GROWTH, CALCIFICATION AND PHOTOSYNTHESIS IN THE COCCOLITHOPHORID *CHRYSOTILA CARTERAE*

BSc, Hon.

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

Jason P. Webb

2015

I declare that this thesis is my own account of my research and contains work which has not been previously submitted for a degree at any tertiary institution

Jason P. Webb

"Usually field work is not in conformity with the plan drawn up.

The experience gained is applied to modify the course of work"

Nils Gunnar Jerlov 1909 – 1990

Oceanographer, Author and leader in ocean optics

N. G. Jerlov (1951) Optical studies of ocean waters, Reports of the Swedish deep-sea expedition1947-1948 Vol III. Physics and Chemistry No 1 (pp 1-59)

ABSTRACT

The mass culture of microalgae for the commercial production of a) low value commodities such as biofuel and food and b) high value products such as polyunsaturated fatty acids, carotenoids, and nano-scaffolds is becoming increasingly attractive. Coccolithophorid algae have been investigated as potential candidates for both low and high value products. This thesis provides data on the specific nutrient and growth requirements in the coccolithophorid, *Chrysotila carterae* (previously *Pleurochrysis carterae*). Via the use of oxygen evolution techniques and PAM fluorometry, it is shown that *C. carterae* is just as susceptible to photoinhibition as some other microalgae with photoinhibition occurring at around 1100-1500 µmol photon m^2 s^{-1}. *C. carterae* also has the ability to recover from short periods of acidification, with recovery from pH 5 when there was no organic carbon assimilation to pH 9 after 20 minutes, Carbon assimilation increased from almost 0, to 3.01 pg C_{ORG} $cell^{-1}$ h^{-1} . This microalga has a fundamental requirement for selenium, with specific growth rates falling from a μ_{max} of 0.6 d^{-1}, with selenium to 0.1 d^{-1} in selenium-limited culture. Selenium is also required for coccolith production. In Se-limited culture coccolith production was almost reduced by half, from 70 $x10^5$ coccoliths mL^{-1} to 3.8 x 10^5 coccoliths mL^{-1}. Diurnal studies of organic and inorganic carbon assimilation showed that *C. carterae* CCMP647 synthesises coccoliths during the day, and then extrudes them onto the cell surface during the last hours of the dark cycle.

Investigations into the effect of various nitrogen sources indicated that with unregulated pH, nitrate achieved the greatest cell density and stable growth: The maximum cell densities reached were nitrate (66.61 x 10^4 ± 8.2 x 10^3 cells mL^{-1}) > urea (34.0 x 10^4 ± 6.2 x 10^3 cells mL^{-1}) = ammonium (36.08 x 10^4 ± 4.2 x 10^3 cells mL^{-1}). Nitrate had the greatest effect on the

culture medium ΔpH, (NO_3^- (0.134 ± 0.003) > urea (0.111 ± 0.003) > NH_4^+ (0.043 ± 0.001) and urea increased the growth rate of *C. carterae* by 150 % from 0.17 0.002 d^{-1} on NO_3^- to 0.44 ± 0.001 d^{-1} on urea. However, coccolith production increased with NO_3^- (73.81± 3.51 ng $CaCO_3$ $cell^{-1}$> NH_4^+ 55.18 ± 0.61 ng $CaCO_3$ $cell^{-1}$ > urea at 12.88 1.62 ng $CaCO_3$ $cell^{-1}$. Organic carbon (C_{ORG}) assimilation using NO_3^- far exceeded that on NH_4^+ and urea (C_{ORG} assimilated with NO_3^- = 7 $x10^3$ pg C_{ORG} $cell^{-1}$ h^{-1} vs Urea at 6 $x10^3$ pg C_{ORG} $cell^{-1}$ h^{-1} and NH_4^+ 5 $x10^3$ pg C_{ORG} $cell^{-1}$ h^{-1} . Inorganic carbon assimilation (C_{INORG}) was also elevated with NO_3^- producing 3 $x10^3$ pg C_{INORG} $cell^{-1}$ h^{-1} vs urea at 2 $x10^3$ pg C_{INORG} $cell^{-1}$ h^{-1} and NH_4^+ at 2 $x10^3$ pg C_{INORG} $cell^{-1}$ h^{-1}. Thus, nitrate provides long term, stable growth with the highest cell overall cell density under unregulated pH.

Under elevated medium pH, urea and ammonium had the highest rate of carbon assimilation far in excess of NO_3^- for both C_{ORG} (Urea 44921.73 ± 2191.08 pg C_{ORG} $cell^{-1}$ h^{-1} > NH_4^+ 22006.22 ± 640.39 pg C_{ORG} $cell^{-1}$ h^{-1} > NO_3^- 773.59 ± 14.8 pg C_{ORG} $cell^{-1}$ h^{-1}) and C_{INORG}, Urea 773.59 ± 14.8 pg C_{INORG} $cell^{-1}$ h^{-1} NO_3^- 569.44 ± 31.4 pg C_{INORG} $cell^{-1}$ h^{-1}. Although carbon assimilation rates were elevated under urea and NH_4^+ at higher pH levels, NO_3^- at pH 8 had the highest Calcifaction to photosynthsdis ratio (C:P) ratio of 0.158, while closely followed by urea at pH 9 (C:P = 0.150).

With enhanced carbon assimilation at pH levels exceeding the pKa of CO_2 in the medium pH indicated that this species must be using HCO_3^- as a carbon source, as cell growth and calcification were elevated at pH levels at which there is a greatly reduced level of CO_2 in the medium which is typically in air equilibrated water approximately 10 μmol L^{-1}.

ACKNOWLEDGMENTS

This Thesis is dedicated to the huge number of people that supported me through my PhD.

First to my principal Supervisor and mentor Professor Michael Borowitzka, words cannot express the gratitude for all your support and constant belief in me. The knowledge that I gained through your guidance is invaluable. Thank you so very much.

To my co-supervisor and very good friend Dr Navid Moheimani, Your constant support and guidance is what does have me through this work. Your enthusiasm and passion for microalgae have been a constant source of motivation and will continue to drive my own passion for microalgae.

My heartfelt gratitude to Dr Mike van Keulen. Thank you for your all your advice, and support throughout my studies.

To Ian McKernan and Mike Taylor, the greatest technicians in the world! Thank you for all your help with the construction the experimental apparatus.

To my very good friends Karen & Lindsay Marshal, your support and friendship has meant so much to me throughout my studies at Murdoch University. The many hours spent in "the spot" were a welcome oasis during the stressful experiential days. Thank you Maxine Dundas for your support and friendship.

I have to give a huge thank you to Sorcha Gillen, dive buddy, drinking buddy and all round awesome person. Your friendship and humour got me though the rough times. I should give a very special, thank to Mathew Lang, thanks mate for being there when needed.

To my partner Riona Park, who suffered though long diurnal experiments, and shared my pain during writing. Thank you for your constant support and for just being there.

Thank you to my awesome sister Lee-Anne Keats, who supported me when I needed it and was a constant source of motivation.

Finally a massive thank you for my parents David and Elaine Keats. Thank you for your unconditional support thought all of my studies. I would not have made if it were not for you helping me every step of the way.

ABBREVIATIONS

μ	Specific Growth rate
atm	atmospheres
C:P	Calcification to Photosynthesis ratio
CA	Carbonic Anhydrase
CA_{ext}	External carbonic anhydrase
CAP	Coccolith associated polysaccharides
Cc	Cells with coccoliths
CCM	Carbon concentrating mechanism
C_{INORG}	Inorganic Carbon Assimilated
Cn	Cells without coccoliths
C_{ORG}	Organic carbon assimilated
CV	Coccolith vesicle
DIN	Dissolved inorganic nitrogen
DMS	Dimethyl sulphide
DMSP	Dimethyl sulphoniopropionate
DON	Dissolved organic nitrogen
EB	Epoxy base
E_k	Minimum light saturation point
ETR	Electron transport Rate
ETR_{max}	Maximum electron Transport rate
Fm	Minimum fluorescence
Fo	Maximum fluorescence
Fv/Fm	Quantum yield of PS_{II}
Fq/Fm’	Effective quantum yield of PS_{II} in light adapted cells
ΔF/Fm	Effective quantum yield of PS_{II} . Replaced by Fq/Fm’
GSH-POD	glutathione peroxidise
LD	Light/Dark Cycle
MS	Magnetic stirrer
NPQ	Non photochemical quenching
PAM	Pulse Amplitude Modulated
PAR	Photosynthetic Active Radiation
PASW	Pacific Artificial Seawater
pCO_2	Partial Pressure of CO_2
PE	pH electrode
PS_{II}	Photosystem II
PS_I	Photosystem I
PSU	Photosynthetic unit
ETR	Electron transport rate
ETR_{max}	Maximum electron transport rate

rETR	Relative electron transport rate (light adapted)
$rETR_{max}$	Maximum electron transport rate (light adapted)
RLC	Rapid Light Curve
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RV	Reaction vessel
SB	Stir Bar
T_{ALK}	Total Alkalinity
TCO_2	Total carbon dioxide concentration
t_d	Doubling time
UALase	Urease amidolyase
WAIO	Western Australian Indian Ocean Seawater
α	The rate of photosynthesis
ΔpH	Rate of change in culture pH

Table of Contents

1 INTRODUCTION

The coccolithophorids are unique among the microalgae in that they have an outer covering of calcite plates (coccoliths). This ability to produce photosynthetically derived calcium carbonate means that the coccolithophorid have a major role in global ocean calcification and the global CO_2 budget (Rost & Riebesell 2004). Recently the coccolithophorids have been investigated as a mechanism of CO_2 sequestration and as a source of biologically derived fuel (biofuel) (Moheimani *et al.* 2012). While *Emiliania huxleyi* is the most dominant species of coccolithophorids in the ocean, with an ecological range from tropical oceans to polar waters (Winter *et al.* 1994), it is the coastal coccolithophorid *Chrysotila carterae* (CCMP647) that has presented as a good candidate for microalgae produced biofuel due to its high lipid content (Moheimani 2005; Moheimani & Borowitzka 2006; Moheimani *et al.* 2011; Moheimani *et al.* 2012). To date, only *Chrysotila* (ex *Pleurochrysis*) *carterae* CCMP637 has been successful cultured in large scale outdoors (Moheimani & Borowitzka 2006). As a result of this study, several physiological anomalies were observed which has formed the basis of this work.

The coccolithophorids role in the global carbon cycle as well as their potential for CO_2 bioremediation has been widely studied for the last 5 decades, (for reviews see (Paasche 1964; Buitenhuis *et al.* 1999; Paasche 2002; Rost & Riebesell 2004; Moheimani *et al.* 2012). While the main focus has been on *Emiliania huxleyi*, there has also been a significant amount of work on the coastal coccolithophorid, *Chrysotila carterae* (T. Braarud & E. Fagerland) Andersen, Kim, Tittley & Yoon) (Israel & Gonzales 1996; Okazaki *et al.* 1998; Moheimani

& Borowitzka 2006). This review is not intended to replicate the large body of exiting reviews on coccolithophorids, but will attempt to bring together the current knowledge on *Chrysotila carterae*, which by itself is a significant part of the global carbon system (Brownlee *et al.* 2004; Brownlee & Taylor 2004).

The Coccolithophyceae (or coccolithophorids) are a unique group of marine phytoplankton that can easily identified by their outer covering of calcium carbonate plates (coccoliths) (Figure 1) (Jordan & Chamberlain 1997; Kleijne *et al.* 2002). The coccoliths are often very intricate and complex resulting in fine, delicate structures that are uniquely identifiable to each species of coccolithophorid (Young & Westbroek 1991; Young & Henriksen 2003; Brownlee *et al.* 2004; Brownlee & Taylor 2004). Coccoliths were first noted by Ehrenberg (1836) in sedimentary rock, then by Thomas Huxley in 1868 in sediment samples from the North Atlantic, as Huxley thought that the small intricate plates resembled the cells of *Protococcus*, he named them coccoliths for convenience (Huxley 1868). It was not until Murray & Blackman (1897) reported the actual living cells that they were able to classify them as an alga. Murray & Blackman (1897) were also the first to comment on the global importance of the coccolithophorids, suggesting that they play a significant role in global geography.

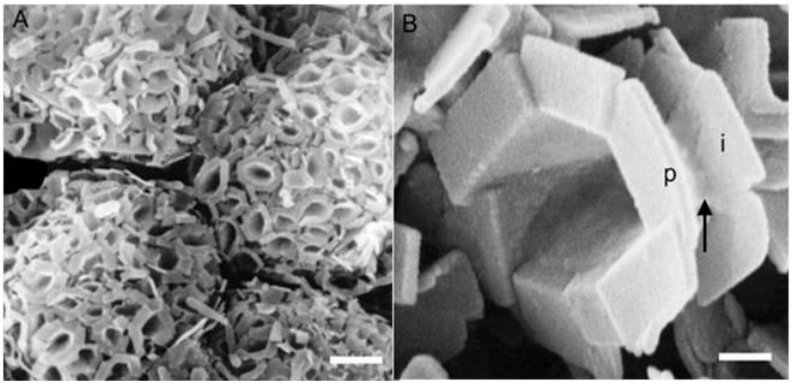

Figure 1 Scanning electron micrographs of *Chrysotila* coccoliths. (A) Three cells with intact coccospheres (Scale bar=1 µm). (B) Narrowing of coccolith crystals at the junction (arrow) of the proximal shield (p) and inner tube (i) similar elements seen by Marsh (1999) in *Chrysotila carterae*. (scale bar = 2 µm) (Hawkins *et al.* 2011).

Without question, *Emiliania huxleyi* (Lohman *et* May) is the most dominant of all coccolithophorids, spanning all of the world's oceans and seas (Brownlee *et al.* 2004). Due to its global dominance, and ability to lock away carbon as calcite (Jordan & Chamberlain 1997; Paasche 2002; Jordan 2012) this species has been the focus of many large scale research projects (i.e. the European Community MAST project EHUX (www.noc.soton.ac.uk). *Emiliania huxleyi* can form extensive blooms, almost continent size (approx. 100,000 km^2), with an average cell density of 10 x 10^3 cells mL^{-1} (Brown & Yoder 1994). It is this large scale representation in the oceans, coupled with the ability to carry out bioremediation CO_2 that makes the coccolithophorids, especially *E. huxleyi*, one of the most significant global producers of calcite (carbon sink) as well as the major contributor of atmospheric oxygen on the planet (Westbroek *et al.*, 2001), and thus has a significant impact on the global carbon cycle. *Chrysotila carterae* can also form extensive blooms (Casareto *et al.* 2009) as can *Chrysotila pseudoroscoffensis* (Reifel *et al.* 2001). *Chrysotila carterae* is

also of special interest as an alternative fuel source and as a mechanism for carbon bioremediation (Moheimani & Borowitzka 2006).

A single large *E. huxleyi* bloom can produce up to 7.2 x 10^4 tonnes of calcite (Holligan *et al.* 1983). This equates to almost 3.2 x 10^4 tonnes of fixed CO_2, considering that $CaCO_3$ contains 44% CO_2. van der Wal *et al* (1995) measured a calcite production of 150 pg calcite $cell^{-1}$ d^{-1} with a peak of 1170 mg calcite m^{-3} in the first 5 m of an *E. huxleyi* bloom. It is this huge amount of carbon that can be locked away from the system that has to have a significant role in any climate model (Pachauri & Reisinger 2007). To put this in perspective, the global CO_2 emissions from fossil fuels and cement production in 2010 were equal to almost 3.06 x 10^{10} tonnes (Friedlingstein *et al.* 2010). That means that a single *E. huxleyi* bloom can fix up to 0.001% of these global CO_2 emissions. The coccolithophorids have an abundant, widespread distribution and are found throughout the world's oceans and in some inland salt water lakes, such as the Salton Sea in North America (Winter *et al.* 1994). The greatest diversity of coccolithophorids occurs around the middle latitudes, however, unlike other phytoplankton groups, coccolithophorid species diversity is greatest in low nutrient waters, such as those around water bodies with limited circulation and oceanic gyres (Winter *et al.* 1994). Only a few species are found in polar waters such as *E. huxleyi*, *Gephyrocapsa caribbeanica G. oceanica* and *Pappomonas weddellensis* (Young & Westbroek 1991; Brownlee *et al.* 2004; Findlay *et al.* 2005).

Considering that the coccolithophorids, are able to fix both organic and inorganic carbon, they are of special interest to researchers on : a) investigating the dominant issues surrounding global carbon cycling (Westbroek *et al.* 1993; Rost *et al.* 2003; Rost & Riebesell 2004; Rokitta & Rost 2012); b) looking into amorphous minerals and crystalline structure

such of porous coccoliths, as potential for lightweight ceramics (Anning *et al.* 1996), catalyst supports and robust membranes for high-temperature separation technology (Walsh & Mann 1995), and more recently by investigating coccolithophorids growth as an alternative energy source and for CO_2 bioremediation (Moheimani and Borowitzka 2006). The increasing recognition of climate change, and the possible dangers of increased levels of atmospheric CO_2 (such as ocean acidification and weather changes) have led to an awareness of the role that calcifying organisms such as corals and coccolithophorids can play in moderating the total levels of planetary carbon, and balancing the global carbon budget. While climate change is a very serious issue, the bigger concern is that the increases in atmospheric CO_2 will cause the oceans to become acidic, with a widely accepted increase of $[CO_2]$ to 1000ppm by the year 2100, leading to drop of 0.74 pH units (Caldeira & Wickett 2003).

In general, calcification by corals (as aragonite) and other invertebrates such as the foraminifera (as calcite) results in a net increase in the partial pressure of atmospheric carbon dioxide (pCO_2) therefore decreasing the pH (Gattuso *et al.* 1999; Gattuso & Buddemeier 2000; Leclercq *et al.* 2000). The same pattern has also been observed in other coccolithophorids such as *E. huxleyi* and *G. oceanica.* Alternatively, calcification and photosynthesis by other genera of coccolithophorids results in a net decrease in pCO_2 and subsequent increase of culture medium pH. *C. carterae* will drive the pH up from an ambient seawater pH of 8.2, to a pH of 9 - 10.5 the during light cycle (Moheimani 2005; Moheimani & Borowitzka 2006; Moheimani *et al.* 2011).

1.1 Biology and Morphology

1.1.1 Taxonomy and Classification of *Chrysotila carterae*

1.1.1.1 Historical classification

Until recently there has been some debate over the classification of the coccolithophores. The effect of this was a cumbersome system within the Haptophyta based on no reasonable phylogenetic basis (Sym & Kawachi 2000). Until recently the coccolithophores were placed within Chrysophyta (Leadbeater 1971) until 1978 when, based on the work of Leadbeater (1971) and Gayral & Fresnel (1976), Tyge Christensen reclassified (1978) *Cricosphaera carterae*, and *Hymenomonas carterae* into the genus *Pleurochrysis,* which was the Genus originally established by Pringsheim in 1955 (Pringsheim 1955; Christensen 1978). In the last few months prior to publication of this Thesis, the 3 morphotypes of *Chrysotila* were once again reclassified into different Genus.

The first person to identify different life stages of *Chrysotila* was Leadbeater (1970), where, working with *Syracosphera carterae* (Von Stosch), he found that the protoplasmic structure of the coccolith bearing stage was identical to that of *Hymenomonas carterae.* He also noted that the *Apistonema* thallus had a covering of organic scales that was similar to those in *Hymenomonas*. Other researches also had believed that Von Stosch's strain (Syracoshera-Panzerflagelen) was similar to *Hymenomonas* (Jordan *et al.* 2004).

1.1.1.2 Phylogeny and Clade Differentiation.

Through molecular systematic studies on the coccolithophorids, a clearer picture is emerging on the classification of the Genus. Saez conducted several detailed investigations into the Pleurochrysidaceae (Sáez *et al.* 2003; Sáez *et al.* 2004; Sáez *et al.* 2008) and found that while there is generally agreement with the current taxonomic classifications, such as within the

strains of *Chrysotila haptonemofera (as Pleurochrysis roscoffensis)*, *C. pseudoroscoffensis* and *C. elongata* strains, there were some differences. Using 18S rDNA techniques Saez (2008) found that there is a quite significant genetic difference between two strains of *C. carterae* (the Von Stosch and HAP1 strains) which were placed into different clades. Sáez (2008) also found that *Hymenomonas coronata* was clearly not genetically similar to *Chrysotila*, even though it shares a very similar life cycle.

1.1.1.3 Current classification of Chrysotila Genus

Recently the Coccolithophorids have undergone wide scale reclassification, and based on small subunit (SSA) rRNA analysis conducted on material collected from the type locality, Andersen *et al.* (2014) re-classified the coccolithophorid Class. The major change was, based on morphology and SSU rRNA, the Genus "*Pleurochrysis*" was removed from the taxonomic record and placed in the older "*Chrysotila*" as described by Anand in (1937). However, keeping true to form, the story of coccolithophorid nomenclature continues to be troublesome and confusing. In the taxonomic authority paper by Andersen et al. (2014), there are errors regarding the epithets of *Chrysotila* (ex *Pleurochrysis)*. Andersen et al. (2014) have already acknowledged this error and have written a Research Note correcting the error (in press). The current classification level listed in Table 1.

Table 1 Current taxonomic classification of *Chrysotila* (ex *Pleurochrysis) carterae* (Andersen *et al*. 2014).

Empire	Eukaryota
Kingdom	Chromista
Phylum	Haptophyta
Class	Coccolithophyceae
Subclass	Prymnesiophycidae
Order	Coccolithales
Family	Pleurochrysidaceae
Genus	*Chrysotila*
Species	*catrerae*

1.1.2 Life Cycle

Three morphotypes of *Chrysotila* have been identified (Figure 2), the diploid heterococcolith stage, the haploid holococcolith stage, and the benthic stage (*Apistonema*) assigned by von Stosch (1955). The *Apistonema* stage was described as having a covering of organic scales (Leadbeater 1971; Gayral & Fresnel 1983; Billard 1994; Nöel *et al.* 2004). There is a wide diversity in the life cycle of the coccolithophorids. The lifecycles of the are yet to be reported, and as yet the only detailed information is on a few of the genera such as *Emiliania* and *Chrysotila* (Jordan 2012) and *Calyptrosphaera sphaeroidea* (Nöel *et al.* 2004).

The four identified life cycle stages of *Chrysotila* (Rayns 1962; Leadbeater 1970, 1971; Pienaar 1994) are:

Diploid motile coccolith-bearing stage (Rayns 1962).

Colonial non-motile coccolith bearing stage (Gayral & Fresnel 1983; Hawkins *et al.* 2011).

Benthic Apistonema stage (haploid) (Leadbeater 1970, 1971).

Benthic filamentous stage (haploid) (Gayral & Fresnel 1976, 1983).

The motile diploid (2n) coccolith-bearing stage generally reproduces by asexual cell division (Figure 3), however, the diploid coccolith-bearing *Chrysotila* also has a colonial stage, consisting of 2-4 cells with an outer covering of coccoliths (Hawkins *et al.* 2011).

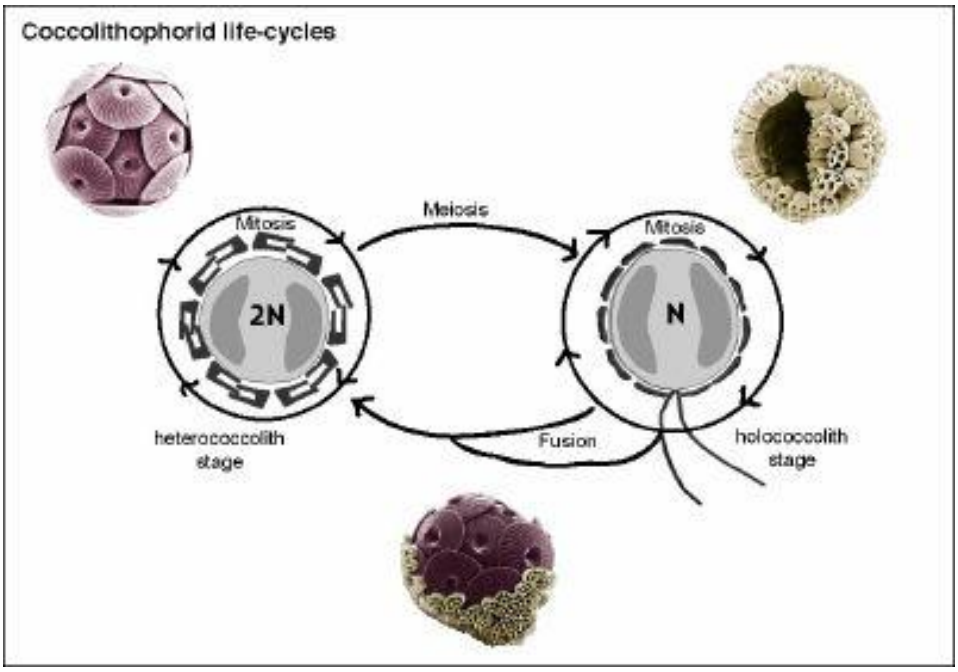

Figure 2 Alternation of generation in *Calyptrosphaera sphaeroidea*. holococcolith stage (n) and heterococcolith stage (2n) (after (Nöel *et al.* 2004).

The benthic haploid filamentous life stage of *Chrysotila* (Figure 4) has an outer covering that resembles a cell wall, however this cell wall is not calcified but made up of organic scales arranged to resemble a cell wall and are held together by a hemicellulose like compound (Pienaar 1994). The haploid, non-motile benthic stage (*Apistonema* stage) is often seen in cultures that have been in stationary phase for an extended period and have become nutrient deficient (Rayns 1962).

Most of the described coccolithophorids have a life cycle involving the alternation of generations (Figure 2, Figure 3, Figure 4), while the heteromorphic, isogametic life cycle in *Chrysotila pseudoroscoffensis* (Gayral & Fresnel 1983), and a *Hymenomonas* sp. are at present the only lifecycle described in any detail. Although the complete life cycle has not been fully described for the Genus *Chrysotila*, it can be assumed that it holds true for *C. carterae,* as the differences in the haploid and diploid scale covering have also been seen in *P. placolithoides* and *Hymenomonas lacuna* (Fresnel & Billard 1991; Billard 1994). Gayral & Fresnel (1983) also described the pseudofilamentous stage in *Chrysotila carterae* (Figure 3) (Fresnel 1994).

Billard (1994**)** proposed that the differences in haploid organic scale morphotypes are common and universal throughout the coccolithophorids. While detailed investigations into the life stages and triggers for morphological changes of *Chrysotila* are yet to made, it is hoped that work being conducted on the genome of the Haptophyta will provide a greater understanding of the complex life history of the Class (Katagiri *et al.* 2010; Hawkins *et al.* 2011). This work is vital to determine the intricate physiological process that is undertaken by the coccolithophorids.

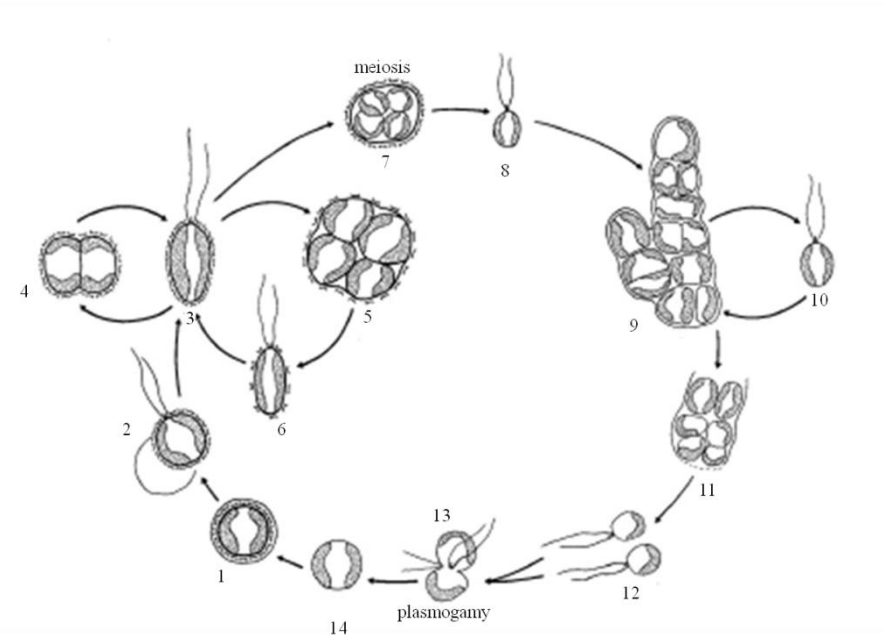

Figure 3 Life cycle of *Chrysotila (Hymenomonas) pseudoroscoffensis* 1, 2n zygote; 2. Release of zygote; 3. Motile coccolith bearing stage; 3-6. Various coccolith bearing stages; 7, meiosis producing colonial cells (up to four haploid cells with coccolith outer layer; 8. Meiospore; 9.pseudofillametous stage (haploid);, 10. Zoospore; 11, gamete formation; 12, motile gametes; 13, plasmogamy; 14, diploid zygote from karyogamy. (Gayral & Fresnel 1983).

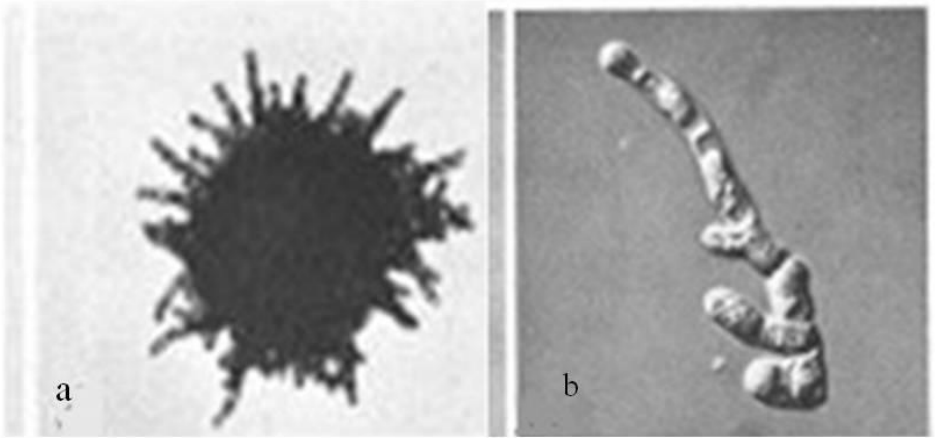

Figure 4 Image of *Chrysotila carterae* benthic filamentous stage at (a = 125x magnification b= 600 x magnification) (Gayral & Frensel 1983)

1.1.3 Colony Formation in *Chrysotila*

The mechanisms and reasons why *Chrysotila* forms colonies (Figure 4b) is not yet understood, however it may be linked to environmental conditions such as nutrient limitation or sexual reproduction (Hawkins *et al.* 2011). Pseudofilamentous colonies have been reported

in *Chrysotila pseudoroscoffensis* (Gayral & Fresnel 1983) and *Chrysotila placolithoides* (Fresnel & Billard 1991), however to date none have been reported in *Chrysotila carterae.* Some cells such the amoebae *Dictyosteium*, aggregate along highly chemical trails by secreting a chemo-attractant macromolecule (cAMP) when under nutrient stress (Kessin 2001)In these chains, there is a polarized organization of filamentous actin (F-actin) at one end of the pseudopods and myosin II at the back end. The same mechanism maybe occurring in *Chrysotila* cells as they have a strong polarity of F-actin (Hawkins *et al.* 2003; Hawkins *et al.* 2011). It is possible that F-actin is involved in the directional movements of *Chrysotila* colonial cells, although further study is required to determine any effect on motility.

1.2 Cell Ultrastructure

The flagella of *C. carterae* consists of 2 unequal length flagella (Figure 5) without any hairs (mastigonemes) or scales and is made up of two or three microtubular roots (Figure 6) (Beech & Wetherbee 1988). *Chrysotila* has a distinctive axosome, which lies proximal to the flagella insertion point, this distinctive feature has also been tentatively reported in other Genera of coccolithophorids such as *Cricosphaera (Chrysotila) roscoffensis* (Beech & Wetherbee 1988; Beech *et al.* 1988; Kawachi & Inouye 1995; Sym & Kawachi 2000)

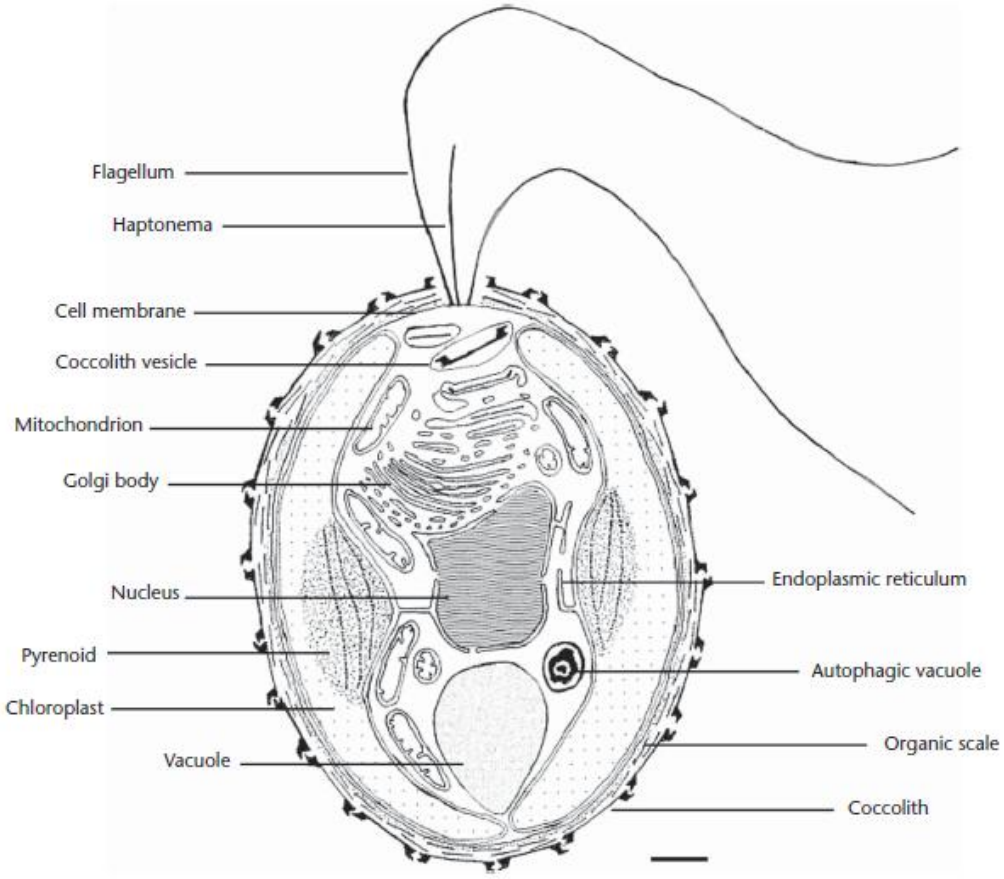

Figure 5 Gross cell structure of *C. carterae* cross section Scale bar = 1µm. Image from Pienaar 1994, after Westbroek *et al.* 1986

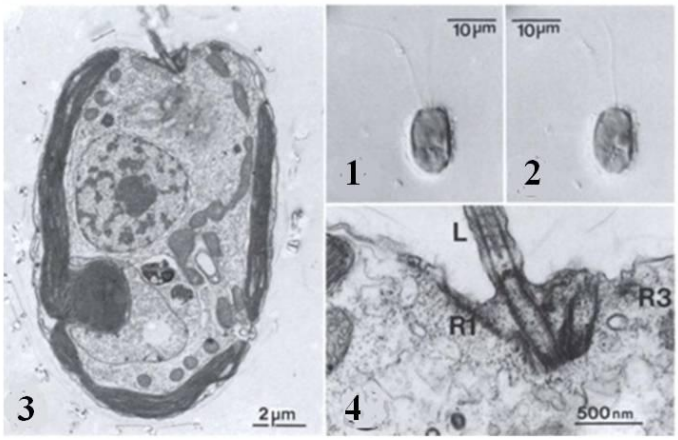

Figure 6 Light micrographs of *C. carterae* flagella (1) shows the longer flagellum while the shorter is shown in (2). TEM section of the same cell with (4) showing the flagellar root arrangement of the left flagella (Beech and Wetherbee 1988).

The haptonema in *Chrysotila carterae* quite distinctive, and can be seen easily with a microscope. There is wide variation in size and shape throughout the coccolithophorids. In *Chrysotila*, it is a rounded organelle approximately 2-4 μm long (Inouye & Pienaar 1985; Pienaar 1994) consisting of four microtubules in the base (Pienaar 1969, 1969; Gayral & Fresnel 1976; Pienaar 1994; Kawachi & Inouye 1995). While the function of the haptonema is still largely unknown, it is believed to be involved with heterotrophic feeding, or possibly a sensory organ (Pienaar & Norris 1979; Kawachi & Inouye 1995).

1.2.1 Coccoliths

Calcification in photosynthetic organisms is relatively rare, occurring mainly in the red algae (Rhodophyta) and few species of green (Chlorophyta) and brown (Heterokontophyta) algae (Borowitzka & Larkum 1976; Borowitzka 1989) as well as invertebrates. The coccolithophorids are unique, in that they have a mechanism of crystallizing the $CaCO_3$ internally through a highly controlled process within a coccolith vesicle (Figure 5), which is a part of the Golgi body (Young *et al.* 1999; Brownlee *et al.* 2004; Brownlee & Taylor 2004). In modern coccolithophorids, the way the coccoliths are attached to the cell surface can be classified into two broad types of coccoliths, heterococcoliths, that appear at different stages of the life cycle. Heterococcoliths appear during the diploid stage are typically formed by a series of complex calcite crystal units of variable shapes (Young *et al.* 1999; Paasche 2002). Holococcolithis are seen in the haploid stage, are as an association of numerous identical euhedral calcite scales (Billard 1994; Young *et al.* 1999; Young & Henriksen 2003, 2003; Frada *et al.* 2009). The coccoliths of *Chrysotila* are constructed of a rim of interlocking calcite scales called and R units (placoliths) (Figure 7). The placoliths of *C. carterae* have often been mistaken for cricoliths, however as Manton & Leedale (1969) pointed out, cricoliths are just placoliths with smaller crystal units. *Chrysotila* coccoliths have a ring of

interlocking calcite crystals; these sections are made-up of alternating V and R type crystal formations (Figure 8) (Marsh 1999; Young *et al.* 1999).

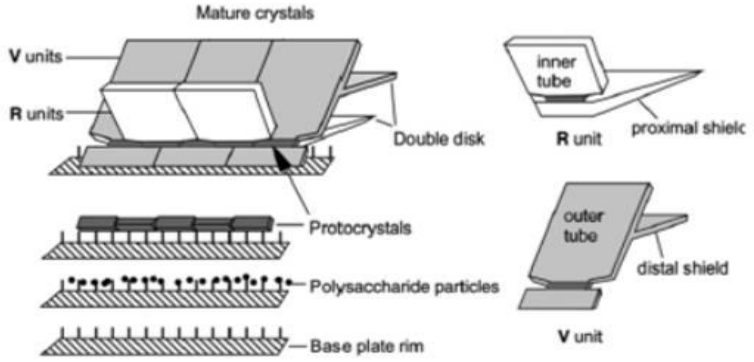

Figure 7 Development and assembly of R and V type coccolith crystals in *C. carterae (*Marsh 2003).

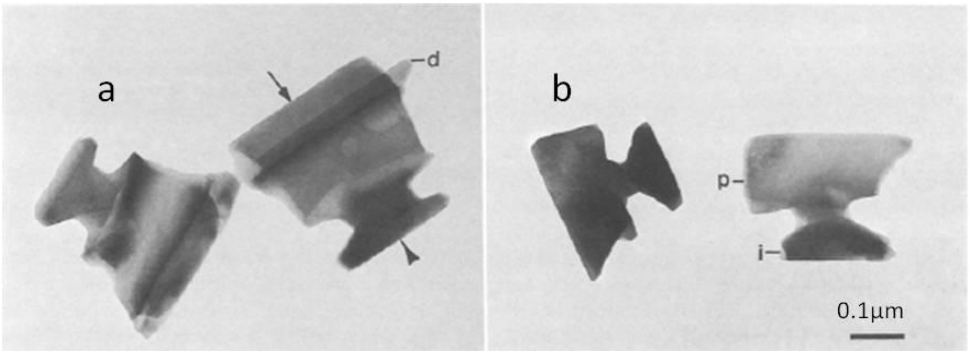

Figure 8 Electron micrograph of *C. carterae* coccolith units (a) V coccolith crystal unit. (b) R coccolith crystal unit (Marsh 1999).

1.2.2 Coccolith Formation

As shown in Figure 9, the outer cell covering of *C. carterae* consist of a layer of $CaCO_3$ plates (coccoliths) with multiple layers of non-mineralized scales underneath (Marsh 1999). For many years it was believed that calcification in the coccolithophorids was linked to light and photosynthesis (Van Der Wal *et al.* 1983). However, the work of Ariovich & Pienaar

(1979) was the first to show that calcification is independent of photosynthesis and this has since been confirmed by many other authors (Buitenhuis *et al.* 1999; Brownlee & Taylor 2004; Suggett *et al.* 2007; Tsuji *et al.* 2009). Formation of coccoliths occurs within the coccolith vesicle (CV) (Figure 10), which is part of the Golgi apparatus (Figure 11). Inside this vesicle the coccolith base plate is formed in a separate vesicle of electron dense microtubules (coccolithosomes) that have a high calcium concentration (at least 6M Ca^{2+}) (van der Wal *et al.* 1983). The vesicle containing the base plate then takes on the shape of the finished coccolith (Figure 10). The coccolithosomes then fuse with the base plate vesicle, where the coccolithosomes dissolve as the calcite rim begins to form (Pienaar 1969, 1971; van der Wal *et al.* 1983; Van Der Wal *et al.* 1983; Pienaar 1994). The complete coccoliths are then transported outside the cell via the coccolith vesicle (Figure 11), where they are attached to the cell surface, forming the coccosphere. Coccolith positioning on the cell surface in *Chrysotila* is believed to be a dense mat layer of columnar/tubular like material, located immediately external to the plasma membrane, (Manton & Leedale 1969; Leadbeater 1970; Pienaar 1994).

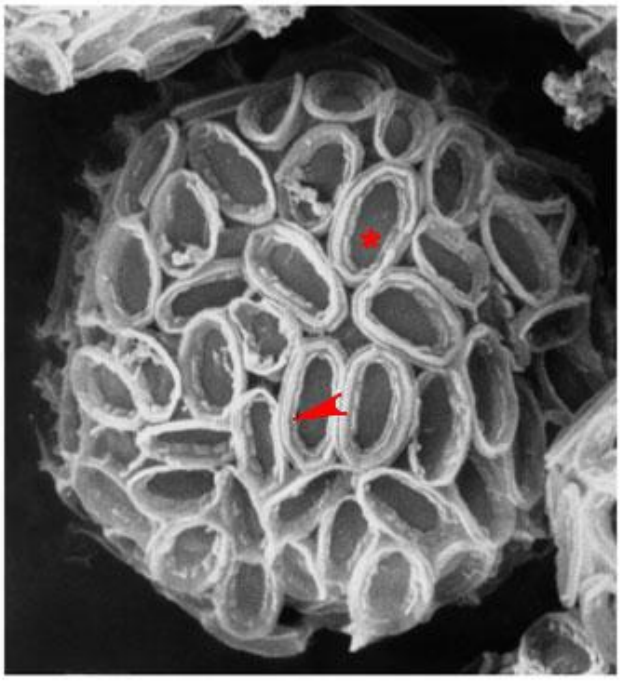

Figure 9 SEM showing coccoliths of *Chrysotila carterae* coccosphere. Arrows indicate individual calcite crystals around the distal ring, forming the coccoliths, (*) shows the organic base plate. Image source http://www.uth.tmc.edu/bmb/faculty/mary-marsh.html

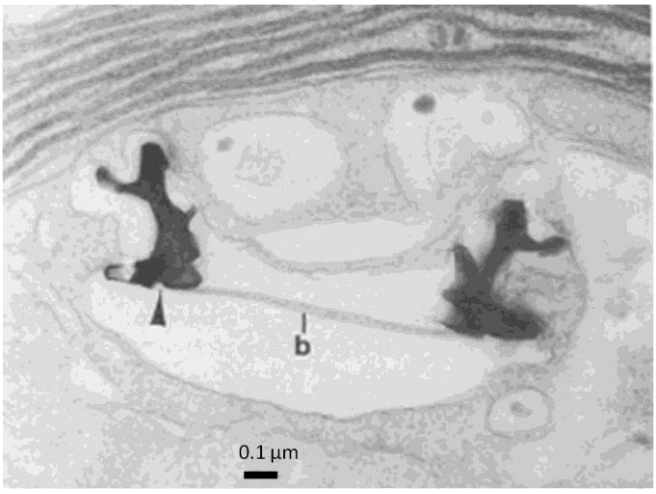

Figure 10 Electron micrograph cross sections of *C. carterae* coccolith vesicle, showing mature coccoliths (arrow) inside the coccolith vesicle (Marsh 1999).

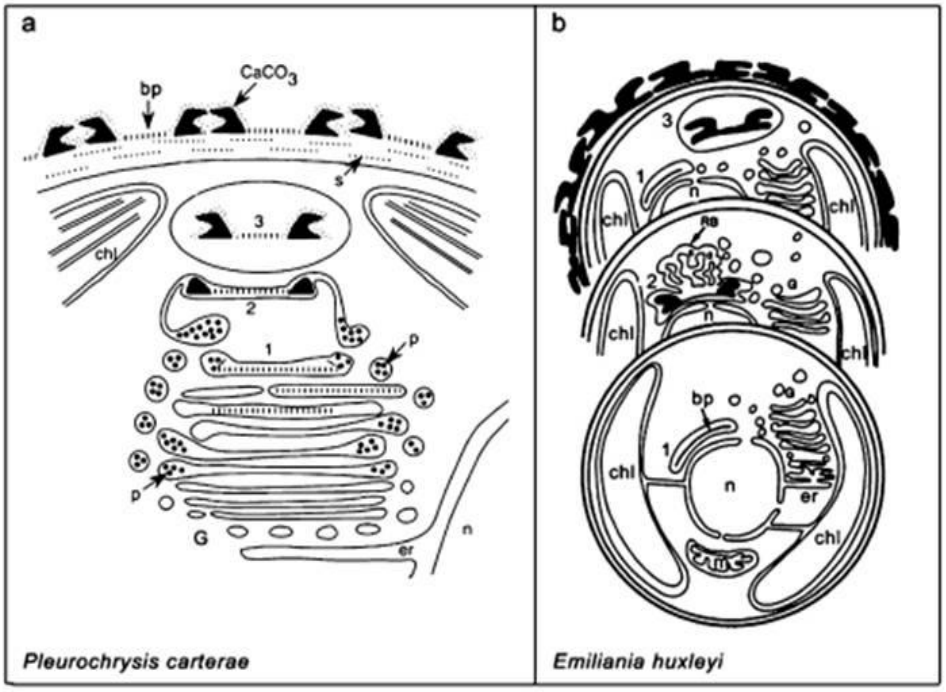

Figure 11 Schematic diagram contrasting the development of coccoliths in *Chrysotila carterae* (a) and *Emiliania huxleyi* (b) illustrating the sequential development of the coccolith,. Mineralizing vesicles at three stages. (1) Before onset of CaCO3 deposition, (2) During calcite growth, (3) after cessation of crystal growth.. (chl) chloroplast, (bp) base plate, (G) golgi body, (er) endoplasmic reticulum, (n) nucleus, (m) mitochondria, (s) unmineralized scales. From Marsh 2003 after (Marsh 1994; de Vrind-de Jong & deVrind 1997).

There are two main coccolith production types within the coccolithophorids, such as the distinction between *Emiliania huxleyi* and *Chrysotila* is, *E. huxleyi* does not have an organic base plate as a substrate of calcium deposition scale (Klaveness 1972, 1972), and *C. carterae* which has a well-defined organic base plate scale with an amorphous distal surface and no ornamentation; as well as a proximal surface with well-defined radiating fibrils. It is the organic base-plate that is the site of $CaCO_3$ deposition (Pienaar 1969) (1969b). As yet there is no real consensus as to why the coccolithophorids have developed this type of calcifying mechanism.

1.2.3 Control of Calcification and coccolith formation

The crystalline coat of *Chrysotila* coccoliths consists mostly of acidic polysaccharides (CAP). In *Chrysotila*, the acidic polysaccharides are essential in coccolith formation, and have been suggested to inhibit the calcification process *in vitro* and thus control the process (Borman *et al.* 1982; Ozaki *et al.* 2001; Hirokawa *et al.* 2005). These polysaccharides appear to be a common trait in the *Chrysotila* Genus as they have also been isolated from *Chrysotila haptonemofera* (Hirokawa *et al.* 2005) and two strains of *C. carterae* (CCMP645 and the LU strain). Coccolith formation in *C. carterae* is controlled by 3 types of acidic polysaccharides (Figure 12); coccolith associated polysaccharides (CAP) designated as PS_1, PS_2 and PS_3 (Marsh 2003). There is a great deal of variation in CAP types within the coccolithophorids. *Emiliania huxleyi* has only one type of CAP, a simple sugar based molecule with many polygalacturonic-acid side chains and ester-bound sulfate groups (Hirokawa *et al.* 2008)**.** The molecule is able to inhibit calcite crystallization and therefore crystal morphology by binding to the Ca^{2+} ions, and thus influence crystal morphology (Henriksen *et al.* 2004). In *Emiliania huxleyi,* a single complex galacturonomannan polysaccharide is involved in coccolith formation (Vliegenthart *et al.* 1981), although this CAP is similar to PS_3, is not incorporated into the calcite structure of the coccolith like those of *C. carterae,* rather is secreted over the mature scale (Marsh *et al.* 2002).

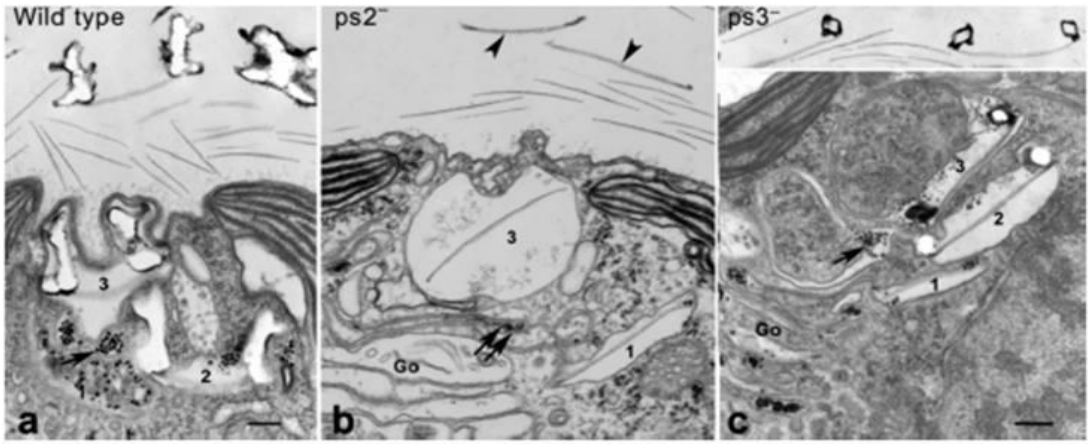

Figure 12 Cross section of *C. carterae* Golgi (Go) showing coccolith formation in (a) natural (wild type) with well-defined coccoliths, (b) a mutant strain that does not express PS_2 ($ps2^-$) and (c) a strain with chemically inhibited PS_3 ($ps3^-$) (Marsh 1994). Mineralizing vesicles labels as per fig 11. Wild type (a) coccoliths have the typical double disc shape, the $ps2^-$ cell has only unmineralized base plate scales (arrow heads on (b)) the $ps3^-$ cell only express protococcoliths (arrow head on (c) (Marsh 2003).

PS_1 has a 1:3 molar ratio of polyuronide with glucuronic and galacturonic acid and PS_2 composed of a 10:1 molar ratio of glucuronic and galacturonic acid, as well D-glucuronic acid and *meso*-tartaric acid and glyoxylic acids in equal ratios, for review see Bilan & Usov 2001. PS_1 and PS_2 are involved coccolith production via crystal nucleation and Ca^{2+} ion transportation (Bilan & Usov 2001; Marsh 2003). The third (PS_3) is an essential requirement for the determination of coccolith morphology. The significance of this CAP can be seen in cell that cannot produce PS_3 as the cells will show a lack of well-developed coccoliths, and only possessing a simple ring of rhombic crystallites (Marsh *et al.* 2002).

1.2.4 Acidic Polysaccharide Function

The CAP may also have a stabilizing effect on the $CaCO_3$, this may be seen in the lower solubility of the coccoliths reported in *Chrysotila* (Takahashi *et al.* 2002) and in cementing

the coccolith to the cell wall (Pienaar 1994). The reduced solubility of the coccolith was due to the CAP, however (Hirokawa *et al.* 2005) asserts that the CAP and calcium ions may cancel each other out, thus stabilizing the $CaCO_3$ in the coccolith.

1.2.5 Function of coccoliths

There have been many theories put forward over the years as to the function of the coccoliths; however, to date we still have no clear idea what purpose they serve. While there are very good reasons for biochemical functions, these hypothesis do not explain why the cell makes such precise and intricate designs. Some of the theories that have been proposed are:

a) *Defense against Grazing*

One of the more logical early assumptions is that the coccoliths provide a defense against grazing, by making the cells unpalatable. This was indicated by sustained growth rates of *E. huxleyi* in grazing trials under bloom conditions (Nejstgaard *et al.* 1997). However there is a great deal of evidence to suggest that the coccosphere does not provide any protection against grazing by zooplankton as even when grazed upon, the cells do not survive intact when passed though the zooplankton (Sikes & Wilbur 1982; Buitenhuis *et al.* 1999).

b) *Cell wall*

The use of the coccosphere to form the cell wall was first proposed by Sikes & Wilbur (1982) where they found the coccosphere prevented changes in cellular volume under variations in external salinity in *C. carterae.* However in the same study they found no evidence of this in *E. huxleyi,* a heterococcolith, with the coccosphere not forming part of the cell wall. The changes are suggested to be due to the way the coccoliths are produced, in *C. carterae* the organic scales are an integral part of the cell covering, and thus any changes to cell volume though osmotic process will also affect the overall cell size (Sikes & Wilbur 1982).

c) *Anti-Viral*

Another theory put forward is that the coccosphere can reduce the risk of viral infections. The work of Evans et al. (2006) on *E. huxleyi* (CCMP1516), has shown that during a viral attack (virus pathogen EhV strain EhV86) the coccoliths provided no real protection against infection. The assumption here is that the coccosphere provides a barrier against vial attack; however the coccosphere is no defense against even very large viruses such as the coccolithovirus Eh V-86 (Allen & Wilson 2006; Kegel et al. 2007; Allen et al. 2008).

d) *Maintaining Position in water column*

Buoyancy control is another common theory, suggesting that the coccoliths allow the cell to sink into the deeper, higher nutrient waters (Eppley *et al.* 1967). The high lipid content of the coccolithophorids may support this theory, The high lipid content of the coccolithophorids may support this theory, work on the effect of nitrogen limitation on the cell size of Emiliania huxleyi has indicated that under N limitation, the cells would have an increased sinking rate which was linked to changed in cell size, coccosphere thickness and lipid contend, which are can all be influenced by changes in nitrogen concentration (Pantoro et al. 2013) The high lipid content of the coccolithophorids may support this theory, work on the effect of nitrogen limitation on the cell size of Emiliania huxleyi has indicated that under N limitation, the cells would have an increased sinking rate which was linked to changed in cell size, coccosphere thickness and lipid contend, which are can all be influenced by changes in nitrogen concentration (Pantoro et al. 2013). The more likely scenario is that species such as *E. huxleyi* are able to adjust their buoyancy by shedding coccoliths as required. However, it is not possible for *Chrysotila* to shed coccolith so this mechanism of buoyancy control may not

apply to the coccolithophorids as a group. Some of the more ornate species, such as *Gephyrocapsa ornata* (Figure 13a) and *Papposphaera spp.* (Figure 13c) may be able to reduce their sinking rate by increasing their surface area via the elongated coccoliths. *E. huxleyi* has a higher sinking rate during periods of maximum light and nutrient stress and actually will increase coccolith production in response to nutrient limitation and this would dramatically increase the sinking velocity (Linschooten *et al.* 1991).

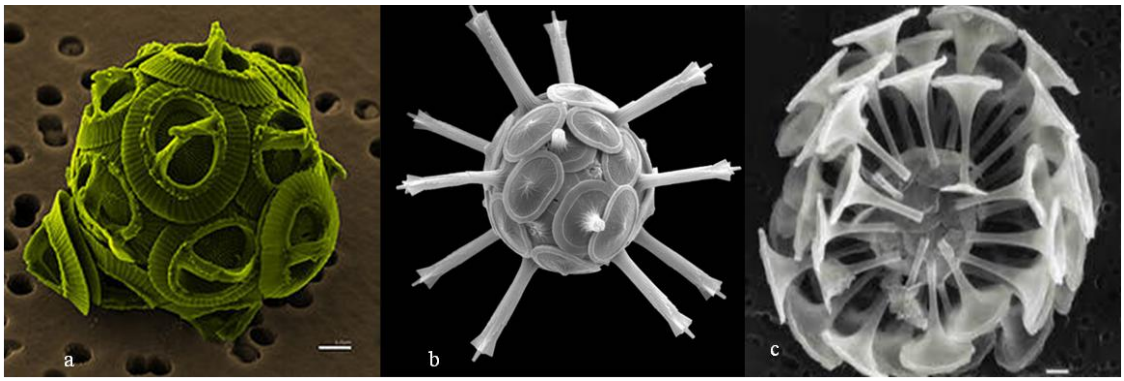

Figure 13 Variation in coccolith diversity. (a) *Gephyrocapsa ornata*, b (c), *Papposphaera spp.* The elongated coccoliths may reduce sinking velocity by increasing surface area. Image source: http://www.uth.tmc.edu/bmb/faculty/mary-marsh.html

e) *Photo-protective function*

Lecourt *et al.* (1996) found that naked strains of *E. huxleyi* had a faster growth rate under saturating irradiance than calcified cells of the same isolate, indicating a possible photo-protective function. It has been suggested that the light scattering effect of the coccoliths may act as a light concentrating mechanism, allowing the cells to survive in deeper water, or disperse light under high irradiance conditions (Paasche 1999). However, the role of the coccosphere in protection against high light was disproved by Nanninga & Tyrrell (1996), where they found that decalcified cells and naked strains cells o*f E. huxleyi* still showed resistance to photoinhibition.

f) *Access to CO_2 from Bicarbonate*

The current leading theory is the "trash can function" theory proposed by Rost & Riebesell (2004) based on the works of (Paasche 1964; Sikes *et al.* 1980; Linschooten *et al.* 1991; Paasche & Brubak 1994; Young *et al.* 1994; Paasche 1999, 2002). Here they suggest that the precipitation of $CaCO_3$ is a mechanism to access HCO_3^- as a source of carbon for photosynthesis. This will also assist in photosynthesis as during the calcification process; One CO_2 molecule is released and made available for photochemistry (Eq. 1) (Zeebe & Wolf-Gladrow 2005).

$$2HCO_3^- + Ca^{2-} \rightarrow CO_2 + CaCO_3 + H_2O \qquad \textbf{Eq. 1}$$

Another advantage is that the coccoliths of *E. huxleyi* appear to provide the cell with a tolerance to lower salinity (Sikes & Wilbur 1982), where calcified cell were able to tolerate much lowers salinities than decalcified cells.

1.3 Ecophysiology

1.3.1 General

The bulk of all coccolithophorid species can be found in stratifies waters ranging from temperate to tropical. A few species including *E. huxleyi* are found in the sub polar regions and polar regions of both the Antarctic and Arctic.

The majority of open ocean sediments are calcareous, and up to 80% of all carbon sequestered in these sediments is in the form of $CaCO_3$. Broecker & Peng (1982) have estimated that the coccolithophorids contribute 20-40% of the sedimentary $CaCO_3$.

1.3.2 DMS Production

Dimethylsulfoniopropinate (DMSP) is significant biogenerated, volatile compound usually produced by marine phytoplankton (Andreae & Barnard 1984). The production of DMS by some species of coccolithophorids (i.e. *Emiliania huxleyi, C. carterae*) is via the splitting of dimethylsulphide (DMS) by the enzyme DMSP-lyase. When DMSP is released into seawater it is immediately converted to the volatile dimethylsulphide (DMS) and acetate. DMSP and the subsequent conversion to DMS within the cell is of global significance. DMS evaporates, and is oxidized to form SO_2 and sulfate aerosols. The sulfate aerosols are a key nucleation source for cloud condensation (Charlson *et al.* 1987; Westbroek *et al.* 1993).

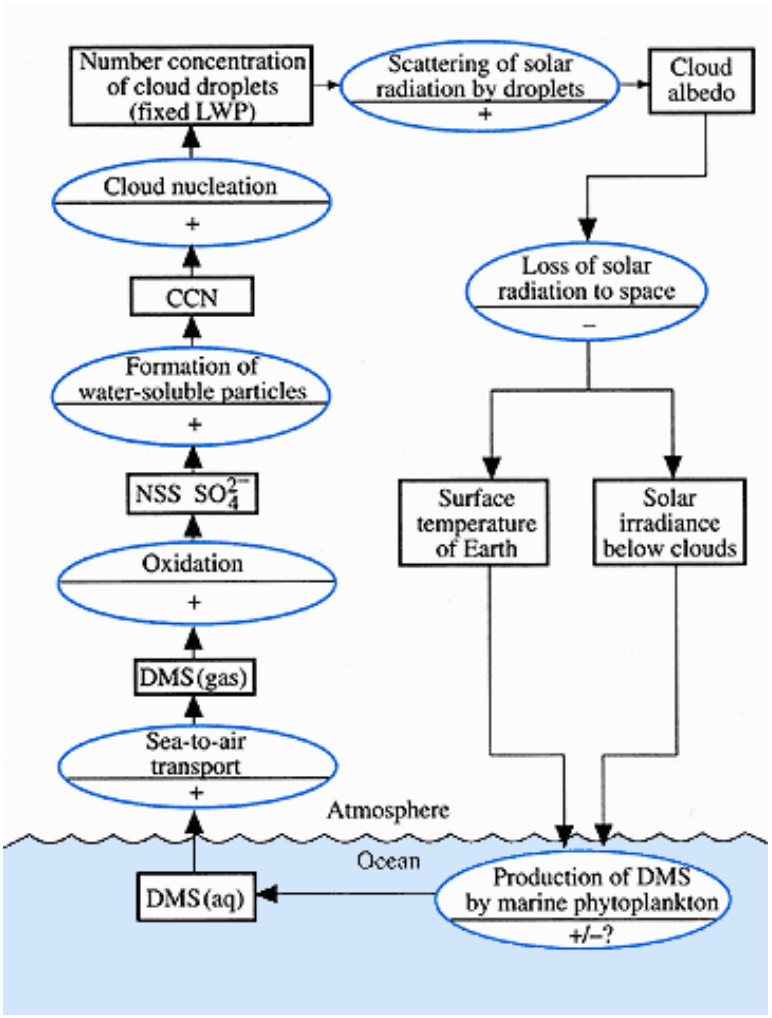

Figure 14 Schematic of hypothesized cloud nucleation though DMS production by marine phytoplankton (Charlson *et al.* 1987).

DMS has been shown to be affective against several types of protist grazers, such as ciliates, heterotrophic dinoflagellates and as deterrent against zooplankton (Strom *et al.* 2003). Evidence of this can be seen in long term outdoor cultures of *C. carterae* (CCMP647), that while being open to the elements, only very minor contamination of the culture was observed (Moheimani 2005; Moheimani & Borowitzka 2006).

The main role of DMSP within the cell is to function as a mechanism for osmoregulation; in *C. carterae* cultures, Vairavamurthy *et al.* (1985) found a 4 fold increase in DMS production

at higher salinity (up to 60‰), and sulfate derived from DMSP has been shown to increase the growth rate of *C. carterae* in culture (Vairavamurthy *et al.* 1985).

It can be inferred that marine phytoplankton and the coccolithophorids in particular, are able to affect the Earth's climate by increasing cloud condensation, and thus increasing the albedo of the Earth. This hypothesis may not be quite correct. Through recent multidisciplinary work on how the marine boundary layer and cloud compensating nuclei interacts with the atmosphere, it has been suggested that there are multiple sources of cloud condensation nuclei, such as salt spray generated by wind and wave action, as well as other biogenerated particles; DMS derived sulfur from marine phytoplankton has been shown to have no significant effect on cloud nucleation. The aerosols derived via inorganic means such as salt spray (Clarke *et al.* 2006) and DMS bound by exopolymer gels which are capable of reaching the upper atmosphere intact, and thus acting as potential cloud nuclei (Decho 1990), have a far greater effect on cloud nucleation (Quinn & Bates 2011) than DMS produced by phytoplankton. Although the impact of marine phytoplankton generated DMS may not play a significant role in cloud nucleation as once thought, there may be an exception;

As *C. carterae* is being investigated as a major candidate for CO_2 bioremediation and potentially as a source of biofuel, a commercial plant will most likely be in excess of 400 h (Chisti 2007). Based on a conservative 12 pg DMS cell released by *C. carterae* (Keller *et al.* 1989) an algal production plant of this size would produce approximately 45 tonnes of DMSP ha $year^{-1}$. The release of this much DMS into the atmosphere from an algal plant built close the coast, may cause an increase in cloud nucleation, and, depending on the average prevailing winds, increase rainfall inland of the plant.

1.4 Growth

1.4.1 Irradiance Effects

Most members of the Haptophyte algae become severely photoinhibited under high light conditions such as irradiances above 750 µmol photons m^{-2} s^{-1} (Grima *et al.* 1996). However some the coccolithophorids are unique, in that they show a high resistance to irradiancies above 800 µmol photons m^{-2} $s^{-1.}$ It is widely believed that *E. huxleyi* has a very high tolerance to photoinhibition; it is not uncommon for *E. huxleyi* to show no signs of photoinhibition at irradiances of approx 2000 µmol photons m^2 s^{-1} (Nimer & Merrett 1993; Nanninga & Tyrrell 1996; Paasche 1999, 2002). Israel & Gonzales (1996) also found no evidence of photoinhibition in *C. carterae* (CCMP299) where at 1200 µmol photons m^2 s^{-1} the net photosynthetic rate remained relatively constant from approximately 600 µmol photons m^2 s^{-1} (85-70 nmol O_2 10^6 $cells^{-1}$ h^{-1}) for both high and low calcifying cells respectively. This does not mean that coccolithophorids are totally resistant to high light. It has been reported that *C. carterae* is very oxygen sensitive, showing signs of oxygen toxicity as irradiance (and thus photorespiration) increases (Israel & Gonzales 1996; Moheimani & Borowitzka 2007). While high oxygen toxicity in *C. carterae* has been reported, Moheimani & Borowitzka (2007) found that when grown outdoors in unregulated raceway ponds, *C. carterae* would adapt and acclimatize to the increased light in as little as 4 weeks.

1.4.2 Trace Nutrients

Chrysotila, like most coccolithophorids, is not a fully autotrophic cell, and has significant requirements for exogenous nutrient supplementation, such as trace metals and vitamin B (Miyamoto *et al.* 2001; Miyamoto *et al.* 2002; Nöel *et al.* 2004) and SeO_2 (Doblin *et al.* 1999).

1.4.3 Selenium

Selenium is a base requirement for many phytoplankton groups (Price *et al.* 1987) and is also an essential trace nutrient for *Chrysotila* which has a very effective Se concentrating mechanism (Obata & Shiraiwa 2004). Selenium is used in the manufacture of selenoproteins. There are several selenium species, however selenite (SeO_3^{2-}) and selenate (SeO_2^{4-}) are the most common occurring equally in the oceans with a molar ratio of 1:1 (Cutter & Bruland 1984). Both forms of Se are accumulated by marine phytoplankton, however selenite is the preferred species as it is much easier to transport into the cell (Price *et al.* 1987). For most Haptophyte algae, selenite is 1000 times more toxic than selenate (Danbara & Shiraiwa 1999), indicating a selenite specific requirement and transport system. In *E. huxleyi*, the uptake of selenite has been reported to be via both a passive and active transport system (Obata *et al.* 2004; Araie *et al.* 2011), and is actively absorbed depending on the H^+ concentration gradient and co-transported with the H^+ via a P-type H^+ -ATPase (Araie *et al.* 2011). Obata *et al.* (2004) reports 3 processes for Se uptake in *Emiliania huxleyi*;

(i) Uptake of selenite via active transport of selenite.

(ii) Immediate fixation and accumulation of Se though the synthesis of as yet undescribed Low Molecular Mass Compounds (LMC).

(iii) Synthesis of selenoproteins from the LMCs.

Obata *et al.* (2004) also found that as accumulation of Se was inhibited by aminooxyacetic acid (AOA), L-Methionine sulfoximine and cycloheximide, that steps (i) and (ii) must be rate limiting.

1.4.3.1 Magnesium

Magnesium has been shown to be a required trace element for all photosynthetic organisims as it is a requirement for chlorophyll production (Ra & Kitagawa 2007). It is also a required micronutrient for coccolith formation in *E. huxleyi*; however, coccolith formation is highly dependent on the Mg^{2+} concentration. Below natural seawater Mg^{2+} concentrations, *E. huxleyi* coccoliths become malformed, however above ambient Mg^{2+} concentrations (29mM $MgCl_2$) there is under-calcification (Herfort *et al.* 2004). Increasing the Mg^{2+} concentration did not affect the composition of the coccolith acidic polysaccharides under normal Ca^{2+} levels and Mg^{2+}/Ca^{2+} of 1:02; however a high Mg^{2+}/Ca^{2+} ratio (1:2.4) does have an effect of coccolith morphology. The high Mg^{2+}/Ca^{2+} has been suggested to repress the synthesis of the PS_2 CAP in *P. haptonemofera* (Katagiri *et al.* 2010).

1.4.3.2 Manganese

Manganese is an unusual metal in that it can exist over a wide range of oxidation states, and is an essential component of the water oxidation complex (Raven *et al.* 1999). As such manganese plays major role in the oxidation states of Photosystem 2 (PS_{II}) (Falkowski & Raven 2007). Brand (1991) showed that *Hymenomonas* (*Chrysotila*) *carterae* growth rate was limited by low Mn concentration (10^{-10}M Mg), growth rates increased with manganese (Mn) concentration, indicating that *Chrysotila* has a significant growth requirement for manganese. This was the only coccolithophorid tested by Brand (1991) that suffered limited growth (50% from baseline growth). The effects were not observed in *E. huxleyi* or other coccolithophores (*Gephyrocapsa oceanica, Cyclococolithina leptopora, Umbilicosphaera sibogae U. hulburtiania*). This may indicate the *C. carterae* has a much higher Mn requirement for photosynthesis than pelagic coccolithophorids.

1.4.3.3 Iron

Iron is considered to be one the 3 main limiting elements in the ocean, along with phosphate and nitrogen (Lenton & Watson 2000). The two main coccolithophorids that have been heavily investigated show a very different requirement for iron. *Emiliania huxleyi* has been shown to have an increase in growth only at high iron concentrations, (in excess of 10^{-7} M when cultured with 10^{-10} phosphate), and had a sustained growth rate within 17% of optimal growth. This was a pattern consistent with other genera of coccolithophores tested by Brand (1991). The only exception was *Hymenomonas* (*Chrysotila*) *carterae*, where at low Fe concentrations, the growth rate of *H. carterae* was reduced with an 80% reduction in daily cell divisions from the optimum (Brand 1991). Muggli & Harrison (1996) in their strain of *E. huxleyi*, isolated from the subarctic Pacific, saw a reduction in cell volume at low (0.8nM Fe) and iron stressed (<0.5nM Fe) conditions in cultures using both nitrate and ammonium as the N source, although cell cultured with ammonium were able to tolerate the extremely low Fe concentration better than cell on nitrate.

1.4.4 Nitrogen

The ultimate fate of all nitrogen species taken up by cells is to be converted to ammonia for protein synthesis (Turner 1979; Young & Beardall 2003). In general, for phytoplankton the preferred species of nitrogen uptake is NH_4^+ ($NH_3 + H^+$) > urea > NO_3^-. The preference for ammonium as a nitrogen source is well documented (Flynn 1991, 2002) as is the ability of ammonium to inhibit nitrate uptake in phytoplankton (Eppley et al. 1969). The preference of *E. huxleyi* for NH_4^+ and to some extent urea over NO_3^-, is well documented (Fernandez *et al.* 1993; Kristiansen *et al.* 1994; Head *et al.* 1998; Rees *et al.* 2002), however, information on urea preference, or the use of amino acids on *C. carterae* is scarce. A good search of the

literature finds very little works on other genus of coccolithophorids, such as *C. carterae.* This makes it difficult to assume that all of the group will have similar metabolic systems. For example, there is evidence to suggest that some Haptophytes, such as *C. carterae,* have a method of amino acid uptake that occurs extracellularly (Palenik & Morel 1990). This method involves surface oxidative deaminase which converts L-amino acids into $NH_4^+ + H_2O$ and α-keto acid and H_2O_2. The NH_4^+ released by this reaction is then assimilated into the cell. It was suggested that this mechanism may be a means of utilising nitrogen in primary amines, without the need to synthesise multiple enzymes to take in the different classes of amino acids (Palenik *et al.* 1989; Palenik & Morel 1990; Antia *et al.* 1991; Palenik & Morel 1991). Strom & Bright (2009) found that in some strains of *E. huxleyi,* there was a preference for urea over ammonium. Nitrogen is one of the most important nutrients for algal growth, second only to CO_2. Nitrogen is usually the most limiting nutrient in marine systems with the exception of iron in some systems (Falkowski 1997) and phosporus such as in the Meditierranian Gyre (Krom *et al.* 1991). When NH_4^+ is the sole Nitrogen source, or in large enough concentrations as to inhibit NO_3^- uptake, there is a much lower energy demand on the cell, when this is coupled with low pH (and thus increased pCO_2), we should see an increase in the rate of photosynthetic carbon uptake and calcification.

The uptake of ammonium will cause the pH of the medium to drop due to the production of protons when NH_4^+ is converted to NH_3. (Eq. 2); if the nitrogen species is NH_3, then uptake will not result in any pH shift. The NH_3^-/NH_4^+ ratio is pH dependent.

$$NH_{4(aq)}^{+} \leftrightarrow NH_{3(aq)} + H^{+} \qquad \textbf{Eq. 2}$$

As growth rates of *E. huxleyi* are often faster when algae are cultured with urea or NH_4^+ (Solomon & Glibert 2008; Strom & Bright 2009), it may appear that this is the more efficient N source, however, the side effects of NH_4^+ use in mass culture can have negative consequences, such as acidification of the culture medium (Eq. 2, Eq. 14), which may result in loss of the culture (Borowitzka & Borowitzka 1990; Borowitzka 1994; Borowitzka 1999).

1.4.5 Urea

Urea is a significant source of regenerated nitrogen for marine phytoplankton, providing up to 50% of total nitrogen uptake in some ecosystems (Varela & Harrison 1999) and is a significant source of nitrogen in coastal and estuarine systems (McCarthy *et al.* 1977; Glibert *et al.* 1991; Glibert *et al.* 2006). It has been suggested that not to include urea in any estimates of the f – ratio (pNO_3^-/Total N uptake) may result in an overestimation of up to 95% depending on seasonal and weather conditions (Tremblay *et al.* 2000; Rees *et al.* 2002). In blooms of *E. huxleyi*, regenerated nitrogen sources such as urea, have been shown to be a major source of N for the alga, providing up to 87% of the total nitrogen requirement (Rees *et al.* 2002). To utilize urea, the cell must convert it to NH_3/NH_4^+, by either a membrane transport system, or *in vitro* hydrolysis via urease (Flynn 1991; Page *et al.* 1999) or by hydrolysing urea via an external urease (Syrett & Bekheet 1977; Rees & Syrett 1979).

The hydrolysis of urea results in a two-fold acidification process, with protons being produced, as well as CO_2 (Eq. 14)

$$(NH_2)_2\,CO_2 + H_2O \leftrightarrow 2NH_3 + H^+ + CO_2 \qquad \text{Eq. 3}$$

Urea metabolism in microalgae occurs though the action of one or both enzyme systems;

Urease, which catalyses –via Eq. 14, or though the action of ATP: Urease amidolyase (UALase) Eq. 4, which catalyses the ATP dependent degradation of urea to NH_3 and CO_3 though the actions of urea carboxylase (urea: CO_2-ligase (ADP) which catalyses:

$$Urea + ATP + HCO_3^- \xrightarrow{Mg^{2+}\ K^+} Allophanate + ADP + P_i \qquad \text{Eq. 4}$$

And (c); allophanate hydrolase which catalyses the reaction in Eq. 5 (Stewart 1980) :

$$Allophanate + 3H_2O + H^+ \rightarrow 2NH_4^+ + 2HCO_3^- \qquad \text{Eq. 5}$$

While urea is quickly hydrolysed to NH_3/NH_4^+ by urease, some groups of microalgae still retain the ability to actively take up urea. While there is limited work on the specific urea uptake mechanism of coccolithophorids gene isolation in *E. huxleyi* (Solomon *et al.* 2010) has revealed that *E. huxleyi* is able to express several genes for urea uptake. *DUR3*, which is present in most eukaryotic cells, encodes a high affinity urea transporter. Under high urea concentrations, a second urea transporter gene (*SLC14A*) may be of greater significance (Solomon *et al.* 2010).

The CO_2 increase generated from the conversation of urea to NH_3 is amplified by the additional of CO_2 evolved during dark respiration, thus increasing the rate of acidification. This may be an issue in mass algal cultures, where acidification may lead to the culture crashing.

There is mounting evidence to suggest that both hetero- and autotrophic phytoplankton compete for Dissolved Inorganic Nitrogen (DIN) and Dissolved Organic Nitrogen (DON) (Berman & Bronk 2003; Bronk *et al.* 2007; Bruhn *et al.* 2010). *E. huxleyi* has been shown to

be able to use both forms of nitrogen, so the ability of coccolithophorids to utilise DIN and regenerated DON has been suggested as one of the reasons for this group's success (Rees *et al.* 2002) and it may be one of the reasons that the coccolithophorids are able to be so successful in low nitrogen waters (Rees *et al.* 2002; Lessard *et al.* 2005). However, the ability of *E. huxleyi* to use the various forms of DON is very strain specific, as not all strains are capable of utilising urea, or other forms of DON such as amides. As yet, it is not known if other coccolithophorids have similar uptake mechanisms (Rees *et al.* 2002; Lessard *et al.* 2005; Langer *et al.* 2009; Strom & Bright 2009).

1.4.6 Effect of pH on nitrogen uptake

The effect of pH on photosynthetic carbon assimilation is widely known (Raven & Johnston 1991; Raven 1997). However, the interactions between media pH (and the resulting changing ratios of HCO^{3-} to CO_3^{2-}) and nitrogen use are still not quite understood. Nitrate is stable at naturally occurring pH concentrations (approx 8.2), while ammonium is slightly more susceptible to pH fluxes with changes to the NH_3/ NH_4^+ (pKa 9.3) ratio. At normal oceanic pH, the main species available to phytoplankton will be NH_4^+ (Stumm & Morgan 1996). In regards to microalgal nutrient uptake, urea has been thought to be a very unstable molecule in aqueous solution, hydrolysing quickly to NH_3/ NH_4^+. Urea usually exists at equilibrium with ammonium cyanate in solution, however, this reaction is weighted towards urea formation (Hagel *et al.* 1971). Under oceanic conditions, (pH ~8 and at 25 ºC, only 0.0225µM ammonium is produced from 10µM urea after 33 days (Antia *et al.* 1991). Hagel *et al* (1971) and Turner *et al* (1979) both found that the rate of ammonium formation will decline with pH and temperature; however, these calculations do not take into account the

rapid hydrolysis of urea via urease generated though microbial metabolism in the media. Price & Harrison (1987) show that in the presence of urease, urea is rapidly hydrolysed with almost all urea hydrolysed in under 1 hour. These authors also demonstrated that this reaction is pH dependent, with the rate of hydrolysis falling as the media becomes more alkaline.

While urea is commonly found in low concentrations in oceanic systems (< 1-2µM-N), it is still a significant form of regenerated nitrogen (Kristiansen 1983). While ammonia is the most common form of nitrogen available in the ocean, urea concentrations are increasing, especially in coastal systems, where there has been a significant increase in anthropogenic urea input (Glibert *et al.* 2006).

It is for this reason that we need to understand how ecologically important microalgal species such as the coccolithophorids, will react to changes in nitrogen sources, as well as the possible changes to oceanic pH due to increased pCO_2 increases in atmospheric CO_2 and acidification by NH_4^+ /urea metabolism.

Shiraiwa (2003) stated that in the EH2 strain of *E. huxleyi* calcification was inhibited when the cells were cultured with sufficient nitrate and phosphate concentrations (Figure 15).

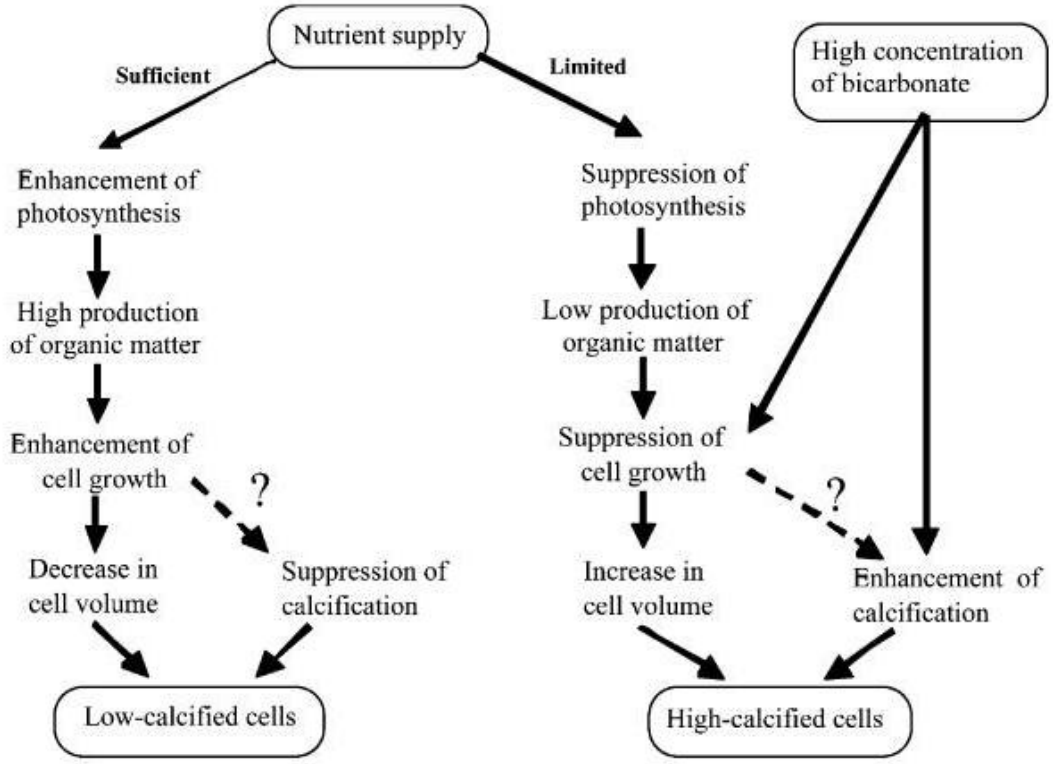

Figure 15 Nutrient source and the effect on regulation and growth of *E. huxleyi* (strain EH2). Low and High calcified cells represent the non-coccolith-bearing and high coccolith bearing stages (Shiraiwa 2003).

What this figure shows is that when the supply of nitrogen & phosphate is in excess, cell growth and photosynthesis is increased to produce a large amount of biomass (bigger cells), and therefore a reduced cell density and the inhibition of calcification. When the EH2 strain is cultured under N & P limited conditions the opposite is true, resulting in the inhibition of cell growth and photosynthesis and increasing calcification.

E. huxleyi has a very low requirement for phosphate and this may be one reason as to why *E. huxleyi* can form such extensive blooms in P limited waters (Paasche & Brubak 1994; Riegman *et al.* 2000).

1.4.7 pCO_2

It has been indicated that when *E. huxleyi* was grown under higher than current atmospheric pCO_2 (at approx pH 8), that there was an increase in both organic and inorganic carbon assimilation (Feng *et al.* 2008; Richier *et al.* 2011). However, the increase only occurred under high irradiance. Casareto *et al.* (2009) also saw increase in the biomass of *Chrysotila* cultures, with a 20% increase in biomass when the cells were exposed to the forecasted 1200 ppm atmospheric CO_2 concentrations. Increased biomass and growth rates were also reported in *C. carterae,* where the increased pCO_2 (via CO_2 addition) will also increase the growth rate and photosynthetic rate of *Chrysotila* (Moheimani & Borowitzka 2011).

Increasing the pCO_2 will result in an increase in the calcification: photosynthesis (C:P) ratio as well (Casareto *et al.* 2009; Moheimani & Borowitzka 2011), however, these results show only slight increase in some cases, and can be dependent on strain and light levels (Table 9)

1.4.8 Effect of pH on growth

Israel & Gonzales (1996) found that a pH of 7.5 *Chrysotila placolithoides* (CCMP299) had the highest photosynthetic rate for both high and low calcifying cells. They also found that as culture pH increased, there was marked decrease in net photosynthesis. The effect was less pronounced on the high calcifying cells, with the decline in oxygen evolution beginning at pH 7 and at pH 9 almost all photosynthetic activity had stopped. A reduction in oxygen evolution rate of approx. 80 nmol O_2 10^6 $cell^{-1}$ h^{-1} to 0.5 nmolO_2 10^6 $cell^{-1}$ h^{-1} was observed in low calcifying cells. The effect was reduced in high calcifying cells, with a drop from 55 to 30 nmolO_2 10^6 $cell^{-1}$ h^{-1}, and there was a reduction of approximately 55% in the high calcifying cells from a peak at pH 5. In other strains, the exact opposite has been seen. It has been observed that at a pH of 9.5 - 11, a cell density of up to 80 x 10^4 cells mL^{-1} with a

specific growth rate (μ) of 0.083, and gross areal productivity of 3.15 g m^{-1} d^{-1} was achieved using *C. carterae* (CCMP647) (Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007).

In almost all mechanisms of biological calcification such as coral, mollusk shells etc, there is a net decrease in pH due to the H^+ evolved during the metabolic processes. However the *Chrysotila carterae* shows a different pattern, in that there is a net increase in pH, while cells actively photosynthesizing and calcifying with pH falling to initial levels during dark respiration. (Moheimani 2005),

1.4.9 HCO_3^- uptake methods

It has been well established that *E. huxleyi* uses bicarbonate (Nimer *et al.* 1991; Nimer & Merrett 1993; Anning *et al.* 1996; Nimer *et al.* 1999; Herfort *et al.* 2002). Herfort *et al.* 2002 also found using O_2 evolution and ^{14}C uptake methods, that *E. huxleyi* suggests may have multiple mechanisms for DIC uptake (AE1 Protein and CA) and that there was no significant difference between calcified and non-calcified cells of the same strain. The authors excluded Ca^{2+} from media at levels that were too low for calcification, but did not impede photosynthesis. This contradicts Nimer *et al.* (1999) who, using the pH drift method on two different strains of *E. huxleyi,* found that there was no external CA detected (Nimer *et al.* 1996; Nimer & Merrett 1996; Nimer *et al.* 1999).

Investigating the variation in calcification processes of *E. huxleyi* by contrasting high and low calcified strains may be problematic according to Herfort *et al.* (2002). Here the authors suggest that to compare strains in relation to calcification rates opens up the possibility of error in that while each strain may have different calcifying capacities there may also be other significant differences such as DIC transport mechanisms (as found in (Elzenga *et al.*

2000). Herfort *et al.* (2002) suggests that is better to compare calcification v photosynthesis (C:P) ratios and to use the same strain and exclude Ca to determine the HCO_3 uptake characteristics.

1.5 Carbonic Anhydrase & Carbon Concentrating Mechanisms (CCM).

The use of external and internal carbonic anhydrase (CA; carbonate-lyse, carbonic dehydrase, EC 4.2.1.1) is very common among the marine phytoplankton. As HCO_3^- is the dominant carbon species in the oceans (Zeebe & Wolf-Gladrow 2005) access to CO_2 for photosynthesis is usually achieved by the enzyme which is also found throughout most living organisms. Carbonic anhydrase's function is to catalyse the reversible reaction between HCO_3^- and CO_2 thus providing CO_2 for cellular processes Eq. 1 (Raven 1997).

$$H^+ + HCO_3^- \overset{CA}{\Leftrightarrow} H_2O + CO_2 \quad \textbf{Eq. 1}$$

Within the coccolithophorids it has been found that *E. huxleyi* has both an internal and external CA (Herfort *et al.* 2002, (Stojkovic *et al.* 2013). However, external CA is only active in *E. huxleyi* when the total flux of DIC in to the cell is sufficient to support the photosynthetic rate observed under carbon replete conditions at optimum PFD (Iglesias-Rodriguez *et al.* 1998). This may explain the importance of regulation of external CA by HCO_3^- + CO_2 in some species but only by CO_2 in others; this would allow phytoplankton to rapidly acclimatise to the variation in DIC within the ecosystem, thus proving an ecological advantage.

Studies conducted by Stojkovic *et al.* (2013) show that several strains of *E. huxleyi* have active CCMs which are used to elevate CO_2 levels at the site of Rubisco, thus allowing for

increased photosynthetic carbon fixation. Stojkovic *et al*. (2013) also found that *E. huxleyi* is capable of using both CO_2 and HCO_3^- for photosynthesis, but suggest that as HCO_3^- is in greater supply, that *E. huxleyi* will used HCO_3^- (via CA_{EXT}) over CO_2 when calcification demands are reduced. Presently, for microalgae to use HCO_3^- as the primary inorganic carbon source, the cells must either use an external CA or have an active HCO_3^- transporter system. External carbonic anhydrase has been detected in *C. carterae* but only under carbon limited conditions (Nimer *et al.* 1999), which may be an indicator that *C. carterae* preferentially take up CO_2 directly and uses HCO_3^- when CO_2 is limited. However, Sikes & Wheeler (1982) suggest that as CA is only detectable in very small concentration in *Chrysotila* (*Hymenomonas*) *carterae* (13.0 ± 2.3 pmol CO_2 s^{-1} µg protein) it role in photosynthesis and calcification is only minor. Sikes & Wheeler (1982) also state that the small role played by CA in the coccolithophorids suggests that the main carbon species used is CO_2. Israel & Gonzales (1996) reported external CA activity in *Chrysotila* at both high and low inorganic carbon concentrations. The alkalization of the medium seen in cultures of *Chrysotila carterae* (Crenshaw 1964; Moheimani & Borowitzka 2006), and the presence of external CA, suggests that this species may use CO_2 as the primary carbon source. It may be suggested that this mechanism indicates that *C. carterae* is converting HCO_3^- to CO_2 at the cell surface, and this line of inquiry need to be perused further.

In other species of *Chrysotila* (such as *C. placolithoides*) carbonic anhydrase was found to be restricted to the chloroplast only, and was not found in any free living cell (Quiroga & Gonzalez 1993) thus limiting its role in inorganic carbon uptake.

CCMs may prove to be critical in countering the increased rate of CO_2 dissolution into the oceans and subsequent reduction in pH (ocean acidification) (The Royal Society, 2005).

The current thinking is that CCMs in genera such as the coccolithophorids, dinoflagellates and the diatoms may be able to consume the increased CO_2 though photosynthesis, in the case of the coccolithophorids (Slobodanka *et al*. (2013), sequester that carbon away as calcite (Biermann & Engel ; Bellerby *et al.* 2008; Bach *et al.* 2013). Bach et al also suggested that calcification in *E. huxleyi* is inhibited at low DIC, not induced as previously believed. In his 202 review, Paasche stated that the coccolithophore will use CO_2 as the primary DIC source for photosynthesis, had HCO_3^- as the DIC source for calcification. This was confirmed by Bach in 2013 whose work with *E. huxleyi* CCM gene expression and CA activity resulted in a very detailed analysis of the function of the CCM in *Emiliania huxleyi*.

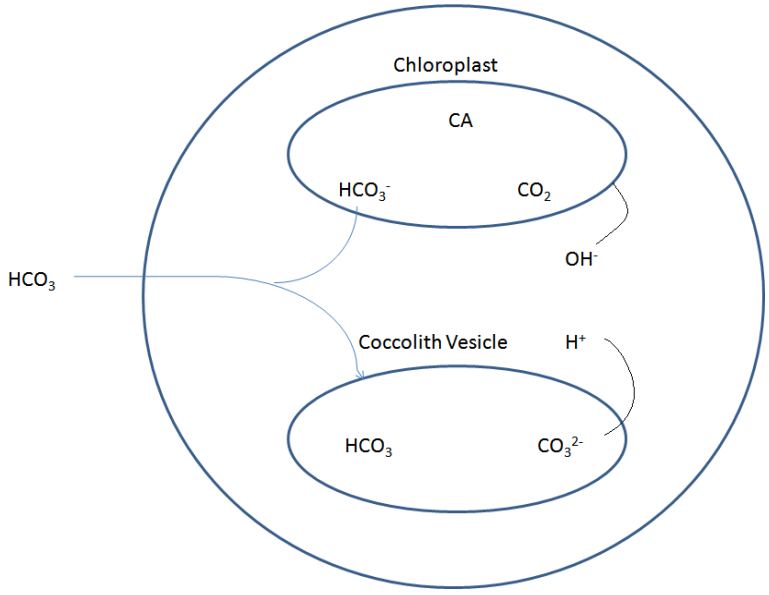

Figure 16 Model showing the path of sequestered DIC in *Chrysotila placolithoides* CA = Carbonic Anhydrase (Quiroga & Gonzalez 1993)

1.6 Economic Benefits

Recently there has been a surge in the mass culture of microalgae for commercial purposes. Microalgae has traditionally been used commercially as a feedstock for the vitamin supplement markets (Krauss 1962) such as *Dunaliella salina* for beta carotene *Chlorella*, *Spirulina* and *Chrysotila* for dietary and vitamin (vitamin B_{12}) supplements (Miyamoto *et al.* 2002; Miyamoto *et al.* 2004) .

For over 40 years there has been interest in using micro algae as an alternative fuel source and for nutrient supplements. However, in the last 10 years there has been an increased interest towards using microalgae as an alternative fuel. Several groups of microalgae such as Eustigmatophyceae (*Nannochloropsis*) and Haptophytes (*Isochrysis* and *Chrysotila*) have been identified as having the best commercial properties; these include ease of culture, high biomass (as aerial productivity) and of course high lipid content. There are many benefits of using microalgae as a source of biofuel, the most significant is that *C. carterae* is sustainable without using large parcels of land (Moheimani *et al.* 2012). The other significant benefit is that if using marine species, very little freshwater is used for culture systems.

While there are many research groups investigating mass culture system most groups are focusing on diatom species, including both fresh and saltwater systems, only a few are looking at the coccolithophorids as a viable candidate for commercial mass culture.

Of the two likely choices within the coccolithophorids (*Chrysotila* and *Emiliania*), *E. huxleyi* is not considered a good choice. While *E. huxleyi* does have a high lipid content (Moheimani *et al.* 2011), it is difficult to culture in large scale open photobioreactors for a long period of time, however it may be possible to cultivate *E. huxleyi* in large plate type reactors (Moheimani 2005). *Chrysotila carterae* (CCMP647) has been shown to be a very good

candidate (Moheimani *et al.* 2012). This clone has been successfully cultured in open outdoor raceway ponds for extended periods, and achieved high areal productivities of 47.65 g m^{-2} d^{-1} in summer and 9.79 g m^{-2} d^{-1} during the cooler months, with a peak lipid content of 71.14 mg L^{-1} d^{-1} during autumn (Moheimani 2005; Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007; Moheimani *et al.* 2011). These productivities are some of the highest recorded to date for any microalga grown in open raceway ponds.

Using coccolithophorids for biofuel has additional advantages. As they are calcifying organising, they not only fix carbon into organic carbon but inorganic carbon as well (as calcite, $CaCO_3$). The resulting calcite can then be buried or used as building materials and thus remove excess carbon from the system.

1.7 Cultivation Systems for *Chrysotila*

Culturing *Chrysotila carterae* in closed photo bioreactors has had mixed success, There have been many types of closed photobioreactors used, with a variety of results for review see Moheimani *et al* (2011).

The greatest success for increasing the biomass was using 12 L carboy systems with low mixing velocity (< 200 rpm) and an impeller Reynolds number (R) of approx. 3200. This yielded a growth rate of 0.64 cells $day^{-1}$and a dry weight production of 0.504 g L d^{-1}.

However, as R increased, growth rate and dry weight decreased, indicating that *C. carterae* can be very sensitive to sheer forces, and thus limiting the ability to “scale up” the culture volume.

This is one of the reasons why growing *C. carterae* in large-scale closed photobioreactors is often difficult, as the amount of energy required to keep the cells in suspension such as in airlift systems, or mechanical stirring will damage the cells (Moheimani *et al.* 2011). This usually occurs by knocking off the flagella or haptonema though the forces generated by bubbles bursting on the surface of the reactor, or though the stirring action. Once the cells have been damaged, this places increase energy-demand on the cells as they attempt to repair damaged structures.

The greatest success in large scale (up to 1000 L^{-1}) has been in open, outdoor raceway ponds (Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007). Using this system the Authors were able to achieve some of the high productivities found to date of any mass cultured microalgae.

1.8 Aims of this project

While there have been several detailed studies on the physiology of *Chrysotila carterae* and investigations into the mass culture of this species (i.e. Moheimani *et al*.2005; 2012), the main source of inorganic carbon used in photosynthesis of this alga is still unknown. Furthermore, very little is known about how *Chrysotila* responds photosynthetically to variations in pH and inorganic nitrogen source. The ability of *Chrysotila* to drive the pH up during the light period and then return to the original medium pH at night with no net increase in the culture pH has been reported previously, however as yet there has been no detailed study into the mechanisms of how *Chrysotila* can achieve this. Therefore, overarching aims of this project are as follows;

(a) Establish a base line for photosynthesis and carbon assimilate (both organic carbon and inorganic carbon),

(b) Investigate the effect of pH on photosynthesis and carbon assimilation

(c) Investigate the response of *Chrysotila carterae* CCMP647 to changes in the nitrogen species available.

In the current study, a detailed literature review is conducted in first chapter. After the Materials and Methods (Chapter 2), the objectives to determine these aims are detailed in the subsequent chapters; Chapter 3 details the establishment of a base line for photosynthesis and carbon assimilation, as well as establishing the previously reported assumption of a selenium requirement. Chapter 4 details the effect of culture pH and diurnal effects of *Chrysotila carterae*. Chapter 5 details the effect of pH and nitrogen source on photosynthesis and carbon assimilation. Finally all results are disused in Chapter 6, along with possible directions to advance the current knowledge of *Chrysotila carterae*.

2 General Methods

2.1 Strain sources and Maintenance

The coccolithophorid alga (Haptophyceae) *Chrysotila carterae* (T.Braarud & E.Fagerland) Andersen, Kim, Tittley & Yoon, strain CCMP647, was obtained from the Centre for Culture of Marine Phytoplankton, Bigelow Laboratory, Maine USA (CCMP).

Seawater for media preparation was from Hilary's Beach, Perth Western Australia, and stored in 10,000 L holding tanks at Murdoch University. To prevent algal growth, the tanks were kept dark. Before use, the seawater was treated with charcoal overnight by adding 1g L^{-1} activated charcoal to the seawater. The seawater was then filtered twice though double thickness Whatman No. 1 filter paper, followed by filtering through a 0.45µm Whatman

nitrocellulose membrane filter. The filtered seawater was then stored in polycarbonate containers in the dark at 4 ºC.

Table 2 Modified f/2 medium (based on Guillard & Ryther 1962)

Compound	Stock Solution (g L^{-1})	f/2 stock added per L medium
NaNO3-	150	0.5 mL
$NaH_2PO4.1H_2O$	10	0.5 mL
Iron Solution		
Na_2EDTA	0.945	0.5 mL
$FeCl_3$.6H2O	1.22	0.5 mL
Vitamin Solution		
Cyanocobalamin	0.001	0.5 mL
Thiamine HCl	2	0.5 mL
Biotin	0.001	0.5 mL
SeO_2	0.0129	0.5 mL
PII Metal Solution		
$MnCl_2.4H_2O$	0.0072	0.5 mL
$ZnSO_4.7H_2O$	0.04	0.5 mL
$CoCl_2.6H_2O$	0.02	0.5 mL
$CuSO_4.5H_2O$	0.0196	0.5 mL

Stock cultures were maintained in 250 mL Erlenmeyer flasks. Cultures were sub-cultured into 100mL fresh medium every 14 days.

2.2 Culture Vessels and Reactors

2.2.1 Plate Photobioreactor

A three-litre plate photobioreactor developed by Moheimani (2005) was used to culture the cells via chemostatic growth. This was achieved by adding a drain at the 3L mark to allow

media to continually flow out of the reactor. Media was added via peristaltic pump with flow rate adjusted to individual experiments. Typical flow rate was 10 mL min^{-1}.

2.2.2 Tube Reactor

This type of reactor was used to determine the effect of selenium. The algae were cultured in 300mL^{-1} small scale bubble columns. To determine any effects on coccolith production, external coccoliths were removed by dissolving with CO_2 (see section 2.3.7). The cells were grown with either selenium limited f/2 medium, or selenium replete f/2 medium at 25°C.

2.3 Culture Conditions and Light History

Light was provided by 10W cool white fluorescent lamps providing a photosynthetically active radiation (PAR) of 150-200 µmol photons m^{-2} s^{-1} at the culture surface on a 12:12 light:dark (LD) cycle. Temperature was maintained at 25ºC in constant temperature growth rooms.

2.3.1 Sterilization

Prior to each experiment, all culture systems, with the exception of carbon uptake incubation systems, were sterilized by autoclaving at 121 ºC for 20 min. As it was not possible to autoclave the plate reactors or 20L carboys, a 5% v/v sodium hypochlorite solution was used. After circulation of the solution for 60 minutes in the reactors it was left overnight. After 24 hours, the apparatus was drained in a laminar flow and rinsed 3 times with sterile de-ionised water and left to dry under laminar flow conditions.

Seawater media and stock solutions were sterilized by either autoclaving or by filter sterilization though a Whatman 0.2 µm membrane filter. To eliminate precipitation of phosphate in the culture medium, the phosphate stock solution was autoclaved separately

then added to the media under a laminar flow cabinet at the same time as the vitamin solution.

Vitamin Solution was prepared in according to the f/2 recipe and then filter sterilized though a Whatman 0.25 µm Cellulose Nitrate membrane filter. All filter apparatus was autoclaved to ensure sterility.

Where it was not practical to autoclave larger volumes of seawater, 10mL L^{-1} 12% sodium hypochlorite was added to the medium and mixed for 1 hour. The medium was left for 12 h in a laminar flow cabinet to allow the NaClO to gas off. After 12 hours any remaining hypochlorite was removed by adding 1mL of 10g L^{-1} Sodium Thiosulphate per 4 L culture medium.

All glassware and tubing used was washed in a 10% detergent solution (Decon 90) for 2 h, rinsed with deionised water then soaked in a 0.1M HCl solution overnight. The equipment was then rinsed 12 times with deionised water and dried in an oven at 70 ºC.

2.3.2 Cell counts

Cell density was determined by counting in a Neubauer haemocytometer. The specific growth rate of culture was determined by first calculating the doubling time in exponential growth phase from semi-log plots. The specific growth rate (µ) was calculated from Eq. 6.

$$\mu = \frac{0.693}{t_2} \qquad \textbf{Eq. 6}$$

Where t_2 is the number of days for cell numbers to double.

2.3.3 pH and Salinity

The pH of cultures was measured with a TPS epoxy pH electrode using an Orion model number 520n bench pH meter.

Constant pH cultivation systems (pH Stat) were monitored by a TPS epoxy pH electrode on a Roche 8001 pH meter/controller. Output of the pH meter was recorded with a PowerLab data recorder (ADI Instruments) with Chart4 software.

Salinity was measured using an Atago PAL-03S digital refractometer, calibrated with DI H_20 prior to use.

2.3.4 Biomass

The biomass was determined as follows:

Whatman 25mm GF/C glass filters were washed in deionised water and dried at 70 ºC for 24 h, then stored over silicate gel under vacuum until required. The filters were then pre weighed to four significant figures. 10 mL of algal culture was filtered until the filter paper was dry. The filter was then washed with 10 mL 0.65M ammonium formate to remove excess salts and dried at 75^{o}C for 5 hours, then placed in a vacuum desiccator overnight. The filters were then weighed to four significant figures and dry weight calculated (g L^{-1}) by subtracting the filter weight from the filter plus sample weight.

Ash free dry weight was determined by ashing the above filters at 450 ºC for 7 h and then cooling under vacuum overnight before re-weighing. The ash free dry weight (AFDW) was calculated as mg L^{-1} by subtracting the weight of the filter from the total weight. This was further subtracted from the total sample weight to give the ash free dry weight. These methods are detailed in (Moheimani & Borowitzka 2006).

2.3.5 Photosynthesis measurements

Photosynthetic activity was assessed using a Pulse Amplitude Modulated (PAM) fluorometer (Water-PAM; Walz GmbH, Effeltrich, Germany). A 2.5 mL aliquot of culture was used for each experiment at a cell density of 1 x 10^5 cells mL^{-1} (unless stated otherwise). The minimum fluorescence (F_O) was determined after a 10 min dark adaption period. Maximum fluorescence (*Fm*) was determined after the application of a 0.6 µs saturation light pulse at 1800 µmol photons m^{-2} s^{-1}.

Photochemical efficiency (Fv/Fm) was determined using the equations of Falkowski & Raven (2007) Eq. 7

$$\frac{(F_m - F_O)}{F_m} \qquad \textbf{Eq. 7}$$

A minimum of five replicate Rapid Light Curves (RLC) from 3 separate batch cultures were used to determine the Relative Electron Transport Rate (r*ETR*). Light levels for the RLC's were 0; 98; 165; 245; 333; 522; 702; 1064; 1547 µmol photons m^2 s^{-1}, as measured with a US-SQS/UB spherical quantum sensor inside the quartz cuvette (Walz GmbH, Effeltrich, Germany). Optimum light exposure time inside the measuring chamber was 15 seconds

2.3.6 Calculation of photosynthetic parameters

Photosynthetic parameters were derived from the Waiting in Line Equations and curve fitting model of (Ritchie (2008) and Ritchie & Bunthawin 2010) Eq. 8.

$$ETR = ETR_{max} . k . E . e^{1-kE} \qquad \textbf{Eq. 8}$$

$$ETR_{max} = \frac{A}{e}$$

The minimum light saturation irradiance (E_k) is calculated from the intercept with α with the maximum photosynthetic rate from Eq. 9

$$E_k = \frac{ETR_{max}}{\alpha} \qquad \textbf{Eq. 9}$$

2.3.7 Coccolith Removal

Where required, coccoliths were removed by gently bubbling (2 L min^{-1}) CO_2 into the culture medium until the desired pH was achieved. pH was monitored via Orion Bench pH meter with 10mm epoxy pH electrode (TPS) calibrated daily. Cells were examined under light microscope to confirm that all coccoliths had been dissolved.

2.4 Carbon Uptake Methods

As coccolith formation occurs inside the cell, it is difficult to separate the organic and inorganic carbon (as $CaCO_3$) that is assimilated during photosynthesis. To measure the molar ratio of calcification to photosynthesis it was necessary to separate the two fractions. This was achieved via a modification to the methods developed by Paasche & Brubak (1994). Here the photosynthetically assimilated organic carbon (C_{ORG}) fraction can be differentiated from the amount of particulate inorganic carbon (C_{INORG}) that can be assumed to be the amount of carbon converted into calcite.

Three time course experiments were conducted to determine the most efficient incubation time, 60 minute, 90 minute and 120 minutes. Sampling was conducted every 15 minutes.

Determination of the carbon 14 equilibrium point, as well as dark carbon assimilation rates were determined by running a 5 h time course sampling every 60 min. Two time courses were run, one commencing 4 h into the light cycle (11am) and running though until 4 pm for a total of 5 h, Dark carbon assimilation commenced 3 h into the dark cycle (9 pm) and finishing at 2 am.

Light incubations were commenced at the same time (2 pm) for all time course experiments. Dark incubations were treated identically to light incubations; however, samples were kept in the dark by covering with aluminium foil and working in a darkened room, with only a dim green light used to work by.

Incubation for general time course was conducted in a 50 mL Eppendorf Combi pipette tip fitted to an Eppendorf Multipette Plus automatic pipette. Light was via 10 W cool white fluorescent lamps at 300 µmol photons m^2 s^{-1}. All time course experiments were conducted at the same time of day (2pm).

The apparatus was secured to a frame that kept the pipette tip containing labelled culture in the light path. The whole rig was gently shaken by hand every 5 min to keep cells suspended and well mixed.

50mL of culture was incubated with 50 µL of 5 mCi $NaH^{14}CO^3$ (Amersham Bioscience, UK) (equal to a final specific activity of 90 µCi) at 25 ºC with a photon flux density of 300 µmol photons m^2 s^{-1} measured with a US-SQS/UB spherical quantum sensor (Walz GmbH, Effeltrich, Germany) inside the pipette tip.

2 mL of labelled culture was filtered through 0.45µm cellulose nitrate membrane filters and washed with 30 mL of unlabelled seawater to remove excess label. 3 replicates were taken for each time sample.

Each membrane filter was then transferred to a 20 mL glass scintillation vial. This gives the organic carbon fraction (C_{ORG}).

A second vial was prepared with 100 µL of ethanolamine to trap the $^{14}CO_2$ from the cells and thus measure the inorganic carbon uptake fraction (C_{INORG}).

The two vials were connected and sealed with a silicone rubber collar and 1 mL of 0.1M H_2SO_4 carefully added to the filter side with a 1 mL syringe and 19 gauge hypodermic needle (Figure 17).

Determination of Sample size for ^{14}C Time Course Experiments (Eq. 10).

$$n \geq \frac{z^2 \sigma^2}{d^2} \qquad \textbf{Eq. 10}$$

- Where z = the value from a standard normal distribution. For a 95% confidence range, z = 1.96 so z^2 = approx 4 (Manly 2001).
- σ^2 = the variance of the sample size (n=5).

All carbon assimilation experiments were replicated 3 times.

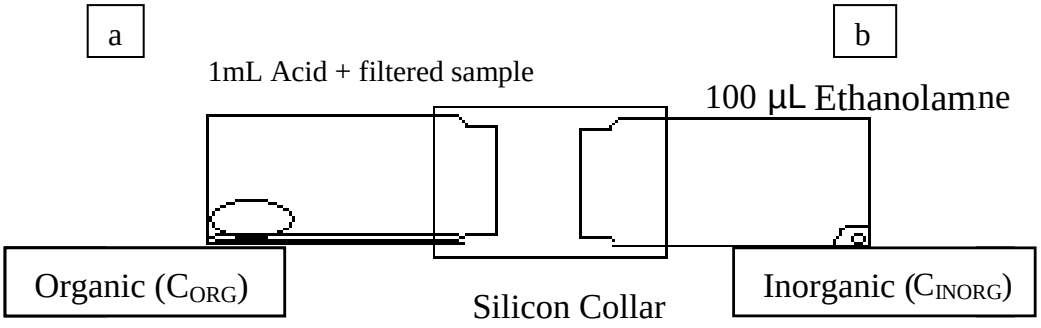

Figure 17 Methods for separating ^{14}C uptake in *C. carterae* carbonate production from photosynthesis. (a) scintillation vial with 25mm cellulose nitrate membrane filter and 0.1M H_2SO_4 (Organic carbon fraction (C_{ORG})). (b) Inorganic fraction (C_{INORG}) Scintillation vial containing 100µL ethanolamine to trap $^{14}CO_2$ from (a).

The sealed vials were placed on their side and then left overnight to allow $^{14}CO_2$ to be absorbed by the ethanolamine.

The vials were then carefully separated and 1 mL of 0.2M NaOH added to the filter side to determine the amount of organic carbon (C_{ORG}), and 2 mL DI H_2O added to the ethanolamine side to determine the amount of inorganic carbon(C_{INORG}) assimilated.

9 mL of Ultima Gold scintillation fluid (Perkin Elmer, Boston USA) was then added to each vial.

The vials were shaken and allowed to stand for 12 h in the dark. Activity was counted in a Beckman LC2500 Liquid Scintillation counter.

Carbon uptake was calculated by Eq. 11 and Eq. 12

$$mmolC.cell = \frac{\left(\frac{Specific\ Activity}{Sample\ Count\ (dpm)}\right)}{Cell\ Number} \qquad \textbf{Eq. 11}$$

$$Specific\ Activity\ (SA) = \frac{Total\ Activity\ (dpm.mL)}{\text{mmol}\ \Sigma CO_2\ X\ 10^{-6}} \qquad \textbf{Eq. 12}$$

Carbon uptake rates were calculated in Sigma Plot for Windows v13 (SPSS, Inc Chicago, Ill.).

2.4.1 pH Regulated Time Course Incubations

100 mL of culture was incubated with 100µL 5 mCi $NaH^{14}CO^3$ at 25 ºC for 90 min with a photon flux density of 300 µmol photon m^2 s^{-1} measured with a spherical quantum sensor (Walz GmbH, Effeltrich, Germany) inside the reactor chamber.

The reactor was constructed from a 200 mL glass jar with plastic screw top lid (Figure 18). The glass jar was acid washed with 0.5M HCl for 2 days prior to first use. pH was monitored a via Roche pH controller and kept constant via addition of either 0.1M HCl in 33.3 g L^{-1} NaCl for pH levels below pH 8, and 0.1m NaOH in 33.3 g L^{-1} for pH 9.00. A 3 point calibration of pH meter was conducted prior to each run. The incubation vessel was stirred with a Teflon magnetic stir bar.

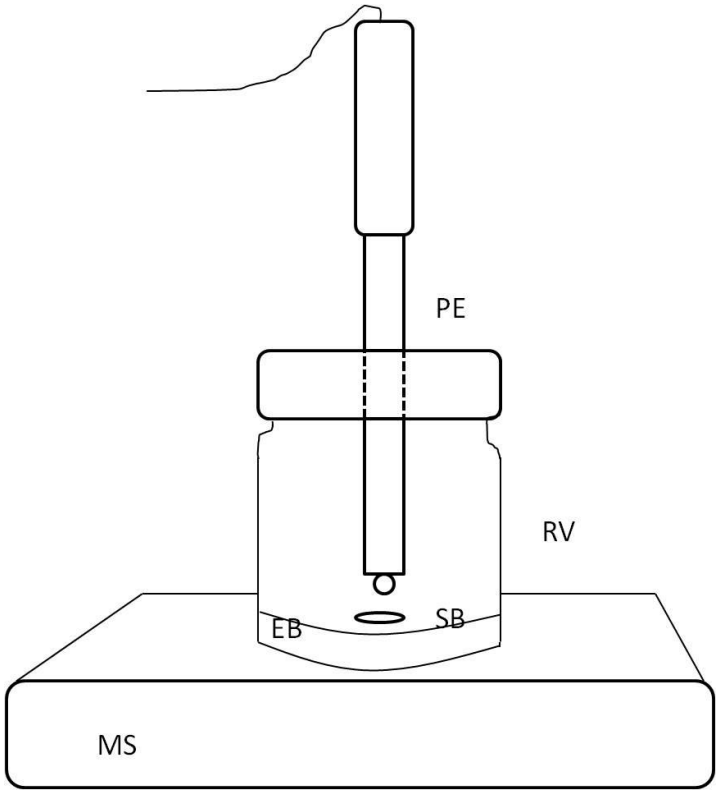

Figure 18 Schematic of pH controlled incubation vessel for constant pH carbon 14 uptake experiments. PE – pH electrode; RV – Reactor vessel; MS – Magnetic Stirrer; SB – Stir Bar; EB- flat, epoxy base.

8 mL aliquots were taken every 15 min with a 10 mL syringe fitted with a 5 cm (4mm id) silicone rubber tube though a small hole in the top of the reactor chamber. The subsample was then transferred to a 20 mL beaker with 30µL formalin to kill the cells and stop any further carbon uptake. The sub sample was then processed as per previous time course methods

2.5 Total Alkalinity and pCO_2 calculation

Alkalinity was determined via methods outlined in (Strickland & Parsons 1972)

200 mL of culture was filtered through a series of 45mm filters (GF/B, followed by GF/C and finally 0.45 µM membrane filters to remove all cells and free-floating coccoliths. Exactly 100 mL of filtered media was placed in a 250 mL polycarbonate Erlenmeyer flask and pH measured to 2 significant figure with TPS epoxy pH electrode by an Orion model number 520n bench pH meter. A three point calibration was conducted before each measurement and accuracy tested with Phthalate Buffer. pCO_2 and other carbon chemistry was calculated from the tables in Strickland and Parson 1972 and the CO2SYS program (http://cdiac.ornl.gov/oceans/co2rprt.html).

2.6 Selenium Requirements

For selenium experiments, Pacific Artificial Seawater (PASW) was used (Borowitzka & Larkum 1976).

2.7 Nitrogen Uptake methods

For the nitrogen source experiment, all nitrogen concentrations were at f/2 levels.

Ammonia 93.2 g L, Urea = 51.7g L and NO_3^- = 150g L

0.5 mL L^{-1} of stock ammonium and nitrate solution was added to the media mix prior to autoclaving. For urea, basic f/2 medium was prepared omitting the urea stock solution. To reduce hydrolisation of urea to NH_3/NH^+_4, fresh stock solution was prepared before each experiment and filter sterilized though a 0.2µm Whatman membrane filter then 0.5mL stock solution per L was added directly to the culture immediately after inoculation.

Cultures were grown on $f/2_{(NO3-)}$ until mid-stationary phase to ensure that all available N had been consumed. This was confirmed using API aquarium test kits (Mars Fishcare USA) for nitrate.

Once inoculated into fresh medium, the cells were acclimatised to new medium for 12 h before pH stat was initiated.

2.7.1 Regulation of Culture pH

Culture pH was monitored using TPS epoxy pH electrodes fitted to Roche 8801 pH controller. pH was monitored and recorded using a Power Lab data recorder (ADI Instruments) set to record every 30 seconds, pH was reduced by addition of 0.1M HCl delivered via a peristaltic pump connected to the pH controller. The pH was raised by addition of 0.5M NaOH in 33.3g L^{-1} NaCl. This was done to eliminate the instant precipitation of phosphates when the NaOH was added. To maintain a constant dissolved inorganic carbon concentration irrespective of pH, no air was added to the culture. Cells were mixed via magnetic stirrer (Figure 19).

The rate of change in the culture pH (ΔpH) was calculated by the determining the slope of the pH rise during the light phase, and dark phase cell respiration.

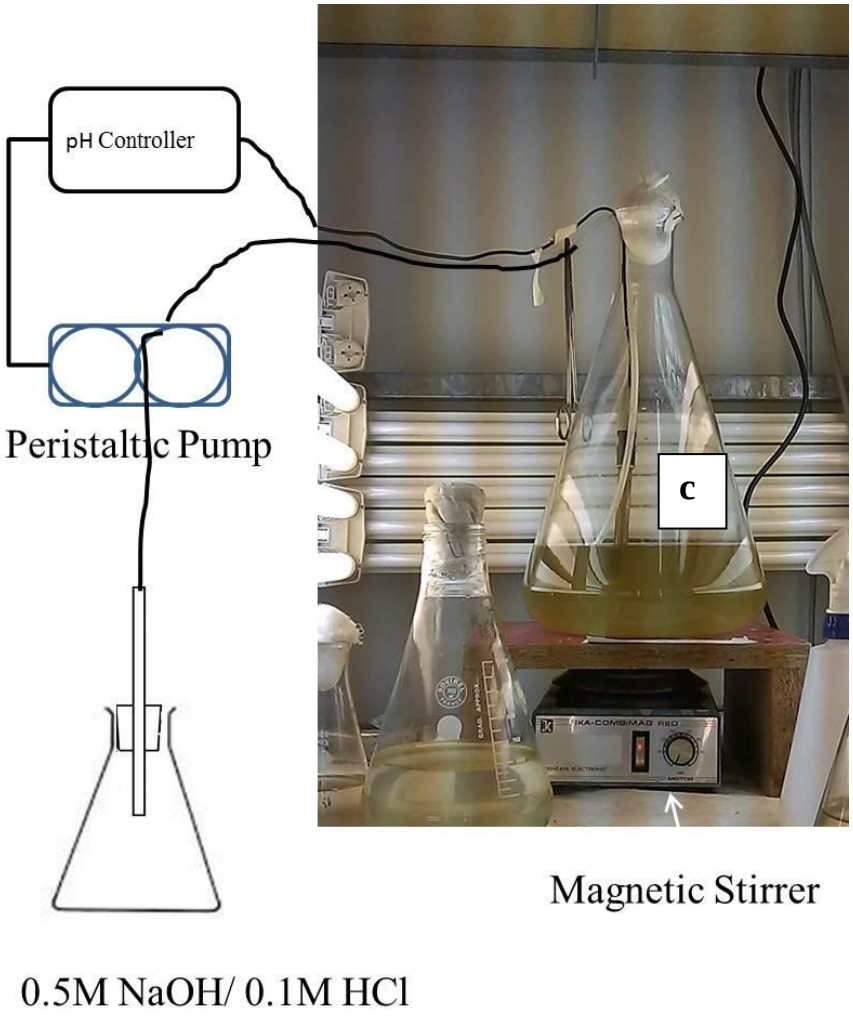

Figure 19 pH control system. pH was maintained by automatic addition of either NaOH or HCl added directly to the culture at a rate of 5mL h^{-1} (b). Addition of acid/base was via peristaltic pump controlled via a Roche pH controller (c= pH probe). Culture was constantly stirred to maintain cell suspension. Light was supplied via 2 banks of cool white fluorescent lamps (300 µmol photons m^2 s^{-1})

3 PHOTOSYNTHESIS AND GROWTH

3.1 Introduction

A challenge when working with coccolithophorids is that the coccoliths, both attached and detached, may adversely affect measurements involving inputting light. The coccosphere has been suggested to reduce light from entering the cell (Young *et al.* 1999; Paasche 2002), while, more practically, the coccoliths (both free and attached) have been shown to scatter the light and so any measurement requiring the accurate measurement of light (such as fluorescence) would be afected. This may have the effect of over, or under estimating the measurement (Balch *et al.* 1993; Gordon & Du 2001; Takahashi *et al.* 2002; Gordon *et al.* 2009).

Pulse Amplitude Modulated (PAM) fluorometry has become one of the most common methods for determining the photosynthetic response of phytoplankton. The technique has the benefit of being able to conduct fast, non-destructive and repeatable measurements on the same plant, or even the same cell (using a microPAM) (Schreiber *et al.* 1997; Beer *et al.* 1998; Komkamp & Forster 2003; Ralph & Gademann 2005).

Photosynthesis measurements in coccolithophorids are further complicated by the fragile nature of *Chrysotila* cells (Cosgrove & Borowitzka 2006). Moheimani & Borowitzka (2011) found that when *C. carterae* is subjected to increased shear forces though either mechanical stirring or via air lift systems, that the cells have a reduced growth rate. The twin flagella and vestigial haptonema are quite fragile (Inouye & Pienaar 1985; Kawachi & Inouye 1995) and can be easily broken off during periods of increased turbulence. Turbulence continues to be a problem for large-scale microalgal cultures and its effect on cell yield within the

photobioreactors have been widely investigated (Silva *et al.* 1987; Barbosa *et al.* 2003; Moheimani *et al.* 2011). The effect of shear forces from culture mixing systems can also pose a problem when using traditional methods of measuring photosynthesis, such as Clark type oxygen electrodes, that require constant stirring in the chamber. The problem is that damage to the cell may negatively affect cell metabolism and place an increased demand for photosynthetic products (Cosgrove & Borowitzka 2006).

One of the benefits of the Walz water PAM over Clark type oxygen electrodes is that the sample in the Water PAM cuvette does not require constant stirring, however here the coccoliths that may pose a potential problem. The coccoliths make the cells heavy, thus many cells will rapidly settle to the bottom of the PAM cuvette, this may result in an underestimation of the Electron Transport Rate (ETR). This problem can be overcome by using the stirrer; however, this will cause the same problems as with the Clark type electrode chamber (Cosgrove & Borowitzka 2006).

A second issue is the light scattering effect of the coccoliths themselves (Gordon & Du 2001; Gordon *et al.* 2009). Within the culture media, there will be both free and attached coccoliths that either have been dislodged from the cell surface or from dead cells. This light scattering may have the effect of changing the light path and intensity within the PAM cuvette, as well as the return fluorescence signal, possibly causing inaccurate results.

The successful, stable growth of coccolithophorids in culture often requires modifications to existing growth medium. The macronutrient requirements for coccolithophorids have been well documented (Obata & Shiraiwa 2004; Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007; Zondervan 2007; Casareto *et al.* 2009; Moheimani *et al.* 2012). However, the effect of trace elements on growth of *C. carterae* (Obata & Shiraiwa 2004) has received

limited attention, there is a great deal of evidence on the selenium requirement on *Emiliania huxleyi* (Danbara & Shiraiwa 1999; Obata *et al.* 2004), details on how selenium affects *C. carterae* are limited. For a detailed review of the selenium requirement in coccolithophorids see the main introduction section: 1.5.2.1).

Personal observations indicated that when selenium was omitted from the culture media the *C. carterae* coccosphere was incomplete. Here we suggest that selenium is an essential requirement of coccolith adhesion.

This series of experiments will investigate how the coccoliths affect photosynthetic measurements which will be used to determine a baseline for future experiments presented in this Thesis. Other physical factors such as pH and how selenium will also be tested to determine the effect on growth and photophysiology, and how these variables may affect coccolith production and cell growth in *C. carterae* CCMP647.

3.2 RESULTS

3.2.1 Baseline photosynthesis

To determine the optimum fluorescence photosynthetic parameters, great care was taken in handling the culture to avoid any damage to external cellular components such as the haptonema and flagellum, which can be easily broken. Figure 20 shows RLCs from which the maximum rETR obtained for *C. carterae*. The best results were obtained with a cell density of 20 x10^4 cells mL (Figure 21) and as such, all PAM readings were taken at this cell density unless otherwise stated.

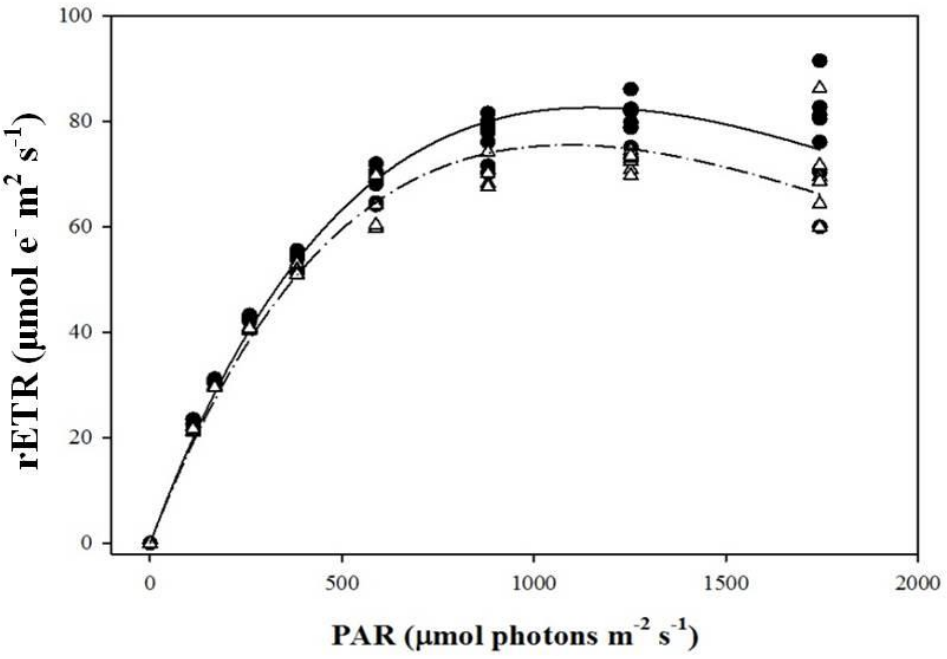

Figure 20 RLC for baseline photosynthetic rates of *C. carterae*. Curves fitted via the waiting in line equations of (Ritchie & Bunthawin 2010) ●= light adapted relative electron transport rate (rETR); △= Dark adapted (ETR). n=15, ± s.e..

To determine the optimum cell density, serial dilutions using f/2 medium were conducted. Figure 21 shows the differences in electron transport rates between the 15 minute dark adapted cells (ETR) and the relative (light adapted) electron transport rate (rETR) cells of *C.*

carterae as related to cell density. The highest ETR was achieved at a cell density of 10 x 10^4 cells $mL^{-1}$and 20 x 10^4 cells mL^{-1}, however the rETR is slightly higher at 20 x10^4 cells mL^{-1} and as such this was the cell density used for all subsequent experiments as rETR is the more useful parameter. While it generally accepted that rETR is independent of cell density as the calculation is based on the yield, what this fig indicates is that the possibility of self shading possibly due to free and attached coccoliths.

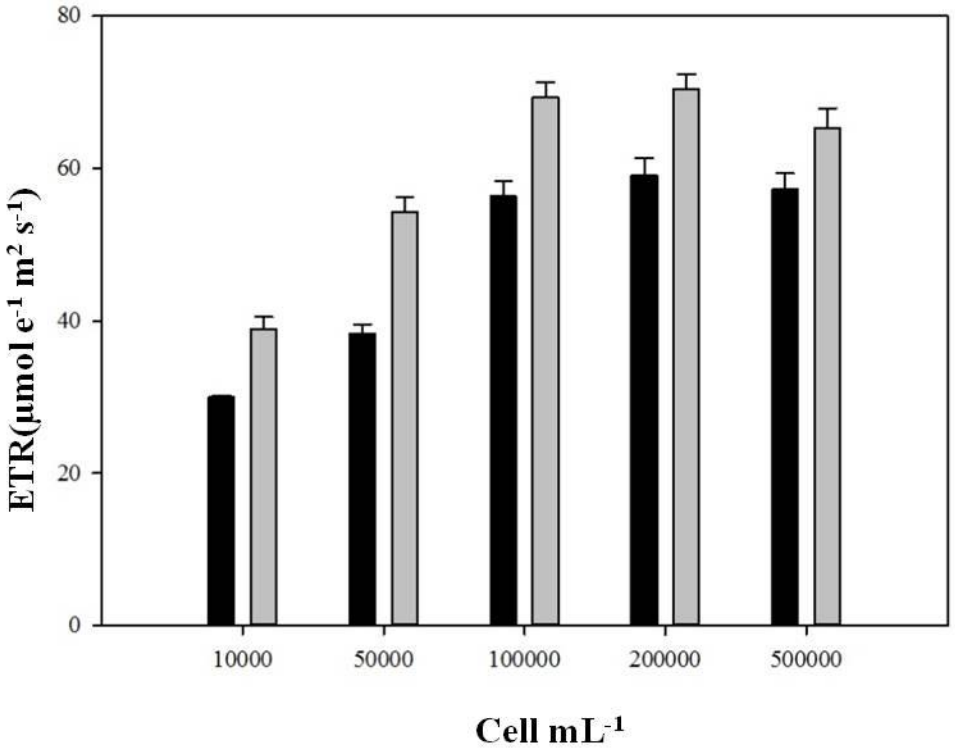

Figure 21 Effect of cell density on the Relative Electron Transport Rate (rETR) of *C. carterae* (dark bars = Dark adapted cells (rETR L), Light Bars = Light adapted (rETR D). n=15 ±s.e.

ETR_{max}, α and Ek of dark and light adapted *C. carterae* is summarised at Table 2. There is a 16% reduction in the maximum electron transport rate (ETR_{max}) of dark adapted cells, and almost a 60% increase in the dark adapted photosynthetic rate (α) (Table 4).

There is a significant difference between the light adapted (rETR) (relative electron transport rate) and dark adapted (ETR) (electron transport rate) (t = -12.957 P = < 0.001). The light harvesting efficiency of *C. carterae* (α µmol e^- µmol photons) in the dark is almost twice that of the light adapted cultures. There is also a significant difference in the light saturation point for both treatments, with E_k dropping to 1032.01 ± 111.54 µmol photons m^{-2} s^{-1} in the dark adapted cells (t = -5.650, P = < 0.001) (Table 3).

Table 3 Key photosynthetic baseline parameters for *Chrysotila carterae*, Light adapted (rETR) and dark adapted (ETR) cultures. n=15, ± s.e..

Light history	ETRmax µmol e^- m^{-2} s^{-1}	α µmol e^- µmol photon	E_k (µmol photons m^{-2} s^{-1})
Light	70.48 ± 1.89	1.89 ± 0.17	1135.16 ± 75.43
Dark	59.1 ± 2.76	2.76 ± 0.16	1032.01 ± 111.54

Maximum oxygen production (P_{max}) (Figure 22) was 1721 ± 98.23 pmol O_2 mg Chl *a* h^{-1} at 1500 (µmol photons m^2 s^{-1}). There is a slight down turn in photosynthesis at this point, indicating slight photoinhibition at approximately 1800 µmol photons m^2 s^{-1} this is a higher irradiance than that causing PS_{II} chlorophyll fluorescence photoinhibition, which occurs at approximately 1100 µmol photons m^{-2} s^{-1} (Figure 20). The photosynthetic light harvesting efficiency (α) for oxygen was 3.53 ± 0.17 µmol O_2 mg Chl *a* h^{-1}, with E_k at 1322.6 ± 203.3 µmol photons m^{-2} s^{-1}) (Table 3).

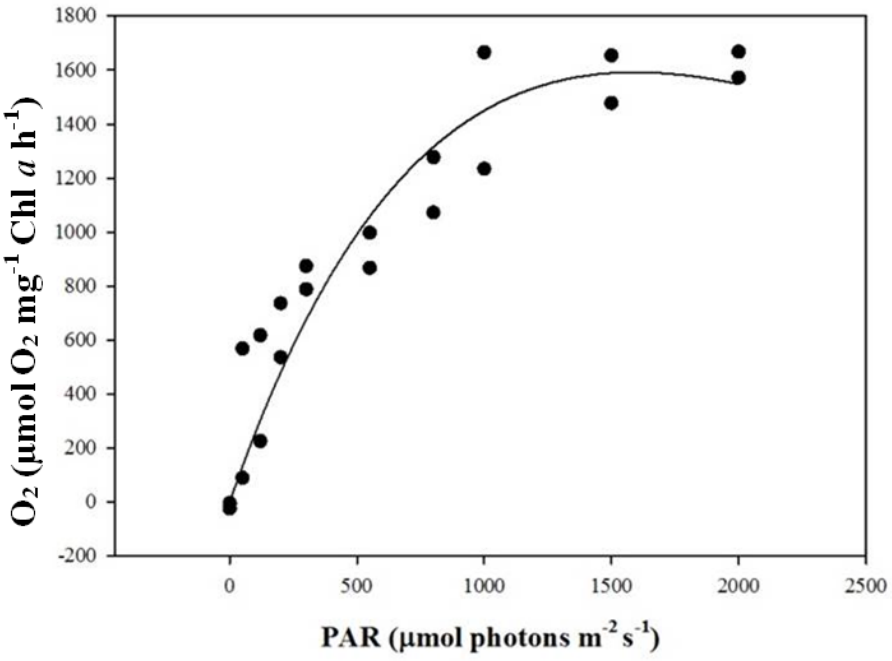

Figure 22 Photosynthesis vs. Irradiance curve for laboratory controlled *C. carterae* as measured with Clark type oxygen electrode.

3.2.2 Settling Effects

Due to the coccoliths, *Chrysotila carterae* cells are very heavy when compared with other phytoplankton. Therefore, the *C. carterae* cells have a tendency to settle out of the water column very quickly. This can potentially affect photosynthetic measurements when PAM fluorometry is used as the cells may not be in the light path for sampling, or the increased cell density toward the bottom of the cuvette may have a self-shading effect. The problems associated with measuring photosynthesis of *C. carterae* can be seen in Figure 23 which shows significant reduction in rETR is seen within the first 5 minutes ($t = 3.203$ $P = 0.0126$) , indicating that a large proportion of the cells in medium have settled out. There is a reduction in both the $rETR_{max}$ and α; with α falling from 0.036 to 0.0207 in 5 minutes. $rETR_{max}$ was reduced by 50%, from an initial peak of 29.06 ± 1.8 µmol $e^- m^{-2} s^{-1}$ to 15.17 ± 0.94 1.8 µmol $e^- m^{-2} s^{-1}$.

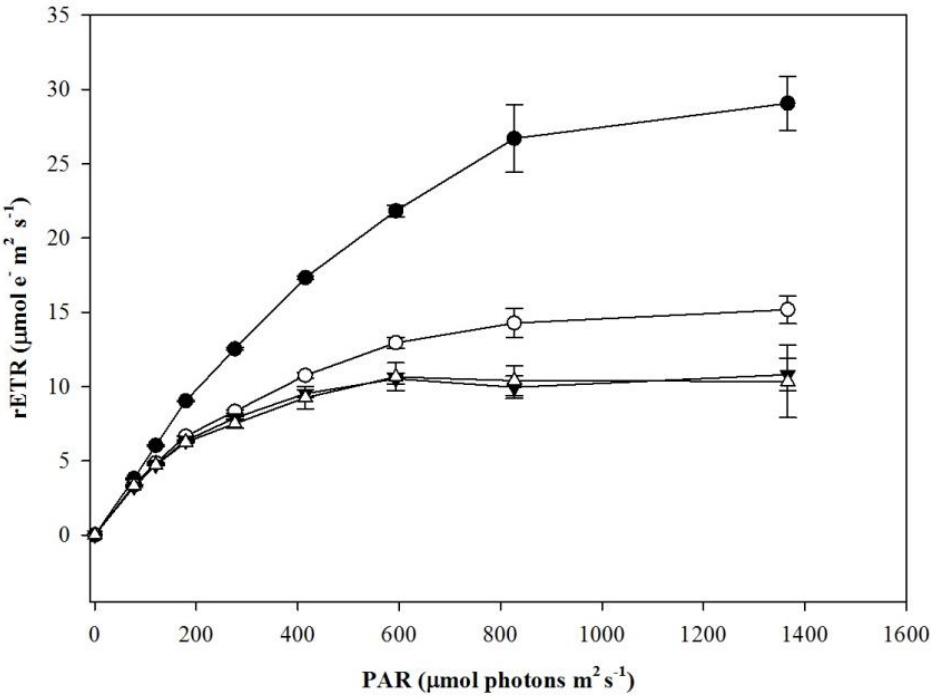

Figure 23 Effect of *C. carterae* settling in Walz Water PAM cuvette on fluoresce measurements (as relative Electron Transport Rates (rETR). ● = sample stirred using Walz PAM cuvette stirrer, ○= 5 min settling time, ▲= 10 min settling time, △= 15 min settling time. (n=7 ± s.e.).

3.2.3 Effect of coccoliths on PAM measurements

To investigate whether the coccoliths have any effect on the accuracy of the PAM fluorescence reading, the coccoliths (both free and attached) were dissolved from the *C. carterae* culture via acidification of the culture medium with CO_2 (see Materials and Methods Section 2.3.3). To reduce possibility of any pH effects, the culture pH was allowed to return to its original pH prior to acidification (average time was 30 min to return to starting pH ± 0.5 pH units).

Table 4 shows that there is a significant difference in the $rETR_{max}$ (t= -2.528, P value = 0.017), however the a 20% increase in the electron transport rate. There was no significant difference in the effective photosynthetic efficiency (Fq/Fm' or alternatively known as

ΔF/Fm) (t = 1.06, P value = 0.3) or the photosynthetic rate (α) (t = 0.4 P value = 0.69). The biggest difference is in the light saturation point (E_k). Here there is a significant increase of 139.15 µmol photons m^{-2} s^{-1} in E_k with the coccoliths removed (t = 1.85, P = 0.102) (Table 4).

Table 4 Effect of coccoliths on photosynthesis of PS_{II} in *C. carterae* (n=15 ± s.e.).

	$rETR_{Max}$ µmol e^- m^2 s^{-1}	**alpha**	**Fq/Fm′**	**E_k µmol photons $m^{-2}s^{-1}$**
With Coccoliths	29.92 ± 0.68	0.2 ± 0.01	0.59 ± 0.01	416.15 ± 14.78
Without Coccoliths	37.47 ± 2.91	0.18 ± 0.03	0.57 ± 0.01	555.3 ± 73.76

The onset of photoinhibition was also not greatly affected by removing the coccosphere (Figure 24), which occurred at approximately 600 µmol photons m^2 s^{-1} for both treatments which is half of what would be expected from the baseline chlorophyll fluorescence and O_2 light curve data (Figure 20 and Figure 22).

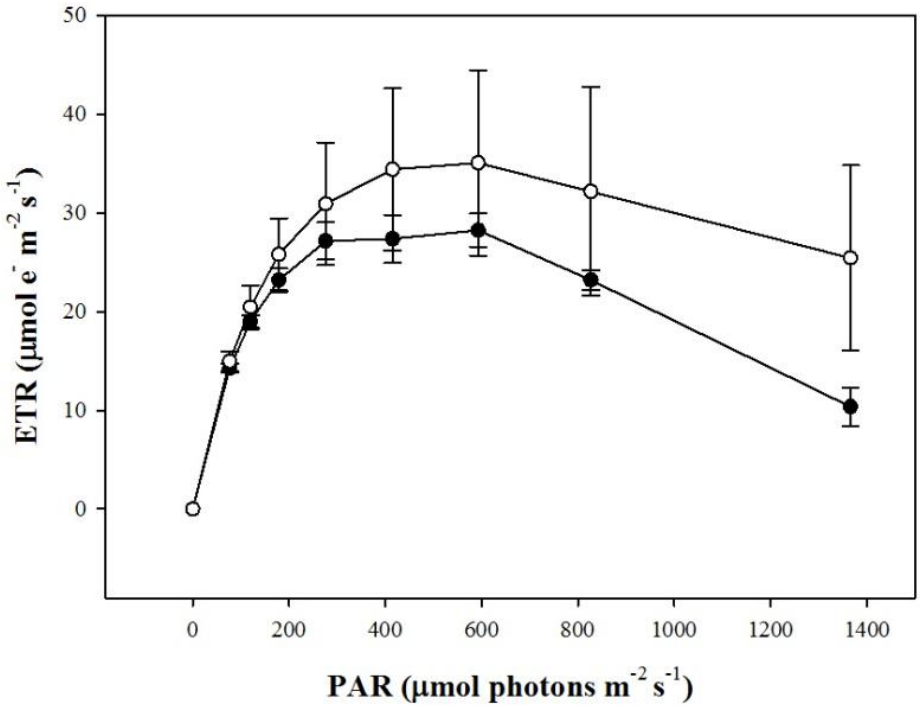

Figure 24 Effect of removing *C. carterae* coccosphere on photosynthesis Rapid Light Curve, ●= no coccosphere, ○= intact coccosphere. (n=15 ± s.e.).

3.2.4 Recovery of Culture after acidification

As the majority of experiments in this thesis are based on pH, it was necessary to determine the recovery of *C. carterae* CCMP647 after acidification.

Figure 25 shows some variation in the onset of photoinhibition with the reduction of pH, with photoinhibition occurring at lower irradiances as pH is reduced and this is reflected in E_k (Table 5). Photosynthesis in *C. carterae* at lower pH 5.5 and 7 both show the expected signs of early onset photoinhibition at an irradiance of 527 µmol photons m^{-2} s^{-1}, while at pH 9, photoinhibition does occur until approximately 800 µmol photons m^{-2} s^{-1}.

The recovery of *C. carterae* after reducing the pH to lethal levels (below pH 6) for 30 min can be seen in Figure 26. At highly acidic pH (5.5) there is no carbon assimilation however,

the same cells 1 hour later show remarkable signs of recovery, with the rate of assimilation into organic carbon rising to 3.01 ± 0.77 pg C_{ORG} $cell^{-1}$ h^{-1}. After acidification, light microscopy was used to monitor the cells, and after 1 day, the cells in culture had complete coccospheres.

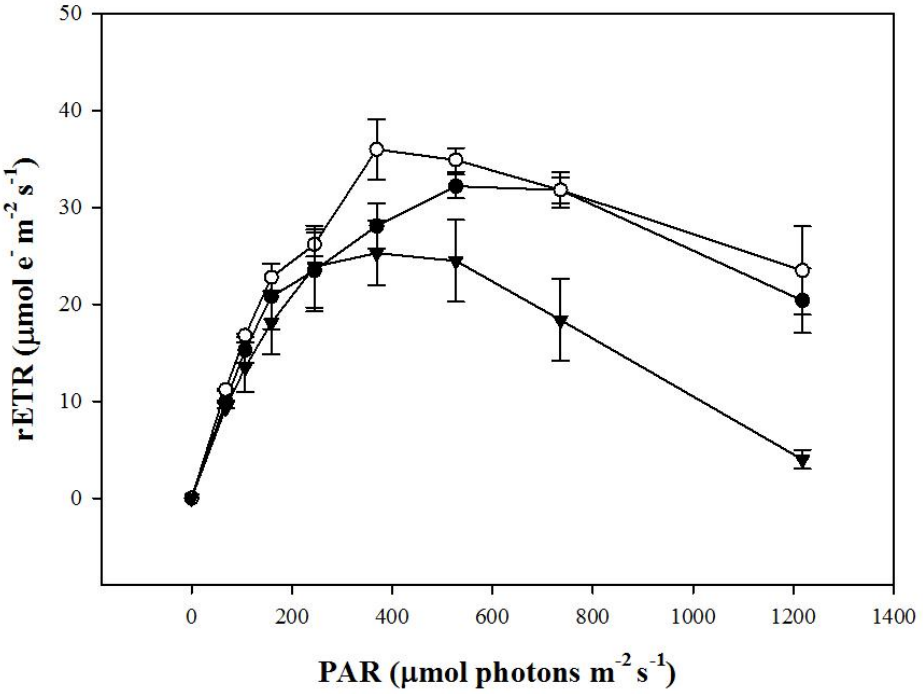

Figure 25 Effect of pH on *C. carterae* photosynthesis. pH reduced via CO_2 addition. (n=15, ± s.e.). ● pH 9, ○ pH 7.8, ▼ pH 5.

The effective quantum yield (Fq/Fm') is significantly different, (One way ANOVA F= 14.992, p value = <0.001) (Table 5).

Table 5 Effect of reducing pH via CO_2 addition on photosynthesis of *C. carterae* (n=15, ±s.e.).

pH	ETR_{max} µmol e^- m^{-2} s^{-1}	α	Ek µmol photons m^{-2} s^{-1}	Fq/Fm'
5.5	24.75 ± 0.85	0.18 ± 0.01	131.52 ± 7.98	0.35 ± 0.01
7.8	35.66 ± 0.74	0.18 ± 0.01	193.29 ± 12.34	0.42 ± 0.01
9.5	32.04 ± 0.64	0.16 ± 0.01	200.39 ± 15.67	0.38 ± 0.01

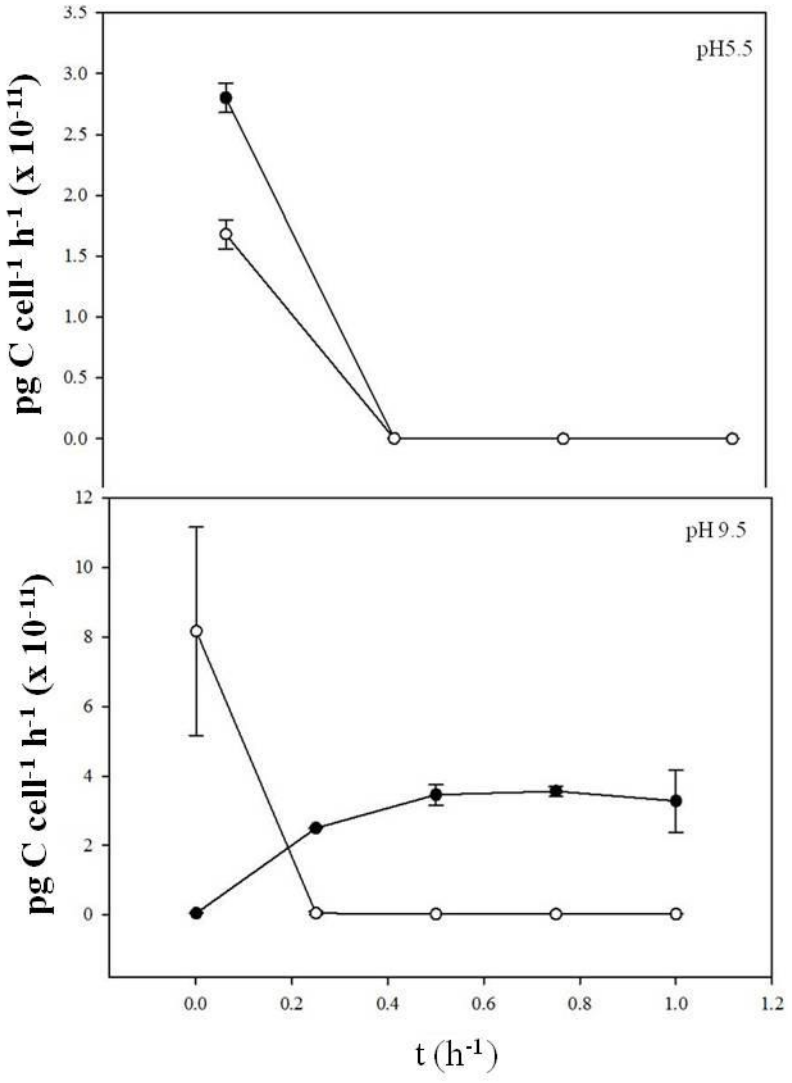

Figure 26 Carbon uptake of *C. carterae* after acidification of media with. ●= Organic carbon (C_{ORG}) uptake, ○= inorganic (C_{INORG}) carbon uptake. (n=9 ± s.e.).

3.2.5 Carbon Uptake

Carbon assimilation rates (Figure 27) were assessed in fully light adapted cells (2 hours into photoperiod) and dark adapted cells, sampled 2 hours into dark photoperiod. The carbon assimilation was determined over a five-hour period, with the light adapted cells being incubated with 300 µmol photons m^2 s^{-1} and the dark adapted cells being incubated with no light (a green lamp was used to provide enough light to work by). There is a significant difference in the rate of carbon uptake between in light and dark adapted cells for both organic ($t = 25.649$, $p= 0.0000137$) and the inorganic ($t = 11.407$, $p = 0.000337$) carbon uptake. The increase seen in the dark assimilation rates on Figure 27 after 4 hours is most likely due to radio labelled carbon ($NaH^{14}CO_3$) reaching equilibrium within the cell. This is where the amount ^{14}C added to the medium has become equal (both intra cellular and intracellular).

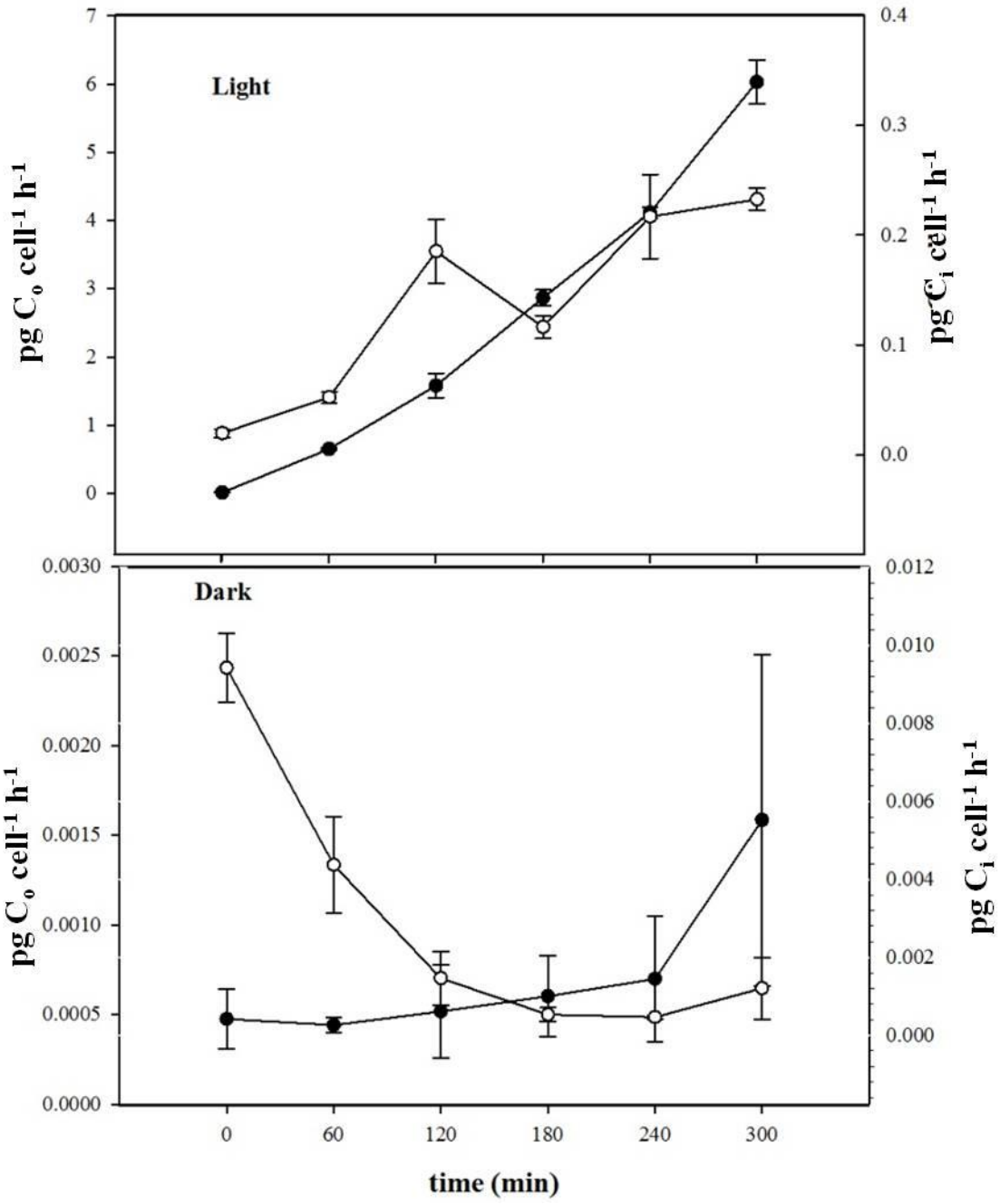

Figure 27 Light incubated (300 µmol photons m^2 s^{-1}, 2 hours into light photoperiod) and dark incubated (no light), 2 hours into dark photoperiod). Carbon uptake of *C. carterae* over 5 hours. ●= Organic Carbon uptake (C_{ORG}); ○= Inorganic Carbon uptake (C_{INORG}). (n=5 ± s.e.).

Table 6 Light and dark Carbon uptake rates and C:P ratio of *C. carterae* over 5 hours (n=5 ± s.e.).

Carbon	Light Rate pg C cell h^{-1}	Dark Rate pg C cell h^{-1}	C:P
Organic C	1193.57 ± 47.5	18.2 ± 0.13	0.035
Inorganic C	42.5 ± 3.85	-1.54 ± 0.34	-8.89

3.2.6 Selenium Requirements

3.2.6.1 Growth Rates

It has been shown that coccolithophorids have a growth requirement for selenium (1.4.3). Here the effect of Selenium (Se) addition to *C. carterae* culture was examined. Small tube photobioreactors and batch cultures were used for cultivation (see method section 1.7). The cells were kept in culture for a total of 11days, however due to the self-concentrating effect of the culture vessel, only data from 9 days are presented in Figure 28.

In selenium limited cultures the maximum cell density only reached a cell density of 36.1 x 10^4 cell mL^{-1} (Figure 28). This is 57% reduction in cell density compared to the selenium replete culture. The specific growth rates of *C. carterae* in the tube photobioreactor are also affected. There is an almost 10 fold reduction in growth rate (from 0.69 day^{-1} to 0.08 day^{-1}) between the cells grown with selenium and the selenium limited culture (Figure 29).

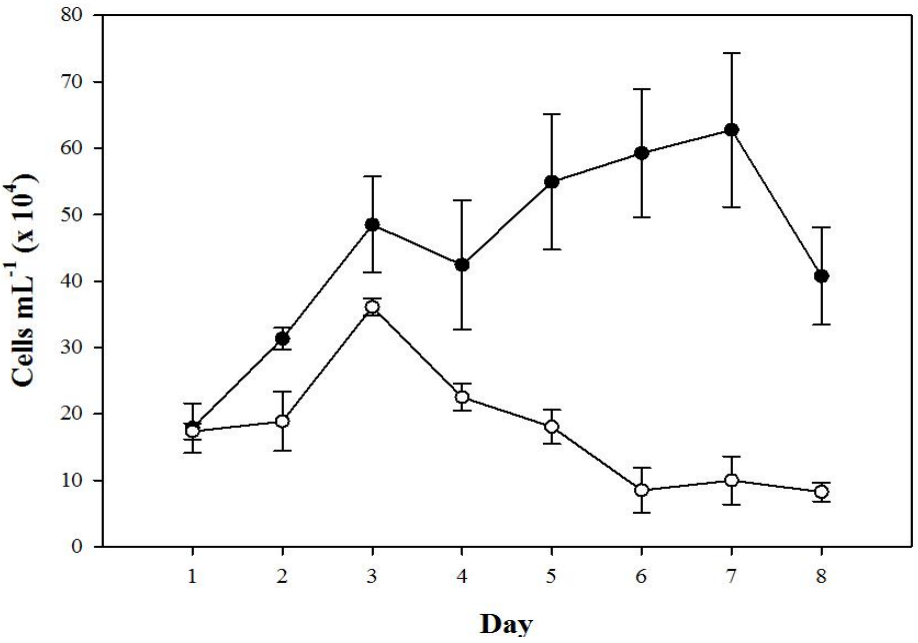

Figure 28 Growth curves of P. *carterae* from Se limited batch cultures. Total length of cells in culture was 11 days. Se depleted (○) and Se replete (●) cultures (n=15 ± s.e.).

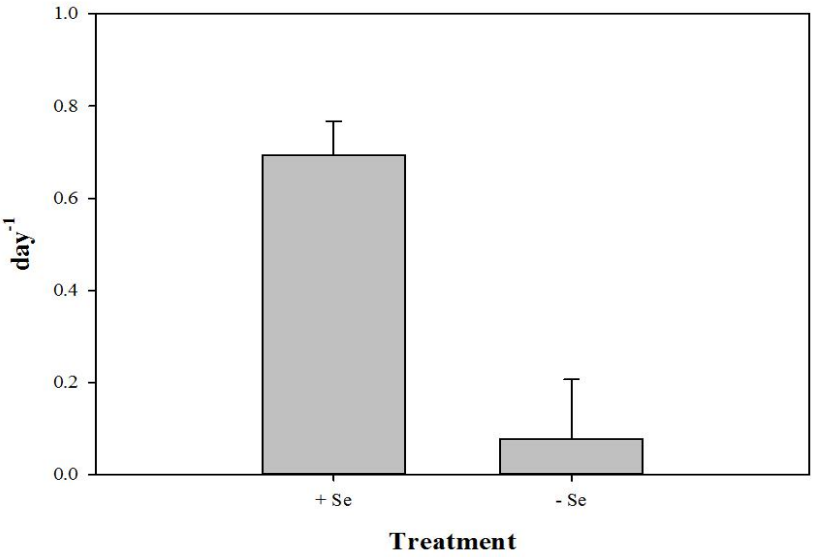

Figure 29 Effect of selenium limitation on specific growth rate (μ_{max} (day^{-1}) of *C. carterae* from tube reactor. (n=15 ± s.e.).

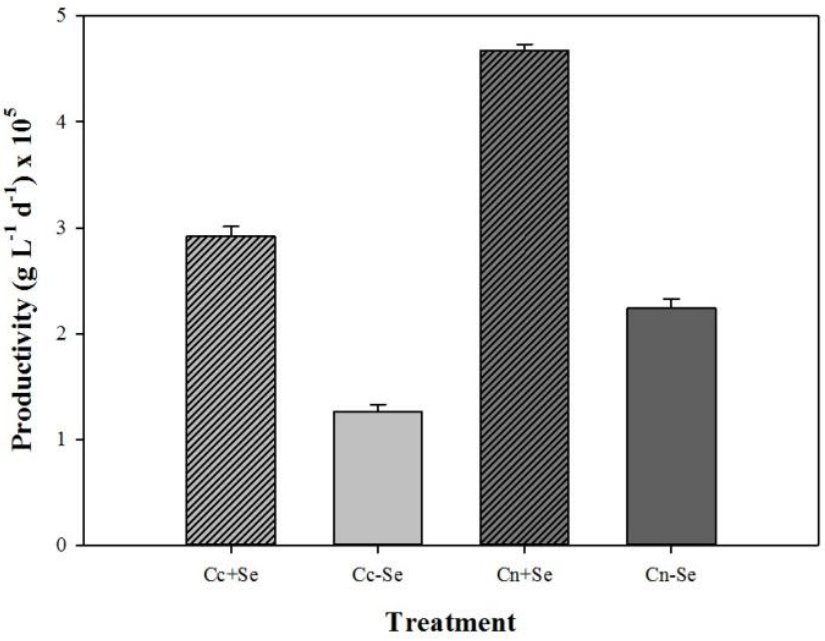

Figure 30 Biomass productivity of *C. carterae* after 4 days with and without coccoliths under selenium replete and limited culture conditions (Cn= incomplete coccosphere, Cc = healthy coccosphere, Cn -Se = cell culture with incomplete or lacking coccosphere. (n=15 ± s.e.).

There is a significant difference in the biomass productivity of *C. carterae* when grown without selenium (One-Way ANOVA F= 324.373 P= < 0.001) (Figure 30). The interesting data here is the large jump in biomass productivity in the cells that started with incomplete coccospheres (Cn) due to being cultured in medium that was low in Se (f/2 media using Western Australian Indian Ocean (WAIO) seawater). Here there is a significant rise in productivity (Tukey test, F= 521.393 P= <0.001) up 48% in the Cn cells with selenium added to the culture medium.

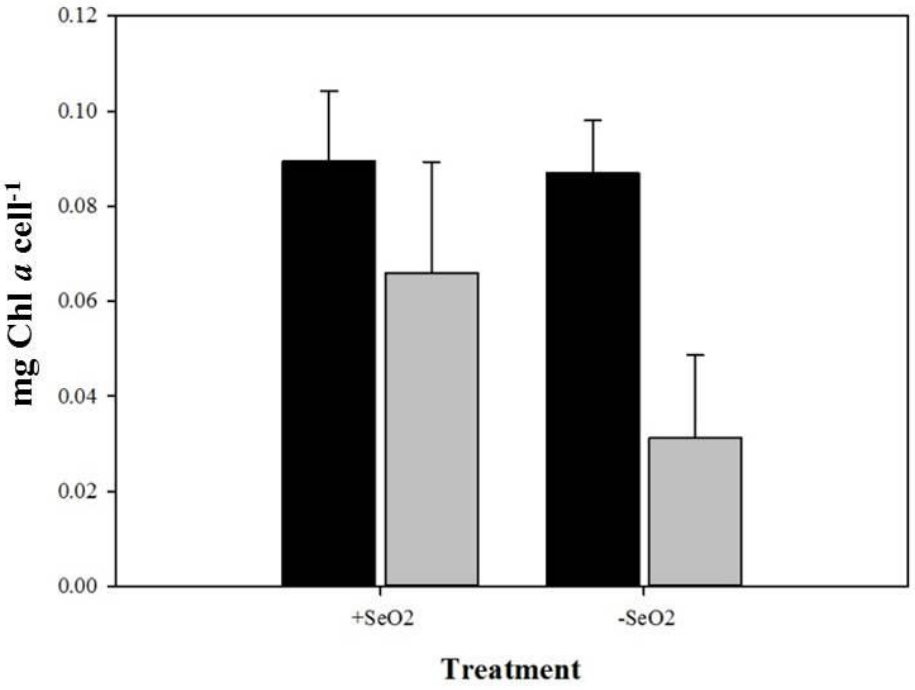

Figure 31 Effect of SeO_2 limitation of chlorophyll concentration on *C. carterae* after 11 days in culture. Black bar = Day 4, Grey bar = Day 11. (n=15 ± s.e.).

Chrysotila carterae (CCMP647) requirement for selenium is further demonstrated in Figure 31, where there is a significant reduction (t= 2.448, p= 0.040) in chlorophyll a concentration over time in the culture grown without selenium. *C. carterae* cells were subcultured from the regular modified f/2 medium and subcultured into PASW f/2 with and without selenium. After 4 days in culture the chlorophyll a concentration in the cells grown with selenium had not changed, while there is a significant reduction (55%) in the selenium limited cells (Tukey Test F=7.075 P = 0.003) falling from 0.08 ± 0.011 mg Chl *a* $cell^{-1}$ to 0.03 ± 0.007 mg Chl *a* $cell^{-1}$.

3.2.6.2 Photosynthesis

Data shown are for cells in log phase grown in semicontinuous culture vessels, as the cells were taken from the same culture (f/2 with SeO_2) day 0 samples were not taken. This was done to ensure that all cells were healthy with full, intact coccospheres.

There appears to be no real effect on photosynthesis (Table 7). The photosynthetic light harvesting efficiency (α) of PS_{II} showed no change when *C. carterae* cells were grown without selenium (t= 0.635, p=0.543). Fv/Fm of *C. carterae* showed no significant difference between the selenium limited and selenium replete medium (t= 1.21, p = 0.260).

Table 7 Effect of selenium dioxide limitation on PS_{II} photosynthesis in *C. carterae* samples were taken on day 4 and day 11 of experiment while all cells were in log phase. 15 min dark adaption. (n=15 ± s.e.).

Day	Treatment	Fv/Fm	$rETR_{max}$ µmol e^- m^{-2} s^{-1}	α	Ek µmol photons m^{-2} s^{-1}
4	+SeO_2	0.502 ± 0.007	72.1 ± 1.95	0.17 ± 0.014	919.53 ± 24.11
	- SeO_2	0.474 ± 0.021	71.42 ± 2.18	0.157 ± 0.014	1080.13 ± 39.95
11	+ SeO_2	0.46 ± 0.01	48.75 ± 1.68	0.19 ± 0.01	689.08 ± 40.92
	- SeO_2	0.42 ± 0.03	38.47 ± 1.8	0.14 ± 0.01	730.92 ± 61.27

3.2.6.3 Selenium and Coccolith Production

When selenium is omitted from *C. carterae* grown in batch cultures, a reduction in the coccosphere is observed usually after or 3 or 4 generations (3 days). Figure 32 shows the requirement of *C. carterae* for selenium in the formation of the coccosphere. Cells that had a healthy coccosphere (Cc) (as determined by light microscopy) and cells with poorly formed coccosphere (Cn) due to being cultured in WAIO seawater. There is the expected increase in coccolith production for both the cells with selenium and the selenium limited culture, however after 4 days; the cells that started without a full intact coccosphere had recovered

totally with fully formed coccospheres. While the total number of coccoliths is reduced in the Cn replete cells had the highest number of coccoliths.

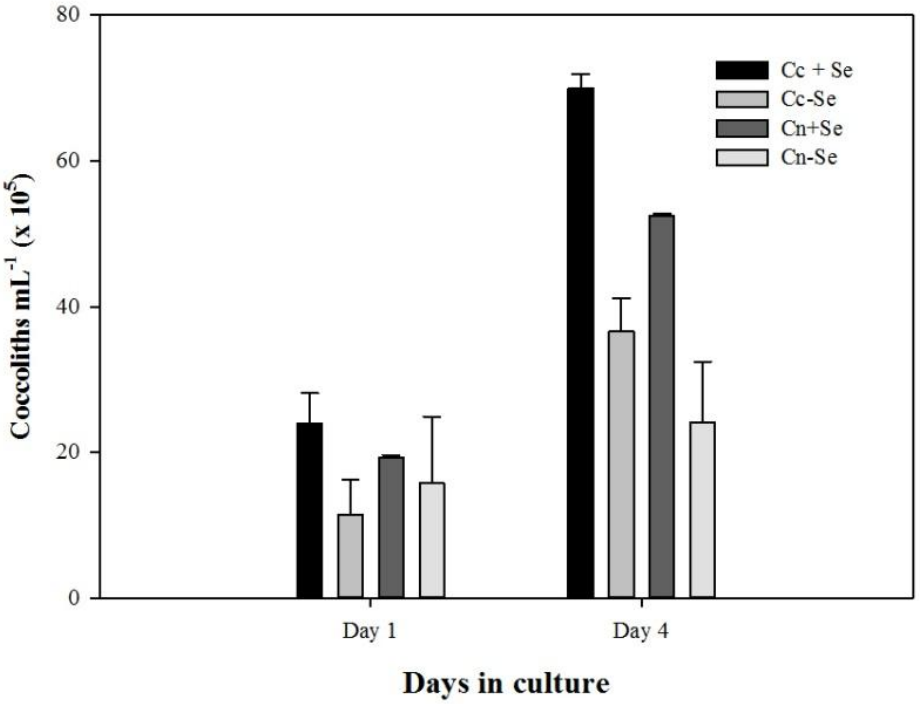

Figure 32 Effect of SeO_2 on total coccolith numbers over 4 days in *C. carterae*. Cc = cells cultured with healthy coccosphere at time of inoculation to experimental media. Cn = cells that have poorly formed coccosphere due to using medium which is low in Se (Western Australian Indian Ocean seawater). +Se = PASW f/2 with selenium dioxide, -Se = PASW f/2 without selenium dioxide. (n=6 ± s.e.).

DISCUSSION

Based on the outcome of these experiments, it is clear that *Chrysotila carterae* (CCMP647) has a significant requirement of selenium to produce coccoliths and form full, intact coccospheres. There is a wide range of marine phytoplankton that requires selenium for growth (for example see Harrison *et al.* 1988; Araie & Shiraiwa 2009). For the most part, selenium is required for the production of selenomethionine and selenocystein and for production of seleno-enzymes such as glutathione peroxidase (GSH-POD) (Boisson *et al.* 1995) which provides a protective function by reducing the damaging effects of hydrogen peroxide under low CO_2 and high light conditions. In cells without GSH-POD, the algae must use catalyse to decompose the H_2O_2 (Yokota *et al.* 1988). Doblin *et al.* (1999) have shown that dinoflagellates have high growth requirement for selenium, with greatly reduced growth rates on Se limited culture medium. These authors also showed that as the concentration of selenium is increased (up to 10^{-7}M Se) there is a significant increase in the chlorophyll *a* concentration in the dinoflagellate cells as well as increased growth rate. The relationship between selenium and chlorophyll is seen in *C. carterae* (Figure 31) where the chlorophyll *a* concentration is reduced when Se is omitted from the growth medium.

In regards to Haptophyte algae, Danbara & Shiraiwa (1999) and Obata *et al.* (2004) have shown that *Emiliania huxleyi* and *Gephyrocapsa oceanica* both have reduced growth rates when selenium is omitted from the culture media. This is also seen in *Hymenomonas elongata* by Boisson (1989), where the authors observed a reduced growth rate when the cells were grown with selenium limited cultures. There are also reports that *Chrysotila elongata* (as *Hymenomonas elongata*) increases the uptake of cadmium into the cell in response to elevated levels of selenite (Boisson *et al.* 1989; Boisson *et al.* 1995) although why the cells would have this pathway appears to be counterproductive as it has been found

that cadmium will interfere with the calcification process (Stillwell & Corum 1982) and reproductive rates (Brand *et al.* 1986) of *Chrysotila* (as *H. carterae*) and *E. huxleyi.*

In batch cultures of *C. carterae*, the growth rate was reduced by omitting selenium from the culture medium. It should be noted that the seawater used for all cultures of *C. carterae* came from the Western Australian Indian Ocean (WAIO), which has been shown to have very limited concentrations of selenium (Buttery 2000). While selenium is not usually associated with photosynthesis, (although selenate has been seen to reduce oxidative stress in *Spirulina platensis* (Wu *et al.* 2012)), it has been shown to be an important trace element for coccolithophorids (see Chapter 1 for review). Danbara & Shiraiwa (1999) have also have shown that there is a marked increase in oxygen respiration as well as cell yield, when selenium (as 10nM selenite) is added to the growth medium on *E. huxleyi*. Unfortunately, while we know that *Chrysotila* and most coccolithophorids have a growth requirement for selenium, the actual metabolic pathway eludes us at present and much more work is required to determine the exact pathways used by *C. carterae* and other coccolithophores.

As mentioned earlier, coccolithophorid algae play a vital ecological role and have high biotechnological potential such as CO_2 bioremediation, food production etc. (Moheimani *et al.* 2012). In regards to coccolithophorids other than *Emiliania huxleyi*, photosynthetic and primary production data is limited for many species such as *Chrysotila*, with *E. huxleyi* dominating the literature (for extensive reviews see: Paasche 1999, 2002; Ragni *et al.* 2008). The reduction in dark adapted rETR seen in *Chrysotila carterae* is consistent with other genera of microalgae (White & Critchley 1999) and in *Emiliania huxleyi* (Ragni *et al.* 2008). Net photosynthetic rates in *C. carterae* (Figure 22) are also consistent with those found by others (Israel & Gonzales 1996; Moheimani & Borowitzka 2006; Moheimani *et al.* 2011).

Photoinhibition is commonly seen in surface waters, where supraoptimal light conditions are prevalent usually at mid-day (Harding *et al.* 1982). High light can lead to a reduction in the photosynthetic rate, as PS_{II} antennae shut down (Raven & Beardall 1981; Falkowski & Raven 2007). In *E. huxleyi*, growth was not inhibited at irradiance above (800 photons m^{-2} s^{-1}) (Harris *et al.* 2005) which is well over the reported optimal light level (E_k) for many species of coccolithophorid (Paasche 2002; Brownlee *et al.* 2004).

It has been suggested previously that coccolithophorids are resistant to photoinhibition, possibly due to the coccosphere (Balch *et al.* 1996; Buitenhuis *et al.* 1999; Brownlee & Taylor 2004). Ragni *et al.* (2008) found that when *E. huxleyi* was cultured under low light (30 µmol photons m^{-2} s^{-1}) and high light (300 µmol photons m^{-2} s^{-1}) that there was no real change in photosynthetic efficiency (Fq/Fm') after 4 hours at well over 10 times the optimal growth irradiance, and only a slight reduction in Fq/Fm' at irradiances over 5000 µmol photons m^{-2} s^{-1}. Ragni *et al.* (2008) suggest that the resistance to observed photoinhibition is that during photoinhibition, inactive or damaged PS_{II} reaction centres (RC_{II}) are still able to transfer energy to the functioning reaction centres. The effect of this is that the PS_{II} antenna size is increased. Photoacclimation is usually heralded by a reduction in chl *a* concentration (Linschooten *et al.* 1991; Zou & Richmond 2000), and the chlorophyll to carbon ratio (Wilbur & Watabe 1963; Brewer & Goldman 1976), as well as the size of PS_{II} (Suggett *et al.* 2007). The size and number of reaction centres are also increased as light decreases (Falkowski *et al.* 1981). Photoinhibition to higher light levels has been reported in *C. carterae* (Israel & Gonzales 1996) and *E. huxleyi*, Moheimani & Borowitzka (2007) found that cells grown in shallow 16cm outdoor ponds had reduced growth rates at high seasonal irradiances which exceed 1800 µmol photons m^{-2} s^{-1}. These authors did not attribute this to photoinhibition however, they suggested that oxygen toxicity and higher temperature played

a role. In *C. carterae* CCMP647 the onset of photoinhibition occurs at approximately 1500 µmol photons m^{-2} $s^{-1}$for both chlorophyll fluorescence Rapid light Curves (Figure 20) and as net photosynthesis though oxygen production (Figure 22). This level of irradiance would be equivalent to a bright sunny day, or found though the lensing effect of surface water waves, thus indicating that in natural waters, *C. carterae* will become photoinhibited. The apparent photoinhibition seen in Figure 20 (occurring at approximately 1500 µmol photons m^{-2} s^{-1}) may be caused by an increase in oxygen due to high light; however, the time that the cells are exposed to the higher irradiances would not be long enough to see any effect.

Previously, the coccolithophorids are thought to be resistant to photo-inhibition (Buitenhuis *et al.* 1999; Brownlee & Taylor 2004). However it is now becoming apparent that the coccolithophorids are just as susceptible to photoinhibition as other phytoplankton (Nimer *et al.* 1991; Nimer & Merrett 1993; Israel & Gonzales 1996; Nimer & Merrett 1996; Paasche 2002). Ragni *et al.*(2008) found that *E. huxleyi* was photoinhibited at ocean surface irradiances (900-1100 µmol photons m^{-2} s^{-1}). Although it should be noted that the light that at which both of these coccolithophore becomes photoinhibited is much greater than for almost any other phytoplankton (for reviews see (Long *et al.* 1994; Baroli & Melis 1998; Marwood *et al.* 2000; Adir *et al.* 2003; Falkowski & Raven 2007). *Chrysotila*, like *E. huxleyi* does show signs of photoprotection of PS_{II} at high irradiances above 1800 µmol photons m^{-2} s^{-1} (Figure 20 and Figure 22). This evidence of photoinhibition may be due to the low cell number, but it is more likely to be a result of negative effect of high oxygen concentration in the medium (Moheimani *et al.* 2011). When algal cells are cultured under high irradiance, several protective mechanisms can come into effect, photoacclimation, photoprotection and photorepair. Photoacclimation involves the modification of the light harvesting reaction centres over time to optimize photosynthesis, and is often accompanied by a reduction in

cellular chlorophyll concentration (Falkowski & Raven 2007) and chlorophyll to carbon ratios (Geider *et al.* 1997; Ragni *et al.* 2008). As well as several changes to the photosynthetic unit (PSU) antenna sizes and size and number of reaction centres and antennae (Suggett *et al.* 2007). Photoprotection mechanisms are those that prevent or reduce damage to the photosystems from excess light. Photoprotection usually occurs at the light harvesting antennae of the PS_{II}, and one the more common ways is to remove excess absorbed excitation energy is as heat, The most common measurable mechanism is non photochemical quenching (NPQ) which is a function of both the reaction centres and light harvesting antennae (Falkowski & Raven 2007; Xu & Gao 2012). While damage to the photosystems is constantly occurring, when the rate of damage exceeds the rate of repair, photoinhibition occurs, usually at the D_1 protein found in PS_{II} (Platt *et al.* 1980; Grima *et al.* 1996; Kato *et al.* 2003). The D_1 protein binds the primary donors and acceptors of PS_{II} electron transport (Adir *et al.* 2003). When exposed to high light (>1500 µmol photons m^{-2} s^{-1}) the result is usually an increase in photoprotection and increased oxygen production that will result in photorespiration (Vonshak & Guy 1992; Vonshak *et al.* 1996). Both processes will result in a loss of culture productivity and possibly the loss of the culture. The observed photoinhibition in may also be due to increased photosynthetically produced oxygen concentrations.

There is strong evidence to suggest that photosynthesis in *Chrysotila* is susceptible to high oxygen concentrations (Israel & Gonzales 1996; Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007; Moheimani *et al.* 2011). The response of *C. carterae* to the negative effects of increased oxygen was investigated by Moheimani & Borowitzka (2007) and these authors showed that *C. carterae* may be able to photo-adapt to increased light in outdoor mass culture systems and adapt to increased oxygen with within a few days. It is important to

recognise that there are two possible mechanisms for the detrimental effects of increased oxygen in the culture medium;

a) One of the effects of high O_2 is increased competition between CO_2 and O_2 at the active site of Rubisco (Laing *et al.* 1974; van Lun *et al.* 2014. This will occur when CCMs are able to maintian the internal concentration of CO_2 a sufficient level to supress photorespiration.

b) High oxygen concentrations can often generate toxic free radicals such as singlet oxygen ($^1O_2$) or superoxide radicals (O_2^-) which have an equivalent toxic effect (Lesser 2006).

In mass microalgal cultures, photo respiration can be a major issue, as it can affect Rubisco activity. The amount of oxygenase activity of Rubisco will depend the species specific kinetics and the steady-state of CO_2 and O_2 concentrations (Falkowski & Raven 2007). When the culture has low CO_2 and high O_2, Rubisco will catalyse Ribulose 1,5 bisphosphate (RuBP) and 2-phosphoglycolate (Hough & Wetzel 1978; Falkowski & Raven 2007).

As summarised by Moheimani & Borowitzka (2007), there is a large amount of evidence to suggest that the oxygen reactions of dioxygen (O_2), and the highly reactive superoxide species ($O^{\cdot}_2$), (which is often associated with a breakdown of the D_1 protein in PS_{II}), Hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($1\Delta g\ O_2$). Morris & Kromkamp (2003) as well as many others have found that photoinhibition and photorespiration are temperature dependent reactions, with increases in temperature speeding up the oxygenase and PS_{II} activity.

In measurements of net photosynthetic oxygen evolution for both calcifying and non-calcifying cells of *Chrysotila*, Israel & Gonzales (1996) saw no photoinhibition at similar irradiances. However, in these experiments, pH was acidic, and thus increased pCO_2, which had been shown to increase the photosynthetic rate (Casareto *et al.* 2009; Moheimani & Borowitzka 2011) may have been a factor as the culture was grown a pH of 7.5.

Chrysotila carterae is well known to have significant levels of free coccoliths when in culture (Young *et al.* 1999; Paasche 2002; Young & Henriksen 2003), the ETR was measured to determine any error caused though light scattering by the coccoliths. From Figure 23 it can be seen that the coccoliths (free and attached) will cause the Water PAM to overestimate the electron transport rate. It must be considered however, that the physiology of the cells could have been adversely affected by the removal of the coccoliths which was done via mild acidification with 0.1M HCl, although no such adverse effects were observed by Israel & Gonzales (1996) using HCl to adjust the culture pH. To overcome the problems of the cells settling, the Water PAM ED unit was gently shaken on an orbital shaker to resuspend the cells in between light pulses. To prevent any signal noise in the photomultiplier, the shaker was stopped during the RLC light pulse.

3.2.7 Chrysotila carterae response to pH

Chrysotila carterae cannot survive for long periods at a pH of less than 7 (Moheimani & Borowitzka 2006), here it was shown that when the pH is reduced below 7 for a short time (15 minutes), that the cells are able to quickly recover to the starting pH via cell metabolism alone. This is a remarkable ability and one that is not common among the phytoplankton, There are of course species that will tolerate very low pH. Swift & Taylor (1966) reported a

strain of *Cricosphaera elongata* that was able to grow at pH 6.4 (Table 4) however at lower pH, there was a significant loss of motility in the cells:

Table 8 The relationship of pH and flagellation (cell motility), cell clumping and cell division rate in *Cricosphaera elongata* (Swift & Taylor 1966).

pH	% Flagellated (motile)	Clumps formed	Division rate (day^{-1})	Coccoliths present
5.8	0	+	0	**
6.4	46	+	0.34	-
7	81	-	0.53	-
7.3	83	-	0.59	+
7.5	**	-	0.68	-
7.8	99	-	0.76	+
8.6	86	-	0.7	-
9	90	-	0.62	+

There are a few other species that are able to tolerate low pH for example *Cylindrotheca* (growth at pH 5.9) and *Nitzschia closterium* (with a growth pH range of 5.9 - 6.3) (Humphrey 1975). Paasche (1964) reported a strain of *Emiliania huxleyi* that was able to grow at pH 6. This strain of *E. huxleyi* is interesting, as above pH 7.5 photosynthetic carbon assimilation was reduced. Of those species that can tolerate pH levels below 7, many will have reduced growth rates and or reduced photosynthetic rates (for a review of marine phytoplankton growth at reduced pH see Hinga (2002)).

The ability of *C. carterae* to recover from such low pH levels may be due its affinity for CO_2 as a carbon source. While Herfort *et al.* (2002) found that *E. huxleyi* uses bicarbonate as a carbon source, there is evidence to suggest that *C. carterae* prefers CO_2 (Moheimani & Borowitzka 2011). As such, the increased pCO_2 may provide the cells the necessary boost to

recover from short periods at low pH. Israel & Gonzales (1996) found in that in response to increasing external inorganic carbon that the $K_{0.5}(CO_2)$ photosynthetic oxygen production at low pH (5.5) was 57 µM, 0.58 mM at pH 7.5, and 0.55 mM at pH 8.5 for a high calcifying strain of *Chrysotila*.

While reduced growth rates and photosynthesis have been seen in *Chrysotila* at low pH, Israel & Gonzales (1996) hypothesised that the reduction in photosynthesis was as a result of substrate competition, CO_2 vs. O_2 for the active sites of Rubisco. This was confirmed when the authors added inorganic carbon to cultures approaching 280µM O_2. Here they observed rapid increase in photosynthesis. The same effect could be achieved by reducing the pH (thus increasing CO_2) but only in a closed system.

The increase in Fq/Fm' at pH 7.8 may be due to the amount of CO_2 available for photosynthesis. The higher pCO_2 has been shown to increase the photosynthetic rate of *C. carterae* quite significantly (Casareto *et al.* 2009; Moheimani & Borowitzka 2011).

At pH 5.5, in low calcifying strain of *Chrysotila* (CCAP961/3), Israel & Gonzales (1996) found increased net photosynthetic rates (as O_2 production) when compared to a high calcifying strain (CCM299). There was an approximate 30% increase in net photosynthesis between the two strains and an overall increase when compared to pH 8.7, providing further evidence of an affinity for CO_2.

Organic and Inorganic carbon assimilation rates and subsequent photosynthesis: calcium carbonate production (C:P) of *C. carterae* CCMP647, production is similar to those found by other researchers.

Dark carbon fixation is required for the "anaplerotic reactions". These reactions replenish intermediates in the metabolic cycles such as those in the tricarboxylic acid cycle or when drawn upon for anabolism or to allow competing pathways such as glycolysis and gluconeogenisis to function at the same time within the cell (Falkowski & Raven 2007).

Without light to drive photosynthesis the carbon uptake in the dark will rapidly decline, however in Figure 27 we see a gradual increase in the rate of dark carbon fixation for the first 4 hours, then a rapid doubling after 5 hours. This dramatic increase maybe contributed to the low cell number present in the incubation chamber at the end of the experiment and the radioactive label entering the cell though diffusion; however, the increase in dark carbon fixation in *C. carterae* is most likely as a result of the anaplerotic reactions.

From this work there are several avenues of inquiry that need to be perused:

1. The role of selenium needs to fully investigated, with emphasis on the metabolic pathways involved in coccolith formation, extrusion and adhesion to the cell wall.
2. Investigate the metabolic processes and pathways that allow *C. carterae* to increase the pH of the culture medium.

4 DIURNAL PHYSIOLOGY

4.1 Introduction

Many species of microalgae have diurnal paters of growth and photosynthesis (Sukenik *et al.* 1991; Flynn *et al.* 1993; Camacho Rubio *et al.* 2003). Previous work on the coccolithophorid *Chrysotila carterae* has noted a strong diurnal pattern in photosynthesis and coccolith extrusion as well as up to a 50% reduction in biomass during the night (Crenshaw 1964; Moheimani & Borowitzka 2011). Cellular weight can also fall from an approximate dry weight of 4 µg cell^{-1} 1 hour after dark to 2 µg cell^{-1} just before sunrise in outdoor raceway ponds, with a very similar pattern occurring in a closed photobioreactor constant temperature (Moheimani *et al.* 2011). It has also been reported that *Chrysotila* extrudes coccoliths in the later stages of the dark cycle (Moheimani & Borowitzka 2007).

Almost all microalgae have diurnal changes in medium pH when in mass culture. Among the coccolithophorids, *Gephyrocapsa oceanica* and *Chrysotila carterae* have displayed diurnal changes in culture pH caused by cell metabolism, however this is not seen in *E. huxleyi* (Moheimani *et al.* 2011). In *E. huxleyi* and *G. oceanica*, there is a net increase in culture pH over time. *Chrysotila* is rare in that when *C. carterae* is cultured in an unregulated pH medium, is that while there are diurnal increases in media pH, there is no net increase in culture pH. This species has been observed to steadily increase the pH of the culture medium during the light phase to unusually high levels for eukaryotic algae. pH levels of 9.2 - 10 have been observed in dense cultures of *C. carterae* (Crenshaw 1964; Moheimani 2005) during the day. This is not

unusual as there are several microalgal genera that can tolerate such high pH levels, *C. carterae* has the unique ability to return to the original "starting" pH and not have a net increase in culture pH (Moheimani & Borowitzka 2007; Moheimani *et al.* 2011). This may be one of the reasons why it is possible to culture *C. carterae* on a large scale without addition of CO_2, as the pCO_2 will remain high during dark respiration when the pH falls to a reported average low pH of 7.5 (Moheimani & Borowitzka 2007).

Several authors have reported changes to media pH via cell metabolism and have also observed a reduction in the pCO_2 levels several hours into the light cycle (Crenshaw 1964; Moheimani 2005). In unregulated pH outdoor cultures Moheimani & Borowitzka (2011) reported almost a 100% reduction in pCO_2 from the start of the light cycle (morning) to late afternoon. Here the pCO_2 fell from 267.3 µatm (pH 8.3) to 2.6 µatm (pH 9.5). In regulated pH cultures the drop in pCO_2 is not as significant, with a 76% fall at pH 8, (603.2 to 460.6 µatm) and 61% fall in pCO_2 at pH 7.7 (1350.2 to 834.6 µatm). *E. huxleyi cultures* however remained relatively constant, with only minor falls in pCO_2. Crenshaw (1964) also saw a drop in pCO_2 from 2.3mM to 1.4mM in a strain of *E. huxleyi*. This lead Crenshaw to hypothesis that the pH changes were due to a release in inorganic carbon (DIC) though photorespiration; this is where Hydrogen ions and hydroxyl ions are produced and consumed though respiration. While there are many genera of microalgae are able to change the pH of the culture medium though photorespiration, this usually results in a net increase in the pH of the culture medium. *Chrysotila carterae* is among one the very few microalgae species that will change the external pH of the culture medium, without a net increase in medium pH (Crenshaw 1964; Moheimani & Borowitzka 2006;

Moheimani & Borowitzka 2007; Moheimani & Borowitzka 2011). This ability of *Chrysotila carterae* (CCMP647) to change the medium pH with no resulting net increase in pH over time may be one of the reason why this species can thrive in mass culture systems without elevated levels of CO_2 (Moheimani & Borowitzka 2011).

Like most other phytoplankton, *Chrysotila carterae* (CCMP647) also displays other circadian traits, it has synchronised cellular division, dividing only once per day usually late in to the dark photoperiod, and a diurnal pattern to the biomass of the cells, with cellular dry weight decreasing during the dark hours and the mitotic ration rising dramatically shortly into the dark cycle (Moheimani 2005; Moheimani & Borowitzka 2007). The results from Moheimani (2005), showed the maximum number of coccoliths being extruded from the cell approximately one hour before the start of the light cycle. There may however, be an overestimation in the diurnal coccolith data from Moheimani (2005). The method used to extract the coccoliths showed both internal and external coccoliths (those already extruded and those still in the coccolith vesicle). As such, this may cause an error in determining the time of coccolith production. Coccolith production and extrusion requires a high amount of energy from the cell, as such it would be assumed that although formation of the coccosphere was observed late into the dark cycle, it is energy derived from photosynthesis that drives coccolith production. If this is the case then a corresponding shift in medium pH should also be observed. This series of experiments will investigate the effect of daily pH change and how it affects carbon assimilation and will show that while the coccoliths are extruded from the coccolith vesicle late into the dark cycle that they are formed during the light phase.

RESULTS

The diurnal pattern in *Chrysotila carterae* can be seen in the changes in medium (f/2) pH throughout the day (Figure 33). During dark respiration there is a dramatic decline in culture pH, with pH falling from an average recorded pH of 9.8, to a minimum of pH 7.8. This cycle is repeated at the onset of the light phase, and no significant net increase in medium pH is observed. As there is such a large change in medium pH, there will be changes in the amount of CO_2 in the medium which should have an effect on photosynthesis.

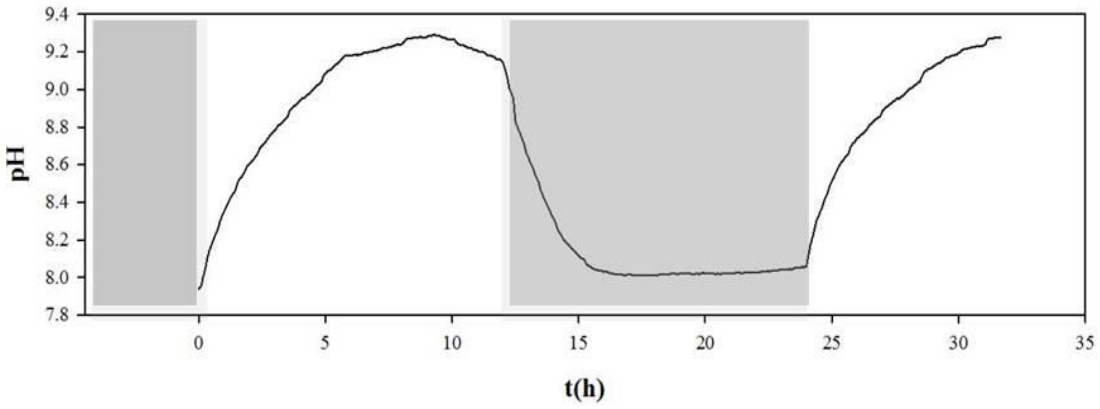

Figure 33 Representative diurnal pH pattern for C. carterae in unregulated culture pH over 24 hours. Shaded area indicate dark period. Time is in hours from start of light cycle (0600-1800)

4.1.1 Photosynthesis

Figure 34 shows the rate of oxygen production in *C. carterae* over a 12 hour period. There is a significant increase in photosynthetic oxygen production with the maximum O_2 rate of 1884.72 ± 87.02 µmol O_2 mg Chl *a* h^{-1} occurring 6 hours into light cycle (12:00) (One Way ANOVA F=41.89 P = < 0.001). The high rates of photosynthesis were not sustainable though out the light period, and fall to 275.87 ±

10.72 µmol O_2 mg Chl *a* h^{-1} at 17:00 (11 hours into light cycle). The 09:00 measurement is anomalous and this pattern is observed throughout the dataset, including PAM and carbon assimilation measurements, and as yet cannot be explained.

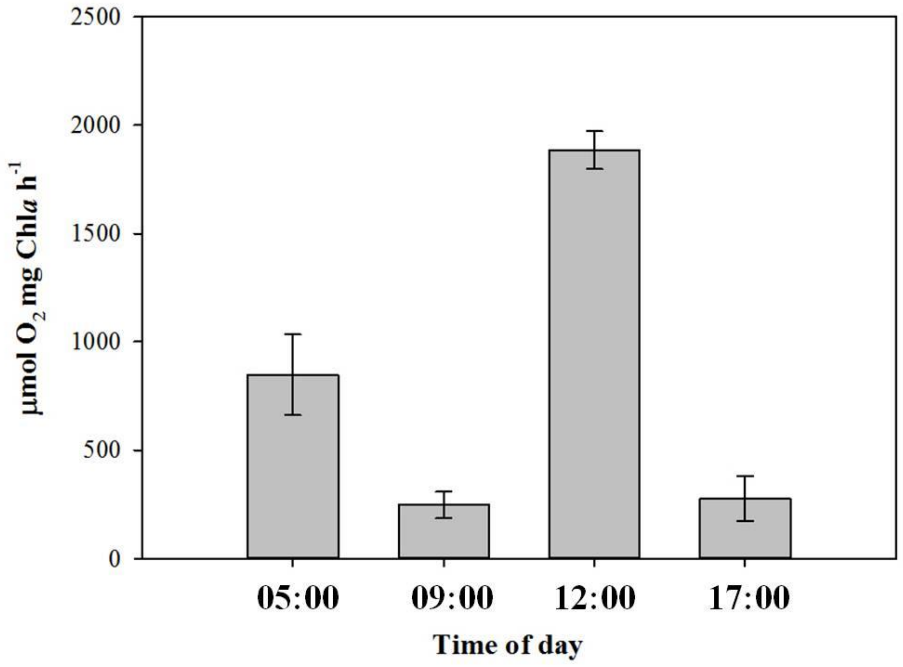

Figure 34 Diurnal photosynthetic oxygen evolution in *Chrysotila carterae* over a 12 hour period. Light period is 06:00-18:00. n=15 ± s.e..

Once again the low photosynthesis measurements are observed 3 hours into the light period. The linear increase in light phase oxygen production is mirrored in the chlorophyll fluorescence (PAM) data for both controlled laboratory conditions and outdoor cultures (Figure 35 & Figure 36). Here the maximum electron transport rate (ETR_{max}) rises steadily from 77.0 ± 8.34 µmol e^- m^{-2} s^{-1} 1.5 hours into light phase, to a peak of 84.616 ± 6.4 µmol e^- m^{-2} s^{-1} (Figure 35a). As a result, the rate of

photosynthesis in the controlled culture also has a linear increase (Figure 35b). The maximum quantum yields (Fv/Fm) (Figure 35c) of *C. carterae* have a very similar pattern to the oxygen exchange rates seen in Figure 34. This correlation is also seen in the Fv/Fm of the outdoor cultures (Figure 36c), although the similarities are not as pronounced.

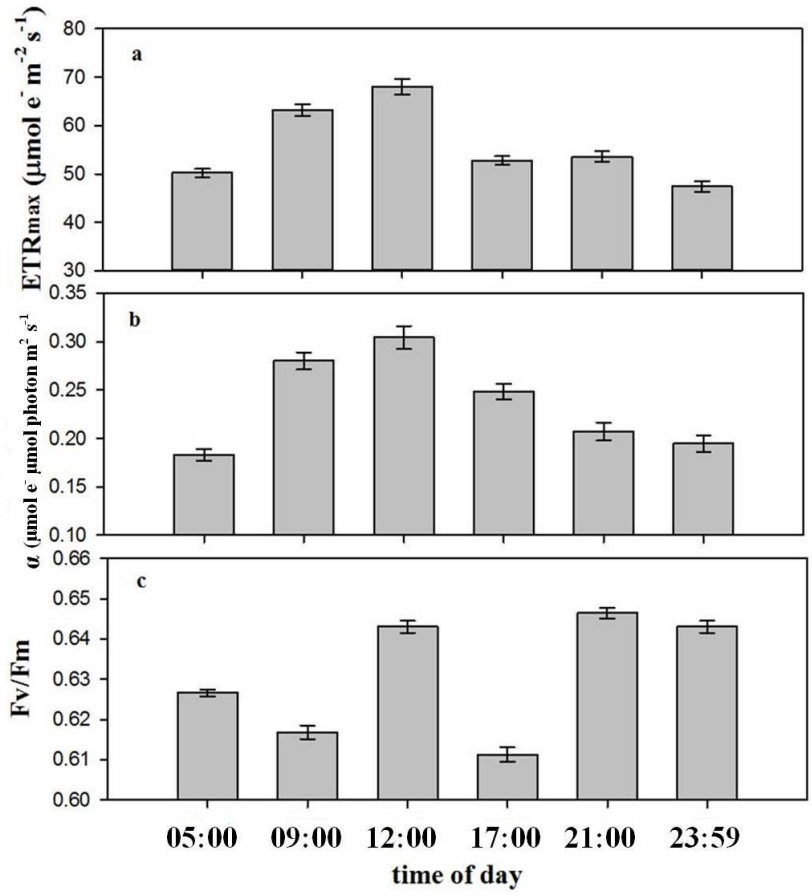

Figure 35 Diurnal photosynthetic response of *C. carterae* in laboratory controlled cultures (3L plate photobioreactor) (Irradiance = 300 µmol photons m^{-2} s^{-1}) on a 12:12 LD cycle commencing at 0600. With 15 min dark adaption. n=15 ± s.e..

The outdoor culture fluorescence data is much more variable, maximum electron transport rates were higher than that of the laboratory batch culture with a peak of 84.61 ± 6.40 µmol e^- m^{-2} s^{-1} to a low of 51.1 ± 4.0 µmol e^- m^{-2} s^{-1} at 04:00. This is 20% higher at 12:00 and 12% higher than the indoor culture at 17:00. Fv/Fm (Figure

36c) of cultures grown in the plate reactor and outdoor raceway ponds show a very different pattern. *Chrysotila carterae* photo-conversion in outdoor raceway ponds has a very similar pattern to the laboratory cultures, however the Fv/Fm is significantly reduced (Repeated Measures One way ANOVA $F = 18.843$; $P = < 0.05$). There is up to 39% reduction in photochemical efficiency at 12:00 and 75% reduction at 17:00.

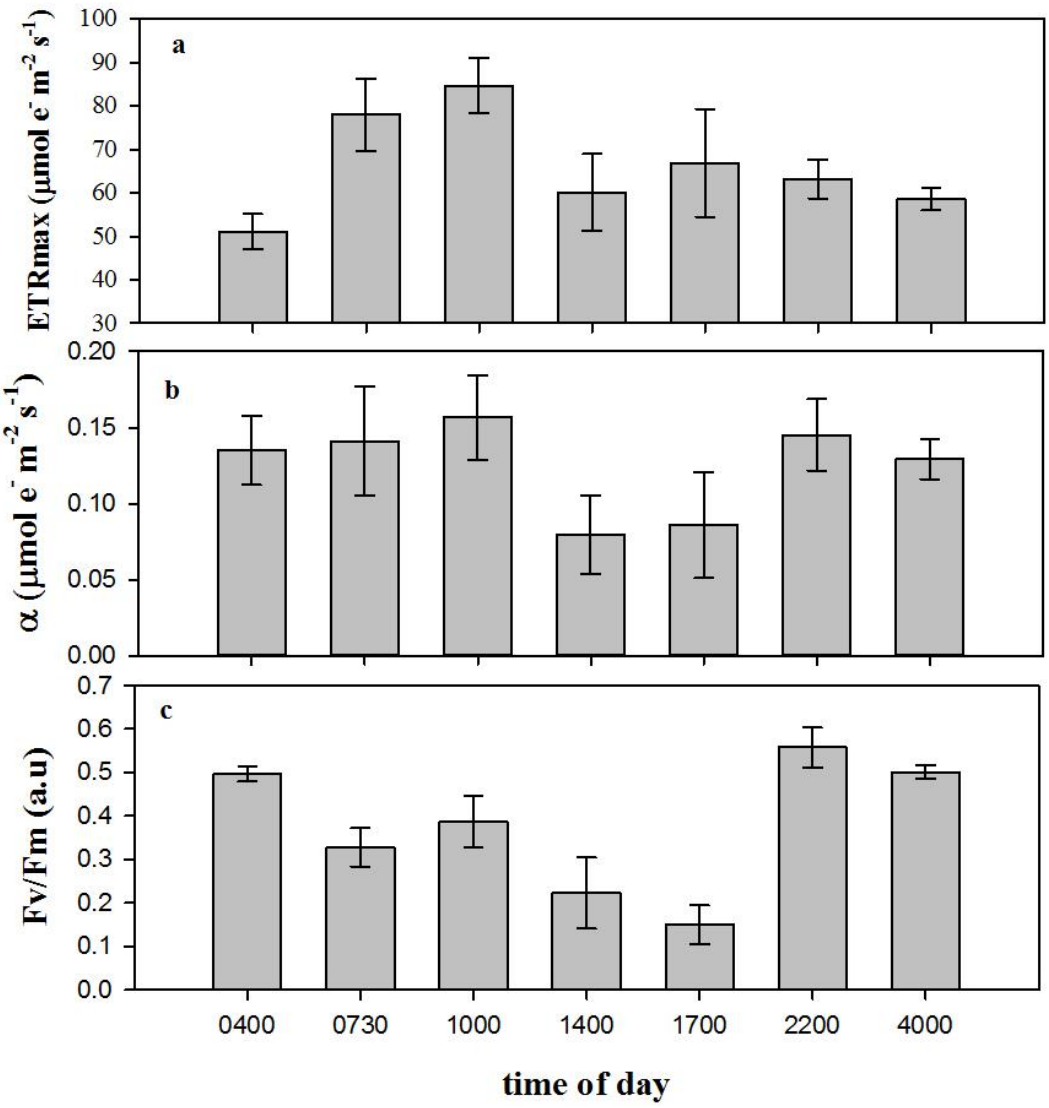

Figure 36 Photosynthetic response of *C. carterae* over 24 hour period in an outdoor raceway pond with natural variable solar irradiance. With 15 min dark adaption. (n=15 ± s.e.).

4.1.2 Carbon Assimilation

The rates of carbon fixed into organic carbon (C_{ORG}) (Figure 37) mirror the diurnal oxygen rates, with the maximum rate of organic carbon assimilation occurring at 12pm (One way ANOVA, Tukey Test F = 27.075 P = < 0.001). Inorganic carbon fraction (calcification) showed a similar pattern; with significant differences

throughout the day (Tukey Test; One Way ANOVA F = 9.853, P = < 0.005). However, there was no significant difference in the rates of uptake into inorganic carbon (C_{INORG}) between at 9am and 12pm.

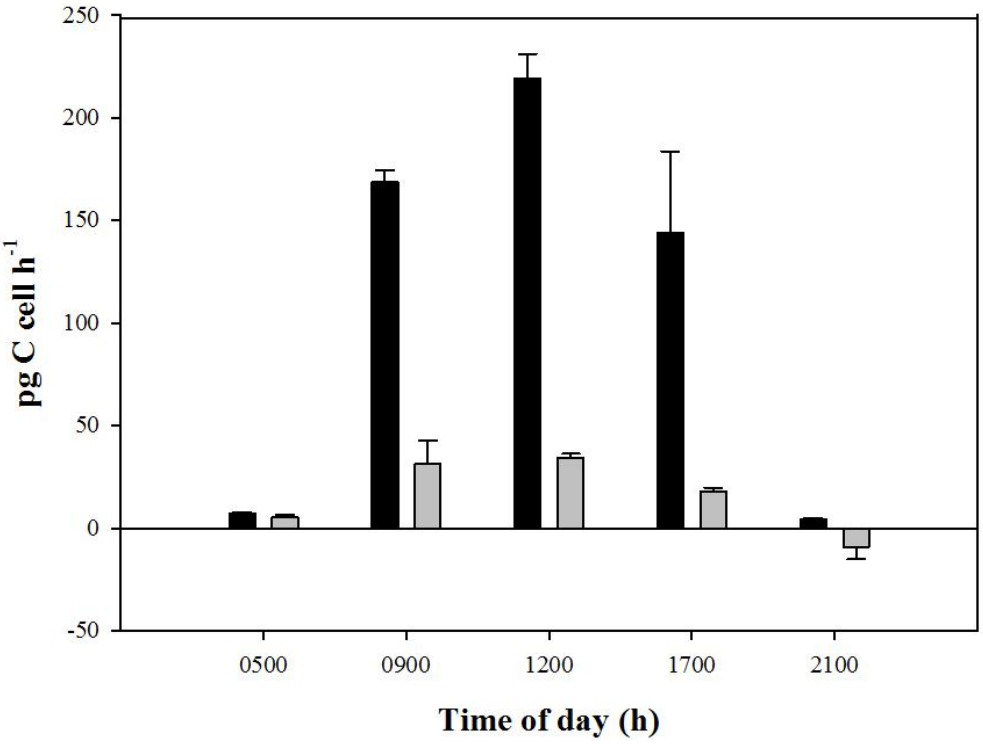

Figure 37 carbon assimilation rates for *Chrysotila carterae* over an 18 hour period. 12 hour L:D cycle commencing at 0600. Dark bars = Organic carbon uptake; Light bars= Inorganic carbon uptake. (n=9 ± s.e.).

4.1.3 Calcification:Photosynthesis (C:P) ratio

The highest C:P ratio was seen one hour before the start of the light cycle at 05:00 (Figure 38), here, the rate of organic carbon and inorganic carbon assimilation (the majority of which will be as $CaCO_3^-$) is almost 1:1 (0.744), this is significantly higher than C:P ratio at 9am (3 hours into light cycle) (t= 4.538 P = 0.0226). After the onset

of the light cycle, the C:P ratio begins to show a linear decline (r^2 = 0.987, P value = < 0.001) to a low of 0.124 at 17:00 (11 hours into light cycle of 12:12 L:D cycle).

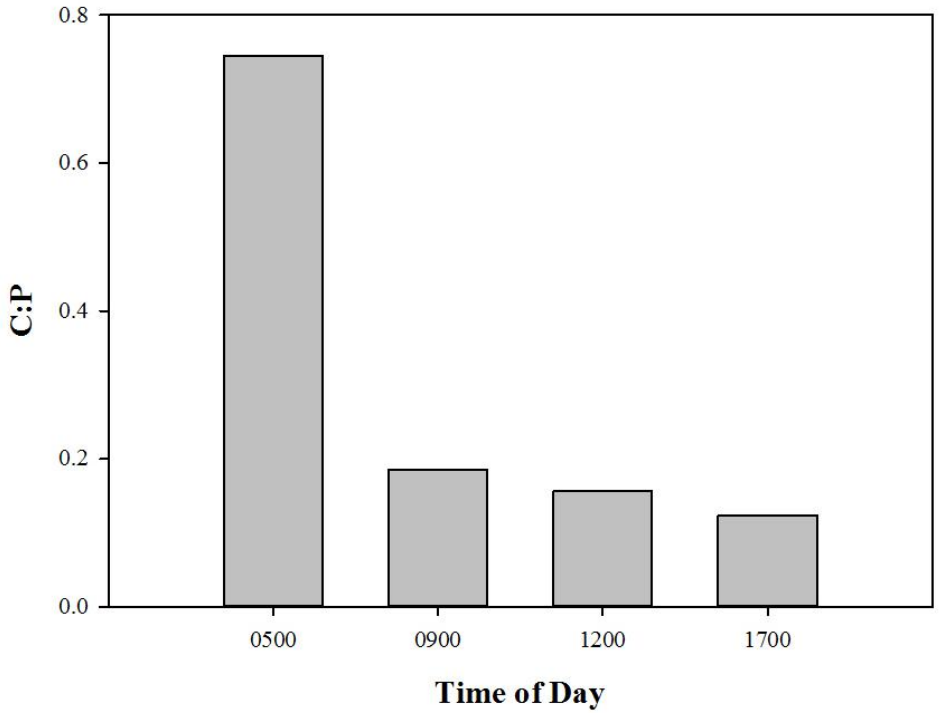

Figure 38 Calcification to Photosynthesis (C:P) ratio for *C. carterae* over 12 hour period commencing 1 hour before start of light period. Due to limitations in methodology the 21:00 sample was removed (Refer to Figure 37). (n=9 ± s.e.).

At 9pm (3 hours into the dark cycle), there is an anomalous measurement. Here the rate of inorganic carbon assimilation becomes negative, with organic carbon at 4.52 pg CORG $cell^{-1}$ h^{-1}, much lower than 17:00, with inorganic carbon assimilation at -9.42 pg C_{INORG} $cell^{-1}$ h^{-1}.

4.2 DISCUSSION

The high net photosynthesis readings observed in *Chrysotila carterae* (CCMP647) between 5am and 12pm (Figure 34) is interesting. The expected pattern would be to have a much lower photosynthetic rate at 12 pm than 9 am due to photosynthesis induced oxygen toxicity (Moheimani *et al*., 2006; Moheimani *et al.,* 2011). However, the fact that *C. carterae* has significantly higher photosynthesis at 12pm (which is a consistent pattern though all measurements) is hard to explain. The lower photosynthesis at 9am when compared to 5am can be explained by the negative effect of high O_2 on the photosynthesis of *C. carterae*, which follows a similar pattern to the carbon assimilation. While high net O_2 production at 05:00 (one hour before the start of the light cycle) could be explained by basic photochemistry mechanisms (i.e. all reaction centres of PS_{II} are open and fully oxidised). The reduction in the O_2 production rate at 09:00 is the interesting one, especially when the Fv/Fm of separately grown cultures under the same conditions (Figure 35c) has the same pattern as the high O_2. The subsequent decrease in the rates of photosynthesis after the start of the light cycle is possibly evidence of diel variation often referred to circadian rhythms (Hastings *et al*. 1961, Borowitzka, 2016). The first reports the diel effect of the light dark cycle on cellular process was by Hastings *et al.* in 1961. Since then it has been found that the rate of oxygen production in microalgae was elevated during the first 3 hours of the light cycle, with the cells maintaining this level of oxygen production for another 3- 4 hours (Kaftan *et al*. 1999, Borowitzka, 2016)

Other researchers have seen similar diurnal patterns in *C. carterae* cells; the work of Moheimani & Borowitzka (2007) saw a decline in *C. carterae* dissolved oxygen 3 hours into the light cycle due to a rapid increase in oxygen, with the result of

photosynthesis being reduced due to oxygen toxicity. The corroborating data from the chlorophyll fluorescence (Figure 35 and Figure 36) and carbon assimilation (Figure 37) support the validity of the data presented in Figure 34. The reduction in photosynthesis seen at 9am is likely due to oxygen toxicity. Moheimani & Borowitzka (2007) found a diurnal dissolved oxygen pattern in outdoor cultures similar to the data in Figure 34, here the Authors saw an sharp increase in DO as soon as light is available, and a small decline in DO a few hours after sunrise, before reaching maximum DO approximately 8 hours after sunrise. This all suggests that there is something very interesting going on and that a great deal more investigation is required to find out what is happening.

A working theory to explain the elevated oxygen production 1 hour (05:00) before the start of the light phase (Figure 34) may be due to the cells being fully dark adapted with reduced levels of Rubisco as they have depleted all energy reserves from coccolith extrusion. The reduction in O_2 evolution seen after 12:00 may be a negative result of high O_2. The rapid increase in gross photosynthesis is the result of the cells adapting to the higher O_2 concentration (Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007). Another, more likely theory is that while the cells begin extruding coccoliths during the dark cycle (Figure 39) (Moheimani & Borowitzka 2007) (which will require energy), by 12pm (6 hours into light cycle), the cells have completed coccolith extrusion and formation of the coccosphere, and are now able to achieve maximum photosynthesis. The reduction in oxygen evolution seen at 2pm (Figure 34) is now due to oxygen toxicity as reported in Moheimani & Borowitzka (2007). Further evidence of light induced oxygen toxicity is seen in the indoor

cultures, where low Fv/Fm and net photosynthesis rates in during light period are most likely due to oxygen toxicity (Moheimani & Borowitzka 2007).

There are two possible scenarios here, 1; the cells are using energy derived from photosynthesis to manufacture the coccoliths, or 2; as photosynthesis increases the increase in DO will bring on oxygen toxicity. The steady decline in Fv/Fm during the light cycle does indicate that reduced photosynthetic efficiency may be due to the increased oxygen concentration. Moheimani & Borowitzka (2007) showed that *C. carterae* is susceptible to oxygen toxicity at irradiance around 1200 µmol photons m^{-2} s^{-1} , however the cells grown in outdoor raceway ponds are less vulnerable to oxygen toxicity. When grown outdoors *C. carterae* has been shown to adapt quickly to high light conditions (Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007; Moheimani *et al.* 2012).

The carbon assimilation data (Figure 37) showing the negative inorganic carbon (C_{INORG}) assimilation rates seen at 2100, are most likely as a result of the declining coccolith concentration previously reported (Moheimani 2005; Moheimani & Borowitzka 2006) (Figure 39) and an increase in CO_2 through respiration. As a result of the increased CO_2, the coccoliths are possibly being dissolved and labelled carbon being gassed off, and thus giving a negative rate. It may also be possible that the increase CO_2 surrounding the cell may be dissolving some of the newly extruded coccoliths, and further testing will be required to determine if this is the case.

The reduction in the rates of inorganic carbon assimilation further supports the hypothesis that the coccoliths are being manufactured during the dark then extruded at night. At one hour before the start of the light cycle (05:00) the C:P ratio (Figure 38)

is almost 1:1(0.744), indicating that there is approximately one half of the carbon assimilation is going to organically derived products, and the other 50% is going to inorganic products (such as calcification). While there are limited data on the C:P ratio of *Chrysotila*, typical C:P values for other coccolithophores such as *E. huxleyi* are much lower (Table 10) with enhanced light and nutrient cycles the maximum C:P obtained was 1.52 by Herfort (2002). Average C:P for *E. huxleyi* is 0.99 (Nimer & Merrett 1992; Nimer et al. 1996). Berry et al. (2002) noticed that the C:P of *E. huxleyi* changed when exposed to variations in nutrient concentration, when under high concentrations of nitrogen and phosphorus, the C:P was greatly reduced (C:P = 0.02), this was reversed under low N and P. where the C:P was significantly greater (C:P = 0.8) (Table 10). C:P ratios often have a high level of variation between the coccolithophorids, and as Paasche (1964) noted, there is even a high level of variation not only between species but within different strains of *E. huxleyi* as well.

Table 9 Summary of calcification to photosynthesis ratios (C:P) showing the variation in different coccolithophorid genera.

Species	Strain	C:P ratio	Reference	Comments
C. carterae	CCAP961/2	0.04	Seki *et al.* 1995	C:P measured under various nitrate conditions.
	CCMP645	0.04	Farby 2007	C:P measured under several light conditions.
	**	0.70	Casareto *et al*. 2009	After 0 days.
	**	0.15	Casareto *et al.* 2009	After 7 days at ambient CO_2.
	**	0.25	Casareto *et al.* 2009	After 7 days at 1200ppm CO_2.
C. placolithoides	CCMP299	0.42	Israel & Gonzalez 1996	C:P measured with high calcifying strain.
E. huxleyi	SMBA279	0.66	Nimer & Merret 1992	
	88E	0.80	Nimer *et al.* 1996	Measured under various nutrient conditions.
	PCC.B11	1.51	Herfort 2002	Measured under various nutrient conditions.
	**	0.2	Berry *et al.* (2002)	High N, High P. pH effects also measured.
	**	0.8	Berry *et al.* 2002	Low N, Low P. pH effects also measured.
	CCMP371	0.56	Feng *et al.* 2008	375 ppm CO_2 @ 20ºC PAR 50 µmol photons m^{-2} s^{-1}
	CCMP371	0.51	Feng *et al.* 2008	750 ppm CO_2 @ 20ºC PAR 50 µmol photons m^{-2} s^{-}
	CCMP371	0.52	Feng *et al.* 2008	375 ppm CO_2 @ 24ºC PAR 50 µmol photons m^{-2} s^{-1}
	CCMP371	0.5	Feng *et al.* 2008	750 ppm CO_2 @ 24ºC PAR 50 µmol photons m^{-2} s^{-1}
	CCMP371	0.3	Feng *et al.* 2008	375 ppm CO_2 @ 20ºC PAR 400 µmol photons m^{-2} s^{-1}
	CCMP371	0.13	Feng *et al.* 2008	750 ppm CO_2 @ 20ºC PAR 400 µmol photons m^{-2} s^{-}
	CCMP371	0.32	Feng *et al.* 2008	375 ppm CO_2 @ 24ºC PAR 400 µmol photons m^{-2} s^{-1}
	CCMP371	0.18	Feng *et al.* 2008	750 ppm CO_2 @ 24ºC PAR 400 µmol photons m^{-2} s^{-1}
	F201	1.35 ± 0.11	Paasche (1964)	
	F401	1.39 ± 0.09	Paasche (1964)	
	F402	0.67 ± 0.03	Paasche (1964)	
	F422	1.22 ± 0.15	Paasche (1964)	

The data presented in Figure 37 are consistent with the findings of (Moheimani & Borowitzka (2011), here the authors found that there was a gradual decrease in the amount of external coccoliths seen immediately after the lights had been turned off (as coccoliths mL) (Figure 39). There is a gradual decalcification occurring at the beginning of the dark cycle due to CO_2 produced via respiration, the decalcification continues for 5 hours into the dark cycle then, the cells begin to calcify again. Throughout the night, there is a gradual acidification of the culture mediums though CO_2 produced via respiration (Crenshaw 1964; Moheimani & Borowitzka 2006; Moheimani *et al.* 2011).

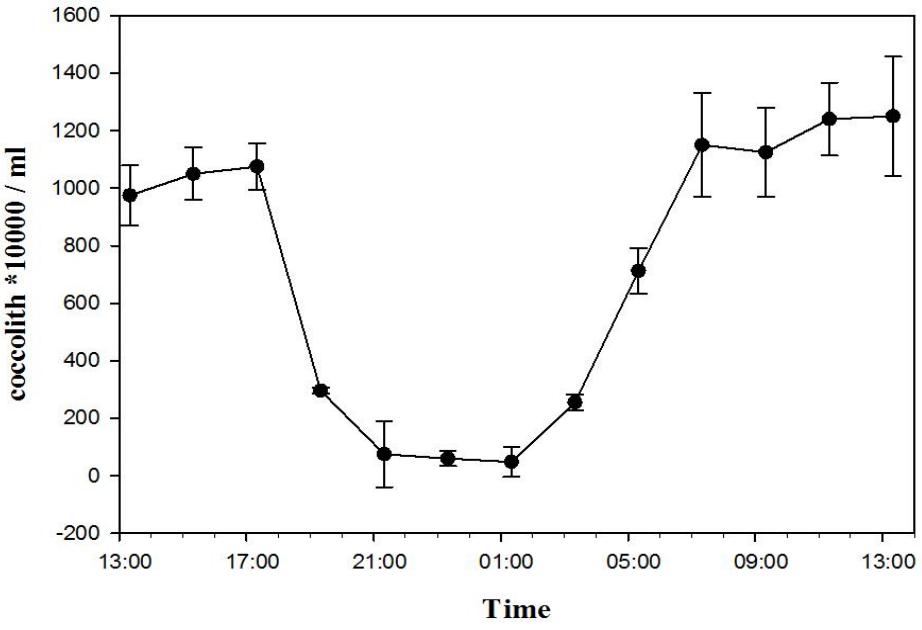

Figure 39 Number of coccoliths extruded by *C. carterae* over 24 hours. This data only shows total complete coccoliths extracted from the coccosphere (Moheimani & Borowitzka 2006).

From the carbon uptake rates results (Figure 37), we can assume that the coccoliths are being formed during the light cycle, and then extruded night.

The findings of Moheimani & Borowitzka (2006) showed coccolith extrusion begins during the later stages of the dark cycle (Figure 39). The method that these authors used (modified from Paasche (1964)) measured complete coccoliths, and not actual coccolith production (refer to section 2.3.7 for full description of method). The data presented in Figure 39 support the assumption that that coccolith production is occurring during the daylight hours (Figure 37), and that what Moheimani & Borowitzka 2006 saw was coccolith extrusion, where the coccoliths are transported out of the coccolith vesicle, onto the coccosphere towards the end of the dark cycle. As this process would require large amounts of energy, this may explain the high oxygen production at 05:00 (one hour before the onset of the light cycle).

5 EFFECT OF N SOURCE & pH ON GROWTH, PHOTOSYNTHESIS AND CARBON UPTAKE

5.1 Introduction

There is a significant amount of work on the nitrogen requirements of microalgae (for reviews see (Antia *et al.* 1991; Flynn 1991). Most of the work on the nitrogen requirements of the coccolithophorids has focussed on *Emiliania huxleyi* (Paasche 1964; Flynn *et al.* 1999; Paasche 2002). To date, there has been very little focus on the effects of nitrogen sources on other coccolithophores such as the coastal coccolithophorid *Chrysotila carterae.*

In general, for phytoplankton the order of preference of the species of nitrogen taken up is NH_4^+ ($NH_3 + H^+$) > Urea > NO_3^- (Flynn 1991). The ultimate fate of all nitrogen species taken up by cells is to be converted to ammonia for protein synthesis (Turner 1979; Young & Beardall 2003). The preference for ammonium as a nitrogen source is well documented (Flynn 1991, 2002) as is the ability of ammonium to inhibit nitrate uptake in phytoplankton (Eppley et al. 1969). The preference of coccolithophorids for NH_4^+ and to some extent urea over NO_3^- in *E. huxleyi* is also well documented (Fernandez *et al.* 1993; Kristiansen *et al.* 1994; Head *et al.* 1998; Rees *et al.* 2002). Interestingly, Strom & Bright (2009) showed that in some strains of *E. huxleyi*, that there was a preference for urea over ammonium. To date information on the effect of other nitrogen sources on other coccolithophorids such as *C. carterae* is scarce.

The small body of work on other genera of coccolithophorids makes it difficult to assume that all coccolithophorids will have similar mechanisms for nitrogen uptake and assimilation.

5.1.1 Type of nitrogen used by phytoplankton

Nitrogen is one of the most important nutrients (after CO_2 and phosphorus) for algal growth, and is one of the most limiting nutrients in marine systems (with the exception of iron) (Falkowski 1997). When NH_4^+ is the sole nitrogen source, or in large enough concentrations as to inhibit NO_3^- uptake, there is a much lower energy demand on the cell, and when this is coupled with low pH, we should see an increase in the rate of photosynthetic carbon uptake and calcification (Greene *et al.* 1991; Kolber *et al.* 1994; Bergmann *et al.* 2002).

There is evidence that some of the coccolithophorids, such as *C. carterae,* can utilise amino acids as a nitrogen source via extracellular enzymes such as L-amino acid oxidase (Palenik & Morel 1990; Palenik & Morel 1991). This process involves a surface oxidative deaminase which converts L-amino acids into $NH_4^+ + H_2O$ and α-keto acids and H_2O_2. The NH_4^+ released by this reaction is then assimilated by the cell. It has been suggested that this mechanism might be a means of utilising nitrogen in the form of primary amines, without the need to synthesise multiple enzymes (Palenik *et al.* 1989; Palenik & Morel 1990; Antia *et al.* 1991; Palenik & Morel 1991). Interestingly, *E. huxleyi* does not seem to possess this enzyme (Palenik & Morel 1990).

5.1.2 Type of N used by Coccolithophorids

The uptake of ammonium will cause the pH of the medium to drop due to the production of protons when NH_4^+ is converted to NH_3 (Eq. 2). However, if the nitrogen species available is NH_3 (at pH lower than 9), then uptake will not result in any significant pH change. The NH_3/NH_4^+ ratio in water is pH dependent (Eq.1);

$$NH_{4(aq)}^{+} \overset{pka\ 9.24}{\longleftrightarrow} NH_{3(aq)} + H^{+} \qquad \textbf{Eq. 13}$$

As growth rates of *E. huxleyi* are often faster when the algae are cultured with urea or NH_4^+ (Solomon & Glibert 2008; Strom & Bright 2009), it may appear that these are the more efficient N sources. However, NH_4^+ use in dense mass cultures can have negative consequences, such as acidification of the culture medium (Eq. 2,), which may result in loss of the culture (Borowitzka 1999).

The hydrolysis of urea results in a twofold acidification process, with protons being produced, as well as CO_2 (Eq. 14).

$$(NH_2)_2\,CO_2 + H_2O \leftrightarrow 2NH_3 + H^+ + CO_2 \qquad \textbf{Eq. 14}$$

For microalgal cultures, this CO_2 increase from urea may be amplified by the additional CO_2 evolved during dark respiration thus increasing the rate of acidification.

Urea is a significant source of regenerated nitrogen for marine phytoplankton providing up to 50% of total nitrogen uptake in some ecosystems (Varela & Harrison 1999) and is a significant source of nitrogen in coastal and estuarine systems (McCarthy *et al.* 1977; Glibert *et al.* 1991; Glibert *et al.* 2006). To utilize urea the cell must convert it to NH_3/NH_4^+, by either a membrane transport system or via *in vitro* hydrolysis via urease (Antia *et al.* 1991; Flynn 1991; Page *et al.* 1999) or by hydrolysing urea via an external urease (Bekheet & Syrett 1979; Rees & Syrett 1979, 1979). Syrett & McCarthy (1972) found that urea utilization involves both passive diffusion though the cell membrane and metabolic processes.

Urea metabolism in microalgae occurs though the action of one or both enzyme systems;

Urease, which catalyses -

$$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2NH_3 \qquad \textbf{Eq. 15}$$

Or through the action of ATP: Urease amidolyase (UALase), which catalyses the ATP dependent degradation of urea to NH_3 and HCO_3 though the actions of urea carboxylase (urea:CO_2-ligase (ADP)) which catalyses :

$$Urea + ATP + HCO_3^- \xrightarrow{Mg^{2+}\,K^+} Allophanate + ADP + P_i \qquad \textbf{Eq. 16}$$

And (Eq. 17); allophanate hydrolase which catalyses:

$$Allophanate + 3H_2O + H^+ \rightarrow 2NH_4^+ + 2HCO_3^- \qquad \textbf{Eq. 17}$$

For more information on this process see (Stewart 1980).

While urea is quickly hydrolysed to NH_3/NH_4^+ by urease, some groups of microalgae still retain the ability to actively take up urea. While there is limited work on the specific urea uptake mechanism of coccolithophorids, Solomon and colleagues (2010) have found that *E. huxleyi* is able to express several genes for urea uptake. The gene *DUR3*, which is present in most eukaryotic cells, encodes a high affinity urea transporter. Under high urea concentrations, a second urea transporter gene (*SLC14A*) may be of greater significance. In addition to the active urea transporter system, *E. huxleyi* also expresses genes to catabolise urea via urease. There is mounting evidence to suggest that both hetero- and autotrophic phytoplankton compete for both dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON) (Berman & Bronk 2003; Bronk *et al.* 2007; Bruhn *et al.* 2010). *E. huxleyi* has been shown to be able to use both forms of nitrogen, so an ability of coccolithophorids to utilise DIN and regenerated DON has been suggested as one of the reasons for this group's success (Rees *et al.* 2002). This may be one of the reasons that the coccolithophorids are able to dominate in low nitrogen waters (Rees *et al.* 2002; Lessard *et al.* 2005).

RESULTS

The effect of different nitrogen sources and external pH was examined in *C. carterae* (CCMP647) in both small-scale (100mL) tube reactors and medium scale (1L) carboys (see Materials and Methods section 1.3). The aim of these experiments was to determine how nitrogen species will affect; 1) The culture medium pH when unregulated (Section 2.4.1), 2). The growth rate and productivities of *C. carterae (*Section 2.3.4) and 3) Photosynthesis and coccolith production of *C. carterae* (Section 2.3.5).

To achieve this *C. carterae* (CCMP647) was grown on f/2 medium using nitrate, ammonium or urea as the sole nitrogen source under unregulated culture pH as well as capping the maximum pH to a pre-determined level using HCl or NaOH to avoid affecting the pCO_2 of the culture medium (see section 2.7 for detailed methods).

5.1.3 Unregulated pH

5.1.3.1 Diurnal pH Pattern.

When the culture was grown without pH control, a distinctive diurnal pattern in the culture pH was seen for all three nitrogen sources (Figure 40). Here the pH increased during light phase, and then dropped immediately when the culture enterd dark phase. The pH continued to decline until the light phase began again (Figure 2). There are significant differences in the pH response between the three nitrogen sources. Addition of urea resulted in an overall net increase in the culture pH over time (Figure 40a), the same pattern was also found in the cultures with ammonium addition, however the increase in pH was reduced (Figure 40b).

However, when nitrate was used there is no net increase in culture pH over time (Figure 40c). The rate of change in culture pH (ΔpH h^{-1}) was measured from the diurnal fluctuation in pH over time (Figure 40, Table 10).

There is a significant difference in the way that the nitrogen source will change the rate of pH change during dark respiration (Table 10). When the pH was left unregulated in the light, nitrate had the greatest effect on the culture medium ΔpH, (NO_3^- (0.134 ± 0.003) > urea (0.111 ± 0.003) > NH_4^+ (0.043 ± 0.001) (One way ANOVA, Tukey test F = 353.53, P = <0.001) (Table 10). However, during dark respiration (i.e. when respiratory CO_2 leads to acidification of the medium) the fastest change in ΔpH was urea (-0.057 ± 0.001) > NH_4^+ (-0.036 ± 0.001) > NO_3^- (-0.035 ± 0.002) (Tukey test, F = 77.17, P = <0.001) (Figure 40, Table 10).

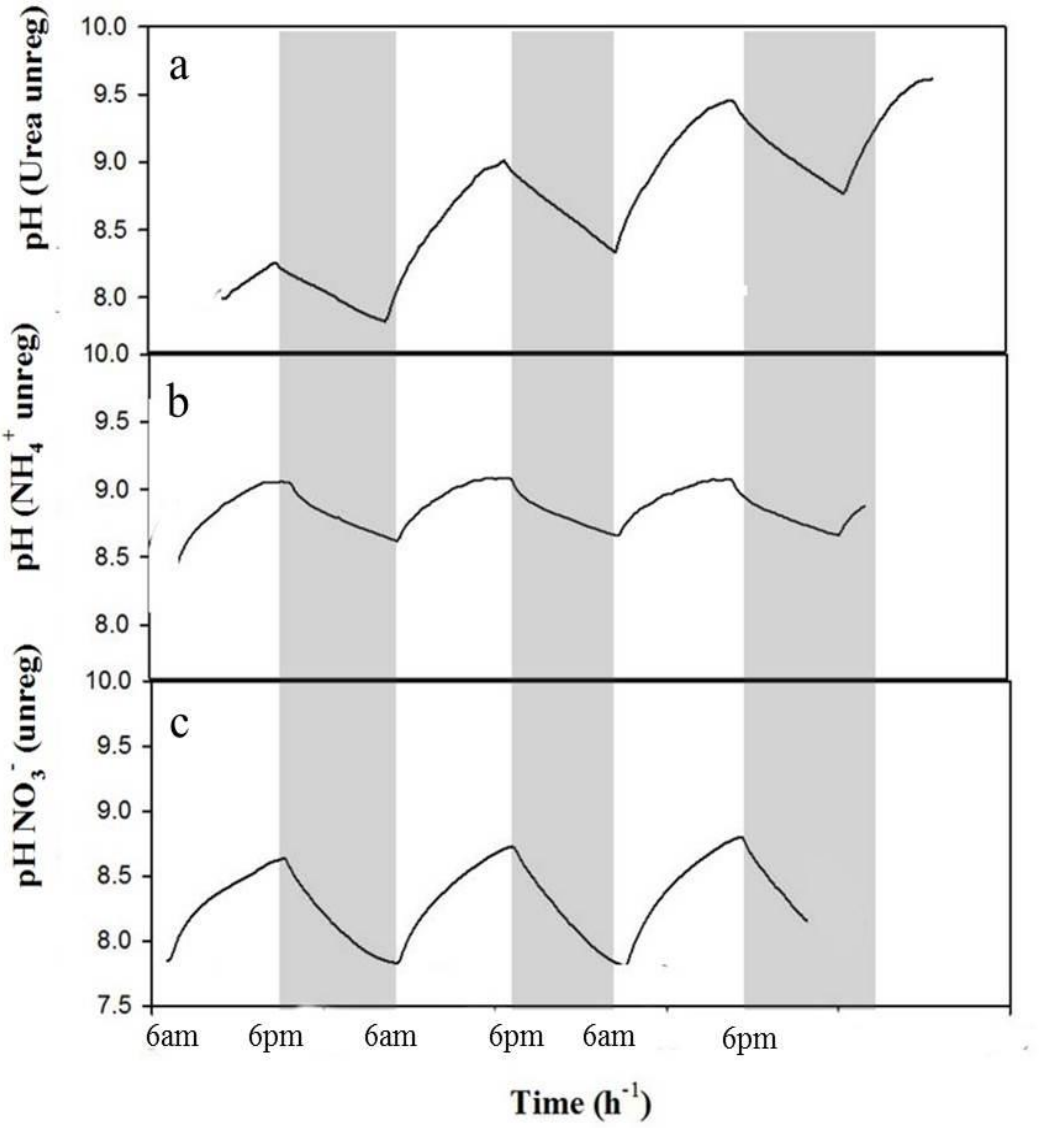

Figure 40 Representative diurnal unregulated pH traces with respect to nitrogen source in *C. carterae.* . a= Urea; b= NH_4^+; c=NO_3^- Time scale time of day. Shaded areas indicate dark period.

Table 10 Acidification and alkalization rates of *C. carterae* media with respect to N source. Dark rate is pH during dark respiration cycle; Light rate shows pH during light cycle (photosynthesis). Bolded numbers indicate highest and lowest rate of pH change and maximum pH obtained for each N source. (un = unregulated pH). (n=5 ± se).

nitrogen Source	pH	Dark Rate (acidification) (ΔpH h^{-1})	Light Rate (alkalization) (ΔpH h^{-1})	Max pH	Min pH	Ave pH
Urea	un	-0.057 ± 0.001	0.111 ± 0.003	**10.14**	8.89	9.5
	7	-0.1 ± 0.001	1.085 ± 0.043	7.71	6.03	6.8
	8	-0.097 ± 0.007	0.395 ± 0.043	8.1	6.71	7.4
	9	-0.116 ± 0.011	0.242 ± 0.007	9.19	7.58	8.3
NO_3^-	un	-0.035 ± 0.002	0.134 ± 0.003	**9.42**	8.05	8.34
	7	-1.03 ± 0.002	1.34 ±0.003	7.12	6.56	6.78
	8	**-0.135 ± 0.021**	1.047 ± 0.103	8.45	6.55	7.6
	9	-1.23 ± 0.004	**2.45 ± 0.002**	9.12	8.01	8.78
NH_4^+	un	-0.036 ± 0.001	0.043 ± 0.001	**9.34**	8.59	8.9
	7	-1.01 ± 0.001	1.123 ± 0.003	7.12	6.03	6.45
	8	-0.114 ± 0.014	0.97 ± 0.09	8.86	6.89	7.65

There is also the expected increase in hydroxyl ions (OH^-) (Table 11) produced though photosynthesis and calcification as well as the H^+ increase during dark cycle respiration (Table 11). The greatest increase in alkalization ($[OH^-]$) is seen when urea is used as the sole N source with 9.06 ± 0.43 mmol kg^{-1} sea water. Here there is an overall net increase in the alkalization of the culture media achieving the highest pH of 10.14. This is in contrast to the maximal pH in the NO_3^- culture of just pH 9.42 (Table 10). The increase in alkalinity under NO_3 (Table12) is most likely due to the release of OH^- via the assimilation of nitrate, and the release of H^+ through the assimilation of NH_4^+ due to pH homeostasis

Table 11 Concentration of carbon species measured during log phase growth as a result of nitrogen source and pH in *C. carterae* calculated via CO2SYS (Lewis & Wallace 1998). n=9 ±se.

Treatment	pCO_2 (µatam)	CO_2 (mmol/kg-1 SW)	OH^- (mmol/kg^{-1} SW)	HCO_3 (mmol/kg^{-1} SW)	CO_3 (mmol/kg^{-1} SW)
Nitrate Unreg	180.31 ± 90.55	5.88	7.99	1611.77	300.72
Nitrate pH 7	**4655.50 ± 127.98**	**151.99**	0.18	961.41	4.14
Nitrate pH 8	320.60 ± 175.89	10.46	3.29	1181.51	90.88
Nitrate pH 9	153.11 ± 27.88	4.9	8.46	1450.53	**286.82**
Ammonia Unreg	616.02 ± 178.77	19.79	***0.015***	***10.20***	***0.004***
Ammonia pH 7	3278.69 ± 753.55	107.04	0.08	325.28	0.67
Ammonia pH 8	571.34 ± 56.99	18.65	2.88	**1844.10**	124.24
Urea Unreg	140.86 ± 27.88	4.59	**9.06**	1427.24	301.85
Urea pH 7	3721.86 ± 144.78	121.51	0.18	486.66	1.33
Urea pH 8	3535.43 ± 215.77	115.42	0.44	1769.60	18.48
Urea pH 9	***128.61 ± 33.77***	***4.2***	8.81	1267.39	260.69

5.1.3.2 Growth

The effect of unregulated pH on the growth rates of *C. carterae* during log phase in the 2L carboys was similar to those in the tube reactor (Table 12). In general the effect of nitrogen source on the growth rate of *C. carterae* was urea > ammonia > nitrate (Tukey Test, P= < 0.001) (Figure 41). It is to be noted that the maximum cell density achieved was: nitrate ($66.61 \times 10^4 \pm 8.2 \times 10^3$ cells mL) > urea ($34.0 \times 10^4 \pm 6.2 \times 10^3$ cells mL) = ammonium ($36.08 \times 10^4 \pm 4.2 \times 10^3$ cells mL) (see Figure 41). Coccolith ($CaCO_3$) production (Table 12) is also affected by the type of nitrogen source available to the cell. The highest coccolith production was seen in the nitrogen starved culture (164.95 ± 26.27 ng$CaCO_3$ cell). In the presence of nitrogen source the coccolith content was nitrate > ammonium > urea.

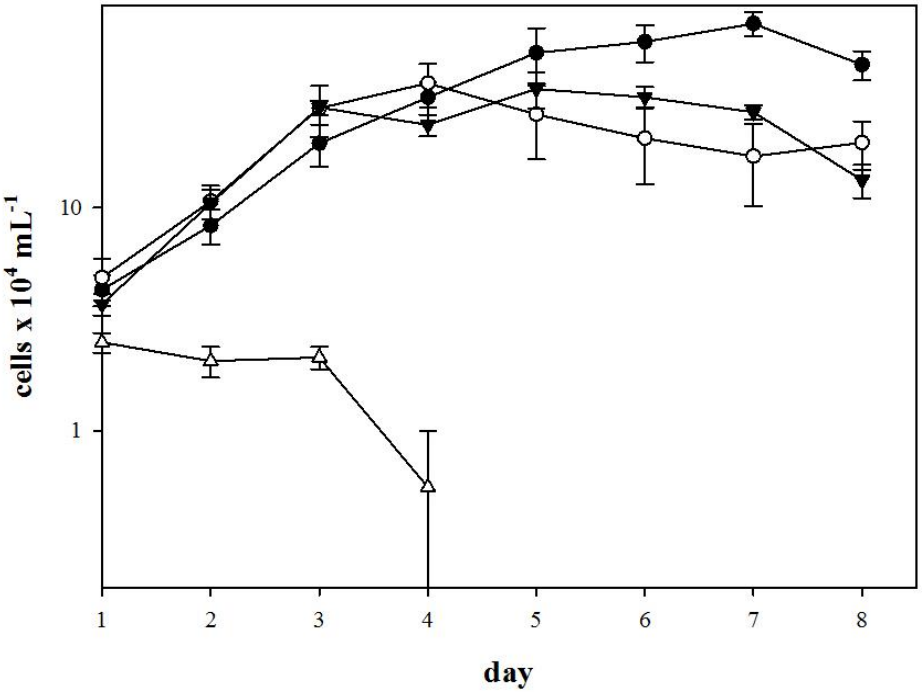

Figure 41 Effect of nitrogen source on the cell growth of *C. carterae* under unregulated pH in 200mL tube photobioreactor. ● = NO_3^-, ○= NH_4^+, ▼= Urea, △= -N. (n=5 ± se).

The specific growth rate (μ (d^{-1})) and coccolith production of *C. carterae* in response to various N sources was first assessed using a 200 mL^{-1} tube reactor with unregulated pH to determine the viability of the cells with different sources of nitrogen. Based on the initial results from the 200mL tube reactor the experiment was then conducted in 2L Erlenmeyer carboys.

Table 12. Effect of N source on the specific growth rates of *C. carterae* and coccolith content of the cell from unregulated pH in 100mL tube photobioreactor and 2L Erlenmeyer Carboy. Superscript indicates significance. (n =5 ± s.e.).

N Source	(tube reactor) μ(d^{-1})	2L Carboy μ(d^{-1})	$CaCO_3$ (ng $cell^{-1}$)
NO_3^-	0.28 ± 0.07	0.17 ± 0.02	73.81 ± 3.51^b
NH_4^+	0.17 ± 0.07	0.31 ± 0.02	55.18 ± 0.61
Urea	1.55 ± 0.1	0.44 ± 0.01	12.88 ± 1.62
- N	-	**	164.95 ± 26.27^a

5.1.3.3 Carbon Chemistry

During logarithmic growth, *C. carterae* was able to alter the pH of the media though photosynthetic and calcification processes. Table 10 shows the changes in pH media and the rate of change of culture pH over time (ΔpH h^{-1}) with different N sources and capped pH maxima. When the culture pH was unregulated, the cultures reached a maximum pH of 9.42 for NO_3^-, 9.34 for NH_4^+ and 10.14 for urea (Table 10). There is an overall net increase in culture pH when urea (and to a lesser extent ammonium) is used as the sole N source with the overall pH increase stronger when urea is the N source. Maximum pH for the nitrate experimental culture was 9.42, with an average culture pH of 8.34 (Figure 40 and Table 10).

The culture pH and nitrogen species available to the cells also had an effect on the carbon species of the culture media (Table 11), with the highest pCO_2 concentration occurring with nitrate (Figure 42) at pH 7 (4655.51 ± 127.98 µatm) which equates to a CO_2 concentration of 151.99 mmol kg^{-1} seawater. These values are significantly higher than those of ammonium and urea grown cultures ($t = 36.38$, $P = <0.001$) (Table 11). The highest HCO_3^- concentration occurred with ammonium grown cell at pH 8 (1844.1 ± 86.88 mmol kg sea water). However, there was no real difference in the HCO_3^- concentrations between the nitrogen species at pH 8. The second highest uptake occurs with cells grown with nitrate and unregulated pH (1611.78 ± 76.48 mmol kg^{-1} sea water. This is significantly higher than that of the ammonium grown culture, which only had a HCO_3^- concentration of 10.21 ± 1.31 mmol kg^{-1} seawater. This is a significant draw down in dissolved organic carbon, which appears to be unrelated to the cell density. Figure 53 shows the large amount of

calcium carbon precipitated out of the culture medium at pH 9. This may account for the huge draw down in DIC observed in the NH_4^+ grown cultures.

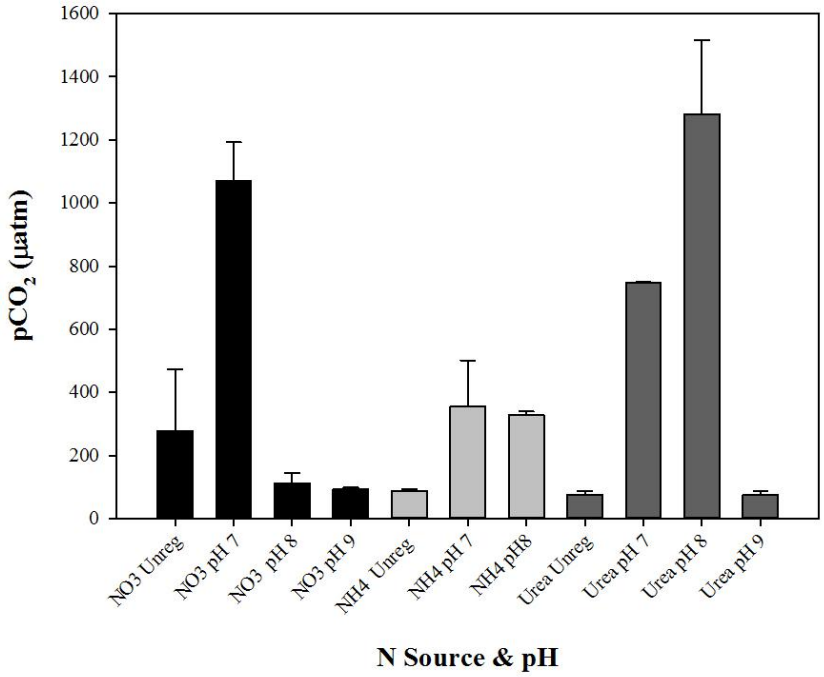

Figure 42 Effect of nitrogen source and pH on the Partial pressure of CO_2 in *Chrysotila carterae* cultures. NO3= Nitrate, NH4=Ammonia, Unreg = Unregulated culture pH. n=5 ± s.e.

5.1.4 Photosynthesis

Chlorophyll a fluorescence of light adapted cells was measured using the Waltz Water PAM (see Materials and Methods section 1.3.4). Dark adaption was not used, as this would result in a change in culture pH that was significantly different to the actual culture. As such, the relative electron transport rate (rETR) was used to avoid incorrect assumptions of absorbance or PS_{II} cross sectional area (σ_{PSII}). As the relationship between rETR and gross photosynthesis in the first 3-4 light intervals, the rates were calculated from these values. The slope (α) of the light limiting region of the rapid light curves (RLC's) was significantly higher when nitrate was used as

the nitrogen source at pH 7 and α of 0.373 ± 0.021 µmol e^- m^2 s^{-1}, the second highest was measured with unregulated pH using nitrate (table 13). These were significantly higher than α for other pH levels and nitrogen sources (t= 15.867 p= < 0.001). However the two highest electron transport rates ($rETR_{Max}$) was seen at pH 8 using urea (122.45 ± 1.67 µmol e^- m^2 s^{-1}, (which was significantly higher than that of nitrate at pH 8 (68.763 ± 0.007 µmol e^- m^2 s^- and ammonium, 93.779 ± 1.66 µmol e^- m^2 s^{-1}) (Figure 43 & (Table 14).

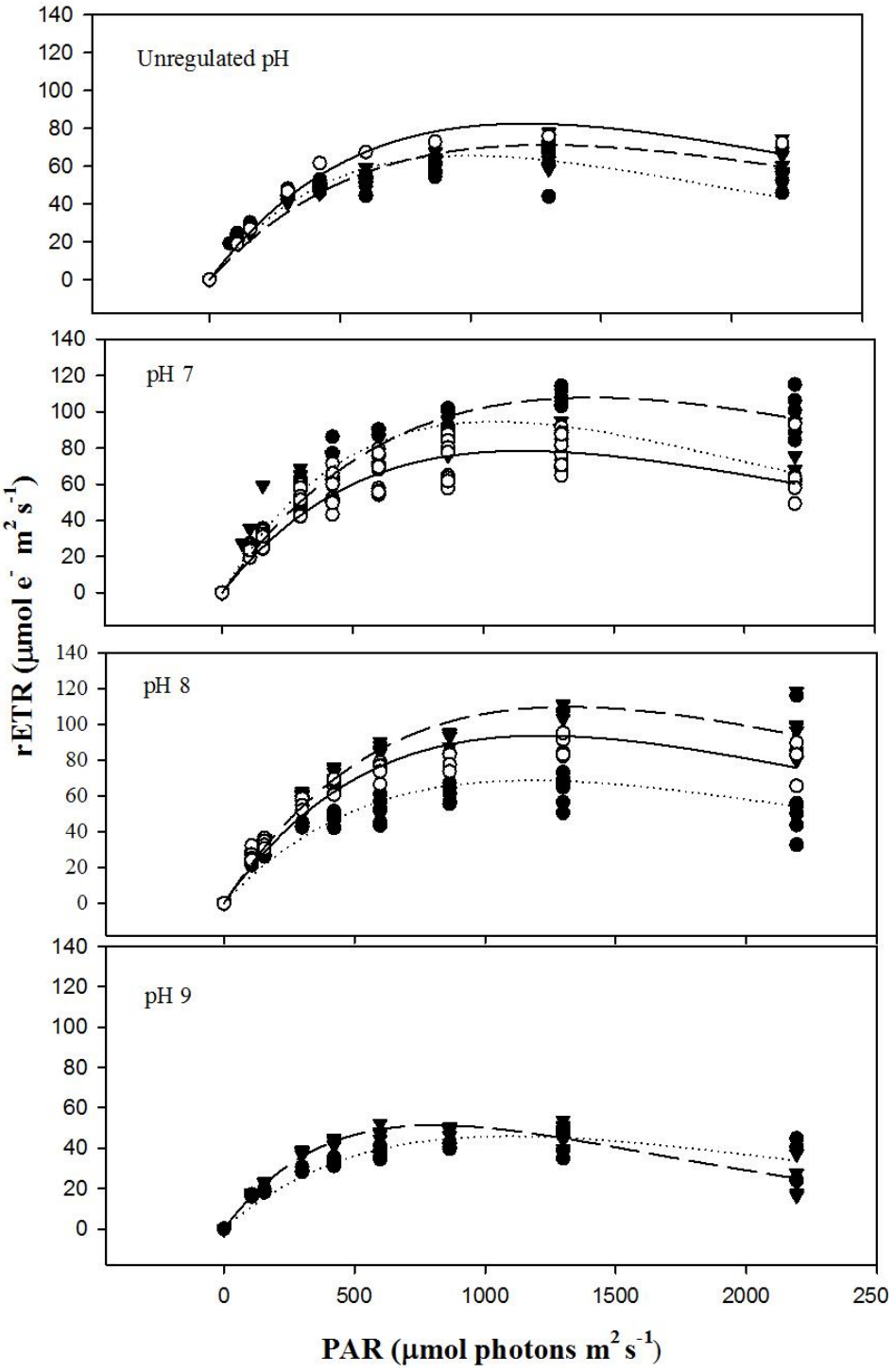

Figure 43 Effect of nitrogen source and pH on the relative electron transport rate (rETR) in *C. carterae.* Curves fitted via waiting in line equations of Ritchie & Bunthawin (2010) n= 5. No data for NH_4^+ at pH 9 as culture was not viable due to heavy carbonate precipitates.□, ···· = NO_3^-;▲, — = NH_4^+ ●, -- -- = Urea

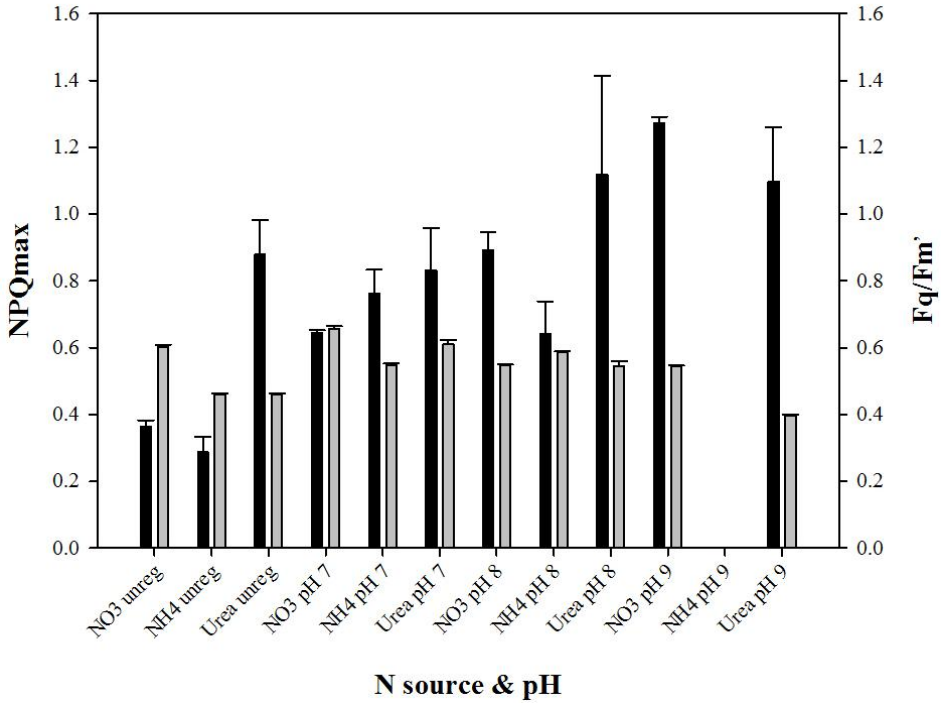

Figure 44 Effect of nitrogen source and pH on the effective quantum yield (Fq/Fm') (light bars) and maximum Non Photosynthetic Quenching value (NPQ) (dark bars) of *C. carterae*. NO3 = Nitrate. NH4= Ammonium Urea = Urea. Unreg indicates unregulated culture pH. (± se, n=5). No data for NH_4^+ at pH 9 as culture was not viable due to heavy carbonate precipitates

Urea has the effect of increasing the irradiance at which photochemistry stops and the cells enter a period of photoprotection (Figure 43 & Figure 45). The E_k values from urea grown cells are significantly higher than those from ammonia or nitrate grown cells. (One way ANOVA, P=<0.001). However, at pH 9, the E_k values drop significantly to 805.32 ± 20.69 µmol photons m^2 s^{-1}, which is the lowest vale overall (Figure 45). This increase in the on-set of photoprotection is also evident by the drop in $rETR_{max}$ of all nitrogen sources, where there is almost a 50% drop in the $rETR_{max}$ (Figure 43).

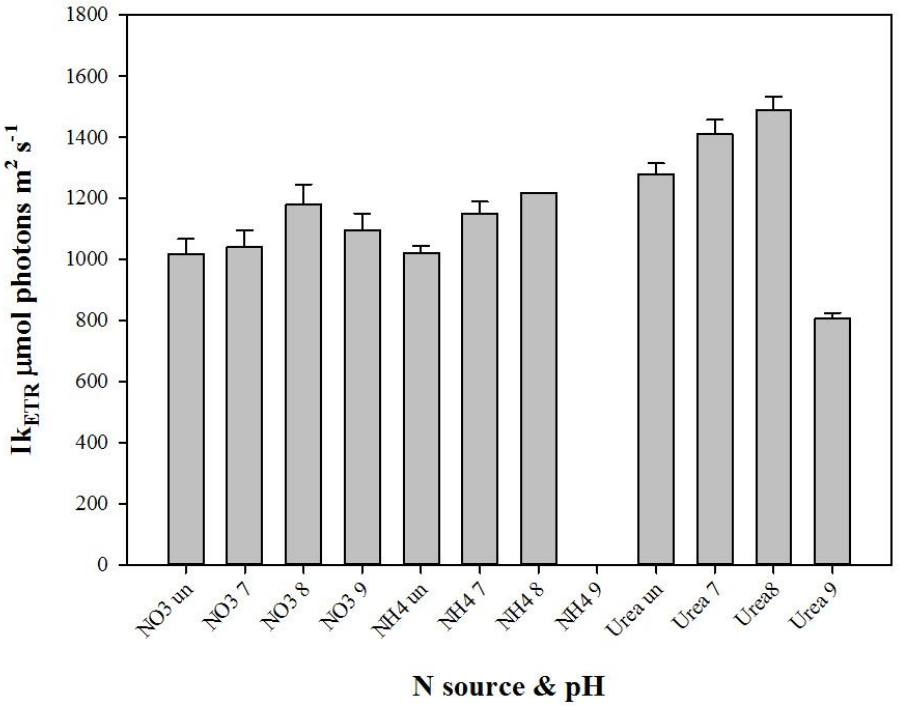

Figure 45 Change in saturating irradiance (Ik) based on rETR measurements with regard to nitrogen source and pH in *C. carterae*. n=5 ± s.e. No data for NH_4^+ at pH 9 as culture was not viable due to heavy carbonate precipitates.

The maximum effective quantum efficiency of photosystem II (Fq/Fm') (Figure 44) indicates that the cells show no real sign of the cells becoming stressed as a result of changes to the pCO_2 of the media via pH or as a result of the type of nitrogen available to the cells. However at lower pH levels (and thus higher pCO_2), there is an increase in the effective quantum yield of *C. carterae*. The Fq/Fm' ranged from a maximum of 0.657 ± 0.007 (nitrate at pH 7) to a low of 0.397 ± 0.003 (urea at pH 9) however the average Fq/Fm' was 5.4 across all other N sources and pH levels. This is reflected in the non-photochemical quenching (NPQ) values, where there is an increase in the NPQ value as Fq/Fm' is decreasing, however there are instances where the NPQ increase is not related to the Fq/Fm' (i.e. at NO_3^- pH 8 and 9, where

there is a NPQ increase from 0.883 ± 0.054 at pH 8, to 1.272 ± 0.019 at pH 9, while the Fq/Fm' remained the same (5.46 ± 0.003 and 5.44 ± 0.002) (Figure 44).

The relative electron transport rate (rETR) tells another story, here the greatest photosynthetic activity was seen with Urea at pH 8 (Figure 47) the reduced photosynthetic performance of nitrate grown cultures (Figure 43) is in contrast to the quantum yield, but does follow the increase in NPQ (Figure 44). Urea grown cultures yielded the greatest photosynthetic rate at pH 8 (122.45 ± 1.67 µmol e^- m^2 s^{-1} with a pCO_2 = 3535.5 ± 215.8 µatm), followed by 107.88 ± 1.77 µmol e^- m^2 s^{-1}) at pH 7 pCO_2= 3721.9 ± 144.78 µatm). The pCO_2 does not seem to have a great effect as the rETR for nitrate grown cells at pH 7 (pCO_2 = 4655.5 ± 90.6 µatm, (Table 11) is 106.3 ± 2.26 µmol e^- m^2 s^{-1}. This is only slightly less than that of urea grown cultures at pH (Table 14). The lowest rate was found at pH 9 with nitrate (45.93 ± 1.22 µmol e^- m^{-2} s^{-1} and urea 51.388± 0.79 µmol e^- m^{-2} s^{-1} pCO_2 =) (Table 14)

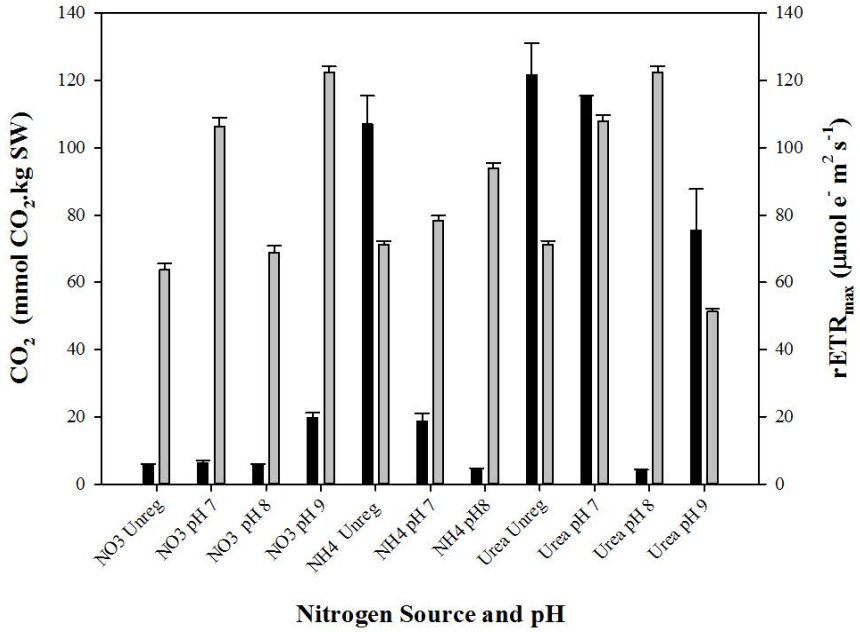

Figure 46 Relationship between nitrogen source and the concentration of CO_2 in the culture medium and the relative electron transport rate (rETR) of *C. carterae*. Black Bar = CO_2, gray bar = rETR. (n=5 ± s.e.).

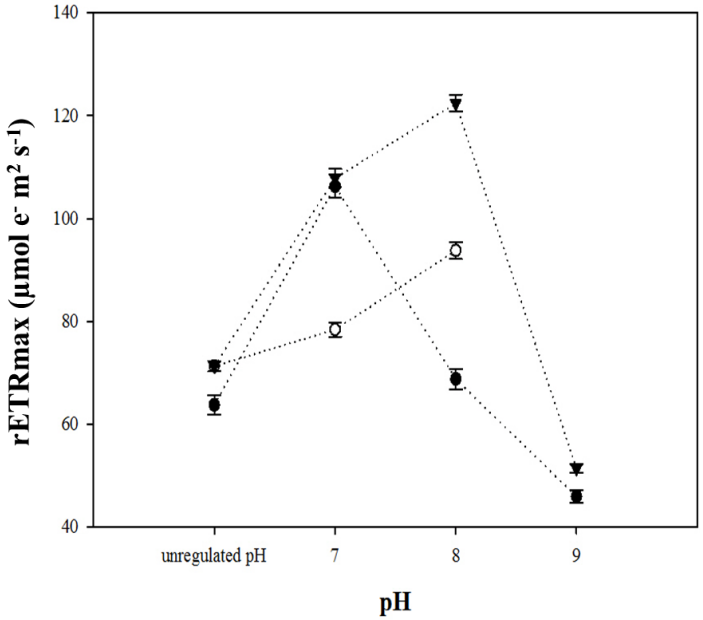

Figure 47 Relative Electron Transport Rate in *Chrysotila carterae* with respect to nitrogen source and pH. Cells were not viable when grown on NH_4^+ at pH 9. ○= NH_4^+, ●= NO_3^-, ▼ = Urea (n=5 ± se). No data for NH_4^+ at pH 9 as culture was not viable due to heavy carbonate precipitates.

5.1.5 Carbon Assimilation

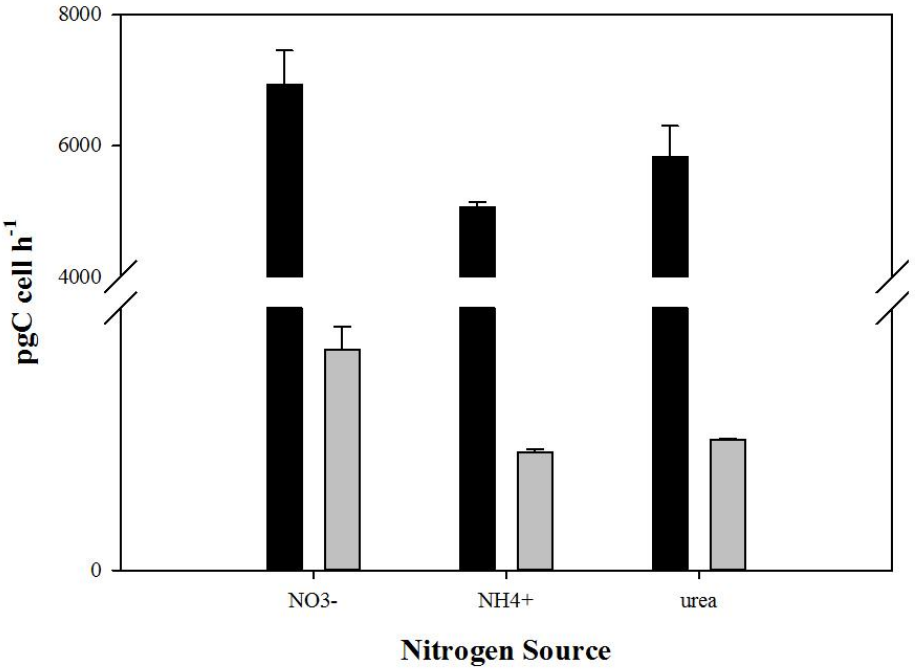

Figure 48 *C. carterae* carbon assimilation in unregulated pH cultures using various nitrogen sources. Dark bars = organic C assimilation, light bars = inorganic C assimilation. ± s.e, n=9

Organic (C_{ORG}) and inorganic (C_{INORG}) carbon assimilation was measured in the unregulated cultures. Here the fastest assimilation rates were seen in the NO_3^- grown cells, with an organic C rate of 6936.95 ± 519.58 pgC_{ORG} $cell^{-1}$ h^{-1}, and inorganic C assimilation rate of 505.3 ± 51.3 pgC_{INORG} $cell^{-1}$ h^{-1}. This is significantly higher that NH_4^+ or urea grown cells (ANOVA, F = 5.32, P=<0.5).

Interestingly, when the pH is left unregulated, the rate of change is much faster that when the media pH is capped. Table 10 shows that when the culture pH is capped, the rate of change in pH due to cell metabolism is altered significantly. The greatest

effect is seen in nitrate grown cells, where the fastest rate of change during dark respiration (acidification of media) occurs when pH is capped at 8 (-0.135 ± 0.001 ΔpH h^{-1}).

5.1.6 Capped Culture pH

To determine the affect of pH on the growth of *C. carterae* with various N sources, the culture pH was capped to a pre-determined maximum (Figure 49), either by addition of 0.1M HCl, or 0.1M NaOH as required (Section 2.4.1).

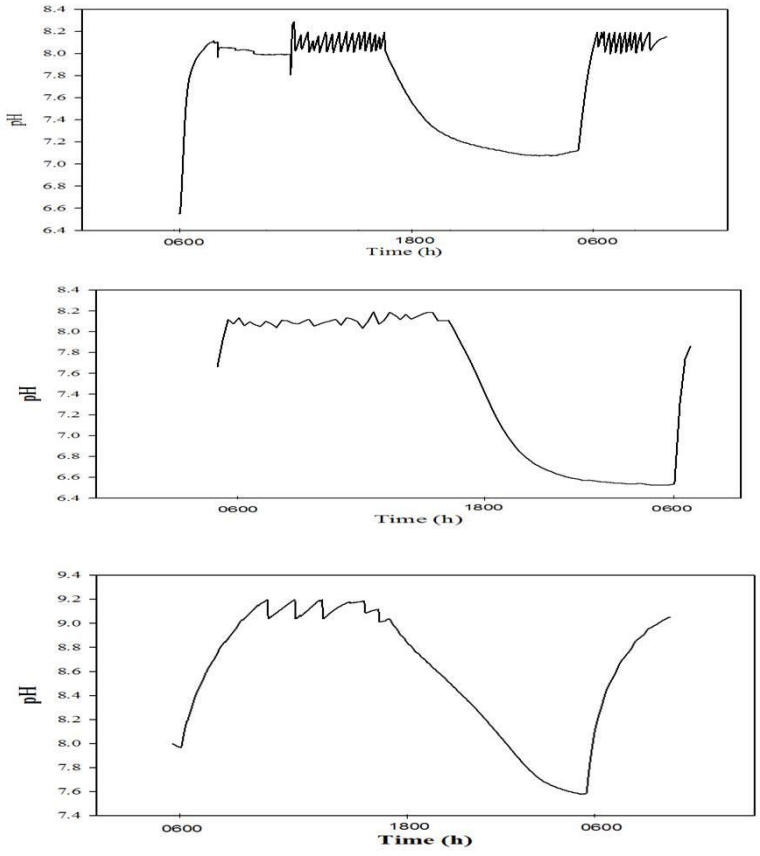

Figure 49 Representative pH trace diagram of *C. carterae* response to capped pH. Figure is to illustrate the response of *C. carterae* to the pHstat system a) NO_3^- capped at pH 7, b) NH_4^+ capped at pH 9, c) Urea capped at pH 9. Dark phase commences at 6:00am and ends at 6:00pm.

When nitrate is used a sole N source, the cultures were stable and could be maintained in batch culture for up to 7 days at pH 7, 8 and 9. However, when ammonia or urea was used, the cultures were unable to be maintained in batch culture, crashing after 4 to 5 days at all pH levels (Figure 51 and Figure 50).

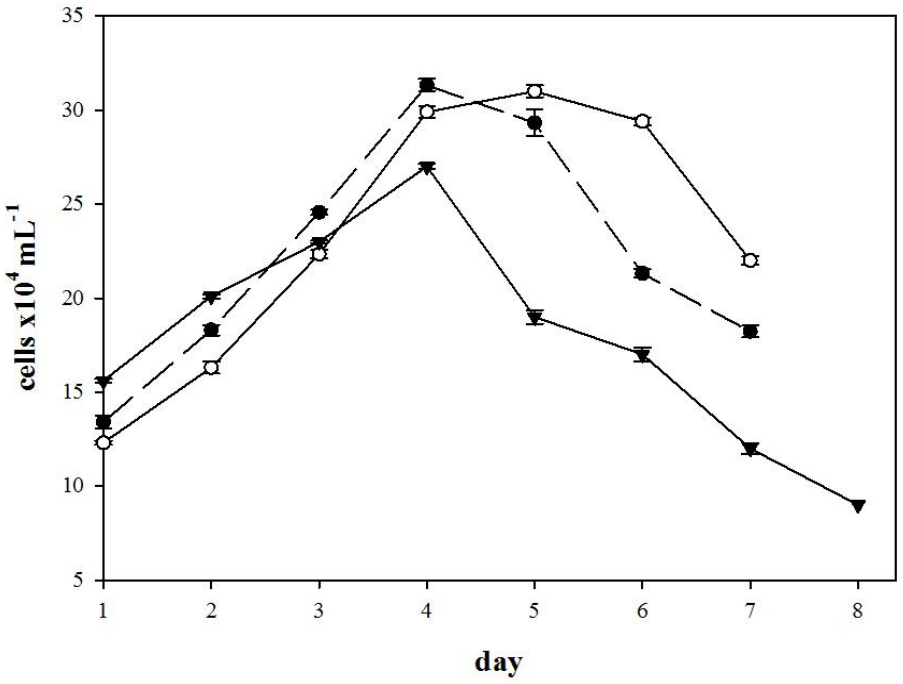

Figure 50 Growth of *C. carterae* at capped pH using urea a sole N source ●= pH 7, ○= pH 8, ▼= pH 9 ± se.

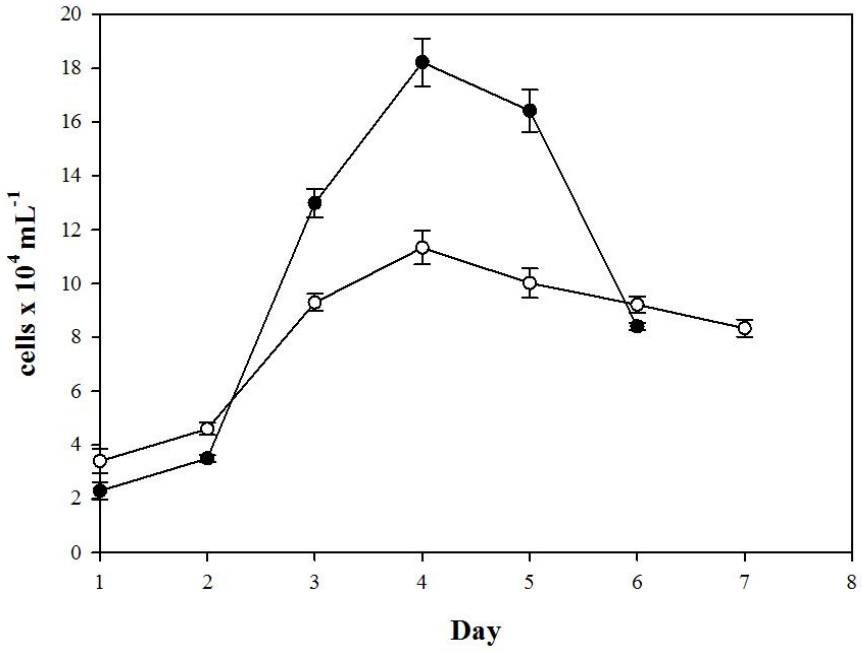

Figure 51 Growth of *C. carterae* in 2L carboy using ammonium at pH 7 (●) and pH 8(○) as sole N source n=5, ± s.e

When ammonia was used as the N source at pH 9 (maintained by adding 0.1M $NaOH^-$), the culture died, apparently due to heavy carbonate precipitates (Figure 52).

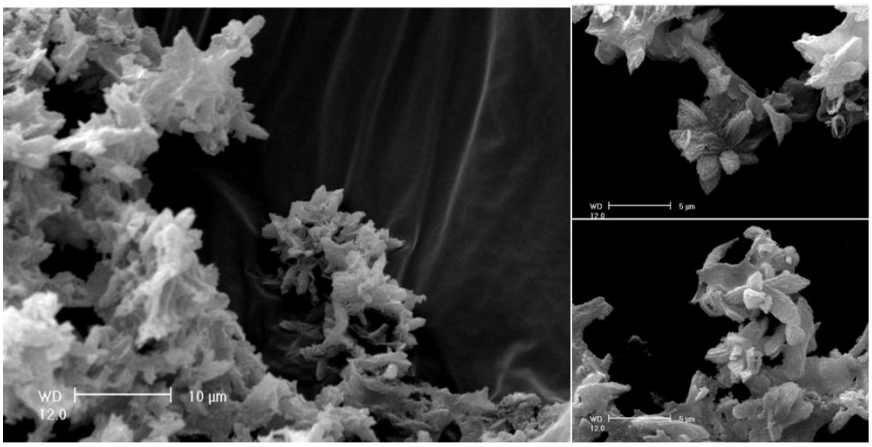

Figure 52 SEM showing heavy carbonate precipitation from culture grown using ammonium at pH 9.

Table 13 Effect of nitrogen source and culture pH on *C. carterae* photosynthesis during log phase growth, photosynthetic carbon uptake and calcification (as inorganic ^{14}C assimilation). Rates are in pg Carbon cell^{-1} h^{-1}. C:P = Calcification: Photosynthesis ratio, α = Photosynthetic rate of PS_{II} Chlorophyll fluorescence, rETR = relative Electron Transport Rate, Fq/Fm' = Quantum Yield of PS_{II} in the light. ** indicates that culture was not viable at pH 9.0 . (± s.d. n=9 for carbon uptake and n=15 ± s.e. for photosynthetic measurements).

	Nitrate			Ammonia			Urea		
pH	**Organic C Uptake Rate**	**Inorg. C Uptake Rate**	**C:P**	**Organic C Uptake Rate**	**Inorg. C Uptake Rate**	**C:P**	**Organic C Uptake Rate**	**Inorg. C Uptake Rate**	**C:P**
unregulated pH	6936.95 ± 519.78	505.66 ± 51.3	0.073	5066.11 ± 80.3	269.66 ± 6.47	0.0532	5831.57 ± 471.45	299.25 ± 2.21	0.0420
7	4178.83 ± 1408.64	428.9 ± 99.2	00.103	3354.54 ± 189.11	224.26 ± 21.71	0.0669	4012.84 ± 182.32	307.85 ± 13.9	0.1380
8	773.59 ± 14.8	122.4 ± 4.13	**0.1582**	22006.22 ± 640.39	1506.69 ± 41.5	0.0685	**44921.73 ± 2191.08**	**7192.83 ± 102.04**	0.1290
9	5191.66 ± 361.25	569.44 ± 31.4	0.1097	**	**	**	6127.20 ± 83.13	773.59 ± 14.8	0.1498

pH	**α**	**rETRmax (µmol photons.m^{2}.s^{-1})**	**Fq/Fm'**	**α**	**rETR max (µmol photons.m^{2}.s^{-1})**	**Fq/Fm'**	**α**	**rETR max (µmol photons.m^{2}.s^{-1})**	**Fq/Fm'**
unregulated pH	0.238 ± 0.015	63.734 ± 1.85	0.602 ± 0.007	0.181 ± 0.005	81.28 ± 1.01	0.459 ± 0.004	0.151 ± 0.004	71.281 ± 1.01	0.459 ± 0.003
7	**0.373 ± 0.021**	106.277 ± 2.26	**0.657 ± 0.007**	0.185 ± 0.007	78.41 ± 1.41	0.548 ± 0.004	0.208 ± 0.007	107.88 ± 1.77	0.611 ± 0.011
8	0.158 ± 0.01	68.763 ± 2.02	0.546 ± 0.003	0.209 ±0.005	93.779 ± 1.66	0.587 ± 0.002	0.223 ± 0.006	**122.45 ± 1.67**	0.545 ± 0.014
9	0.114 ± 0.006	45.93 ± 1.22	0.544 ± 0.002	**	**	**	0.173 ± 0.005	51.388 ± 0.79	0.397 ± 0.003

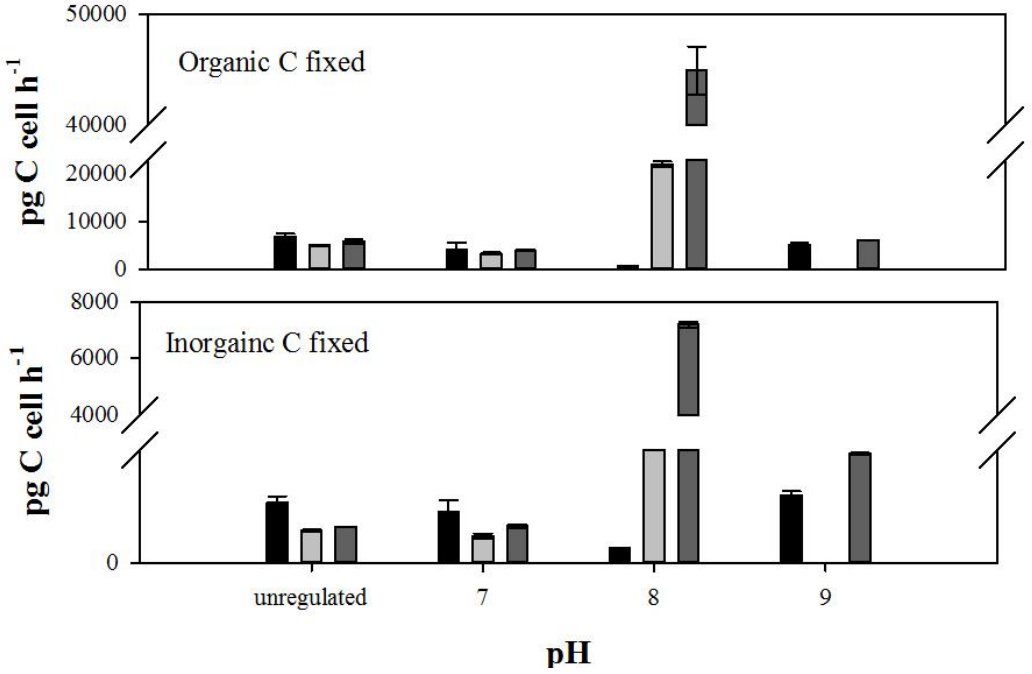

Figure 53 Organic (C_{ORG}) and Inorganic (C_{INORG}) carbon fixation rates of *C. carterae* with various nitrogen sources, grown under unregulated and capped pH culture conditions. Cells were not viable when grown on NH_4^+ at pH 9. Black Bar = NO_3^-, Light Grey = NH_4^+ Dark Grey = Urea. n=9, ± s.d. No data for NH_4^+ at pH 9 as culture was not viable due to heavy carbonate precipitates (Figure 52).

Inorganic carbon assimilation was approximately 1-8% of the total carbon assimilated, however cells grown using nitrate at pH 7 saw a significant increase (33%) in the amount of carbon synthesised into inorganic compounds.

Carbon fixation and inorganic carbon assimilation was greatly affected by pCO_2 and the type of nitrogen available to the cells (Figure 53). The greatest assimilation of organic (C_{ORG}) and inorganic (C_{INORG}) carbon occurred with urea grown cells at pH 8 with 44921.73 ± 2191.08 pgC_{ORG} $cell^{-1}$ h^{-1} and 7192.83 ± 102.04 pgC_{INORG} $cell^{-1}$ h^{-1}

(Figure 53) which is assumed to be predominantly calcium carbonate production. This is significantly higher than that of any other treatment (Tukey test, F = 1148.70, P = < 0.001).

There is a strong relationship between both organic (C_{ORG}) and inorganic (C_{INORG}) assimilation and pH, with the greatest interactions occurred at pH 8 for all N species (Figure 54). The calcification to photosynthesis ratio (C:P) was greatest in nitrate grown cells when pH was capped to 8 (C:P= 0.158), closely urea at pH 9 (0.149) and followed by pH 8 (C:P= 0.129) average C:P was approximately 0.06 (Table 14)

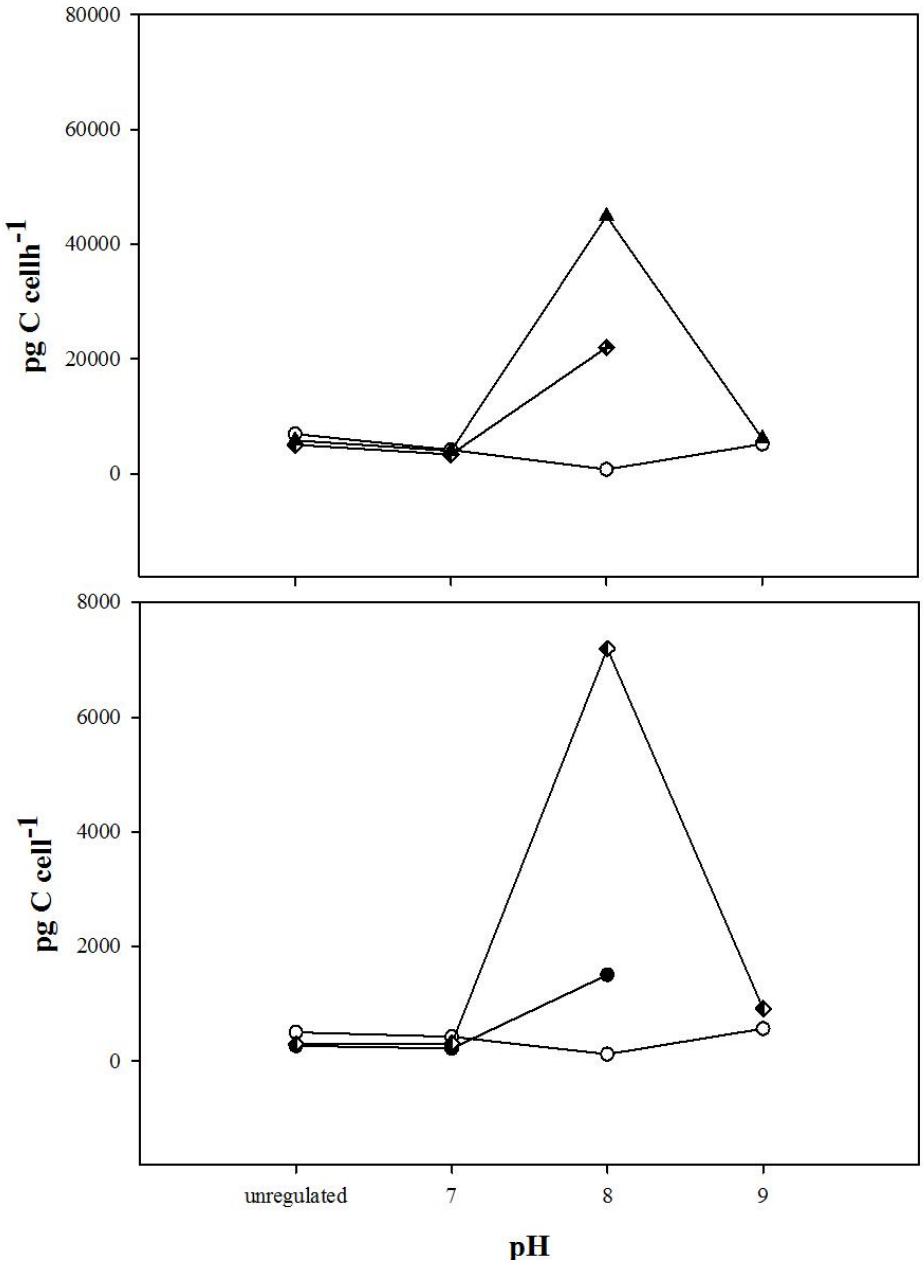

Figure 54 Statistical interaction plot for mean inorganic carbon fixation rates (a) and mean organic carbon uptake rate (b) interaction between nitrogen species and pH in *C. carterae.* Cells were not viable when grown on NH_4^+ at pH 9. n=9 ± se. No data for NH_4^+ at pH 9 as culture was not viable due to heavy carbonate precipitates.

$NO3^-$ = ○, NH_4^+ = ●, urea = ◘

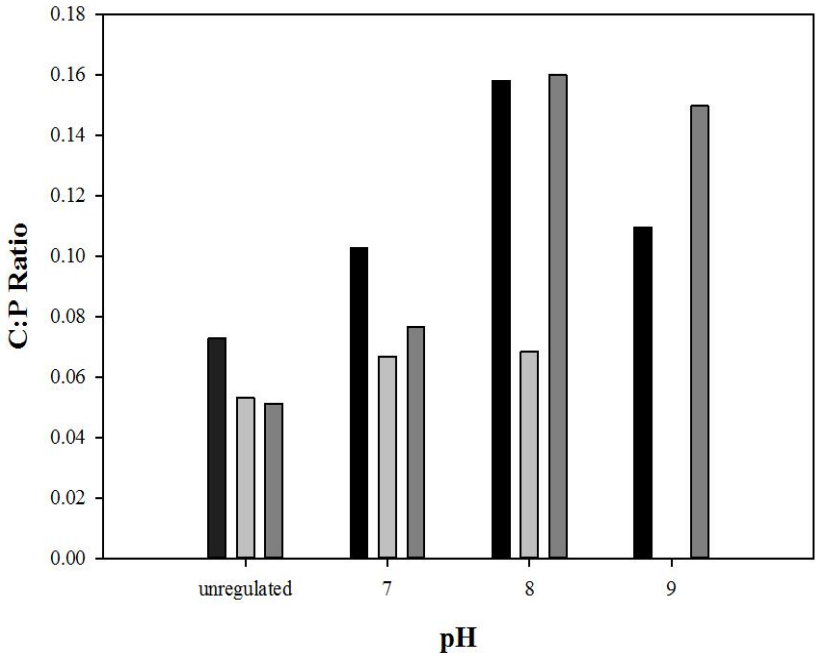

Figure 55 Effect of nitrogen source and external pH on the Calcification to Photosynthesis (C:P) ratio of *C. carterae*, calculated from the carbon uptake rates in Table 14.

Black bar = NO_3^-, Light Grey = NH_4^+, Dark Grey = Urea. No data for NH_4^+ at pH 9 as culture was not viable due to heavy carbonate precipitates.

Figure 54 shows the interactions between pH and the type of nitrogen available to the cells, clearly there is a very high interaction when urea is used at pH 8. Here we can see the greatest carbon uptake rate and inorganic carbon assimilation.

Figure 55 shows there is a reduction in the maximum C:P at pH 8 for both nitrate (C:P = 0.158) and urea (C:P = 0.161) to C:P. to 0.109 and 0.150 respectively. This is a 30% drop in the amount of inorganically derived carbon via photosynthesis in nitrate and a 6.5% drop for urea. Ammonium C:P however is reduced even at pH 7 and 8.

5.2 DISCUSSION

5.2.1 Cell Growth

The best cell growth was achieved under unregulated pH and using nitrate as the sole N source, although the growth rate was accelerated using urea and NH_4^+Table 12), the most stable growth and highest cell density was achieved with nitrate and as the N source. These findings are in agreement with those other works on *Chrysotila carterae* (CCMP647) cultured on a large scale and for extended periods outdoors (Moheimani 2005; Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007; Moheimani *et al.* 2011). While there is accelerated growth when urea and NH_4^+ are used, after 3-4 days of rapid growth the culture crashes (Figure 50 & Figure 51). This response to ammonia and urea is also seen in mass cultures of *Dunaliella salina*, where after several days grown with ammonia the culture collapses. Azov & Goldman (1982) reported the mild inhibition of carbon uptake in *Dunaliella tertiolecta* and the diatom *Phaeodactylum tricornutum* when the cells were exposed to 10mM NH_3 at pH 8.0, however when the pH was increased to 8.9, these authors observed dramatic reduction in carbon assimilation. The reported inhibitory effects of NH_3 on microalgal photosynthesise are not supported by the data here. In *Chrysotila carterae*, at pH 8 (at which the NH_3:NH_4^+ concentration would be shifting toward NH_3) photosynthesis is not significantly affected; the data presented here suggests that when urea is used, the electron transport rate is increased when compared to NO_3^- (Figure 43). The maximum electron transport rate (ETR_{max}) was high for urea at pH 8 and a similar rate with nitrate at pH9 (Figure 47). Other indicators of increased photosynthesis under urea are the high carbon assimilation

rates at pH for both urea and NH_4^+ and a higher C:P with urea at pH 8 and 9. This indicates that *C. carterae* photosynthesis is not inhibited but NH_3. The interesting results presented here indicate that growth of *C. carterae* is much faster when using urea or ammonium as than source. It is widely reported that most microalgae have an affinity for ammonium over nitrate and will take up ammonium in preference to nitrate due to the energy requirements in nitrate conversion (Flynn 1991; Berges & Mulholland 2008; Jenkins & Zehr 2008). Within the coccolithophorids, *Emiliania huxleyi* (strain L NIOZ) has been reported to use both nitrate and ammonium simultaneously under the right conditions (Page *et al.* 1999). Increased growth rates have also been reported in *E. huxleyi* when ammonium and urea are used as an N source. Page *et al.* (1999) reported a lower concentration of ammonium required to inhibit nitrate assimilation (half saturation point (*Kt)* of 0.2µM). These authors suggest that due to the higher growth rate reported when cultured with ammonium (Eppley *et al.* 1969; Muggli & Harrison 1996; Page *et al.* 1999), that *E. huxleyi* may be adapted to using regenerated N sources. Nitrogen assimilation in the coccolithophorids is complicated by the wide diversity between different strains, for example Paasche (1964) found large variation in the C:P (see Chapter 2 Table 9) between different strains of *Emiliania huxleyi*, while more recently the conflicting results in the response of *E. huxleyi* to an increase in pCO_2 (Riebesell *et al.* 2000; Iglesias-Rodriguez *et al.* 2008) have sometimes confused the literature. Strom & Bright (2009) found significant variation in the growth rates for *E. huxleyi* under various N sources, (Table 15).

What is significant here is that under urea, the growth rate of the two strains (CCMP373 isolated from the Sargasso sea in 1960 and CCMP379 isolated from the English Channel in 1957) were significantly reduced, while urea outperformed nitrate in the other two strains (Table 15).

Table 14 Summary of growth rates (d^{-1}) of non-coccolith forming stains of *Emiliania huxleyi* grown with different nitrogen species as sole N source. ±SD. (Strom & Bright 2009).

	***Emiliania huxleyi* Strain μ(d^{-1})**					
N source	**CCMP370**	**CCMP373 Exp. 1**	**CCMP373 Exp.2**	**CCMP374 Exp. 1**	**CCMP374 Exp.2**	**CCMP379**
Nitrate	0.13 ± 0.001	0.1 ± 0.005	0.28 ± 0.033	0.21 ± 0.011	0.23 ± 0.006	0.18 ± 0.013
Ammonium	0.15 ± 0.003	0.05 ± 0.022	0.29 ± 0.025	0.23 ± 0.01	0.24 ± 0.003	0.15 ± 0.015
Urea	0.19 ± 0.001	0.09 ± 0.005	0.05± 0.005	0.33 ± 0.010	0.32± 0.008	**0.07 ± 0.007**

However all of these strains were non coccolith forming, and as such any comparison to calcified stains of other coccolithophorids must be regarded and tenuous as the calcification mechanisms will have significant effect on photosynthesis and N metabolic pathways. This is evident by the results of Palenik & Henson (1997) who found that in a calcified strain of *E. huxleyi* (CCMP371) growth on acetamide was faster than growth on nitrate, contrary to Strom & Bright (2009) who found reduced growth on acetamide in the non-calcifying strains. Kaffes (2010) also found similar growth rates when using limiting ($\mu = 1.1 \pm 0.1\ d^{-1}$) and replete ($\mu = 1.2 \pm 0.1\ d^{-1}$) levels of NO_3^-. Kaffes (2010) main finding however was that with *E. huxleyi* NO_3^- availability has a significant impact on *E. huxleyi* C and N fluxes, but does not change the C:N ratio, or the growth rates of the cells. Kaffes goes on to suggest that in *E. huxleyi*, the CO_2 and NO_3^- concentrations are highly regulated in order to maintain the balance between fluxes of C and N assimilation pathways.

The loss of the culture after 3-4 days (Figure 50 & Figure 51) poses some interesting questions: There are several possible mechanisms that may lead to the crash of the culture in such a short time; the most obvious is that the cells are running out of carbon due to the rapid growth. However, as reported in Table 11, the amount of CO_2 available to the cells in the culture medium is still available to the cells at all pH levels, although at pH 9 with urea the CO_2 concentration is very low at 4.2 mmol kg^{-1} sea water and is the most likely reason for the reduced growth and cell density. While the amount of CO_2 in the medium may have been in excess, recent work by Slobodanka *et al*. has suggested that in the absence of a functioning CCM, *C. carterae* may have become carbon limited due to the $_{KM}(CO_2)$ values for Rubisco which have been shown to have a $_{K1/2}$ DIC range from approximately 100 µM to 700 µM depending on the strain (Slobodanka *et al*. 2013) . Another theory is that in converting urea to NH_3 and the assimilation of NH_4^+ for conversion to NH_3, that there is a build-up of NH_4^+ in the cytoplasm (Morel et al. 2003) which may lead to ammonium toxicity and thus loss of the culture. There is also the chance that the cells are running out of trace nutrients such as nickel. In diatoms and other phytoplankton nickel is required in the formation of urease (Oliveira & Antia 1984), however in the coccolithophorids, the type of urease produced is UALase, and as such does not have a requirement for Ni (Dyhrman & Anderson 2003; Muyssen *et al.* 2004). We are only beginning to develop the full picture regarding the assimilation of nutrients and how the mechanisms of uptake of major nutrients and subsequent growth with different molecular species can be affected by trace elements such as nickel. Oliveira & Antia (1984) found that the diatom *Cyclotella cryptic* also had a

high growth requirement for nickel when cultured using urea as the sole N source. It may also be possible that due to the increased photosynthetic rates seen under NH_4^+ and urea that the culture is being affected by oxygen toxicity (Moheimani & Borowitzka 2006, 2011). What is clear is that much more work is required to determine what is causing *Chrysotila carterae* to crash under ammonium and urea.

5.2.2 Effect of external pH on growth

While many species of phytoplankton alter the pH of the growth medium during growth for a wide range of reasons, (carbon uptake and H^+ transfer through metabolic processes are the most common). *Chrysotila carterae* is one of only a very few species of microalgae that will increase the medium pH during photosynthesis to levels that are usually unsuitable for other marine species, with culture pH as high as pH 11 being recorded (Crenshaw 1964; Moheimani & Borowitzka 2006; Moheimani & Borowitzka 2007). At these high pH levels, ans in a sealed system, CO_2 is almost no existent in the culture medium (Stumm & Morgan 1996). This gives rise to the probability that *Chrysotila carterae* using CO_2 as the main carbon source, via external carbonic anhydrase. The absence of any reported active bicarbonate transporter such that the one reported in *Emiliania huxleyi* (Herfort *et al.* 2002) further suggests the presence of an external CA. What is different between the two species is that during dark respiration, the culture pH is reduced back to the original "starting pH" of the culture (Moheimani & Borowitzka 2006). This diurnal pattern is repeated throughout the life of the life of the culture

(Chapter 3, Figure 33). The diurnal pH response reported in Moheimani & Borowitzka (2006) was achieved using unregulated pH and with NO_3^- as the sole nitrogen source. The findings presented here show a different diurnal pattern when urea and NH_4^+ are used; under unregulated pH there is an overall net increase in the pH of the culture over time, and the effect is greater with urea (Figure 40), which is surprising as the urea conversion process should result in acidification of the culture medium due to the production of H^+ and CO_2 (Eq. 2). The increased rate of change in culture pH is also faster in urea, however this is to be expected due to the increased photosynthetic rate, as well as the extra CO_2 produced during conversion of urea to NH_4^+ via external urease (Table 5, Eq. 2). Using urea, with unregulated culture pH, the pCO_2 of the culture is significantly reduced (Figure 42), this is surprising as it would be expected to increase due to the extra CO_2 produced via Eq. 2. This is most likely explained by the increase in photosynthesis (Figure 46).

It is difficult to explain the different diurnal patterns observed under the various N sources, the most likely mechanism is that increased photosynthetic rates on urea and to a lesser extent NH_4^+ when compared to NO_3^- is resulting in a much faster uptake of CO_2 from the culture medium. The decline in culture pH may be due to the cells taking up the components required for calcification, and this may account for the stable diurnal pH pattern seen on NO_3^-. The calcification rate is greater when NO_3^- is used (Figure 48), is significantly reduced with urea. Interestingly when Benner (2008) cultured *Coccolithus pelagicus* on urea and nitrate they saw an increase in the calcification rate as well as a decline in incomplete coccoliths. To fully explain the mechanisms behind the different diurnal pH patterns, much more work on the

photosynthetic response will be required, with a focus on fine scale sampling to accurately determine the processes responsible. Attention also needs to be directed to the cell boundary layer pH, which may have an influence on the N species being assimilated under urea and NH_4^+. Above pH 9.3, all NH_3/NH_4^+ will be in the form of NH_3. This will account for the increased photosynthetic rates observed under NH_4^+ and urea as the energy costs to assimilate NH_3 for protein synthesis would be reduced (Antia *et al.* 1977; Bekheet & Syrett 1977; Solomon & Glibert 2008; Allen *et al.* 2011). For the most part it has been assumed that the coccolithophorids take up urea via active transport mechanisms then use urease to convert it to NH_3 and CO_2 (Leftley & Syrett 1973). The use of external urease in converting urea has been reported in *Emiliania huxleyi,* as has the use of a urea transporter (DUR3A-C and SLC14A) (Bekheet & Syrett 1977; Solomon *et al.* 2010), however, there is very little information about what mechanisms *Chrysotila* utilises to convert urea. From the data presented here, it is most likely that *Chrysotila carterae* (CCMP647) is using an external urease and as such, if the cellular boundary pH is greater than 9.24, then again all nitrogen available would be in the form of NH_3. This may account for the increased carbon assimilation rates observed at pH 9 when compared to lower pH levels (Figure 53) as well as increased photosynthetic rate (Figure 47). For the most part, urea results in reduced growth rates for other calcifying coccolithophorids (Antia *et al.* 1977; Palenik & Koke 1995; Benner 2008), and the increased growth observed herein poses some interesting questions. The reported lack of a nickel-urea co-limitation and stable growth in the calcifying coccolithophorids, *Coccolithus leptoporus* and *Emiliania huxleyi* (Benner 2008), gives some insight into the

possibility that *Chrysotila carterae* may be utilising another form of external urease that requires nickel, and this may be another reason for the loss of the culture after 3-4 days. It again highlights the huge inter-species variation within the coccolithophorids, and requires much more work to determine the exact reasons why *Chrysotila* displays an initial rapid growth response to urea and NH_4^+. It may be that due to its ability to drive the pH up past pH 10, and back to the starting pH of 8, that the cells are capable of utilising both CO_2 and HCO_3, or there is a yet undiscovered effect occurring within the cell boundary layer that is affecting the pH and thus the type of nitrogen being assimilated. This is only possible if, like *E. huxleyi*, *Chrysotila* uses external urease to utilise urea. As stated above, the nitrogen species available for assimilation is often affected by the medium pH, While nitrate is stable at most pH levels, at pH 9.3, NH_3 is in equilibrium with NH_4^+ and there is no real distinction between the ionic NH_4^+ and free NH_3 (Azov & Goldman 1982), as the pH increases so does the NH_3 concentration. For marine phytoplankton NH_4^+ is made available through N recycling, and must be converted in the cell by nitrate reductase to NH_3 for protein synthesis (for details see section 5.1.1).

5.2.3 Effect of nitrogen source on photosynthesis and calcification

The type of nitrogen available to *Chrysotila carterae* (CCMP647) had a wide effect on the photochemistry and carbon assimilation (Table 14) as did the medium pH. For the most part, photosynthesis was not significantly affected by the N species in unregulated pH cultures (Figure 43). It has been reported that NH_4^+ can inhibit

photosynthesis in *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* (Azov & Goldman 1982) although the data presented herein suggests that *C. carterae* is able to successfully utilise both NH_4^+ and urea when the pH is allowed to be controlled by the cells. However, evidence of any effect of urea on photosynthesis or calcification in the coccolithophorids is scarce. Here we demonstrate that using urea we can increase the calcification rate of *C. carterae* (Figure 53), although only at pH 8. This may have something to do with the calcification to photosynthesis ratio (C:P) which is much higher at alkaline pH (Figure 55), and is similar for both NO_3^- and Urea. However the C:P under NH_4^+ is greatly reduced. The reduction in C:P of *E. huxleyi* in response to excess nitrogen with increased pCO_2 had been well documented, with inhibition of PIC and increased POC at pCO_2 levels above 100 ppm (Buitenhuis *et al.* 1999; Riebesell *et al.* 2000; Sciandra *et al.* 2003). Previous studies have reported a reduction in calcification rate in most marine calcifiers when exposed to elevated CO_2 concentrations (Gattuso *et al.* 1999). In *Emiliania huxleyi* (strains; 88E, 1779 Ga, DWN 53/74/6 and a wild type isolated from an Icelandic Basin) has an been reported to have an absolute NO_3^- requirement for calcification (Merrett *et al.* 1993), with the inhibition of photosynthesis and calcification at high NO_3^- levels (100µM +) have also been reported (Merrett *et al.* 1993). Sciandra *et al.* (2003) found that in nitrogen limited cells (using NO_3^-) the calcification rate of *E. huxleyi* TW1 exposed to elevated pCO_2 (7000 µatm) will increase the C:P ratio as well as suppressing calcification by 25%. Sciandra *et al.* (2003) showed that at low pCO_2 (4000 µatm) that calcification was enhanced. The data presented in Figure 53 is in agreement with these reports, where an increase in calcification and C:P (Figure 55) is reported

(when compared to NO_3^-) under both urea and NH_4^+ with "low level" pCO_2 (Table 11). In light of these findings, it may be possible that the culture of *Chrysotila* used for these experiments is becoming C limited, and this may explain the increase in calcification observed at lower pH levels. The mechanisms behind this are reviewed extensively in Sciandra *et al.* (2003). Berry (2002) and Shiraiwa (2003) both reported that at low N and P that photosynthesis was not "inhibited" but there was an absolute increase in the rate of calcification. It is therefore possible that due to the rapid growth rate observed with urea and NH_4^+, that the culture is reducing the N and P thus increasing the calcification rate in response (Zondervan *et al.* 2002). Further testing of the nitrogen and phosphorus assimilation rates across the N sources and pH levels need to be done to determine if this is indeed the cause of the increased C:P observed in *Chrysotila carterae* (CCMP647).

From the data presented here, it is clear that the type of nitrogen available to *Chrysotila carterae* (CCMP647) will have an effect on photosynthesis and carbon uptake. Investigations into the effect of nutrients on the composition of *Emiliania huxleyi* coccoliths have also suggested that there is a rapid uptake of both N and P during the first 4 days of growth supporting the results presented in Chapter 5.1.3.2, (Hariskos, I., personal communication). Although this work on *E. huxleyi* is only in the early stages, the fact that this pattern has been observed in three different strains of *E. huxleyi* may suggest that *C. carterae* is also rapidly depleting the available nitrogen and phosphorus. Evidence to support this theory can be seen in the high photosynthetic rates when NH_4^+ and urea are used. At pH 9 (pKa 9.3), the NH_3/NH_4^+ balance is shifted to NH_3 which may have a toxic effect on the cells and

result in the collapse of the culture. While the loss of the culture after 3 days due to depletion of N and P when NH_4^+/urea is used cannot be excluded, the most likely explanation is that the culture is becoming carbon limited. In the Haptophyte *Isochrysis galbana*, similar growth patterns have been recorded to those of *C. carterae*. For example, when *I. galbana* was cultured using similar N sources to this study, growth rates were not significantly different; however greater cell densities were achieved when using $NaNO_3^-$ under nutrient limited conditions).

Table **15**).

Investigations into the effect of nitrogen source in *E. huxleyi* have indicated that NO_3^- will enhance calcification, while NH_4^+ will reduce calcification (Lefebvre *et al.* 2012); this is consistent with the results presented in Chapter 3. Lefebvre *et al*. 2012 hypothesis that the increase in calcification observed under NO_3^- is linked to a change in the redox state of the cell due to the extra amount of reductive power required to reduce NO_3^- , which is supported by (Shiraiwa 2003). These authors go on to propose that this effect may be increased under low pCO_2 or alternatively would be that cell has to remove excess protons generated during NH_4^+ assimilation.

There is link between pCO_2 and NO_3^- assimilation in *Emiliania huxleyi* (NZEH(CAWPO 6)). It has been reported that when under nutrient limitation there is a positive correlation between the amount of particulate organic nitrogen (PON) and the amount of particulate organic carbon (POC). As PON increases, so does the amount of POC; however, the amount of nitrate reductase will drop at PON concentrations above 0.2 pmol $cell^{-1}$ (Rouco *et al.* 2013). These authors also

reported that as pCO_2 increases, the C:P ratio of *E. huxleyi* NZEH will be reduced, from approximately 9.5 at 300 µatm, to 8.5 µatm pCO_2. A significant finding that is highlighted in the work of Rouco *et al.* (2013) is that the C:P ratio in *E. huxleyi* NZEH is significantly increased when the cells are under nutrient limitation (from 0.95 under nutrient replete conditions, to 1.2 under nutrient limited conditions).

Table 15 Growth data of *Isochrysis galbana* (GenBank accession number JX868515.1) from Roopnarian *et al*. (2015). Data is from reported 15 day growth curves. Culture medium was f/2. (-) indicates that culture was still in exponential growth phase after 14 days. N sources used in this study are in bold font.

N Source	Max cell density Cells L^{-1}	Days to enter stationary Phase	Growth phase after 15 days
Sodium Nitrate	7.5×10^9	-	Exponential
Potassium Nitrate	7.1×10^9	-	Entering Stationary
Ammonium Nitrate	6.0×10^9	10	Stationary
Ammonium Chloride	5.0×10^9	10	Death Phase after 14 days
Ammonium Sulphate	5.0×10^9	10	Death phase after 10 days
Ammonium Bicarbonate	4.5×10^9	11	Death phase after 12 days

What is notable about the data shown in the nutrient limited condition outlined in Table 15 is that the greatest cell density was achieved when nitrate was used as the main N source, and that when NH_4^+ is used, the culture of *I. galbana* declined much earlier than when grown on nitrate based compounds. This further supports the finding that nitrate is the most effective N source to use when culturing coccolithophorids, and *Chrysotila carterae* in particular.

6 GENERAL DISCUSSION

This thesis looked at how the pH of the culture medium and nitrogen source supplied to the cells affected the growth and calcification rate of *C. carterae*. From the data presented here, it is clear that NO_3^- is the most effective N source, but more significantly, the assumption can be made that *C. carterae* CCMP647 is using HCO_3^- directly as the main carbon source. Indications of this were reported in Moheimani & Borowitzka (2006) and Crenshaw (1964) where they recorded high biomass levels in both large scale and laboratory cultures of *Chrysotila carterae* that were allowed to reach pH 11 by cellular metabolism alone, and without the addition of an external carbon source. These results show elevated calcification rates as the pH increases, with urea and NH_4^+ increasing the amount of inorganic carbon assimilated at pH 8. This work builds on the knowledge of coccolithophorid culture by showing that *Chrysotila carterae* has a specific requirement for selenium in the same way as previously reported for *Emiliania huxleyi* (Boisson *et al.* 1989; Danbara & Shiraiwa 1999; Obata *et al.* 2004; Obata & Shiraiwa 2004).

While it was common belief that the coccoliths provide protection against high light, this work shows that *C. carterae* CCMP647 is just as susceptible to irradiances greater the 900 µmol photons m^2 s^{-1} as other algae, including *Emiliania huxleyi*. In general net photosynthetic rates in *C. carterae* CCMP647 were similar to other coccolithophorids and photoinhibition occurs at approximately 1500 µmol photons m^{-2} s^{-1}.

It has been reported previously that *Chrysotila carterae* cannot survive for long periods at a pH of less than 7 (Moheimani & Borowitzka 2006). Here it was shown that when the pH is reduced below 7 for a short time (15 minutes), the cells are able to quickly recover to the starting pH via cell metabolism alone. Moheimani and Borowitzka (2006) previously reported that *C. carterae* was producing coccoliths at night; however, the results of diurnal experiment here indicated that this alga is using the energy derived from photosynthesis to manufacturing the coccoliths during the day and then extruding them onto the cell surface at night. The reduction in medium carbon concentration (as CO_2) is a possible reason for the increase in pH observed during the light cycle. The acidification of the culture medium during dark respiration is most likely due to the increase in medium CO_2 via several mechanisms, 1) normal dark respiration, 2) an increase in external coccolith associated polysaccharides that are slightly acidic, and a small increase due to the dissolution of the coccoliths into the medium that would result in extra CO_2 being released.

The significant finding of this study is that out of the different nitrogen sources applied, nitrate was found to be the best N source for stable growth. It was also found that there is an interaction between pH and nitrogen updake, especially when ammonium compounds were used.

The growth of *C. carterae* at pH levels above pH 9 must indicate the presence of a carbon concentrating mechanism (CCM) based on HCO_3^-. In seawater, at pH above 9 the dominant carbon species is CO_3^-, with much less HCO_3^- and negligible CO_2 (Stumm & Morgan 1996). If HCO_3^- is the main external carbon source and CO_2 is

subsequently used in the carboxylation process, then this must involve the use of carbonic anhydrase to speed up the reaction rate. The use of HCO_3^- as the carbon source in *Emiliania huxleyi* has been widely reported (Sikes & Wheeler 1982; Nimer *et al.* 1991; Nimer *et al.* 1994; Nimer & Merrett 1996) although it was not until Herfort (2002) that the mechanism of carbon uptake in *E. huxleyi* was initially found. The presence of carbonic anhydrase (CA) has been detected in *C. carterae* by Sikes & Wheeler (1982) and Quiroga & Gonzalez (1993) and in *Chrysotila elongata* (CCAP961/3) with greater activity in the high calcifying strain of *C. placolithoides* (Israel & Gonzales 1996). To date, only Israel & Gonzales (1996) and Elzenga *et al.* (2000) have reported an external carbonic anhydrase in *Chrysotila*. Israel & Gonzales (1996) also found that CA was only expressed inside the chloroplasts and was not detected outside of the cell. With CA only found inside the chloroplast, these authors suggest that this CA has a role in the calcification process, although this link may be tenuous and further study required and CA has only been detected in the chloroplast, and may not have any influence on calcification, which occurs in the coccolith vesicle located inside the Golgi body. This assumption is based on the two possible pathways of HCO_3^- in *Emiliania huxleyi* as outlined below (Sikes *et al.* 1980).

HCO_3^- is channelled into the calcification pathway via:

$$HCO_3^- + Ca^{2+} \rightarrow CaCO_3 + H^+$$

CO_2 is made available for photochemistry via:

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O$$

It is a recurring point throughout this body of work that care must be taken when making general assumptions about the coccolithophorids due to the high degree of inter-species (and inter-strain) variation. With this in mind, the use of an external carbonic anhydrase may be why *C. carterae* (CCMP647) is able to be successful at high pH. The alternative would require an active HCO_3^- transporter similar to the one found by Herfort (2002) in *E. huxleyi*, however as yet no such transporter has been found. Inorganic carbon assimilation is proving an elusive question. In 2002 Herfort *et al.* give a very detailed description of the potential carbon assimilation of *E. huxleyi*. Here the authors suggest that *E. huxleyi* use a HCO_3^- transporter system (via AE1 protein) as well as yet undetected external CA process, although in 2002 Elzenga *et al.* reported external CA in a low calcifying strain of *E. huxleyi* The presence of an external CA has also been alluded to in *Isochrysis galbana*, where Bhatti *et al*. (2002) and Coleman *et al.* (2002) detected external CA in air grown cells as well as an active HCO_3^- transporter. The use of CA_{ext} in marine haptophytes has been suggested by Bhatii *et al*. (2002), to be an adaption to the higher levels of HCO_3^- in the modern oceans. These authors go on to suggest that CA_{ext} is a mechanism to maintain the equilibrium concentrations of CO_2 at the plasma membrane.

As this mechanism would appear to be common within the haptophytes, (such as *Isochrysis* and *Phaeocystis*), it would not be unrealistic to assume that *Chrysotila carterae* has a similar mechanisms, although to date external CA has only been

reported in *Crysotila* by Israel & Gonzales (1996), where a reduction in carbon assimilation was observed when carbonic anhydrase was inhibited via addition of 0.1 mM acetazolamide. Based on the evidence presented in this work it is suggested that *Chrysotila carterae* (CCMP647) also has a biphasic carbon uptake mechanism. This would be one explanation as to the success of this species at pH levels above 9.6 and may be one possible mechanism as to how this species can alter the medium pH. The diurnal change in external culture medium is consistent with other genera of phytoplankton that contain CCM's (Shiraiwa *et al.* 1993), however *Chrysotila* is among one of a very few species of microalgae that is able to alkalize the external culture medium during the day, then to return to the original (ambient) pH at night. The truly remarkable thing is that under normal conditions there is no net increase in pH over the life of the culture, which is not often seen within the phytoplankton. In contrast, *Emiliania huxleyi* does not display any change in external pH thoughout the life of the culture.

One deviation from previous reports is that it is now clear that *C. carterae* is using the energy derived from photosynthesis to assimilate inorganic carbon, which is subsequently converted to $CaCO_3$; the coccoliths are then extruded at night, which is a possible reason for increased cellular metabolic activity one hour before the start of the light cycle. It can be hypothesized that the cells have depleted all energy reserves during coccolith extrusion and, as all PS_{II} reaction centres would be open, photochemistry would be at a maximum.

6.1 Future directions:

The economic benefits of using coccolithophorids and in particular *Chrysotila carterae* CCMP647 as an alternative fuel source or for carbon sequestration have been widely reported and studied (Moheimani & Borowitzka 2011; Moheimani *et al.* 2011; Moheimani *et al.* 2012).

One key area that requires much more investigation is the internal pH of *Chrysotila carterae* and to look the interactions between cell compartments and pH. It was not the scope of this thesis to investigate these interactions, but it is a key question that must be answered to further advance out knowledge of the coccolithophorids. Another key element is the cell boundary layer pH. While external enzymes such as external urease and carbonic anhydrase have been detected in his alga, how they affect the boundary layer pH and thus, what type of carbon species can be used is another key area that required great endeavour. As part of this line of inquiry, the presence of carbonic anhydrase needs to be investigated across a wide range of stains (and genera) as well as the circumstances by which it is produced.

To date, *Chrysotila carterae* is the only coccolithophorid to be reliably grown in large scale outdoors for use with commercially valuable resources such as bio fuels, high value lipids such as polyunsaturated fatty acids (PUFA) bio-scaffolding and as a source of carbon bioremediation for an extensive review of the commercialization and high value product generated from coccolithophorids and other microalgae see Moheimani *et al.* (2012) and Borowitzka (2013). It is for these reasons that we need further investigations into the interaction between nitrogen and growth, in particular,

how the nitrogen species will affect growth and calcification. It is clear from this work that ammonium based compounds increase growth, but via what mechanism? Therefore, a detailed look at the nitrogen pathway and how it interacts with the carbon cycle and calcification is desperately required. It was not the focus of this work to investigate the effects of nitrogen sources on lipid production in *C. carterae*; however, this is a key element that will need further study. This will be vital information for any attempt to produce massive, commercial scale culture of *C. carterae*.

6.2 General conclusion

From this work, it is suggested that for *Chrysotila carterae* (CCMP647), nitrate is the most effective N source for successful mass culture. While nitrate provides the most stable growth, should a rapid increase in cell growth be required, is suggested that the addition of urea to the medium would provide a quick increase in cell numbers. This species also has an absolute requirement for selenium. Diurnal carbon uptake shows that this species is manufacturing coccoliths during the light period using the energy derived from photosynthesis, and then extruding the coccoliths at night.

7 REFERENCES

Adir, N., Zer, H., Shochat, S. Ohad, I. (2003). Photoinhibiton - a historical perspective. Photosynthesis Research 76: 343-370.

Allen, A. E., Dupont, C. L., Obornik, M., Horak, A., Nunes-Nesi, A., McCrow, J. P., Zheng, H., Johnson, D. A., Hu, H., Fernie, A. R. Bowler, C. (2011). Evolution and metabolic significance of the urea cycle in photosynthetic diatoms. Nature 473 (7346): 203-207.

Allen, M. J., Howard, J. A., Lilley, K. S. Wilson, W. H. (2008). Proteomic analysis of the EhV-86 virion. Proteome Science 6.

Allen, M. J. Wilson, W. H. (2006). The coccolithovirus microarray: an array of uses. Briefings in Functional Genomics & Proteomics 5 (4): 273-279.

Anand, P. L. (1937). A taxonomic study of the algae of the British chalk-cliffs. Journal of Botany (London) 75 (2): 1-51.

Andersen, R. A., Kim, J. I., Tittley, I. Yoon, H. S. (2014). A re-investigation of *Chrysotila* (Prymnesiophyceae) using material collected from the type locality. Phycologia 53 (5): 463-473.

Andreae, M. O. Barnard, W. R. (1984). The marine chemistry of Di-methyl Sulfide. Marine Chemistry 14 (3): 267-280.

Anning, T., Nimer, N., Merret, M. J. Brownlee, C. (1996). Cost and benifit of calcification in coccolithophorids. Journal of Marine Systems 9: 45-56.

Antia, N. J., Berland, B. R., Bonin, D. J. Maestrini, S. Y. (1977). Effects of urea concentration in supporting growth of certain marine micro planktonic algae. Phycologia 16 (1): 105-112.

Antia, N. J., Harrison, P. J. Oliveira, L. (1991). The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia 30 (1): 1-89.

Araie, H., Sakamoto, K., Suzuki, I. Shiraiwa, Y. (2011). Characterization of the Selenite Uptake Mechanism in the Coccolithophore *Emiliania huxleyi* (Haptophyta). Plant and Cell Physiology 52 (7): 1204-1210.

Araie, H. Shiraiwa, Y. (2009). Selenium utilization strategy by microalgae. Molecules 14 (12): 4880-4891.

Ariovich, D. Pienaar, R. N. (1979). The role of light in the incorporation and utilization of Ca^{++} ions by *Hymenomonas carterae* (Braarud et Fagerl.) Braarud (Prymnesiophyceae). British Phycological Journal 14 (1): 17-24.

Azov, Y. Goldman, J. C. (1982). Free ammonia inhibition of algal photosynthesis in intensive cultures. Appl. Environ. Microbiol. 43 (4): 735-739.

Bach, L. T., Mackinder, L. C. M., Schulz, K. G., Wheeler, G., Schroeder, D. C., Brownlee, C. Riebesell, U. (2013). Dissecting the impact of CO_2 and pH on the mechanisms of photosynthesis and calcification in the coccolithophore *Emiliania huxleyi*. New Phytologist 199 (1): 121-134.

Balch, W., Fritz, J. Fernandez, E. (1996). Decoupling of calcification and photosynthesis in the coccolithophore *Emiliania huxleyi* under steady-state light-limited growth. Marine Ecology Progress Series 142: 87-97.

Balch, W. M., Kilpatrick, K., Holligan, P. M. Cucci, T. (1993). Coccolith production and detachment by *Emiliania huxleyi* (Prymnesiophyceae). Journal of Phycology 29 (5): 566-575.

Barbosa, M. J., Albrecht, M. Wijffels, R. H. (2003). Hydrodynamic stress and lethal events in sparged microalgae cultures. Biotechnology and Bioengineering 83 (1): 112-120.

Baroli, I. Melis, A. (1998). Photoinhibitory damage is modualted by the rate of photosynthesis and the photosystem II light-harvesting chlorophyll antenna size. Planta 205: 288-296.

Beech, P. L. Wetherbee, R. (1988). Observations on the flagellar apparatus and peripheral endoplasmic reticulum of the coccolithophorid, *Pleurochrysis carterae* (Prymnesiophycea). Phycologia 27 (1): 142-158.

Beech, P. L., Wetherbee, R. Pickett-Heaps, J. D. (1988). Transformation of the flagella and associated flagellar components during cell division in the coccolithophorid *Pleurochrysis carterae*. Protoplasma 145 (1): 37-46.

Beer, S., Ilan, M., Eshel, A., Weil, A. Brickner, I. (1998). Use of pulse amplitude modulated (PAM) fluorometry for in situ measurements of photosynthesis in two Red Sea faviid corals. Marine Biology 131 (4): 607-612.

Bekheet, I. A. Syrett, P. J. (1977). Urea degrading enzymes in algae. British Phycological Journal 12 (2): 137-143.

Bekheet, I. A. Syrett, P. J. (1979). The uptake of urea by *Chlorella*. New Phytologist 82 (1): 179-186.

Bellerby, R. G. J., Schulz, K. G., Riebesell, U., Neill, C., Nondal, G., Heegaard, E., Johannessen, T. Brown, K. R. (2008). Marine ecosystem community carbon and nutrient uptake stoichiometry under varying ocean acidification during the PeECE III experiment. Biogeosciences 5 (6): 1517-1527.

Benner, I. (2008) The utilization of organic nutrients in marine phytoplankton with emphasis on coccolithophores. Bremen University, Ph.D.

Berges, J. A. Mulholland, M. R. (2008). Enzymes and nitrogen cycling. Nitrogen in the Marine Environment, Capone, D. G., Bronk, D. A., Mulholland, M. R. (Eds), 1385-1444. Elsevier Inc.

Bergmann, T., Richardson, T. L., Paerl, H. W., Pinckney, J. L. Schofield, O. (2002). Synergy of light and nutrients on the photosynthetic efficiency of phytoplankton populations from the Neuse River Estuary, North Carolina. Journal of Plankton Research. 24 (9): 923-933.

Berman, T. Bronk, D. A. (2003). Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquatic Microbial Ecology 31 (3): 279-305.

BermanFrank, I., Kaplan, A., Zohary, T. Dubinsky, Z. (1995). Carbonic anhydrase activity in the bloom-forming dinoflagellate *Peridinium gatunense.* Journal of Phycology 31 (6): 906-913.

Berry, L., Taylor, A. R., Lucken, U., Ryan, K. P. Brownlee, C. (2002). Calcification and inorganic carbon acquisition in coccolithophores. Functional Plant Biology 29 (3): 289-299.

Bhatti, S., Huertas, I. E. Colman, B. (2002). Acquisition of inorganic carbon by the marine Haptophyte *Isochrysis galbana* (Prymnesiophyceae). Journal of Phycology 38 (5): 914-921.

Biermann, A. Engel, A. Effect of CO_2 on the properties and sinking velocity of aggregates of the coccolithophore *Emiliania huxleyi*. Biogeosciences 7 (3): 1017-1029.

Bilan, M. I. Usov, A. I. (2001). Polysaccharides of calcareous algae and their effect on the calcification process. Russian Journal of Bioorganic Chemistry 27 (1): 2-16.

Billard, C. (1994). Life Cycles. In: The Haptophyte Algae. J. C. Green and B. S. C. Leadbeater (Eds). Oxford University, Oxford pp 446

Boisson, F., Gnassia-Barelli, M., Chiaverini, J. Romeo, M. (1989). Effect of selenium on the uptake of cadmium by the marine microalga *Hymenomonas eongata*. Marine Environmental Research 28 (1-4): 465-470.

Boisson, F., Gnassiabarelli, M. Romeo, M. (1995). Toxicity and accumulation of selenite and selenate in the unicellular marine alga *Cricosphaera elongata*. Archives of Environmental Contamination and Toxicology 28 (4): 487-493.

Borman, A. H., de Jong, E. W., Huizinga, M., Kok, D. J., Westbroek, P. Bosch, L. (1982). The role in $CaCO_3$ crystallization of an acid Ca^{2+}-binding polysaccharide associated with coccoliths of *Emiliania huxleyi*. Eur. J. Biochem. 129 - 179.

Borowitzka, L. J. (1994). Commercial pigment from algae. In Moi, P.S, Kun, L. Y., Borowitzka, M. A., Winton, B.A. (Eds). The First Asia-Pacific Conference on Algal Biotechnology, Institute of Advanced Studies, University of Malaya, Kuala Lumpur.pp 82-84

Borowitzka, L. J. Borowitzka, M. A. (1990). Commercial production of β-carotene by *Dunaliella salina* in open ponds. Bulletin of Marine Science 47: 244-252.

Borowitzka, M. A. (1989). Carbonate Calcification in Algae - Initiation and Control. Biomineralization: chemical and biochemical perpectives. S. Mann, J. Webb and R. J. P. Williams, 63-64. VCH**:** Weinheim.

Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. Journal of Biotechnology 70 (1-3): 313-321.

Borowitzka, M. A. (2013). High-value products from microalgae-their development and commercialisation. Journal of Applied Phycology 25 (3): 743-756.

Bororwitzka, M.A, (2016) Algal physiology and large-scale outdoor cultures of microalgae. In, Borowitzka, M.A., Beardall, J., Raven, J.A (Eds) The physiology of microalgae. Springer, Dordecht.

Borowitzka, M. A. Larkum, A. W. D. (1976). Calcification in the Green Alga *Halimeda*: II. The exchange of Ca^{2+} and the occurance of age gradients in calcification and photosynthesis. J. Exp. Bot. 27 (5): 864-878.

Brand, L. E. (1991). Minimum iron requirements of marine plhytoplankton and the implications for the biogeochemical control of new production. Limnology and Oceanography 36 (8) 1756 – 1771.

Brand, L. E., Sunda, W. G. Guillard, R. R. L. (1986). Reduction of marine phytoplankton reproduction rates by copper and cadmium. Journal of Experimental Marine Biology and Ecology 96 (3): 225-250.

Brewer, P. G. Goldman, J. C. (1976). Alkalinity changes generated by phytoplankton growth. Limnology and Oceanography 21 (1): 108-117.

Broecker, W. S. Peng, T. H. (1982). Tracers in the sea. Columbia Universiity, Palisades, New York.

Bronk, D. A., See, J. H., Bradley, P. Killberg, L. (2007). DON as a source of bioavailable nitrogen for phytoplankton. Biogeosciences 4 (3): 283-296.

Brown, C. W. Yoder, J. A. (1994). Coccolthiophorid blooms in the global ocean. Journal Geophysical Research (99) 7467-7482.

Brownlee, C., Taylor, A., Thierstein, H. Young, J. R. (2004). Coccolithophores - From Molecular Scale Processes to Global Impact. Springer, Heidleberg.GmbH

Brownlee, C. Taylor, A. R. (2004). Calcification in coccolithophores: A cellular perspective. Coccolithophores, from molecular processes to global impact. H. R. Thierstein and J. R. Young. Pp 31 -50. Springer**:** Berlin Heidelberg. GmbH

Bruhn, A., LaRoche, J. Richardson, K. (2010). *Emiliania huxleyi* (Prymnesiophyceae): nitrogen-metabolism genes and their expression in response to external nitrogen sources. Journal of Phycology 46 (2): 266-277.

Buitenhuis, E. T., de Baar, H. J. W. Veldhuis, M. J. W. (1999). Photosynthesis and calcification by *Emiliania huxley*i (Prymnesiophyceae) as a function of inorganic carbon species. Journal of Phycology 35 (5): 949-959.

Buttery, M. J. (2000) Culture studies of two toxic dinoflagellate species, *Alexandrium minutum* and *Gymnodinium catenatum*. Murdoch University, Perth Ph.D.

Caldeira, K. Wickett, M. E. (2003). Oceanography: Anthropogenic carbon and ocean pH. Nature 425 (6956): 365-365.

Camacho Rubio, F., Garcia Camacho, F., Fernandez Sevilla, J. M., Chisti, Y. Molina Grima, E. (2003). A mechanistic model of photosynthesis in microalgae. Biotechnology and Bioengineering 81 (4): 459-473.

Casareto, B. E., Niraula, M. P., Fujimura, H. Suzuki, Y. (2009). Effects of carbon dioxide on the coccolithophorid *Pleurochrysis carterae* in incubation experiments. Aquatic Biology 7 (1-2): 59-70.

Charlson, R. J., Lovelock, J. E., Andreae, M. O. Warren, S. G. (1987). Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. Nature 326 (6114): 655-661.

Chisti, Y. (2007). Biodiesel from microalgae. Biotechnology Advances 25 (3): 294-306.

Christensen, T. (1978). Annotations to a text book of phycology. Botanisk Tidsskift 73 65-70.

Clarke, A. D., Owens, S. R. Zhou, J. C. (2006). An ultrafine sea-salt flux from breaking waves: Implications for cloud condensation nuclei in the remote marine atmosphere. Journal of Geophysical Research-Atmospheres 111 (D6).

Colman, B., Huertas, E., Bhatti, S. Dason, J. S. (2002). The diversity of inorganic carbon acquisition mechanisms in eukaryotic microalgae. Functional Plant Biology. 29: 261 - 271.

Cosgrove, J. Borowitzka, M. (2006). Applying Pulse Amplitude Modulation (PAM) fluorometry to microalgae suspensions: stirring potentially impacts fluorescence. Photosynthesis Research 88 (3): 343-350.

Crenshaw, M. A. (1964) Coccolith formation by two marine coccoliths, *Coccolithus huxleyi* and *Hymenomonas carterae*. Duke Univversity, Ph.D

Cutter, G. A. Bruland, K. W. (1984). The Marine Biogeochemistry of Selenium: A Re-Evaluation. Limnology and Oceanography 29 (6): 1179-1192.

Danbara, A. Shiraiwa, Y. (1999). The requirement of selenium for the growth of marine coccolithophorids, *Emiliania huxleyi*, *Gephyrocapsa oceanica* and *Helladosphaera* sp (Prymnesiophyceae). Plant and Cell Physiology 40 (7): 762-766.

de Vrind-de Jong, E. W. deVrind, J. P. M. (1997). Algal deposition of carbonates and silicates. Geomicrobiology: Interactions between Microbes and Minerals. J. F. Banfeild and K. H. Nealson, 267-307. Mineralogical Scociety of America**:** Washington.

Decho, A. W. (1990). Microbial exopolymer secretions in ocean environments - their role(s) in food webs and marine processes. Oceanography and Marine Biology 28: 73-153.

Doblin, M. A., Blackburn, S. I. Hallegraeff, G. M. (1999). Comparative study of selenium requirements of three phytoplankton species: *Gymnodinium catenatum*, *Alexandrium minutum* (Dinophyta) and *Chaetoceros* cf. *tenuissimus* (Bacillariophyta). Journal of Plankton Research 21 (6): 1153-1169.

Dyhrman, S. T. Anderson, D. M. (2003). Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. Limnology and Oceanography 48 (2): 647-655.

Ehrenberg, D. C. G. (1836). Bemerkungen über feste mikroskopische, anorganische Formen inden erdigen und derben Mineralien. Berlin.in: Bericht über die zur Bekanntmachung geeigneten Verhandlungen der Königl. Preuß. Akademie der Wissenschaften zu Berlin. S.84-85

Elzenga, J. T. M., Prins, H. B. A. Stefels, J. (2000). The role of extracellular carbonic anhydrase activity in inorganic carbon utilization of *Phaeocystis globosa* (Prymnesiophyceae): A comparison with other marine algae using the isotopic disequilibrium technique. Limnology and Oceanography 45 (2): 372-380.

Eppley, R. W., Coatsworth, J. L. Solorzano, L. (1969). Studies of nitrate reductase in marine phytoplankton. Limnology and Oceanography 14 (2): 194-205.

Eppley, R. W., Holmes, R. W. Strickland, J. D. H. (1967). Sinking rates of marine phytoplankton measured with a fluorometer. J. Exp. Mar. Biol. Ecol 1(2): l91-208.

Evans, C., Malin, G., Mills, G. P. Wilson, W. H. (2006). Viral infection of *Emiliania huxleyi* (Prymnesiophyceae) leads to elevated production of reactive oxygen species. Journal of Phycology 42 (5): 1040-1047.

Falkowski, P. G. (1997). Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. Nature 387 (6630): 272-275.

Falkowski, P. G., Owens, T. G., Ley, A. C. Mauzerall, D. C. (1981). Effects of growth irradiance levels on the ratio of reaction centers in two species of marine phytoplankton. Plant Physiology 68 (4): 969-973.

Falkowski, P. G. Raven, J. A. (2007). Aquatic Photosynthesis. Princeton University Press, New Jersey.

Feng, Y., Warner, M. E., Zhang, Y., Sun, J., Fu, F.-X., Rose, J. M. Hutchins, D. A. (2008). Interactive effects of increased pCO_2, temperature and irradiance on the marine coccolithophore *Emiliania huxleyi* (Prymnesiophyceae). European Journal of Phycology 43 (1): 87 - 98.

Fernandez, E., Boyd, P. W., Holligan, P. M. Harbour, D. S. (1993). Production of organic and inorganic carbon within a large-scale coccolithophore bloom in the northeast Atlantic Ocean. Marine Ecology Progress Series 97: 271-285.

Findlay, C. S., Young, J. Scott, F. J. (2005). Haptophytes: Order Coccolithophorales. Antarctic Marine Protists. F. J. Scott and H. J. Marchant. AAD Hobart, ABRS Canberra**:** Canberra.pp. 256-276.

Flynn, K., Flynn, K. J. Jones, K. J. (1993). Changes in dinoflagellate intracellular amino-acids in response to diurnal changes in light and N-supply. Marine Ecology Progress Series 100 (3): 245-252.

Flynn, K. J. (1991). Algal carbon-nitrogen metabolism: a biochemical basis for modelling the interactions between nitrate and ammonium uptake. J. Plankton Res. 13 (2): 373-387.

Flynn, K. J. (2002). How critical is the critical N : P ratio? Journal of Phycology 38 (5): 961-970.

Flynn, K. J., Page, S., Wood, G. Hipkin, C. R. (1999). Variations in the maximum transport rates for ammonium and nitrate in the prymnesiophyte *Emiliania huxleyi* and the raphidophyte *Heterosigma carterae*. Journal of Plankton Research 21 (2): 355-371.

Frada, M., Percopo, I., Young, J., Zingone, A., de Vargas, C. Probert, I. (2009). First observations of heterococcolithophore-holococcolithophore life cycle combinations in the family Pontosphaeraceae (Calcihaptophycideae, Haptophyta). Marine Micropaleontology 71 (1-2): 20-27.

Fresnel, J. (1994). A heteromorphic life cycle in two coastal coccolithophorids, *Hymenomonas lacuna* and *Hymenomonas coronata* (Prymnesiophyceae). Canadian Journal of Botany 72 (10): 1455-1462.

Fresnel, J. Billard, C. (1991). *Pleurochrysis placolithoides* sp. nov. (Prymnesiophyceae), a new marine Coccolithophorid with remarks on the status of criocolith-bearing species. British Phycological Journal 26: 67-80.

Friedlingstein, P., Houghton, R. A., Marland, G., Hackler, J., Boden, T. A., Conway, T. J., Canadell, J. G., Raupach, M. R., Ciais, P. Le Quere, C. (2010). Update on CO_2 emissions. Nature Geosci 3 (12): 811-812.

Gattuso, J.-P., Allemand, D. Frankignoulle, M. (1999). Photosynthesis and Calcification at Cellular, Organismal and Community Levels in Coral Reefs: A Review on Interactions and Control by Carbonate Chemistry. Amer. Zool. 39 (1): 160-183.

Gattuso, J. P., Allemand, D. Frankignoulle, M. (1999). Photosynthesis and Calcification at Cellular, Organismal and Community Leveles in Coral Reefs: A Review on Interaction and Control by Carbonate Chemistry. American Zoology 39: 160-183.

Gattuso, J. P. Buddemeier, R. W. (2000). Calcification and CO_2. Nature 407: 311-313.

Gayral, P. Fresnel, J. (1976). New observations on 2 marine coccolithophoraceae *Cricosphaera roscoffensis* new combination and *Hymenomonas globosa* new combination. Phycologia 15 (3-4): 339-356.

Gayral, P. Fresnel, J. (1983). Description sexuality and developmental cycle of a new coccolithophorid Prymnesiophyceae *Pleurochrysis pseudoroscoffensis* new-species. Protistologica 19 (2): 245-262.

Geider, R. J., MacIntyre, H. L. Kana, T. M. (1997). Dynamic model of phytoplankton growth and acclimation: Responses of the balanced growth rate and the chlorophyll a:carbon ratio to light, nutrient-limitation and temperature. Marine Ecology Progress Series 148 (1-3): 187-200.

Glibert, P. M., Garside, C., Fuhrman, J. A. Roman, M. R. (1991). Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. Limnology and Oceanography 36 (5): 895-909.

Glibert, P. M., Harrison, J., Heil, C. Seitzinger, S. (2006). Escalating worldwide use of urea - a global change contributing to coastal eutrophication. Biogeochemistry 77 (3): 441-463.

Gordon, H. R. Du, T. (2001). Light scattering by nonspherical particles: Application to coccoliths detached from *Emiliania huxleyi*. Limnology and Oceanography 46 (6): 1438-1454.

Gordon, H. R., Smyth, T. J., Balch, W. M., Boynton, G. C. Tarran, G. A. (2009). Light scattering by coccoliths detached from *Emiliania huxleyi*. Applied Optics 48 (31): 6059-6073.

Greene, R. M., Geider, R. J. Falkowski, P. G. (1991). Effect of iron limitation on photosynthesis in a marine diatom. Limnology and Oceanography 36(8): 1772-1782.

Grima, E. M., Sevilla, J. M. F., Perez, J. A. S. Camacho, F. G. (1996). A study on simultaneous photolimitation and photoinhibition in dense microalgal cultures taking into account incident and averaged irradiances. Journal of Biotechnology 45 (1): 59-69.

Hagel, P., Gerding, J. J. T., Fieggen, W. Bloemendal, H. (1971). Cyanate formation in solutions of urea part 1 calculation of cyanate concentrations at different temperature and pH. Biochimica et Biophysica Acta 243 (3): 366-373.

Harding, L. W., Jr., Prézelin, B. B., Sweeney, B. M. Cox, J. L. (1982). Diel oscillations of the photosynthesis-irradiance (P-I) relationship in natural assemblages of phytoplankton. Marine Biology 67 (2): 167-178.

Harris, G. N., Scanlan, D. J. Geider, R. J. (2005). Acclimation of *Emiliania huxleyi* (Prymnesiophyceae) to photon flux density. Journal of Phycology 41 (4): 851-862.

Harrison, P. J., Yu, P. W., Thompson, P. A., Price, N. M. Phillips, D. J. (1988). Survey of selenium requirements in marine phytoplankton. Marine Ecology Progress Series 47: 89-96.

Hastings, J. W. Astracham, L., Sweeney, B.M. (1961). Persistant daily rhythm in photosynthesis. J. Gen Physiol. 45:69-76

Hawkins, E. K., Lee, J. J. Correia, M. (2003). Polar localization of filamentous actin in cells of the scale-forming alga *Pleurochrysis* sp. Protoplasma 220 (3-4): 233-236.

Hawkins, E. K., Lee, J. J. Fimiarz, D. K. (2011). Colony formation and sexual morphogenesis in the coccolithophore *Pleurochrysis* sp. (Haptophyta)1. Journal of Phycology 47 (6): 1344-1349.

Head, R. N., Crawford, D. W., Egge, J. K., Harris, R. P., Kristiansen, S., Lesley, D. J., Maranon, E., Pond, D. Purdie, D. A. (1998). The hydrography and biology of a bloom of the coccolithophorid *Emiliania huxley*i in the northern North Sea. Journal of Sea Research 39 (3-4): 255-266.

Henriksen, K., Stipp, S. L. S., Young, J. R. Marsh, M. E. (2004). Biological control on calcite crystallization: AFM investigation of coccolith polysaccharide function. American Mineralogist 89 (11-12): 1709-1716.

Herfort, L., Loste, E., Meldrum, F. Thake, B. (2004). Structural and physiological effects of calcium and magnesium in *Emiliania huxleyi* (Lohmann) Hay and Mohler. Journal of Structural Biology 148 (3): 307-314.

Herfort, L., Thake, B. Roberts, J. (2002). Acquisition and use of bicarbonate by *Emiliania huxleyi*. New Phytologist 156 (3): 427-436.

Hinga, K. R. (2002). Effects of pH on coastal marine phytoplankton. Marine Ecology Progress Series 238: 281-300.

Hirokawa, Y., Fujiwara, S., Suzuki, M., Akiyama, T., Sakamoto, M., Kobayashi, S. Tsuzuki, M. (2008). Structural and physiological studies on the storage β-polyglucan of Haptophyte *Pleurochrysis haptonemofera*. Planta 227 (3): 589-599.

Hirokawa, Y., Fujiwara, S. Tsuzuki, M. (2005). Three Types of Acidic Polysaccharides Associated with Coccolith of *Pleurochrysis haptonemofera* Comparison with & *Pleurochrysis carterae*; and Analysis Using Fluorescein-Isothiocyanate-Labeled Lectins. Marine Biotechnology 7 (6): 634-644.

Holligan, P. M., Viollier, M., Harbour, D. S., Camus, P. Champagne-Philippe, M. (1983). Satellite and ship studies of coccolithophore production along a continental shelf edge. Nature 304 (5924): 339-342.

Hough, R. A. Wetzel, R. G. (1978). Photorespiration and CO_2 compensation point in *Najas flexilis*. Limnology and Oceanography 23 (4): 719-724.

Humphrey, G. F. (1975). The photosynthesis: Respiration ratio of some unicellular marine algae. Journal of Experimental Marine Biology and Ecology 18 (2): 111-119.

Huxley, T. H. (1868). On some organisms living at great depths in the North Atlantic Ocean. Quaterly Journal of Micoscopical Society. 8: 203-212.

Iglesias-Rodriguez, M. D., Halloran, P. R., Rickaby, R. E. M., Hall, I. R., Colmenero-Hidalgo, E., Gittins, J. R., Green, D. R. H., Tyrrell, T., Gibbs, S. J., von Dassow, P., Rehm, E., Armbrust, E. V. Boessenkool, K. P. (2008).

Phytoplankton Calcification in a High-CO2 World. Science 320 (5874): 336-340.

Iglesias-Rodriguez, M. D., Nimer, N. A. Merrett, M. J. (1998). Carbon dioxide-concentrating mechanism and the development of extracellular carbonic anhydrase in the marine picoeukaryote *Micromonas pusilla*. New Phytologist 140 (4): 685-690.

Inouye, I. Pienaar, R. N. (1985). Ultrastructure of the flagellar apparatus in *Pleurochrysis* class Prymnesiophyceae. Protoplasma 125 (1-2): 24-35.

Israel, A. A. Gonzales, E. L. (1996). Photosynthesis in inorganic carbon utilization in *Pleurochrysis* sp. (Haptophyta), a coccolithophorid alga. Marine Ecology Progress Series 137: 243-250.

Jenkins, B. D. Zehr, J. P. (2008). Molecular approaches to the nitrogen cycle. In: Nitrogen in the Marine Environment, Capone, D.G., Bronk, D. A., Muholland, M. R.., Carpenter, E.J. (Eds). 1303-1344. Elsevier Inc. Burlington USA.

Jordan, R. W. (2012). Haptophyta. eLS. John Wiley & Sons, Ltd**:** Chichester.

Jordan, R. W. Chamberlain, A. H. L. (1997). Biodiversity among haptophyte algae. Biodiversity and Conservation V6 (1): 131-152.

Jordan, R. W., Cros, L. Young, J. R. (2004). A revised classification scheme for living haptophytes. Micropaleontology 50 (Suppl 1): 55-79.

Kaffes, A., Thoms, S., Trimborn, S., Rost, B., Langer, G., Richter, K.-U., Köhler, A., Norici, A. Giordano, M. (2010). Carbon and nitrogen fluxes in the marine coccolithophore *Emiliania huxleyi* grown under different nitrate concentrations. Journal of Experimental Marine Biology and Ecology 393 (1-2): 1-8.

Kaftan, D., Meszaros, T., Whitmarsh, J., Nedbal, L., (1999). Characerization of photosystem II activity and hererogeneity during the cell cyle of the green alga, *Scenedemus quadricauda*. Plant Physiology 120: 433-443

Katagiri, F., Takatsuka, Y., Fujiwara, S. Tsuzuki, M. (2010). Effects of Ca and Mg on Growth and Calcification of the Coccolithophorid *Pleurochrysis haptonemofera*: Ca Requirement for Cell Division in Coccolith-Bearing Cells and for Normal Coccolith Formation with Acidic Polysaccharides. Marine Biotechnology 12 (1): 42-51.

Kato, M. C., Hikosaka, K., Hirotsu, N., Makino, A. Hirose, T. (2003). The excess light energy that is neither utililzed in photosynhtesis nor dissipated by photoprotective mechanisms determines the rate of photoinactivation in photosysntem II. Plant and Cell Physiology 44 (3): 318-325.

Kawachi, M. Inouye, I. (1995). Functional roles of the haptonema and the spine scales in the feeding process of *Chrysochromulina spinifera* (Fournier) Pienaar et Norris (Haptophyta Prymnesiophyta). Phycologia 34 (3): 193-200.

Kegel, J., Allen, M. J., Metfies, K., Wilson, W. H., Wolf-Gladrow, D. Valentin, K. (2007). Pilot study of an EST approach of the coccolithophorid *Emiliania huxleyi* during a virus infection. Gene 406 (1-2): 209-216.

Keller, M. D., Bellows, W. K. Guillard, R. R. L. (1989). Dimethyl Sulfide Production in Marine Phytoplankton. Biogenic Sulfur in the Environment.

Saltzman, E. S., Cooper, W. J. (Eds), pp 167-182. American Chemical Society.

Kessin, R. H. (2001). *Dictyostelium* Evolution, cell biology, and the development of multicellularity. Cambride University Press, Cambeidge UK.

Klaveness, D. (1972). *Coccolithus huxley* (Lohm.) Kamptn. 2. The flagellate cell, aberrant cell types, vegetative propagation and life cycles. British Phycolological Journal. 7 (3): 309-318.

Klaveness, D. (1972). *Coccolithus huxleyi* Part 1 morphological investigations on the vegetative cell and the process of coccolith formation. Protistologica 8 (3): 335-346.

Kleijne, A., Jordan, R. W., Heimdal, B. R., Samtleben, C., Chamberlain, A. H. L. Cros, L. (2002). Five new species of the coccolithophorid genus *Alisphaera* (Haptophyta), with notes an their distribution, coccolith stucture and taxonmy. Phycologia 40 (6): 583-601.

Kolber, Z., Barber, R. T., Coale, K. H., Fitzwater, S. E., Greene, R. M., Johnson, K. S., Lindley, S. Falkowski, P. G. (1994). Iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. Nature 317: 145-148.

Komkamp, J. C. Forster, R. M. (2003). The use of variable fluorescence measurements in aquatic ecosystems: differences between multiple and single turnover measuring protocols and suggested terminology. European Journal of Phycology 38: 103-112.

Krauss, R. W. (1962). Mass culture of algae for food and other organic compounds. Amer Jour Bot 49 (4): 425-435.

Kristiansen, S. (1983). Urea as a nitrogen source for the phytoplankton in the Oslofjord. Marine Biology 74 (1): 17-24.

Kristiansen, S., Thingstad, T. F., Vanderwal, P., Farbrot, T. Skjoldal, E. F. (1994). An *Emiliania huxley*i dominated subsurface bloom in Samnangerfjorden, Western Norway - importance of hydrography and nutrients Sarsia 79 (4): 357-368.

Krom, M.D., Kress, N., Brenner, S., *and* Gordon, L.I. *(*1991*)* Phosphorus limitation of primary productivity in the eastern Mediterranean Sea. Limnol Oceanogr 36*:* 424–432

Laing, W. A., Ogren, W. L. Hagman, R. H. (1974). Regulation of soybean net photosynthetic CO_2 fixation by the Interaction of CO_2, O_2 and Ribulose 1, 5-Diphosphate Carboxylase 1.2. Plant Physiology 54: 678-685.

Langer, G., Nehrke, G., Probert, I., Ly, J. Ziveri, P. (2009). Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry. Biogeosciences 6 (11): 2637-2646.

Leadbeater, B. S. C. (1970). Preliminary observations on differences of scale morphology at various stages in the life cycle of *Apistonema syracosphaer* sensu von Stosch. Br. Phycol. J 5 (1): 57-69.

Leadbeater, B. S. C. (1971). Observations on the Life History of the Haptophycean Alga Pleurochrysis-Scherffelii with Special Reference to the Micro Anatomy of the Different Types of Motile Cells. Annals of Botany (London) 35 (140): 429-439.

Leclercq, N. I. c., Gattuso, J. E. A. N. P. Jaubert, J. E. A. N. (2000). CO_2 partial pressure controls the calcification rate of a coral community. Global Change Biology 6 (3): 329-334.

Lecourt, M., Muggli, D. Harrison, P. J. (1996). Comparison of growth and sinking rates of non-coccolith and coccolith-forming strains of *Emiliania huxleyi* (Prymnesiophyceae) grown under differnet irradiances and nitrogen sources. Journal of Phycology 32: 17-21.

Lefebvre, S. C., Benner, I., Stillman, J. H., Parker, A. E., Drake, M. K., Rossignol, P. E., Okimura, K. M., Komada, T. Carpenter, E. J. (2012). Nitrogen source and pCO_2 synergistically affect carbon allocation, growth and morphology of the coccolithophore *Emiliania huxleyi*: Potential implications of ocean acidification for the carbon cycle. Global Change Biology 18 (2): 493-503.

Leftley, J. W. Syrett, P. J. (1973). Urease and ATP - urea amidolyase activity in unicellular algae. Journal of General Microbiology 77 (JUL): 109-115.

Lenton, T. M. Watson, A. J. (2000). Redfield revisited: 1. Regulation of nitrate, phosphate, and oxygen in the ocean. Global Biogeochem. Cycles 14 (1): 225-248.

Lessard, E. J., Merico, A. Tyrrell, T. (2005). Nitrate : phosphate ratios and *Emiliania huxleyi* blooms. Limnology and Oceanography 50 (3): 1020-1024.

Lesser, M. P. (2006). Oxidative stress in marine environments: Biochemistry and physiological ecology. Annual Review of Physiology. 253-278.

Linschooten, C., van Bleijswijk, J. D. L., van Emburg, P. R., de Vrind, J. P. M., Kempers, E. S., Westbroek, P. de Vrind-de Jong, E. (1991). Role of the light-dark cycle and medium composition on the production of coccolithis by *Emiliania huxleyi* (Haptophyceae). Journal of Phycology 27: 82-86.

Linschooten, C., Vanbleijswijk, J. D. L., Vanemburg, P. R., Devrind, J. P. M., Kempers, E. S., Westbroek, P. Devrinddejong, E. W. (1991). Role of the light-dark cycle and medium composition on the production of coccoliths by *Emiliania huxleyi* (Haptophyceae). Journal of Phycology 27 (1): 82-86.

Long, S. P., Humphries, S. Falkowski, P. G. (1994). Photoinhibition of photosynthesis in nature. Annual Review of Plant Physiology and Plant Molecular Biology. 45: 633- 662.

Manly, B. F. J. (2001). Statistics for Enviromental Science and Management. Chapman & Hall/ CRC, Boca Raton, Florida.

Manton, I. Leedale, G. F. (1969). Observations on the micro anatomy of *Coccolithus-pelagicus* and *Cricosphaera-carterae* with special reference to the origin and nature of coccoliths and scales inst light microscopy inst electron microscopy. Journal of the Marine Biological Association of the United Kingdom 49 (1): 1-16.

Manton, I. Leedale, G. F. (1969). Observations on the micro anatomy of *Coccolithus pelagicus* and *Cricosphaera carterae* with special reference to the origin and nature of coccoliths and scales inst light microscopy inst electron microscopy. Journal of the Marine Biological Association of the United Kingdom 49 (1): 1-16.

Marsh, M. E. (1994). Polyanion-mediated mineralization - assembly and reorganization of acidic polysaccharides in the golgi system of a

coccolithophorid alga during mineral deposition. Protoplasma 177 (3-4): 108-122.

Marsh, M. E. (1999). Coccolith crystals of *Pleurochrysis carterae*: crystallographic faces, organization, and development. Protoplasma 207 (1-2): 54-66.

Marsh, M. E. (2003). Regulation of $CaCO_3$ formation in coccolithophores. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 136 (4): 743-754.

Marsh, M. E., Ridall, A. L., Azadi, P. Duke, P. J. (2002). Galacturonomannan and Golgi-derived membrane linked to growth and shaping of biogenic calcite. Journal of Structural Biology 139: 39-45.

Marwood, C. A., Smith, R. E. H., Furgal, J. A., Charlton, M. N., Solomaon, K. R. Greenburg, B. M. (2000). Photoinhibition of natural phytoplankton asemblages in Lake Erie exposesd to solar ultraviolet radiation. Canadian Journal of Fisheries and Aquatic Sciences 57 (2): 371-379.

McCarthy, J. J., Taylor, W. R. Taft, J. L. (1977). Nitrogenous nutrition of the plankton in the Chesapeake Bay part 1. nutrient availability and phytoplankton preferences. Limnology and Oceanography 22 (6): 996-1011.

Merrett, M. J., Dong, L. F. Nimer, N. A. (1993). Nitrate availability and calcite production in *Emiliania huxleyi* Lohmann. European Journal of Phycology 28 (4): 243 - 246.

Miyamoto, E., Watanabe, F., Ebara, S., Takenaka, S., Takenaka, H., Yamaguchi, Y., Tanaka, N., Inui, H. Nakano, Y. (2001). Characterization of a vitamin B-12 compound from unicellular coccolithophorid alga (*Pleurochrysis carterae*). Journal of Agricultural and Food Chemistry 49 (7): 3486-3489.

Miyamoto, E., Watanabe, F., Takenaka, H. Nakano, Y. (2002). Uptake and physiological function of vitamin B12 in a photosynthetic unicellular coccolithophorid alga, *Pleurochrysis carterae*. Bioscience Biotechnology and Biochemistry 66 (1): 195-198.

Miyamoto, E., Watanabe, F., Yamaguchi, Y., Takenaka, H. Nakano, Y. (2004). Purification and characterization of methylmalonyl-CoA mutase from a photosynthetic coccolithophorid alga, *Pleurochrysis carterae*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 138 (2): 163-167.

Moheimani, N. R. (2005) The culture of coccolithophorid alge for bioremediation. Murdoch University, Perth Ph.D

Moheimani, N. R. Borowitzka, M. A. (2006). The long-term culture of the coccolithophore *Pleurochrysis carterae* (Haptophyta) in outdoor raceway ponds. Journal of Applied Phycology 18 (6): 703-712.

Moheimani, N. R. Borowitzka, M. A. (2007). Limits to productivity of the alga *Pleurochrysis carterae* (Haptophyta) grown in outdoor raceway ponds. Biotechnology and Bioengineering 96 (1): 27-36.

Moheimani, N. R. Borowitzka, M. A. (2011). Increased CO_2 and the effect of pH on growth and calcification of *Pleurochrysis carterae* and *Emiliania huxleyi* (Haptophyta) in semicontinuous cultures. Applied Microbiology and Biotechnology 90 (4): 1399-1407.

Moheimani, N. R., Isdepsky, A., Lisec, J., Raes, E. Borowitzka, M. A. (2011). Coccolithophorid algae culture in closed photobioreactors. Biotechnology and Bioengineering 108 (9): 2078-2087.

Moheimani, N. R., Webb, J. P. Borowitzka, M. A. (2012). Bioremediation and other potential applications of coccolithophorid algae: A review. Algal Research 1 (2): 120-133.

Morel, F. M., Milligan, A. J. Saito, M. A. (2003). Marine bioinorganic bhemistry: The role of trace metals in the oceanic cycles of major nutrients. Treatise on Geochemistry. H. Elderfield. 6, 113-143. Elsevier.

Morris, E. P. Kromkamp, J. C. (2003). Influence of temperature on the relationship between oxygen- and fluorescence- based estimates of photosynthetic parameters in a marine benthic diatom (*Cylindrotheca closterium*). European Journal of Phycology 38: 133-142.

Muggli, D. Harrison, P. J. (1996). Effects of iron and nitrogen source on the sinking rate, physiology and metal composition of an oceanic diatom from the subarctic Pacific. Marine Ecology Progress Series 130: 255-267.

Murray, G. Blackman, V. H. (1897). Coccospheres and Rhabdospheres. Nature lvi: 511.

Muyssen, B. T. A., Brix, K. V., DeForest, D. K. Janssen, C. R. (2004). Nickel essentiality and homeostasis in aquatic organisms. Environmental Reviews 12 (2): 113-131.

Nanninga, H. J. Tyrrell, T. (1996). Importance of light for the formation of algal blooms by *Emiliania huxleyi* Marine Ecology Progress Series [. 136: 195-203.

Nejstgaard, J. C., Gismervik, I. Solberg, P. T. (1997). Feeding and reproduction by *Calanus finmarchicus*, and microzooplankton grazing during mesocosm blooms of diatoms and the coccolithophore *Emiliania huxleyi*. Marine Ecology Progress Series 147: 197-217.

Nimer, N., A., Dixon, G. K. Merrett, M. J. (1991). Utilization of inorganic carbon by the coccolithophorid *Emiliania huxleyi* (Lohman) Kamptner. New Phytologist 120: 153-158.

Nimer, N., A., Merret, M. J. Brownlee, C. (1996). Inorganic carbon transport in relation to culture age and inrganoc carbon concentration in a high-calcifing strain of *Emiliania huxleyi* (Prmnesiophyceae). Journal of Phycology 32: 813-818.

Nimer, N., A. Merrett, M. J. (1992). Calcification and utilization of inorganic carbon by the coccolthiophorid *Emiliania huxleyi* Lohman. New Phytologist 121: 173-177.

Nimer, N., A. Merrett, M. J. (1993). Calcification rate in *Emiliania huxleyi* (Lohman) in response to light, nitrate and availability of inorganic carbon. New Phytologist 123: 673-677.

Nimer, N. A., Brownlee, C. Merrett, M. J. (1994). Carbon dioxide availability, intracellular pH and growth rate of the coccolithophore *Emiliania huxleyi*. Marine ecology progress series. 109: 2-3.

Nimer, N. A., Brownlee, C. Merrett, M. J. (1999). Extracellular carbonic anhydrase facilitates carbon dioxide availability for photosynthesis in the marine dinoflagellate *Prorocentrum micans*. Plant Physiology 120 (1): 105-111.

Nimer, N. A. Merrett, M. J. (1993). Calcification rate in *Emiliania huxleyi* Lohmann in response to light, nitrate and availability of inorganic carbon. New Phytologist 123 (4): 673-677.

Nimer, N. A. Merrett, M. J. (1996). The development of a CO_2 concentrating mechanism in *Emiliania huxleyi*. New Phytologist 133 (3): 383-389.

Nöel, M., Kawachi, M. Inouye, I. (2004). Induced dimorphic life cycle of a coccolithophorid, *Calyptrosphaera sphaeroidea* (Prymnesiophyceae, Haptophyta). Journal of Phycology 40 (1): 112-129.

Obata, T., Araie, H. Shiraiwa, Y. (2004). Bioconcentration Mechanism of Selenium by a Coccolithophorid, *Emiliania huxleyi*. Plant and Cell Physiology 45 (10): 1434-1441.

Obata, T. Shiraiwa, Y. (2004). What are microelements indispensable to breeding of *Pleurochrysis carterae*? Improvement of marine environment using marine microalgae with element concentration ability. (Translated from Japanese). Kagaku to seibutsu 42 (6): 354-356.

Okazaki, M., Sato, T., Mutho, N., Wada, N. Umegaki, T. (1998). Calcified scales (coccoliths) of *Pleurochrysis carterae* (Haptophyta): Structure, crystallography, and acid polysaccharides. Journal of Marine Biotechnology 6 (1): 16-22.

Oliveira, L. Antia, N. J. (1984). Evidence of nickel ion requirement for autotrophic growth of a marine diatom with urea serving as nitrogen source. British Phycological Journal 19 (2): 125-134.

Ozaki, N., Sakuda, S. Nagasawa, H. (2001). Isolation and some characterization of an acidic polysaccharide with anti-calcification activity from coccoliths of a marine alga, *Pleurochrysis carterae.* Bioscience Biotechnology and Biochemistry 65 (10): 2330-2333.

Paasche, E. (1964). A tracer study of the inorganic carbon uptake during formation and photosynthesis in the coccolithophorid *Coccolithus huxleyi*. Physiologia Plantarum 3: 5-82.

Paasche, E. (1999). Reduced coccolith calcite production under light-limited growth: A comparative study of three clones of *Emiliania huxleyi* (Prymnesiophyceae). Phycologia 38 (6): 508-516.

Paasche, E. (2002). A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification- photosynthesis interactions. Phycologia 40 (6): 503-529.

Paasche, E. Brubak, S. (1994). Enhanced calcification in the coccolithophorid Emiliania huxleyi (Haptophyceae) under phosphorus limitation. Phycologia 33 (5): 324-330.

Pachauri, R. K. Reisinger, A. (2007). Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC Fourth Assessment Report (AR4). R. K. Pachauri and A. Reisinger. Geneva, Switzerland. , IPCC. 104.

Page, S., Hipkin, C. R. Flynn, K. J. (1999). Interactions between nitrate and ammonium in *Emiliania huxleyi*. Journal of Experimental Marine Biology and Ecology 236 (2): 307-319.

Palenik, B. Henson, S. E. (1997). The use of amides and other organic nitrogen sources by the phytoplankton *Emiliania huxleyi*. Limnology and Oceanography 42 (7): 1544-1551.

Palenik, B., Kieber, D. J. Morel, F. M. M. (1989). Dissolved organic nitrogen use by phytoplankton the role of cell-surface enzymes. Biological Oceanography 6 (3-4): 347-354.

Palenik, B. Koke, J. A. (1995). Characterization of a Nitrogen-Regulated Protein Identified by Cell Surface Biotinylation of a Marine Phytoplankton. Appl. Environ. Microbiol. 61 (9): 3311-3315.

Palenik, B. Morel, F. M. M. (1990). Amino-acid utilization by marine-phytoplankton - a novel mechanism. Limnology and Oceanography 35 (2): 260-269.

Palenik, B. Morel, F. M. M. (1991). Amine Oxidases of Marine Phytoplankton. Appl. Environ. Microbiol. 57 (8): 2440-2443.

Pienaar, R. N. (1969). The fine structure of *Cricosphaera carterae* I. External morphology. Journal of Cell Science 4 (2): 561-567.

Pienaar, R. N. (1969). The Fine Structure of *Cricosphaera carterae* Part 2 Observations on Scale and Coccolith Production. Journal of Phycology 5 (4): 321-331.

Pienaar, R. N. (1971). Coccolith production in *Hymenomonas carterae*. Protoplasma 73 (2): 217-224.

Pienaar, R. N. (1994). Ultrastructure and calcification of coccolithophores. In: Coccolithophores. (Winter, A. and Siesser, W. G. eds). Cambridge University Press, Cambridge, pp: 13-37

Pienaar, R. N. Norris, R. E. (1979). The ultrastructure of the flagellate *Chrysochromulina spinier* new combination prymnesiophyceae with special reference to scale production. Phycologia 18 (2): 99-108.

Platt, T., Gallegos, C. L. Harrison, W. G. (1980). Photoinhibition of photosynthesis in natural assemblages of marine phytoplanton. Journal of Marine Research 38 (4): 687-701.

Price, N. M. Harrison, P. J. (1987). Comparison of methods for the analysis of dissolved urea in seawater. Marine Biology 94 (2): 307-317.

Price, N. M., Thompson, P. A. Harris, R. P. (1987). Selenium: An essential element for growth of the coastal marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). Jounal of Phycology 23 (1): 1-9.

Pringsheim, E. G. (1955). Kleine Mitteilungen uber Flagellaten und Algen. I. Algenartige *Chrysophyceen* in Reinkultur.[Short report on flagellates and algae. I. Alga-like *Chrysophyceae* in pure culture]. Archiv fur Mikrobiologie 21 (4): 401-410.

Quinn, P. K. Bates, T. S. (2011). The case against climate regulation via oceanic phytoplankton sulphur emissions. Nature 480 (7375): 51-56.

Quiroga, O. Gonzalez, E. L. (1993). Carbonic anhydrase in the chloroplast of a coccolithophorid (Prymnesiophyceae). Journal of Phycology 29 (3): 321-324.

Ra, K. Kitagawa, H. (2007). Magnesium isotope analysis of different chlorophyll forms in marine phytoplankton using multi-collector ICP-MS. Journal of Analytical Atomic Spectrometry 22 (7): 817-821.

Ragni, M., Airs, R. L., Leonardos, N. Geider, R. J. (2008). Photoinhibition of PSII in *Emiliania huxleyi* (Haptophyta) under high light stress: the roles of photoacclimation, photoprotection, and photorepair. Journal of Phycology 44 (3): 670-683.

Ralph, P. J. Gademann, R. (2005). Rapid light curves: A powerful tool to assess photosynthetic activity. Aquatic Botany 82 (3): 222-237.

Raven, J. A. (1997). Putting the C in phycology. European Journal of Phycology 32 (4): 319-333.

Raven, J. A. Beardall, J. (1981). Respiration and Photorespiration. Canadian Journal of Fisheries and Aquatic Sciences 210: 55-82.

Raven, J. A., Evans, M. C. W. Korb, R. E. (1999). The role of trace metals in photosynthetic electron transport in O_2 evolving organisms. Photosynthesis Research 60 (2-3): 111-149.

Raven, J. A. Johnston, A. M. (1991). Mechanisms of inorganic-carbon acquisition in marine phytoplankton and their implications for the use of other resources. Limnology and Oceanography 36 (8): 1701-1714.

Rayns, D. G. (1962). Alternation of generations in a coccolithophorid, *Cricosphaera carterae* (Braarud & Fagerl.) Braarud. Journal of the Marine Biological Association of the United Kingdom 42: 481-484.

Rees, A. P., Malcolm, E., Woodward, S., Robinson, C., Cummings, D. G., Tarran, G. A. Joint, I. (2002). Size-fractionated nitrogen uptake and carbon fixation during a developing coccolithophore bloom in the North Sea during June 1999. Deep-Sea Research Part II-Topical Studies in Oceanography 49 (15): 2905-2927.

Rees, T. A. V. Syrett, P. J. (1979). Mechanisms for urea uptake by the diatom *Phaeodactylum tricornutum*: the uptake of thiourea. New Phytologist 83 (1): 37-48.

Rees, T. A. V. Syrett, P. J. (1979). The uptake of urea by the diatom, *Phaeodactylum.* New Phytologist 82 (1): 169-178.

Reifel, K., McCoy, M., Tiffany, M., Rocke, T., Trees, C., Barlow, S., Faulkner, D. J. Hurlbert, S. (2001). *Pleurochrysis pseudoroscoffensis* (Prymnesiophyceae) blooms on the surface of the Salton Sea, California. Hydrobiologia 466 (1): 177-185.

Richier, S., Fiorini, S., Kerros, M. E., von Dassow, P. Gattuso, J. P. (2011). Response of the calcifying coccolithophore *Emiliania huxleyi* to low pH/high pCO_2 from physiology to molecular level. Marine Biology 158 (3): 551-560.

Riebesell, U., Zondervan, I., Rost, B., Tortell, P. D., Zeebe, R. E. Morel, F. M. M. (2000). Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 407 (6802): 364-367.

Riegman, R., Stolte, W., Noordeloos, A. A. M. Slezak, D. (2000). Nutrient uptake and alkaline phosphatase (ec 3:1:3:1) activity of *Emiliania huxleyi* (prymnesiophyceae) during growth under N and P limitation in continuous cultures. Journal of Phycology 36 (1): 87-96.

Ritchie, R. (2008). Fitting light saturation curves measured using modulated fluorometry. Photosynthesis Research 96 (3): 201-215.

Ritchie, R. J. Bunthawin, S. (2010). Photosynthesis in Pineapple *(Ananas comosus comosus* [L.] Merr) Measured Using PAM (Pulse Amplitude Modulation) Fluorometry. Tropical Plant Biology 3 (4): 11.

The Royal Society, (2005). Ocean acidification due to increasing atmospheric carbon dioxide, The Royal Society, London.

Rokitta, S. D. Rost, B. (2012). Effects of CO_2 and their modulation by light in the life-cycle stages of the coccolithophore *Emiliania huxleyi*. Limnology and Oceanography 57 (2): 607-618.

Roopnarain, A., Sym, S. Gray, V. M. (2015). Effect of nitrogenous resource on growth, biochemical composition and ultrastructure of *Isochrysis galbana* (Isochrysidales, Haptophyta). Phycological Research 63 (1): 43-50.

Rost, B. Riebesell, U. (2004). Coccolithophorids and the biological pump: responses to enviromental changes. Molecular processes to global impact. H. R. Thierstein and J. R. Young, 79-99. Spinger**:** New York.

Rost, B., Riebesell, U., Burkhardt, S. Sultemeyer, D. (2003). Carbon acquisition of bloom-forming marine phytoplankton. Limnology and Oceanography 48 (1): 55-67.

Rouco, M., Branson, O., Lebrato, M. Iglesias-Rodriguez, M. D. (2013). The effect of nitrate and phosphate availability on *Emiliania huxleyi* (NZEH) physiology under different CO_2 scenarios. Frontiers in Microbiology 4: 11.

Sáez, A. G., Probert, I., Geisen, M., Quinn, P., Young, J. R. Medlin, L. K. (2003). Pseudo-cryptic speciation in coccolithophores. PNAS 100 (12): 7163-7168.

Sáez, A. G., Probert, I., Young, J. R., Edvardsen, B., Eikrem, W. Medlin, L. K. (2004). A review of the phylogeny of the Haptophyta. Cocclithophores- From Molecular Processessto Global Impact. H. R. Thiersten and J. Young. Springer**:** Berlin.pp 251 – 270.

Sáez, A. G., Zaldivar-Riverón, A. Medlin, L. K. (2008). Molecular systematics of the Pleurochrysidaceae, a family of coastal coccolithophores (Haptophyta). Journal of Plankton Research 30 (5): 559-566.

Schreiber, U., Gademann, R., Ralph, P. J. Larkum, A. W. D. (1997). Assesment of Photosynthetic Performance of *Prochloron* in *LIssoclinum patella* in hospite by Chlorophyll Fluorescence Measurements. Plant and Cell Physiology 38 (8): 945-951.

Sciandra, A., Harlay, J., Lefevre, D., Lemee, R., Rimmelin, P., Denis, M. Gattuso, J. P. (2003). Response of coccolithophorid *Emiliania huxleyi* to elevated partial pressure of CO_2 under nitrogen limitation. Marine Ecology Progress Series 261: 111-122.

Shiraiwa, Y. (2003). Physiological regulation of carbon fixation in the photosynthesis and calcification of coccolithophorids. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 136 (4): 775-783.

Shiraiwa, Y., Goyal, A. Tolbert, N. E. (1993). Alkalization of the medium by unicellular green algae during uptake dissolved inorganic carbon. Plant and Cell Physiology 34 (5): 649-657.

Sikes, C. S., Roer, R. D. Wilbur, K. M. (1980). Photosynthesis and coccolith formation: Inorganic carbon sources and net inorganic reaction of deposition. Limnology and Oceanography 25: 248-261.

Sikes, C. S. Wheeler, A. P. (1982). Carbonic Anhydrase and Carbon Fixation in Coccolithophorids. Journal of Phycology 18 (3): 423-426.

Sikes, C. S. Wilbur, K. M. (1982). Functions of coccolith formation. Limnology and Oceanography 27 (1): 18-26.

Silva, H. J., Cortinas, T. Ertola, R. J. (1987). Effect of hydrodynamic stress on *Dunaliella* growth. Journal of Chemical Technology and Biotechnology 40 (1): 41-49.

Solomon, C. M., Collier, J. L., Berg, G. M. Glibert, P. M. (2010). Role of urea in microbial metabolism in aquatic systems: a biochemical and molecular review. Aquatic Microbial Ecology 59 (1): 67-88.

Solomon, C. M. Glibert, P. M. (2008). Urease activity in five phytoplankton species. Aquatic Microbial Ecology 52 (2): 149-157.

Sotoj, A. R., Zheng, H., Shoemaker, D.. Rodriguez, J., Read, B. A., Wahlund, T. M. (2006). Identification and preliminary characterization of two cDNAs encoding unique carbonic anhydrases from the marine alga *Emiliania huxleyi*. Applied and Environmental Microbiology. 72(8) 5500-5511

Stewart, W. D. P. (1980). Transport and utilization of nitrogen sources by algae. Microorganisms and Nitrogen Sources. J. W. Payne, pp 10- 20. John Wiley & Sons**:** London.

Stillwell, E. F. Corum, K. E. (1982). Effects of cadmium and low magnesium on cell division and calcification in the marine coccolithophorid *Cricosphaera (Hymenomonas) carterae*. Marine Biology 66 (3): 227-230.

Strickland, J. D. H. Parsons, T. R. (1972). A practical handbook of seawater analysis. Fisheries Board of Canada, Ottawa.

Strom, S. L. Bright, K. J. (2009). Inter-strain differences in nitrogen use by the coccolithophore *Emiliania huxleyi*, and consequences for predation by a planktonic ciliate. Harmful Algae 8 (5): 811-816.

Stumm, W. Morgan, J. J. (1996). Aquatic Chemistry; Chemical equilibria and rates in natural waters. Wiley-Interscience, United States of America.

Suggett, D. J., Le Floc'H, E., Harris, G. N., Leonardos, N. Geider, R. J. (2007). Different strategies of photoacclimation by two strains of *Emiliania huxleyi* (Haptophyta). Journal of Phycology 43 (6): 1209-1222.

Sukenik, A., Levy, R. S., Levy, Y., Falkowski, P. G. Dubinsky, Z. (1991). Optimizing algal biomass production in an outdoor pond - a simulation-model. Journal of Applied Phycology 3 (3): 191-201.

Swift, E. Taylor, W. R. (1966). The effect of pH on the division rate of the Coccolithophorid *Cricosphaera elongata*. Journal of Phycology 2 (3): 121-125.

Sym, S. Kawachi, M. (2000). Ultrastructure of *Calyptrosphaera radiata*, sp nov (Prymnesiophyceae, Haptophyta). European Journal of Phycology 35 (3): 283-293.

Syrett, P. J. Bekheet, I. A. (1977). The uptake of thiourea by *Chlorella*. New Phytologist 79 (2): 291-297.

Takahashi, J., Fujiwara, S., Kikyo, M., Hirokawa, Y. Tsuzuki, M. (2002). Discrimination of the Cell Surface of the Coccolithophorid *Pleurochrysis haptonemofera* from Light Scattering and Fluorescence After Fluorescein-Isothiocyanate-Labled Lectin Staining Measured by Flow Cytometry. Marine Biotechnology 4: 94-101.

Tremblay, J. E., Legendre, L., Klein, B. Therriault, J. C. (2000). Size-differential uptake of nitrogen and carbon in a marginal sea (Gulf of St. Lawrence, Canada): Significance of diel periodicity and urea uptake. Deep-Sea Research Part ii-Topical Studies in Oceanography 47 (3-4): 489-518.

Tsuji, Y., Suzuki, I. Shiraiwa, Y. (2009). Photosynthetic carbon assimilation in the coccolithophorid Emiliania huxleyi (Haptophyta): evidence for the predominant operation of the C3 cycle and the contribution of beta-carboxylases to the active anaplerotic reaction. Plant and Cell Physiology 50 (2): 318-329.

Turner, M. F. (1979). Nutrition of some marine micro algae with special reference to vitamin requirements and utilization of nitrogen and carbon sources. Journal of the Marine Biological Association of the United Kingdom 59 (4): 535-552.

Vairavamurthy, A., Andreae, M. O. Iverson, R. L. (1985). Biosynthesis of Dimethylsulfide and Dimethylpropiothetin by *Hymenomonas carterae* in Relation to Sulfur Source and Salinity Variations. Limnology and Oceanography 30 (1): 59-70.

van der Wal, P., de Jong, E. W., Westbroek, P., de Bruijn, W. C. Mulder-Stapel, A. A. (1983). Ultrastructual polysaccharide localization in calcifying and naked cells of the Coccolithophorid *Emiliania huxleyi*. Protoplasma 118: 157-168.

Van Der Wal, P., De Long, L., Westbroek, P. De Bruijn, W. C. (1983). Calcification in the coccolithophorid alga *Hymenomonas carterae*. Hallberg, R. 251-258.

van der Wal, P., Kempers, E. S. Veldhuis, M. J. W. (1995). Production and downward flux of organic matter and calcite in a North Sea bloom of the coccolithophore *Emiliania huxleyi* Marine Ecology Progress Series 126: 247-265.

van Lun, M., Hub, J. S., van der Spoel, D. Andersson, I. (2014). CO_2 and O_2 distribution in rubisco suggests the small subunit functions as a CO_2 reservoir. Journal of the American Chemical Society 136 (8): 3165-3171.

Varela, D. E. Harrison, P. J. (1999). Seasonal variability in nitrogenous nutrition of phytoplankton assemblages in the northeastern subarctic Pacific Ocean. Deep Sea Research Part II: Topical Studies in Oceanography 46 (11-12): 2505-2538.

Vliegenthart, J. F. G., Fichtinger-Schepman, A. M. J., Kamerling, J. P. Versluis, C. (1981). Structural studies of the methylated, acidic polysaccharide associated with coccoliths of Emiliania huxleyi (Lohmann) Kamptner. Carabohydrate Research 93 (1): 105-123.

von Stosch, H. A. (1955). Alternation of morphological phases in a Coccolithophorid (*Ein morphologischer Phasenwechsel bei einer Coccolithophoride)*. Naturwiss 42 ((14)): 423.

Vonshak, A. Guy, R. (1992). Photoadaptation, photoinhibition and productivity in the blue-green-alga, *Spirulina platensis* grown outdoors. Plant Cell and Environment 15 (5): 613-616.

Vonshak, A., Kancharaksa, N., Bunnag, B. Tanticharoen, M. (1996). Role of light and photosynthesis on the acclimation process of the cyanobacterium *Spirulina platensis* to salinity stress. Journal of Applied Phycology 8 (2): 119-124.

Walsh, D. Mann, S. (1995). Fabrication of hollow porous shells of calcium carbonate from self-organizing media. Nature 377 (6547): 320-323.

Westbroek, P., Brown, C. W., Vanbleijswijk, J., Brownlee, C., Brummer, G. J., Conte, M., Egge, J., Fernandez, E., Jordan, R., Knappertsbusch, M., Stefels, J., Veldhuis, M., Vanderwal, P. Young, J. (1993). A model system approach to biological climate forcing - the example of *Emiliania huxleyi*. Global and Planetary Change 8 (1-2): 27-46.

White, A. J. Critchley, C. (1999). Rapid light curves: A new fluorescence method to assses the state of the photosyntheitic apparatus. Photosynthesis Research 59: 63-72.

Wilbur, K. M. Watabe, N. (1963). Experimental studies on calcification in molluscs and the alga *Coccolithus huxleyi*. Annals of the New York Academy of Sciences 109 (1): 82-112.

Winter, A., Jordan, R. Roth, P. H. (1994). Biogeography of living coccolithophores in ocean waters. In: Coccolithophores. (Winter, A. and Siesser, W. G. eds). Cambridge University Press, Cambridge, pp: 161-177.

Wu, H.-l., Chen, T.-f., Yin, X. Zheng, W. (2012). Spectrometric characteristics and underlying mechanisms of protective effects of selenium on *Spirulina platensis* against oxidative stress (In Chinese, Abstact only). Guang pu xue yu guang pu fen xi 32 (3): 749-754.

Xu, K. Gao, K. S. (2012). Reduced calcification decreases photoprotective capability in the Coccolithophorid *Emiliania huxleyi*. Plant and Cell Physiology 53 (7): 1267-1274.

Yokota, A., Shigeoka, S., Onishi, T. Kitaoka, S. (1988). Selenium as inducer of glutathione-peroxidase in low-CO_2 grown *Chlamydomonas reinhardtii.* Plant Physiology 86 (3): 649-651.

Young, E. B. Beardall, J. (2003). Rapid ammonium- and nitrate-induced perturbations to chl a fluorescence in nitrogen-stressed *Dunaliella tertiolecta* (Chlorophyta). Journal of Phycology 39 (2): 332-342.

Young, J. R., Brown, P. R. Burnett, J. A. (1994 Palaeontological perspective. In: The Haptophyte Algae. (Green, J. C. and Leadbeater, B. S. C. eds). Clarendon Press, Oxford, pp: 393-412

Young, J. R., Davis, S. A., Bown, P. R. Mann, S. (1999). Coccolith ultrastructure and biomineralisation. Journal of Structural Biology 126 (3): 195-215.

Young, J. R. Henriksen, K. (2003). Biomineralization within vesicles: The calcite of coccoliths Biomineralization. Reviews in Mineralogy and Geochemistry. P.

Dove, J. J. De Yoreo and S. Weiner. 54, 189-216. Mineralogical Society of America Geochenical Society**:** USA.

Young, J. R. Westbroek, P. (1991). Genotypic variation in the coccolithophorid species *Emiliania huxleyi*. Marine Micropaleontology 18 (1-2): 5-23.

Zeebe, R. E. Wolf-Gladrow, D. (2005). CO_2 In Seawater: Equilibrium, Kinetics, Isotopes. Elsevier, Amsterdam.

Zondervan, I. (2007). The effects of light, macronutrients, trace metals and CO_2 on the production of calcium carbonate and organic carbon in coccolithophores. A review. Deep Sea Research Part II: Topical Studies in Oceanography 54 (5-7): 521-537.

Zondervan, I., Rost, B. Riebesell, U. (2002). Effect of CO_2 concentration on the PIC/POC ratio in the coccolithophore *Emiliania huxleyi* grown under light-limiting conditions and different daylengths. Journal of Experimental Marine Biology and Ecology 272 (1): 55-70.

Zou, N. Richmond, A. (2000). Light-path length and population density in photoacclimation of *Nannochloropsis* sp. (Eustigmatophyceae). Journal of Applied Phycology 12 (3-5): 349-354.