THE IMPORTANCE OF OSMO-MIXOTROPHY IN THE NUTRITION OF THE DIATOM *THALASSIOSIRA OCEANICA* UNDER IRON LIMITATION

by

Afrah Alothman

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ABSTRACT

This thesis addresses the potential for osmo-mixotrophy within the diatom species *Thalassiosira oceanica* under iron-limited and iron-replete conditions. Urea, a simple organic molecule, can provide both carbon and nitrogen to fulfill the cellular requirements of diatoms. Laboratory experiments were conducted with a pure culture of *T. oceanica* to investigate the relative contribution of urea to carbon and nitrogen uptake in iron-limited and iron-replete diatoms. Isotope labeling experiments with ^{13}C-HCO3onate, ^{15}N-nitrate, and dual labeled ^{13}C/^{15}N urea showed that both N and C from the urea molecule were assimilated into cellular biomass by *T. oceanica*, though C uptake was at a lower rate than photosynthetic CO_2 fixation; the C/N uptake ratio was 0.15, which is less than the 0.5 C/N stoichiometric ratio of the urea molecule. In addition, the C uptake from urea under iron-limited and iron-replete conditions contributed from 5% ± 3.72 to 11% ± 1.71 of total carbon assimilation. This was proportional to the fraction of urea-C relative to the total pool of dissolved carbon in the medium. In addition, the batch culture experiments suggested that, at equal dissolved N concentrations, urea supported a higher biomass than nitrate in iron-limited cultures. There was no significant difference in photosynthetic carbon fixation and nutrient uptake between the iron-limited and the iron-replete cultures.

LIST OF ABBREVIATIONS USED

Abbreviation	Description
ASW	Artificial seawater
ATP	Adenosine triphosphate
Cyt (b6c)	Cytochrome complex
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4,6-Diamidino-2-phenylindole
DOM	Dissolved organic matter
DON	Dissolved organic nitrogen
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron Transport Chain
Fv/Fm	Maximum chlorophyll fluorescence
HNLC	High nutrient, low chlorophyll
HSD	Honest Significant Difference
ILE	Isotope labeling experiments
IRMS	Isotope ratio mass spectrometry
ISIP	Iron-starvation-induced proteins
L1	Ligand 1
L2	Ligand 2
LNLC	Low nutrient, low chlorophyll
N.A.	Not applicable
n.s.	Not significant

NADPH	Nicotinamide adenine dinucleotide phosphate
POM	Particulate organic matter
PSI	Photosystem I
PSII	Photosystem II
RuBPCase	1,5-bisphosphate carboxylase/oxygenase
SOIREE	Southern Ocean Iron Release Experiment
TCA	Tricarboxylic acid
TM	Trace metals

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Chapter 1: Introduction

1.1 General introduction:

Marine diatoms are vitally important to human life because of their importance to ecology, and marine productivity and the fisheries economy. Diatoms play an essential role in marine ecosystems contributing about 40% of marine primary productivity and producing up to 20% of the oxygen that we breathe (Lommer *et al*, 2011). In addition, diatoms have potential for numerous biotechnological and industrial applications such as biofuel (Kim *et al*, 2016, Zheng *et al*, 2013), additives for the food and construction industries, and nanotechnologies (Hildebrand *et al*, 2012; Poulsen & Kroger 2008).

Diatoms bloom when the balance between light and macronutrients (nitrate, phosphate, silicate) is optimal. In most of the oceanic surface waters, nitrate and phosphate concentrations are very low throughout the euphotic zone and accumulate at the nutricline at the bottom of the euphotic zone. Nonetheless, one of the diatoms' most distinctive characteristics is their ability to survive for long periods under environmentally-adverse conditions that may affect photosynthesis and growth (Armbrust, 2009). Fixed nitrogen, generally in the form of ammonium or nitrate, is an essential macronutrient for the synthesis of nucleic acids, proteins and chlorophyll (Schaffner *et al*, 2007), which are necessary for photosynthesis and other cellular functions in diatoms. However, in a few specific oceanic regions defined as high-nutrient, low-chlorophyll (HNLC), iron limitation prevents the surface depletion of nitrate and phosphate by phytoplankton. The HNLC regions include the Southern Ocean, Northeast Subarctic Pacific, and the Equatorial Pacific (Strong *et al*, 2009). In contrast, there are also low-nutrient, low-chlorophyll regions (LNLC) where phosphorus and nitrate are the limiting nutrients, including the North Pacific Ocean and South Atlantic Ocean (Williamson *et al*, 2012).

Numerous ocean fertilization experiments conducted in the last two decades have demonstrated the importance of iron as a limiting nutrient in HNLC regions, while laboratory experiments have shed light on the physiological adaptation of phytoplankton, and in particular diatoms, to iron limitation (Williamson *et al*, 2012). In general, photosynthetic carbon fixation by phytoplankton contributes significantly to carbon sequestration and storage in the deep ocean through the biological pump (Martin, 1990). Martin (1990) put forward the "iron hypothesis" that the rapid changes in CO_2 concentration in the atmosphere observed on geological timescales could be due to low phytoplankton productivity caused by iron deficiency in the HNLC regions. Martin's hypothesis predicted that adding large amounts of iron to HNLC areas would improve phytoplankton growth, leading to enhanced carbon absorption from the atmosphere and, as a result, would reduce the greenhouse effect and cool the earth (Martin, 1990). Several *in situ* iron fertilization experiments of HNLC waters, such as IronEx I (Martin *et al*, 1994), IronEx II (Coale *et al*, 1996), and SOIREE (Boyd *et al*, 2000), provided clear evidence in support of iron-limited conditions; phytoplankton responded to iron enrichment by increasing in biomass, chlorophyll concentration and photosynthesis rate, while chemical analyses of the seawater in the iron-enriched patch indicated a decrease in nitrate, and CO_2 concentration in the surface waters, demonstrating that primary productivity is limited by iron in these regions.

1.2 Iron (Fe) requirements of diatoms:

Most living organisms require iron (Fe) for growth. Marine microbes are no exception to this rule, as it is essential in carbon reduction, nitrate/nitrite reduction, and chlorophyll synthesis (Twining & Baines, 2013); photosynthetic microorganisms, in particular, have a high Fe demand because the photosynthetic apparatus requires 23 moles of iron per mole of reaction centre (Raven, 1990). Iron plays an essential role in cellular processes such as the photosynthetic

electron transport chain (ETC; Geider & LaRoche, 1994) and the ETC of cellular respiration in mitochondria (Berg *et al*, 2002). Specifically, iron is an essential component of iron-sulfur complexes, cytochromes, ferredoxin, and several enzymes important in energy production and nutrient acquisition such as nitrate/nitrite reductase, chelatase, and nitrogenase (reviewed in Twining & Baines, 2013). Iron deficiency in phytoplankton can lead to decreased photosynthetic rate, chlorophyll biosynthesis, and C assimilation (Lommer *et al*, 2012). A decrease in chlorophyll molecules affects the light-harvesting complexes and reaction centers, which in turn decreases the overall ETC capacity of photosystem complexes, and carbon fixation (Behrenfeld & Milligan, 2013). The effects of decreasing the ETC are manifested in a decrease in ATP and NADPH, which are sources of chemical energy and reductant, respectively, necessary for inorganic carbon uptake, carbon fixation, and nitrate assimilation (Petrou *et al*, 2014). In diatoms, the cellular iron quota is variable, and to some extent the cellular demand reflects the iron concentration typical of their habitat. Thus, oceanic diatoms have a lower cellular iron quota relative to coastal diatoms, and the growth rate of oceanic diatoms is higher than that of coastal diatoms when both are grown at low Fe concentration (Strzepek & Harrison, 2004).

1.2.1 Iron limitation in the ocean and in the laboratory

In HNLC regions, iron is found in the surface (<20m depth) at very low nanomolar concentrations (means of 0.15 ± 0.16 nmol; More1 & Braucher, 2007) due to the insolubility of its Fe^{+3} stable oxidation state (Martin *et al*, 1991). The solubility and availability of iron is affected by the complexity of its behavior in seawater. Dissolved Fe concentrations are 10-1000 times higher in coastal oceans (10 nM-1 μM) compared to open ocean water (≤ 0.1 nM; Sunda *et al*, 1991; Sunda & Huntsman, 1995). However, dissolved organic matter (DOM) can play an important role in the nutrition of phytoplankton by indirectly serving as a chelator or ligand to

increase the solubility of essential trace metal nutrients such as iron (Morel & Price, 2003). Trace metals are present in the ocean either as metal ions or as different inorganic/organic bound ligands; therefore, pH, metal iron concentration, and the concentration of ligands could affect the metal speciation (Weng *et al*, 2002). Iron bio-availability is controlled by the concentration and binding strength of the organic ligands that are present in the ion buffering system. In the ocean, there are two classes of naturally-occurring ligands, represented by the L_1 and L_2 ligands. The strong L_1 ligands found in the mixed layer are likely siderophores produced by prokaryotes, and the weaker L_2 ligands found in deeper waters are likely produced by the degradation of organic matter (Hunter & Boyd, 2007).

The study of iron limitation in the laboratory requires control of the growth rate and total biomass by iron availability and total dissolved concentration, respectively. This is difficult to achieve because the preferred oxidation state of iron (Fe^{3+}) has a low solubility that leads to its precipitation as iron oxyhydroxides, thus becoming unavailable to phytoplankton (Rich & Morel 1990). Consequently, iron in culture medium must be complexed with a chelator (iron-binding ligand) such as EDTA (ethylenediaminetetraacetic acid), rendering it more soluble in seawater and preventing precipitation (Guillard & Ryther, 1962). Mimicking the natural environment where iron is bound to an organic chelator, the high concentration of EDTA creates an ion buffer system, in which high chelator concentrations are in excess of the low metal ion concentrations. There are several other growth conditions that may affect iron limitation experiments in phytoplankton, including nitrogen source, light intensity, and photoperiod duration. Iron requirement varies among phytoplankton grown with the assimilation of different nitrogen sources. In addition, under light limitation, iron requirements would increase because of the photo-redox process of binding ligands to iron (Andersen, 2005), and because of more

photosynthetic proteins that have iron content needed in low light.

1.3 Adaptation mechanisms of diatoms to Fe limitation

Research on coastal and oceanic species has shown that oceanic diatoms require much lower iron levels for cellular processes compared to coastal species (Brand *et al*, 1983), although the uptake rate of iron is quite similar (Sunda *et al*, 1991). The responses of phytoplankton, and of diatoms in particular, to iron limitation vary across species. Some species have remarkably low iron demands for their cellular processes (Allen *et al*, 2008; Lommer *et al*, 2012). For example, the coastal diatom *Phaeodactylum tricornutum* can grow in culture with iron levels 50 times lower than those required by another coastal diatom *Thalassiosira pseudonana* (Kustka *et al* 2007). Moreover, the oceanic species *Thalassiosira oceanica* can grow at even lower iron concentrations than *P. tricornutum* (Lommer *et al*. 2012). The low iron demands of these two species reflect their adaptation to iron limitation.

Diatoms are very plastic in their physiology and can acclimate to nutrient-limited conditions by down-regulating specific physiological processes in order to minimize demands for certain elements or nutrients, including iron (Chappell *et al*, 2015). Several adaptation mechanisms that allow diatoms to acclimate and survive in a low iron environment have been identified, and can be classified into three broad categories. Firstly, cellular iron requirements can be decreased by replacing Fe- containing proteins, such as cytochrome c6 and ferredoxin, with proteins using alternate co-factors that do not require iron, such as plastocyanin and flavodoxin (LaRoche *et al*, 1996; Raven *et al*, 1999; Peers & Price, 2006). Secondly, metabolic remodeling can reduce dependence on iron-rich metabolic pathways such as photosynthesis (Calvin cycle) and nitrate assimilation, or generally reduce chloroplast processes while increasing mitochondrial processes such as the TCA cycle (Allen *et al*, 2008; Lommer *et al*, 2012). Additionally, several genes

encoding cell surface enzymes involved in the degradation of organic matter, such as glycoside hydrolase and metallo-protease, are up-regulated under iron limitation in some diatoms, suggesting a switch to an osmo-mixotrophic lifestyle with respect to metabolism (Lommer *et al*, 2012). These mechanisms of compensating for iron limitation have been detected in several diatoms, including *T. oceanica* and *P. tricornutum* (Allen *et al*, 2008, Lommer *et al*, 2012). A third approach to iron-limited conditions is an increased uptake of iron from the environment, either through increased expression of a high affinity iron uptake system comprised of specific cell membrane proteins and extracellular enzymes (Morrissey *et al*, 2015), or through the use of siderophores, molecules with high affinity for extracellular ferric iron that are then transported into the cell.

1.3.1 *Thalassiosira oceanica* adaptation to iron limitation

Thalassiosira oceanica is a centric diatom, living mostly in the open ocean (Sakshaug *et al*, 1987), which has shown noticeable tolerance to low iron conditions (Lommer *et al*, 2012). Strzepek and Harrison (2004) identified one mechanism through which *T. oceanica* is able to reduce iron demand during growth under low iron conditions: Iron is of central importance to cytochrome and iron-sulphur protein co-factors within photosynthetic complexes, thus a particular species' iron requirement depends greatly on the relative abundance of the photosynthetic complexes photosystem II (PSII), photosystem I (PSI), and cytochrome (Cyt) b_6c. A comparison study between *T. oceanica* and the coastal diatom *Thalassiosira weissflogii* showed that *T. oceanica* has a high PSII/PSI ratio, which is maintained in cells grown in high Fe. PSII needs only two iron atoms per complex, while PSI and Cyt b_6c require 12 and six atoms per complex, respectively (Strzepek & Harrison, 2004). Thus, the tolerance of *T. oceanica* to low iron conditions may result partially from its unique ratio of photosynthetic complexes, which

minimizes overall iron demands. However, the overall high tolerance of *T. oceanica* to iron limitation results from a combination of several mechanisms that act in concert, as demonstrated by the presence of plastocyanin, a copper containing protein (Peers & Price, 2006), a suite of ISIP proteins implicated in a novel iron uptake mechanism (Lommer *et al*, 2012; Morrisey & Bowler, 2012), and lastly a suspected diversion of energy acquisition away from chloroplast to mitochondrial processes such as TCA cycle and degradation of organic substrates, leading to a down-regulation of photosynthesis (Allen *et al*, 2008; Lommer *et al*, 2012).

1.4 Possible role of osmo-mixotrophy in diatoms:

Planktonic organisms are generally classified as photoautotrophs if they use inorganic nutrients and light for energy, or chemoheterotrophs if they use complex organic compounds in the form of either dissolved organic molecules or prey (Tittel *et al*, 2003). However, when an organism has the ability to combine the use of both strategies, it is known as a mixotroph (Tittle *et al*, 2003). Mixotrophy has been defined in several ways over the last three decades, with a broad definition encompassing the uptake and utilization of DOM and stricter definitions encompassing only the ingestion of prey (Mitra *et al*, 2016). More recently, some have argued that the stricter definition adopted by Mitra *et al* (2016), defining mixotrophy as the combination of phototrophy and phagotrophy should be more widely accepted, arguing that most photoautotrophs have access to DOM as a nutrient and energy source through the process of osmotrophy. A recent review supported this concept (Stoecker *et al*, 2017), also describing a mixotroph as an autotroph which can ingest prey to provide the carbon and nitrogen requirements as an alternative form of inorganic nutrients. Others have reviewed the concept of mixotrophy more broadly, defining three types of mixotrophy in phototrophic organisms: Firstly, ‘osmo-mixotrophy’ was defined as the utilization of organic substrates available in the

environment (labile organic substrates); secondly, 'symbiotic mixotrophy' entails the utilization of organic substrates that come from interaction with other organisms, and lastly, 'predatory mixotrophy' utilizes organic substrates provided by predation on other organisms (Selosse *et al*, 2016). Most of the published work on mixotrophy reports on the ability of phototrophic phytoplankton, including diatoms, to utilize dissolved organic compounds (Garcia *et al*, 2005; Kamjunke & Tittle, 2008; Wan *et al*, 2011; Zheng *et al*, 2013, Benavides *et al*, 2017,). Since this work focuses on the growth of a diatom species in different dissolved organic nitrogen and carbon sources, the concept of osmo-mixotrophy is used in this thesis.

Since 1953, it has been known that some autotrophic algae species have the ability to utilize organic substrates in the light under limited nutrients conditions (Saunders, 1957). There are two major observations that support a role for osmo-mixotrophy in photosynthetic organisms: Firstly, assimilation of organic carbon substrates happens when there is light limitation (Zilliges & Dau, 2016), and secondly, the uptake of organic nitrogen and phosphorus substrates relies on inorganic macronutrient limitation (Matantseva & Skarlato, 2013). Many studies have supported these observations regarding the use of organic compounds and inorganic nutrients by obligate photosynthetic organisms **(Table 1.1)**.

Table 1.1: Non-exhaustive review of organic substrates (C or N) utilized by obligate phototrophic organisms:

Species name.	Phylum/Class	Organic substrate(s)	References
Chlorella	Chlorophyta/Tredouxiophyceae	Glucose (C)	(Lalucate *et al*, 1984)
Cyanidium caldarium	Rhodophyta/Phodophyceae	Glucose (C)	Steinmuller & Zetch, 1984)
Emiliania huxleyi	Haptophyta/Coccolithophyceae	Alanine, leucine (N)	(Ietswaart *et al*, 1994)
Aureococcus anophagefferens	Ochrophyta/Pelagophyceae	Urea (N)	(Berg *et al*, 1997)
Thalassiosira weissflogii	Bacillariophyta/Mediophyceae	|Glucose (C)	Radchenko & Fedeove, 2004)
Phaeodactylum tricornutum	Bacillariophyta/Bacillariophyta	Urea/glycine/glycerol/glucose/ acetate(C/N)	(Garcia *et al*, 2004)
Micractinium pusillum	Ochrophyta/Phaeophyceae	Glucose, and Acetate	Bouarab *et al* i, 2004)
Galderia sulphuraria	Rhodophyta/Cyanidiophytina	Glucose (C)	(Oesterrelt *et al*, 2007)
Phaeodactylum tricornutum	Bacillariophyta/Bacillariophyta	Glucose (C)	(Liu *et al*, 2008)
Prochlorococcus	Cyanobacteria/Cyanophyceae	D-glucose (C)	(Baena *et al*, 2008)
Several algae species		Leucine (C)	(Kamjunke & Tittle, 2008)
Anabaena	Cyanophyta/Cyanophyceae	D-glucose (C)	(Ungerer *et al*, 2008; Stebegg *et al*, 2012)
Chlorella sorokiniana	Chlorophyta/Tredouxiophyceae	Glucose (C)	(Wan *et al*, 2011)
Phaeodactylum tricornutum	Bacillariophyta/Bacillariophyta	Glucose (C)	(Zheng *et al*, 2013)
Synechococcus	Cyanobacteria/Cyanophyceae	D-glucose (C)	(McEwen *et al*, 2013)
Chlorella	Chlorophyta/Tredouxiophyceae	Urea (N)	(Lie *et al*, 2013; Muthuraj *et al*, 2014)
Phaeodactylum tricornutum	Bacillariophyta/Bacillariophyta	Glycine, glucose, and glycerol (C)	(Huang *et al*, 2015)
Ochromonas	Ochrophyta/Chrysophyceae	Glucose	(Zhang *et al*, 2016)
Tetraselmis	Chlorophyta/Chlorodendrophycea e	Yeast extract/glycine/urea (N)	(Kim *et al*., 2016)
Chlorella sorokiniana	Chlorophyta/Tredouxiophyceae	Acetate (C)	(Xie *et al*, 2016)
Trichodesmium	Cyanobacteria/Cyanophyceae	Carbohydrate (C), amino acids (N)	(Benavides *et al*, 2017)

Photosynthetic organisms may switch to use available organic substrates under conditions of environmental stress, the best-known example being algae exposed to low solar radiation (Tuchman *et al*., 2006). Sea ice algae are located under the polar ice where they experience chronically low light conditions, and thus cannot meet their energy requirements from photosynthesis alone; in such circumstances, algae may supplement their energy acquisition mechanism by metabolizing organic carbon substrates in addition to photosynthesis (Qing *et al*, 2003). These sources include the organic substrates acetate, fatty acids, amino acids, and glycerol (e.g., Neilson & Lewin, 1974).

Researchers have investigated osmo-mixotrophy in a variety of algal species, but little is yet known about its role in diatoms, specifically. In general, glucose metabolism plays an important role in algal osmo-mixotrophy. For example, *Chlorella sp*. grown osmo-mixotrophically on glucose had a higher yield of cells than those grown without glucose (autotrophically) or with glucose heterotrophically (Lalucat *et al*, 1984). In *Cyanidium sp.* grown osmo-mixotrophically, glucose acted as a strong inhibitor of RuBPCase, the enzyme that catalyzes CO_2 reduction in the Calvin cycle (Steinmuller & Zetche 1984). In the red alga *Galdieria sulphuraria*, assimilation of glucose as an organic carbon source reduced PSII capacity, and the number of reaction center proteins (Oesterhelt *et al*, 2007).

Of the diatoms, *P. tricornutum* has been the most studied with regard to osmo-mixotrophy because of its potential use in biotechnology and biofuel production (Zheng *et al*, 2013). Garcia *et al* (2005) stated that the possibility of osmo-mixotrophic growth within diatom species depends on the nature of the substrate, the initial concentration utilized, and time. After exploring the effect of different organic substrates and concentrations on *P. tricornutum* growth, they concluded that *P. tricornutum* can grow on all the different organic substrates tested (starch,

lactic acid, glycine, glycerol, and urea), except acetate, but the optimal concentration for growth varied with the substrate. For example, urea together with glycerol significantly supported growth at 0.01M concentration compared to the control (nitrate). In addition, glucose had a pronounced effect on growth rate; this is perhaps expected, since glucose, the product of photosynthesis, likely plays an important role in diatom metabolism (Garcia *et al*, 2004). Another study of growth on organic carbon substrates showed that acetate, glycerol, and glucose at 0.1M concentration supported *P. tricornutum* growth, and that the photosynthetic O_2 evolution rate was lower in mixotrophic cultures relative to the rates observed under strictly photoautotrophic growth conditions (Liu *et al*, 2008). Further work by Bender *et al* (2012) investigated how the urea cycle might be integrated into nitrogen metabolism in *T. pseudonana* by using different nitrogen sources - nitrate, urea, and ammonium - grown under different light intensities; by examining the transcript abundance of genes encoding enzymes involved in the urea cycle and in N assimilation, it was found that transcripts for mitochondrial enzymes were predominant in the dark, whereas transcripts for plastid enzymes were highest in the light for all tested conditions. Zheng *et al* (2013) investigated the use of glucose by *P. tricornutum* under different light/dark and dark conditions; using isotope-labelling experiments to track glucose uptake, they found that *P. tricornutum* can consume glucose as a primary carbon source throughout the light/dark cycle.

1.4.1 Urea as a nutrient on land and in the ocean:

Nitrogen is an essential nutrient for all living organisms because it is constituted of amino acids, proteins, and nucleic acids (Novoa & Loomis, 1981). Phytoplankton can utilize several chemical forms of inorganic and organic nitrogen including nitrate NO_3^-, nitrite NO_2^-, ammonium NH_4^+, dissolved organic nitrogen such as urea $(NH_2)_2CO$, and amino acids (Collos &

Berges, 2003). The use of urea as a nitrogen fertilizer for land plants and water applications has been increasing globally over four decades (Glibert *et al*, 2006). On land, urea is estimated to account for 50% of nitrogen-based soil fertilizers, particularly for corn, sweet potatoes, and vineyards, and it is preferred over nitrate for rice production (Glibert *et al*, 2006). There is evidence that urea supports phytoplankton growth, especially in coastal waters where excess urea can cause noxious algal blooms (Glibert *et al*, 2005). Urea, with two nitrogen atoms, is comprised of 46% nitrogen and 20% carbon by weight, and as such could be an effective nitrogen source for organisms capable of utilizing it (Collos & Berges, 2003). While nitrate assimilation by phytoplankton requires iron as a co-factor in the nitrate reductase enzyme, urease utilizes nickel as a co-factor to hydrolyze urea into carbon dioxide and ammonium (Oliveira & Antia, 1984); thus, a switch from nitrate to urea utilization could effectively reduce cellular iron requirements. At concentrations of 1 μM in open ocean water and 25 μM in coastal water (Collos & Berges, 2003), urea could be most effective source of nitrogen in iron-limited waters where nitrate is accumulating in the surface; productivity there could be driven by regenerated products such as ammonium and urea (Allen *et al*, 2011).

1.5 Rationale for the study:

The primary goal of this thesis was to assess the possibility that an osmo-mixotrophic lifestyle could lead to a competitive advantage for diatom populations that grow in oceanic regions chronically low in bioavailable iron. *Thalassiosira oceanica* CCMP1005 has been selected as a model diatom species in this study for the following reasons: Few experiments have investigated the possibility of osmo-mixotrophy in open ocean diatoms, *T. oceanica* has shown a remarkable tolerance toward low iron conditions (Lommer *et al*, 2012), the entire *T. oceanica* genome sequence is available, and a urease gene sequence - “urea amidohydrolase” - has been

identified in the *T. oceanica* genome (Baker, Gobler, & Collier, 2009) indicating that it is likely able to grow on urea as a sole source of nitrogen.

Demonstrating a shift to osmo-mixotrophic growth in iron-limited environments would provide a framework to explain diatom adaptation and survival in iron-limited regions of the oceans. If there is a significant switch to osmo-mixotrophy under iron limitation, then this pathway will need to be incorporated into models of primary productivity in the ocean, which are increasingly important in assessing how climate change might affect productivity of diatoms, both generally and under conditions of nutrient stress and Fe limitation in particular.

1.6 Hypothesis

The overall hypothesis of this work is that under iron-limited conditions, the diatom *T. oceanica* will optimize growth by shifting to osmo-mixotrophy in the presence of dissolved organic substrates to address their nitrogen and carbon requirements. I further hypothesize that when grown on urea, iron-limited *T. oceanica* will incorporate both the carbon and nitrogen atoms, and that this may lead to a reduction in photosynthetic carbon fixation. Thus, the main objective of this thesis is to determine whether urea can be utilized as a source of both carbon and nitrogen by *T. oceanica* and whether urea utilization could play a significant role in alleviating iron limitation in this diatom.

Chapter 2: Materials and Methods

2.1 Medium preparation and diatom culture conditions:

2.1.1 Preparation of ASW medium

Artificial seawater (ASW) (Guillard and Ryther, 1962) with f/2 nutrients was used (Table 2.1), with modifications for manipulating the fixed nitrogen concentration and source. Chemicals used in these experiments were obtained from Fisher Scientific.

Table 2.1: Final concentrations of macro- and micro-nutrients in nitrate and urea culture media (Guillard and Ryther, 1962).

Chemical	Final concentration (mM*)
NaCl	400.1
KBr	1.7
$MgSO_4$	20.0
$CaCl_2 \cdot 2H_2O$	7.5
KCl	10.1
$MgCl_2 \cdot 6H_2O$	20.0
$NaHCO_3$	2.0
H_3BO_3	0.20
$NaNO_3$**	0.20
Urea**	0.10
Vitamin	(f/2 formulation; Appendix A: supplemental material Table A.1)
$NaH_2PO_4 \cdot H_2O$	0.036
$Na_2SiO_3 \cdot 9H_2O$	0.106
Trace metal mix	(Modified from f/2; Appendix A: supplemental material Table A.1)
Selenium (H_2SeO_3)	0.01µM
$NiCl_2 \cdot 6H_2O$	100nM

*Concentrations in mM unless otherwise indicated; ** Either urea or nitrate was used in the culture medium.

The standard ASW dissolved salts solution was prepared according to the recipe listed in **Table 2.1.** The pH of the medium was adjusted to 8.0 using 1M NaOH and HCl and autoclaved for 45-80 minutes, dependent on volume. Separate PO_4, SiO_3, and trace metal mix with SeO_3 solutions were autoclaved for 15 minutes. Once the dissolved salts returned to room

temperature, the sterilized pre-aliquoted nutrients were added to the autoclaved medium under sterile conditions. Freshly prepared HCO3onate solution was mixed with the vitamins, and added directly into the medium using a single-use 0.2 μm filter and a syringe.

2.1.2 Preparation of iron-limited ASW medium:

The major macronutrient solutions used in the media - PO_4, NO_3, SiO_3, urea – and the urea and nitrate isotope solutions were treated with Chelex 100 (50-100 mesh; Sigma) to remove contaminating divalent cations. The Chelex resin was regenerated each time by washing with 2 bed volumes of 1N HCl, 5 bed volumes of ultrapure water (Milli-Q), 2 bed volumes of 1N NaOH, and 2 bed volumes of ultrapure water. Strict trace-metal (TM)-clean techniques were followed to prevent any iron from being introduced into the media. Polycarbonate bottles were filled with 1:1000 dilution of Citranox (Sigma Aldrich) in ultrapure water; after 24 hours, the bottles were rinsed with ultrapure water 6-8 times until all the soap was removed. The bottles were then half-filled with low trace metal grade 10% HCl; after one week, the bottles were rinsed with ultrapure water and kept in double plastic bags to avoid any iron contamination until used. Bottles to be used for culturing were cleaned as above using TM-clean techniques, and sterilized by filling 2/3 with ultrapure water with three cycles of heating to 95°C and cooling to 80°C in microwave to avoid any iron addition from the steam of the autoclave. All pipette tips used for low-iron work were rinsed three times each with 10% HCl and pH 2.0 ultrapure water, as were plastic spoons used to dispense low-TM grade reagents.

For the preparation of the iron-limited medium, low-TM salts were used to minimize iron contamination **(Table 2.1)**. The pH was adjusted to 8.0 using TM-1M NaOH; the salt mixture was not Chelexed and was sterilized in the microwave to avoid any potential iron contamination from the autoclave steam. Microwave sterilization requires three cycles of heating to 95°C,

followed by cooling to 80°C. The temperature was checked using an infrared thermometer. Separate solutions of PO_4, Si, iron-free trace metal-mix, and Se were sterile-filtered directly into the medium. The sterilization of the salts ASW medium and adding the nutrients for iron-limited medium were under TM-clean strict environment. The EDTA used in the iron-limited trace metal solutions is 100 times greater than the iron concentration to avoid iron precipitation; 100 μM EDTA was combined with 400 μM NaOH to lower the pH prior to adding the iron-free trace metal solution. For iron-replete medium, the iron was added separately at the time of culture initiation. Vitamins were added to the $NaHCO_3$ solution and then sterile-filtered into the cooled sterilized medium. Bottles were stored in double plastic bags until used for culture experiments.

2.1.3 Diatoms strains and growth conditions

Phaeodactylum tricornutum strain CCMP 2561 and *Thalassiosira oceanica* CCMP 1005 were used for this study. Stock cultures were kept at 22°C, 100 μM m^{-2} s^{-1} light intensity under 14hr light/10hr dark photocycles (with one exception, described below). Stock cultures were transferred weekly; 60 mL flasks (Thermo Scientific) containing 30 mL of culture medium were inoculated with 30 μL of *T. oceanica* containing approximately $1x10^4$ cells/mL. To assess whether *T. oceanica* was able to grow heterotrophically on organic substrates in continuous darkness, cultures were kept inside a dark box in the incubator to prevent light exposure.

2.2 Design of preliminary experiments

The role of osmo-mixotrophy in diatom growth was assessed in *T. oceanica*, and *P. tricornutum* on a series of organic carbon and nitrogen substrates. 0.05M acetate ($C_2H_3O_2$), 0.01M glycine ($C_2H_5NO_2$), 0.01M glycerol ($C_3H_8O_3$), 0.02M glucose ($C_6H_{12}O_6$), and 0.01M urea ($[NH_2]_2CO$) were chosen to examine the ability of *T. oceanica* to grow osmo-mixotrophically in the presence of organic nitrogen and carbon in addition to inorganic substrates; batch culture was

chosen over semi-continuous culture (**Appendix B: Figure B.1**). Urea and glycine were also tested as the sole source of fixed nitrogen in the medium to determine whether these nitrogen compounds could sustain growth in the absence of nitrate. With the exception of urea, the concentration of each substrate was taken from Garcia *et al* (2005). Different concentrations of urea – 100µM, 200µM, 400µM, and 800µM – were tested to determine the concentration that supported the highest cell density of *T. oceanica*. Since nickel (Ni) is important to urea assimilation and is not present in f/2 ASW, Ni was added to the medium at a concentration of 100nM (Oliveria & Antia, 1984; Price & Morel, 1991). Triplicate 40mL cultures were grown for each of the substrates, transferring a 1×10^4 cells/mL with daily cell counts measurements. Each culture was checked for bacterial contamination using DAPI staining as described below in section 2.3.4.

T. oceanica and *P. tricornutum* cultures were grown with different organic substrates with dark/light and dark cycles. For these trials, 40 mL of medium were inoculated with 1×10^4 cells/mL in triplicate. A dark box was used to keep the dark-treatment cultures in complete darkness in the 22˚C incubation. Daily cell count measurements and DAPI staining for bacterial contamination were conducted.

2.3 Experimental design:

T. oceanica was grown in two different media, one with urea and the other one with nitrate (control), using the growth conditions described in section 2.1.3. Twelve 500mL polycarbonate bottles were used, comprising triplicate iron-limited/iron-replete conditions for both nitrate and urea treatments. Throughout the growth period, a series of measurements were conducted. Cell counts, and Fv/Fm were measured daily from the 500 mL bottles. When the cells reached mid-exponential phase (range from $40 \times 10^4 – 60 \times 10^4$ cells/ml), samples were collected

for isotope labeling experiments (ILE). From each replicate culture, a 40mL aliquot was filtered onto a pre-combusted (8 hours, 400°C) 25 mm GF/F filter to measure the initial natural abundance of ^{13}C and ^{15}N in the particular organic matter (POM). An additional 40 mL aliquot from each culture with nitrate was used to initiate an ILE with both ^{13}C-HCO_3 and ^{15}N-NO_3 for both iron-replete and iron-limited conditions; the ILE for cultures with urea required separate incubations for ^{13}C-HCO_3 and the dual labelled ^{13}C/ ^{15}N-urea. The samples for the ILE were incubated for 24 hours to cover a full light/dark cycle in order to avoid circadian cycle bias in nutrient uptake **(Figure 2.1).** At the end of the incubation period, the 40mL cultures were filtered onto pre-combusted GF/F filters and rinsed three times with 5 mL of nitrogen-free, sterile-filtered artificial seawater; filters were dried overnight at 50ºC, packed in tin cups (Elemental Microanalysis) and stored in a 96-well plate for further analysis with the isotope ratio mass spectrometer (IRMS). At the termination of the ILE incubations, the final cell density was measured for all samples, and 10 mL of the filtrate from the natural abundance samples was collected for nutrient analysis (nitrate and urea). Samples for 4,6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) staining were collected from the 500 mL cultures to ensure that the cultures were not contaminated by bacteria prior to the initiation of the ILE measurements (as described below).

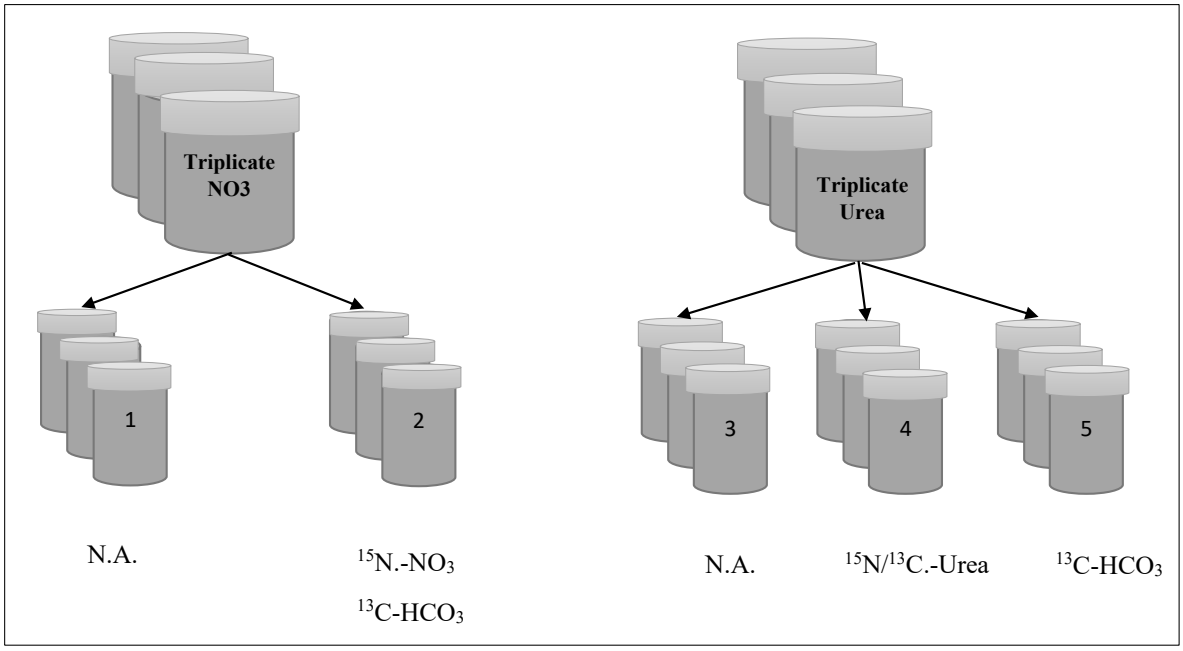

Figure 2.1: Schematic of the experimental design to investigate the effect of both iron-limited and iron-replete conditions on *T. oceanica* grown in nitrate vs urea. For each growth condition, triplicate 500mL *T. oceanica* cultures were grown with either nitrate or urea as a nitrogen source.

2.3.1 Stable isotope labeling experiments (ILE)

The ILEs were designed to measure uptake and assimilation of inorganic and organic carbon and nitrogen by cells grown in iron-replete and iron-limited conditions with either nitrate or urea as inorganic nitrogen and organic nitrogen source, respectively. Culture media for iron-limited plus nitrate, iron-limited plus urea, iron-replete plus nitrate, and iron-replete plus urea were prepared as described in sections 2.1.1 and 2.1.2. Cells were pre-conditioned in their respective growth medium for at least 6 generations to allow the cells to acclimate before transferring an equal number of cells (approximately $1x10^4$ cells/mL) to 500mL of equivalent medium in preparation for the stable isotope labeling experiments (ILE). After the inoculation of the 500 mL bottles, cell density and Fv/Fm were monitored daily until the cultures reached a cell

density range from $30 \times 10^4 – 50 \times 10^4$ for iron-replete cultures or $15 \times 10^4 – 20 \times 10^4$ for iron-limited cultures, as the latter tended to decrease in cell density beyond this level. The cultures were harvested and samples were then collected for the ILEs as shown in **Figure 2.1** as well as for cell density measurements, Fv/Fm, initial natural abundance of ^{13}C and ^{15}N in the POM, and DAPI-staining for potential bacterial contamination. The ILEs were initiated by transferring 40 mL of each 500 mL culture to 60 mL disposable culture flasks (Thermo Scientific). In total, five treatments were incubated for a 24-hour period at 22°C with the same light/dark cycle as in the pre-conditioning. ILE 1 and 3 represented controls for measuring the natural abundance (N.A.) of ^{13}C and ^{15}N in the (+/- iron) nitrate grown and (+/- iron) urea grown cells **(Figure 2.1)**. For the nitrate cultures, ^{15}N- nitrate (Sigma) and ^{13}C-HCO3onate (Sigma) were added together to the ILE 2 incubations to a final 11 atom% and 10 atom%, respectively **(Figure 2.1)**. For the urea cultures, two separate ILE incubations were required to measure the assimilation of both organic carbon and nitrogen from urea (ILE 4), and photosynthetic carbon fixation (ILE 5), because HCO3onate is an essential component of seawater, and photosynthetic carbon fixation is expected to continue in the presence of urea. Thus ^{13}C-HCO3onate (99 atom %) was added to ILE 5 to a final concentration of 10 atom% at the onset of the incubation. The assimilation of carbon and nitrogen from urea was measured by adding dual $^{13}C/^{15}N$-labeled urea (Sigma) to a final 40 atom%. The isotope-labelled cultures were incubated for 24 hours at 22°C before harvesting the cells as described in section 2.2. Filters with natural abundance, ^{13}C-HCO3onate, and ^{15}N-nitrate were dried in separate ovens.

2.3.1.1 Preparation of the stable isotope stock solutions

A stock solution of ^{15}N-sodium nitrate (117mM; 98 at% ^{15}N) was used to prepare a working solution of 20mM ^{15}N-nitrate (98 at%) in order to obtain a final concentration of 20 μM

^{15}N-nitrate in the ILE, or a 9.8 at% ^{15}N enrichment of nitrate. Similarly, a 99 at% ^{13}C-HCO3onate and a 98 at% $^{15}N_2/^{13}$C-urea stock solutions were added to the appropriate ILEs **(Figure 2.1)** to final concentrations of 0.2 mM and 68 µM, respectively yielding final ^{13}C at% of 9.9 for HCO3onate additions and $^{15}N/^{13}$C at% of 39.7 at% for the urea addition.

2.4 Measurements

2.4.1 Cell counts

A suite of physiological parameters were measured **(Table 2.2)**, including cell counts**.** Cells were initially counted using both the Accuri C6 flow cytometer (BD) and a hemocytometer; the two methods showed similar results **(Figure 2.2)** with a correlation coefficient of 0.89, so the faster flow cytometry was used for daily cell counting**.** Proper cytometer function was confirmed by daily use of 8-peak and 6-peak validation beads (Spherotech) as directed by the cytometer manufacturer (BD Accuri C6 software manual, p. 9-13). The cells present in 100µL of culture were enumerated daily as cells/mL.

Table 2.2.: Physiological parameters measured in iron-limited and iron-replete *T. oceanica.*

Parameter	**Method**
1. Physiological measurements	
Fv/Fm measurements	PAM fluorometer
Cell density (Cells mL^{-1})	Accuri C6 Flow cytometer
Visualization of bacteria with DAPI	AXIO-Imager.M2 microscopy
NO_3 and urea concentrations (µM)	Skalar SAN++ Continuous Flow Nutrient Analyzer
POC and PON concentration (µM)	Vario Micro Cube elemental micro analyzer
2. ^{13}C and ^{15}N tracer experiment (ILE)	
^{13}C-HCO_3, ^{15}N-NO_3, $^{15}N/^{13}$C-urea (at%)	Isoprime 100 Isotope Ratio Mass Spectrometry

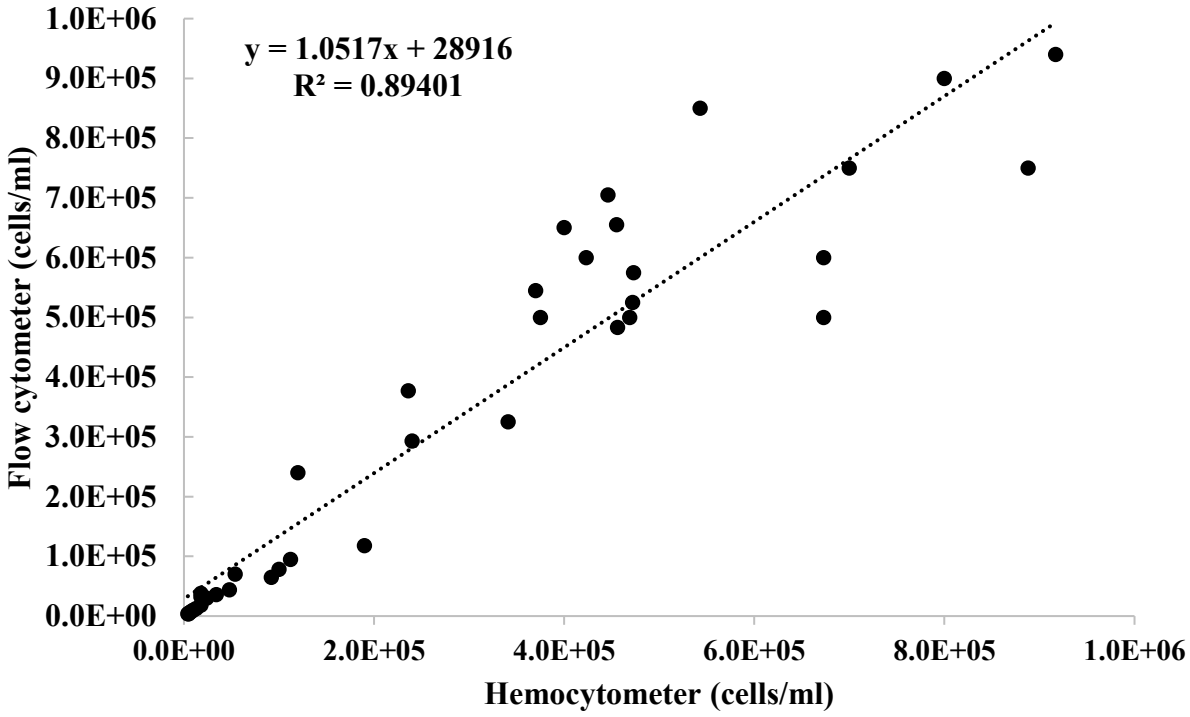

Figure 2.2: *T. oceanica* cells counted by flow cytometer and hemocytometer show a high correlation between the results from two methods

2.4.2 Fv/Fm measurements

The degree of iron limitation was assessed daily by measuring Fv/Fm (DUAL-PAM-100, P700 & chlorophyll fluorescence measuring system; Walz), which measures the PSII photosynthetic efficiency. 3 mL subsamples of the cultures were pre-adapted in the dark for 20 minutes before measuring the Fv/Fm as recommended by the manufacturer.

2.4.3 Nitrate and urea concentration measurements

Nitrate and urea concentrations in the cultures were measured using the San ++ Continuous Flow Analyzer (CFA; Skalar). The nitrate concentration was calculated by

subtracting the NO_2 measurements from the combined NO_2+NO_3 measurements. Urea concentrations were determined by measuring the ammonia resulting from the enzymatic hydrolysis of urea into carbon dioxide and ammonia. Urease (Sigma-Aldrich) was added to filtered culture media at a concentration of 10 units per 10mL of sample; the samples were incubated for 10 minutes at 50°C, and immediately processed by the CFA (Parsons, 2013). A calibration curve with known urea concentrations was generated **(Figure 2.3)**.

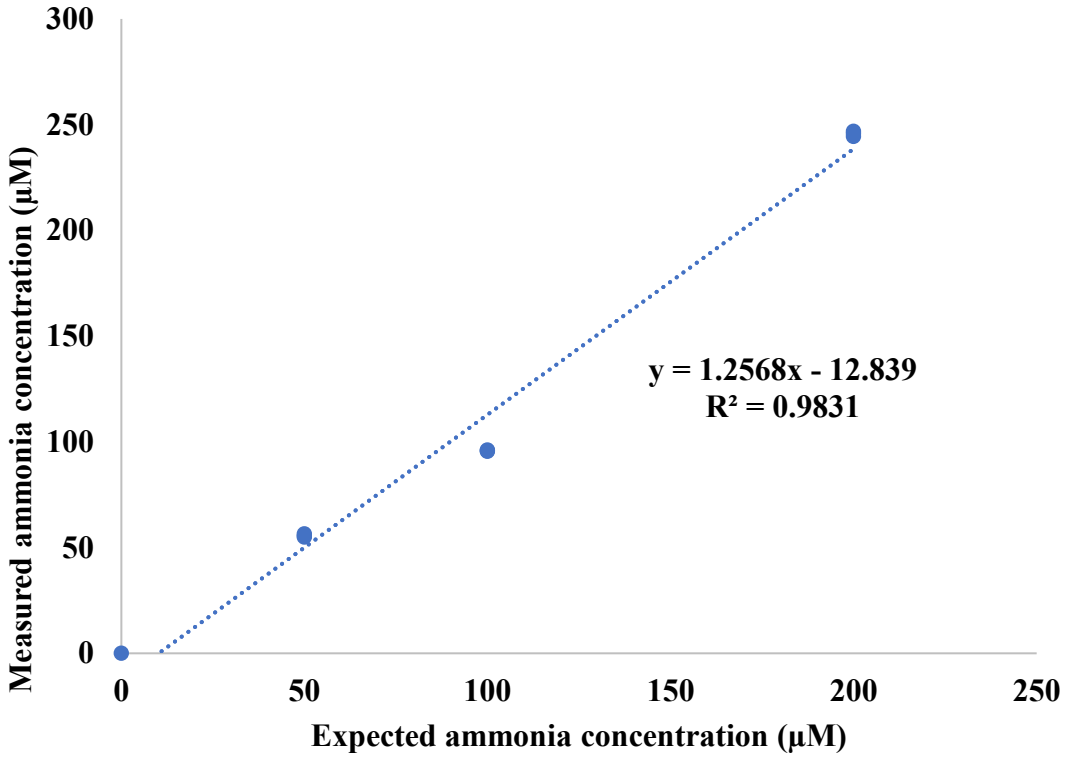

Figure 2.3: Calibration curve for the ammonia measured from hydrolyzed urea relative to expected concentrations**.**

2.4.4 Assessment of bacterial contamination

Bacterial contamination of the diatom cultures was assessed in each experiment; 0.5 mL of culture was mixed with 3.5 mL of sterile ASW and fixed with 1% paraformaldehyde solution

(Alfa Aesar) at room temperature for one hour. The fixed cells were filtered onto 25mm black polycarbonate track etched membranes (Cyclopore; Whatman) and dried at room temperature in the dark for 15 min. The filter was cut with a scalpel; half was mounted on a slide with 20µL of DAPI solution (4mM DAPI/220mM 1,4-Diazabicyclo[2.2.2]octane (DABCO) in Mowiol mounting medium) under a glass coverslip, while the other half was kept in the dark for later use. Bacterial contamination was assessed under the 60X oil immersion lens of the AXIO-Imager.M2 (Zeiss) with ZEN 2 pro software for fluorescent detection of DNA and chlorophyll.

Bacterial contamination was also assessed by inoculating 5 mL of sterile marine broth with 1 mL of medium or culture and incubating at 22°C for several days; turbidity was taken to be indicative of bacterial contamination. Contaminated cultures were discarded.

2.4.5 Isotope ratio mass spectrometry (IRMS)

To determine stable isotope ratios and particulate organic carbon (POC) and nitrate (PON), samples were filtered onto pre-combusted (8 hours, 400°C) 25mm GF/F filters (Whatman) using acid-washed filter cups and a vacuum manifold (5 mm Hg). The filter cups were rinsed with distilled ultrapure water between each triplicate treatment. The filtrate from the natural abundance samples was collected and frozen at -20°C for later analysis in the CFA. Filters were stored in individual petri dishes, dried for 24h at 50°C, packed into tin cups (Elemental Microanalysis), and stored in a 48 well plate prior to analysis in the Vario Micro Cube elemental micro analyzer (EA) in line with the Isoprime 100 Isotope Ratio Mass Spectrometer (IRMS; Elementar). POC and PON were measured by the EA, while stable isotope ratios were measured by the IRMS. To calibrate the IRMS, two blank samples and ten standards (duplicate caffeine (Alfa Aesar), L-glutamic acid (Sigma Aldrich), glycine (Sigma Aldrich), sulfanilamide (Elementar), and nicotinamide (Sigma Aldrich), respectively), were run before the experimental

samples and duplicate nicotinamide standards were run at the end of the set of samples.

2.4.6 Rate calculations and statistical analyses

The following equation was used to calculate the uptake of carbon and nitrogen by each sample, thereby estimating the effect of the presence of organic carbon and nitrogen substrates in the absence and presence of iron. The equations 1-4 (Großkopf *et al*, 2012) were used to calculate the nitrogen uptake rate. The same equations were used to calculate the C uptake rate by replacing the nitrogen isotope data by the carbon stable isotope results.

$$N\ Uptake\ rate\ (\mu M\ N\ h^{-1}) = \frac{APE^{PN}{}_{sample}}{APE^{N}{}_{substrate}} \times \frac{PN}{\Delta t} \quad (1)$$

$$= \frac{(At\%^{PN}{}_{sample} - At\%^{PN}{}_{NA-control})}{(At\%^{N}{}_{substrate} - At\%^{PN}{}_{NA-control})} \times \frac{PN}{\Delta t} \quad (2)$$

$$At\%^{N}{}_{substrate} = \frac{[S]_{added} * 99}{[S]_{added} + [S]_{ambient}} \quad (3)$$

$$At\%^{PN}{}_{sample} = 1 + \left[\frac{R_{sample}}{R_{sample}+1}\right] \times 100 \quad (4)$$

$$R_{sample} = \left[\frac{\delta}{1000} + 1\right] \times R_{standard}$$

$$R_{standard} = \left[\frac{\delta}{1000} + 1\right]$$

$$^{\delta}N = 0.003664$$

$$^{\delta}C = 0.011179$$

.

APE = at% excess stable isotope (^{15}N or ^{13}C) in PON or POC, respectively (at% of either ^{15}N or ^{13}C in PON/POC of the enriched sample - at% of the un-enriched N.A. sample).

[PN] = Particulate organic nitrogen (µmoles N litre $^{-1}$).

$[S]_{added}$ = Amount of isotope added (µmoles N or C litre $^{-1}$).

$[S]_{ambient}$ = Ambient substrate concentration (µmoles)

Time = Incubation time (h).

($^{\delta}$) $R_{standard}$ = $^{\delta}N$ (atmospheric air) or $^{\delta}C$ (Pee Dee Belemnite)

($^{\delta}$) R_{sample} = $^{\delta}15N/14N$ (Mass spectrometry data)

After calculating the C and N uptake rates (µmoles C or N litre^{-1}h^{-1}) from the equation above, the C or N specific uptake rates (h^{-1}) were calculated by normalizing the C and N uptake rate to the biomass (POC or PON), respectively. In addition, the C or N cellular uptake rates (fmole cell^{-1}h^{-1}) were calculated by dividing the C and N uptake rate by the cell density. Standard deviation was calculated for each of the measured parameters whenever appropriate. Statistically significant differences between the treatments were assessed using an ANOVA with a Tukey's

Honest Significant Difference (HSD) post hoc test using R (version 3.2.3) (R Core Team, 2015). Comparison of the treatment means were considered significant when the adjusted *p* value (p-value adj.) was < 0.05.

For the percentage of urea-C incorporation of the total carbon assimilated, standard error was calculated using propagation standard error formulas (Pezzullo, 2013), as described below:

$$\%\ Urea-C\ uptake\ rate = \frac{Urea-C\ cellular\ uptake\ rate}{Urea-C\ cellular\ uptake\ rate + HCO^{-}_{3}\ cellular\ uptake\ rate} \times 100 \quad (1)$$

$$SE\ (x+y) = \sqrt{SE\ (x)^2 + SE\ (y)^2} \quad (2)$$

$$SE\ (xy) = xy\sqrt{\left(SE(x)/x\right)^2 + \left(SE(y)/y\right)^2} \quad (3)$$

SE = Standard Error

x= Urea-C cellular uptake

y = HCO_3^- cellular uptake rate

Chapter 3: Results

The hypothesis of the thesis was that under iron-limited conditions, *T. oceanica* will optimize growth by shifting to osmo-mixotrophy in the presence of dissolved organic substrates to fulfill their nitrogen and carbon requirements. Further, it was hypothesized that when grown on urea, iron-limited *T. oceanica* will incorporate both the carbon and nitrogen atoms, and that this may compensate for previously observed down-regulation of photosynthetic carbon fixation in iron-limited conditions (Lommer *et al,* 2012). In order to test this hypothesis, the thesis addressed three main objectives: 1) Determine whether *T. oceanica* can grow on a simple organic nitrogen molecule, 2) determine whether *T. oceanica* can assimilate the carbon as well as the nitrogen atoms from the simple organic substrate, and 3) determine whether the assimilation of carbon and nitrogen from an organic molecule could contribute to the relief of iron limitation.

3.1 *Thalassiosira oceanica* can grow osmo-mixotrophically

The first step in testing the hypothesis was to identify a simple organic substrate that could provide a combined organic carbon and nitrogen source. A suite of organic carbon and nitrogen sources was used to investigate whether *T. oceanica* could grow osmo-mixotrophically. Because dissolved inorganic carbon (DIC) is an intrinsic component of seawater, it was not possible in these experiments to determine the assimilation of dissolved organic carbon (DOC) in the absence of DIC. The organic carbon sources tested in the presence of DIC and nitrate were acetate ($C_2H_3O_2$), glycerol ($C_3H_8O_3$), and glucose ($C_6H_{12}O_6$). In addition, two mixed carbon and nitrogen compounds, glycine ($C_2H_5NO_2$), and urea (CH_4N_2O) were explored as potential organic nutrient candidates for testing the main hypothesis.

Urea was identified early on as a potentially suitable substrate for these experiments because it is simple molecule that contains both carbon and nitrogen atoms. As the urease

enzyme, essential for the assimilation of urea, contains Ni as a co-factor (Oliveria & Antia, 1984; Price & Morel, 1991), a range of initial urea concentration (100µM, 200µM, 400µM, and 800µM) with and without Ni addition in the trace metal mix were tested to identify the concentration that best promoted growth in *T. oceanica* **(Figure 3.1).**

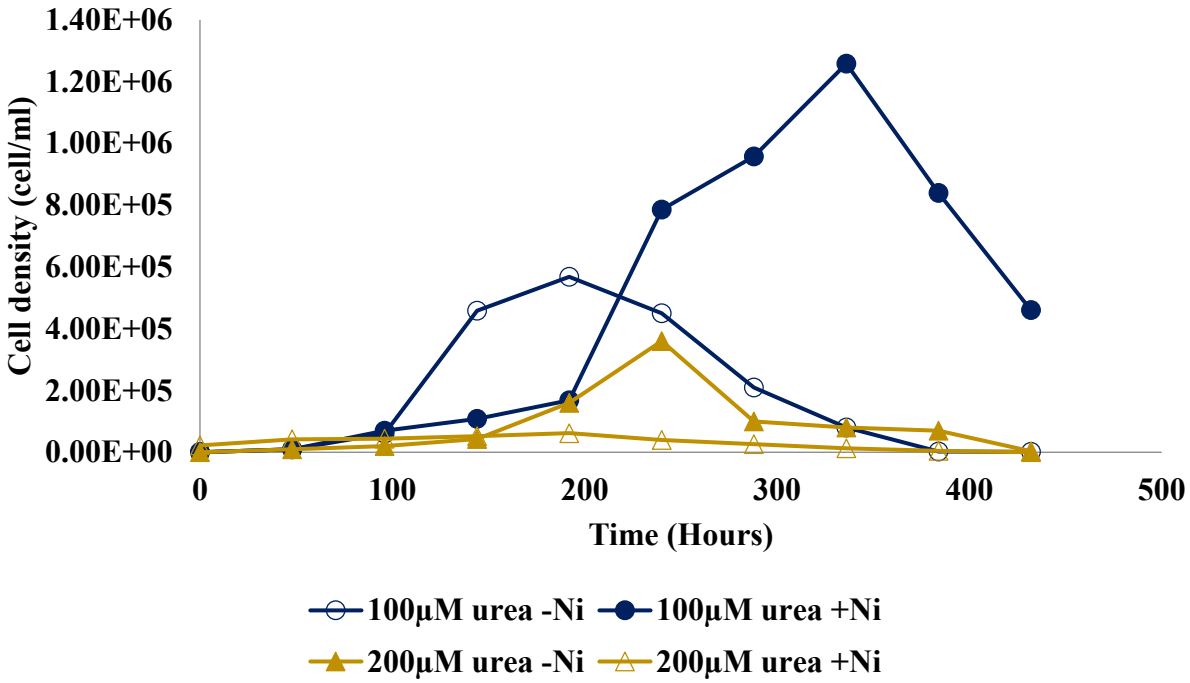

Figure 3.1: Growth curves of *T. oceanica* in media containing varying concentrations of urea and nickel.

The results showed that 100 µM urea and 100 nM nickel best supported growth of *T. oceanica,* confirming the results of Oliveria & Antia (1984), while urea concentrations of 200 µM and higher inhibited the growth of *T. oceanica*. A series of growth curves with different organic substrates **(Figure 3.2)** showed that *T. oceanica* supplemented with glycine reached the highest cell density, even exceeding that of the control (nitrate). The elemental analyzer was used to measure the biomass within the glycine, glucose and glycerol cultures (**Appendix B: Table B.2**). While the cell yield was higher for glucose and glycerol amended cultures, it is not possible to

determine whether or not the organic carbon was assimilated in the absence of isotope labeling experiments. In contrast, acetate inhibited *T. oceanica* growth. The growth and cell yield of *T. oceanica* with urea as a sole source of nitrogen was comparable to the nitrate control **(Figure 3.2**).

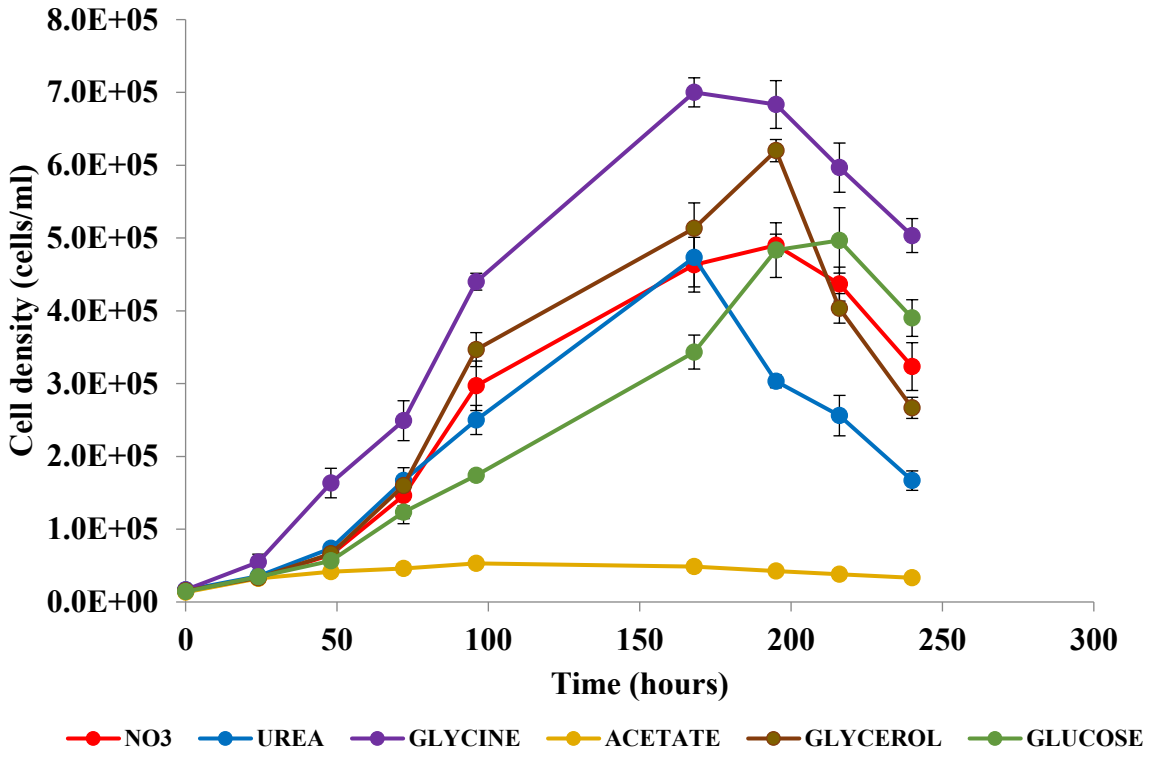

Figure 3.2: *Thalassiosira oceanica* grown on 0.05M Acetate ($C_2H_3O_2$), 0.01M glycine (CH_2-COOH), 0.01M glycerol ($C_3H_8O_3$), 0.02M glucose ($C_6H_{12}O_6$), and 0.01M urea ($[NH_2]_2CO$), in presence of NO_3^-, and urea alone vs NO_3^- alone. Error bars represent the standard error of measurements from biological triplicate cultures.

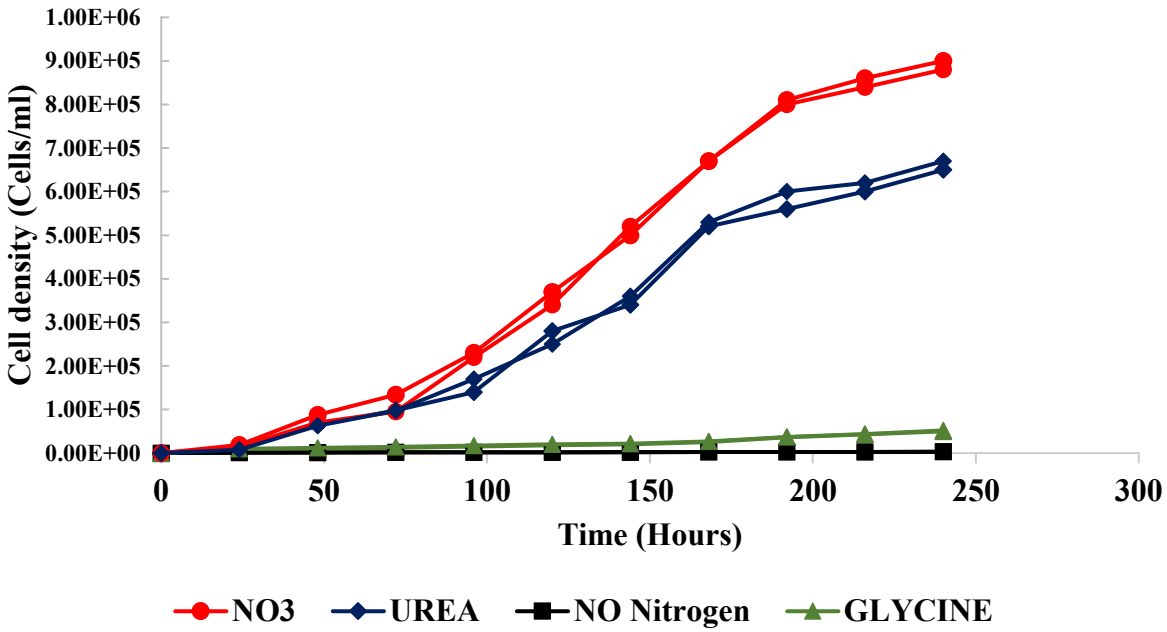

Figure 3.3: *Thalassiosira oceanica* grown with dissolved organic and inorganic nitrogen substrates as sole source of nitrogen. Biological duplicate cultures were analysed; the duplicate plots for glycine overly one another.

Glycine, one of the two tested organic substrates that contained both carbon and nitrogen, was added to culture medium containing 200 µM nitrate **(Figure 3.2).** Although the glycine-supplemented culture yielded the highest cell density, glycine on its own as an organic nitrogen source did not support the growth of *T. oceanica* and was therefore not pursued further **(Figure 3.3).** In these substrate assessment comparisons, *P. tricornutum* was the control species as it is already known to grow osmo-mixotrophically in the light (Garcia et al, 2005), thus it was used to compare *T. oceanica* grown osmo-mixotrophically on the same substrates that *P. tricornutum* could grow on. The results presented in **Figure 3.4** show that in contrast to *T. oceanica*, acetate did not inhibit the growth in *P. tricornutum*. In addition, amendment with glycine resulted in the highest cell density production in the growth experiments. The *P. tricornutum* cultures survived for several months in the presence of glycine as a substrate (data not shown).

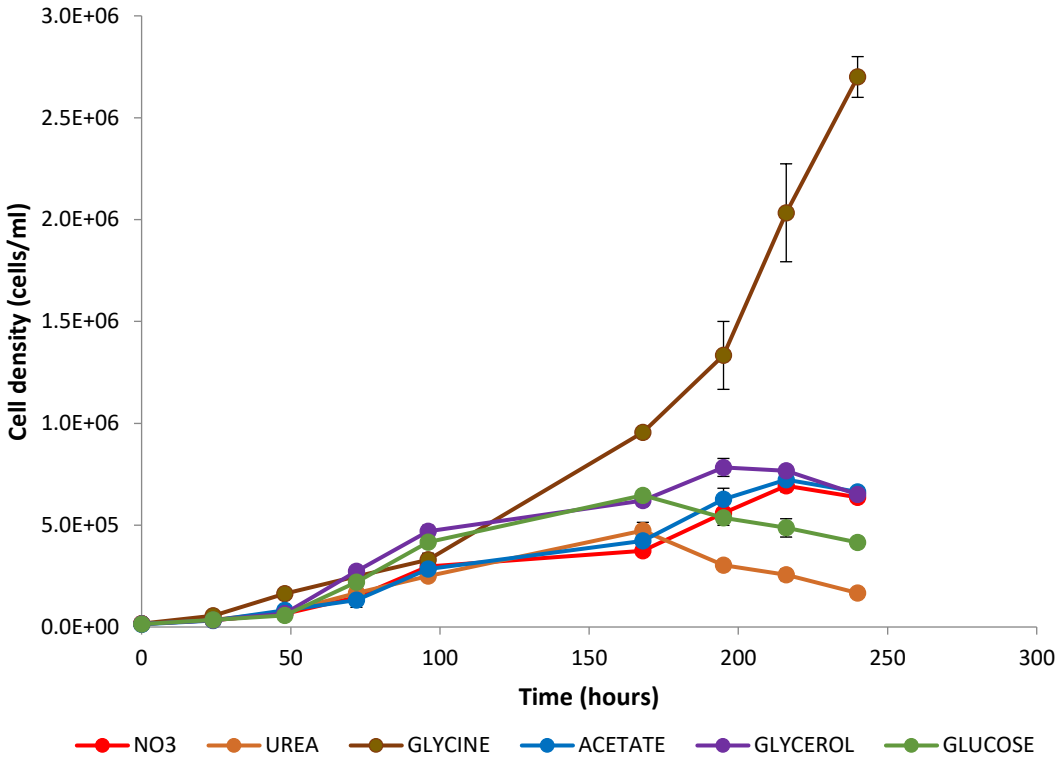

Figure 3.4: *Phaeodactylum tricornutum* grown on the organic carbon sources glycine, glycerol, glucose, and acetate in presence of nitrate, and with the organic carbon and nitrogen source urea vs nitrate alone.

Experiments were performed with the same organic substrates to compare growth under a light/dark (14/10) regime and in complete darkness to determine whether the two species of diatoms could grow heterotrophically. Although, it was possible to maintain *T. oceanica* axenically in the dark-grown cultures **(Figure 3.5)**, several attempts to grow *P. tricornutum* under the same dark conditions and with the same culture medium, resulted in heavy bacterial contamination as seen in **Figure 3.6**. Therefore, the growth curves for the *P. tricornutum* are not presented here.

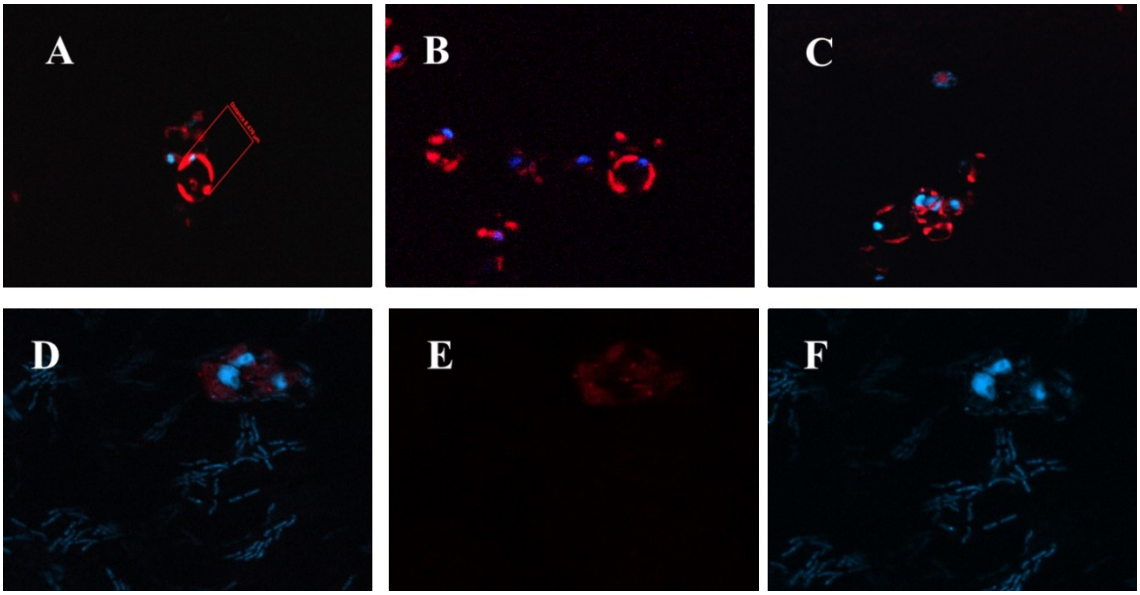

Figure 3.5: Fluorescence micrographs of fixed DAPI-stained *T. oceanica* cultures grown on different substrates. Chlorophyll appears as red and DAPI-stained nucleic acids as blue. A, B, and C show uncontaminated cultures. D shows a bacterially contaminated culture; where E and F are the individual chlorophyll and DAPI images, respectively.

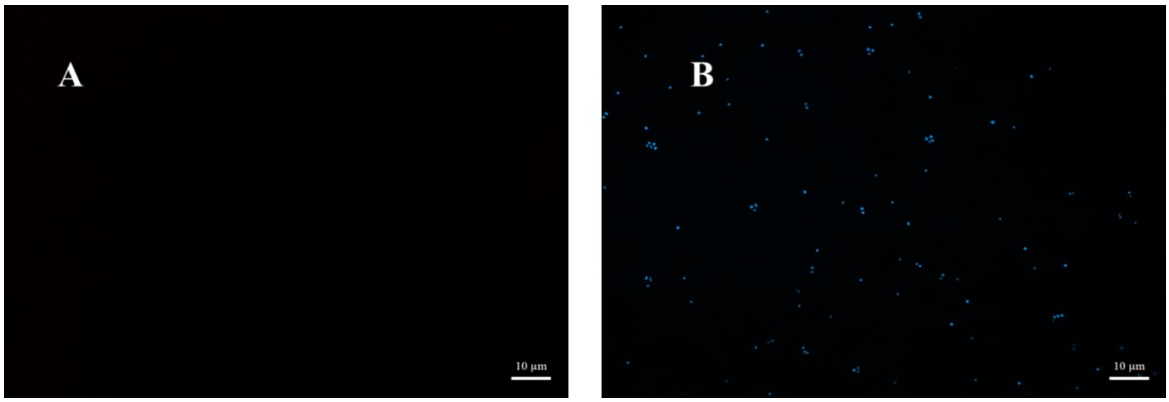

Figure 3.6: Fluorescence micrographs of fixed DAPI-stained *P. tricornutum* cultures grown on urea. Chlorophyll (A) appears as red and DAPI-stained nucleic acids (B) as blue.

The light/dark and dark grown culture experiments showed that *T. oceanica* cannot grow heterotrophically on the suite of substrates used in this study but that significant cell density can be maintained when grown on a light/dark cycle, as shown from **Figure 3.7**

Thus, the preliminary experiments with organic carbon and nitrogen substrates demonstrated that urea was a suitable nutrient for osmo-mixotrophic growth of *T. oceanica*.

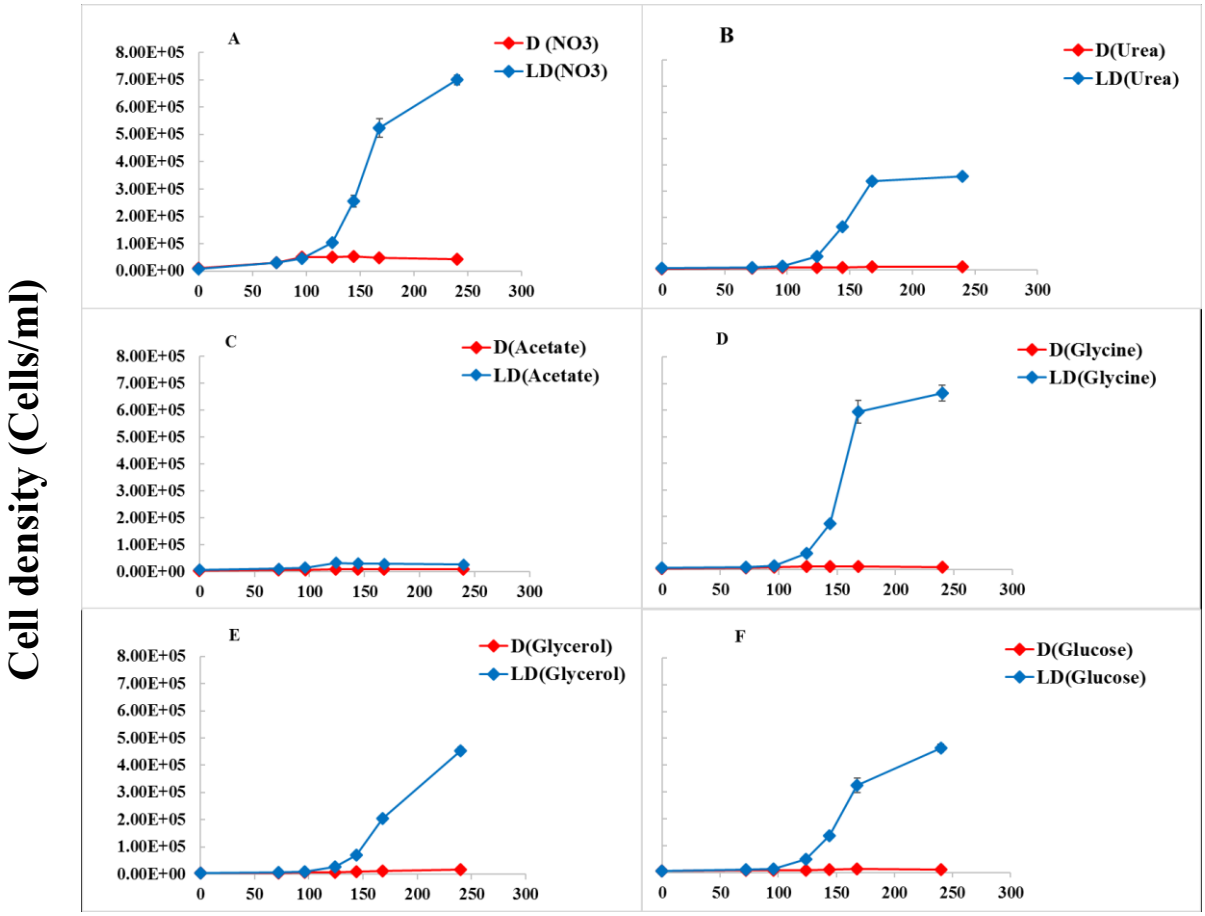

Figure 3.7: Comparison of *T. oceanica* grown in the dark (D; red symbols) and on a 14h-light/10h-dark cycle (LD; blue symbols). A and B show *T. oceanica* grown on nitrate and urea, respectively, as the sole nitrogen source. C, D, E, and F show acetate, glycerol, glycine and glucose, respectively, in the presence of nitrate as an inorganic nitrogen source.

3.2 The effect of dissolved nitrogen sources on *T. oceanica* growth under iron-limited vs iron-replete conditions

The effects of supplemental dissolved organic carbon and nitrogen compounds on diatom growth were investigated under iron-limited conditions to identify a potential shift to osmo-mixotrophy under these conditions. Iron-limited cultures were first established with nitrate as a nitrogen source. **Figure 3.8** shows that growth of *T. oceanica* responds to increasing dissolved iron concentration by increasing the cell density; the growth of *T. oceanica* increased with increasing iron concentration up to 10μM, which was the highest concentration tested. Measurements of Fv/Fm taken daily increased with the initial iron concentration in each treatment, indicating that iron was limiting photosynthesis and growth **(Figure 3.9).** Also, addition of 10 μM iron to iron-limited cultures resulted in an increase in cell density compared to no addition or addition of urea **(Figure 3.10**), further confirming that the medium and growth conditions led to the growth of cultures that were iron-limited. Taken together the cell density measurements and nutrient (iron or urea) addition bioassays indicated that the cultures were growing in iron-limited conditions. In this study, Fv/Fm lower or equal to 0.4 were considered iron-limited, and an increase in Fv/Fm from 0.4 to 0.6 was taken as an indicator of relief of the iron-limited growth conditions.

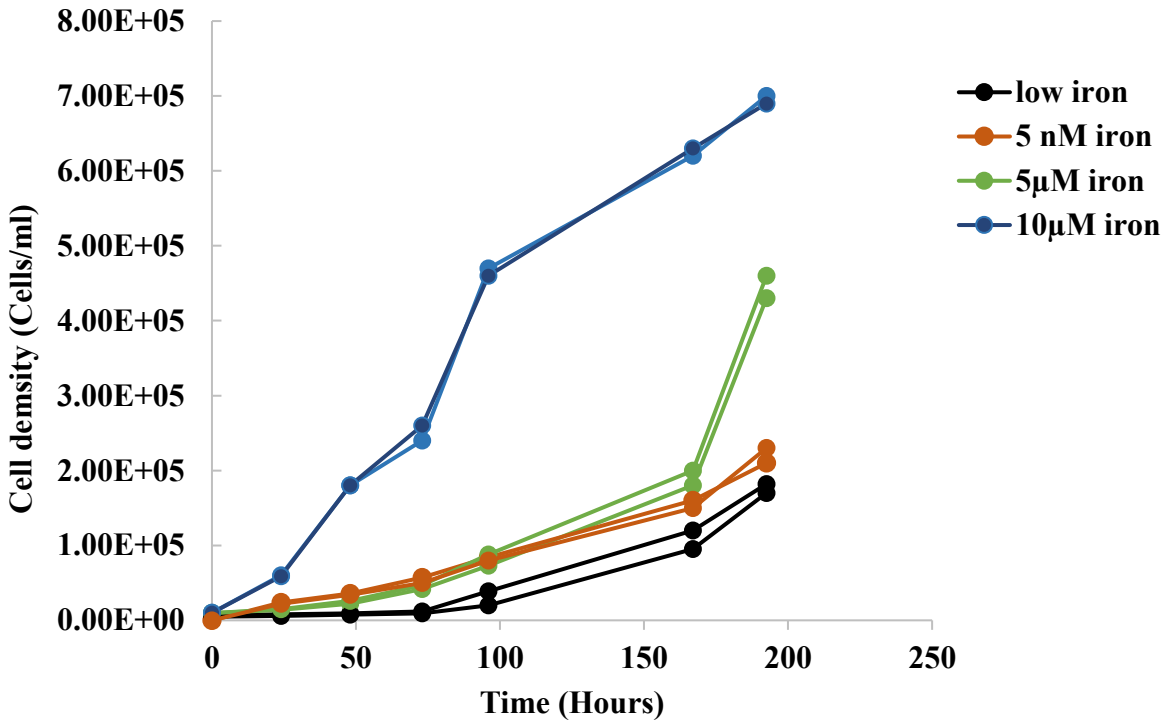

Figure 3.8: *Thalassiosira oceanica* growth response to four different iron concentrations (no iron, 5nM, 5µM, and 10µM) with nitrate as a nitrogen source.

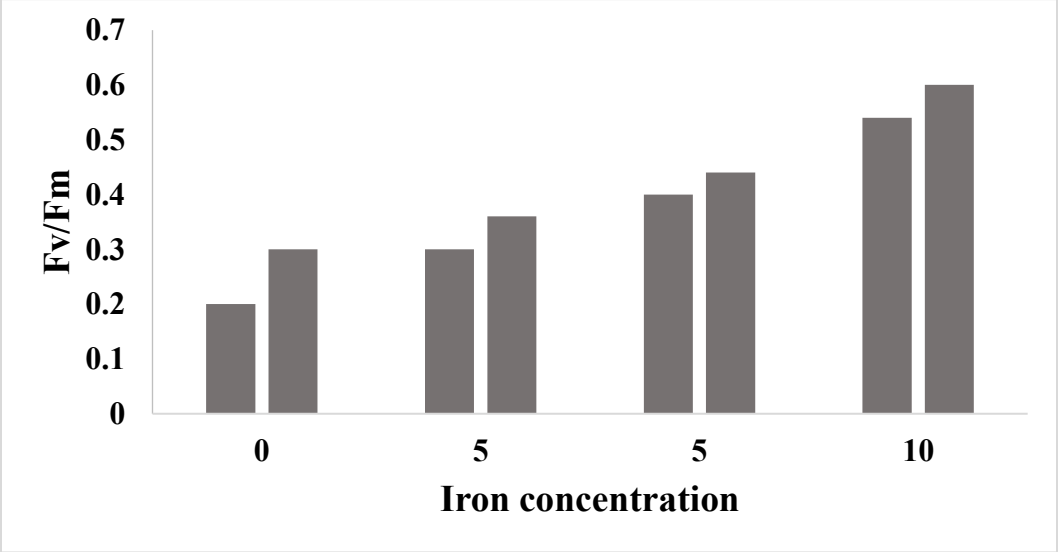

Figure 3.9: Fv/Fm measurements as an indicator of iron-limited vs iron-replete cultures. Biological duplicates are shown.

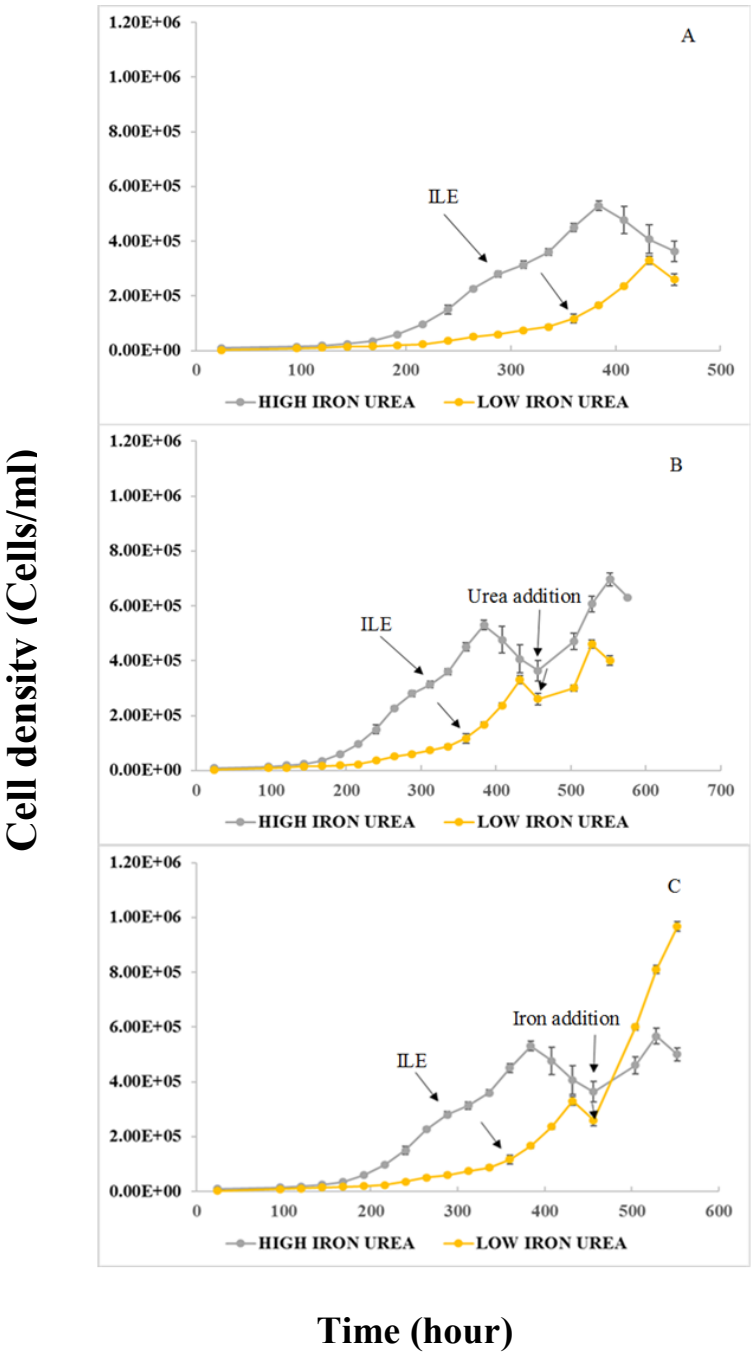

Figure 3.10: Iron-limited and iron-replete growth of *T. oceanica* with urea as a sole source of nitrogen. A shows *T. oceanica* grown on urea under iron-limited and iron-replete conditions. B shows the response of the iron-limited and the iron-replete cultures to the addition of urea (100 µM). C shows the response of iron-limited and iron-replete cultures to the addition of iron (10 µM). The arrow indicates the time point in the growth curve where the ILEs were initiated

Although urea supported *T. oceanica* growth under iron-replete conditions as a sole nitrogen source, growth on nitrate resulted in a higher cell density **(Figure 3.11).** In contrast, under iron-limited conditions, *T. oceanica* grown on urea reached a higher cell density **(Figure 3.12).** However, nitrate cultures had higher Fv/Fm than urea under both iron-limited and iron-replete conditions **(Table 3.1)**.

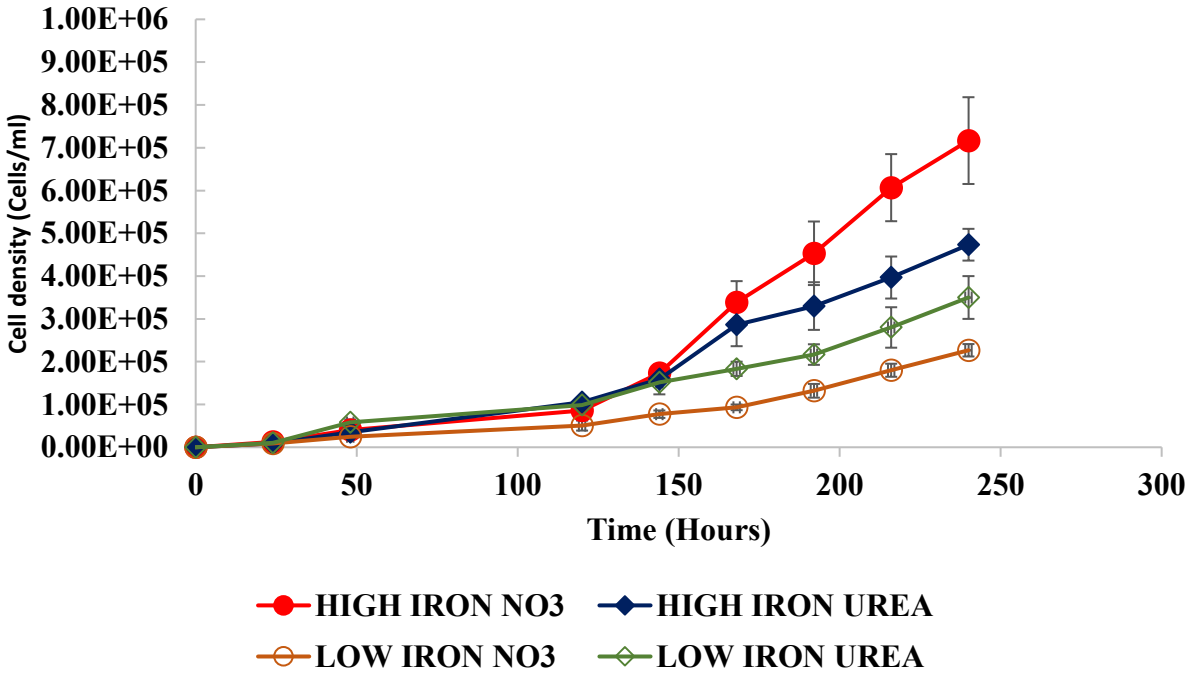

Figure 3.11: Growth of *T. oceanica* in iron-limited (open) vs iron-replete (filled) on urea (rhombus) and nitrate (circle).

Table 3.1: Fitness indicators of *T. oceanica* grown with inorganic and organic nitrogen sources with high or low iron showed the range of the Fv/Fm measurements.

Treatment	Fv/Fm
High iron NO_3	0.56-0.67
High iron Urea	0.55-0.63
Low iron NO_3	0.32-0.40
Low iron Urea	0.33-0.40

3.3 Isotope labeling experiments (ILE):

Because seawater inherently contains dissolved inorganic carbon (DIC) as the primary carbon source for photoautotrophs, assessing the relative contribution of DIC and urea-C to the overall carbon assimilation during the growth of the cultures required the use of the stable isotope ^{13}C for the organic (urea) and inorganic carbon sources. Likewise, nitrate and urea enriched with ^{15}N were used to measure the uptake and assimilation of these nutrients. The use of dual-labeled $^{13}C/^{15}N$ urea allowed the simultaneous determination of C and N assimilation from urea. Isotope labeling experiments (ILE) were carried out, tracing the incorporation and uptake rate of the carbon and the nitrogen in the urea and nitrate cultures under both iron-limited and iron-replete growth conditions. The goal of these experiments was twofold: 1) determine whether both the C and N atoms from urea were incorporated in the *T. oceanica* algal biomass, and 2) determine the relative contribution of the urea-carbon relative to photosynthetic carbon assimilation (DIC) under iron-limited and iron-replete growth conditions. The results of these ILEs are presented in **Table 3.2, Table 3.3,** & **Table 3.4.** As expected, the C/N uptake ratio of DIC and NO_3 was within a standard deviation of the C/N Redfield ratio of 6.6 (6.12 ± 0.042 and 5.77 ± 3.85 for iron-limited and iron-replete, respectively; **Table 3.2**). The cellular C/N uptake molar ratio for C and N derived from the urea molecule was 0.12 ± 0.02 and 0.15 ± 0.01, in iron-limited and iron-replete conditions respectively; this is well below the 0.5 stoichiometric C/N ratio of the urea molecule, which contains 2 N atoms for every C atom. However, the overall results indicate that urea C and N were assimilated within the biomass. Specifically, the ILE experiments demonstrated that the urea-C assimilation could contribute between 5-11% of the total carbon (DIC + Urea-C) assimilated during the 24-hour incubation period.

ANOVA Tukey's HSD test between the different treatments under both iron conditions indicates that, contrary to the main hypothesis of this thesis, no statistically significant differences were detected between the different treatments under both iron conditions (**Appendix B: Tables B.3, B.4, B.5, B.6** & **B.7**). Iron did not affect the specific uptake rate of N or C from urea or nitrate, as there was no statistical difference between iron-limited and iron-replete conditions for either nitrate or urea individually **(Table 3.3)**. However, the ANOVA test results indicate that there were statistically significant differences between urea and nitrate-N specific uptake rate; the N uptake rate from urea was higher than from nitrate under both iron-limited and iron-replete conditions (0.0115 ± 0.0012 and 0.0127 ± 0.0004), respectively. In contrast, the uptake of C from urea and bicarbonate were significantly different, where urea-C uptake was much lower than the HCO_3^- specific uptake rate **(Table 3.2** & **Table 3.3)**.

The C/N molar ratio from the biomass was monitored in all of the ILE incubations under both iron conditions (**Table 3.4)**, and the overall results suggest that the C/N ratio does not differ between the iron-replete and iron-limited nitrate-grown cultures with means of 7.1 ± 1.5 and 7.3 ± 2.0, respectively. The C/N molar ratio in the urea grown cultures was slightly higher at 7.9 ± 0.2 for the iron-limited and 8.5 ± 0.4 for the iron-replete. However, the differences in the biomass C/N ratios are not statistically significant due to the large standard deviations in the nitrate grown cultures.

Table 3.2: Carbon and nitrogen specific uptake rates and cellular uptake rates calculated from the ILEs with the stable isotope ^{13}C and ^{15}N. ILE results for urea and nitrate grown cultures under both iron-limited and iron-replete conditions where DIC indicates (^{13}C-HCO_3). Mean ± standard deviation are presented for all the data except for the % of C uptake from urea-C for which the standard error was calculated by propagating the error as described in the methods.

Stable isotope	DIC-^{13}C		Urea-^{13}C		Urea-^{15}N or nitrate-^{15}N			
N in culture medium	**C-Specific uptake rate (h^{-1})**	**Uptake rate (fmol C $cell^{-1}$ h^{-1})**	**C-Specific uptake rate (h^{-1})**	**Uptake rate (fmol C $cell^{-1}$ h^{-1})**	**N-Specific uptake rate (h^{-1})**	**Uptake rate (fmol N $cell^{-1}$ h^{-1})**	**C/N uptake* (molar ratio)**	**% of C uptake from urea-C****
High Iron Condition								
UREA	0.0037 ± 0.0009	7.0 ± 1.9	0.00022 ± 0.00001	0.86± 0.12	0.0127 ± 0.0004	5.93 ± 0.38	0.15 ± 0.01	11% ± 1.71
NO_3	0.0035 ± 0.0006	7.2 ± 2.9	N/A	N/A	0.0062 ± 0.0030	1.55 ± 0.62	5.77 ± 3.85	
Low Iron Condition								
UREA	0.0044 ± 0.0016	15.0 ± 3.6	0.00018 ± 0.00005	0.79 ± 0.41	0.0115 ± 0.0012	6.26 ± 2.71	0.12 ± 0.02	5% ± 3.72
NO_3	0.0043 ± 0.0006	27.4 ± 20.5	N/A	N/A	0.0052 ± 0.0011	4.55 ± 3.58	6.12 ± 0.04	

*Calculated from the DIC and NO_3 uptake rates for NO_3 cultures and from Urea-C and Urea-N uptake rates for urea cultures

**Calculated from the Urea-C cellular uptake rate (Urea-C cellular uptake rate + DIC-C cellular uptake

Table 3.3: Pairwise comparison of the specific uptake rate of carbon and nitrogen (h^{-1}) for all substrate additions and iron conditions. Statistical analyses using ANOVA with Tukey's Honest Significant Difference (HSD) post hoc (R version 3.2.3).

Treatment (1)	Mean Specific uptake rate (h^{-1})	Treatment (2)	Mean Specific uptake rate (h^{-1})	p-value adj.
Low iron + Urea HCO_3-^{13}C	0.00449 ± 0.00162	High iron + Urea HCO_3-^{13}C	0.00370± 0.00090	0.63810
Low iron + NO_3 HCO_3-^{13}C	0.00434 ± 0.00064	High iron +NO_3 HCO3-^{13}C	0.00359 ± 0.00062	0.87472
Low iron + NO_3 NO_3-^{15}N	0.00516 ± 0.00110	High iron + NO_3 NO_3-^{15}N	0.00617 ± 0.00301	0.91086
low iron + Urea Urea-^{15}N	0.01152 ± 0.00120	High iron + Urea Urea-^{15}N	0.01272 ± 0.00044	0.90146
Low iron + Urea Urea-^{13}C	0.00018 ± 0.00005	High iron + Urea Urea-^{13}C	0.00022 ± 0.00001	1.00000
Low iron + NO_3 HCO_3-^{13}C	0.00434 ± 0.00064	Low iron + Urea HCO_3-^{13}C	0.00449 ± 0.00162	0.99992
High iron + NO_3 HCO_3-^{13}C	0.00359 ± 0.00062	High iron + Urea HCO_3-^{13}C	0.00370 ± 0.00090	0.99993
Low iron + Urea HCO_3-^{13}C	**0.00449 ± 0.00162**	**Low iron + Urea Urea-^{13}C**	**0.00018 ± 0.00005**	**0.00002**
High iron + Urea HCO_3-^{13}C	**0.00370 ± 0.00090**	**High iron + Urea Urea-^{13}C**	**0.00022 ± 0.00001**	**0.00020**
Low iron + NO_3 NO_3-^{15}N	**0.00516 ± 0.00110**	**Low iron + Urea Urea-^{15}N**	**0.01152 ± 0.00120**	**0.01793**
High iron + NO_3 NO_3-^{15}N	**0.00617 ± 0.00301**	**High iron + Urea Urea-^{15}N**	**0.01272 ± 0.00044**	**0.00581**

Table 3.4: Mean cellular carbon and nitrogen content at the end of the 24h incubations for ILEs with addition of ^{13}C-HCO_3, ^{15}N-NO_3 or $^{13}C/^{15}N$-Urea. The cellular contents were calculated from elemental analysis of the particulate matter collected on the filters for IRMS. Note that twice as much data is available for the urea cultures because separate incubations were carried out for ^{13}C-HCO_3 and $^{13}C/^{15}N$-urea. Mean ± standard deviation are presented

ILE addition	^{13}C-HCO_3			$^{13}C/^{15}N$-Urea		
Nutrient	**(pmole C/cell)**	**(pmole N/cell)**	**C/N (molar ratio)**	**(pmole C/cell)**	**(pmole N/cell)**	**C/N (molar ratio)**
High Iron Condition						
UREA	2.61 ± 2.01	0.53 ± 0.19	7.34 ± 2.00	3.98 ± 0.35	0.54 ± 0.04	8.53 ± 0.18
NO_3	1.98 ± 0.57	0.27 ± 0.07	7.24 ± 0.31	N/A	N/A	N/A
Low Iron Condition						
UREA	3.75 ± 1.72	0.28 ± 0.08	7.08 ± 1.96	4.18 ± 1.34	0.47 ± 0.20	7.91 ± 0.42
NO_3	5.96 ± 3.58	0.82 ± 0.46	7.21 ± 0.32	N/A	N/A	N/A

Chapter 4: Discussion

4.1 *Thalassiosira oceanica* can grow osmo-mixotrophically

Many published reports have shown that several species of microalga can grow osmo-mixotrophically using different organic carbon and nitrogen sources such as acetate, glycerol, glucose, and urea in addition to their ability to grow phototrophically (Jeon, Cho & Yun, 2006; Garcia *et al*, 2005; Liu *et al*, 2009). Using these studies as guidance for the work presented in this thesis, several organic carbon and nitrogen sources were tested for their ability to support growth in *T. oceanica*. With the exception of acetate, all of the organic carbon and nitrogen compounds used in this study supported the osmo-mixotrophic growth of *T. oceanica* in the presence of HCO3onate and nitrate. However, only urea could support the growth of *T. oceanica* as a sole source of fixed nitrogen (**Figure 3.3).** Adaptation time, initial concentration, and light intensity could be factors that contribute to the potential assimilation of acetate in photoautotrophic algae and a more thorough study designed to optimize the growth conditions with acetate would be needed to definitively determine whether or not acetate is toxic to *T. oceanica* (Garcia *et al*, 2005). For example, the green algae *Haematococcus pluvialis* grown in different acetate concentrations and light intensity showed that 30mM acetate under continuous light intensity was the appropriate acetate concentration needed to enhance the cell biomass (Jeon, Cho & Yun, 2006). Therefore, it is possible that the concentration of acetate used in this study was too low to support significant growth of *T. oceanica*.

4.2 The effect of dissolved nitrogen sources on *T. oceanica* growth under iron-limited vs iron-replete conditions

Overall, the results presented here do not conclusively support the main hypothesis of the thesis which is that under iron-limited conditions, the diatom *T. oceanica* will optimize growth

by shifting to osmo-mixotrophy in the presence of dissolved organic substrates to fulfill their nitrogen and carbon requirements. The results of the growth curves from **Figure 3.11** suggested that iron-limited urea-grown cultures increase in cell density faster than the iron-limited nitrate-grown cultures, and this may indicate that urea provides a slight advantage under iron-limited growth conditions. However, the hypothesis implies that under iron limitation, photosynthetic carbon assimilation should be down-regulated as was previously observed for *P. tricornutum* (Allen *et al*, 2008). A reduction in photosynthetic carbon uptake was not detected in the experiments presented here (Table 3.2 & Table 3.3). The assimilation of urea under iron-limited growth conditions would clearly be advantageous for acquiring nitrogen for growth. In contrast to the NO_3 assimilation pathway that requires iron as a cofactor for the nitrate reductase and nitrite reductase enzymes, the urea assimilation pathway does not require iron, instead incorporating Ni as a cofactor for the urease enzyme (Oliveira & Antia, 1984). A study by Peers *et al* (2000) confirmed that the urease enzyme, involved in the hydrolysis of urea into ammonia and CO_2, was active in the diatoms *T. pseudonana* and *Thalassiosira weissflogii* independent of iron availability. According to Morel *et al* (1991), a photosynthetic species grown on urea instead of nitrate may reduce the overall cellular iron requirement by 5 μmole Fe per mole of carbon, based on the economy of Fe provided by the replacement of nitrate reductase with urease. Whether the urea-C assimilation also would provide an advantage for iron-limited diatoms depends on the C-assimilation pathway. In previous studies, CO_2 release from the hydrolysis of urea has been assumed to be through recapture by the photosynthetic pathway (Ignatiades, 1986); this would not provide a real advantage under iron limited growth conditions because photosynthetic carbon fixation is linked to the high demand for iron by the

photosynthetic reaction centers and therefore would not contribute to the relief of iron limitation (Raven, 1990)

4.3 Both C and N atoms from urea are assimilated by *T. oceanica*

In general, the experiments demonstrated clearly that *T. oceanica* can incorporate both the carbon and nitrogen atoms from urea during a 24 hour incubation. Genome sequencing has shown that diatoms in general – and *T. oceanica* specifically – possess genes that code for urea transporters and the urease enzyme (Armbrust *et al* 2004; Bowler *et al* 2008; Lommer *et al* 2012). Moreover, the ILE experiments of this study indicated that the fraction of urea-C incorporated into the biomass is proportional the ratio of urea to total dissolved carbon pool (HCO_3^- pool (2000μM) + urea-C (168 μM)), with measured % urea-C contribution to the total carbon assimilated ranging between 5% ± 3.72 and 11% ± 1.71, in low iron and high iron respectively. The demonstration that urea-C is incorporated into biomass after 24-hour incubations is an important result because studies of urea uptake in phytoplankton have been primarily focussed on urea as a source of fixed nitrogen, even in cases where the urea uptake was measured with dual labeled ^{13}C / ^{15}N substrate (Hu *et al*, 2014). It has been shown that the ornithine urea cycle (OUC) of diatoms can serve a point of entry for anaplerotic CO_2 assimilation (Allen *et al*, 2011). Further, it has been shown that urea uptake is high in the light phase of the growth cycle, but in contrast to HCO3onate uptake which is very low in the dark, urea-C uptake maintains significant rates (Mitamura & Saijo, 1980). Experiments comparing urea-C uptake during the light phase and the dark phase of the daily light cycle could provide additional information to clarify the importance of urea-C assimilation through the anaplerotic pathway. The ILE results indicated that N assimilation from urea is higher than the N assimilation of nitrate under both iron conditions when presented as sole nitrogen source in the media, indicating

that regardless of the iron nutritional status, urea may be the preferred source of fixed nitrogen relative to nitrate

4.3.1 The importance of *T. oceanica* globally and in iron-limited oceanic regions.

Thalassiosira oceanica is an important diatom in oceanic environments (Aizawa, Tanimoto & Jordan, 2005). Although *T. oceanica* can be found in high abundance in eutrophic and upwelled conditions (> 1×10^4 cell L^{-1}) such as the coastal waters of the North Pacific Ocean, it is also predominant in the subarctic Pacific gyre, known as a prime region for iron limitation of primary productivity (Chappell *et al*, 2013 & 2015). A recent report located along the well-studied line P in the Subarctic eastern Pacific has shown that the *T. oceanica* is prominent in this area and is increasingly iron-limited in offshore stations (Chappell *et al*, 2013). This diatom has additionally been found to be widely distributed in the world's ocean and is clearly of importance globally (Garcia & Odebrecht, 2009). Previous transcriptomics studies have indicated that the down-regulation of the photosynthesis might be compensated by osmo-mixotrophy (Lommer *et al*, 2012) These results could support that urea assimilation is important within the cellular metabolism of diatoms, as it could serve as a recycling centre for inorganic C and N and contribute to increased growth rate and metabolism when presented as a fixed nitrogen source. In *P. tricornutum,* the OUC is found to be active regardless of the nitrogen source available to the cells (Allen *et al*, 2011). However, the genes involved in the degradation of organic substrates in *T. oceanica* found to be upregulated under iron-limited conditions (Lommer *et al.* 2012) might also be indicative of internal protein recycling rather than osmo-mixotrophy.

4.3.2 The importance of urea as a carbon and nitrogen source for diatoms in the global ocean

Although the concentration of urea in the ocean can vary from undetectable to 13 µM, the average concentration of urea in the open ocean is 300 nM, which is at least one order of magnitude lower than the ammonia concentrations (Bronk, 2002). A study along the latitudinal transect from 50°N to 50°S in the Atlantic Ocean (Painter *et al,* 2008) confirmed the estimates reported by Bronk (2002). However, urea is found in higher concentrations (5.0µM-9.3µM) in lakes and coastal waters, and could thus provide a major source of fixed nitrogen and carbon for phytoplankton (reviewed in Berman & Bronk, 2003). Even at the lower nM concentrations, urea is highly bioavailable as a nitrogen source in the ocean (reviewed in Berman & Bronk, 2003). In their study of urea uptake in the North Atlantic Ocean, Painter *et al* (2008) observed that the uptake rates of urea were inversely correlated to ambient urea concentrations, pointing to an *in situ* depletion resulting from the high demand for fixed nitrogen by the phytoplankton in this area. In areas of iron limitation, NO_3 accumulates in the surface ocean leading to the formation of HNLC regions (Boyd *et al,* 2007). The Eastern Subarctic Pacific is a well identified HNLC region (Tsuda & Takeda, 2005) and there chronically high NO_3 concentrations result from the inability of the phytoplankton to assimilate this fixed nitrogen source due to the high iron requirement of the nitrate and nitrite reductases. Productivity in these areas can be driven by regenerated production, for example through the uptake of NH_4 and urea. In contrast to NO_3, urea and NH_4 accumulate in the surface waters as a result of local processes that degrade organic matter, and in the case of urea, from excretion by zooplankton (Painter *et al*, 2008). Therefore, it is likely that urea is an important source of both nitrogen and carbon allowing *T. oceanica* to persist, and survive in the HNLC waters of the subarctic Pacific region where it is at times abundant (Chappell *et al*, 2015).

4.4 Recommendations

Although this thesis shows that a molecule of urea can be assimilated by the *T. oceanica* diatom, further investigation of the importance of urea assimilation for *T. oceanica* under iron-limited and iron-replete growth is needed. It is important to determine whether the urea-C assimilation pathway is via photosynthesis or anaplerotic CO_2 assimilation, or some combination; this can be done by using ILE to measure the urea-C and HCO3onate uptake rates separately in the light and in the dark under both iron conditions. Furthermore, combining urea and nitrate in one growth medium could be used to estimate the diatom preference for each nitrogen source, by employing the ILE technique described in this thesis.

It is also important to keep in mind that the down-regulation of photosynthesis and the gene expression patterns observed by Lommer *et al* (2012) may involve internal recycling of the cellular protein rather than the assimilation of external dissolved organic carbon and nitrogen substrates, as hypothesized here. Evidence for the internal recycling or proteins under various types of nutrient limitation is mounting (Levitan *et al.* 2015; Allen *et al*, 2011; Schoefs & Kroth, 2017). Thus, transcriptomics should be used to assess the expression patterns of the genes in the urea cycle under both iron conditions. More complex organic matter could also be tested as potential sources of carbon and nitrogen.

4.5. Conclusion

Although the original hypothesis of this study – that osmo-mixotrophy could relieve iron-limitation in *T. oceanica* – was not directly supported by the results, a number of significant conclusions can be drawn from this thesis. Foremost, this studied showed that *T. oceanica* can grow on urea, a simple organic molecule, as a sole source of fixed nitrogen. In addition, it was

established that urea-C assimilation contributed between 5-10% of the total C assimilation, and that the relative contribution of urea-C to the total carbon assimilation was proportional to the fraction of urea-C in the total pool of dissolved carbon in the medium. Although additional experiments would be necessary to confirm this finding, the batch culture experiments suggested that, at equal dissolved N concentrations, urea supported a higher biomass than nitrate in iron-limited cultures. Moreover, the uptake rate of N from urea was higher than from nitrate in both iron-limited and iron-replete cultures, and the urea-C was assimilated by *T. oceanica* but at a lower rate than photosynthetic CO_2 fixation.

Appendix A Supplemental Material

Table A.1 Vitamin and metal mix solutions in f/2 ASW

Chemical	Concentration (µM)
Vitamin	
Biotin	0.002
B12	0.0004
Thiamine-HCl	0.30
Metal-Mix	
$FeCl_3 \cdot 6H_2O$	11.65
$Na_2EDTA \cdot 2H_2O$	11.71
$CuSO_4 \cdot 5H_2O$	0.039
$Na_2MoO_4 \cdot 2H_2O$	0.026
$ZnSO_4 \cdot 7H_2O$	0.077
$CoCl_2 \cdot 6H_2O$	0.042
$MnCl_2 \cdot 4H_2O$	0.91

Appendix B: Supplemental Data

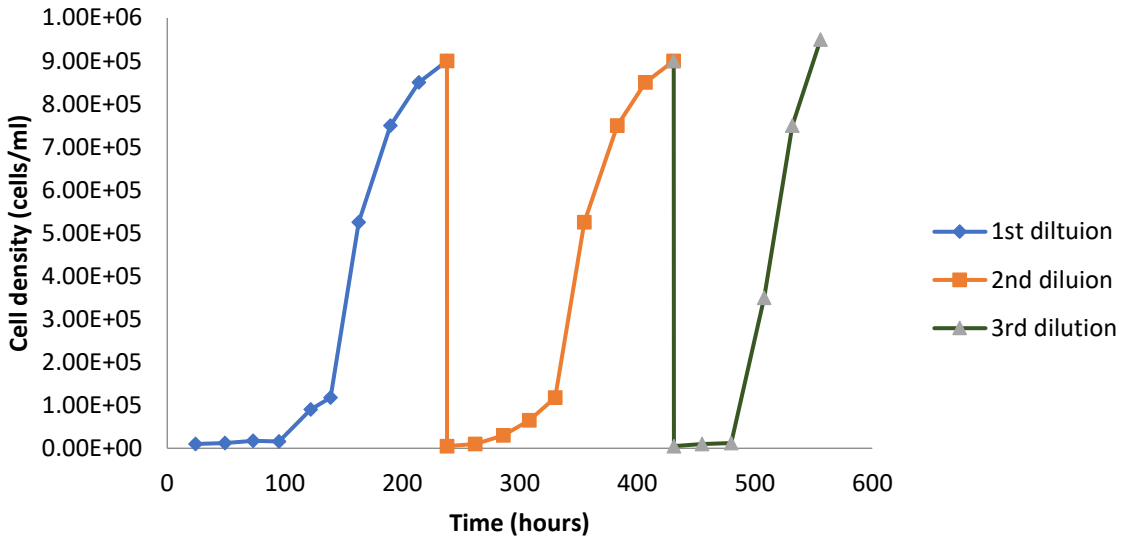

Figure B.1: *T. oceanica* grown osmo-mixotrophically on glycine in the presence of nitrate in semi-continuous cultures

Table B.2: Elemental analyzer results (POC/PON) of *T. oceanica* grown on glycerol, glycine, and glucose with 200 µM NO_3^- and 0.2M HCO_3^-

Treatment	C (µmol/L)	N (µmol/L)	C/N molar ratio	C (pmol/cell)	N (pmol)/cell	Cells/ml
Glucose	**2237.7**	**106.6**	**20.8**	**3.76**	**0.18**	**595000**
Glycine	**2185.8**	**209.6**	**10.5**	**2.33**	**0.22**	**940000**
Glycerol	**1790.0**	**101.6**	**17.8**	**2.67**	**0.15**	**670000**

Table B.3: Pairwise comparison of the cellular uptake rate (fmole cell^{-1} h^{-1}) of carbon and nitrogen for all substrate additions and iron conditions. Cellular uptake rate was calculated by normalizing the uptake rate (μmol h^{-1}) to the cell number. Statistical analyses were done with ANOVA with Tukey's Honest Significant Difference (HSD) post hoc (R version 3.2.3). Low iron indicates iron-limited conditions and high iron indicates iron-replete conditions. Bold indicates statistically significant differences between treatments.

Treatment (1)	Mean Uptake rate (fmole cell^{-1} h^{-1})	Treatment (2)	Mean Uptake rate (fmole cell^{-1} h^{-1})	p-value adjusted
Low iron + Urea HCO_3-^{13}C	15.01 ± 3.63	High iron + Urea HCO_3-^{13}C	7.02 ± 1.97	0.210
High iron + Urea HCO_3-^{13}C	7.02 ± 1.97	High iron + Urea Urea-^{13}C	0.86 ± 0.12	0.704
Low iron + NO_3 NO_3-^{15}N	4.55 ± 3.58	High iron + NO_3 NO_3-^{15}N	1.55 ± 0.62	0.196
low iron + Urea Urea-^{15}N	6.26 ± 2.71	High iron + Urea Urea-^{15}N	5.93 ± 0.38	0.996
Low iron + Urea Urea-^{13}C	0.79 ± 0.41	High iron + Urea Urea-^{13}C	0.86 ± 0.12	1.000
Low iron + NO_3 HCO_3-^{13}C	27.42 ± 20.58	Low iron + Urea HCO_3-^{13}C	15.01 ± 3.63	0.104
High iron + NO_3 HCO_3-^{13}C	7.26 ± 2.99	High iron + Urea HCO_3-^{13}C	7.02 ± 1.97	1.000
Low iron + NO_3 NO_3-^{15}N	4.55 ± 3.58	Low iron + Urea Urea-^{15}N	6.26 ± 2.71	0.712
High iron + NO_3 NO_3-^{15}N	**1.55 ± 0.62**	**High iron + Urea Urea-^{15}N**	**5.93 ± 0.38**	**0.040**
Low iron + Urea HCO_3-^{13}C	**15.01 ± 3.63**	**Low iron + Urea Urea-^{13}C**	**0.79 ± 0.41**	**0.045**
Low iron + NO_3 HCO_3-^{13}C	**27.42 ± 20.58**	**High iron +NO_3 HCO3-^{13}C**	**7.26 ± 2.99**	**0.002**

Table B.4 Comparison of cellular N uptake rates (fmol cell^{-1} h^{-1}) between different treatments under iron-limited and iron-replete conditions. Cellular uptake rate was calculated by normalizing the uptake rate (μmol h^{-1}) to the cell number. Statistical analyses were done with ANOVA with Tukey's Honest Significant Difference (HSD) post hoc (R version 3.2.3). Low iron indicates iron-limited conditions and high iron indicates iron-replete conditions.

Treatment (1)	Treatment (2)	difference of the mean cellular N uptake rate (fmol cell^{-1} h^{-1})	lower confidence level	upper confidence level	p-adjusted
Low iron + NO_3 NO_3-^{15}N	High iron + NO_3 NO_3-^{15}N	2.99978	-1.19201	7.19157	0.19612
High iron + NO_3 NO_3-^{15}N	High iron + Urea Urea-^{15}N	4.37698	0.18520	8.56877	0.04006
High iron + NO_3 NO_3-^{15}N	Low iron + Urea Urea-^{15}N	4.71293	0.52115	8.90472	0.02676
Low iron + NO_3 NO_3-^{15}N	High iron + Urea Urea-^{15}N	1.37720	-3.46306	6.21746	0.82672
Low iron + NO_3 NO_3-^{15}N	Low iron + Urea Urea-^{15}N	1.71315	-3.12711	6.55341	0.71651
High iron + NO_3 NO_3-^{15}N	Low iron + Urea Urea-^{15}N	0.33595	-4.50431	5.17621	0.99658

Table B.5 Comparison of cellular C uptake rates (fmol cell^{-1} h^{-1}) between different treatments under iron-limited and iron-replete conditions. Cellular uptake rate was calculated by normalizing the uptake rate (μmol h^{-1}) to the cell number. Statistical analyses were done with ANOVA with Tukey's Honest Significant Difference (HSD) post hoc (R version 3.2.3). Low iron indicates iron-limited conditions and high iron indicates iron-replete conditions.

Treatment (1)	Treatment (2)	difference of the mean cellular C uptake rate (fmol cell^{-1} h^{-1})	lower confidence level	upper confidence level	p-adjusted
Low iron + NO_3 HCO_3-^{13}C	High iron + NO_3 HCO_3-^{13}C	20.16426	6.12419	34.20432	0.00212
High iron + Urea HCO_3-^{13}C	High iron + NO_3 HCO_3-^{13}C	-0.23233	-10.69717	10.23252	1.00000
Low iron + Urea HCO_3-^{13}C	High iron + NO_3 HCO_3-^{13}C	7.75146	-3.71221	19.21512	0.32502
High iron + NO_3 HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-6.39234	-20.43241	7.64772	0.72217
High iron + NO_3 HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-6.47093	-20.51100	7.56913	0.71211
Low iron + NO_3 HCO_3-^{13}C	High iron + Urea HCO_3-^{13}C	-20.39659	-33.63369	-7.15949	0.00095
Low iron + NO_3 HCO_3-^{13}C	Low iron + Urea HCO_3-^{13}C	-12.41280	-26.45287	1.62726	0.10492
Low iron + NO_3 HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-26.55660	-42.76867	-10.34453	0.00045
Low iron + NO_3 HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-26.63519	-42.84726	-10.42312	0.00044
High iron + Urea HCO_3-^{13}C	Low iron + Urea HCO_3-^{13}C	7.98378	-2.48106	18.44863	0.21042
High iron + Urea HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-6.16001	-19.39711	7.07709	0.70400
High iron + Urea HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-6.23861	-19.47571	6.99849	0.69315
Low iron + Urea HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-14.14380	-28.18386	-0.10373	0.04760
Low iron + Urea HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-14.22239	-28.26246	-0.18233	0.04584
High iron + Urea Urea-^{13}C	Low iron + Urea Urea-^{13}C	-0.07859	-16.29066	16.13348	1.00000

Table B.6: Comparison of specific N uptake rates (h^{-1}) between different treatments under iron-limited and iron-replete conditions Specific uptake rate was calculated by normalizing the uptake rate (μmol h^{-1}) to the biomass (μmol). Statistical analyses were done with ANOVA with Tukey's Honest Significant Difference (HSD) post hoc (R version 3.2.3). Low iron indicates iron-limited conditions and high iron indicates iron-replete conditions.

Treatment (1)	Treatment (2)	difference of the mean specific N uptake rate (h^{-1})	lower confidence level	upper confidence level	p-adjusted
Low iron + NO_3 NO_3-^{15}N	High iron + NO_3 NO_3-^{15}N	-0.00100	-0.00558	0.00358	0.91087
High iron + NO_3 NO_3-^{15}N	High iron + Urea Urea-^{15}N	0.00657	0.00199	0.01115	0.00573
High iron + NO_3 NO_3-^{15}N	Low iron + Urea Urea-^{15}N	0.00537	0.00079	0.00995	0.02107
Low iron + NO_3 NO_3-^{15}N	High iron + Urea Urea-^{15}N	0.00757	0.00228	0.01286	0.00581
Low iron + NO_3 NO_3-^{15}N	Low iron + Urea Urea-^{15}N	0.00637	0.00108	0.01166	0.01793
High iron + NO_3 NO_3-^{15}N	Low iron + Urea Urea-^{15}N	-0.00120	-0.00649	0.00409	0.90147

Table B.7 Comparison of specific C uptake rates (h^{-1}) between different treatments under iron-limited and iron-replete conditions. Specific uptake rate was calculated by normalizing the uptake rate (μmol h^{-1}) to the biomass (μmol). Statistical analyses were done with ANOVA with Tukey's Honest Significant Difference (HSD) post hoc (R version 3.2.3). Low iron indicates iron-limited conditions and high iron indicates iron-replete conditions.

Treatment (1)	Treatment (2)	difference of the mean specific C uptake rate (h^{-1})	lower confidence level	upper confidence level	p-adjusted
Low iron + NO_3 HCO_3-^{13}C	High iron + NO_3 HCO_3-^{13}C	0.00070	-0.00136	0.00287	0.87473
High iron + Urea HCO_3-^{13}C	High iron + NO_3 HCO_3-^{13}C	0.00010	-0.00147	0.00168	0.99993
Low iron + Urea HCO_3-^{13}C	High iron + NO_3 HCO_3-^{13}C	0.00080	-0.00083	0.00262	0.60050
High iron + NO_3 HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-0.00330	-0.00548	-0.00125	0.00064
High iron + NO_3 HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-0.00340	-0.00552	-0.00129	0.00056
Low iron + NO_3 HCO_3-^{13}C	High iron + Urea HCO_3-^{13}C	-0.00060	-0.00264	0.00135	0.91285
Low iron + NO_3 HCO_3-^{13}C	Low iron + Urea HCO_3-^{13}C	0.00010	-0.00197	0.00226	0.99994
Low iron + NO_3 HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-0.00410	-0.00656	-0.00168	0.00031
Low iron + NO_3 HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-0.00410	-0.00660	-0.00172	0.00027
High iron + Urea HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	0.00070	-0.00079	0.00236	0.63810
High iron + Urea HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-0.00340	-0.00547	-0.00149	0.00020
High iron + Urea HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-0.00350	-0.00551	-0.00152	0.00017
Low iron + Urea HCO_3-^{13}C	High iron + Urea Urea-^{13}C	-0.00420	-0.00638	-0.00215	0.00003
Low iron + Urea HCO_3-^{13}C	Low iron + Urea Urea-^{13}C	-0.00430	-0.00642	-0.00219	0.00002
High iron + Urea Urea-^{13}C	Low iron + Urea Urea-^{13}C	-0.00004	-0.00248	0.00240	1.00000

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