grow faster under 24h of light compared to 14h (~0.95 in 70-3, and ~0.85in 124-3). *C. pelagicus* growth rates remained unchanged.

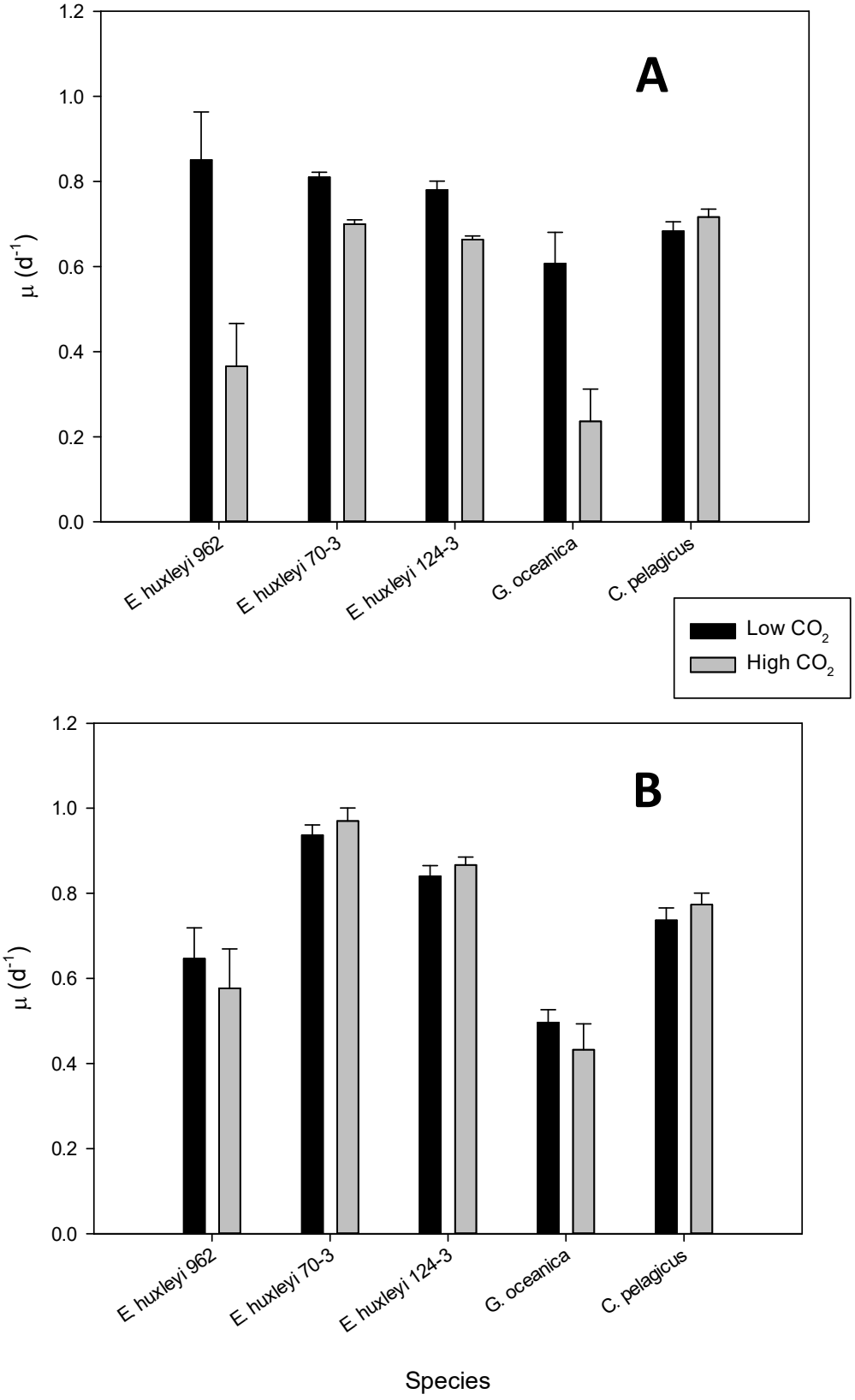

Fig. 3.3.1. The effect of photoperiod and pCO_2 on growth rates in five different species of coccolithophores. A is cultures grown under a 14:10h L:D cycle, and B is cultures grown under 24h of light. Error bars are +/- S.D., *n*=3.

3.4.2. PIC and POC

In the 14h cultures, PIC cell^{-1} decreased by over 50% with increased CO_2 in the two sub-tropical species (3.2pg to 0.98pg in *E. huxleyi* 962; 4.7pg to 1.7pg in *G. oceanica*). In contrast, all other species showed an increase in PIC cell^{-1} with increased CO_2. These trends were accompanied by a significant increase in POC cell^{-1} for all species; consequently, PIC:POC decreased markedly in all species with increased CO_2. The species with the lowest PIC:POC at ambient conditions was *E. huxleyi* 70-3, indicating it is the weakest calcifier of all the tested organisms. The highest was *E. huxleyi* 962, though the standard error is large.

Once again, the influence of elevated CO_2 upon PIC and POC content was less under 24h than 14h daylength. *E. huxleyi* 962, *G. oceanica* and *C. pelagicus* exhibited a small decrease, whilst the two temperate isolates showing a slight increase of PIC cell^{-1} with elevated CO_2. POC cell^{-1} changed little with CO_2 in *E. huxleyi* 70-3, 124-3 and *C. pelagicus*, while the two tropical species showed an increase in POC cell^{-1}. Consequently, in contrast to the 14h daylength, PIC:POC was no longer significantly affected by CO_2 under continuous light. The PIC:POC of all species under the high CO_2 conditions increased from the high CO_2 PIC:POC of the 14h cultures. *E. huxleyi* 962 and *G. oceanica* had a lower PIC:POC under ambient CO_2 conditions compared to the 14h cultures, whereas *E. huxleyi* 70-3 and 123-4 rose. *C. pelagicus* showed only a slight decrease in ambient PIC:POC compared to the 14h cultures.

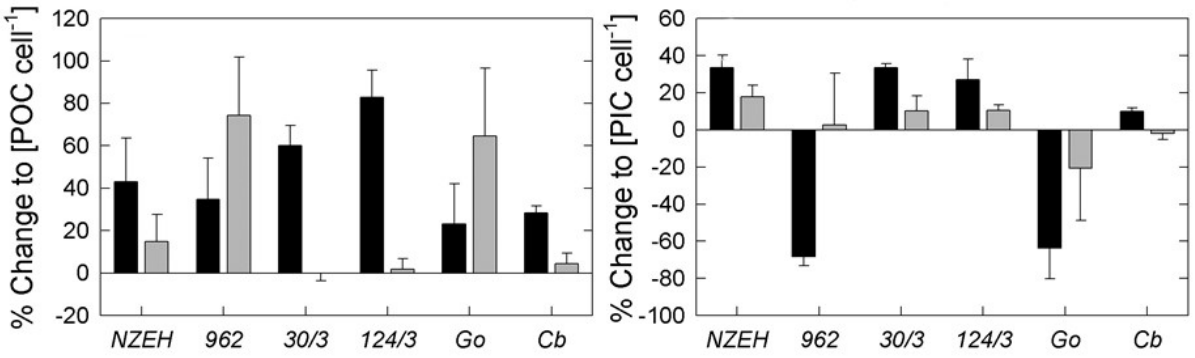

Fig. 3.3.2. The percent change in POC and PIC cell^{-1} in 6 different coccolithophore taxa (*E. huxleyi* NZEH, 962, 70-3 and 124-3; *G. oceanica* and *C. pelagicus*) in response to CO_2 when grown under 14h light (black bars) and 24h light (grey bars). Error bars are +/- S.D., n=3.

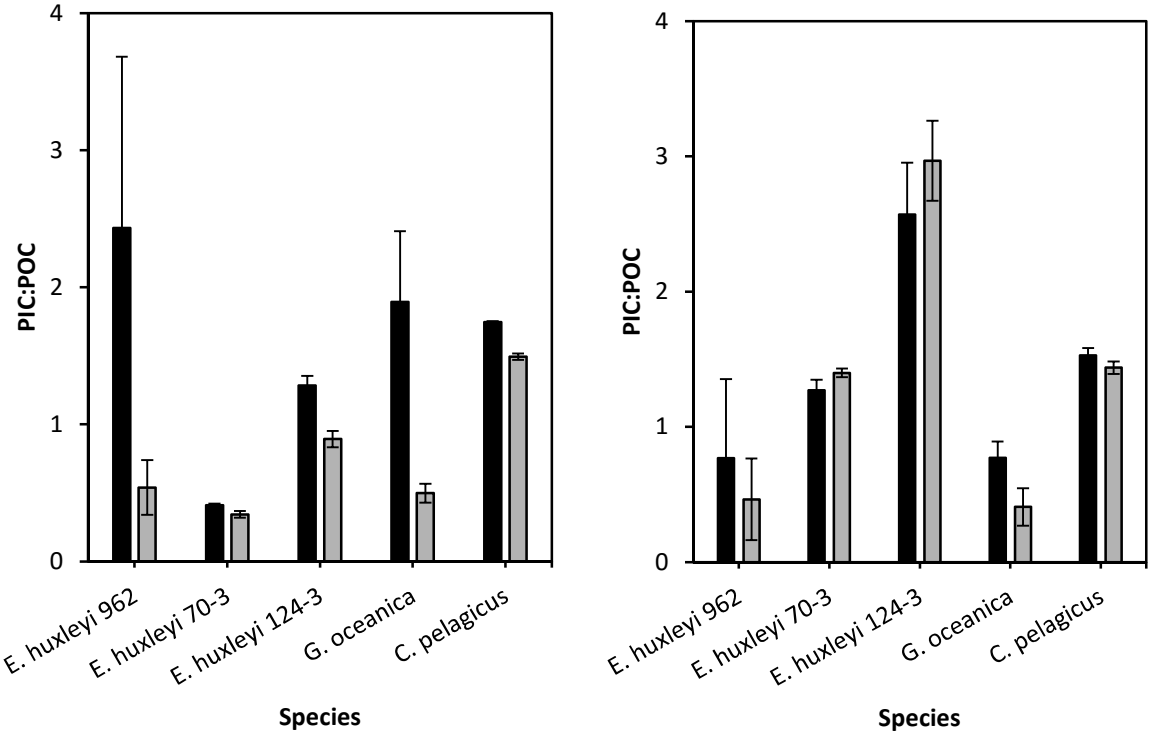

Fig. 3.3.3. PIC:POC ratios in five different coccolithophore taxa (*Emiliania huxleyi* 962, 70-3 and 124-3, *Gephyrocapsa oceanica* and *Coccolithus pelagicus*) when grown under 14h (A) and 24h (B) of light. Black bars are low CO_2, and grey bars are high CO_2, error bars are +/- S.E., *n*=3.

Table 3.3.1. Summary of 2-way ANOVA examining the influence of genotype (coccolithophore species, *E. huxleyi* isolate, n=6) and photoperiod (14:10h and 24h) upon the percentage change in response to variable CO_2. Significant outcomes and interactions are highlighted in bold.

Source of variation	df	Growth		PIC		POC		PIC:POC	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Genotype	5	55.7	**<0.001**	27.1	**<0.001**	3.16	**0.025**	37.0	**<0.001**
L:D Cycle	1	30.6	**<0.001**	3.81	0.060	5.35	**0.030**	38.8	**<0.001**
Genotype x L:D	5	4.19	**0.007**	12.6	**<0.001**	10.4	**<0.001**	2.35	0.072

In order to reconcile the differences in OA response amongst species and light treatments, I subsequently examined the percent change in PIC:POC between CO_2 treatments as a function of the ambient CO_2 growth rate (Fig 3.3.4).

While there was a decrease in the PIC:POC of all species, the two sub-tropical ones showed the most dramatic (~70%, compared to 16-30% in the other 3 species). Post-hoc tests from a 1-way ANOVA confirm that there is no significant difference between *E. huxleyi* 962 and *G. oceanica*, but both are significantly different from the other three species – and vice versa. This is only true for the 14h cultures. Under 24h, there is no significant species-specific response.

When plotted as a function of ambient (CO_2) growth rate, there is a strong positive correlation (R^2=0.68), i.e. faster growth rates show less of a decrease in PIC:POC, and in some cases even an increase with CO_2, but only in the 24h cultures. The two slowest growing species at 24h are both sub-tropical species, *E. huxleyi* 962 and *G. oceanica*.

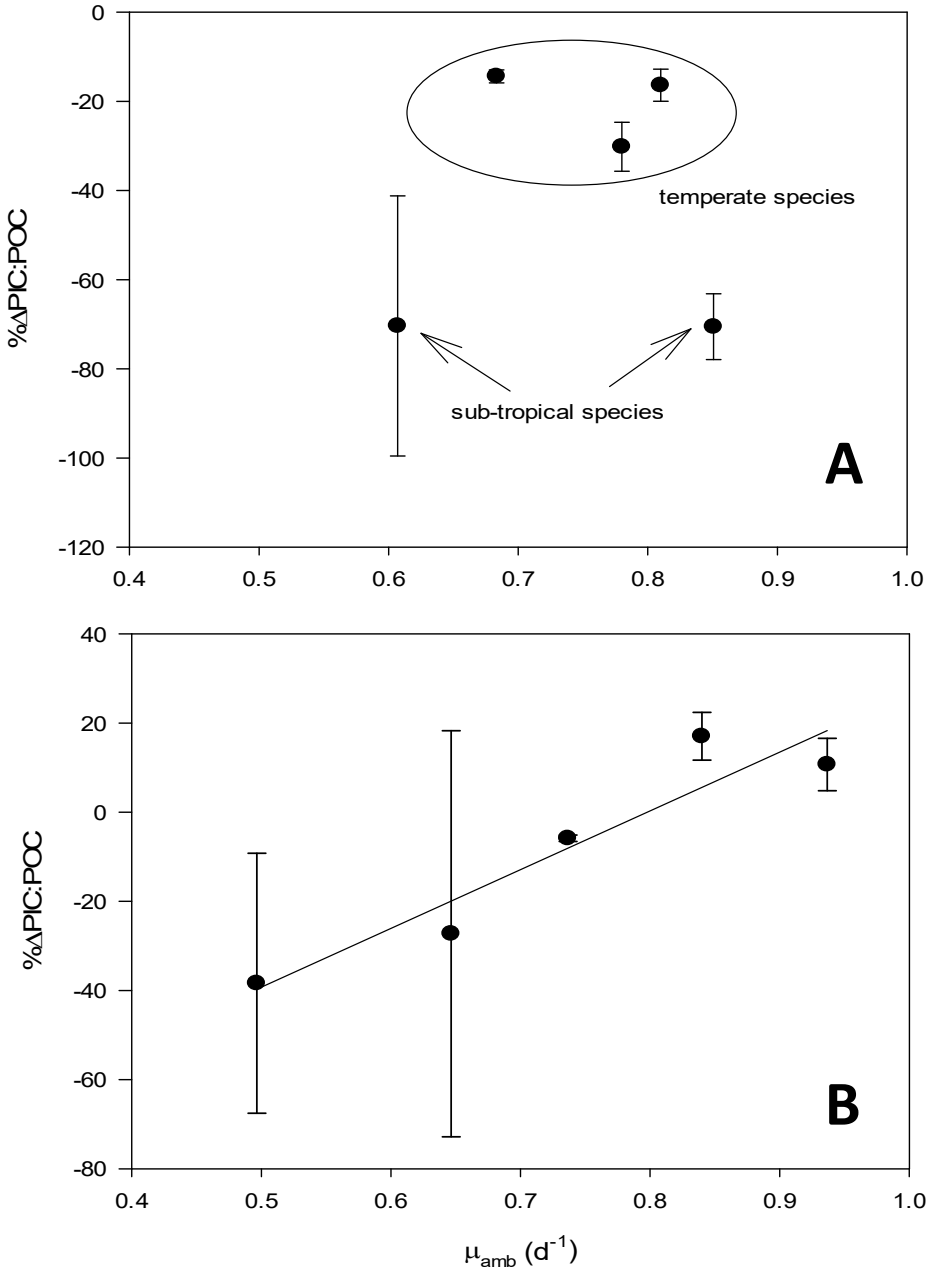

Fig. 3.3.4. The percent difference in PIC:POC from ambient to high CO_2, plotted as a function of the ambient growth rate. Cultures were grown under 14h (A) and 24h (B) of light. Error bars are +/- S.E., *n*=3.

3.5 Discussion

The two temperate isolates of *E. huxleyi*, 70-3 and 124-3, exhibited responses consistent with that of NZEH reported in Chapter 2. Growth rates were negatively affected by CO_2 under the 14h light regime, but were unaffected when grown under continuous light (Fig. 3.3.1). POC cell^{-1} was enhanced by CO_2 under the 14h light regime only, and PIC cell^{-1} demonstrated no CO_2 response, but was enhanced when grown under continuous light (Fig. 3.3.2). This means that overall PIC:POC values were greatly increased with continuous light (Fig. 3.3.3). As such, it appears that continuous light induces a stronger relationship between photosynthesis and calcification in these two isolates, as it does in NZEH (Section 2.4). It is interesting that such a consistent response is observed between these three isolates, as intra-specific responses to CO_2 within *E. huxleyi* have been established (Langer *et al.* 2009). These three isolates come from temperate locations at comparable latitudes (see Table 3.2.1), which could indicate some degree of conservation in the way temperate coccolithophores respond to daylength.

The two tropical coccolithophores, *E. huxleyi* 962 and *G. oceanica*, had a response to CO_2 and L:D cycle that differed from the three temperate *E. huxleyi* strains. In general, these two isolates exhibited greater sensitivity to CO_2 under 14h of light, particularly in growth rates and PIC:POC. This would seem counter-intuitive as tropical organisms would be more accustomed to shorter daylength periods that vary little over the course of the year. As L:D cycle affects the preferential carbon source in *E. huxleyi* (Rost *et al*. 2006), it is possible that preference of bicarbonate over CO_2 is dictated in a different fashion in these tropical isolates. However, PIC:POC ratios decrease overall under continuous light (Fig. 3.3.3), which would indicate that 24h light is not an optimum condition for these strains.

These two different responses are likely a result of the differences in biogeographic origin, resulting in two groups of coccolithophores that are pre-adapted to thrive in different

environmental conditions. Considerable phenotypic variability is known to exist between isolates of *E. huxleyi* (Iglesias-Rodriguez *et al.* 2006; Poulton *et al.* 2011; Smith *et al.* 2012), and phenotypic plasticity in response to changing environmental factors can be observed even within a single strain (Paasche 2001). It is unclear what exactly drives the phenotypic diversity in response to CO_2, though several avenues have been investigated. There are clear differences in the physiologies between strains isolated from oceanic ecosystems and coastal ones (Brand 1982), which again suggests that biogeographical origin, and thus the need to adapt to very specific environmental conditions, is responsible. However, Langer *et al*. (2009) found that CO_2 responses in 4 different *E. huxleyi* isolates did not correspond to carbonate chemistry typically found at their respective biogeographical origins, nor to the morphotype. The two strains with similar responses come from locations that are connected via the Antarctic circumpolar current, and thus the possibility of gene flow between populations might be a controlling factor in the phenotypic variability. However, information on strain-specific physiology is limited, and more experimental work on a variety of isolates is required before any solid conclusions can be made on the matter. Certainly, the results here back the idea of biogeography being an important factor in dictating differences to environmental factors (CO_2 and L:D cycle).

Biogeography is a pertinent consideration for this thesis beyond driving intra-specific physiology, since local L:D cycles change depending on latitude. As a result of a changing climate, phytoplankton species have already begun migrating to higher latitudes as the temperatures in the waters around the Equator increase beyond a liveable threshold (Thomas *et al.* 2012). A few specific cases have been documented, such as incursions of tropical species of the dinoflagellate *Ceratium* in the North Sea and the waters around Scotland (Barnard *et al.* 2004). The range of *E. huxleyi* has also expanded northward into the polar regions, led by a few distinct populations that can tolerate colder temperatures (Winter *et al.* 2014). Phytoplankton can also be displaced through currents that have been altered due to increasing temperatures, which

has seen tropical radiolarians transplanted into the Arctic Circle (Bjørklund *et al.* 2012). Since the tropical strains appeared to be less sensitive to CO_2 under continuous light, moving away from the equator may provide a refuge from ocean acidification, though at a cost to PIC production and growth rates.

It is important to note that the two tropical strains were growing at a higher temperature (20°C) than the temperate ones. For *G. oceanica* in particular, this is below the optimum growth temperature of 25°C (Rhodes *et al.* 1995; Buitenhuis *et al.* 2008), and may have had an impact on the data collected from these strains. It has been established for some time that elevated temperatures have an overall stimulating effect on phytoplankton metabolism and physiology (Lund 1949; Talling 1955; Eppley 1972), fewer studies have focused on the combined effects of CO_2 and temperature (so called "greenhouse" conditions). In the field, greenhouse conditions have been reported to both reduce (Coello-Camba *et al.* 2014) and enhance (Feng *et al.* 2009) primary productivity. These responses appear to differ between locations, and indicate that flexibility in thermal tolerance is dependent on the starting community. Certainly, laboratory experiments report a variety of responses to greenhouse conditions across several groups of phytoplankton (Fu *et al.* 2007; Hyun *et al.* 2014; Tew *et al.* 2014; Xu *et al.* 2014).

With regards to coccolithophores, several experimental studies have demonstrated that *E. huxleyi* and *G. oceanica* achieve maximum growth rates at temperatures up to 10°C warmer than those found at their location sites (Rhodes *et al.* 1995; Conte *et al.* 1998; Sett *et al.* 2014), and may explain the low growth rates reported in the tropical strains here. Elevated temperatures have also been reported to decrease the PIC $cell^{-1}$ in coccolithophores (Langer *et al.* 2007; Feng *et al.* 2009), but not consistently (Feng *et al.* 2008; Sett *et al.* 2014). Sett *et al*. (2014) investigated the effect of temperature on the CO_2 response in *E. huxleyi* and *G. oceanica*, and found that elevated temperatures cause the optimum CO_2 range for growth, C fixation and calcification to shift towards higher concentrations. This effect was much stronger in *E. huxleyi*,

in part because *G. oceanica* appeared to have a much more narrow CO_2 tolerance range. Thus, elevated temperatures may help coccolithophores in acclimating to OA conditions, which may explain why the CO_2 effect was much stronger in the tropical strains.

C. pelagicus did not show any significant changes in its physiology in response to either daylength or CO_2. This lack of response is consistent with other published findings, though this organism has not been tested extensively.

Conclusions

The temperate strains of *E. huxleyi* responded to daylength and CO_2 in a manner consistent with the findings for NZEH in the previous chapter, indicating a uniform response to changes in daylength in temperate isolates. Two tropical coccolithophores showed a distinct response that differed from the temperate isolates, and were overall more sensitive to CO_2 under the shorter photoperiod, though growth rates and PIC:POC were lower with continuous light. The mechanisms driving this different response are unclear, but could be related to differences in biogeographical origins. This highlights the point that OA will affect coccolithophore populations differently around the world. As tropical organisms move away from the equator towards longer daylength periods, they could find refuge from OA.

However, temperature may have played an important role in driving some of the different responses. Elevated temperatures push the optimum CO_2 range for *E. huxleyi* and *G. oceanica* to higher concentrations, and as the tropical strains were grown at sub-optimal temperatures, they may have been more vulnerable to OA conditions than they might otherwise be *in situ*.

Chapter 4: Regulation of coccolithophore photobiology in response to ocean acidification and light availability

4.1. Introduction

As mentioned in Chapter 1, there are two main marine biological processes that utilise inorganic carbon; calcification and photosynthesis. While OA can negatively impact calcification, it also has the potential to increase marine primary productivity by alleviating CO_2 limitation (Rost *et al.* 2003). As previously discussed in Section 1.5, phytoplankton are C-limited in the modern ocean because of biological constraints imposed by RubisCO (Tortell 2000). While many species have evolved means for concentrating CO_2 (Giordano *et al.* 2005), OA studies on marine primary producers show that there will be winners and losers when it comes to enhanced biology.

Seagrasses appear to be among the biggest winners. Elevated CO_2 yields higher photosynthetic rates (Invers *et al.* 2001; Russell *et al.* 2013) and increased biomass and growth rates (Palacios and Zimmerman 2007; Martin *et al.* 2008), while increases in abundance tend to only happen in species with dense below-ground biomass such as *Cymodocea serratus* (Russell *et al.* 2013). Rates of photosynthesis increase so much, that there is a significant increase in pH and Ω_{Ar} in seawater surrounding seagrass meadows, which could provide refuge from OA for calcifying epibionts or downstream coral reefs (Unsworth *et al.* 2012; Hendriks *et al.* 2014). In areas where CO_2 vents create a gradient of carbonate chemistry, intertidal communities lose species diversity at high CO_2, where they have become dominated by seagrasses (Hall-Spencer *et al.* 2008). Work on CO_2 vents has also provided evidence that OA stimulates productivity and growth rates in macroalgae, such as *Padina* spp. (Johnson *et al.* 2012), and benthic biofilm communities (Johnson *et al.* 2013; Russell *et al.* 2013).

Among phytoplankton, large diatoms benefit the most from elevated CO_2 (Rost *et al.* 2003; Wu *et al.* 2014), and frequently become the dominating group during *in situ* experiments (Tortell *et al.* 2002; Tortell *et al.* 2008). It is thought that this is due to relieving CO_2 limitation, as nutrient limitation is generally greater in large cells because of their small SA:V ratio, and allowing resources to be reallocated from CCM production to other functions such as growth and synthesis of photosynthetic apparatus. However, most phytoplankton employ efficient enough CCMs that many studies have reported no changes in primary productivity or C uptake at high CO_2 levels (Rost *et al.* 2003; Beardall and Raven 2004; Martin and Tortell 2006; Tortell *et al.* 2010). *In situ* primary productivity has been increasing in areas such as the Arctic (Arrigo and van Dijken 2011), but this is a result of less sea ice cover and earlier bloom onset (Kahru *et al.* 2011) rather than an increase in primary productivity per unit area. In fact, because of the simultaneous warming associated with rising CO_2 levels, and hence increased stratification, changes to primary production will depend on geographic location, with some areas, such as the Atlantic, declining by 20%, while the Arctic is indeed predicted to see a nearly 60% increase by the end of the century (Yool *et al.* 2013).

Work in the South China Sea revealed decreased primary productivity in phytoplankton exposed to simultaneous increase of CO_2 and light intensity (Gao *et al.* 2012), and an inability to tolerate light stress. This is particularly pertinent to the notion that photoperiod can change the observable OA response in phytoplankton. With this in mind, it is important to explore the effects of L:D cycle and OA on coccolithophores not just on calcification, but their photophysiology as well. Coccolithophore photobiology is still poorly understood, as it is not yet known how calcification and the coccosphere affect the internal bio-optical environment of the cells. Coccolithophores, and specifically *E. huxleyi*, are known to possess very inefficient CCMs (Rost *et al.* 2003), and it was long thought that the process of calcification plays some role as an additional CO_2 source for photosynthesis to make up for this (Sikes *et al.* 1980; Nimer and

Merrett 1992). However, it has since been demonstrated that there is no direct dependency of photosynthesis on calcification (Trimborn *et al.* 2007; Leonardos *et al.* 2009).

One proposed function of coccoliths is the scattering of radiation, providing some refuge from high light intensities in surface waters. Nielsen (1995) found higher light-saturated rates of photosynthesis at a given DIC concentration in heavily calcified cells compared to light calcifiers. When Ca^{2+} availability is changed, *E. huxleyi* cells exhibit both a decrease in calcification and photosynthetic carbon fixation rates (Nimer *et al.* 1996). Long-term exposure to Ca^{2+} limitation also leads to lower calcification and C fixation rates compared to cultures grown under normal conditions (Xu *et al.* 2011). When exposed to a sudden increase in light intensity, *E. huxleyi* is able to dissipate the excess excitation energy by increasing its calcification rates (Ramos *et al.* 2012). In addition, when calcification is reduced by limiting Ca^{2+}, *E. huxleyi* cells are less efficient at both photo- and non-photochemical quenching in response to stressful light intensities, suggesting that the coccosphere plays a photoprotective role (Xu and Gao 2012). Similarly, *E. huxleyi* suffers more damage from UV radiation as calcification decreases with elevated CO_2 (Gao *et al.* 2009). Similarly, a lack of photoinhibition has also been observed when comparing lightly and heavily calcified strains of *E. huxleyi* (Paasche and Klaveness 1970; Nanninga and Tyrrell 1996; Trimborn *et al.* 2007). Leonardos *et al.* (2009) observed no correlation between light-dependent O_2 evolution and calcification, while Trimborn *et al.* (2007) found that the process of calcification does not provide any means of energy dissipation under high irradiances. In addition, some studies indicate that coccoliths may in fact be used to focus light to plastids in deeper waters (Nanninga and Tyrrell 1996; Raven and Waite 2004). The use of calcite crystals to focus radiation has been observed in extant ophiuroids (brittlestars) (Aizenberg *et al.* 2001) and extinct trilobites (Gál *et al.* 2000), albeit in a sensory capacity rather than an energetic one.

Advancements in active chlorophyll *a* fluorescence allow this idea to be investigated in a quick and non-invasive fashion. Initial studies showed that *in situ* measurements of chlorophyll fluorescence could allow rapid assessment of the physiological state of phytoplankton as they exist in nature (Kolber *et al.* 1990; Geider *et al.* 1993; Schreiber *et al.* 1995). Active chlorophyll *a* fluorescence measurements evaluate the efficiency with which light is being utilised by PSII. Light energy absorbed by PSII can be used to drive the transfer of electrons from the reaction centre chlorophyll, P680, to the primary quinone acceptor (Q_A), and thus the light-dependent reactions of photosynthesis. However, the energy may also be lost through either chlorophyll fluorescence, or dissipated as heat (Butler 1978). These three processes (photochemistry, fluorescence, heat) are in direct competition for excitation energy, and therefore proportional to each other, i.e. if the rate of one of these processes increases, the rates of the other two drop. As the rate of heat loss only changes under certain circumstances, measuring chlorophyll fluorescence can yield information about photochemical processes occurring in the cell.

The aim of this chapter is to investigate further the effect that L:D cycle has on the observed OA response of a range of coccolithophores, specifically focussing on changes in photobiology as measured using fast repetition rate (FRR) fluorometry, and to assess whether these changes were associated with changes to the coccosphere.

4.2. Methods

4.2.1. Culturing of Strains

The six strains of coccolithophore used here are four types of *E. huxleyi* (NZEH, PML70-3, PML124-3, RCC962), one strain of *G. oceanica* (RCC1804) and one strain of *C. pelagicus* (PLY182). Details of the selection of these strains can be found in Section 3.2. Cultures were grown at ambient (400µatm) and high (1000µatm) CO_2 levels, under both a L:D cycle (14:10h) and continuous light. All strains were cultured as detailed in Sections 2.2 and 3.2.

4.2.2. Fast Repetition Rate Fluorescence

An aliquot from each vessel was removed at 08:00 (i.e. 1 hour after light on), dark acclimated for 20 minutes and assessed for PSII activity using a Fast*tracka* II FRR fluorometer housed within a bench top Fast*act* base-unit (*Chelsea Technologies Group*, UK). The Fast*tracka* II was programmed to deliver a 200 ms single turnover (ST) fluorescence induction sequence, using 100 x 1 ms flashlets each separated by a 1 ms interval, followed by a 2.5 ms relaxation sequence of 50 x 1 ms flashlets each separated by 49 ms. The Fast*tracka* LED intensity was initially modified to optimise the rate of PSII reaction centre for subsequent fitting of the ST-saturation model (e.g. (Oxborough *et al.* 2012). A set of 12 consecutive fluorescence transients, each separated by an interval of 100 ms, were then acquired per sample. Each transient was fit to the biophysical (KPF) model of Kolber *et al.* (1998) describing ST-saturation and –relaxation of PSII to generate the minimum (F_0) and maximum (F_m) fluorescence yields, and PSII photochemical efficiency (F_v/F_m, dimensionless), as well as the average effective absorption cross section (s_{PSII}, nm^2) and minimum turnover time (1/t, e^- ms^{-1}) for the PSII pool. Note that s_{PSII} is weighted according to the Fast*tracka* LED spectra, which for our instrument (model) was centred at ca. 450 nm. All fluorescence-based parameters were averaged from the 12 acquisitions per sample.

Measurements of σ and F_0 were used to determine the concentration of functional PSII reaction centers [RCII] (mol m^{-3}) following Oxborough *et al*. (2012) based on a previous calibration of this Fast*tracka* II against measures of [RCII] from a range of phytoplankton cultures (see Robinson et al. subm.). FRRf parameters and [RCII] determinations from each acquisition were subsequently used to derive the light saturation intensity (E_K, mmol photons m^{-2} s^{-1}), maximum light utilization efficiency (α, m^{-1}) and maximum rate (ETR^{max}, mmol e^- m^{-2} s^{-1}) for PSII electron transport, specific to the FRR fluorometer wavelength used to yield s, according to Sakshaug *et al*. (1997) (see also (Behrenfeld *et al.* 1998; Moore *et al.* 2006):

$$E_K = \left[\Phi_{RC} \cdot 1.66 \cdot 10^6\right] / \left[\sigma \cdot \tau\right] \quad [1]$$

$$\alpha = [\text{RCII}] \cdot \sigma \cdot \left(F_m / F_v\right) \cdot 6.023 \cdot 10^{-4} \quad [2]$$

$$ETR^{max} = E_K \cdot \alpha = \Phi_{RC} \cdot \left([\text{RCII}]/\tau\right) \cdot \left(F_m / F_v\right) \cdot 10^3 \quad [3]$$

Where Φ_{RC} accounts for the assumption that within the PSII pool every e^- generated is the result of a single photon being delivered, i.e. e^- photon^{-1} = 1, and the factors (i) 1.66 · 10^6 accounts for the conversion of nm^2 to m^2, ms to s, quanta to mol quanta, and mol photons to mmol photons for E_K (Eq. 1), (ii) 6.023 · 10^{-4} accounts for the conversion of quanta to mol quanta for the PSII pool governing [RCII], conversion of nmol RCII to mol RCII and nm^2 to m^2 for a (Eq. 2) (e.g. Suggett et al. 2009), and hence (iii) 10^3 accounts for conversion of nmol RCII to mol RCII and ms to s. The term F_m/F_v (= 1/ F_v/F_m) is included to further account for the decoupling between s and a from non-radiative losses (Suggett et al. 2007, 2009).

4.3. Results

4.3.1. General Photophysiology

In all species, continuous light caused a decrease in F_v/F_m under ambient CO_2 conditions, and only in *E. huxleyi* 962 and *C. pelagicus* was this change not significant. In the cases of NZEH, 70-3 and *G. oceanica*, σ consistently decreased under continuous light, whereas *C. pelagicus* exhibited a decrease under CO_2 enrichment, regardless of L:D cycle. F_v/F_m was positively correlated with σ, though it is important to note this is largely driven by *G. oceanica*, *C. pelagicus* and NZEH, compared to the other strains of *E. huxleyi*.

Table 4.3.1. Summary of the changes in photobiology in six different coccolithophore species when grown under 14h light and continuous light.

Species	**Treatment**	F_v/F_m	σ (nm^2)	τ (μs)	[RCII] ($nmol\ m^{-3}$)	E_K ($\mu mol\ photons\ m^{-2}\ s^{-1}$)
E. huxley NZEH	LO-14h	0.453	4.67	482	97	762
	LO-24h	0.408	3.68	630	78	717
	HI-14h	0.419	4.72	519	82	691
	HI-24h	0.421	3.86	722	62	602
E. huxleyi 70-3	LO-14h	0.491	4.89	473	96	740
	LO-24h	0.447	4.55	460	84	796
	HI-14h	0.494	4.94	546	82	616
	HI-24h	0.424	4.03	602	81	688
E. huxleyi 124-3	LO-14h	0.490	4.59	590	127	615
	LO-24h	0.431	4.69	526	61	675
	HI-14h	0.469	4.67	632	102	563
	HI-24h	0.451	4.32	572	44	672
G. oceanica	LO-14h	0.447	4.61	514	33	650
	LO-24h	0.433	4.28	595	18	957
	HI-14h	0.438	4.37	552	23	707
	HI-24h	0.431	3.32	695	18	904
E. huxleyi 962	LO-14h	0.456	4.64	546	153	677
	LO-24h	0.448	4.92	576	109	593
	HI-14h	0.448	4.21	558	98	719
	HI-24h	0.466	4.59	559	105	656
C. pelagicus	LO-14h	0.414	3.96	639	164	657
	LO-24h	0.402	3.86	714	117	608
	HI-14h	0.398	3.74	640	145	694
	HI-24h	0.339	3.75	676	122	657

In all species, continuous light caused a decrease in F_v/F_m under ambient CO_2 conditions, and only in *E. huxleyi* 962 and *C. pelagicus* was this change not significant. In the cases of NZEH, 70-3 and *G. oceanica*, σ consistently decreased under continuous light, whereas *C. pelagicus* exhibited a decrease under CO_2 enrichment, regardless of L:D cycle. F_v/F_m was positively correlated with σ, though it is important to note this is largely driven by *G. oceanica*, *C. pelagicus* and NZEH, compared to the other strains of *E. huxleyi*.

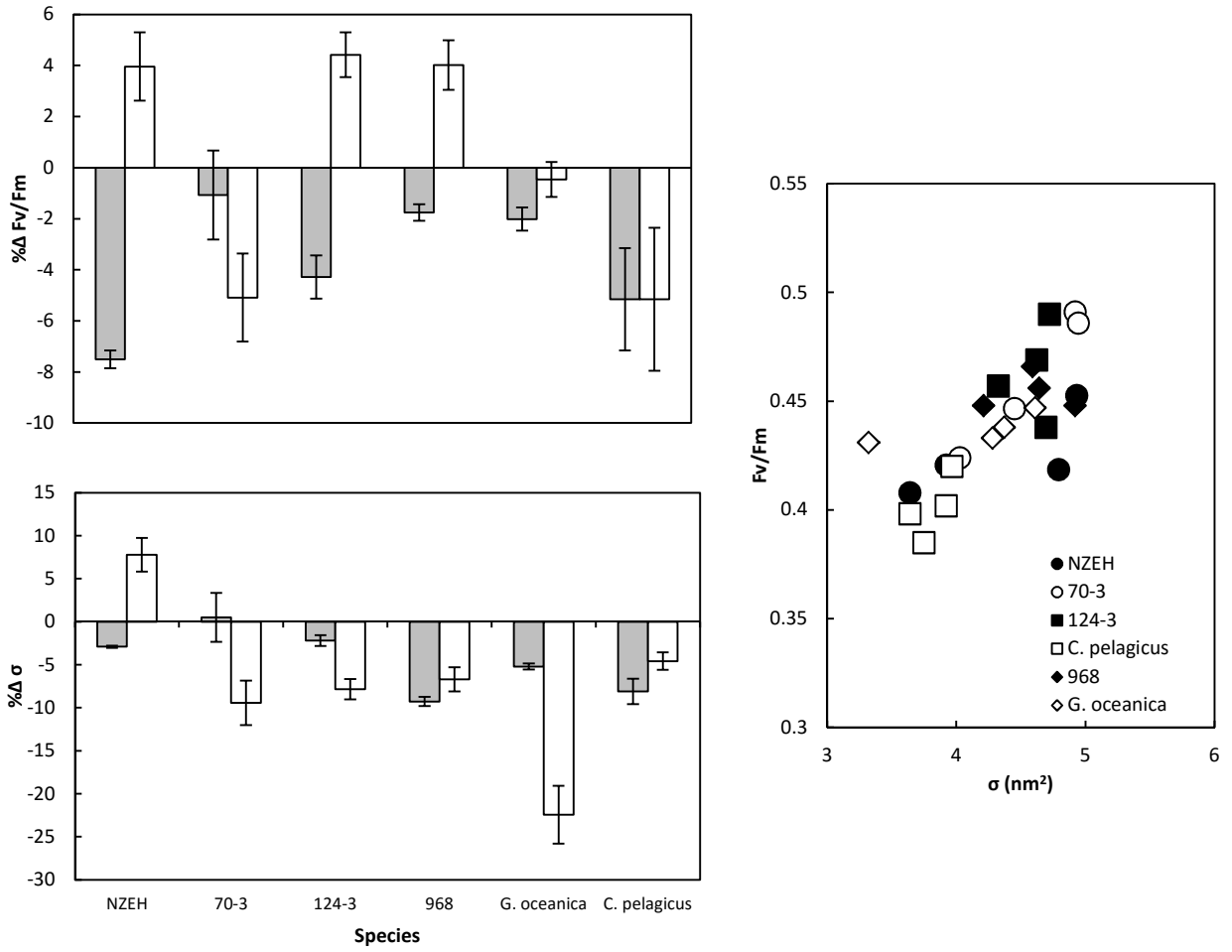

Fig. 4.3.1. Percent change in F_v/F_m and σ in response to elevated CO_2 when grown under 14h (grey) and 24h (white) light regime in six different coccolithophore species (+/- S.D., n=3). F_v/F_m is also plotted againt σ to assess photoacclimation response.

[RCII] was highly varied, ranging from as low as 18nmol m^{-3} (*G. oceanica*) to 145nmol m^{-3} (*C. pelagicus*). While no overall correlation was observed between [RCII] and σ, there were two responses observed among the organisms tested. NZEH, 70-3 and *G. oceanica* exhibited very constant values of [RCII] while σ varied. *C. pelagicus* and 124-3 showed much more plasticity in [RCII], but σ was highly conserved. *E. huxleyi* 962 had a [RCII] value of 153nmol m^{-3} under ambient CO_2 and 14h of light, for all other treatments it ranged between 98 and 109nmol m^{-3} (i.e. conserved across the other three treatments), with σ varying between 4.21 and 4.2 nm^2.

As τ varied considerably (between 460 and 722μs), there was very little correlation with [RCII]. Again, there were some species/strains that conserved [RCII] while τ was highly varied. This is exceptionally pronounced in NZEH and *G. oceanica*, where τ ranged from 482 to 722us and 514 to 695μs respectively. 70-3 also exhibited this response, but over a smaller range of 460 to 601μs. 124-3 showed significant decreases in both τ and [RCII] with CO_2 enrichment under both light regimes. Under continuous light, τ was higher compared to cultures grown under the same CO_2 treatment with 14h of light, while [RCII] was lower. Thus, there is a slight positive correlation between the two. *C. pelagicus* exhibited a negative correlation between τ and [RCII], while 962 had high plasticity in [RCII] with τ changing very little.

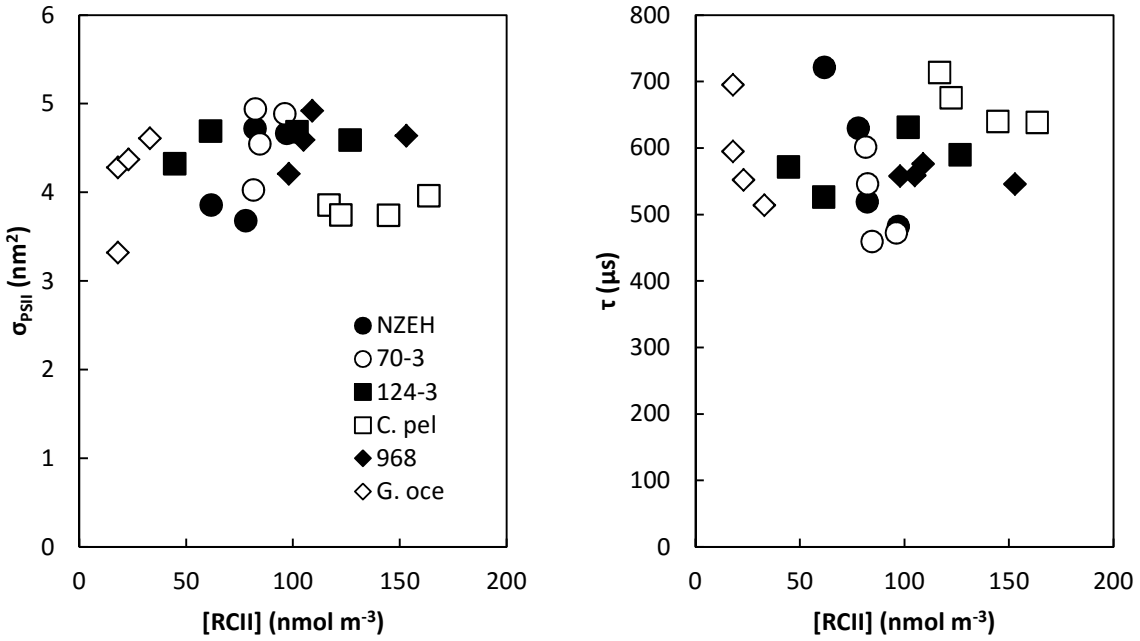

Fig. 4.3.2. Changes in σ and τ with PSII pool size ([RCII]) in six different coccolithophore species grown under two CO_2 treatments and two different light regimes (14h and 24h).

4.3.2. The effect of coccosphere on photophysiology

As cell size showed taxonomic variation, coccosphere thickness is presented as percentage of total volume (cell and coccosphere). Coccosphere thickness was weakly positively correlated with σ, though this was driven mostly by *G. oceanica* and 962, and, to a lesser extent,

by *C. pelagicus*. In fact, coccosphere thickness in NZEH and 70-3 was strongly negatively correlated with σ.

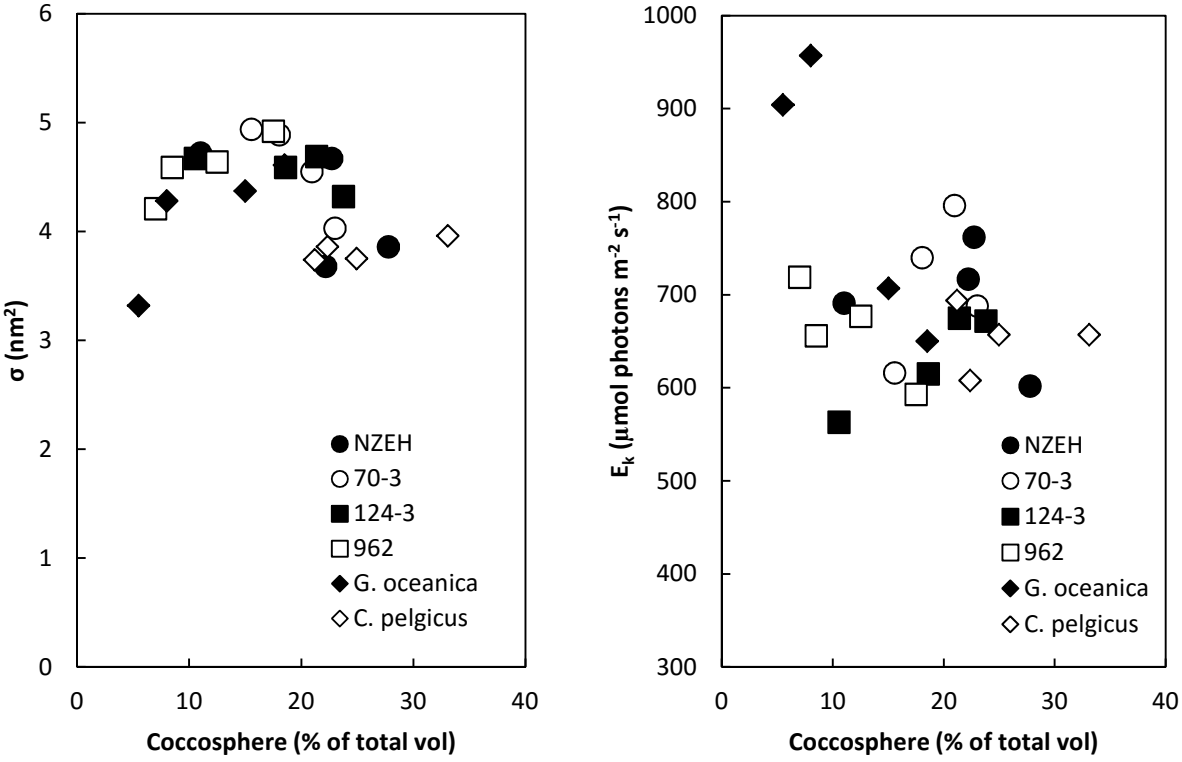

Fig. 4.3.3. Changes in σ and E_k, and how they relate to coccosphere size in 6 coccolithophore species grown under two CO_2 treatments and two different light regimes (14h and 24h). Coccosphere size is reported as a percentage of the total cell volume.

Table 4.3.2. Coccosphere thickness as a percentage of total cell volume (+/- S.E., *n*=3) for six coccolithophore cultures grown under different CO_2 levels (LO=400µatm; HI=1000µatm) and L:D cycles (14=14:10h; 24=continuous light).

	NZEH	**70-3**	**124-3**	**962**	***G. oceanica***	***C. pelagicus***
LO-14	22.70	18.03	18.59	12.48	18.70	33.08
	(3.71)	(1.32)	(0.67)	(1.34)	(0.81)	(3.54)
HI-14	27.76	22.99	23.78	17.62	8.17	24.94
	(4.39)	(2.26)	(1.65)	(2.77)	(0.32)	(2.82)
LO-24	11.01	15.55	10.54	7.02	15.26	21.19
	(1.88)	(3.49)	(2.51)	(1.87)	(1.52)	(1.33)
HI-24	22.18	20.94	21.38	8.42	5.48	22.36
	(2.83)	(2.22)	(2.66)	(2.27)	(0.32)	(1.26)

4.4. Discussion

NZEH, 70-3, and *G. oceanica* all exhibited a similar photophysiological response, whereby ambient CO_2 and/or light regime yielded higher values of F_v/F_m and σ, versus elevated CO_2 and/or continuous light that yielded lower values of F_v/F_m and σ. This parallels acclimation to low and high light respectively (Suggett *et al.* 2007; Suggett *et al.* 2009), suggesting that CO_2 and long daylength periods induce photophysiological adjustments to a high light state. This is further supported by the simultaneous increase in τ with a reduction in σ, resulting in higher E_k values (Moore *et al.* 2006) under CO_2 enrichment and continuous light. The plasticity of both τ and σ appear to be traded against a relatively constant PSII pool size ([RCII]) for these species. In contrast, 124-3, 962 and *C. pelagicus* exhibited more plasticity in the PSII pool size - reducing it with CO_2 enrichment and/or continuous light - over changes to σ or τ, suggesting an alternative photoacclimation strategy. Work on natural assemblages of diatoms in the South China Sea is consistent with this, where cells CO_2 induced a rapid decrease in F_v/F_m (Gao *et al.* 2012), but not all laboratory studies support this. CO_2 has no effect on σ and F_v/F_m in *Skeletoneta costatum* (Chen and Gao 2004), *Phaeodactylum tricornutum* (Wu *et al.* 2010) and *T. pseudonana* (Crawfurd *et al.* 2011; McCarthy *et al.* 2012; Li and Campbell 2013). CO_2 has also been reported to increase susceptibility to PSII inactivation in *T. pseudonana* (Sobrino *et al.* 2008; McCarthy *et al.* 2012; Li and Campbell 2013). In the cyanobacteria taxa *Synechococcus* and *Prochlorococcus*, CO_2 on its own does not have a significant effect on photophysiology, but when combined with elevated temperatures it in fact increases F_v/F_m and, in the case of *Synechococcus*, maximum photosynthetic rates (Fu *et al.* 2007).

Past studies have established that a diversity of photoacclimation strategies exists among *E. huxleyi* isolates (Leonardos and Harris 2006; Suggett *et al.* 2007), though have also demonstrated a light-driven positive correlation between F_v/F_m and σ (as seen in NZEH, 70-3 and *G. oceanica*). While it is likely that these alternative photoacclimation strategies exist

because of different evolutionary histories, it is difficult to ascertain what particular advantages are conferred without further characterisation of pigment content and the stoichiometry of components involved in photosynthetic apparatus. The general photoacclimation response in microalgae is to decrease chlorophyll content, while increasing cellular carbon content, resulting in a decrease in the effective area for light absorption relative to RubisCO content (Fisher *et al.* 1989; Falkowski and LaRoche 1991; Geider *et al.* 1998). There is often a rearrangement of pigments over the course of photoacclimation, with the ratio of photosynthetic pigments to non-photosynthetic pigments decreasing in order to reduce the amount of excitation energy reaching the photosystems (Harris *et al.* 2005). As these changes result in lower F_v/F_m values and photosynthetic rates, it suggests that maintaining maximal carbon fixation is not the main priority of photoacclimation (MacIntyre *et al.* 2002).

Thicker coccospheres yielded higher σ, F_v/F_m and E_k values in *G. oceanica* and *E. huxleyi* 962 (the tropical isolates), comparable to a "low light" acclimation response (Moore *et al.* 2006; Suggett *et al.* 2007). The other four (temperate) isolates showed little pattern with changes to the coccosphere. Both of the tropical isolates produced consistently thinner coccospheres than the temperate ones, and calcification was reduced more with CO_2 enrichment in these cultures (see Chapter 3). It is possible that in the tropical isolates, the coccosphere becomes thin enough to change the internal light environment of the cells, and forces some photophysiological change. In the case of *G. oceanica*, very thin coccospheres (~5.5% total volume) yield the lowest σ and highest τ values recorded for any of the organisms in this experiment. It is therefore likely that the coccosphere plays a photoprotective role, allowing the cells to maintain lower values of τ and higher σ.

The temperate isolates maintain relatively thick coccospheres relative to cell size, usually over 20% of the total cell volume, thus maintaining the photoprotection it provides. Interestingly, the coccosphere becomes thinner in all organisms with CO_2, but only under the shorter

photoperiod. Under continuous light, the coccosphere thickness is comparable to that under ambient conditions (no CO_2 enrichment and 14h of light), sometimes even thicker, and there is no CO_2 effect. This indicates that under continuous light, cell resources are reallocated to maintain calcification even at high CO_2 levels. These results are consistent with previous investigations into the photoprotective capacity of the coccosphere, whereby decreased calcification resulted in a light stress response in *E. huxleyi* (Xu and Gao 2012; Jin *et al.* 2013). As calcification requires energy, it could be used as a process to drain electrons from PSII under stressful conditions (Xu and Gao 2012) (i.e. long photoperiod), as well as providing physical protection from high light.

Interestingly, calcification is not maintained at high CO_2 to same degree as ambient cultures under 14h light. There is recent evidence that OA enhances stress signals and decreases primary production in phytoplankton in general, not just coccolithophores (Gao *et al.* 2012), and causes increased photorespiration (Yang and Gao 2012), indicating that elevated CO_2 induces changes to photophysiology. Cultures here still showed decreases in F_v/F_m and E_k at high CO_2 under 14h of light, but as calcification was not maintained, the mechanism behind these signals is perhaps different. Rather than causing a build-up of electrons, elevated CO_2 on its own may disrupt the proton gradient across the thylakoid membrane. An increase in $[H^+]$ outside the membrane would lower the potential across the membrane, making it more difficult to synthesise ATP. In order to move the excess protons, more energy may be required to synthesise more proton pumps.

It is unclear why the two tropical coccolithophores produce the thinnest coccospheres, as solar radiation is strongest around the tropics, and seawater is more super-saturated with $CaCO_3$ compared to higher latitudes (Takahashi *et al.* 2014). Higher latitudes have more variation in daylength, however, and during the summer months the photoperiod can range from 16 to 20h between 50 and 60° in either hemisphere (Forsythe *et al.* 1995). Perhaps these longer

photoperiods are more stressful than 12h of higher light intensities, and thus require more investment into calcification to remove excess electrons and/or physically protect the cell.

Conclusions

Modification of two key growth conditions (daylength and CO_2) generally induces a "high light" acclimation strategy. In the two tropical isolates, this conforms to changes in the coccosphere, and is likely due to changes in the internal light field. In general, coccosphere thickness decreases with CO_2 under the short photoperiod, but is maintained under continuous light, supporting the idea that in general the coccosphere has a photoprotective role. This builds on previous work that different photoacclimation strategies exist for *E. huxleyi* (and other coccolithophores). The results presented here demonstrate that there are two main responses to changes in CO_2 and light availability; changing the PSII pool size ([RCII]) while maintaining constant values for σ and τ, or varying σ and τ while keeping the PSII pool size constant. Differences in photoacclimation strategies and stress responses perhaps reflect adaptations to different environmental conditions, in particular, differences in evolutionary history, but better characterisation of the photophysiology of these isolates would be required to conclude what advantages these strategies offer.

Chapter 5: Assessing the impact of ocean acidification on natural populations

5.1. Introduction

Much of our understanding of the OA response in phytoplankton comes from laboratory studies. While this provides a highly controlled environment and allows for a mechanistic analysis, care must be taken when applying this to real world scenarios. Monocultures are not representative of the marine ecosystem, and miss out on inter-specific interactions that may occur in nature that could affect how the subject organism responds to OA. Laboratory studies have shown that different species exhibit different responses to elevated CO_2, and as such will affect competition between plankton groups. Because the phytoplankton community composition has profound effect on the local food web and biogeochemical cycles, it is important to understand exactly what the net change of these individual responses actually is.

Very few OA field studies have been published to date. While there have been several studies in the Equatorial Pacific and Southern Ocean that look at C uptake of phytoplankton communities (Tortell *et al.* 2002; Tortell *et al.* 2008; Tortell *et al.* 2010), these were not specific OA studies, so do not always use "useful" CO_2 levels (e.g. comparing 150 and 750 µatm pCO_2).

Marine CO_2 vents provide a unique opportunity to study ecosystem-level effects of ocean acidification. Many of these volcanic vents eject toxic sulphur compounds at high temperatures, but in some areas, they are at ambient seawater temperatures and only release CO_2. Such vents are prevalent in the Mediterranean, in particular around Italy and Greece. In many cases, these vents have existed for millennia, and offer established ecosystems that exist in a gradient of CO_2 conditions. Research facilities have been set up around a few of these sites, in particular on the island of Ischia. Characterisation of the rocky shores around this volcanic island show clear succession of different communities along the CO_2 gradient, with coralline crustose algae (CCA)

and calcifying invertebrates being abundant in ambient waters (pH 8.0-8.1), but disappearing as pH falls (pH ~7.6) and are eventually taken over by extensive seagrass beds (Hall-Spencer *et al.* 2008). Invertebrates, in particular snails and limpets, also showed pitting of the shell when relocated to the low pH areas. Further experimentation shows that larval recruitment of many invertebrate species is adversely affected by ocean acidification (Cigliano *et al.* 2010). Due to the tidal nature of these sites, it is not possible to investigate the effects on phytoplankton, though there has been some work published on benthic algae. The brown macroalgae genus *Padina* is a calcifying organism that, unlike CCAs, shows an increase in abundance along a CO_2 gradient (Johnson *et al.* 2012). *Padina spp*. are not obligate calcifiers, and are therefore probably more less sensitive to changes in carbonate chemistry than other calcifying algae. The increased abundance was also partly attributed to the fact that sea urchin populations disappeared altogether at high CO_2 sites, which contribute to the top-down control of *Padina spp*. through grazing pressure. Benthic microalgae communities also showed changes in response to CO_2, with diatom abundance increasing in biofilms formed at higher CO_2 sites (cyanobacteria abundance remained unchanged) (Johnson *et al.* 2013). The community composition changed dramatically with CO_2, ultimately becoming less diverse and dominated by one or two genera.

The first big initiative to study the effects of CO_2 on pelagic plankton *in situ* was the Pelagic Ecosystem CO_2 Enrichment (PeECE) project, which consisted of 3 mesocosm experiments at the Large-Scale Mesocosm Facilities of the University of Bergen, Norway. These were carried out in 2001, 2003 and 2005, and yielded mixed results. For example, biogenic calcification was found to be negatively affected by CO_2 in one mesocosm (Delille *et al.* 2005), but no CO_2 effect was observed in the following experiment (Bellerby *et al.* 2008). Perhaps the most interesting result from the PeECE project was that there was no observable CO_2 effect on many aspects of the experiment. This includes grazing by microzooplankton (Suffrian *et al.* 2008), inorganic nutrient utilisation (Bellerby *et al.* 2008; Schulz *et al.* 2008) despite an increase

in DIC consumption (Riebesell *et al.* 2007), bacterial abundance (Allgaier *et al.* 2008) and phytoplankton community composition (Paulino *et al.* 2008). It is difficult to say whether this denotes resilience to elevated CO_2, or that not enough time had elapsed for any CO_2 response materialise. On the other hand, DMS production was reduced by CO_2 (Hopkins *et al.* 2010), and there was some shift in phytoplankton community, with picoeukaryotes becoming more abundant and *Synechococcus* becoming less so in high CO_2 treatments (Paulino *et al.* 2008). For a full review of the Bergen mesocosm experiments, see Riebesell *et al*. (2008).

It is important to remember when comparing field studies that carbonate chemistry varies depending on location. The pH of the global ocean ranges from 7.9-8.2, but warmer waters are supersaturated by aragonite by a factor 4.2, compared to just 1.2 in polar regions (Takahashi *et al.* 2014). The polar seas are some of the most extreme environments on Earth in terms of both temperature and light availability, but are also the most vulnerable when it comes to changing climate and elevated CO_2 (McNeil and Matear 2008; Shadwick *et al.* 2013; Constable *et al.* 2014; Overland *et al.* 2014; Turner *et al.* 2014). Arctic sea ice is declining much faster than originally predicted (Stroeve *et al.* 2007), and has seen a 75% net loss by volume since the 1980s (Overland and Wang 2013). This increased melting enhances ocean acidification by flushing the Arctic Basin with freshwater, diluting $CaCO_3$ and decreasing Ω further (Li *et al.* 2009). Changes to Antarctic sea ice appear to be regionally specific, with the Amundsen Sea showing a trend of warming and declining sea ice coverage (Stammerjohn *et al.* 2008), while the Ross Sea has experienced an increase in both the extent and duration of sea ice coverage (Comiso *et al.* 2011). Even so, the projection is an overall 33% decrease of sea ice surface area by the end of the 21st century (Bracegirdle *et al.* 2008).

Perturbation experiments on natural communities have the potential to provide more insight about the complex ecosystem-level response to OA and climate change, and give greater environmental relevance than monocultures (Tortell *et al.* 2002; Delille *et al.* 2005; Engel *et al.*

2005; Feng *et al.* 2008; Gao *et al.* 2012). However, the interpretation of results can be complicated due to multiple biogeochemical feedbacks and food web interactions that are characteristic in any natural assemblage (Riebesell *et al.* 2008; Krause *et al.* 2012; Brussaard *et al.* 2013). Laboratory experiments also allow for greater replication (and therefore statistical power), while time and resources can limit the scope for this in field studies, particularly in mesocosms (Schulz *et al.* 2013).

Perhaps the major limiting factor for field work is the timescale for incubations. In the context of OA, this can certainly be a limiting factor for all experimental work, including cultures grown in a laboratory. The timescale on which OA occurs will be equivalent to many thousands of microbial generations, suggesting that evolutionary adaptation (rather than acclimation) may dictate ecosystem-level responses (Reusch and Boyd 2013; Sunday *et al.* 2014). To date, a few long term (between 6 and 18 months) laboratory culture studies have been performed, which indicate some capacity to adapt and restore – or even enhance – growth, calcification and C fixation rates over prolonged exposure to CO_2 (Crawfurd *et al.* 2011; Lohbeck *et al.* 2012; Jin *et al.* 2013). Due to constraints on both time and resources, field work cannot feasibly cover long time scales. Realistically, shipboard experiments cannot last longer than several days (see Tortell *et al*. 2002), so while such experimentation can account for changes to community composition resulting from high inter- and intraspecific plasticity in CO_2 response (Schaum *et al.* 2013), great care must be taken when extrapolating these results to long term trends.

The work in previous chapters has focused solely on laboratory cultures. It is not clear how well the observed trends might apply to natural assemblages of phytoplankton. Therefore the aims of this chapter are to assess whether or not changes in CO_2 and daylength (i.e. latitude) have any effect on natural phytoplankton communities, and how well (or not) these responses conform to previous laboratory observations.

5.2. Methods and Materials

5.2.1. Study Sites

Two cruise tracks were covered in the polar regions, one in the Atlantic sector of the Arctic Circle (JR271) during June and July 2012, and one in the Southern Ocean (JR274) during January and February 2013. These tracks encompassed areas of natural environmental gradients such as $CaCO_3$ saturation, temperature and salinity. These cruise tracks also encountered phytoplankton blooms, occurring off the southern coast of Iceland, in the Barents Sea, to the north of South Georgia and to the east of the South Sandwich Islands.

5.2.2. Shipboard Bioassays

Bioassays were set up in 5 different locations during cruise JR271, and 4 locations for JR274, along the cruise track with different initial environmental conditions, reflecting both spatial variability within the study region and areas where phytoplankton blooms were likely to occur.

Surface seawater was collected from titanium Niskin bottles (24x10L) over three successive casts in order to provide enough water for the large number of final measurements. Once on deck, the Niskin bottles were immediately transfer in a positive pressure Class-100 filtered trace metal clean container to avoid contamination. Unfiltered water containing the unperturbed full suite of microbial groups was dispensed into 4.5L polycarbonate incubation bottles using acid-cleaned silicon tubing and closed, pending carbonate chemistry manipulation. A further set 6x4.5L polycarbonate bottles were filled in parallel and grown under a shorter L:D cycle by placing them in a blacked out box inside the on-deck incubator.

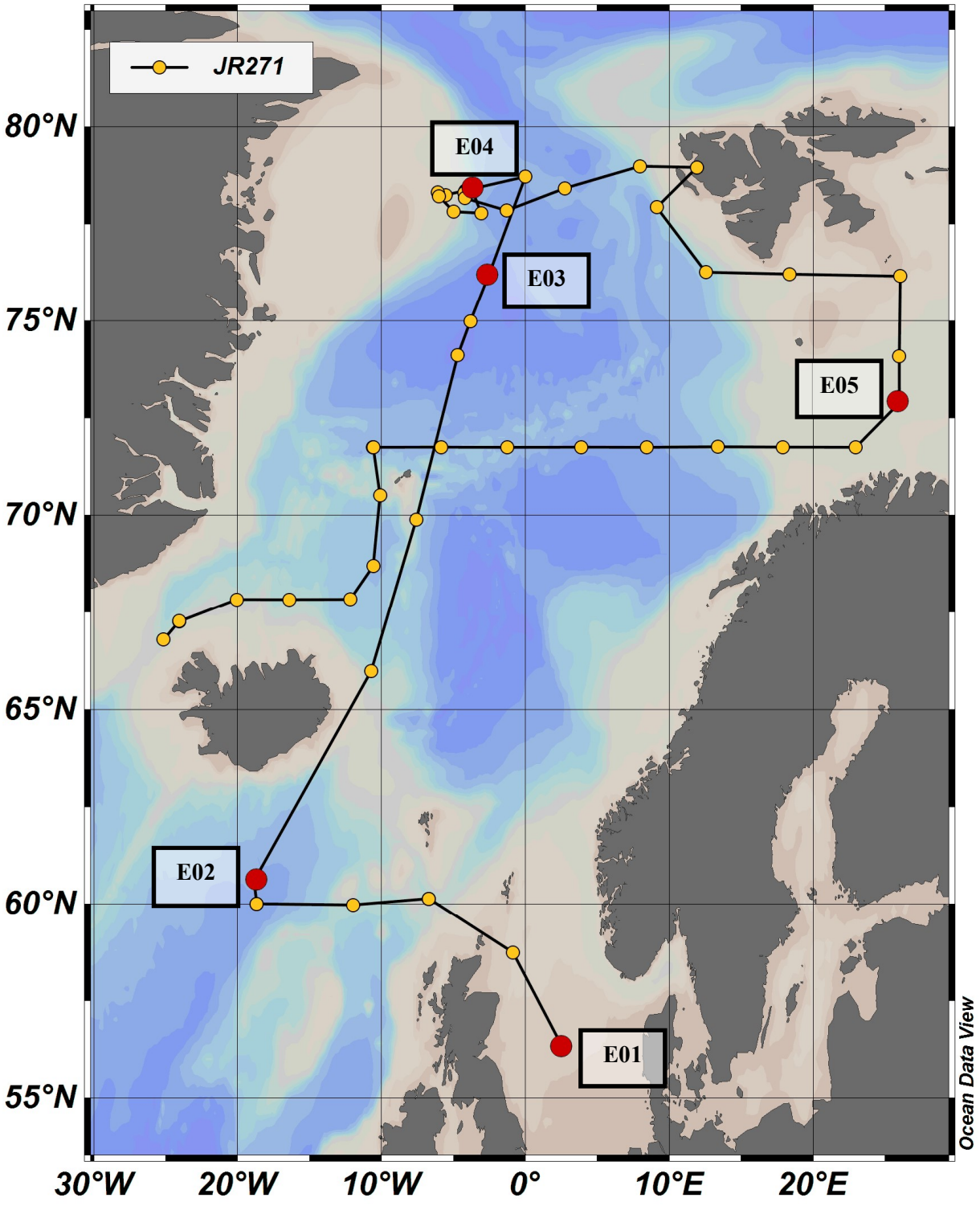

Fig. 5.2.1. Cruise track for the Arctic cruise JR271, conducted June-July 2012. Red points indicate stations where water for bioassays was collected. E02 was a coccolithophore bloom station, E03 and E04 were sea ice stations.

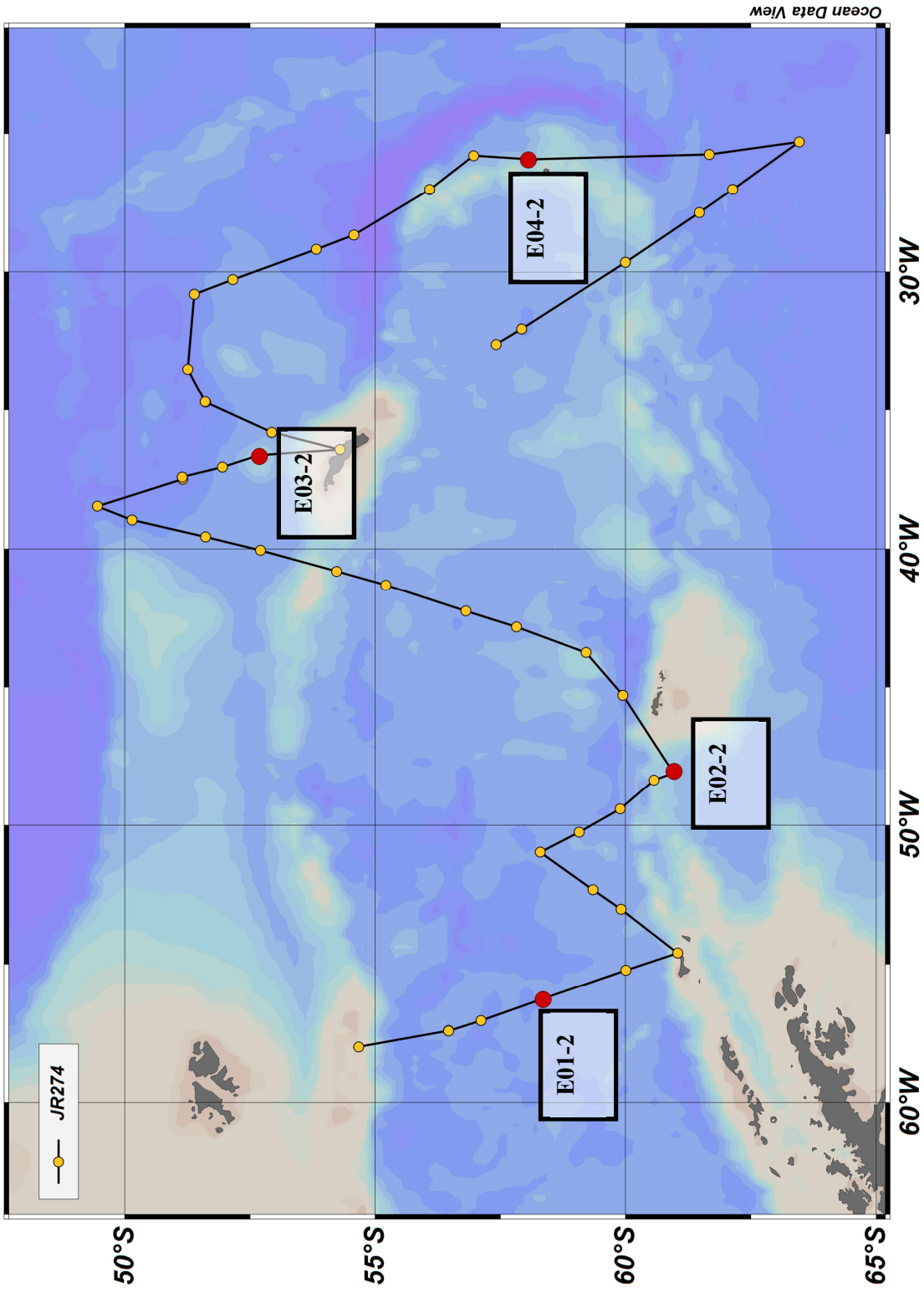

Fig. 5.2.2. Cruise track for Southern Ocean cruise JR274, conducted during austral summer during January-February 2013. Red points indicate bioassay stations. E02-2 was a sea ice station.

Each experimental bottle was individually manipulated to achieve different conditions. In the Arctic cruise (JR271), there were 4 different target CO_2 levels; ambient, 550, 750 and 1000 µatm. In the Antarctic cruise, the 4 different treatments were ambient, Fe addition, 750 µatm CO_2, and 750 µatm with Fe additions.

The manipulation of the carbonate system was achieved through additions of $NaHCO_3$- + HCl (Borowitzka, 1981; Gattuso and Lavigne, 2009; Schulz et al., 2009), and immediately verified by total alkalinity (TA) and DIC analyses. Following manipulation of pCO_2, bottles were sealed with septum lids, parafilmed and incubated. The incubation was performed within a purpose-built experimental laboratory container allowing precise temperature and light control. The temperature was adjusted to the in situ value at the time of the water collection. Temperature within a dummy incubation bottle was monitored using a traceable thermometer, while two recording thermometers were used to monitor air temperature in the incubator. The light conditions in the incubator were set up with a 10/14h light dark/cycle for the first two experiments (E01 and E02) and no dark phase for the 3 successive experiments which were sampled under conditions of 24 hour sunlight during summer within the Arctic Circle. Each experiment was run for 96h total including three collection time points: T0, T1 (48h) and T2 (96h). Each condition was run in triplicate bottles. Detailed records were kept of the CTD and Niskin bottle used to fill each of the experimental bottles. Additionally records were kept of the people who filled each bottle. A total of 72 bottles were incubated within each of the main experiments, consisting of 9 bottles required for all the water utilized at each timepoint (3 sets of triplicates for different sets of analyses) and the two post initial timepoints.

Before sampling work was carried out in the polar regions, the bioassay method (set up, maintenance and sample collection) was tested on a cruise track sampling the European shelf seas (D366) during June and July 2011. Five bioassays were performed, each 4 days long, but on the deck of the ship rather than in a trace-metal clean containerised laboratory as trace-metal

contamination is less of a concern in coastal shelf seas. The initial data from this cruise indicated that the biological and chemical variables measured across the set of bottles were highly constrained, and standard deviations from biological triplicates were typically <10% of the mean values. Additionally, the carbonate system was well constrained, allowing for successful increases in [DIC] with no concurrent changes to TA. For a review of this method and initial data set, see Richier *et al*. (2014).

5.2.3. General FRRF Protocol

All samples were incubated in the dark for 15-20 minutes in a water bath kept at the *in situ* temperature. After incubation, a 2mL sub-sample was pipetted into the FRRF for single turnover acquisitions to obtain general photophysiological parameters. In addition, a rapid light curve (RLC) was also carried out on some samples to further assess photophysiology. PAR values used in the RLC ranged between 0-1400µmol photons m^{-2} s^{-1}. In order to run blanks, 10mL of each sample was filtered using 0.2µm syringe filter, and gently gravity filtered to minimise the amount of cells lysing and contaminating the filtrate with chlorophyll.

5.3. Results

5.3.1. Arctic Circle – Chlorophyll

Total chlorophyll a concentration was used as a proxy for biomass (see Fig 5.3.1). Station E04 had the highest initial biomass at 3.03mg m^{-3} (the northern-most station), and E01 the lowest at 0.31mg m^{-3} (the southern-most station).

Bioassay 1 – very low biomass, which does not increase either over time or with CO_2 enrichment. About 50% of the biomass was in the <10µm size fraction, which also did not change over the course of the experiment, indicating that there were no significant changes in community.

Bioassay 2 – a coccolithophore bloom was occurring at this station, initial chlorophyll concentration was 1.82mg m-3. Biomass increased over time in all treatments, but by 96h, chlorophyll concentrations were higher than the ambient cultures in all other CO_2 conditions (3.14mg m^{-3} compared to 3.78–4.07mg m^{-3}). The 1000µatm cultures exhibited a much earlier "bloom", peaking at 4.37mg after 48h, but falling to similar values as the other cultures by the end of the experiment. About 65% of the biomass was in the smaller size fraction, consistent with the high concentration of coccolithophores in the initial population.

Bioassay 3 – a relatively low biomass station, with a starting biomass of 0.90mg m^{-3}. The chlorophyll concentration rose steadily in all cultures, to more than double by the end of the experiment (2.34–2.50mg m^{-3}), though the 750µatm cultures reached over 3mg. Around 2/3 of the biomass was in the small size fraction, but at 96h the amount of small biomass actually fell, indicating not only that the larger organisms had proliferated, but out-competed the smaller ones.

Bioassay 4 – the highest biomass station (3.03mg m^{-3}), the chlorophyll increased steadily over time, with the 750µatm treatment producing the most biomass. The 1000µatm cultures were about 1mg lower in concentration than the others. Only ~20% of the initial population was in the smaller size fraction, and by 48h effectively 0% was in the <10µm fraction. However, at the 96h time point, the smaller population had recovered to its initial concentration.

Bioassay 5 – nearly all the biomass in this station was in the <10µm fraction (~90%), starting at 1.20mg m^{-3} and finishing at 2.0–2.5mg.

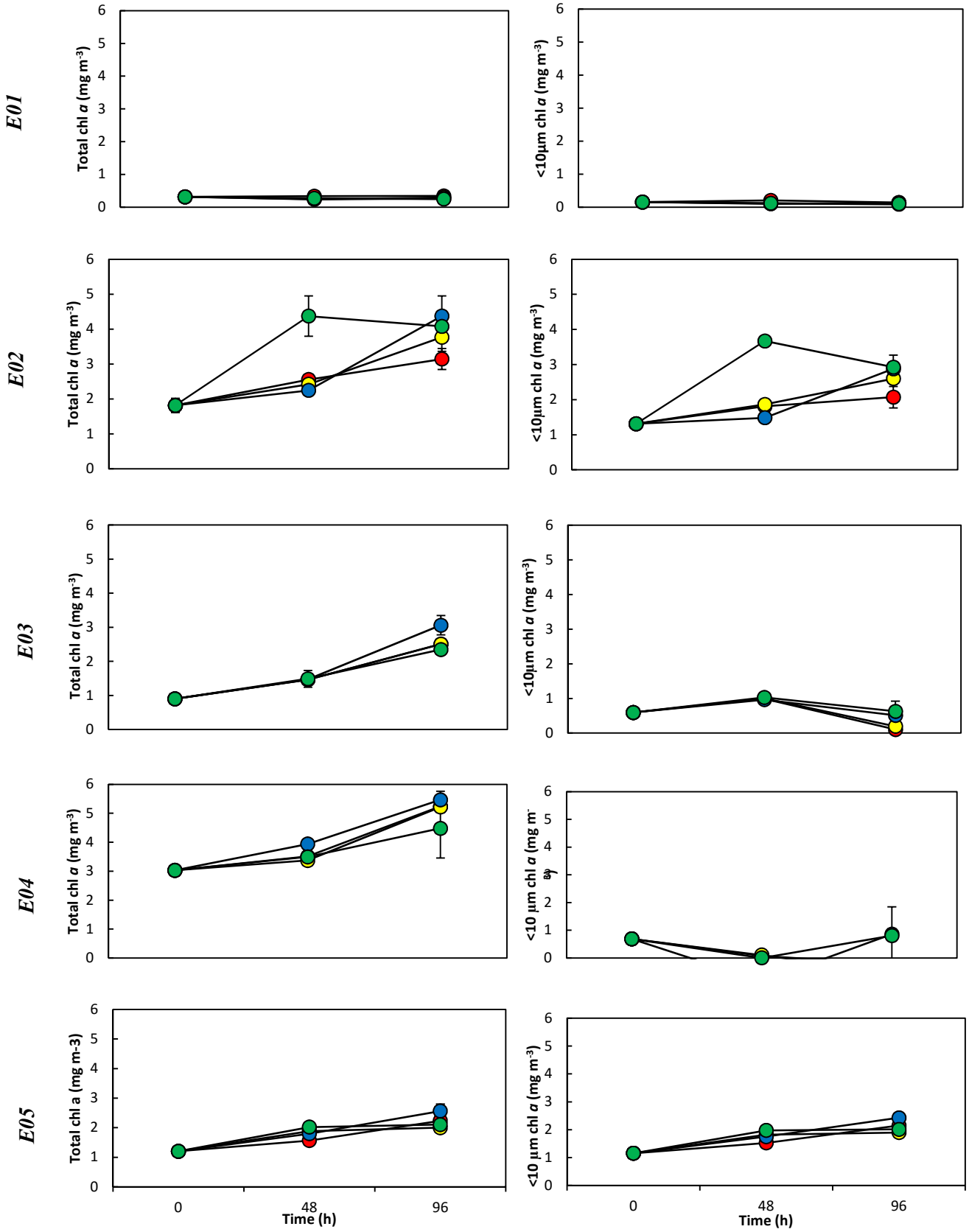

Fig 5.3.1. The total chlorophyll *a* and <10µm fraction chlorophyll *a* over time in each bioassay experiment conducted in the Arctic Circle. The four CO_2 treatments are ambient conditions (red), 550µatm (yellow), 750µatm (blue) and 1000µatm (green). Error bars are +/- S.E., n=3.

5.3.2. Arctic Circle – Photophysiology

Initial F_v/F_m varied between 0.29 and 0.39, with the highest value found at the site with the highest starting biomass, E04 (Fig. 5.3.2).

Bioassay 1 – second highest initial F_v/F_m, which begins dropping in the CO_2 enriched cultures over time. The 1000µatm cultures show a significant fall in F_v/F_m compared to the other treatments, dropping to 0.29. The absorption cross-section is relatively small, beginning at 3.11nm. In all cultures except the 550µatm, σ showed an overall decrease (though the ambient cultures displayed an increase at the 48h time point). There was an increase of τ in the 550µatm from 771 to 925µs by the end of the experiment. In the ambient cultures, it dropped to below 600µs at 48h, before returning to around the initial value by 96h. The two highest CO_2 treatments showed very little change over time.

Bioassay 2 – one of the lowest initial F_v/F_m values at 0.29, however this increased to ~0.38 by 48h, and did not change much from there to the end of the experiment (though all cultures except the 750µatm showed a slight decrease). Initial σ values were high at 4.68nm, and while they dipped slightly to ~4.50nm, they increased to 4.90-5.20nm by the end of the experiment – with the exception of the 750µatm cultures that didn't change at all over time. The τ values started high at 1009µs, but fell to 702-742µs by 48h. There was a slight recovery by 96h, but it does not return to initial values. 1009µs, but fell to 702-742µs by 48h. There was a slight recovery by 96h, but it did not return to initial values.

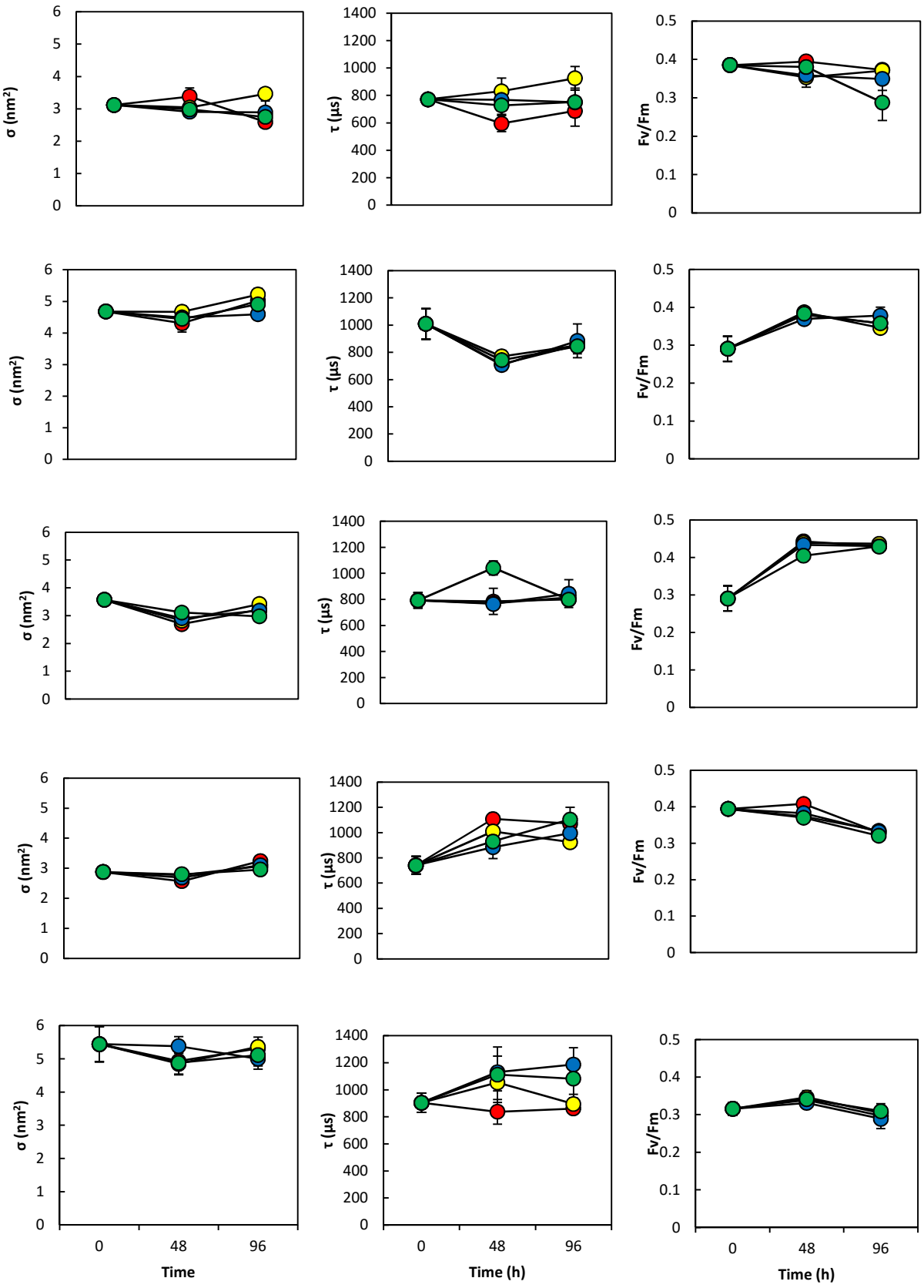

Fig 5.3.2. Changes in σ, τ and F_v/F_m in response to CO_2 over time in bioassays conducted in the Arctic Circle. The four CO_2 treatments are ambient conditions (red), 550µatm (yellow), 750µatm (blue) and 1000µatm (green). Error bars are +/- S.E., n=3.

Bioassay 3 – low initial F_v/F_m (0.29) that increased to ~0.43 by 48h, though only 0.40 in the 1000µatm cultures. It remained stable through to 96h, by which time even the 1000µatm cultures were at ~0.43. Absorption cross-section sizes were initially 3.57nm, and all cultures but the 1000µatm dropped below 3.0nm by 48h. At 96h they had recovered to between 3.18 and 3.42nm, but the 1000µatm had fallen to 2.97nm. For the duration of the experiment, τ remained between 765 and 845µs in all cultures, except for a large spike to 1042µs in the 1000µatm cultures at 48h.

Bioassay 4 – F_v/F_m was initially high (0.39), but already started to fall slightly by 48h in all cultures except for the ambient ones. At the end of the experiment, F_v/F_m had decreased to ~0.32 in all cultures. The σ values were initially 2.87nm, and in ambient cultures, there was a slight decrease at 48h (2.57nm), but an increase at 96h to higher than the starting values (3.24nm). All other treatments remained stable throughout the experiment. The τ values started at 741µs, and then followed one of two responses; either increasing to over 1000µs at 48h, and then decreasing slightly by 96h, though still much higher than initial values (ambient and 550µatm), or, steadily increasing over time to ~1000µs at the final time point (750 and 1000µatm).

Bioassay 5 – F_v/F_m was relatively low at this station, starting at 0.31, and not changing much over time. At 48h it had risen slightly to 0.33-0.34, but fell back to initial values by 96h. The highest initial σ values were observed here, at 5.44nm. At 48h, values had fallen to ~4.88nm, but had recovered somewhat to 5.11-5.36nm by the second time point. The exception is the 750µatm CO_2 treatment, which remained stable until the final time point, where it dropped to ~5nm. At T0, τ was at 904µs, which was maintained throughout the experiment in ambient cultures and increased to over 1000µs in the other CO_2 treatments. The 550µatm cultures dropped back to initial values at 96h, but remained high in the 750 and 1000µatm cultures.

5.3.3. Arctic Circle – Primary Productivity and Calcification Rates

Primary productivity (PP) increased with an increase in latitude (moving further north). The lowest initial PP was 1.03mmol C m^{-3} d^{-1} at station E01, followed closely by E02 with 1.67mmol C m^{-3} d^{-1} (Fig. 5.3.3). By contrast, the highest was 6.13mmol C m^{-3} d^{-1} at E04, the northern-most bioassay station. Calcification rates followed an inverse relationship with latitude, where the more southern stations exhibited higher calcification rates relative to the northern ones. The highest calcification rate was recorded at E02, of 69.33µmol C m^{-3} d^{-1}, and the lowest at E03, which was 5.00µmol C m^{-3} d^{-1}.

Bioassay 1 – PP was very low initially, but dropped to virtually zero over time in all treatments. Calcification rates were relatively high in the starting population at 22.93µmol C m^{-3} d^{-1}, but this also dropped significantly over time to between 1 and 3µmol (again, in all treatments).

Bioassay 2 – PP was again low here, but did increase over the course of the experiment. PP more than doubled by 48h in ambient and 550µatm cultures to ~4mmol, and continued to 5.90mmol in ambient cultures and 4.82mmol in the 550µatm cultures. The PP in the two higher CO_2 treatment cultures increased less than the others at 48h, but the 750µatm cultures exhibited a sharp increase to 7.1mmol at 96h, whereas the 1000µatm cultures increased to only 4.80mmol. In comparison, calcification was very high in the initial population, decreasing slightly in all treatments at 48h, except for 550µatm cultures where it nearly doubled to 128.0µmol C m^{-3} d^{-1}. By 96h, the ambient and 750µatm cultures had increased to 194.6µmol and 224.0µmol C m^{-3} d^{-1} respectively. The 550µatm cultures had fallen to 94µmol at this point, and 1000µatm remained similar to starting conditions at 65.33µmol C m^{-3} d^{-1}.

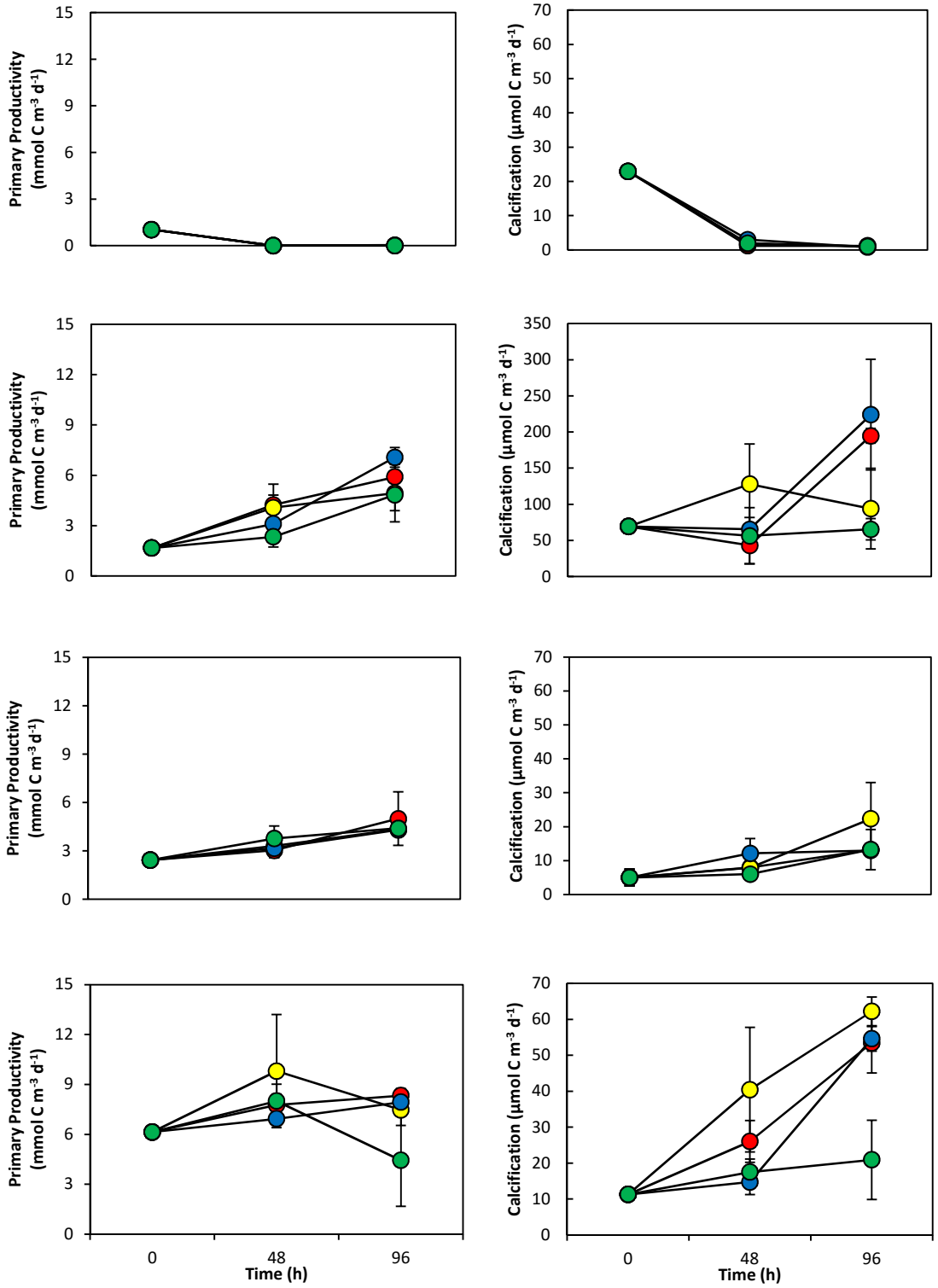

Fig 5.3.3. Primary productivity and calcification rates over time in each bioassay experiment conducted in the Arctic Circle. The four CO_2 treatments are ambient conditions (red), 550µatm (yellow), 750µatm (blue) and 1000µatm (green). Data is unavailable for bioassay 5 due to technical problems on board the ship. Error bars are +/- S.E., n=3.

Bioassay 3 – starting PP was higher than the previous two sites at 2.43mmol C m^{-3} d^{-1} and increased steadily over the course of 96h to between 4 and 4.5mmol, with no discernible CO_2 effect. Calcification was very low in the starting population. In both ambient and 1000µatm cultures, these calcification rates remain unchanged until 96h, where they more than doubled their initial values (~13µmol C m^{-3} d^{-1}), while the 550µatm cultures increased to 22.3µmol C m^{-3} d^{-1}. The 750µatm calcification rates doubled at the 48h time point (12.13µmol C m^{-3} d^{-1}), and remained stable through to 96h.

Bioassay 4 – the highest PP rates were recorded at E04 (6.13mmol C m^{-3} d^{-1}). PP increased in all cultures at 48h to between ~7 and 10mmol. The final rates in all the cultures were higher than the initials, with the exception of the 1000µatm cultures, which had fallen to 4.43mmol C m^{-3} d^{-1}. In contrast, calcification was relatively low, starting at 11.27µmol C m^{-3} d^{-1} but increased significantly over time (54.4–62.2µmol), even doubling in the 1000µatm cultures.

5.3.4. Southern Ocean – Chlorophyll

The lowest chlorophyll *a* concentration was observed at E02-2, 0.51mg m^{-3}, which is comparable to the lowest value in the Arctic Ocean at 0.31mg m^{-3} (E01) (Fig 5.3.4). The highest, 4.19mg m^{-3}, at E04-2 was greater than the highest recorded value in the Arctic (3.03mg m^{-3} at E04). There was little difference between the average initial biomass of the Arctic and the Southern Ocean bioassay stations (1.45mg m^{-3} and 1.91mg m^{-3} respectively).

Bioassay 1 – biomass started at 2.31mg m^{-3} and steadily increases through the course of the experiment in all vessels, with the ambient cultures having the highest chlorophyll concentration at the end of the experiment (5.69mg m^{-3}) followed by +Fe (5.10mg m^{-3}), Fe with 750µatm CO_2 (4.60mg m^{-3}), and finally the 750µatm CO_2 cultures (4.26mg m^{-3}). The amount of biomass in the small size fraction was effectively zero at the start of the experiment, but by 96h ~8% of the chlorophyll was in the <10µm fraction.

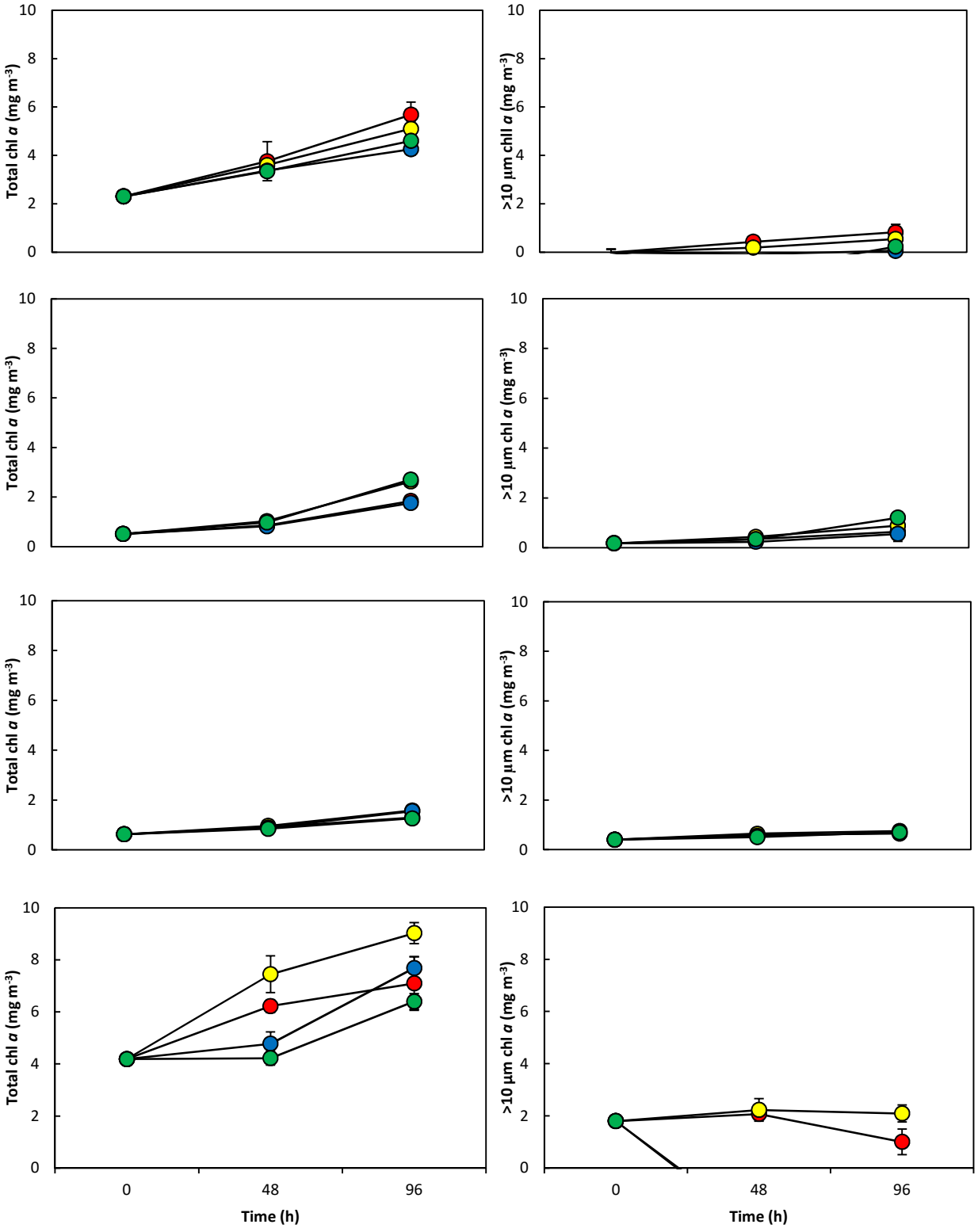

Fig. 5.3.4. The total chlorophyll *a* and <10µm fraction chlorophyll *a* over time in each bioassay experiment conducted in the Southern Ocean. The four treatments are ambient conditions (red), +Fe in bioassays 1 and 2, 750µatm CO_2 in bioassays 3 and 4 (yellow), 750µatm CO_2 in bioassays 1 and 2, and 1000 µatm CO_2 in bioassays 3 and 4 (blue) and Fe+750 µatm in bioassays 1 and 2, and 2000µatm CO_2 in bioassays 3 and 4 (green).

Bioassay 2 – a low biomass station, with an initial chlorophyll concentration of 0.51mg m^{-3}. There was a small increase to ~0.9mg m^{-3} at 48h, but at 96h there were two different responses. The ambient and CO_2 enriched cultures rose to ~1.75mg m^{-3}, but the two Fe enriched cultures increased to ~2.70mg m^{-3}. More of the biomass was made up of smaller organisms at this site than the previous, with about 35% of the starting community in the <10µm fraction, and this remained the case for the rest of the experiment.

Bioassay 3 – similar to Bioassay 2, low biomass station with a starting chlorophyll *a* concentration of 0.62mg m^{-3}. This bioassay displayed a similar rate of increase to Bioassay 2, but with a much less pronounced response at 96h. Unlike the previous experiment, it was the ambient and 2000µatm cultures that had the lower values at the final time point (1.26 and 1.29mg m^{-3} respectively), and the 750µatm and 1000µatm cultures that increased the most (1.55 and 1.57mg m^{-3} respectively).

Bioassay 4 – the highest biomass station, with an initial chlorophyll concentration of 4.19mg m^{-3}. At 48h, the ambient and 750µatm cultures had increased significantly to 6.22 and 7.44mg m^{-3} respectively, whereas the two higher CO_2 enrichments remained relatively unchanged. By the second time point, the ambient and 750µatm cultures had continued their growth in biomass at a slow rate, with final concentrations of 7.09 and 9.02mg m^{-3}, but the higher CO_2 cultures had increased to rates comparable to the lower CO_2 cultures at 48h (7.68 and 6.40mg m^{-3}). ~45% of the initial population was found in the <10µm fraction, but over time the larger organisms took over and dominated the community. In the higher CO_2 enrichment treatments (1000µatm and 2000µatm), there was no recordable biomass in the <10µm fraction at either time point.

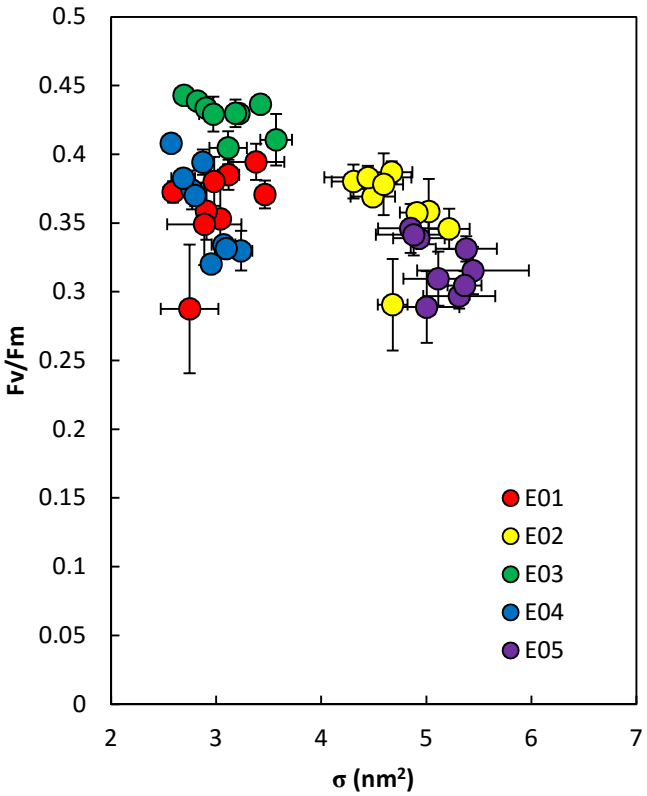

Fig. 5.3.5. Values of the maximum PSII photochemical efficiency (F_v/F_m, dimensionless) and effective absorption cross-section (σ, nm^2) for the five bioassays in the Arctic Circle. Data are from all CO_2 treatments and time points. Error bars are +/- S.E., *n*=3.

5.3.5. Southern Ocean – Photophysiology

Initial F_v/F_m values were, on average, much lower in the Southern Ocean compared to the Arctic (0.26 compared to 0.33). The highest initial F_v/F_m values were found at E04-2, a bloom station to the east of the South Sandwich Islands, and the lowest were found at E03-2, just off the north coast of South Georgia.

Bioassay 1 – F_v/F_m values started at 0.25, and increased to between 0.35 and 0.38 by 48h. At 96h, there were two observable responses; ambient and CO_2 enriched cultures had F_v/F_m values of ~0.38, whereas Fe enrichments exhibit higher values of ~0.41.

Bioassay 2 – relatively high F_v/F_m for the Southern Ocean, with initial values of 0.29. These values increased to 0.32–0.35 by 72h, and finally showed two different responses at 144h, where the ambient and CO_2 enriched cultures exhibited values of ~0.33, while the Fe enriched cultures reached ~0.43.

Bioassay 3 – the lowest initial F_v/F_m values were recorded here, at 0.18. By the first time point, F_v/F_m values had reached 0.3–0.32, and remained stable into the second time point. There was no discernible CO_2 effect.

Bioassay 4 – the highest initial F_v/F_m values were recorded here at 0.34, but is also the only bioassay where F_v/F_m decreased. The ambient cultures decreased at the first time point to ~0.30, and by the second time point ambient F_v/F_m had fallen to the lowest value of all the treatments (0.19), followed by 750µatm, 1000µatm and then 2000µatm (0.26).

5.4. Discussion

In general, there were no trends with CO_2 across the dataset. Biomass typically increased over time in the bioassays, but this was independent of CO_2. There was one coccolithophore-dominated station in the Arctic, E02, which produced very high rates of calcification (Fig. 5.3.3). Interestingly, at the end of the experiment, calcification rates had increased significantly in both the ambient and 750µatm treatments (to ~200µmol C m^{-3} d^{-1}), but remained close to T0 rates in 550 and 1000µatm treatments. However, the 550µatm treatment induced the highest rates of calcification at T1. This indicates an optimum CO_2 range, with ambient and 750µatm on the outer edges of the range, and 550µatm close to the optimum. The decrease in calcification rates by T2 in the 550µatm treatment could be indicative that the community depleted another key resource, causing the population to crash. CO_2 optima for calcification in coccolithophores have been identified for individual species (Langer *et al.* 2006; Iglesias-Rodriguez *et al.* 2008), which

would enable blooms to continue calcifying even as *in situ* CO_2 levels increase due to elevated respiration rates. This perhaps indicates a certain degree of flexibility in coccolithophore assemblages to deal with CO_2 changes. Past field work on calcification in coccolithophore blooms grown under OA conditions has produced mixed results. Some indicate tolerance to high CO_2 concentrations (Bellerby *et al.* 2008; Feng *et al.* 2009) while others have shown definite sensitivity to such conditions (Delille *et al.* 2005; Engel *et al.* 2005). The results from this bioassay perhaps indicate that unless CO_2 conditions are pushed past an optimum range, no CO_2 effect will be observed.

Interestingly, there was also a strong increase in calcification in bioassay 4, which was an Arctic sea ice station dominated by large diatoms, mainly from the genera *Fragilariopsis*, *Thalassiosira*, and *Chaetoceros*. There were some coccolithophore species present at E04, with the main species being *Coccolithus pelagicus*, *Algirosphaera robusta*, *Calciopappus caudatus* and *Syrachosphaera dilatata*. With the exception of *C. pelagicus*, very little is known about these species in general, or how they contribute to the PIC pool. The one study on calcification rates in *A. robusta* shows that it calcifies more than *E. huxleyi*, and has comparable calcification rates to *Gephyrocapsa oceanica* (Ziveri *et al.* 2003), but would not explain the high rates seen here. *C. caudatus* usually grows at low concentrations in Arctic waters (Baumann *et al.* 2000), but has in the past has been recorded as the dominant species in certain sites, such as off the coast of Jan Mayen (Ramsfjell 1960). This is mainly attributed to alleviation of light limitation due to mixing. Regardless, cell densities of coccolithophores at this station were an order of magnitude smaller than those found at E02, so it is extremely unlikely that they are the source of calcification. As chlorophyll does not increase very much, and primary productivity decreases over time (Fig. 5.3.3), it could be possible that the source of calcification is from zooplankton. Pteropod snails were encountered infrequently over the course of the Arctic cruise track, mainly around the Svalbard archipelago, but the calcification rates are not consistent with published

literature, especially over the short time scale (Comeau *et al.* 2010). It is interesting to note that there is some degree of sensitivity to CO_2 demonstrated here, as calcification rates increase in all treatments except for 1000 µatm. Pteropods have consistently been shown to be very sensitive to OA, in particular as they use aragonite for calcification (Comeau *et al.* 2010; Bednaršek *et al.* 2014; Busch *et al.* 2014). However, as the zooplankton community was not characterised in these cultures, it is not possible to say with any certainty what the source of calcification might be at this site.

The first two bioassays in the Southern Ocean tested CO_2 and Fe enrichment, an interaction that has largely been ignored. At 96h, there was a significant difference in chlorophyll a and F_v/F_m between the Fe enriched cultures and the ambient/CO_2 enriched cultures, with values being higher with Fe enrichment. This demonstrates that phytoplankton in the Southern Ocean are not CO_2 limited, and/or Fe is a more limiting factor. There is also no synergistic effect of Fe and CO_2, which does imply that CO_2 is simply not a limiting resource to Southern Ocean phytoplankton. This is consistent with incubation experiments using water from the Weddell Sea (Hoppe *et al.* 2013).

It has been demonstrated that ocean acidification decreases the bioavailability of Fe, and slows Fe uptake rates of phytoplankton (Shi *et al.* 2010; Sugie *et al.* 2013). So while there is no synergistic "fertilisation" effect of combining Fe and CO_2, ocean acidification itself may well exacerbate Fe limitation in the Southern Ocean.

Photophysiology was highly variable between stations, and showed no clear trend with either CO_2 enrichment or daylength, i.e. latitude (Fig. 5.3.2). Trends across the whole data set for the Arctic Circle for F_v/F_m and σ conform to the general trend seen across phytoplankton taxa, that F_v/F_m negatively co-varies with σ (Suggett *et al.* 2009). Much of the variability of phytoplankton photophysiology reported in the literature is as a result of different "taxonomic

signatures" (Suggett *et al.* 2004; Moore *et al.* 2005; Fishwick *et al.* 2006; Moore *et al.* 2006). This stems, in part, from the importance of light absorption as a key driving factor in the evolution of phytoplankton taxa (Wood 1985; Stomp *et al.* 2007; Polimene *et al.* 2013). As such, great care should be taken when analysing FRR fluorescence data from natural communities, and it cannot be interpreted on the basis of physiological stress only (Prasil *et al.* 2008; Suggett *et al.* 2009).

Fig. 5.3.5 demonstrates distinct groups of phytoplankton that correspond well to known taxonomic signatures (see Suggett *et al*. 2009). Low σ values with higher F_v/F_m values as observed in bioassays 1, 3 and 4 are typical of well-mixed areas dominated by large diatoms, while higher σ values with lower F_v/F_m values from bioassays 2 and 5 are indicative of areas with enhanced water column stability and dominance of smaller eukaryotes such as coccolithophores and dinoflagellates. Chlorophyll fractionation (Fig. 5.3.1) shows larger cells dominated bioassays 1, 3 and 4, while taxonomic observation confirmed that large, chain-forming diatoms formed most of the phytoplankton. Bioassays 2 and 5 were characterised by smaller cells, and a dominance of either coccolithophores (E02) or *Phaeocystis sp*. (E05). While other taxonomic groups were identified at each station, such as cyanobacteria, changes in F_v/F_m and σ are always dictated by the dominant group of phytoplankton (Suggett *et al.* 2004). These results confirm that the photophysiology derived from the FRR fluorometer is an effective method of identifying distinct groups of phytoplankton.

However, with regard to OA and natural assemblages, these results show that community composition was far more important in dictating physiological response than CO_2. It is important to note that laboratory culture work on OA has been able to induce significant changes to photophysiology in several different groups of phytoplankton (Wu *et al.* 2010; Brading *et al.* 2011; Xu and Gao 2012; Zheng *et al.* 2015), which perhaps indicates some methodological constraints, specifically that the bioassays were not incubated long enough for CO_2 to induce any

major changes to the organisms. The experimental set up for this project prioritised replication and range in geographic location, which meant that incubations longer than six days were impractical. A mesocosm set up would allow for longer incubation, and indeed some CO_2-driven responses have been observed in mesocosm experiments (Riebesell *et al.* 2008; Riebesell *et al.* 2013), but this limits the scope of the study to one location. Ultimately, these results offer insight as to the nature of phytoplankton communities in a range of locations, but are limited in what they can offer about how they might respond to OA in the future.

Conclusions

There were few clear trends across the data set, and most of the observations were driven by community composition over anything else. Two photoacclimation strategies were identified in the Arctic Circle, with diatoms showing high F_v/F_m and low σ values, while dinoflagellates and coccolithophores had low F_v/F_m and high σ values, demonstrating the effectiveness of using FRR fluorometer readings to identify taxonomic signatures. No CO_2 effect was observed, but as laboratory studies have shown photophysiological sensitivity to OA, it is probably that incubation times were not long enough to induce these changes. Calcification rates from the coccolithophore bloom station in the Arctic Circle suggest that there is an optimum CO_2 range for calcifying, and that coccolithophore assemblages are flexible in their tolerances to CO_2 levels.

It is difficult to compare these results to the laboratory experiment results from Chapters 2 to 4 as most of the responses are driven by community, and coccolithophore-dominated stations were limited. Longer incubation times are likely required to see any CO_2-driven response, but would be difficult to put into practise given the high volume of material used in the bioassays. However, these results are a reminder that phytoplankton communities can differ

substantially between geographic locations, and as such, OA and climate change will not have a uniform effect on the global ocean.

Chapter 6: General Discussion

6.1. Main Findings

L:D cycle has an effect on the OA response in the coccolithophores tested, in particular on growth rates. It is unclear why continuous light would remove the CO_2 effect on growth, or why, at 14h light, CO_2 has a negative impact on growth. Continuous light changes the preferred carbon source in *E. huxleyi* (Rost *et al*. 2006), and it is possible that this had an impact on the way the isolates tested in this thesis reallocated resources in response to CO_2 under the different light regimes. Overall, L:D cycle is a more important variable than CO_2 in determining some physiological aspects of coccolithophores, particularly PIC $cell^{-1}$, and hence PIC:POC. Continuous light not only enhanced PIC $cell^{-1}$, but caused over-production of PIC to the point of coccolith shedding (though not in the tropical strains). This could be a photoprotective response, by either scattering light with the loose coccoliths (Tyrrell *et al*. 1999), or promoting aggregation of larger particles to sink out to darker waters (Chow *et al*. 2015), or as a result of changes in EP and/or TEP production. Additionally, this indicates a stronger link between calcification and photosynthesis under continuous light, and it is possible that calcification is being used as CCM to allow cells to access both bicarbonate and CO_2 under this light regime.

There was a distinct "temperate" versus "tropical" response observed across the isolates. L:D cycle changed the OA response of these organisms, with *E. huxleyi* 962 and *G. oceanica* showing greater sensitivity to CO_2 under 14h light. However, continuous light in general caused lower growth rates, PIC $cell^{-1}$, and PIC:POC, indicating this is not an optimum growth condition. Strain-specific responses to CO_2 have been observed (Langer et al. 2009), and these results support the idea that this phenotypic variability stems from their biogeographic history. As phytoplankton move away from the equator, they will encounter longer photoperiods, which could therefore provide some refuge from OA but at the cost of metabolic and calcification rates.

It should also be noted that the differences in growth temperature might have played large role in the OA response of these organisms (Sett *et al*. 2014).

Both continuous light and elevated CO_2 induce a "high light" acclimation response in coccolithophores, with two different strategies observed; conserving the PSII pool size ([RCII]) while varying τ and σ, or varying the PSII pool size while conserving σ and τ. Changes to photophysiology correspond with coccosphere thickness, especially in *G. oceanica* and *E. huxleyi* 962. Thicker coccospheres resulted in lower τ and higher σ values, denoting a "low light" acclimation response. In the other taxa, coccosphere thickness decreased with CO_2 under 14h of light, but this was not seen under continuous light. Therefore the coccosphere possibly has a photoprotective role, and any changes to calcification have the potential to lower coccolithophores' abilities to cope with changes in light availability.

These trends did not carry over into field work on natural assemblages of polar phytoplankton. This is mostly due to the low abundance of coccolithophores encountered, and methodological constraints. However, FRR fluorometry data was effective at identifying dominant taxonomic groups at each bioassay station, demonstrating the presence of taxonomic signatures in photophysiology.

6.2. Wider Implications

The notion that L:D cycle has an impact on the OA response in coccolithophores has two main implications. The first is the need to standardise, or at least consider, L:D cycle and other environmental variables across OA experiments. L:D cycle on its own can drive changes in phytoplankton physiology (Nielsen 1992; Foy and Gibson 1993; Nielsen 1997; Thompson 1999; Rost *et al.* 2006) (see also Chapter 2 and 3), thus comparing studies where organisms are grown under different light regimes is unfair. Debates that have arisen in the OA literature, notably following the publication of Iglesias-Rodriguez *et al*. (2008) and the subsequent response from

Riebsell *et al*. (2008), focus on asserting which results are correct, while the discussion should be about why the results are different. In the case of comparing that work to the Riebesell *et al*. (2000) paper, both clearly use different methods for controlling the carbonate system, as well as using different strains of coccolithophores. Subsequent studies on NZEH used longer photoperiods which, as already discussed in Chapter 2, affect the observed response to CO_2. This highlights the need to include the nature of other environmental variables when making comparisons and interpretations about other OA studies.

As discussed in Section 2.4, another issue with growing organisms under continuous light is that cells become unsynchronised (Müller *et al*. 2008). This has the potential to affect measurements by obscuring physiological responses in more noise, and must be considered when reporting weak or no feedback to CO_2 (or any other variable). Calcification is strongly linked to cell cycle, so anything that impacts growth rates has the potential to change the way coccolithophores calcify. It is unclear how cell cycle is altered by continuous light, though periods of darkness may be important in regulating calcification. Ultimately, the time of day a culture is sampled is no doubt very important, particularly when growing phytoplankton in L:D cycles, and should be reported with results. As PIC:POC ratios increased with longer photoperiods, it appears that calcification and photosynthesis also have a stronger link than previously thought. There is evidence for calcification having a role as both a CCM and acting as a form of photoprotection. The function of calcification in coccolithophores is still poorly understood, but resolving how much it is linked to photosynthesis can be vital in better comprehending why coccolithophores respond to CO_2 the way they do.

L:D cycle varies with latitude, and increasing temperatures that accompany elevated CO_2 also enhance stratification (Steinacher *et al.* 2010; Hofmann *et al.* 2011), creating longer effective photoperiods by not allowing phytoplankton to sink out of the euphotic zone. As the work here has established the importance of L:D cycle on the OA response, models that aim to

predict the nature of changes to coccolithophore populations in the global ocean need to take it into consideration. Since the tropical isolates responded so uniquely to CO_2 and L:D cycle, it is also very likely that OA will affect coccolithophores differentially depending on location. Importantly, phytoplankton are towards higher latitudes as temperatures become sub-optimal in their original location (Thomas *et al.* 2012; Winter *et al.* 2014), which means they will experience more variation in L:D cycle. It is therefore important to understand how coccolithophores from a range of locations will react to longer photoperiods in order to predict changes to food webs, primary productivity and biogeochemical cycles with climate change.

6.3. Future Work

In order to help contextualise the response of *E. huxleyi* to changes in photoperiod, more characterisation is needed of its daily cell cycle, and the role periods of darkness plays in controlling it. This could be achieved by running the same experiments, but sampling at regular intervals throughout the day to measure the diurnal fluctuations in PIC:POC, coccosphere thickness and cell size. Knowing how coccolithophore physiology is affected by time of day could help to standardise sampling methods, and provide explanations for the impacts of changing L:D cycle. Additionally, the carbon source (bicarbonate or CO_2) used by the isolates over the course of the experiments could also be recorded. This can be done using carbon isotope fractionation, described in detail in Tortell and Morel (2002). It would confirm whether or not changes in L:D cycle are changing the way *E. huxleyi* preferentially uptakes inorganic C from the media, and would provide insight as to whether it changes in response to CO_2 availability as well. In order to further investigate the link between photosynthesis and calcification, and whether or not calcification is used as a CCM under continuous light, naked strains of *E. huxleyi* could be used in future work. If there is a link, then it is possible that a CO_2 response might be observed under 24h light, or growth rates might not be maintained at such high rates, in these strains.

Since there were distinct responses based on biogeography among the coccolithophores tested here, more strains could be tested in the future to ascertain the link between CO_2 response and geographic origin, as suggested by Langer *et al*. (2009). Understanding how different strains respond to CO_2 can help further our understanding of why CO_2 affects coccolithophores the way it does. Recently, more studies on coccolithophores have focussed on understanding the genetic basis of how they respond to CO_2 (Lefebvre *et al.* 2010; Richier *et al.* 2010; Rokitta *et al.* 2012; Jones *et al.* 2013). Greater resolution of the genetic diversity in coccolithophore isolates would help in understanding why some populations respond similarly (or uniquely), as well as indicating where more gene flow occurs between populations. As such, genetic analysis might also be helpful, but physiological studies are necessary in order to understand the phenotypic variability present within coccolithophores.

Optimising the experimental set up is another avenue to explore. Semi-continuous cultures were used into order to keep careful control of the carbonate chemistry, but there were limitations to this method. Firstly, culture volume had to be kept low (1L) because of the nature of keeping cultures semi-continuously. It involved diluting six cultures every day, often replacing around half the media with freshly prepared seawater. If the culture vessels were any larger, it would have been unfeasible to prepare such big quantities of media. This was further limited by needing to keep cell densities relatively low, meaning sample volumes needed to be bigger to be able to detect things such as chlorophyll, POC and PIC. This is a limitation that is difficult to overcome, but if more manpower was available to make the artificial seawater in much larger batches, bigger culture vessels could be used.

Additionally, better control of the "light dose" could be used in future experiments. 300µmol photons over 14h versus 300µmol photons over 24h is subjecting organisms grown under continuous light to much more excitation energy over a photoperiod. Decreasing the light intensity so that cultures are receiving the same amount of light per photoperiod could tell us

more about their ability to cope with changing L:D cycles. In fact, better characterisation of how L:D cycle affects coccolithophore physiology in general would be beneficial. Comparing "long" photoperiods with "short" photoperiods has been done in a somewhat arbitrary fashion, and experimental analysis of where the threshold is for long and short (i.e. at what photoperiod length do we start observing different photoacclimation strategies) would make these comparisons more robust.

6.4. Concluding Remarks

L:D cycle has a controlling effect on the observed OA response in coccolithophores, and on coccolithophore physiology in general. These results indicate a stronger relationship between photosynthesis and calcification than previously thought, and calcification may act as a CCM under continuous light. Differences in L:D cycle can explain some (but not all) of the variation in the response of *E. huxleyi* reported within the OA literature, and certainly indicate that we need a better understanding of how other environmental factors interact with CO_2 to give a more insightful view of how climate change will affect coccolithophores. As the L:D cycle and CO_2 response differed between groups of biogeographically distinct isolates, it is likely that OA will have specific localised effects on coccolithophore populations, a key fact when predicting how they will respond on a global scale to climate change. The interaction between L:D cycle and CO_2 is of particular importance as tropical groups of phytoplankton migrate away from the equator. The tropical isolates lost the CO_2 response under continuous light, but growth and PIC:POC were overall lower, so while longer L:D cycles might offer some refuge, it comes at a metabolic cost. More data is needed to understand how and why L:D cycle affects the OA response, and coccolithophore physiology as a whole. This in turn will enable us to better predict the fate of this widespread and biogeochemically important group of phytoplankton as the global climate continues to change throughout the coming decades.

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