THE INFLUENCE OF IRON AVAILABILITY ON NITROGEN ASSIMILATION IN THE MARINE DIATOM *THALASSIOSIRA WEISSFLOGII*

by

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ABSTRACT

Since the recognition of iron-limited, high nitrate, low chlorophyll (HNLC) regions of the ocean, iron limitation has been hypothesized to limit the assimilation of nitrate by diatoms. To determine the influence of iron availability on nitrogen assimilatory enzymes, *in vitro* enzyme assays were developed and optimized for urease and nitrite reductase. Both assays were able to account for 100% or more of the incorporated nitrogen when growing on urea or nitrate as the sole nitrogen source. Cultures of *Thalassiosira weissflogii* (Grunow) Fryxell *et* Hasle (clone Actin) were grown under non-steady-state Fe-limited and Fe-replete conditions using artificial seawater medium. Iron-limited cultures suffered from decreased efficiency of photosystem II as indicated by variable fluorescence (F_v/F_m). Under Fe-replete conditions, *in vitro* nitrate reductase (NR) activity was rate limiting to nitrogen assimilation and *in vitro* nitrite reductase (NiR) activity was 100-fold higher. Under iron limitation, cultures excreted up to 100 fmol NO_2^- $cell^{-1}$ d^{-1}, and NiR activities declined by 50-fold while internal NO_2^- pools remained relatively constant. Activities of both NR and NiR remained in excess of nitrogen incorporation rates throughout iron limited growth indicating that the supply of reductant to NiR may be responsible for the limitation of nitrogen assimilation at the nitrite reduction step. Urease activity showed no response to iron limitation. Carbon:nitrogen ratios were equivalent in both iron conditions indicating that relative to carbon, nitrogen was incorporated at similar rates whether iron was limiting growth or not. Steady-state long-term iron limitation of *T. weissflogii* using the artificial culture medium Aquil produced similar results, although NO_2^- efflux rates were much lower (10 fmol NO_2^- $cell^{-1}$ d^{-1}). If inferences from a single species may be applied to a mixed assemblage then, according to this work, diatoms in HNLC regions are not deficient in their ability to

assimilate nitrate when Fe-limited. Rather, it appears that diatoms are limited in their ability to process photons within the photosynthetic electron transport chain which results in nitrite reduction becoming the rate limiting step in nitrogen assimilation. This appears to be the case whether limiting iron is supplied either in non-steady or steady states.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic Acid
Hepes	N-[2-Hydroxyethyl]piperazine-N′-[4-butanesulfonic acid]
HNLC	High Nutrient (or Nitrate) Low Chlorophyll
KDa	KiloDalton
NiR	Nitrite Reductase
NR	Nitrate Reductase
PETS	Photosynthetic Electron Transport System
PS I	Photosystem I
PS II	Photosystem II
PVP	Polyvinyl Pyrolidone
Tris-HCl	Tris(hydroxymethyl) amino ethane - HCl

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1. General Introduction

1.1 Primary production in marine ecosystems

Photosynthesis supplies energy to aquatic, oceanic and terrestrial food webs and has been considered to be the most important biological process affecting the atmosphere. Through photosynthesis, the atmosphere was converted from anaerobic to aerobic resulting in the most dramatic global change in earth's history (see Margulis 1981). Currently, the earth is faced with the possibility of another dramatic global change, namely global warming, as a result of increased levels of CO_2 from anthropogenically supplied fossil fuel carbon. Although atmospheric CO_2 levels have been much higher in the geological past, the current rate of increase in atmospheric CO_2 is unprecedented. The recent concern over increasing levels of atmospheric CO_2 has renewed interest in determining accurate open-ocean rates of primary production, which for the purposes of this thesis, is defined as the amount of inorganic carbon converted or "fixed" into organic molecules per unit area by photoautotrophs (see Flynn 1988 for discussion).

According to Broecker *et al.* (1979), approximately 45% of anthropogenic carbon can not be accounted for in atmospheric measurements. Therefore, there are unknown sinks for anthropogenic carbon which need to be assessed if efforts to model increases in atmospheric CO_2 and subsequent global warming are to be successful. Oceanographic research is now directed at further resolving CO_2 losses such as rates of primary productivity and rates of carbon transport from surface waters to the deep ocean via sinking of biogenic material.

1.2 Limits to primary production

Rates of primary production in the ocean are dependent on the supply rate of the limiting nutrient. The concept of a limiting nutrient, termed the "law of the minimum" was first applied to single species crop yields by Liebig (1840). In the simplest terms, the final biomass of a particular species is determined by the concentration of the single nutrient which is at a minimum concentration relative to it's requirement by that species. The limiting nutrient for a particular body of water has traditionally been identified by deviations from the expected ratio of 106:16:1, carbon:nitrogen:phosphorus (Redfield 1958), or by bioassay. For example, nitrogen would be the suspected limiting nutrient if the dissolved inorganic N:P ratio (in moles) was below the ideal ratio of 16. In a bioassay, the suspected limiting nutrient is added to sub-samples of water which are monitored to determine whether biomass and or production rate increases. Nutrients which have been identified as limiting in marine systems are nitrogen (Ryther & Dunstan 1971), phosphorus (Harrison *et al.* 1990), iron (Martin *et al.* 1989) and specifically for diatoms, silicon (Nelson & Dortch 1996). Carbon limitation of primary production has been hypothesized but not observed in oceanic systems (Riebesell *et al.* 1993). In most cases nitrogen is believed to be limiting to phytoplankton biomass and growth rates (e.g. Ryther & Dunstan 1971, Glibert & McCarthy 1984, Kolber *et al.* 1990).

1.3 Biomarkers for nutrient limitation

Physiological measurements of photosynthetic parameters as well as specific response proteins have also been used to infer specific nutrient limitations. The utility of this approach is that incubations with associated bottle effects and disruption of marine food webs are avoided.

1.3.1 Variable Fluorescence

Fluorescence emission has been used in both lab and field studies to infer nutrient limitation by nitrogen and iron (Kolber *et al.* 1988, Kolber *et al.* 1990, Greene *et al.* 1994). In fluorescence-emission studies electron transfer from photosystem II to plastiquinone is blocked causing *in vivo* fluorescence of PS II to be enhanced (see Krause & Weis 1991 for review). Fluorescence is measured before (F_o) and after (F_m) the addition of the blocking agent. The maximum quantum yield of fluorescence ($\Delta\phi_m$) is then calculated as F_v/F_m where $F_v = F_m - F_o$. Values are commonly reported as $\Delta\phi_m$ or F_v/F_m. Blocking agents most commonly employed are (3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or a saturating high-intensity light flash. An *in situ* instrument which is based on using a saturating light flash called a fast repetition rate (FRR) fluorometer has been developed for identification of nutrient limitation of natural populations of marine phytoplankton (Kolber & Falkowski 1993). Values for F_v/F_m range from 0.17 for both nitrogen and iron-limited phytoplankton to 0.68 for phytoplankton grown with excess amounts of these nutrients (Kolber *et al.* 1990, Greene *et al.* 1991).

Measurements of variable fluorescence using an *in situ* FRR fluorometer have verified phytoplankton iron limitation in the equatorial Pacific and the alleviation of iron limitation following iron fertilization of an area of the equatorial Pacific in the IronEx II experiment (Behrenfeld *et al.* 1996).

1.3.2 Nutrient specific protein expression

Specific proteins have been identified which are expressed during nutrient limitation. For instance, the iron-containing electron donor ferredoxin is replaced by flavodoxin in iron-stressed

microalgae (Zumft & Spiller 1971, La Roche *et al.* 1993 Doucette *et al.* 1996). The replacement of ferredoxin with flavodoxin in phytoplankton is currently being used to verify iron limitation in field samples although McKay *et al.* (1997) warn that flavodoxin is expressed so early during the response of phytoplankton to low iron that it is not an appropriate indicator of iron limitation of primary production.

Several probes of plasmalemma proteins have been developed which identify specific nutrient limitations. For instance, a fluorogenic substrate for alkaline phosphatase can be used to assay plasmalemma activity in single cells of phosphate-starved phytoplankton (González-Gil *et al.* 1998).

1.3.3 Co-limitation

The concept of co-limitation of primary production in marine systems was first introduced by Price & Morel (1991). The principle of co-limitation is that the expression of an enzyme system necessary for acquisition of a nutrient is limited by the availability of another nutrient. An example is the strict requirement of the enzyme urease for nickel (Ni). Using cultures of the marine diatom *Thalassiosira weissflogii* supplied with low Ni, Price & Morel (1991) demonstrated that cultures were unable to metabolize urea. The addition of either Ni or nitrogen in the form of NH_4^+ elicited a growth response. This system can be defined as co-limited by Ni and nitrogen in any form other than urea. Co-limitation must be defined by nutrient addition experiments rather than by physiological indicators. In the case of Ni and N co-limitation, cells would appear nitrogen limited based on physiological measurements such as C:N ratio alone. The term co-limitation has been incorrectly applied to bioassays of marine systems in which the greatest growth response is elicited by the addition of more than one nutrient. The term co-

limitation can only be correctly applied to systems where either one or another nutrient elicits a growth response.

Limitation of primary production by iron and light has also been suggested to occur in marine systems (Sunda & Huntsman 1997) and terrestrial vascular plants (Terry 1983). The ability of an algal cell to photoacclimate to low light is dependent on the availability of iron for photosystem I and II, cytochromes and iron-sulfur proteins of the photosynthetic electron transport chain. When cells enter into low light there is an increase in cellular pigment concentrations, photosynthetic electron carriers and iron quotas (Sunda & Huntsman 1997, Strzepek & Price submitted). Other suggested co-limitations which appear in the literature are: CO_2 and zinc (Zn) due to the Zn requirement of carbonic anhydrase (Morel *et al.* 1994) and nitrogen and iron (Fe) due to the requirement of Fe in the enzymes nitrate reductase and nitrite reductase (Price *et al.* 1991). To date the only example of co-limitation in a natural marine system is for Fe and nitrogen. Price *et al.* (1994) found that an enhancement of algal biomass could be attained either by additions of Fe or NH_4^+ to bioassays performed in the equatorial Pacific.

1.4 Primary production and nitrogen sources

Nitrogen is considered to be most common nutrient limiting primary production in marine systems (e.g. Ryther & Dunstan 1971, Glibert & McCarthy 1984, Kolber *et al.* 1990). For this reason, nitrogen cycling through biotic and abiotic systems has been studied extensively by oceanographers (e.g. Carpenter & Capone 1983). Nitrogen chemistry in marine systems is complex. Sources of nitrogen include nitrogen (N_2) fixation, rain, terrestrial runoff, and upwelling, while sinks include sinking of biogenic material and denitrification. The forms of

nitrogen readily available to phototrophic organisms can be broken into two groups based on oxidation state: the oxidized forms (nitrate and nitrite) are the major source of nitrogen to primary producers in highly productive areas, while the reduced forms (ammonium and urea) are the major source in open ocean, low productivity areas. In addition, a significant amount of nitrogen can be found in reduced, organic forms such as amino acids, purines, pyrimidines and adenylates. However, their importance to primary production is poorly understood (see Antia *et al.* 1991 for review).

1.5 New and regenerated production

The amount of carbon lost from the surface waters of the ocean is dependent on the rate of carbon fixation and the subsequent transport of fixed carbon via sinking. The rate of carbon fixation in the ocean is, in many cases, dependent on the supply rate of nitrogen through upwelling of oxidized forms (NO_3^- and NO_2^-) and the recycling rate of reduced forms (NH_4^+, urea and other organic nitrogen forms) through microbial and heterotrophic activity. Dugdale and Goering (1967) defined the carbon fixation fueled by oxidized forms of nitrogen as "new" production and carbon fixation fueled by reduced forms of nitrogen as "regenerated" production. Under steady-state conditions, and assuming nitrification can be ignored, new production represents carbon which can be lost from the system via sinking, and regenerated production represents carbon which is recycled in surface waters through grazing and remineralization.

The amount of new and regenerated production is estimated from nitrogen uptake rates using the heavy nitrogen isotope ^{15}N, typically in the form of $^{15}NO_3^-$ (new), $^{15}NH_4^+$ and occasionally urea ($CO(^{15}NH_2)_2$) (regenerated). Uptake rates for each nitrogen source can be used to calculate an "f" ratio (f = new / new + regenerated, Eppley and Peterson 1979). The f

ratio ranges from 0.01 for an oligotrophic oceanic ecosystem such as the Sargasso Sea, to 0.7 for an active upwelling area such as the coast of Peru. An f ratio of 0.7 would indicate that 70% of the production is fueled by oxidized nitrogen (NO_3^-) and by assuming steady state, 70% of the production is transported out of surface waters via sinking. Because the f ratio is critical in estimating the loss of carbon to deep waters and is dependent on the nitrogen preference of the phytoplankton assemblage, it is important to understand the physiological factors affecting nitrogen preference.

1.6 Nitrogen Preference

When phytoplankton are nitrogen-starved their ability to take up and assimilate various forms of nitrogen generally increases, particularly for NH_4^+ (McCarthy & Goldman 1979, Syrett & Peplinska 1988, Luque *et al.* 1994). This is due to ammonia inhibition of uptake and/or assimilation of other nitrogen sources including nitrate (NO_3^-), nitrite (NO_2^-) and urea ($CO(NH_2)_2$) (Antia *et al.* 1991, Glass & Siddiqi 1995). The proposed explanation for preferential uptake of ammonium is the lower requirement for enzyme systems and reductant energy (e.g. Thompson *et al.* 1989, Levasseur *et al.* 1993). The reduction of nitrate requires the equivalent reductant energy of 8 electrons, while urea requires no reduction, but does require the activity of the enzyme urease.

Preference for NH_4^+ is not a universal phenomenon in phytoplankton. In a review by Dortch (1990), the presence of NH_4^+ has been reported to inhibit, have no effect on, or even enhance NO_3^- uptake. Maximum growth rates also vary depending on the form of nitrogen which is supplied. For example, diatoms have been shown to have higher, equal or lower growth

rates on NO_3^- compared to NH_4^+. In many cases, culture or environmental conditions affect preference for nitrogen forms. For example, nitrate uptake is enhanced in diatoms growing at low temperatures (Lomas & Glibert in press).

The most common explanation for preferential uptake of ammonium is the lower requirement for enzyme systems and reductant; however, the additional metabolic costs required to assimilate NO_3^- or urea have not been observed in marine microalgae. Growth rate comparisons of several marine phytoplankton using NO_3^-, urea and NH_4^+ as nitrogen sources and saturating and sub-saturating irradiances, have shown that NH_4^+ does not always yield the highest growth rates (Thompson *et al.* 1989, Levasseur *et al.* 1993). At saturating irradiance, algal growth rates should be similar because energy is not limiting. However, it was found that at saturating irradiance, the diatom *Chaetoceros gracilis* had the highest growth rates using NO_3^-, while NH_4^+ yielded lower growth rates and urea yielded the lowest growth rates. At low irradiances (energy limitation), the growth of *C. gracilis* on NO_3^- and NH_4^+ was not significantly different even though NO_3^- assimilation has additional energy requirements. Higher growth rates using NO_3^- at high irradiance may be related to the additional sink for photosynthetically derived electrons when NO_3^- is reduced to NH_4^+ (Lomas & Glibert in press).

Growth rate comparisons of *Thalassiosira pseudonana* under saturating irradiance show higher growth rates for NH_4^+ grown cultures than those grown on NO_3^- (urea was not tested for this species) (Levasseur *et al.* 1993). While at subsaturating irradiances, growth on NO_3^- or NH_4^+ for *T. pseudonana* was the same. This is counter-intuitive because **saturating** irradiance

should provide the excess energy required to perform the extra reduction steps of NO_3^- assimilation. When light is **limiting** there is no difference in growth rates between *T. pseudonana* growing on NO_3^- or NH_4^+, even though cellular reducing power should be low and limiting to NO_3^- assimilation. The results from both *C. gracilis* and *T. pseudonana* have lead to the hypothesis that under saturating irradiances either cross-membrane transport of NO_3^- and urea or assimilatory enzyme activities are limiting the growth rate of NO_3^- and urea grown cultures (Levasseur *et al.* 1993).

1.7 Iron limitation in the sea

There is accumulating evidence that iron rather than nitrogen may be limiting to phytoplankton biomass in some areas of the Pacific Ocean, namely the subarctic North Pacific (Martin *et al.* 1989, Boyd *et al.* 1996), the equatorial Pacific (Price *et al.* 1994) and the Southern Ocean (Helbling *et al.* 1991). These areas have been termed high nitrate (or nutrient), low chlorophyll (HNLC) regions (see Hutchins 1997 for review). In each of these areas, nitrogen is always present at high concentrations (5-30 μM), however, iron is usually in the nM-fM (10^{-9}-10^{-12}) range (Martin *et al.* 1989). Iron limitation in the equatorial Pacific has been verified by the large scale enrichment experiments, IronEx I (Martin *et al.* 1994) and IronEx II (Coale *et al.* 1996). Future, iron enrichment experiments are also planned for the Southern Ocean and the subarctic North Pacific (Boyd pers. comm., Harrison pers. comm.)

Correlations of atmospheric iron loads and CO_2 concentrations suggests that during glacial periods, when atmospheric CO_2 was low, iron supplies to the ocean in the form of atmospheric dust were approximately 50 times higher than during interglacial periods (Martin 1990). This

correlation has been used to formulate the hypothesis that iron controls oceanic primary production in HNLC regions and consequently affects global atmospheric CO_2 concentrations.

1.8 Influence of iron availability on photosynthesis

Iron limitation has been equated with energy limitation because iron is an important component of photosystems I and II and photosynthetic electron transport system (PETS) cytochromes. At least 26 Fe atoms (4 Fe in PS II, 5 Fe in cytochrome b_6f, 2 Fe-S centers, 12 Fe in PS I, 1 Fe in cytochrome c_{553} and 2 Fe in ferredoxin) are required in a single functional photosynthetic electron transport chain, nearly half of which are contained within PS I (Raven 1988). Iron limitation has also been shown to increase PSII:PSI stoichiometry in the marine diatom *Phaeodactylum tricornutum* (Greene *et al.* 1991) and the freshwater cyanobacterium *Aphanocapsa* sp. (Sandmann 1985). The function of PS II is severely impaired by iron limitation and results in light harvesting pigments for PS II becoming isolated from the reaction center of PS II in the green marine alga *Dunaliella tertiolecta* (Vassiliev *et al.* 1995). Vassiliev (1995) also found indirect evidence that PS I may be decreased to a greater extent than PS II. Berges *et al.* (1996) found the opposite case for nitrogen limitation where PS II activity is decreased while PS I activity is relatively unaffected both in *Dunaliella tertiolecta* and *Thalassiosira weissflogii*.

1.9 Influence of iron availability on nitrogen assimilation

Iron limitation has been hypothesized to affect nitrogen utilization based on the high iron requirements of the assimilatory enzymes nitrate (two atoms Fe) and nitrite reductase (five atoms Fe), as well as the additional energetic requirements of nitrate/nitrite reduction. Theoretical calculations by Raven *et al.* (1992), estimated that algae growing on NO_3^- should require 60%

more iron than those growing on NH_4^+. These calculations have been verified by Maldonado & Price (1996) using laboratory cultures of several coastal and open ocean diatoms. They found that NO_3^--grown cultures require about 1.8 times more iron than NH_4^+-grown cells. Price *et al.* (1994) also found that an enhancement of algal biomass and growth rate could be attained either by additions of iron or NH_4^+ to bioassays performed in the HNLC region of the equatorial Pacific suggesting that this environment may be co-limited by iron and reduced forms of nitrogen.

The prevailing hypothesis to explain the ecology of HNLC regions is that small (<5 μm) phytoplankton dominate the biomass, use NH_4^+ as a nitrogen source and are not iron-limited. Large diatoms are rare and iron-limited in these areas, preventing the use of NO_3^- as a nitrogen source, and are essentially co-limited by iron and reduced forms of nitrogen (Price *et al.* 1991).

In laboratory cultures, iron-limited cultures grown on NO_3^- do not have consistently higher carbon:nitrogen (C:N) ratios relative to cultures grown on NO_3^- under iron replete conditions (La Roche *et al.* 1993, Maldonado & Price 1996). A high C:N ratio would be expected if cells were deficient in their ability to metabolize NO_3^-. Nitrogen limited diatoms have C:N ratios as high as 20 - 40 (La Roche *et al.* 1993); however Fe-limited diatoms have C:N ratios similar to nitrogen replete diatoms (4 - 8) (Maldonado & Price 1996). In addition, comparison of diatom growth rates in cultures with either NO_3^- or NH_4^+ as the sole nitrogen source show little or no difference between nitrogen sources (Maldonado & Price 1996).

To date only one study has investigated the influence of iron on nitrate assimilatory enzymes but this study was limited to nitrate reductase (NR) (Timmermans *et al.* 1994). They found that NR activity decreased under Fe-limited growth in several marine phytoplankton. Growth rates

were not reported in their study making it impossible to distinguish between the decrease of NR activity due to lower growth rates or due to Fe-limitation. The influence of iron limitation on the enzyme nitrite reductase (NiR) which has a higher iron requirement (five atoms Fe in NiR vs. two atoms Fe in NR), has not been investigated. Several studies have indicated that nitrite reduction may be the limiting step in nitrogen assimilation of algae and cyanobacteria based on the efflux of nitrite into the culture medium when either light (Martinez 1991) or carbon (Krämer *et al.* 1988, Suzuki *et al.* 1995) is limiting. Due to the higher iron requirement of NiR and additional energetic costs of NO_2^- reduction over NO_3^-, the reduction of NO_2^- is likely to be more affected by iron limitation than the reduction of NO_3^-. Indeed if iron influences nitrogen assimilation, then it is reasonable to expect that alternative nitrogen assimilatory pathways would be induced in order to fulfill cellular nitrogen demands. Of the main sources of alternate nitrogen available to marine phytoplankton, NH_4^+ and urea are likely to fulfill this demand. The main enzyme for NH_4^+ assimilation, glutamine synthetase, is common to all three nitrogen sources but the assimilatory enzyme for urea, urease, is exclusive and a likely indicator of nitrogen limitation.

1.10 Objectives

In a recommendation for future research drafted by the American Society of Limnology and Oceanography, researchers suggested future work be directed towards "studies of the comparative physiology and biochemistry of iron utilization in phytoplankton isolated from coastal, oligotrophic, and nutrient-rich [iron-limited] oceanic ecosystems"(ASLO Symposium Report 1991). This thesis investigates the biochemistry of nitrogen assimilation as it relates to iron supply.

The research objectives and hypotheses of this thesis are as follows:

1. To develop and optimize an *in vitro* urease assay for marine phytoplankton (Chapter 2).
2. To develop and optimize an *in vitro* nitrite reductase assay for marine phytoplankton (Chapter 3).
3. To test, under non-steady state iron supply the following three null hypotheses: **A)** nitrite reductase is not the limiting step in the nitrate reduction pathway when the availability of dissolved iron is low, **B)** the addition of iron to iron-limited cultures does not specifically enhance the activity of nitrite reductase, and **C)** iron limitation does not induce the activity of urease, an iron-free alternative nitrogen assimilatory pathway. Batch cultures of the marine diatom *Thalassiosira weissflogii* with sufficient iron served as a control and low iron cultures served as treatments (Chapter 4).
4. To test the above null hypothesis (3A) under steady state iron supply, iron was supplied to the marine diatom *Thalassiosira weissflogii* growing at steady state using the trace metal-buffered culture medium Aquil (Chapter 5).

2. Urease enzyme assay optimization and activity in marine diatoms

2.1 Introduction

Nitrogen in the marine environment occurs primarily as nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and urea ($CO(NH_2)_2$). The occurrence and uptake of these four forms of nitrogen have been well characterized in the open ocean (e.g. Eppley *et al.* 1971, Varela & Harrison in press). Phytoplankton in most algal classes have the ability to grow on urea as the sole nitrogen source (see Antia *et al.* 1991 for extensive review). In addition, a significant amount of dissolved organic nitrogen (DON) can be found in forms such as amino acids, purines, pyrimidines and adenylates, but their importance to primary production is poorly understood (Antia *et al.* 1991).

While urea concentrations in seawater are low (typically < 1 μM), turnover rates are high. Price & Harrison (1988a) report turnover times as short as 6-8 h for the oligotrophic waters of the Sargasso Sea and North Atlantic, while turnover times as long as 140 days have been observed for urea in the North Pacific (Mitamura & Saijo 1980), but the methodology used in the latter study has been questioned (Price & Harrison 1988a, Antia *et al.* 1991).

2.1.1 Urea uptake

Urea uptake can either be passive or active. Active uptake of urea is accomplished by a high affinity system, which is dependent on ATP (Rees & Syrett 1979a,b). Uptake is thought to be a sodium (Na)-dependent process and is inhibited by lithium (Li) and potassium (K) (Rees *et al.* 1980). Urea is an uncharged N species and can diffuse through the plasmalemma. At concentrations > 70 μM, uptake can be explained as passive diffusion. Uptake of urea in natural

populations of phytoplankton is likely to be active as the highest urea concentration reported in marine systems is 23 μM (Antia *et al.* 1991).

2.1.2 Urea assimilation

After urea is taken up it is metabolized to ammonium and carbon dioxide through one of two pathways in marine algae: urease or ATP:urea amidolyase (UALase). Single organisms have not been reported to contain both enzymes (Antia *et al.* 1991). The presence of UALase is exclusive to several classes of the Chlorophyceae (Leftley & Syrett 1973; Bekheet & Syrett 1977). In this two step enzyme mediated pathway, urea is carboxylated via urea carboxylase (urea:CO_2 ligase (ADP); EC 6.3.4.6) according to the equation:

$$NH_2 \cdot CO \cdot NH_2 + ATP + HCO_3^- \rightarrow NH_2 \cdot CO \cdot NH \cdot COO^- + ADP + H_2PO_4 \qquad (2.1)$$

Allophanate ($NH_2 \cdot CO \cdot NH \cdot COO^-$) is then hydrolyzed by allophanate hydrolase (allophanate amido hydrolase; EC 3.5.1.13) which catalyzes the reaction:

$$NH_2 \cdot CO \cdot NH \cdot COO^- + 2H_2O + OH^- \rightarrow 2\,HCO_3^- + 2\,NH_3 \qquad (2.2)$$

All algal classes other than the chlorophyceae contain the enzyme urease. Urea is hydrolysed via urease (urea amidohydrolase; EC 3.5.1.5) according to the equation:

$$NH_2 \cdot CO \cdot NH_2 + 2H_2O \rightarrow NH_3 + NH_2COOH \qquad (2.3)$$

to yield ammonia and carbamate, which spontaneously hydrolyses to ammonia and carbonic acid:

$$NH_2COOH \rightarrow NH_3 + H_2CO_3 \quad (2.4)$$

Following hydrolysis, the two ammonia molecules become protonated and form ammonium and carbonic acid loses a proton resulting in a net increase in pH. Urease is a nickel-ligated metalo-protein present in bacteria, algae, fungi and vascular plants (Mobely & Hausinger 1989). In marine phytoplankton, urease is believed to be a constitutive enzyme with its activity dependent on the supply of nickel (Price & Morel 1991). Many reports of phytoplankton being unable to use urea as a nitrogen source were the result of culturing without the addition of nickel to the medium (Antia *et al.* 1991).

2.1.3 Ammonium assimilation

Following urea hydrolysis, $NH4^+$ can be assimilated through two alternate pathways, the glutamate dehydrogenase (GDH) pathway, or the glutamine synthetase, glutamate oxoglutarate aminotransferase (GS-GOGAT) pathway. In the GDH pathway, $NH4^+$ reacts with α-ketoglutarate to form glutamate. In the GS-GOGAT pathway, $NH4^+$ reacts with glutamate to form glutamine, followed by the transfer of an amine group from glutamine to α-ketoglutarate to form two molecules of glutamate. This is thought to be the predominant pathway in marine phytoplankton (Zehr & Falkowski 1988).

2.1.4 Urease expression inferred from urea uptake

Much of current knowledge regarding urease expression in marine diatoms has been inferred from urea uptake. For instance, measurements of urea uptake using thiourea, a non-metabolizable sulfur analogue of urea, by the marine diatom *Phaeodactylum tricornutum* shows low uptake (8 nmol 10^8 $cells^{-1}$ h^{-1}) in NH_4^+-grown cultures and high uptake (31.5 nmol 10^8 $cells^{-1}$ h^{-1}) in NO_3^- and urea-grown cultures (Rees & Syrett 1979a). Urea uptake is also enhanced within 24 h of nitrogen starvation and declines after 48 h of starvation in the same organism (Rees & Syrett 1979a,b). Inhibition of urea uptake by NH_4^+ has been observed for *Thalassiosira pseudonana* and *Skeletonema costatum* (Horrigan and McCarthy 1982). In *Thalassiosira pseudonana* urea uptake occurs simultaneously with NO_3^- uptake and is enhanced following 24 h of NO_3^- starvation (Price & Harrison 1988b). Based on these studies and assuming urea uptake and assimilation are coordinated, one would predict that in marine diatoms NH_4^+ represses urease activity and nitrogen starvation induces urease.

Past measurements of urease activity have not attempted to account for the assimilated nitrogen of the test organism (e.g. Rees & Bekheet 1982). In addition, assays performed using the same method and organism give widely different activities. Rees & Bekheet (1982) measured urease activity 200 times greater than activities measured by Leftley & Syrett (1973) for the diatom *Phaeodactylum tricornutum.* Price & Morel (1991) were unable to account for the incorporation rate of nitrogen in urea assays performed on the marine diatom *Thalassiosira weissflogii* and attributed this to unfavorable assay conditions.

This study presents a simple colorimetric method for the assay of urease activity in marine diatoms using crude homogenates which can account for all the incorporated nitrogen in *Thalassiosira weissflogii* and twice the nitrogen incorporation rate in *Thalassiosira pseudonana*.

2.2 Materials and Methods

2.2.1 Culture conditions

Unialgal cultures of *Thalassiosira pseudonana* (clone 3H, NEPCC #58) and *Thalassiosira weissflogii* (clone Actin, NEPCC #741) were obtained from the Northeast Pacific Culture Collection (NEPCC), Department of Botany, University of British Columbia. Axenic cultures of *Thalassiosira pseudonana* (clone 3H, CCMP #1335) and *Thalassiosira weissflogii* (clone Actin, CCMP #1336) were obtained from the Provasoli - Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, USA. Cells were grown in ESAW artificial seawater (Harrison *et al.* 1980) with the following modifications. Selenium and nickel were added at final concentrations of 10 nM and 63 nM respectively (Price *et al.* 1987, Price & Harrison 1988b). For urease assay optimization experiments, 150 μM urea was supplied as the sole nitrogen source. For activity experiments, either 150 μM urea, 300 μM NO_3^- or 300 μM NH_4^+ were supplied as sole nitrogen sources. The medium was sterilized either by filtering (bacterized cultures) using a series of autoclaved nitrocellulose membranes (pore size 0.8, 0.45 & 0.2 μm) or by autoclaving (axenic cultures). Cultures were grown in 1 L flat-bottom boiling flasks at 18.5 ± 0.5°C under a constant illumination of 180 μmol m^{-2} s^{-1} (Vitalite) measured with a QSL - 100 meter (4π collector, Biophysical Instruments Inc.). To ensure mixing and an adequate supply of CO_2, cultures were bubbled with air that was scrubbed through 5% sulfuric acid, distilled water

and a 0.8/0.2 μm filter set. Axenic cultures were not bubbled. Instead $NaHCO_3$ was added when dissolved inorganic carbon (DIC) levels fell below 1.7 mM. To ensure carbon saturation, total dissolved inorganic carbon was measured daily using an infrared gas analyzer (Analytical Development Co. 225-MK3) calibrated against a 449 ppm CO_2 gas standard.

2.2.2 Growth and biomass measurements

Cell counts were obtained using a Coulter particle counter (Model TA II). Growth rates were calculated from cell counts using the formula:

$$\mu = \ln (C_1/C_0)/(t_1-t_0) \tag{2.5}$$

where μ is the intrinsic rate of growth in units of per day, C_0 is the cell count at day zero (t_0), and C_1 is the cell count at day one (t_1). Cellular carbon and nitrogen quotas were obtained by filtering 5 ml aliquots of culture onto pre-combusted (460°C for 4 h) 13 mm AE (Gelman) glass fiber filters and analyzed with a Carlo Erba NCS analyzer NA 1500. Carbon and nitrogen were calculated against a standard curve generated from sulfanilamide standards. The particulate nitrogen growth rate was calculated using the formula:

$$\mu PN = \mu \times (N\ cell^{-1}) \tag{2.6}$$

where μPN is the intrinsic rate of increase of particulate nitrogen in units of pg day^{-1} and N is cellular nitrogen in grams.

Bacterial cell counts were performed on cells harvested using 0.2 μm, 25 mm polycarbonate filters (Poretics) and counted visually using acridine orange stain and epifluorescence microscopy.

Dissolved external NH_4^+ concentrations were determined colorimetrically according to Parsons *et al.* (1984). Dissolved external NO_3^- was determined using the spongy cadmium method of Jones (1984). Dissolved external urea concentrations were determined colorimetrically using the method of Price & Harrison (1987).

2.2.3 Urease Enzyme Assay

Triplicate aliquots (30-40 ml) of culture were filtered onto pre-combusted (4 h at 460°C) 25 mm A/E glass fiber filters. Filters were quickly folded, placed into 1.5 ml microcentrifuge tubes (Fisher), frozen and stored in liquid nitrogen. Samples were stored for no more than 10 d. Filters were ground in a 5 ml glass tissue homogenizer with a Teflon® pestle in 1 ml of ice cold homogenization buffer while immersed in an ice slurry. The buffer, which was optimized for use with marine diatoms (Peers *et al.* unpubl. results See Appendix A), consisted of 150 mM potassium phosphate buffer adjusted to a range of pH (5-12), with additions of 5 mM ethylenediaminetetracetic acid (EDTA) and 0.3% w/v polyvinyl pyrolidone (PVP) and 0.1% v/v Triton X-100. Homogenates were spun for 5 s at 15,000 rpm in an Eppendorf 5414 centrifuge to remove glass particulates. Clarified homogenate (200 μl) was added to 750 μl of buffer solution minus additives in 1.5 ml clear microcentrifuge tubes. Incubations (15-20 min) were started with the addition of 50 μl of 150 mM urea prepared in potassium phosphate buffer and run in the same incubator that was used for cultures. Time zero samples (t_0) and incubations were stopped by the addition of 20 μl of 4 N HCl prior to the addition of urea. Incubation periods were stopped by the addition of 20 μl of 4 N HCl with subsequent neutralization using 20 μl of 4N NaOH.

Ammonium production was measured using the indophenol method (Solorzano 1969) where phenol and nitroprusside reagents were mixed 1:1 as recommended by Chaney & Marback (1962). Samples were reacted with ammonium reagents in the following proportion and order: 300 µl sample : 200 µl phenol-nitroprusside solution : 400 µl alkaline solution in 1.5 ml disposable cuvettes (Fisher). Following the addition of each reagent, samples were mixed by vortexing. The absorbance at 640 nm was read on a LKB Ultraspec II against a blank of buffer and ammonium reagents. Linearity of the method was checked using algal homogenates spiked with a range of NH_4^+ concentrations.

Urease assays were considered "optimized" when enzyme activity was maximal and/or accounted for 100% or more (excess activity) of the nitrogen incorporation rate defined as the particulate nitrogen growth rate. Particulate nitrogen growth rate (µPN) is defined as the intrinsic rate of increase of the particle associated nitrogen which is functionally defined as that which can be retained by a Gelman A/E glass fiber filter (nominal pore size 1.0 µm). Particulate nitrogen growth rate was obtained by multiplying the growth rate (µ) by the cellular nitrogen quota (pg N $cell^{-1}$). This value provided a check of the enzyme assay efficiency. For instance, the activity of urease given in units of g N $cell^{-1}$ d^{-1} can be directly compared to the particulate nitrogen growth rate given in units of g N $cell^{-1}$ d^{-1}.

2.3 Results

2.3.1 Urease assay optimization

The indophenol method for NH_4^+ detection was found to be linear for the range of NH_4^+ concentrations typically found in incubated enzyme assays (Fig. 2.1). Absorbance in the majority of assays fell between 0.1 and 0.3 absorbance units (640 nm).

Urease activity had a pH optimum of 7.9 for *Thalassiosira weissflogii* (Fig. 2.2) using potassium phosphate buffer, and a broad pH optimum for *Thalassiosira pseudonana* using both potassium phosphate and HEPES buffer (Fig. 2.3).

Urease activity within crude homogenates, stored on ice, was found to be stable for up to 1 h (Fig. 2.4). By 2 h the activity of urease declined to approximately 70% of its original value. Subsequent assays were all processed within 1 h of the grinding step.

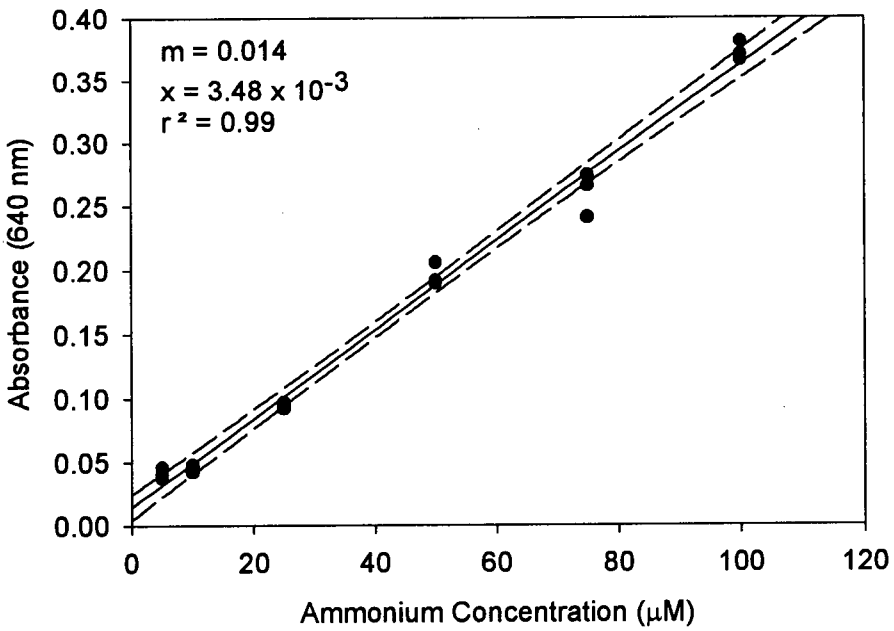

Figure 2.1. Ammonium measurement in algal homogenates using the indophenol method. A linear regression with 95% confidence limits is shown. Homogenates were spiked with NH_4Cl.

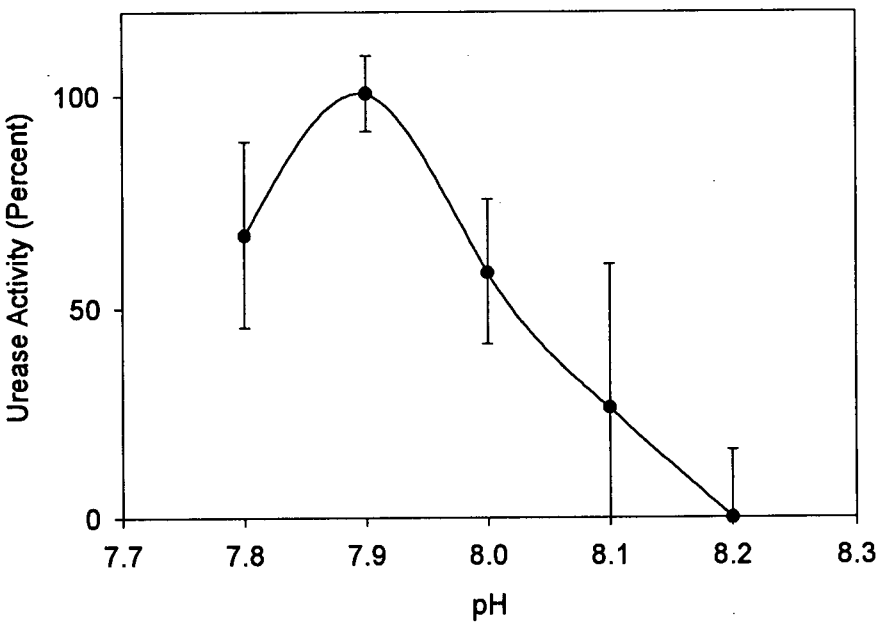

Figure 2.2. Urease pH optimum for *Thalassiosira weissflogii*. Error bars are ± 1 SD calculated from three replicate samples from a single culture.

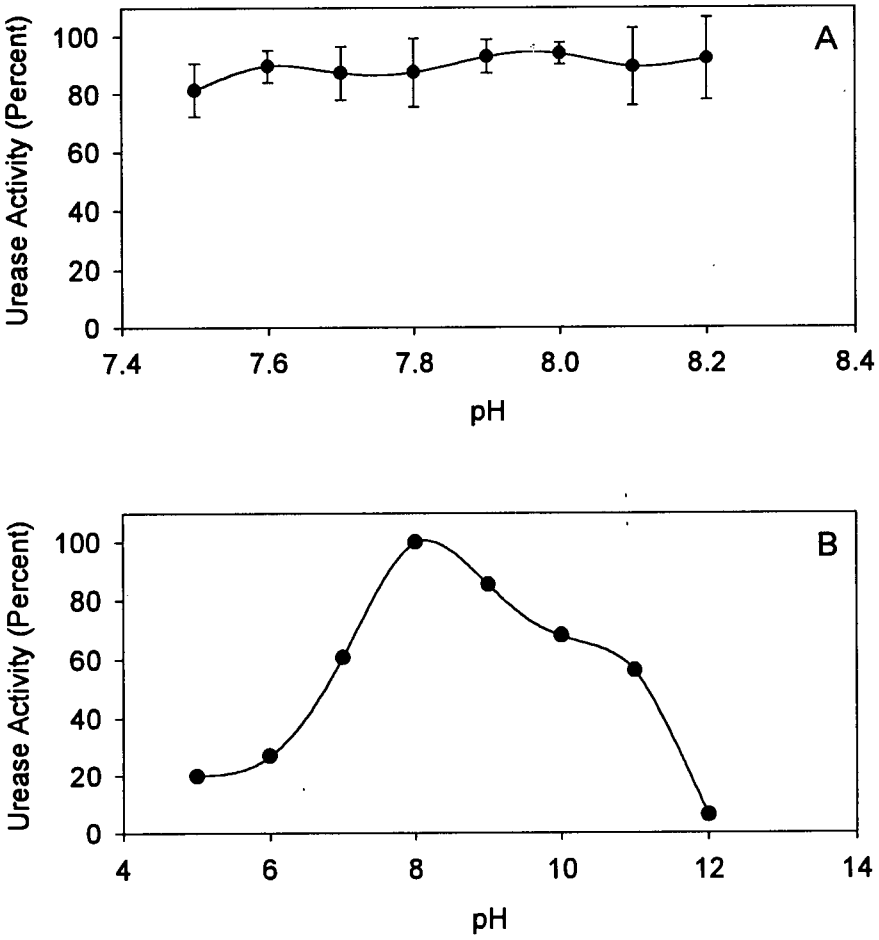

Figure 2.3. Urease pH optimum for *Thalassiosira pseudonana*. A) Potassium phosphate buffer and a narrow pH range. Error bars are ±1 SD calculated from three replicate samples from a single culture. B) Hepes buffer and a broad pH range (n = 1).

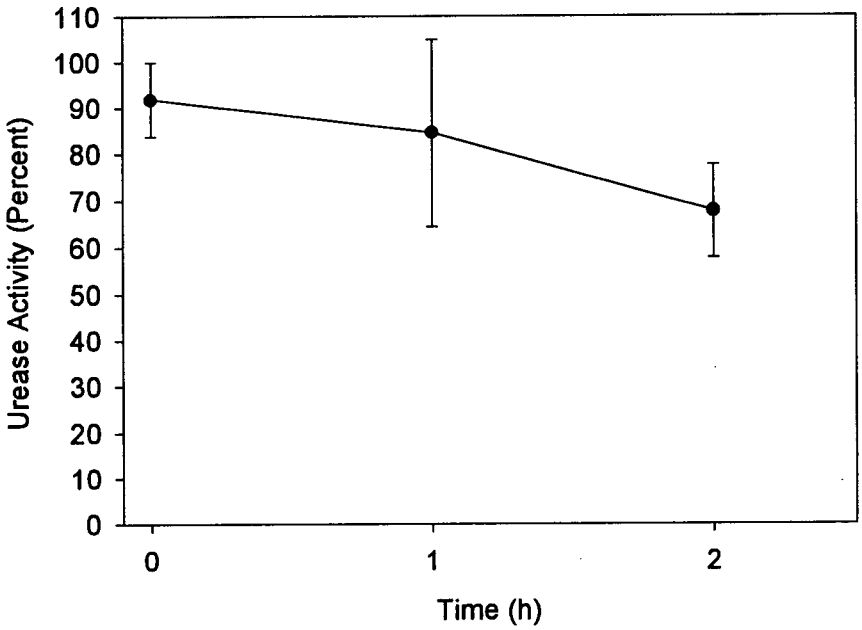

Figure 2.4. Urease stability following extraction in homogenization buffer for *Thalassiosira pseudonana*. Homogenization buffer contained potassium phosphate buffer (pH 7.9) with 0.1% PVP and 5 mM EDTA. Error bars are ±1 SD calculated from three replicate samples from a single culture.

2.3.2 Urease activity in Thalassiosira pseudonana

The urease activity of axenic cultures of *T. pseudonana* depended on nitrogen supply. Urea-grown cultures had the highest activities, which were roughly twice the nitrogen incorporation rate (μPN = 3.91; SD = 0.32) (Fig. 2.5). Nitrate-grown cultures expressed urease at levels roughly equivalent to the incorporation rate (μPN = 3.33; SD = 0.35) while NH_4^+-grown cultures expressed a basal level of urease activity at roughly 25% of the nitrogen

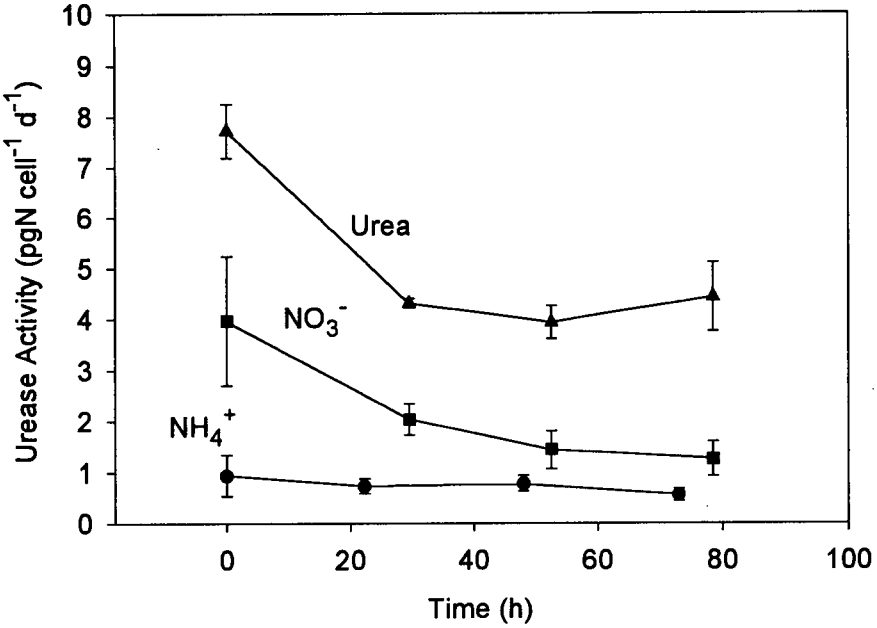

Figure 2.5. Urease activity in axenically grown *Thalassiosira pseudonana* under three nitrogen sources (NH_4^+ (●), NO_3^- (■), and urea (▲)). Time zero represents nitrogen replete conditions. External nitrogen concentrations reached zero at approximately 20 h. Error bars are 95% confidence intervals (CI) calculated from three replicate cultures.

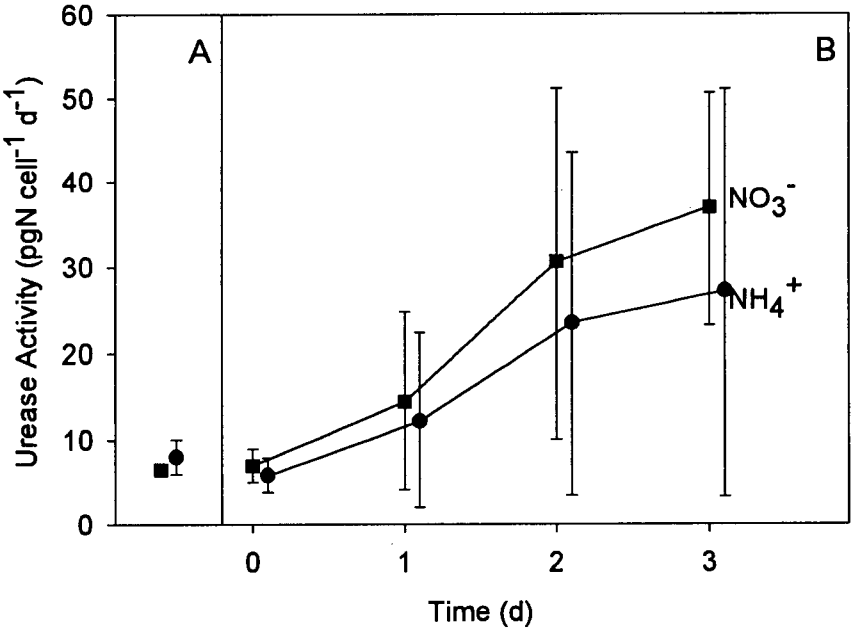

Figure 2.6. Urease activity in *Thalassiosira pseudonana* cultures containing bacteria under two nitrogen sources (NH_4^+ (●), NO_3^- (■)). A) Nitrogen replete cultures, and B) Separate experiment with cultures entering nitrogen limitation. External nitrogen concentrations reached zero between day one and two. Error bars are 95% CI calculated from three replicate cultures.

incorporation rate (μPN = 3.55; SD = 0.37) (Fig. 2.5). Following nitrogen depletion, urease activities dropped in all cultures, but were maintained at the highest levels in urea grown cultures.

In bacterized cultures of *T. pseudonana*, activities of nitrate grown cultures were similar to axenic cultures, while NH_4^+ grown cultures expressed activities approximately four times higher (Fig. 2.6). Urease activities as well as variance around the mean increased dramatically in bacterized cultures following nitrogen depletion of the medium.

2.3.3 Urease activity in Thalassiosira weissflogii

Axenic nitrogen replete cultures of *T. weissflogii* expressed urease at similar levels regardless of nitrogen supply (Fig. 2.7A). Activities in all nitrogen sources were roughly equivalent to nitrogen incorporation rates (μPN = 36.4; SD = 0.4). After two days of nitrogen exhaustion, urease activities increased for urea grown cultures only, while both NO_3^- and NH_4^+ grown cultures maintained similar activities to nitrogen replete conditions (Fig. 2.7B). On the third day of nitrogen starvation both urea and NO_3^- grown cultures had increased urease activities while NH_4^+-grown cultures maintained levels of activity similar to NH_4^+-replete conditions. During the starvation stage axenic cultures became contaminated with bacteria. Increased activities in axenic cultures corresponded to increases in the abundance of contaminating bacteria, although the correlation between bacterial numbers and urease activity was poor ($p = 0.15$).

In nitrogen-replete bacterized cultures, urease activities behaved similarly. No differences in activity were observed among the three nitrogen sources (Fig. 2.8). Activities for urea grown cultures decreased significantly following nitrogen exhaustion, while NH_4^+-starved

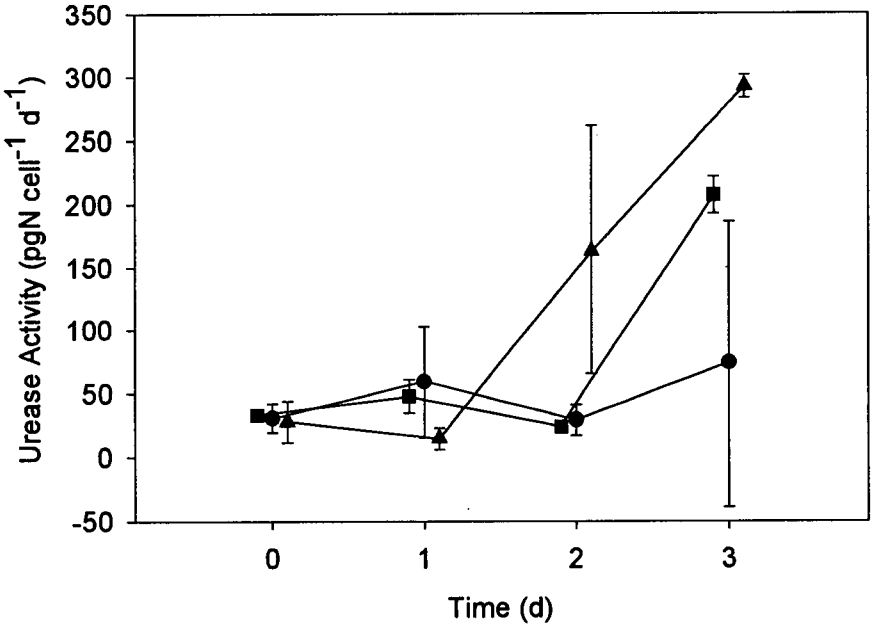

Figure 2.7. Urease activity in axenically grown *Thalassiosira weissflogii* under three nitrogen sources (NH_4^+ (●), NO_3^- (■), and urea (▲)).Time zero represents nitrogen replete conditions. External nitrogen concentrations reached zero between day zero and one. Error bars are 95% CI calculated from three replicate cultures.

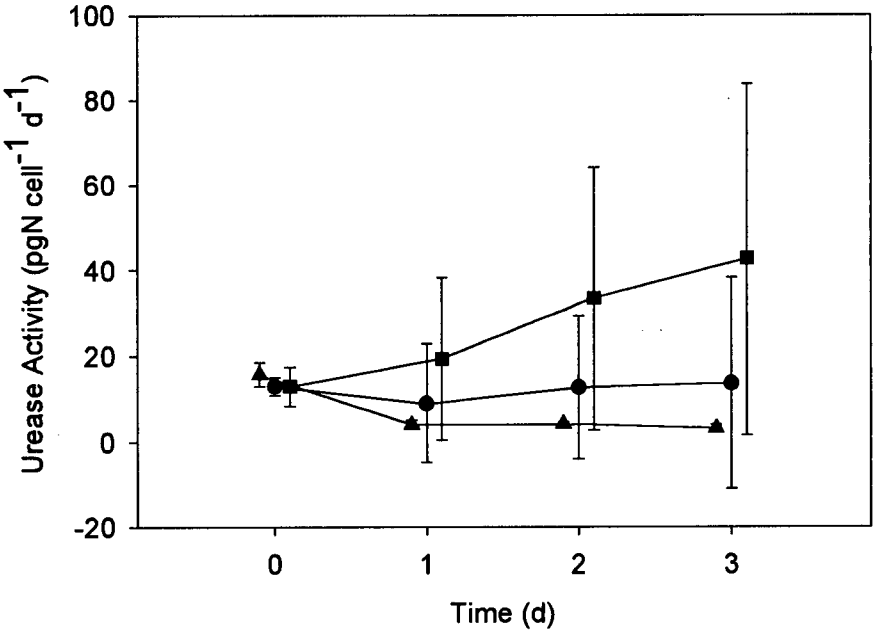

Figure 2.8. Urease activity in *Thalassiosira weissflogii* cultures containing bacteria, grown with three nitrogen sources (NH_4^+ (●), NO_3^- (■), and urea (▲)).Time zero represents nitrogen replete conditions. External nitrogen concentrations reached zero between day zero and one. Error bars are 95% CI calculated from three replicate cultures. Error bars not visible are smaller than symbols.

cultures remained similar to NH_4^+-replete conditions, although variance increased dramatically. Both activities and variance increased in nitrate grown cultures which corresponded to an increase in bacterial biomass.

2.4 Discussion

2.4.1 Urease enzyme assay

Urease was successfully measured in two marine diatoms using a novel homogenization buffer and simple colorimetric measurement for the production of NH_4^+ in crude homogenates. This study is the first report of a method which successfully accounts for the rate of nitrogen incorporation in marine algae grown on urea.

The common method previously used to measure urease in marine algae utilized the method of Leftley & Syrett (1973) which was modified from a method by Adams (1971). This method utilizes radioactive urea ($^{14}CO(NH_2)_2$) and tris (tris(hydroxymethyl) amino ethane) - HCl buffer (pH 8.4) in sealed bottles in which the hydrolysis of urea liberates $^{14}CO_2$ and is trapped using a solution of KOH (Leftley & Syrett 1973, Price & Morel 1991). The method employed in this study avoids the complications of using radioactive isotopes and allows manipulation of the pH to values which are physiologically relevant, but where liberation of CO_2 is problematic. A buffer pH of 7.9 was found to be appropriate for use with both species of diatoms.

Although an indophenol assay has not been used for phytoplankton, it has been applied to prokaryotes. In a review of urease assay techniques, Mobley & Hausinger (1989) recommended

the indophenol method for its ease and accuracy. The indophenol assay is linear and accurate despite the complicating factors of algal internal NH_4^+ pools and reactive amines.

Price & Morel (1991) found that urease activity in *T. weissflogii* (the same clone used in this study) was <50% of their calculated N incorporation rate. They attributed this low activity to unfavorable enzyme assay conditions. Their assay buffer was modeled after that of Leftley & Syrett (1973) and therefore was buffered at a sub-optimal pH (8.4) and contained 1.4 μM dithiothreitol (DTT). Thiols, such as DTT or 2-mercaptoethanol competitively inhibit jack bean urease by binding to the nickel ion in the active site of the enzyme (Mobley & Hausinger, 1989). The inclusion of dithiothreitol (DTT) in the homogenization buffer used in this study also decreased urease activity (Peers *et al.* unpubl. results, Appendix A). The method reported here can account for 100% (±20%) of the incorporated nitrogen in *T. weissflogii.*

Crude homogenates were found to be stable within the typical range of processing times (ca. 1 h) used in this study. Two hours of storage on ice resulted in a decrease in activities of approximately 70%. Urease can be inactivated by heavy metals and is commonly stabilized by the addition of EDTA and thiols (Mobely & Hausinger 1989). Thiols were not included in the homogenization buffer due to their negative impact on activity, but EDTA was included and improved activity (Peers *et al.* unpubl. results, Appendix A).

Enzyme activities were normalized to cell number in this study rather than protein, which is used predominantly in the literature (e.g. Singh 1990 & 1992, Leftley & Syrett 1973). In addition, many techniques employ the measurement of $^{14}CO_2$ liberated from radiolabeled urea. This makes it difficult to directly compare results between studies conducted with different groups of phytoplankton in the past, but rough comparisons can be made. Using a protein content of 4.5 pg $cell^{-1}$ (Berges 1993) for *T. pseudonana* and assuming that cells grown on urea do not alter

their protein constituents significantly from those grown on NO_3^-, urease enzyme activity was normalized to protein and converted to carbon units. The converted activity measured in this study, for *T. pseudonana* grown on urea, is 2.7×10^3 nmol C mg protein^{-1} h^{-1} with a converted nitrogen incorporation rate of 1.38×10^3 nmol C mg protein^{-1} h^{-1}. Leftley & Syrett (1973) measured urease activities of 2.22 nmol C mg protein^{-1} h^{-1} for the diatom *Phaeodactylum tricornutum.* However, using the same method, Rees & Bekheet (1982) were able to measure activities of up to 450 nmol C mg protein^{-1} h^{-1} in the same species grown on NO_3^-. Singh (1990) measured a urease activity of 399.2 nmol C mg protein^{-1} h^{-1} in the freshwater cyanobacterium *Anabaena dolium.*

Price & Harrison (1988b) found that the uptake of urea by *T. pseudonana* resulted in the rapid efflux of NH_4^+ into the medium presumably due to high rates of urea hydrolysis and excretion of ammonia which could not be assimilated. The high activity of urease (twice the nitrogen incorporation rate) for *T. pseudonana* grown on urea measured in this study supports this observation.

2.4.2 Urease activity under nitrogen-replete conditions

Urease was found to be expressed regardless of the nitrogen source in the two diatoms studied, although activities showed distinctly different patterns according to the form of nitrogen supplied. Under nitrogen-replete conditions, urease activity in *T. pseudonana* was expressed constitutively when grown on NH_4^+ and upregulated when grown on NO_3^- or urea. In *T. weissflogii*, urease activity was expressed constitutively regardless of the nitrogen source and showed no upregulation.

Similar variability in urease activity according to nitrogen supply has been observed by others. In the freshwater cyanobacteria *Anabaena doliolum, Anacystis nidulans* and *Nostoc muscorum*, Singh (1990 & 1992) found that urease activity remained the same when grown on NO_3^- and urea as the sole N source, but decreased in the presence of NH_4^+. Ge *et al.* (1990) found that urease remained at a constitutive level in the freshwater cyanobacterium *Anabaena variabilis*, regardless of N source.

Variations in urease activity which do not agree with findings of this study have also been observed. For instance, in the marine cyanobacterium *Synechococcus*, Collier & Palenik (1996) found that urease activity remained similar when grown on NH_4^+ or urea, but growth on NO_3^- showed increased urease activity. In two different studies, the marine diatom *Phaeodactylum tricornutum* (clone 1052/6) expressed higher levels of urease when grown on NO_3^- as the sole nitrogen source (Rees & Bekheet 1982) than when grown on urea (Leftley & Syrett 1973).

Urease activity in axenic and bacterized cultures *T. pseudonana* showed no difference for nitrate-grown cultures, however, bacterized ammonium-grown cultures had higher urease activities than axenic cultures. Clearly the contribution of bacterial urease activity was high enough to make inferences about nitrogen source regulation of urease impossible even though bacterial biomass is a minor fraction of total biomass of the culture.

2.4.3 Urease activity under nitrogen starvation

When axenic *T. pseudonana* cultures entered nitrogen starvation, urease activity declined but activities remained similar relative to each nitrogen source indicating at least a 3 day influence from growth on each nitrogen source. Bacterized cultures showed dramatic upregulation of urease with high variability between replicates indicating the variability in bacterial activity in each

flask. Algal cell death and an increase in dissolved organic compounds most likely contributed to the enhanced bacterial urease activity.

Axenic cultures of *T. weissflogii* showed no change in urease activity under nitrogen starvation with the exception of urea-grown cultures which developed bacterial contamination and had increased activity. Bacterized cultures of *T. weissflogii* showed a similar pattern with the exception of NO_3^--grown cultures which developed bacterial contamination.

In neither species tested, was urease activity upregulated by nitrogen starvation. This observation appears contrary to the observation that several marine algal species induce high uptake rates of urea following nitrogen starvation. However, urease expression and uptake are likely separately regulated systems in marine diatoms. For instance, uptake but not assimilation of urea is inhibited by the presence of NH_4^+ in *Phaeodactylum tricornutum,* a marine diatom (Syrett & Leftley 1976, Rees & Syrett 1979a). With the exception of NH_4^+-grown *T. pseudonana,* urease activity remains at levels high enough to satisfy the nitrogen demands of nitrogen starved cells. Steady state nitrogen limited growth was not tested in this work and could potentially give different results. Cultures experienced complete exhaustion of nitrogen which can result in remobilization of proteins such as RUBISCO and pigment proteins (Geider *et al.* 1993). If limiting nitrogen were to be made available under steady state conditions using a continuous culture apparatus, urease may be upregulated.

2.5 Conclusions

This is the first report of a urease assay which can account for 100% of the nitrogen incorporated by urea-grown marine diatoms. This assay was able to discriminate between levels of urease activity in two marine diatoms grown on the three major nitrogen sources available in the marine environment. The evidence of a three day influence of the nitrogen source used for growth, following nitrogen starvation is unprecedented.

While urease activity is not upregulated under nitrogen starved conditions, the question of whether urease is upregulated under nitrogen-limited growth conditions remains to be resolved.

3. Enzyme assay optimization of nitrite reductase in marine phytoplankton

3.1 Introduction

In photoautotrophs, NO_3^- assimilation is accomplished through an enzymatic pathway which requires uptake of NO_3^- across the cell membrane and two reduction steps to reduce NO_3^- to NH_4^+. Uptake of NO_3^- and NO_2^- is active and accomplished through at least three transport systems in vascular plant roots: a constitutive high affinity system, a constitutive low affinity system and a high affinity inducible system (Glass & Siddiqi 1995). The transport of NO_3^- and NO_2^- across the plasmalemma is accomplished via a proton/nitrogen symport in which transport of NO_3^- and NO_2^- is accompanied by the transport of two H^+ ions. This decreases cellular pH and must be balanced by the efflux of protons through a translocating ATPase, which consumes ATP. In marine phytoplankton, the transport processes are thought to be similar to those of vascular plants; however, it has been hypothesized that marine algae have a sodium (Na^+) symport rather than a proton symport and a Na-ATPase (Rees *et al.* 1980).

3.1.1 Nitrate assimilation

Nitrate assimilation is facilitated through the catalytic function of two iron-containing enzymes, nitrate and nitrite reductase. Following uptake, NO_3^- undergoes a $2e^-$ reduction via nitrate reductase (NR, EC 1.6.6.1) to NO_2^- according to the equation:

$$2H^+ + NO_3^- + 2e^- \rightarrow NO_2^- + H_2O \quad (3.1)$$

Nitrate reductase is a large (200 - 300 KDa) soluble cytoplasmic enzyme with FAD and iron-containing cytochrome b557 prosthetic groups, which requires NADH and/or NADPH as an electron donor and molybdenum as a cofactor (Guerrero *et al.* 1981). Functional NR is in the form of a homodimer and therefore requires two atoms of Fe per functional enzyme. Following transport into the chloroplast, nitrite (NO_2^-) undergoes a $6e^-$ reduction to NH_4^+ via nitrite reductase (NiR, EC 1.7.7.1) according to the equation:

$$8H^+ + NO_2^- + 6e^- \rightarrow NH_4^+ + 2H_2O \quad (3.2)$$

Nitrite reductase, a soluble 63 KDa chloroplastic enzyme, is a metalo-protein with five Fe atoms per active enzyme molecule, which requires photosynthetically reduced ferredoxin as an electron donor (Guerrero *et al.* 1981). Alternatively, the replacement of ferredoxin with the electron donor flavodoxin (an electron donor with no iron, Zumft & Spiller 1971), could partially mitigate the effects of iron limitation in the nitrate assimilatory pathway. Production of flavodoxin has been shown to occur under iron limitation in several marine phytoplankton (La Roche *et al.* 1993, Doucette *et al.* 1996).

In phytoplankton, under certain conditions, nitrite reduction may become the limiting step in nitrate assimilation resulting in the excretion of NO_2^- into the culture medium due to its intracellular toxicity (Laws & Wong 1978, Serra *et al.* 1978, Collos 1982, Martinez 1991, Sciandra & Amara 1994, Flynn & Flynn 1998). Reliable assay methods for NR and NiR are necessary to investigate the regulation of nitrate assimilatory enzymes under conditions where

NiR may become the rate limiting step in nitrate reduction. A nitrate reductase assay has been developed and optimized for use with marine phytoplankton (Berges & Harrison 1995).

Development and optimization of a NiR assay for marine phytoplankton was the goal of this study. The method presented below was developed using three species of phytoplankton. Both *Actinocyclus* sp. and *Emiliania huxleyi* were used to determine the ideal conditions for the homogenization buffer. *Thalassiosira weissflogii* was used in further experiments because of the large data set available from other researchers regarding iron limitation in this species. During the course of experimentation, I found that much of the NiR activity remained associated with the glass fiber filters used to harvest cultures. The method below includes an attempt, which was unsuccessful, to remove this particle-bound activity using *T. weissflogii*. Further NiR measurements included the glass-bound NiR in an effort to estimate maximum enzyme activities.

3.2 Materials and Methods

3.2.1 Culture conditions

Unialgal cultures of the prymnesiophyte *Emiliania huxleyi* (NEPCC #732) and the marine diatoms *Actinocyclus* sp. (NEPCC #751) and *Thalassiosira weissflogii* (clone Actin, NEPCC #741) were obtained from the North East Pacific Culture Collection (NEPCC), Department of Botany, University of British Columbia. Cultures were maintained using ESAW artificial seawater medium (Harrison *et al.* 1980) with several modifications. Selenium and nickel were added at final concentrations of 10 nM and 63 nM respectively (Price *et al.* 1987, Price & Harrison 1988). Medium was filter-sterilized using autoclaved nitrocellulose membranes (pore size 0.8, 0.45 and 0.2 μm).

Cultures were maintained at 18.5 ± 0.5°C using acid-cleaned 3 L flat-bottom boiling flasks. Flasks were sterilized by autoclaving. Continuous saturating (Strzepek & Price submitted) irradiance (24 h) was provided at a photon flux density of 160 μmol m^{-2} s^{-1} using fluorescent bulbs (Vitalite) on either side of the cultures. To remove potentially contaminating atmospheric ammonia, cultures were bubbled with air filtered through a 5% sulfuric acid bath, distilled water and a 0.8/0.2 μm sterile filter set.

3.2.2 Growth and biomass measurements

Duplicate cell counts from each replicate flask were obtained using a Coulter particle counter. Daily growth rates were calculated from cell counts using the formula:

$$\mu = \ln (C_1/C_0)/(t_1-t_0) \quad (3.3)$$

where μ is the intrinsic rate of growth in units of per day, C_0 is the cell count at day zero (t_0) and C_1 is the cell count at day one (t_1). Cellular carbon and nitrogen quotas were obtained by filtering duplicate 5 ml aliquots of culture from each replicate flask onto pre-combusted (460°C, 4 h) 13 mm A/E (Gelman) glass fiber filters and analyzed with a Carlo Erba NCS analyzer NA 1500. Carbon and nitrogen were calculated against a standard curve generated from sulfanilamide standards. Nitrogen incorporation rate was calculated using the formula:

$$\mu PN = \mu \times (N\ cell^{-1}) \quad (3.4)$$

where μPN is the intrinsic rate of increase of particulate nitrogen in units of pg $cell^{-1}$ d^{-1} and N is cellular nitrogen in grams.

3.2.3 Enzyme Assay

Triplicate aliquots of culture were filtered onto a variety of filter types (Table 3.1). Filters were quickly folded, placed into 1.5 ml microcentrifuge tubes (Fisher), frozen and stored in liquid nitrogen. Samples were stored for no more than 10 d. Filters were ground in a 5 ml glass tissue homogenizer with a Teflon® pestle in 1 ml of ice cold homogenization buffer while immersed in an ice slurry.

Table 3.1. Filters used to harvest cultures for NiR assays.

Filter Type	Source	Pore Size	Treatment
25 mm Glass fiber A/E	Gelman	1.0 μm (nominal)	combusted (460°C for 4 h) and non-combusted
25 mm Glass fiber A/E	Gelman	1.0 μm (nominal)	combusted (460°C for 4 h) and silinized with Sigmacote®
25 mm Glass fiber GF/F	Whatman	0.7 μm (nominal)	combusted (460°C for 4 h) and non-combusted
25 mm Polycarbonate	Nuclepore	5 μm	none

Nitrite reductase activity was determined using a modified vascular plant assay (Vega *et al.* 1980). The NiR homogenization buffer consisted of 100 or 200 mM potassium phosphate buffer, at a range of pH with additions given in Table 3.2. Homogenates were either spun for 5 s at 15,000 rpm in an Eppendorf 5414 centrifuge to remove glass particulates or shaken to suspend

glass particles. Either clarified homogenate or homogenate containing glass particles was split between time zero and incubation tubes using 200 ml of homogenate including glass fibers. Assays were run in 1.5 ml microcentrifuge tubes (Fisher). Additions consisted of 2 mM KNO_2 and 1 mM methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dichloride) in a total of 0.8 ml phosphate buffer. Incubations were started by the addition of 200 µl of sodium dithionite at 1 mg ml^{-1} dissolved in a 0.29 M sodium bicarbonate solution. Time zero tubes were immediately stopped by oxidizing dithionite by shaking. All tubes were incubated in the light for approximately 15-20 min in the same temperature-controlled bath used for cultures. Incubations were stopped by oxidizing dithionite by shaking.

In assays performed on *T. weissflogii*, protein was precipitated in an effort to avoid NH_4^+ measurement error associated with the use of BSA in the homogenization buffer. Protein was precipitated using 70 µl of perchloric acid and centrifuged at 7,200 x g for 6 min. Perchloric acid was removed by precipitation of $KClO_4$ using 100 µl of 5N KOH (resulting pH ca. 7) and centrifuged at 7,200 x g for 4 min. The production of ammonium in NiR assays for all species tested was measured using the indophenol method (Solorzano 1969) with several modifications. Phenol and nitroprusside reagents were combined in equal proportions as recommended by Chaney and Marback (1962). Aliquots were reacted with ammonium reagents in a proportion of 300 µl sample : 200 µl phenol-nitroprusside solution : 400 µl alkaline solution in 1.5 ml disposable cuvettes. Samples were mixed using a vortex mixer following the addition of each reagent. Absorbance at 640 nm was read on a LKB Ultraspec II against a blank of phosphate buffer plus ammonium reagents. Linearity of the method was checked using algal homogenates spiked with a range of NH_4^+ concentrations.

Table 3.2. Compounds and concentrations of additives included in NiR homogenization buffer.

Compound	Source	Concentration	Function
BSA (bovine serum albumin) Fraction V Protease, γ globulin free Cat #A-3059	Sigma	30 mg ml^{-1}	Protease substrate
DTT (dithiothreitol)	Sigma	2 mM	protects S-H groups from oxidation
Na-EDTA (ethylenediaminetetracetic acid)	Sigma	5 mM	Protease inhibitor Protects from heavy metal attack
PVP (polyvinyl pyrolidone)	Sigma	3 mg ml^{-1}	Binds phenolic compounds
Detergents			Detergent Type
MTA (mixed alkyl-trimethylammonium bromides)	Sigma	10 ug ml^{-1}	Cationic
OGP (*n*-octyl-β-D-gluco-pyranoside)	Calbiochem	40 mM	Non-ionic
Triton X-100	Sigma	0.1% and 10%	Non-ionic
SDS (sodium dodecyl sulfate)	Sigma	0.6 mM	Anionic

Nitrite reductase assays were considered "optimized" when enzyme activity was maximal and/or accounted for 100% or more (excess activity) of the nitrogen incorporation rate defined as the particulate nitrogen growth rate (See Chapter 3 Methods).

3.3 Results

3.3.1 Ammonium method

As with the indophenol method for urease (Chapter 2), NH_4^+ detection was found to be linear for the range of NH_4^+ concentrations typically found in incubated enzyme assays (Fig. 3.1). Absorbance in the majority of assays fell between 0.1 and 0.5 absorbance units (640 nm).

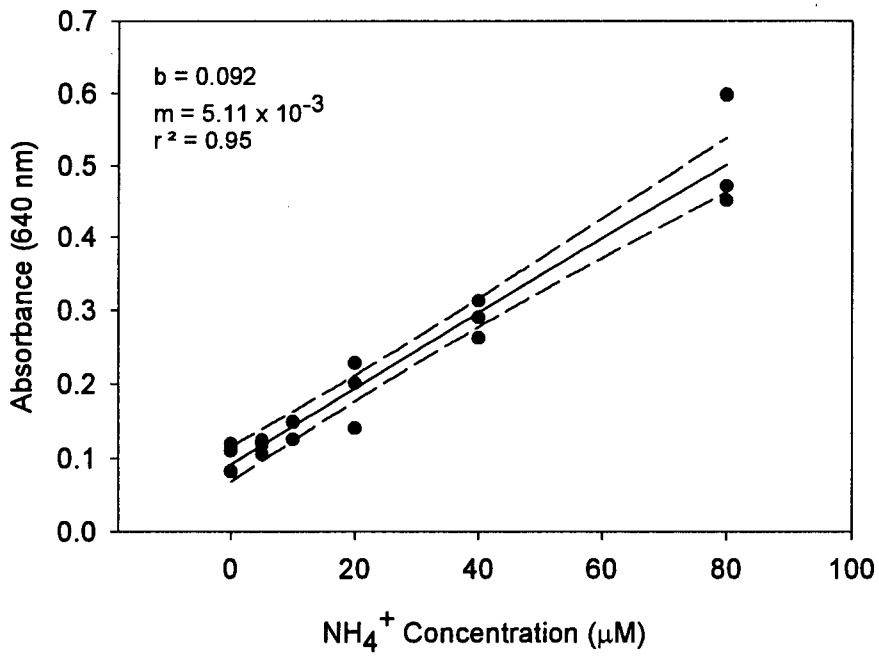

Figure 3.1. Ammonium measurement for NiR assay in algal homogenates using the indophenol method. A linear regression with 95% confidence limits is shown. Homogenates were spiked with NH_4Cl.

3.3.2 Homogenization buffer additions

Activity of NiR in *Actinocyclus* sp. was low or undetectable in all buffers used except when using buffer 2 (100 mM potassium phosphate buffer (pH 7.9), 3% BSA, 0.1% Triton X-100) where activity was much greater than the nitrogen incorporation rate (Fig. 3.2). Inclusion of EDTA in the homogenization buffer resulted in complete loss of activity which appears to be partially mitigated by the presence of PVP and DTT. In *Emiliania huxleyi*, NiR activity was observed in all buffers employed but activity was highest when using buffer 2. In all cases, activity exceeded nitrogen incorporation rates (Fig. 3.3). The negative impact of EDTA is absent in *E. huxleyi*. Further work employed buffer 2 (100 mM potassium phosphate buffer (pH 7.9), 3% BSA, 0.1% Triton X-100).

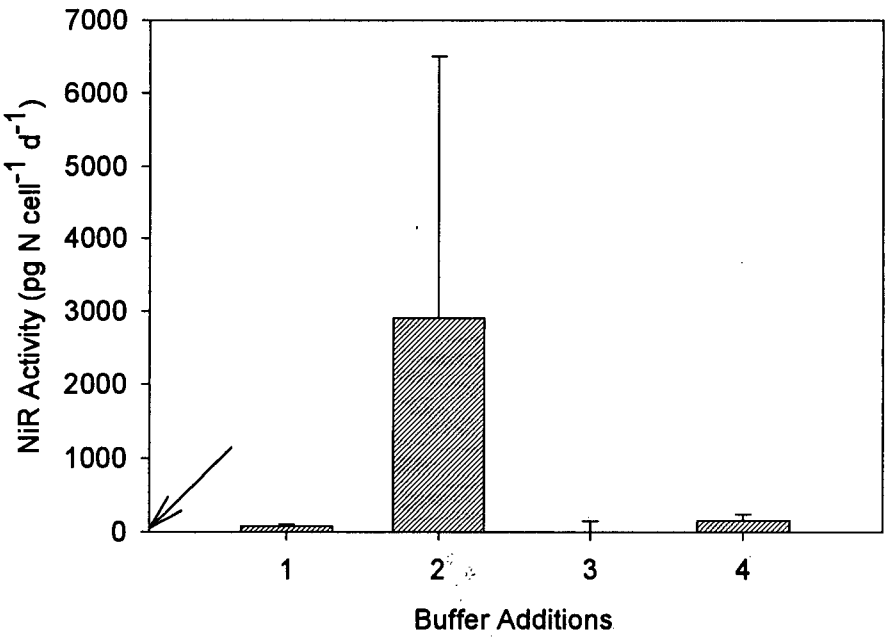

Figure 3.2. Buffer additions to NiR assay performed on *Actinocyclus* sp. Phosphate buffer (pH 7.9) with additions of: 1) Triton X-100 (0.1%), 2) BSA (3%) and Triton X-100 (0.1%), 3) BSA (3%), EDTA (5 mM) and Triton X-100 (0.1%), 4) BSA (3%), EDTA (5 mM), PVP (3 mg ml^{-1}), DTT (2 mM) and Triton X-100 (0.1%). The nitrogen incorporation rate (μPN) is indicated by the arrow (47 pgN $cell^{-1}$ d^{-1}). Error bars are ± 1 SD (n = 3).

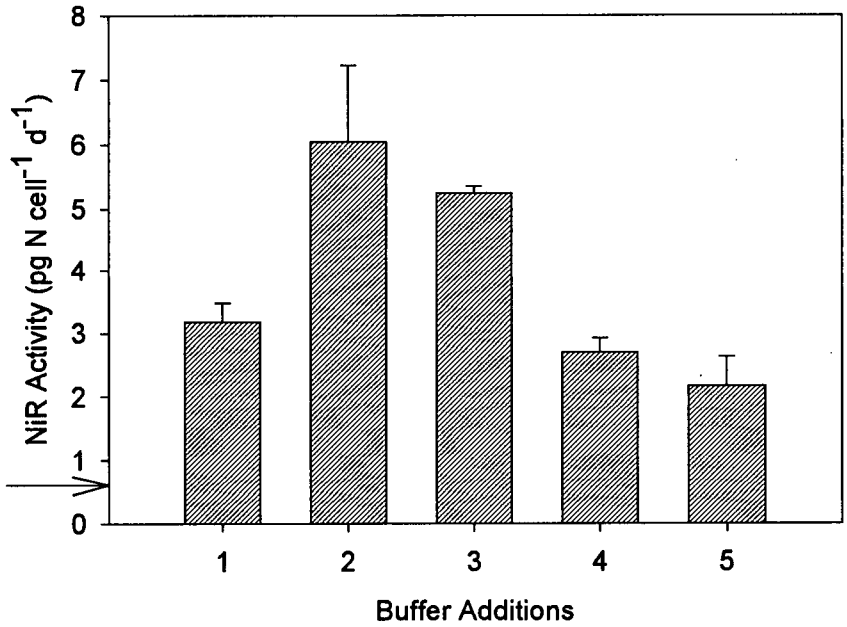

Figure 3.3. Buffer additions to NiR assay performed on *Emiliania huxleyi*. Phosphate buffer (pH 7.9) with additions of: 1) Triton X-100 (0.1%), 2) BSA (3%) and Triton X-100 (0.1%), 3) BSA (3%), DTT (2 mM) and Triton X-100 (0.1%), 4) BSA (3%), EDTA (5 mM), DTT (2 mM) and Triton X-100 (0.1%), 5) BSA (3%), EDTA (5 mM), DTT (2 mM) PVP (3 mg ml^{-1}) and Triton X-100 (0.1%). Nitrogen incorporation rate (μPN) indicated by arrow (0.6 pgN cell^{-1} d^{-1}). Error bars are ± 1 SD (n = 3).

3.3.3 Filters

When the homogenization buffer was applied to *T. weissflogii*, much of the NiR activity remained associated with ground glass fiber filter particles. Microscopic observation using epifluorescence revealed no intact chloroplasts but did show large (ca. 50 μm) masses of fluorescent photosynthetic membranes which could potentially contain NiR.

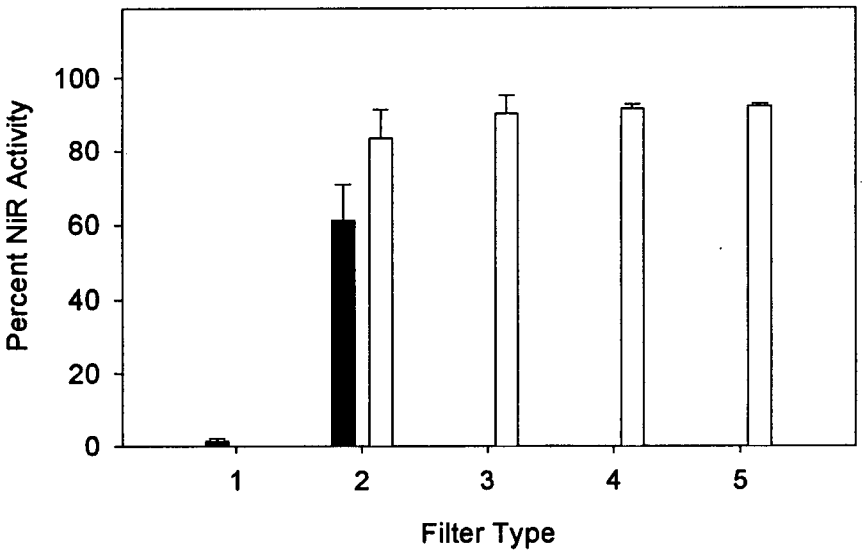

Figure 3.4. Activity of NiR in assays performed using different filter types on *Thalassiosira weissflogii* and either clarified homogenate (solid) or homogenate with ground filter particles included (open). Cells were harvested on: 1) polycarbonate filters (5 μm), 2) combusted and silinized AE glass fiber filters, 3) non-combusted AE glass fiber filters, 4) combusted AE glass fiber filters, 5) and non-combusted GF/F glass fiber filters. All assays were performed using buffer 2. Solid bars not present have zero activity. Error bars are ± 1 SD (n = 3).

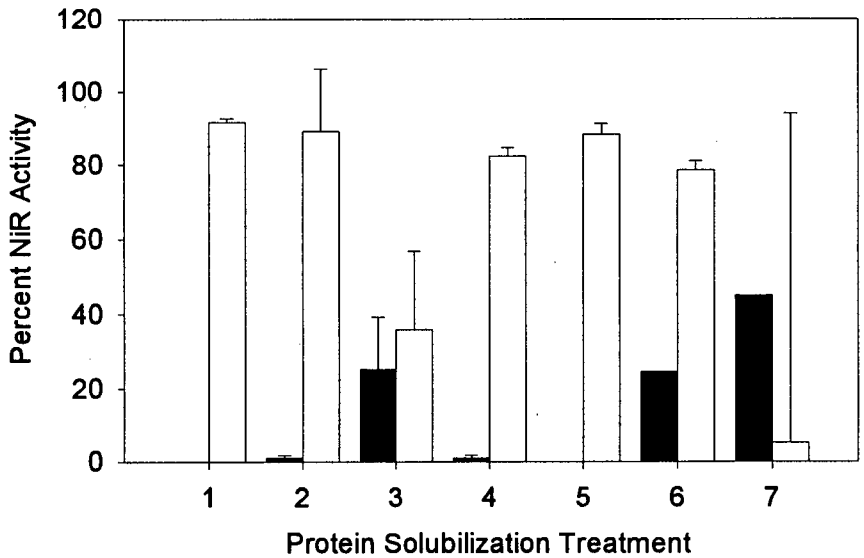

Figure 3.5. Activity of NiR in assays performed using different protein solubilization treatments on *Thalassiosira weissflogii* using either clarified homogenate (solid) or homogenate with ground filter particles included (open). Treatments to buffer 2 were: 1) No detergent 2) Triton X-100 (0.1%) 3) Triton X-100 (10%) 4) MTA (10 μg ml^{-1}) 5) OGP (40 mM) 6) SDS (0.6 mM) and 7) Toluene (10 μl ml^{-1}). Solid bars not present have zero activity. Error bars are ± 1 SD (n = 3). Error bars are too small to be resolved where not visible.

Of the several types of filters used (Table 3.1), only silinization of glass fiber filters resulted in removal of approximately 60% of NiR activity from glass fibers (Fig 3.4). In all treatments, higher NiR activities were associated with glass fibers. No activity was associated with the filter when polycarbonate filters were used as the cells were rinsed from the filter before homogenization, but poor activities were obtained.

3.3.4 Detergents and ionic strength

Use of several types of detergents (Table 3.2) as well as toluene facilitated removal of glass associated NiR activity to different degrees (Fig. 3.5). Of the methods employed, toluene removed the largest fraction of NiR but also gave the largest variance. Interestingly, a low concentration of the denaturing detergent SDS did not eliminate activity and removed the largest amount of NiR activity from glass fibers without strongly inhibiting overall activity.

Ionic strength of the homogenization buffer was altered by increasing potassium phosphate concentration to 200 mM. Thylakoid appression is enhanced by the presence of Mg^{+2}. To reduce total free Mg^{+2} concentration, 2 mM EDTA was added to 100 mM potassium phosphate buffer. Addition of EDTA to the homogenization buffer resulted in removal of glass associated NiR, but resulted in low activities as seen for *Actinocyclus* sp. (Fig. 3.6). Increasing the ionic strength of the homogenization buffer did not facilitate removal of NiR activity, but resulted in a decrease in activity and an increase in variability (Fig. 3.6).

For further work, buffer 2 (100 mM potassium phosphate buffer (pH 7.9), 3% BSA, 0.1% Triton X-100) was used and glass fibers were included in all assays in an effort to estimate maximum enzyme activities.

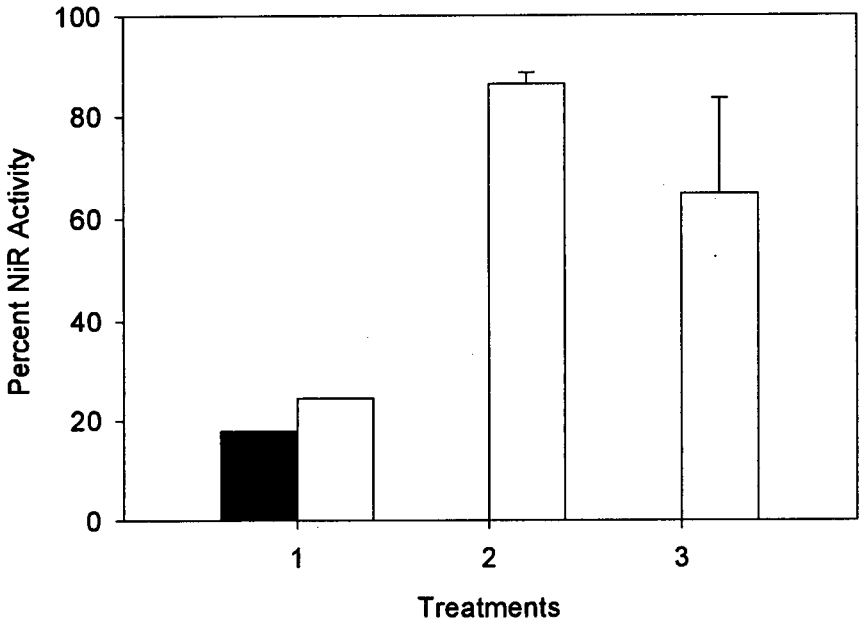

Figure 3.6. Activity of NiR in assays performed on *Thalassiosira weissflogii* using either clarified homogenate (solid) or homogenate with ground filter particles included (open). Treatments were made to buffer 2 using: 1) EDTA (5 mM) 2) low concentration phosphate buffer (100 mM) 3) high concentration phosphate buffer (200 mM). Solid bars not present have zero activity. Error bars are ± 1 SD (n = 3). Error bars are too small to be resolved where not visible.

3.3.5 Optimizing NiR for use with T. weissflogii

Nitrite reductase activity had a pH optimum of 7.9 in *Thalassiosira weissflogii* using potassium phosphate buffer (Fig. 3.7). Activity within crude homogenates stored on ice was found to be stable for up to two hours with some improvement in activity after one hour (Fig. 3.8). Variability was high immediately after grinding, but decreased after one hour and increased by the second hour of storage. Subsequent assays were run approximately one hour after the grinding step.

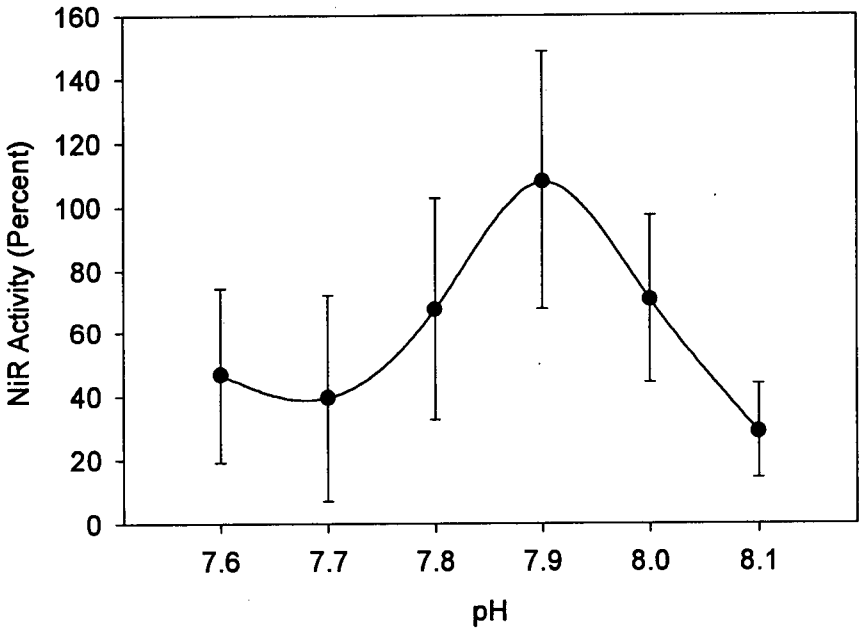

Figure 3.7. Optimization of NiR activity for pH in *Thalassiosira weissflogii*. Assays were performed using buffer 2. Error bars are ± 1 SD (n = 4).

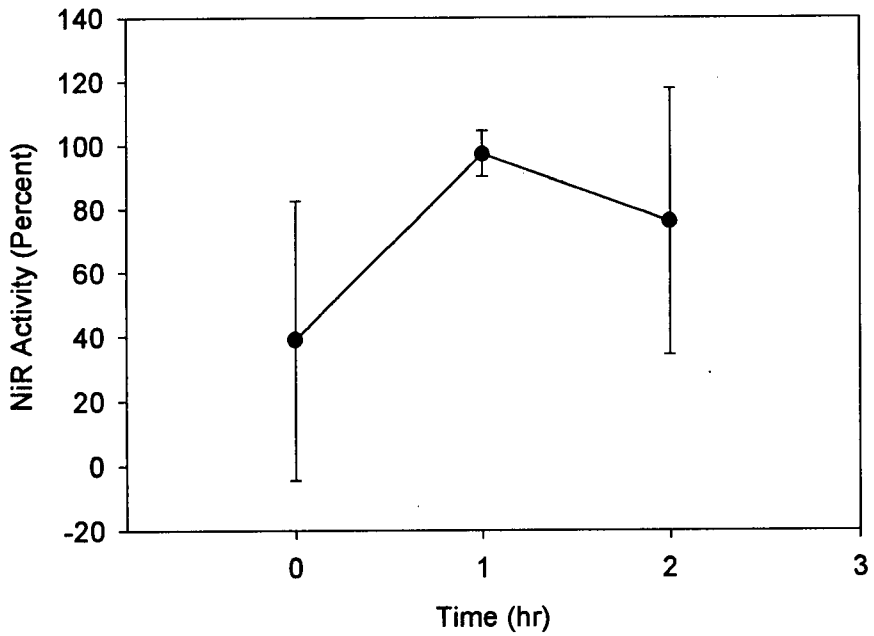

Figure 3.8. Stability of NiR in crude homogenates from *Thalassiosira weissflogii* stored on ice. Assays were performed using buffer 2. Error bars are ± 1 SD (n = 3).

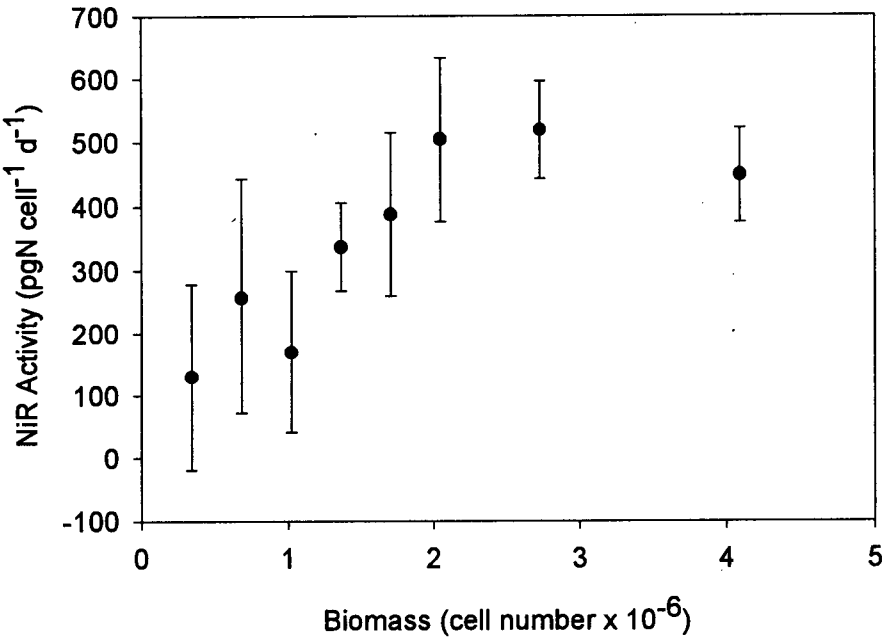

Figure 3.9. Biomass dependency of NiR activity in *Thalassiosira weissflogii*. Assays were performed using buffer 2. Error bars are ± 1 SD (n = 3).

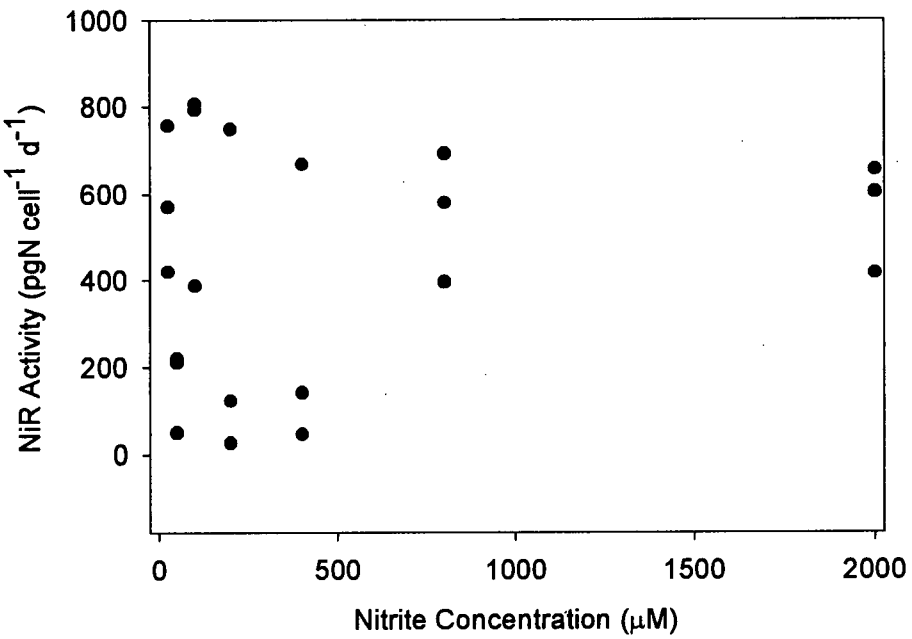

Figure 3.10. Substrate dependency of NiR activity in *Thalassiosira weissflogii*. Assays were performed using buffer 2.

The biomass harvested for NiR assays had an effect on the maximum activity. A cell biomass of 2-4 x 10^6 cells was found to give maximum enzyme activities (Fig. 3.9).

An attempt to obtain Michaelis-Menten kinetic parameters K_m and V_{max} was not successful, but did demonstrate that the use of 2 mM NO_2^- was sufficient to saturate NiR (Fig. 3.10).

3.4 Discussion

Nitrite reductase activity was measured in several marine phytoplankton using a modified homogenization buffer system. Measured activities were up to several orders of magnitude greater than nitrogen incorporation rates in these species.

Enzyme assays for vascular plant and algal NiR are almost exclusively based on the use of methyl viologen as an artificial electron donor in place of ferredoxin, the natural electron donor (Vega *et al.* 1980). Occasionally benzyl viologen is also used and is an effective artificial electron donor to NiR (Migge *et al.* 1997). The buffers employed are commonly potassium phosphate, Tris-HCl, or Hepes, but rarely are additions made in an effort to maximize or stabilize NiR. In addition, all NiR assays which are based on colorimetric methods measure the disappearance of NO_2^- rather than the production of NH_4^+. Obtaining sensitive measurements of the disappearance of NO_2^- requires the use of low concentrations of NO_2^- (e.g. 0.5 μM; Herrero & Guerrero 1986) and the possibility that substrate saturation of NiR will not be obtained. The half-saturation constant for NiR is particularly variable between species with values of 1 - 810 μM

reported (Guerrero *et al.* 1981). The method developed in this work measured the production of NH_4^+ and allows the use of high concentrations of NO_2^- (2 mM) to assure enzyme saturation.

Additions to the NiR homogenization buffer in this study had negative effects on NiR activity with the exception of bovine serum albumin (BSA) which gave maximal activities. Bovine serum albumin may act as a protease substrate and may also decrease binding on glass fibers used to harvest cells. In a review of NiR assay methods, Vega *et al.* (1980) recommended no additions to homogenization buffers. Krämer *et al.* (1989) included 100 μM EDTA in their NiR assay, but use of EDTA in this study resulted in decreased activities. Several investigators use a homogenization buffer designed for the measurement of NR and split the homogenate into NR and NiR assays. The addition of EDTA in the optimized NR assay method of Berges & Harrison (1995) precludes this approach.

Removal of NiR activity from glass fibers was the largest obstacle encountered using the method presented here. Of the several types of filters used, only silinization of glass fiber filters resulted in removal of some NiR activity from glass fibers, although higher activities remained associated with glass fibers. Silinization coats the glass fibers with a silicon-fluoride polymer which renders them hydrophobic and decreases nominal pore size, making it difficult to filter large volumes. Poor activities were obtained using polycarbonate filters indicating that glass fibers are important in breaking cells when a Teflon® tissue homogenizer is used.

Activity of NiR could not be removed from glass fibers using several detergents or by increasing the ionic strength of the homogenization buffer. Only the use of 0.6 mM SDS resulted in solubilization of some activity (30%) without a concomitant dramatic decrease in overall activity. Enzyme activities of NiR were measured by including the glass fibers in all incubations throughout the remainder of this thesis.

3.4.1 Enzyme activities in T. weissflogii

The pH optimum for NiR was determined to be 7.9 which is higher than the reported optimum of 7.5 for vascular plants, fungi and algae (Guerrero *et al.* 1981). Activity in crude homogenates was found to be dependent on storage time with one hour of storage on ice giving the highest and least variable activities. This most likely reflects the action of the detergent (Triton X-100) in breaking up observed membrane masses and solubilization of NiR. Hereafter, enzyme assays were performed approximately 1 h after grinding.

An attempt to obtain Michaelis-Menten kinetic parameters K_m and V_{max} was not successful. The lowest concentration of NO_2^- used was 25 μM which could potentially be saturating to NiR. Reported K_m values for NiR are as low as 1 μM (Guerrero *et al.* 1981).

Activity of NiR is often normalized to different biomass proxies. Activity in *T. weissflogii* was converted to some commonly reported units and is presented in Table 3.3 for comparison with others. Activity of NiR in *Chlorella fusca* (Krämer *et al.* 1988) compares well with the reported activity for *T. weissflogii* in this study. However, activity of NiR in this study is much greater than activities reported for pea chloroplasts, two species of cyanobacteria and two species of green algae (Table 3.3). Activity of NiR is dependent on the nitrogen demand of each organism which is dependent on the nitrogen content and growth rate. The nitrogen content differences were partly corrected for by using cellular protein to normalize NiR activities, and differences in growth rate were unlikely to account for NiR activities which were up to nine orders of magnitude lower than those measured in this study.

3.5 Conclusions

This is the first report of NiR activities in marine diatoms and of activities that can account for 100% or more of the nitrogen incorporated by nitrate-grown marine algae. Activities of NiR for *T. weissflogii* are among the highest reported values, but are within the same order of magnitude as those reported for a green microalga.

Removing NiR activity from the glass fibers in ground homogenates proved to be problematic. Although several detergents were employed without success many more are available that could potentially solublize the protein. In addition, the use of high energy sonication would preclude the use of filters and could potentially prove more effective at solublizing the protein.

Table 3.3. Comparison of NiR activity in exponentially growing *Thalassiosira weissflogii* with reported values from the literature. Activities were normalized using a protein content for *T. weissflogii* of 115.9 pg cell^{-1} (Peers pers. comm.). Reported activities from each study are for organisms grown in nutrient replete conditions.

Reported units	NiR activity in *T. weissflogii*	Organism	Reported activities from literature
µmol N (mg protein)$^{-1}$ h^{-1}	58.0	*Chlorella fusca* (freshwater green microalga)[1]	20.1
nmol N (mg Chl *a*)$^{-1}$ 5 min^{-1}	1.9 x 10^{5}	*Pisum sativum* chloroplasts (Pea)[2]	90
nmol N (mg protein)$^{-1}$ min^{-1}	966	*Anacystis nidulans* (cyanobacterium)[3]	34.1
nmol N (mg protein)$^{-1}$ 15 min^{-1}	1.45 x 10^{4}	*Anabaena flos-aquae* *Scenedesmus bijugatus* (both freshwater green microalgae)[4]	41 81
nmol N (mg protein)$^{-1}$ 15 min^{-1}	966	*Agmenellum quadruplicatum* (cyanobacterium)[5]	7.9 x 10^{-6}

1) Krämer *et al.* 1988, 2) Brunswick & Cresswell 1988a, 3) Herrero & Guerrero 1986, 4) Verstreate *et al.* 1980, 5) Hardie 1983.

4. Effects of non-steady state iron limitation on nitrogen assimilatory enzymes in the marine diatom *Thalassiosira weissflogii*

4.1 Introduction

There is accumulating evidence that iron rather than nitrogen may be limiting to phytoplankton biomass in the North Pacific gyre (Martin *et al.* 1989, Boyd *et al.* 1996), the equatorial Pacific (Price *et al.* 1994) and the Southern Ocean (Helbling *et al.* 1991). These areas have been termed high nitrate (or nutrient), low chlorophyll (HNLC) areas. In each of these areas, macronutrients are present at high concentrations (μM), however, iron is usually in the nM-fM (10^{-9} M-10^{-12} M) range (Martin *et al.* 1989).

Iron limitation has been hypothesized to affect nitrogen utilization in marine phytoplankton based on the theoretical calculations of Raven *et al.* (1992), where they estimated that algae growing on nitrate (NO_3^-) should require 60% more iron than those growing on ammonium (NH_4^+). These calculations have been verified by Maldonado & Price (1996) using laboratory cultures of several coastal and open ocean diatoms. They found that NO_3^--grown cultures require approximately 1.6 times more iron than NH_4^+-grown cells. However, iron-stressed cultures grown on NO_3^- did not have consistently higher carbon:nitrogen (C:N) ratios relative to cultures grown on NO_3^- under iron-replete conditions (Maldonado & Price 1996). A high C:N ratio would be expected if cells were deficient in their ability to metabolize NO_3^-.

Nitrate assimilation is mediated through the catalytic function of two Fe-containing enzymes, nitrate and nitrite reductases. The reduction of NO_3^- to NO_2^- by NR is thought to be the limiting step of nitrogen assimilation in phytoplankton based on the observation that NO_3^- can often accumulate in internal pools, while NO_2^- is not detected (Dortch *et al.* 1984). In vascular plants, the maximal activity for NiR is much higher (up to five times) than the activity for NR and its half-saturation constant (K_m [NO_2^-]) is much lower than the half-saturation constant (K_m [NO_3^-]) for NR (Layzell 1990).

In phytoplankton, under certain conditions, nitrite reduction may become the limiting step in nitrate assimilation resulting in the excretion of NO_2^- into the culture medium due to its intracellular toxicity. For example, cultures of a marine dinoflagellate excrete NO_2^- when supplied with NO_3^- following nitrogen starvation presumably due to induction of NR faster than NiR induction (Sciandra & Amara 1994). *Synechococcus* sp. (PCC7942) has been shown to excrete NO_2^- under CO_2 limitation as a result of decreased activity of NiR for up to 100 h suggesting that NiR limitation of nitrate assimilation can occur over long periods (Suzuki *et al.* 1995). Krämer *et al.* (1988) report NO_2^- excretion under CO_2 limitation in *Chlamydomonas fusca* and suggest that transport of NO_2^- into the chloroplast rather than NiR activity limits the reduction of NO_2^- to NH_4^+. The high iron requirement of NiR, as well as the higher reductant requirement of NO_2^- reduction support the hypothesis that nitrate assimilation may become rate limited at the NO_2^- reduction step during iron-limited growth. The goal of this chapter is to explore this possibility.

The major forms of nitrogen in marine systems are nitrate, ammonium and urea. Under Fe limitation, phytoplankton could potentially avoid the reduction costs of nitrate and utilize nitrogen from ammonium and urea. If Fe limitation causes a nitrogen stress then alternative nitrogen assimilatory pathways may be utilized. Enhancement of urea uptake has been reported for nitrogen starved cultures of *Phaeodactylum tricornutum* (Rees & Syrett 1979), although urease activity has not been measured in nitrogen starved diatoms.

I report here that under Fe limitation, nitrogen incorporation rates are not decreased due to activities of NR or NiR in the marine diatom *Thalassiosira weissflogii*. Under Fe- replete conditions NR is rate limiting to nitrate assimilation. Under Fe limitation the activity of NiR is dramatically decreased and nitrite reduction becomes the rate limiting step in the nitrate assimilatory pathway resulting in the excretion of nitrite. Activities of both NR and NiR remained in excess of nitrogen incorporation rates indicating that the supply of reductant to NiR rather than the amount of enzyme was responsible for the limitation of nitrate assimilation at the nitrite reduction step.

4.2 Materials and Methods

4.2.1 Experimental Design

A continuous time-series experiment was performed to evaluate algal physiological response to non-steady state Fe-limitation and recovery from Fe-limitation following an Fe addition. Under the culture conditions presented below, Fe-limitation can only be achieved by the cellular depletion of Fe in internal and external pools. In order to define Fe-limitation and recovery, Fe-replete cultures were also monitored and used to illustrate Fe-replete performance.

4.2.2 Culture conditions

A unialgal culture of the marine diatom *Thalassiosira weissflogii* (clone Actin, NEPCC #741) was obtained from the North East Pacific Culture Collection (NEPCC), Department of Botany, University of British Columbia. Cultures were maintained using ESAW artificial seawater medium (Harrison *et al.* 1980) with several modifications. Both nitrate and silicate were added at final concentrations of 200 μM. Selenium and nickel were added at final concentrations of 10 nM and 63 nM respectively (Price *et al.* 1987, Price & Harrison 1988b). Iron-replete medium was filter-sterilized using a series of autoclaved nitrocellulose membranes (pore size 0.8, 0.45 and 0.2 μm). Low-Fe medium was filter-sterilized using an acid-cleaned (10% reagent grade HCl followed by 0.1% quartz distilled HCl, Seastar) nitrocellulose membrane (pore size 0.2 μm) and acid-cleaned Teflon® filter housing. Low Fe medium was prepared in a metal-free class 100 flow hood using distilled deionized water (18MΩ · cm). No effort was made to remove contaminating Fe from stock solutions. Iron-replete cultures contained 15 μM Fe as $FeCl_3$ and 14.8 μM ethylenediaminetetracetic acid (EDTA) while Fe-limited cultures contained 22 nM Fe (SD = 4.2 n = 6) (measured using the ferrozine method of Stookey (1970)) and 8.3 μM EDTA. Iron addition following stress increased the Fe concentration to 15 μM and the EDTA concentration to 14.8 μM.

Triplicate Fe-replete and Fe-limited cultures were maintained at 18.5 ± 0.5°C using acid cleaned 3 L flat bottom boiling flasks. Flasks were sterilized by autoclaving. Continuous saturating irradiance (24 h) was provided at a photon flux density of 160 μmol m^{-2} s^{-1} using fluorescent bulbs (Vitalite) on either side of the cultures (Strzepek & Price submitted). All culture preparation was carried out in a metal-free class 100 flow hood. Cultures were bubbled with air filtered through a 5% sulfuric acid bath, distilled water and a 0.8/0.2 μm sterile filter set.

Total dissolved inorganic carbon was measured daily using an infrared gas analyzer (Analytical Development Co. 225-MK3) calibrated against a 449 ppm CO_2 gas standard to ensure carbon saturation.

4.2.3 Growth and biomass measurements

Duplicate samples for cell counts and cell volume from each replicate flask were obtained using a Coulter particle counter. Daily growth rates were calculated from cell counts using the formula:

$$\mu = \ln (C_1/C_0)/(t_1-t_0) \quad (4.1)$$

where μ is the intrinsic rate of growth in units of per day; C_0 is the cell count at day zero (t_0) and C_1 is the cell count at day one (t_1). Cellular carbon and nitrogen quotas were obtained by filtering duplicate 5 ml aliquots of culture from each replicate flask onto pre-combusted (460^{o}C, 4 h) 13 mm A/E (Gelman) glass fiber filters and analyzed with a Carlo Erba NCS analyzer NA 1500. Carbon and nitrogen were calculated against a standard curve generated from sulfanilamide standards. Nitrogen incorporation rate was calculated using the formula:

$$\mu PN = \mu \times (N\ cell^{-1}) \quad (4.2)$$

where μPN is the intrinsic rate of increase of particulate nitrogen in units of pg cell^{-1} d^{-1} and N is cellular nitrogen in grams. Chl *a* was determined fluorometrically on a Turner fluorometer model 10-AU calibrated against pure chl *a* (Sigma).

4.2.4 Variable fluorescence assay

Variable fluorescence (F_v/F_m) was determined using the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Triplicate 7 ml aliquots from each replicate culture flask were dark adapted for approximately 10 min in a temperature controlled bath. *In vivo* fluorescence before (F_O) and after (F_m) the addition of 75 μM DCMU was determined on a Turner fluorometer. Variable fluorescence was calculated according to the formula:

$$F_V/F_m = (F_O - F_m)/F_m \quad (4.3)$$

4.2.5 Carbohydrate and lipid measurements

Triplicate samples (20 ml) for cellular carbohydrate quotas were harvested from each replicate flask onto precombusted 25 mm A/E glass fiber filters stored frozen at -20°C and measured according to Kochert (1978). Cellular lipid quotas were obtained by filtering duplicate 100-200 ml aliquots from each replicate flask onto precombusted 25 mm A/E glass fiber filters and analyzed according to (Bligh & Dyer 1959).

4.2.6 Enzyme Assays

Samples for enzyme assays were all processed in the same manner. Triplicate aliquots of culture from each replicate flask were filtered onto pre-combusted 25mm A/E glass fiber filters. Filters were quickly folded, placed into 1.5 ml microcentrifuge tubes (Fisher), frozen and stored in liquid nitrogen. Samples were stored for no more than 10 d. Filters were ground in a 5 ml glass tissue homogenizer with a Teflon® pestle in 1 ml of ice cold homogenization buffer while immersed in an ice slurry.

Nitrite reductase activity was determined using a vascular plant assay which was optimized for use with diatoms (See Chapter 3). The NiR homogenization buffer consisted of 100 mM potassium phosphate buffer (pH 7.9) with 3% bovine serum albumin (BSA) and 0.1% Triton X-100.

Methyl viologen can serve as an electron donor for both eukaryotic and prokaryotic NiR. Therefore, NiR activities were corrected for contaminating bacteria by performing size fractionated enzyme assays. Bacteria were separated from *T. weissflogii* by filtering through a 5 μm polycarbonate membrane filter and collected on 25 mm A/E glass fiber filters. Bacterial cells caught on glass fiber filters were determined using cell counts before and after filtering. Bacterial cell counts were performed on cells harvested using 0.2 μm, 25 mm polycarbonate filters (Poretics) and counted visually using acridine orange stain and epifluorescence microscopy.

Nitrate reductase activity was determined according to Berges & Harrison (1995), except a 100 mM potassium phosphate buffer (pH 7.9) was used rather than the 200 mM potassium phosphate buffer recommended by Berges & Harrison (1995), to avoid precipitation at 0°C. Urease activity was determined according to Peers *et al.* (Appendix A). The homogenization buffer consisted of 150 mM phosphate buffer (pH 7.9) with 0.1% w/v polyvinyl pyrolidone and 5 mM EDTA. Urease activity of contaminating bacteria was corrected for by size fractionation. Bacteria were separated from *T. weissflogii* by filtering through a 5 μm polycarbonate membrane filter and collected on 25 mm A/E glass fiber filters.

4.2.7 Internal and external nitrogen measurements

Internal pools of NO_3^- and NO_2^- were determined on 0.2 ml sub-samples obtained from urease homogenates diluted to 1 ml with distilled deionized water. Both internal and external

NO_2^- concentrations were determined colorimetrically according to Parsons *et al.* (1984).

Internal and external NO_3^- was determined using the spongy cadmium method of Jones (1984).

Efflux of nitrite was corrected for the bacterial contribution by size fractionation. Bacteria were separated from *T. weissflogii* by filtering through a 5 μm polycarbonate membrane filter incubated in 30 ml sterilized borosilicate tubes in the same incubator used for culturing. Nitrite concentration was monitored over a 24 h period.

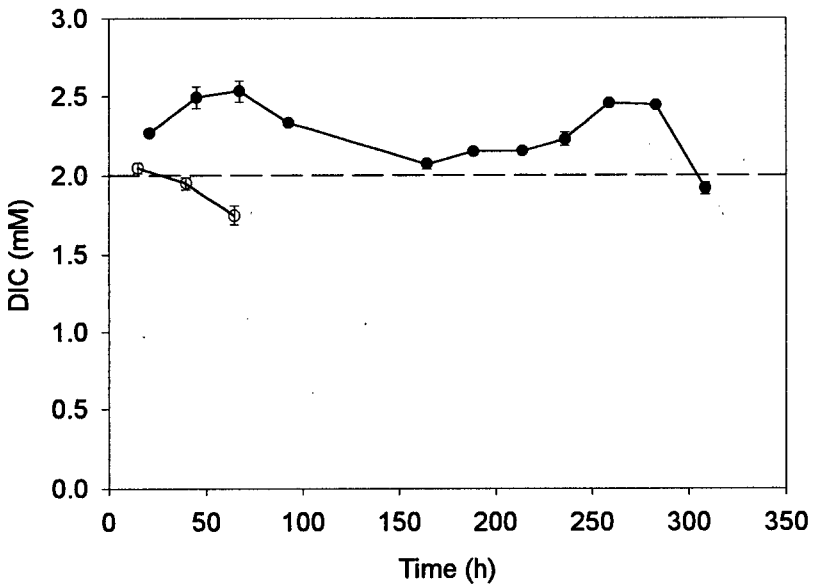

Figure 4.1. Total dissolved inorganic carbon (DIC) in cultures of *T. weissflogii* grown under Fe-limited (●) and Fe-replete (○) conditions. The broken line represents DIC saturation (2 mM) of seawater at a salinity of 30 and a pH 8.2. Error bars are ±1 SD calculated from three replicate cultures. Error bars not visible are smaller than symbols.

4.3 Results

During the course of the experiment dissolved inorganic carbon (DIC) levels never became limiting to growth. Cultures remained around 2.25 mM DIC (range ± 0.25) for Fe-limited cultures and 1.9 mM DIC (range ± 0.15) for Fe-replete (control) cultures (Fig. 4.1)

The time course of culture response to Fe-limitation and recovery is shown in the DCMU induced fluorescence signal F_v/F_m (Fig. 4.2A). During growth in the iron-limited culture medium, cells became iron stressed as indicated by the drop in variable fluorescence from 0.67 to 0.48. At 170 hr, cultures reached maximum biomass and were diluted with Fe-deplete medium (Arrow 1, Fig 4.2A). Cultures become increasingly Fe stressed and variable fluorescence decreased to a minimum of 0.32 at 267 h. Upon the addition of 15 μM Fe, variable fluorescence began to recover and increased from 0.32 to 0.63. Cultures reached maximum biomass at 295 hr and were diluted with Fe-replete medium (Arrow 2, Fig. 4.2A). By 310 hr (48 hr following Fe addition) variable fluorescence recovered fully to 0.68. In Fe-replete cultures maximum biomass was attained within 65 hr. Throughout Fe-replete growth, variable fluorescence remained around 0.67 (Fig. 4.2B).

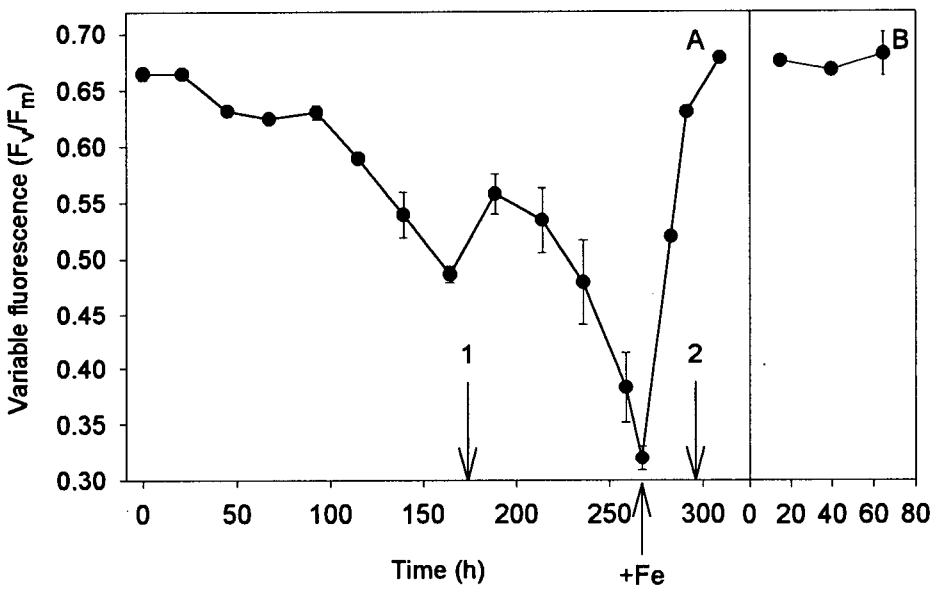

Figure 4.2. DCMU induced variable fluorescence in A) Fe-limited (growth period 0-350 h) and B) Fe-replete cultures (growth period 0-80 h). Iron-limited cultures were diluted with 1) Fe-free medium and 2) Fe-replete medium at indicating arrows. Chelated Fe (15 μM Fe-EDTA) was added to Fe-limited cultures at 267 h (+Fe arrow). Error bars are 95% CI calculated from three replicate cultures. Error bars not visible are smaller than symbols.

Under Fe-limited conditions growth rate and cell volume decreased by approximately 40% (Table 4.1). Carbon and nitrogen quotas also decreased by 30 and 23% in Fe-limited cultures respectively, however, C:N ratios were not different. Chl *a* $cell^{-1}$ and carbohydrate and lipid quotas also decreased under Fe limitation, but carbon normalized values were equivalent in both Fe-replete and Fe-limited cultures.

Cellular nitrite excretion during Fe-limited growth increased to a maximum of 100 fmol $cell^{-1}$ d^{-1} while in Fe-replete cultures, nitrite excretion remained low or was negative (Fig. 4.3).

Contaminating bacteria took up nitrite rather than effluxing it, but rates were insignificant when compared to nitrite changes in cultures (data not shown). Following maximum nitrite excretion, excretion decreased during Fe-limited growth and upon the addition of Fe,

Table 4.1. Comparison of growth rate and cellular parameters in Fe-replete and Fe-limited conditions for *Thalassiosira weissflogii*. Iron-limited values do not include measurements made following iron addition.

	+Fe (95% CI)	-Fe (95% CI)
Growth rate μ (d^{-1})	0.85 (0.04)	0.47 (0.15)
Cell volume (μm^3)	505.6 (22.2)	342.6 (25.6)
C quota (pmol · $cell^{-1}$)	13.76 (1.47)	9.67 (2.35)
N quota (pmol · $cell^{-1}$)	2.12 (0.45)	1.63 (0.35)
C:N (molar)	6.66 (1.00)	6.25 (0.86)
Carbohydrate (pg · $cell^{-1}$)	21.6 (2.9)	15.6 (7.6)
Lipid (pg · $cell^{-1}$)	42.7 (1.6)	26.1 (4.0)
Chl *a* (pg · $cell^{-1}$)	2.95 (0.26)	2.20 (0.32)

and NO_2^- excretion decreased further to levels observed for Fe-replete cultures. Internal pools of NO_3^- in Fe-limited cells varied between 1 and 3 fmol $cell^{-1}$ and were significantly positively correlated ($R = 0.50$, $p < 0.01$) to external NO_3^- concentrations, while internal NO_2^- pools remained relatively constant and were not correlated with external NO_2^- concentrations ($R = 0.01$, $p = 0.57$) (Fig. 4.4).

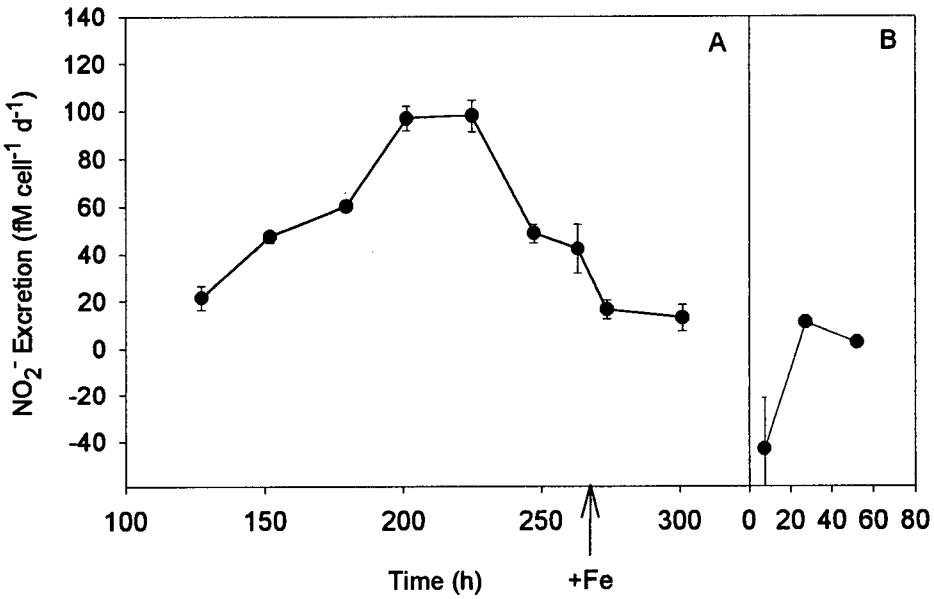

Figure 4.3. Nitrite excretion rates in A) Fe-limited and B) Fe-replete cultures. Chelated Fe (15 μM Fe-EDTA) was added to Fe-limited cultures at 267 h (+Fe arrow). Error bars are 95% CI calculated from three replicate cultures. Error bars not visible are smaller than symbols.

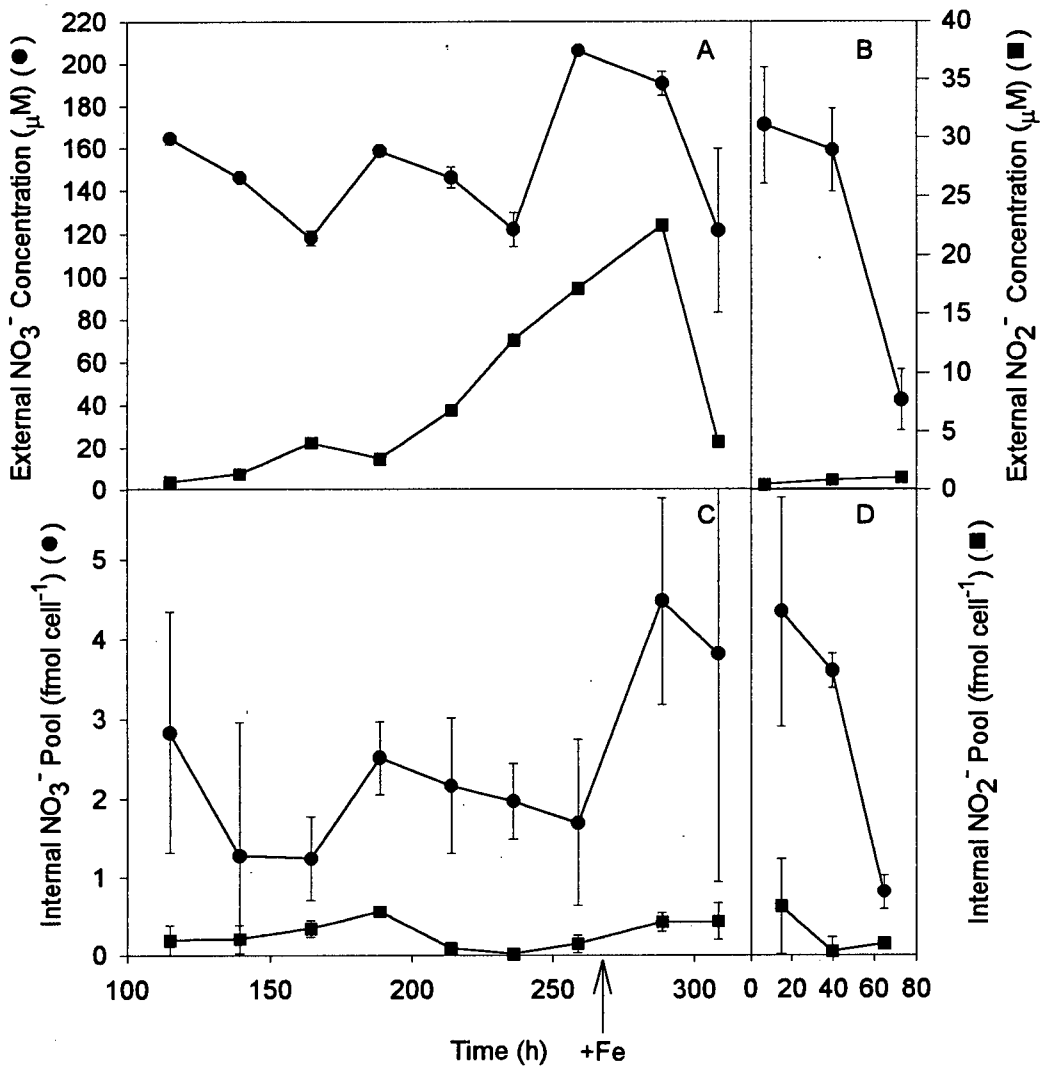

Figure 4.4. External and internal dissolved nitrogen concentrations. A) External NO_3^- (●) and NO_2^- (■) in Fe-limited cultures. B) External NO_3^- (●) and NO_2^- (■) in Fe-replete cultures. C) Internal NO_3^- (●) and NO_2^- (■) in Fe-limited cultures. D) Internal NO_3^- (●) and NO_2^- (■) in Fe-replete cultures. Chelated Fe (15 μM Fe-EDTA) was added to Fe-limited cultures at 267 h (+Fe arrow). Error bars are95% CI calculated from three replicate cultures. Error bars not visible are smaller than symbols.

Following the addition of 15 μM Fe, internal NO_3^- pools increased to levels similar to controls while internal NO_2^- pools remained unchanged.

Contaminating bacteria contributed to NiR activity (about 15%) and all reported NiR activities are corrected for this contaminating amount. In Fe-replete cultures, the activity of NiR remained roughly two orders of magnitude higher than NR activities (Fig. 4.5B). Under Fe-limiting conditions, however, NiR activities decreased dramatically (30-50 fold) (Fig. 4.5A). After the Fe addition, NiR activity increased, but did not recover to control levels within 48 h. Throughout Fe-limited growth, activities of both NR and NiR remained higher than the nitrogen incorporation rate (μPN). The ratio of NR:NiR activity ranged from 0.01 to 0.07 in Fe-replete cultures (Fig. 4.6B) while in Fe-limited cultures NR:NiR activity ranged from 0.27 to 0.45 (Fig. 4.6A). After the addition of 15 μM Fe, NR:NiR activity decreased dramatically, primarily due to the large increase in NiR activity. Changes in the NR:NiR ratio did not correspond to changes in the excretion rate of nitrite. Urease activity showed no response to Fe limitation as activities under Fe-limited conditions remained similar to controls (Fig. 4.7), however activities were similar to required nitrogen incorporation rates (Fig. 4.5A).

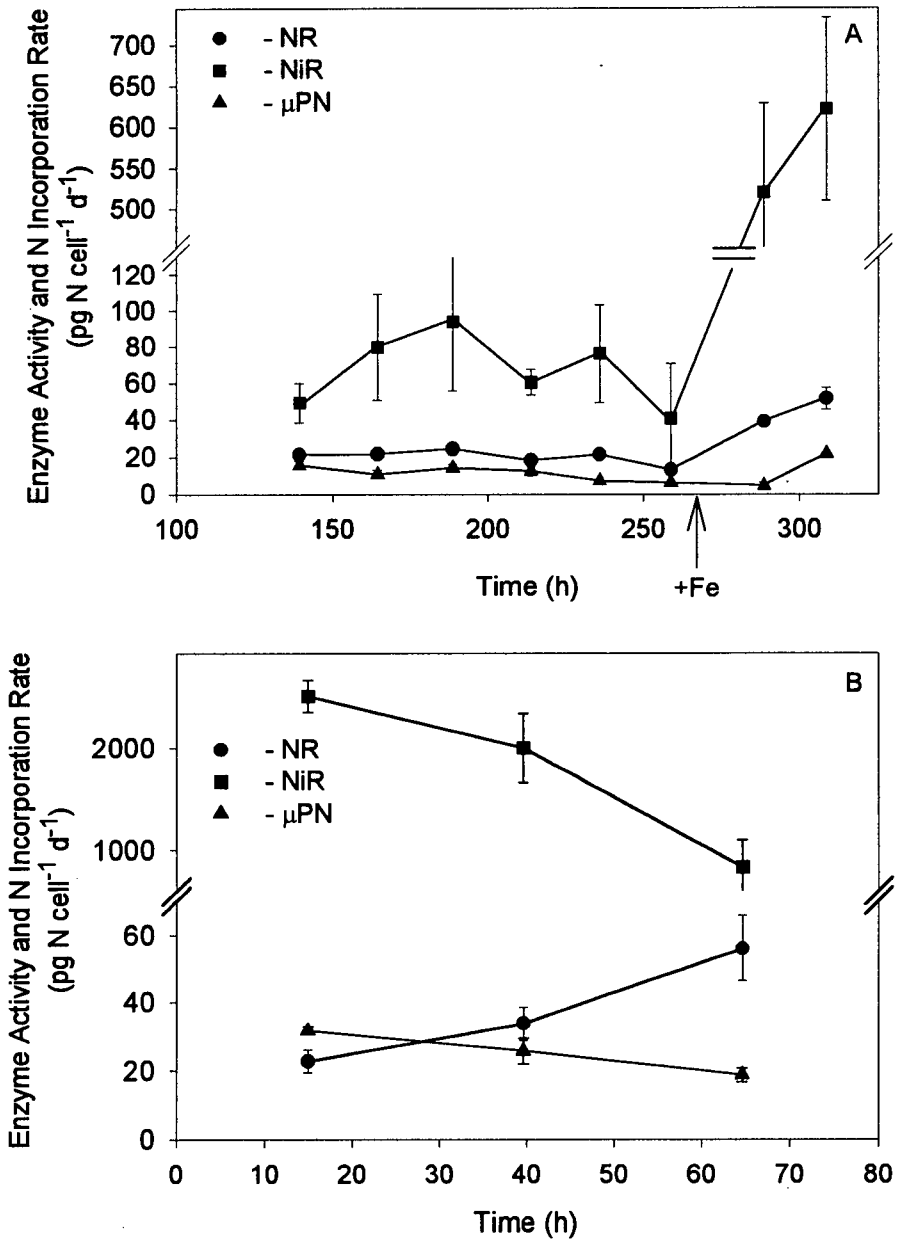

Figure 4.5. A) Enzyme activities of NR (●), NiR (■) and nitrogen incorporation rate (μPN, ▲) in Fe-limited cultures. Chelated Fe (15 μM Fe-EDTA) was added to Fe-limited cultures at 267 h (+Fe arrow). B) Enzyme activities of NR (●), NiR (■) and nitrogen incorporation rate (μPN, ▲) in Fe-replete cultures. Error bars are 95% CI calculated from three replicate cultures. Error bars not visible are smaller than symbols.

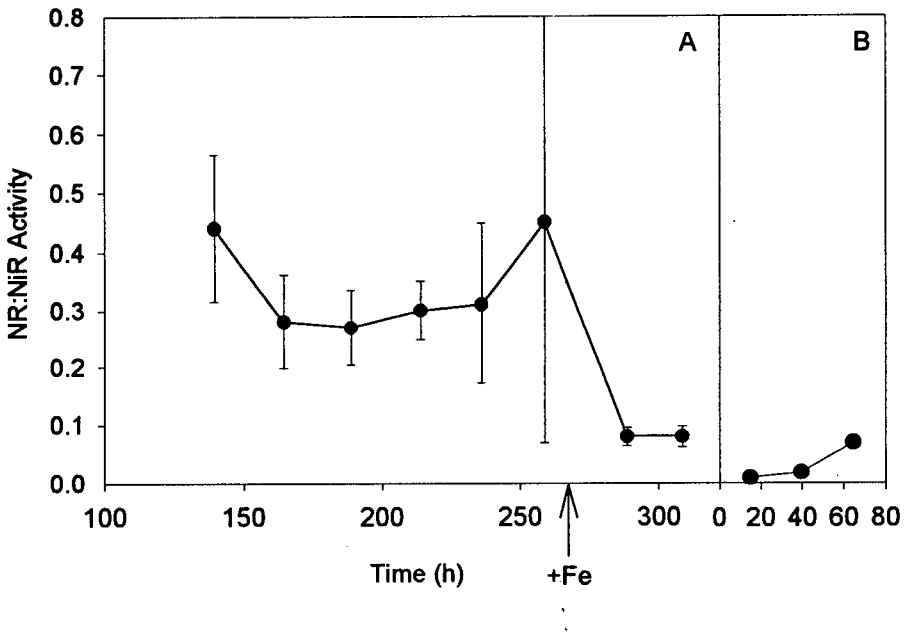

Figure 4.6. Activity ratios of NR:NiR in A) Fe-limited and B) Fe-replete cultures. Chelated Fe (15 μM Fe-EDTA) was added to Fe-limited cultures at 267 h (+Fe arrow). Error bars are 95% CI calculated from three replicate cultures. Error bars not visible are smaller than symbols.

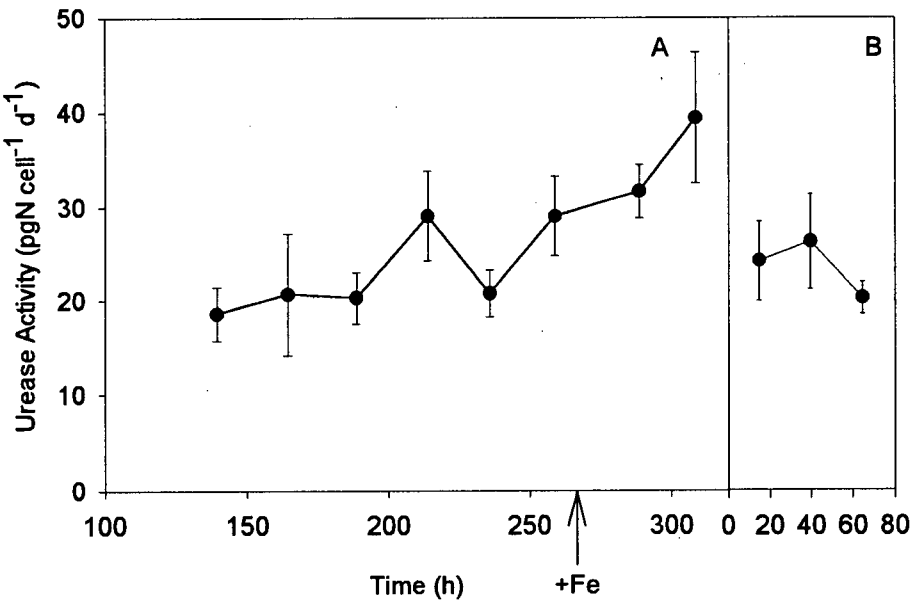

Figure 4.7. Urease activity in A) Fe-limited and B) Fe-replete cultures. Chelated Fe (15 μM Fe-EDTA) was added to Fe-limited cultures at 267 h (+Fe arrow). Error bars are 95% CI calculated from three replicate cultures. Error bars not visible are smaller than symbols.

4.4 Discussion

Since the recognition of Fe-limited HNLC regions of the ocean, Fe limitation has been hypothesized to limit the use of nitrate by diatoms (See Hutchins 1995 for review). Carbon:nitrogen data from laboratory cultures (Maldonado & Price 1996) as well as field samples from HNLC regions do not support this hypothesis. Timmermans *et al.* (1994) has demonstrated decreased activity of NR in several marine phytoplankton grown under Fe-limited conditions, however, a decrease in growth rate, which was not reported in this study, could also be responsible for the observed decreases in NR activity.

My measurements of *in vitro* NR activity agree with results obtained for *Emiliania huxleyi* by Timmermans *et al.* (1994). Activity of NR decreased by roughly 60% under Fe limitation, however, *in vitro* NiR activity decreased by approximately 50-fold. Activities of both enzymes were sufficient to meet the nitrogen demands of the cell. A decrease in NiR activity due to low Fe availability has been observed for green algae as well. When Kessler & Czygan (1968) used the Fe chelator EDTA to control Fe availability, they found that low Fe availability specifically decreased the activity of NiR, but not NR in the green alga *Ankistrodesmus braunii*. Nitrite reductase has a high requirement for Fe (five Fe atoms per active enzyme vs. two Fe atoms per active enzyme in NR).

The decrease in NiR activity could supply Fe to other parts of the cell. Using a range of specific activities for purified NiR of 52 - 108 U (mg protein)$^{-1}$ (Guerrero *et al.* 1981) and assuming five Fe atoms per enzyme, approximately 68 - 141 amol (10^{-18} moles) Fe would be made available from the observed decrease in NiR enzyme activity. Using an average Fe:C ratio of 45 μmol mol^{-1} from Sunda (1988/89) and Maldonado & Price (1996) and the carbon quota

from this study (Table 1), an Fe quota of 624 amol cell^{-1} was calculated. The change in NiR activity could make 11 - 22% of the total cellular Fe available for other purposes. Activity of NiR in excess of nitrogen incorporation rate requirements represents a significant pool of Fe in *T. weissflogii*. Since specific activities for purified NiR in diatoms are not available, an underestimation of NiR specific activity results in an overestimation of the Fe pool associated with NiR.

In vitro activity of NiR remained well above nitrogen incorporation rates during Fe-limited growth. Assuming that NiR activities were not overestimated due to methodology, high activity relative to the nitrogen incorporation rate suggests that either reductant supply to NiR or transport of NO_2^- into the chloroplast limited the assimilation of nitrite rather than the amount of enzyme. Limitation of nitrite assimilation at the transport step into the chloroplast has been suggested for *Chlorella fusca* and is hypothesized to involve regulation at a NO_2^- specific chloroplastic porter (Krämer *et al.* 1988).

Reductant supply to NiR in the form of ferredoxin or flavodoxin is dependent on the rate of electron flow through the photosynthetic electron transport system (PETS) and could be controlled by Fe limitation. Iron limitation has been shown to decrease electron transfer efficiency within PSII (Vassiliev *et al.* 1995) and between PSII and PSI due to the loss of cytochrome b6/f in *Dunaliella tertiolecta* (Greene *et al.* 1992). Iron limitation has also been shown to increase PSII:PSI stoichiometry in the marine diatom *Phaeodactylum tricornutum* (Greene *et al.* 1991) and the freshwater cyanobacterium *Aphanocapsa* sp. (Sandmann 1985) and has been indirectly observed for the marine green alga *Dunaliella tertiolecta* (Vassiliev *et al.* 1995). A decrease in both the electron transfer efficiency to PSI and numbers of PSI reaction centers can result in a decrease in the production of reductant. The variable fluorescence data for

T. weissflogii is consistent with the idea that Fe limitation causes a decrease in electron transfer efficiency within the PETS.

Under Fe-replete conditions, ferredoxin supplies the reducing power to NiR. Under low Fe supply, ferredoxin is replaced by the non-iron containing flavodoxin (La Roche *et al.* 1993 Doucette *et al.* 1996), which has been shown to be a less efficient electron donor in some enzyme systems (Zumft & Spiller 1971). A decrease in NiR activity due to electron transfer efficiency of flavodoxin is unlikely as the expression of flavodoxin is an early response to low Fe availability and is most likely present throughout the Fe-limited growth period where NO_2^- efflux varies by an order of magnitude. The marine diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* both produce flavodoxin as an early response to low Fe, while low growth rates and other biological indicators of Fe stress are absent (McKay *et al.* 1997). In the green alga *Chlorella fusca*, flavodoxin is produced under Fe limitation while maintaining normal cellular levels of cytochromes and NiR activity (Zumft & Spiller 1971).

Internal pools of NO_3^- were correlated ($p < 0.01$) to the external concentration of nitrate although only 25% ($R^2 = 0.25$) of the variation in internal pools could be explained by variation in external concentrations. The internal pool of NO_2^- appeared to be tightly regulated as there was little change in the size of the pool under Fe limitation or sufficiency, while excretion rates of nitrite varied by an order of magnitude. Nitrite excretion has been observed in a number of marine phytoplankton (Laws & Wong 1978, Serra *et al.* 1978, Collos 1982, Martinez 1991, Sciandra & Amara 1994, Flynn & Flynn 1998). Phytoplankton excretion of nitrite is thought to be responsible for the upper nitrite maximum observed throughout the oceans where concentrations have been observed to reach as high as 5 μM (Wada & Hattori 1971). Nitrite

excretion is hypothesized to be caused by an imbalance between nitrate and nitrite reduction. Several possible mechanisms have been suggested to cause nitrite release: 1) uptake and reduction of NO_3^- under low light conditions where supply of photosynthetically derived reductant is limiting NO_2^- reduction (Martinez 1991), 2) uptake and reduction of NO_3^- under low CO_2 supply where carbon skeletons for the synthesis of amino acids limit the assimilation of ammonium and, through an unknown feedback mechanism, NO_2^- reduction (Krämer *et al.* 1988, Suzuki *et al.* 1995) and 3) uptake and reduction of NO_3^- by previously nitrogen starved cells where *de novo* synthesis and/or activation of NR may be faster than NiR activity (Sciandra & Amara 1994, Flynn & Flynn 1998). Case 3 does not apply to the experimental conditions of this study.

Nitrite excretion during Fe-limited growth most likely resembles limitation of NO_2^- reduction by supply of photosynthetically derived reductant and is supported by variable fluorescence data. Both bulk carbohydrate and lipid pools were unaffected by Fe limitation in *T. weissflogii* and total dissolved inorganic carbon levels remained near saturation (saturation = 2 mM) throughout the experiment suggesting that supply of carbon skeletons for nitrogen assimilation did not become limiting.

Excretion rates of nitrite are not correlated with either NR:NiR ratios or NiR activity in *T. weissflogii* indicating that activities of these enzymes would make poor indicators of NO_2^- efflux in field samples, although they may be useful as biological indicators of Fe limitation. Excretion rates of nitrite declined prior to the addition of Fe indicating acclimation of the nitrate assimilatory system to low Fe availability. Given more time to acclimate, or growing cultures under steady-

state Fe supply by using trace metal buffered Aquil medium (Price *et al.* 1988/89), may result in balanced growth and lower NO_2^- excretion rates.

Activity of urease under Fe limiting conditions is not different from controls indicating that there is no induction of an alternative nitrogen assimilation pathway. However, no induction is necessary as urease activity is high enough to assimilate all the nitrogen necessary for growth. Transport of urea was not investigated, but it could potentially give different results. Transport of urea has been reported to increase under nitrogen starvation (Rees & Syrett 1979).

Nitrogen incorporation rates in Fe-limited cultures do not appear to be decreased relative to carbon incorporation rates as indicated by the similarity in C:N ratios. Others have routinely observed little difference in C:N ratios between NO_3^--grown phytoplankton under Fe-limited and Fe-replete conditions. Differences between C:N ratios have been reported to range from 3.5 units lower for Fe-limited *Thalassiosira pseudonana* (clone 3H) to 2 units higher for Fe-limited *Thalassiosira oceanica* (clone 13.1, Maldonado & Price 1996). Muggli & Harrison (1996) report an increase in C:N ratios from 8.6 to 13 for *Emiliania huxleyi* grown under low Fe; however, there was no difference between NH_4^+ or NO_3^--grown cultures indicating that the Fe effect did not specifically involve the nitrate assimilatory pathway. Doucette & Harrison (1991) found that the C:N ratio of the marine dinoflagellate *Gymnodinium sanguinium* was much higher under Fe-limited growth on NO_3^- (C:N = 6.3) vs. NH_4^+ (C:N = 3.8), but there was no statistical difference in C:N ratios between NO_3^--grown Fe-deplete (C:N = 6.3) and Fe-replete (C:N = 7) cultures, again suggesting that the Fe effect on NH_4^+ and NO_3^--grown cultures did not specifically involve the nitrate assimilatory pathway.

Despite the increased iron requirements of nitrogen assimilation, the limitation of nitrogen incorporation by low Fe availability has not been observed in C:N ratios or growth rates when comparing NH_4^+ and NO_3^--grown eukaryotic (Maldonado & Price 1996) and prokaryotic (Kudo & Harrison 1997, Henley & Yi 1998) phytoplankton. This work suggests that nitrate assimilatory enzyme activities are in excess of the nitrogen requirements of *T. weissflogii* and that the primary effect of iron limitation is on the light reactions of photosynthesis. Iron-limited phytoplankton are essentially energy limited, not Fe-nitrogen co-limited. Limitation at the point of the PETS can explain a decrease in phytoplankton growth rates without a change in C:N ratios. Observations of nitrogen incorporation patterns by Reuter & Ades (1987) agree with this finding. They found that the iron-limited freshwater green alga *Scenedesmus quadricauda* preferentially incorporated nitrogen into protein which is similar to the nitrogen incorporation pattern observed in light limited algae (Morris *et al.* 1974, Glover 1977).

The experimental conditions of this work provide Fe under non-steady state conditions. Excretion rates of NO_2^- in *T. weissflogii* declined prior to the addition of Fe indicating cultures may be acclimating to low Fe availability. The supply of Fe under steady state conditions by using trace metal buffered Aquil medium (Price *et al.* 1988/89) may result in balanced growth and lower NO_2^- excretion rates. Chapter 5 explores this possibility.

5. Effects of steady state iron limitation on nitrogen assimilatory enzymes in the marine diatom *Thalassiosira weissflogii*

5.1 Introduction

Nitrogen metabolism in phytoplankton is influenced by the availability of iron. While non-steady state iron limitation does not influence the ability of *T. weissflogii* to incorporate nitrogen, it does influence nitrite reduction resulting in nitrite efflux (Chapter 4). In the previous chapter, excretion rates of NO_2^- declined prior to the addition of iron, indicating an acclimation of the nitrate assimilatory system to low iron availability. The goal of this chapter is to investigate the influence of steady state iron limitation on nitrogen metabolism and assimilation.

5.1.1 Iron uptake in marine phytoplankton

Uptake of most trace metals is believed to be controlled by the concentration of the uncomplexed free metal ion which is in equilibrium with complexed species (Sunda & Guillard, 1976). In the case of iron, the major dissolved inorganic species in seawater are Fe-hydroxides. Available iron is in equilibrium with organic and inorganic ligands and can be written simplistically as:

$$Fe^{3+} + 2L^- \leftrightharpoons FeL_2^- \qquad (5.1)$$

where L denotes a ligand. In seawater at pH 8.2, the major inorganic ligand is OH^-. The free Fe^{3+} is available for biological uptake, which is the process by which the reaction is driven to the

left. Recent work in the North Pacific Ocean has shown that very little of the total iron in seawater is in the form of inorganic Fe-hydroxides and that 99.9% of iron is bound to organic ligands with stability constants which suggest that these ligands are siderophores (Rue & Bruland 1995). This is also the case for copper (Cu) (Coale & Bruland 1988) and suggests that the speciation of trace metals in the ocean is controlled by biological production of ligands. Several marine cyanobacteria have been shown to produce siderophores in response to low iron availability (Wilhelm *et al.* 1996) and produce Cu chelators in response to high copper concentrations (Moffet & Brand 1996, Lawrence 1998).

The production of siderophores is limited to prokaryotic systems and eukaryotic phytoplankton are generally believed to be at the mercy of the kinetically controlled release of ligated Fe (Hudson & Morel 1993, Sunda & Huntsman 1995). A process which is responsible for enhancing Fe availability at rates greater than that predicted by kinetic release is photodissolution. One mechanism proposed is photoinduced ligand to metal charge transfer (O'Sullivan *et al.* 1991) which proceeds according to the equation:

$$Fe^{3+} \cdot L \xrightarrow{h\nu} Fe^{2+} + \cdot L \qquad (5.2)$$

The reduction of Fe in a metal-ligand complex effectively lowers the stability constant of the complex and results in release of Fe^{2+} which is several orders of magnitude more soluble than Fe^{3+}. In the absence of organic chelators, the stability of Fe^{2+} in oxygenated seawater at a pH of 8.2 is low with a half-life of 2.2 min (O'Sullivan *et al.* 1991). In oxygenated seawater, photoreduction can produce Fe^{2+} at concentrations as high as 2 nM which approach 20 - 25% of

the total iron in seawater (O'Sullivan *et al.* 1991). The poor stability of Fe^{2+} and rapid reoxidation to Fe^{3+} suggests a high production rate of Fe^{2+} through photoreduction.

Biological reduction of Fe has also been hypothesized but has been considered unimportant in Fe acquisition by marine phytoplankton until recently. Based on kinetic arguments, Fe^{2+} should be four orders of magnitude slower at reacting with cellular uptake ligands than Fe^{3+} (Morel & Gering 1993). However this argument assumes that free Fe^{3+} is as equally available as Fe^{2+} and that the metal-ligand exchange takes place in the seawater phase immediately adjacent to the plasmalemma, rather than between plasmalemma bound ligands.

Biological reduction of Fe may be an absolute requirement of Fe uptake when one considers that essentially all iron is bound to organic ligands. Evidence of biological reduction has come from several observations. For example, relatively high concentrations (0.5 nM) of reduced iron have been observed at night (in the absence of photoreduction) in the equatorial Pacific (O'Sullivan *et al.* 1991). The marine diatom *Thalassiosira weissflogii* exhibits plasmalemma redox activity for Cu and Fe (Jones *et al.* 1987) and has been hypothesized to involve the NAD(P)H:diaphorase subunit of nitrate reductase (Jones & Morel 1988). The freshwater green alga *Chlorella vulgaris* has also been shown to exhibit an Fe reductase and is able to reduce Fe bound to several siderophores and chelators (Allnutt & Bonner 1987a,b).

5.1.2 Steady state iron limited growth

Steady state iron-limited phytoplankton growth is attained using the trace metal buffered culture medium, Aquil (Morel *et al.* 1979, Price *et al.* 1988/89). Contamination by trace metals in the salts and macronutrients of this medium is minimized using an ion exchange resin (Chelex).

Known concentrations of metals and chelator (EDTA) are then added and the free ferric ion concentration is calculated using a computer-based thermodynamic equilibrium model (Mineql) which ignores metal complexation with biologically derived organic compounds and photoreductive dissolution. Essentially, the speciation of all ions in the medium are defined until it is used to grow organisms. Despite these simplifying assumptions steady-state growth rates and by inference, Fe availability, can be attained (e.g. Sunda & Huntsman 1995).

5.2 Methods

5.2.1 Culture conditions

A unialgal culture of the marine diatom *Thalassiosira weissflogii* (clone Actin, NEPCC#741) was obtained from the North East Pacific Culture Collection (NEPCC), Department of Botany, University of British Columbia. Cultures were maintained using Aquil artificial seawater medium (Morel *et al.* 1979, Price *et al.* 1988/89). Medium and culture flasks were microwave sterilized according to Keller *et al.* (1988) in Teflon® bottles. All medium preparation and sample handling was carried out in a metal-free class 100 flow hood. All plasticware was acid-cleaned by soaking in 10% reagent grade HCl for 24 h, rinsed five times with 18 MΩ · cm deionized distilled water (DDW), soaked in 0.1M quartz distilled acetic acid (Seastar) for 24 h, rinsed with DDW (5X) and finally soaked in 0.1% quartz distilled HCl (Seastar) for 2-5 d and rinsed with DDW (5X). Free ferric ion concentration was calculated using the thermodynamic equilibrium program Mineql (Westall *et al.* 1976).

Triplicate high-Fe (pFe 18) and low-Fe (pFe 21) cultures and a single culture with no-added-Fe were maintained at 18.5 ± 0.5°C using acid-cleaned polycarbonate Erlenmeyer flasks. The no-Fe culture contained 2 nM Fe (measured using competitive ligand cathode stripping

voltametry) from contamination prior to the addition of trace metals and EDTA (Lawrence pers. comm.). The culture with zero added iron was sampled for NR and NiR activities only. Continuous saturating (Strzepek & Price submitted) irradiance (24 h) was provided at a photon flux density of 160 μmol m^{-2} s^{-1} using fluorescent bulbs (Vitalite) below the cultures. To ensure carbon saturation total dissolved inorganic carbon was measured at harvest using an infrared gas analyzer (Analytical Development Co. 225-MK3) calibrated against a 449 ppm CO_2 gas standard.

5.2.2 Growth and biomass measurements

Duplicate samples for cell counts and cell volume from each replicate flask were obtained using a Coulter particle counter. Growth was monitored fluorometrically on a Turner fluorometer model 10-AU. Long-term growth rates were calculated by regression of the natural log transformed fluorescence values vs. time. Cellular carbon and nitrogen quotas were obtained by filtering duplicate 5-10 ml aliquots of culture from each replicate flask onto pre-combusted (460^{o}C, 4 h) 13 mm A/E (Gelman) glass fiber filters and analyzed with a Carlo Erba NCS analyzer NA 1500. Carbon and nitrogen were calculated against a standard curve generated from sulfanilamide standards. Nitrogen incorporation rate was calculated using the formula:

$$\mu PN = \mu \times (N\ cell^{-1}) \qquad (5.3)$$

where μPN is the intrinsic rate of increase of particulate nitrogen in units of pg $cell^{-1}$ d^{-1} and N is cellular nitrogen in grams. Chlorophyll *a* was determined fluorometrically on a Turner fluorometer model 10-AU calibrated against pure chl *a* (Sigma).

5.2.3 Variable fluorescence assay

Variable fluorescence (F_V/F_m) was determined using the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Triplicate 7 ml aliquots from each replicate culture flask were dark adapted for approximately 10 min in a temperature controlled bath. *In vivo* fluorescence before (F_o) and after (F_m) the addition of 75 μM DCMU was determined on a Turner fluorometer. Variable fluorescence was calculated according to the formula:

$$F_V/F_m = (F_o - F_m)/F_m \quad (5.4)$$

5.2.4 Carbohydrate measurements

Triplicate samples (20 ml) for cellular carbohydrate quotas were harvested from each replicate flask onto precombusted 25 mm A/E glass fiber filters stored frozen at -20°C and measured according to Kochert (1978).

5.2.5 Enzyme Assays

Samples for enzyme assays were all processed in the same manner. Triplicate aliquots of culture from each replicate flask were filtered onto pre-combusted 25mm A/E glass fiber filters. Filters were quickly folded, placed into 1.5 ml microcentrifuge tubes, frozen and stored in liquid nitrogen. Samples were stored for no more than 10 d. Filters were ground in a 5 ml glass tissue homogenizer with a Teflon® pestle in 1 ml of ice cold homogenization buffer while immersed in an ice slurry.

Nitrite reductase activity was determined using a vascular plant assay which was optimized for use with diatoms (See Chapter 3). The NiR homogenization buffer consisted of 100 mM potassium phosphate buffer (pH 7.9) with 3% bovine serum albumin (BSA) and 0.1% Triton X-100.

Nitrate reductase activity was determined according to Berges & Harrison (1995), except a 100 mM potassium phosphate buffer (pH 7.9) was used rather than the 200 mM phosphate recommended by Berges & Harrison (1995), to avoid precipitation at 0°C.

5.2.6 Internal and external nitrogen measurements

Internal pools of NO_3^- and NO_2^- were determined on 0.2 ml sub-samples as in chapter 4. Both internal and external NO_2^- concentrations were determined colorimetrically according to Parsons *et al.* (1984). Internal and external NO_3^- was determined using the spongy cadmium method of Jones (1984).

5.3 Results

During the course of culture growth, dissolved inorganic carbon (DIC) levels did not become limiting to growth. High-Fe (pFe 18) cultures drew DIC levels from saturation (2 mM) down to 1.6 mM (range ± 0.09), while low-Fe (pFe 21) cultures remained saturated at 1.99 mM DIC (range ± 0.08).

Under steady-state Fe-limited (pFe 21) conditions, growth rate and cell volume decreased by approximately 74 and 54% respectively (Table 3.1). Carbon and nitrogen samples were lost due to a malfunction of the Carlo Erba NCS analyzer, but values were obtained from Maldonado & Price (1996) who used the same clone and growth conditions. Both carbon and nitrogen

quotas decreased by roughly 50%, but C:N ratios remained similar. Chl *a* $cell^{-1}$ and carbohydrate quotas were also decreased in low Fe (pFe = 21) cultures even when normalizing values to either cell volume or carbon.

Table 5.1. Comparison of growth rate and cellular parameters (95% C.I.) in Fe-replete (pFe 18) and Fe-limited (pFe 21) conditions for *Thalassiosira weissflogii* grown using Aquil.

	pFe 18	pFe 21
Growth rate μ (d^{-1})	1.11 (0.08)	0.29 (0.05)
Cell volume (μm^3)	508.9 (11.0)	233.8 (7.8)
Carbohydrate (pg · $cell^{-1}$)	14.90 (5.9)	2.22 (3.02)
Carbon (pmol · $cell^{-1}$)*	5.24	2.64
Nitrogen (pmol · $cell^{-1}$)*	0.73	0.35
C:N Ratio (molar)*	7.56	7.2
Chl *a* (pg · $cell^{-1}$)	6.71 (2.32)	1.20 (0.09)

* From Maldonado and Price (1996). Values are corrected for differences in cell volumes between this work and that of Maldonado & Price (1996).

In high Fe cultures, variable fluorescence remained around 0.69 and decreased to 0.56 in low Fe (Fig. 5.1). Cellular nitrite excretion during growth in low-Fe cultures was significantly ($p < 0.05$) greater at 10.9 fmol $cell^{-1}$ d^{-1} (95% C.I. = 4.6), while in high-Fe cultures, nitrite excretion remained low or was negative (-1.53 fmol $cell^{-1}$ d^{-1}, 95% C.I. = 3.47). Internal pools of nitrate

remained similar in both iron treatments, but nitrite pools were significantly lower ($p < 0.05$) in low-Fe-grown cells (Fig. 5.2).

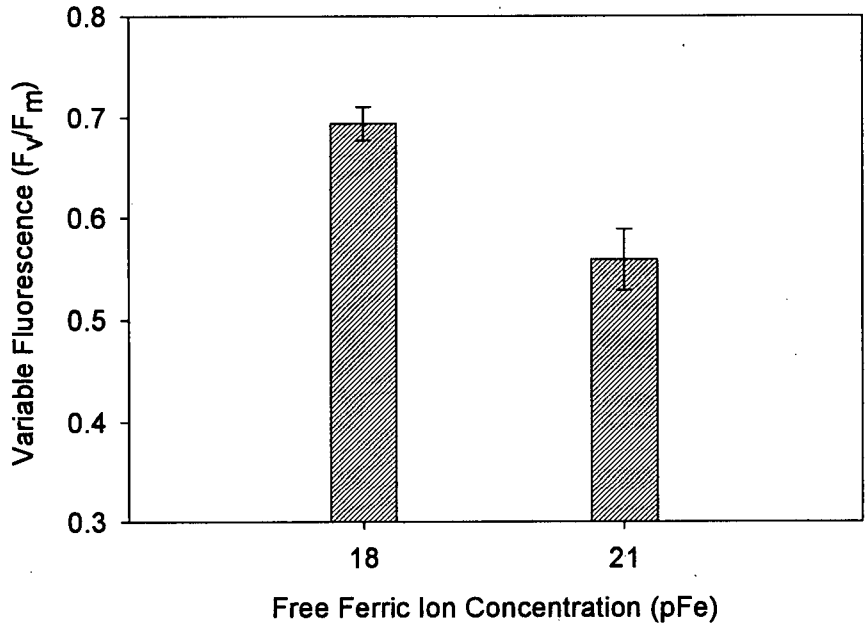

Figure 5.1. Variable fluorescence of *Thalassiosira weissflogii* grown under steady state high (pFe 18) and low (pFe 21) iron supply. Error bars are 95% confidence intervals.

Activity of NiR remained higher than or equal to NR activity in all iron treatments (Fig. 5.3). In both high and low Fe cultures, activities of both NR and NiR remained well in excess of the nitrogen incorporation rates (μPN).

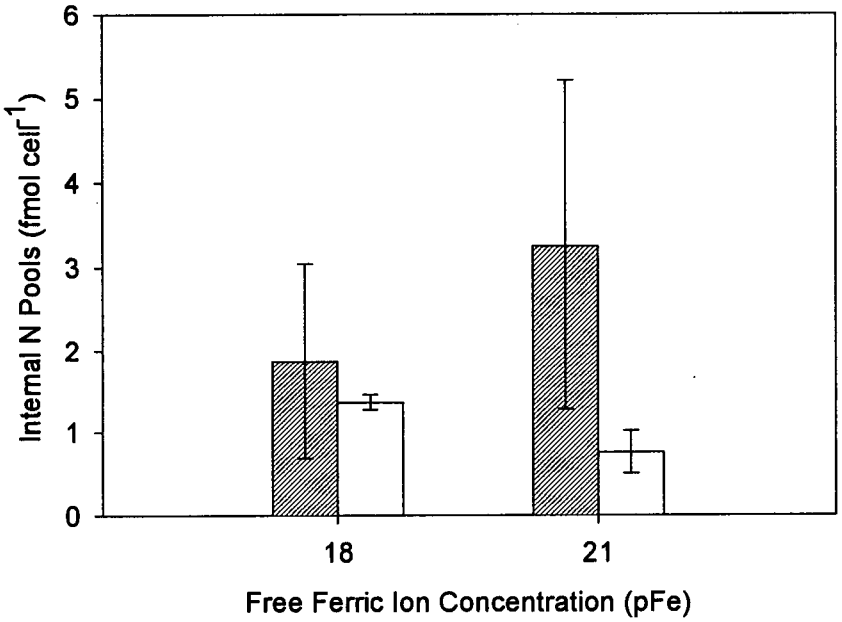

Figure 5.2. Internal NO_3^- (hatched) and NO_2^- (open) pools in *Thalassiosira weissflogii* grown under steady state high (pFe 18) and low (pFe 21) iron supply. Error bars are 95% confidence intervals calculated from three replicate cultures.

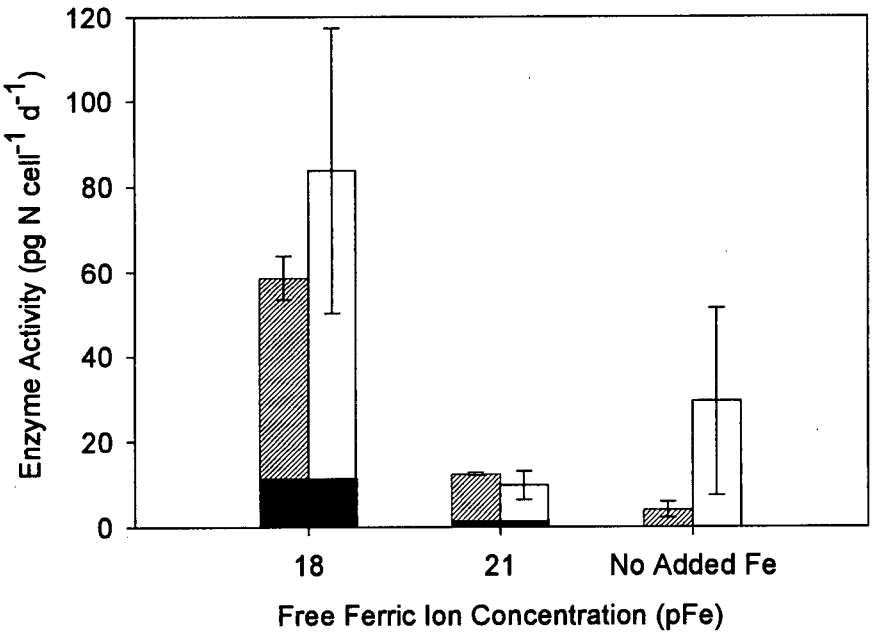

Figure 5.3. Enzyme activities of NR (hatched) and NiR (open) and nitrogen incorporation rate (solid) in *Thalassiosira weissflogii* grown under steady state high (pFe 18) and low (pFe 21) iron supply, and NR and NiR activity in no-added-Fe culture. Error bars are 95% confidence intervals calculated from three replicate cultures for pFe 18 and 21, and calculated from three replicate measurements from a single no-added-Fe culture.

5.4 Discussion

As in non-steady state Fe limitation, steady state Fe limited growth on nitrate results in an efflux of nitrite into the culture medium. Rates of efflux are an order of magnitude lower in steady state Fe limitation than in non-steady state Fe limitation, but are still large compared to iron replete conditions.

In high Fe cultures, NiR activity was expected to be on the order of 2×10^3 pg N cell^{-1} d^{-1} rather than the observed 80 pg N cell^{-1} d^{-1}. Either reduced iron availability or low DIC concentrations could be explain lower NiR activity while growth is maximal. Iron supply at pFe 18 is saturating to growth of *T. weissflogii* (Actin) as observed by several investigators (Harrison & Morel 1986; Maldonado & Price 1996), although it is unknown if this iron level allows for full activity of NiR. While growth rate showed no decrease at the time of sampling, the DIC concentration decreased to 1.6 mM. Induction of the carbon concentrating mechanism has been observed at DIC concentrations of 1.7 or lower and should supply the necessary carbon for nitrite assimilation. It is unknown whether NiR will remain fully expressed during operation of the carbon concentrating mechanism.

As in chapter 4, excretion of nitrite can be explained as the limitation of nitrite assimilation at the nitrite reduction step. The decrease in DCMU fluorescence indicates that photosynthesis is impaired at low iron supply and that nitrite reduction is likely limited by the supply of reductant to NiR rather than by the amount of enzyme as the *in vitro* activity remains higher or equal to NR activity. Unlike chapter 4 results, carbohydrate pools are much lower under Fe-limited growth and these differences cannot be reconciled by normalizing to cell volume or to carbon data from Maldonado & Price (1996), suggesting carbon assimilation may be impaired under Fe limitation. Without carbon data from these experiments a definitive statement is not possible.

5.5 Conclusions

Both steady state and non-steady state Fe limitation produce similar responses in NO_2^- excretion and in nitrogen assimilatory enzymes. However, the excretion rate in steady state Fe limitation is an order of magnitude lower indicating an acclimation of the nitrogen assimilatory pathway to Fe limitation. Several possible mechanisms by which Fe may regulate nitrogen assimilation are proposed in chapter 6.

6. General Conclusions

6.1 The influence of iron on phytoplankton physiology

The prevailing hypothesis to explain the ecology of high nutrient, low chlorophyll (HNLC) areas is that small (<5 μm) phytoplankton dominate the biomass, use NH_4^+ as a nitrogen source and are not iron-limited. Large diatoms are rare and iron-limited in these areas, preventing the use of NO_3^- as a nitrogen source.

If inferences from a single species may be applied to a mixed assemblage then, according to this work, diatoms in HNLC regions are not deficient in their ability to assimilate nitrate when iron availability is limited. Rather, it appears that diatoms are limited in their ability to process photons within the photosynthetic electron transport chain which results in nitrite reduction becoming the rate limiting step in nitrogen assimilation. This appears to be true whether iron is supplied either in non-steady (Chapter 4) or steady states (Chapter 5).

This finding has been hypothesized by others based on C:N ratios of iron limited phytoplankton and is verified here by measurement of both NR and NiR in iron-limited marine phytoplankton. This work represents the first report of NiR measurements in marine phytoplankton and the first measurements of both enzymes under conditions where efflux of nitrite was measured.

6.2 Iron regulation of nitrogen assimilation

Based on the data available from chapters 4 and 5, a hypothetical, conceptual model of carbon and nitrogen assimilation was constructed (Fig. 6.1). In this scheme, a NO_2^- specific porter located at the chloroplast membrane is considered a key regulatory step in the reduction of NO_2^- to NH_4^+. The limitation of NiR under iron stress is hypothesized to be through the inactivation of the nitrite porter through a signal from photosynthetic electron transport system activity and not due to the cell's ability to supply carbon skeletons in the form of glutamate.

The flux of nitrite into the chloroplast has been hypothesized to occur by several mechanisms: 1) the activity of NiR determines the passive flux through a NO_2^- specific channel (Brunswick & Cresswell 1988b); 2) the flux of NO_2^- is active through a NO_2^- specific porter and can be greater than the flux through NiR allowing for the build up of a chloroplastic NO_2^- pool; and 3) the flux of NO_2^- is determined by the thermodynamic rate constant of the formation of HNO_2 (pKa NO_2^- / HNO_2= 3.6) which is uncharged and may freely diffuse across the chloroplast membrane, as well as the flux through NiR. This mechanism is unlikely based on the alkaline pH optimum (7.8) of NO_2^- uptake in pea (*Pisum sativum*) chloroplasts (Brunswick & Cresswell 1988a) and the high K_m (450 μM) of HNO_2 transport in cyanobacteria (Flores *et al.* 1987).

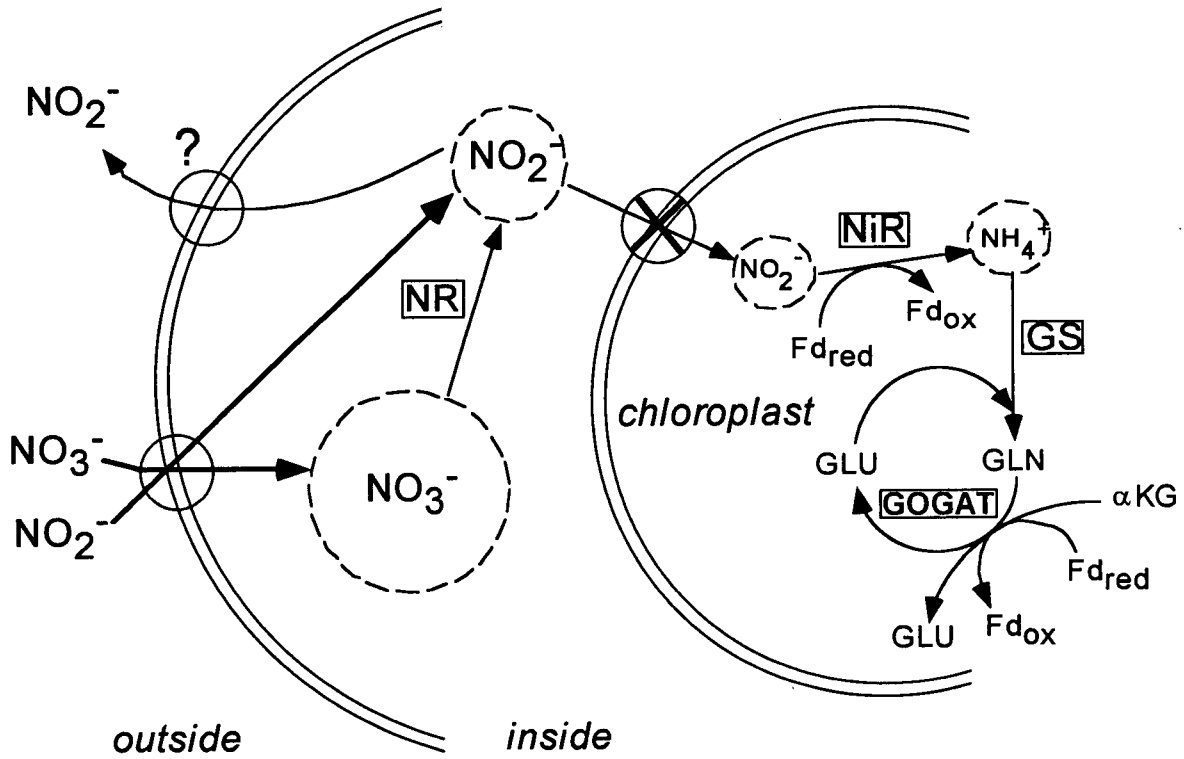

Figure 6.1. Conceptual model of nitrate assimilation and regulation in *Thalassiosira weissflogii* under iron-replete and limited conditions. Arrows represent fluxes of components. Broken circles represent dissolved pools of nitrogen. Circles within membranes represent porters. The hypothesized porter at the chloroplast membrane is indicated with an X. The possible efflux porter for NO_2^- is indicated with a question mark (?). Catalyzing enzymes are indicated by abbreviation within boxes. NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; GOGAT, glutamate synthase.

The internal concentration of NO_2^- and the activity of NiR support the hypothesis that a specific chloroplastic porter has a regulatory role in nitrite assimilation. Under iron limitation, NO_2^- efflux is greatest at the point of lowest internal NO_2^- concentration (Figs. 4.3 & 4.4) consistent with the hypothesis that a block of NO_2^- transport at the chloroplast membrane is present. Otherwise, a passive flux through the chloroplast membrane based on NiR activity should result in the highest efflux rates being associated with the highest internal NO_2^- concentrations (i.e. the system should back up at NiR). However, the observed NO_2^- efflux rates are also consistent with the hypothesis that a NO_2^- specific plasmalemma efflux porter decreases the cytoplasmic pool of NO_2^-, and transport across the chloroplastic membrane is unaffected, passive and maintained by NiR activity. While there are no studies which report a nitrite specific plasmalemma efflux porter in plants, a specific uptake porter has been reported in the freshwater green alga *Chlamydomonas reinhardtii* (Cordoba *et al.* 1986) and could potentially serve as an efflux porter through a back reaction.

A chloroplastic NO_2^- porter has also been reported in several studies. In the freshwater green microalga *Chlorella fusca,* a chloroplastic protein which binds NO_2^- has been observed (Krämer *et al.* 1989). Protein density on the chloroplastic membrane is lowest when CO_2 supply is decreased and efflux of nitrite is highest while levels of NiR remain unaffected, suggesting regulation of NO_2^- reduction at the site of transport into the chloroplast. The use of the

photosynthetic electron transport inhibitor DBMIB (dibromothymoquinone), which prevents electron flow to PSI but does not prevent PSII photophosphorylation, decreases NO_2^- uptake of pea chloroplasts (Brunswick & Cresswell 1988b) suggesting a feedback from PSI activity to NO_2^- transport possibly through ferredoxin.

6.3 Possible regulatory signals of nitrite transport into the chloroplast

Several regulatory signals which control transport of nitrite into the chloroplast can be envisioned. Assuming a single regulatory signal exists, its response should be similar under the conditions in which NO_2^- efflux has been observed. Efflux of NO_2^- occurs under low light (Martinez 1991), limited CO_2 supply (Krämer *et al.* 1988, Suzuki *et al.* 1995) and low iron (this work). A recent hypothesis by Lomas & Glibert (in press) suggests that NO_2^- efflux may also occur when light is saturating for photosynthesis at low temperatures. Low temperatures decrease carbon flow through the dark reactions of photosynthesis, but do not affect the rate of light reactions. Lomas & Glibert (in press) hypothesize that the reduction of NO_3^- and subsequent NO_2^- efflux may be used as a sink of excess photosynthetically derived energy. Regulatory signals which can account for these various processes are discussed below.

6.3.1 Glutamine-glutamate ratio regulation

The balance between carbon and nitrogen metabolism has been hypothesized to be controlled by the ratio of glutamine:glutamate GLN:GLU. Glutamine is formed from NH_4^+ and glutamate through the catalytic function of GS. Under low carbon supply, the formation of

glutamate from glutamine and α–ketoglutarate via GOGAT can become limiting to nitrogen assimilation resulting in a high GLN:GLU ratio. Regulation of the chloroplastic nitrite transport through the ratio of GLN:GLU can not explain both the efflux of NO_2^- under carbon limitation (high GLN:GLU), and the efflux of NO_2^- under low iron supply as the carbon stores of *T. weissflogii* were unaffected (low GLN:GLU).

6.3.2 Ferredoxin-thioredoxin regulation

Many light-mediated enzymatic reactions are regulated by the redox state of thioredoxin. Thioredoxin is reduced by ferredoxin through the catalytic function of ferrodoxin-thioredoxin reductase (Macdonald & Buchanan 1990). High activity of the PETS causes a drop in the pool of reduced thioredoxin resulting in activation of enzymes of the pentose phosphate pathway, adenosine 5′triphosphatase-synthetase complex, and translation of chloroplast proteins (Levings & Siedow 1995). The observations that NO_2^- efflux can occur either during high (Lomas & Glibert in press) or low (this work) PETS activity do not support regulation via thioredoxin. The thioredoxin pool would be highly reduced under high PETS activity and oxidized under low PETS activity.

6.3.3 Plastiquinone redox state

The redox state of the plastiquinone pool has recently been shown to regulate the transcription of several proteins in the PETS (Escoubas *et al.* 1995) and GS activity in a cyanobacterium (Reyes *et al.* 1995). Plastiquinone is the first electron acceptor in the PETS following PS II and is the slowest step in electron transfer between PS II and PS I. Under conditions of high light or inhibition of electron transfer downstream from plastiquinone, the

plastiquinone pool is highly reduced. A decrease in PS I function by iron limitation (Greene *et al.* 1991) and the reduced fixation of carbon under low CO_2 supply (Krämer *et al.* 1988, Suzuki *et al.* 1995) both result in highly reduced plastiquinone pools as well. Assuming that a single signal controls NO_2^- efflux, regulation of NO_2^- reduction can be explained through the redox state of the plastiquinone pool under high light, low iron, or low CO_2 supply. However, the efflux of NO_2^- under conditions of low light cannot be directly explained by this mechanism.

6.3.4 Caveats

The signals described above do not account for regulation of nitrogen assimilation in the absence of light which occurs under nitrogen stress and is believed to be controlled through the C:N status of the cell (Huppe & Turpin 1994). Redox signaling is envisioned to occur in addition to regulation by C:N status. Reyes *et al.* (1995) hypothesize a single phosphorylation mechanism which regulates the activity of GS through both the plastiquinone redox status and the C:N status although, as yet, no evidence is available to support this mechanism.

6.4 Oceanic implications of iron influence on nitrogen assimilation

Under natural conditions, the influence of iron, light, and carbon limitations and high-light stress all produce the same response of nitrite efflux, but the response of NR and NiR may be specific to iron limitation. Nitrite reductase contains a significant amount of iron (11-22% of total cellular iron). A decrease in NiR can be used to supply iron to other parts of the cell when iron becomes limiting (Chapter 4). The ratio of NR:NiR could potentially serve as a biological indicator of iron limitation, however, the response of NR:NiR under limitation by light or other nutrients has not been investigated.

Nitrite efflux has been considered a potential complicating factor in estimates of new production using ^{15}N (Collos 1982, Flynn & Flynn 1998). Incubations for new production estimates in iron-limited regions would likely suffer the complicating problem of nitrite efflux. Nitrite efflux in my study represented a maximum of approximately 20% of the nitrogen assimilated by *T. weissflogii* in a day. Considering the difficulty in estimating NO_3^- uptake rates from the use of ^{15}N, the added complication of NO_2^- efflux in iron limited cells is likely to be significant for short term (2-4 h) incubations due to isotope dilution. During long-term incubations (1 d), errors due to NO_2^- efflux will be a function of the external NO_3^- concentration as NO_3^- competitively inhibits NO_2^- uptake (Collos 1982).

The efflux of DON during ^{15}N incubations can also result in an underestimate of new production rates where efflux rates average around 25 to 40% of the NO_3^- uptake rate (Bronk *et al.* 1994). Nitrite efflux rates are on the order of these effluxes and could potentially compound errors in estimates of new production rates in low iron HNLC regions.

6.5 Future studies

6.5.1 Urea uptake and enzyme activity

Much of the past work on urea uptake and metabolism has suffered from the lack of an optimized enzyme assay. Measurements of urea uptake and urease activity when cultures are supplied with alternative nitrogen sources would establish whether urea utilization is regulated at the transport step as has been implied in many studies or at the assimilation step.

6.5.2 Nitrite reductase assay

Removing NiR activity from the glass fibers in ground homogenates proved to be problematic. Although several detergents were employed without success, others should be investigated for their potential to solublize the protein. In addition, the use of high energy sonication would preclude the use of filters and could potentially prove more effective in solublizing the protein.

6.5.3 The influence of iron limitation on nitrogen metabolism

Detailed studies of iron limitation and nitrogen assimilation as presented here are generally lacking in the literature. This study supports the observations of others that C:N ratios are not affected by iron limitation, but does not validate the assertion that iron primarily influences photosynthesis. An approach which involves measurement of nitrogen fluxes through the enzymes and dissolved pools of the nitrate assimilatory pathway combined with measurements of the key iron-containing components of the photosynthetic electron transport chain may prove useful in establishing the responses of both systems. Ultimately the question of how carbon and nitrogen balance is maintained may need to be answered to establish how iron limitation influences cellular metabolism.

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Appendix A

Urease enzyme assay optimization and activity in two marine diatoms

Abstract

An *in vitro* urease enzyme assay was developed for the marine diatoms *Thalassiosira pseudonana* Hasle et Heimdal (clone 3H) and *Thalassiosira weissflogii* (Grunow) Fryxell *et* Hasle (clone Actin). This assay involves the colormetric measurement of ammonium following the hydrolysis of urea in crude cell homogenates and can account for the rate of nitrogen assimilation in both species grown on urea as the sole nitrogen source.

Urease was found to be expressed regardless of the nitrogen source, although activities showed distinctly different patterns depending on the species examined and form of nitrogen supplied. Under nitrogen-replete conditions, urease activity in *T. pseudonana* was expressed constitutively when grown on NH_4^+ and upregulated when grown on NO_3^- or urea. In nitrogen-replete *T. weissflogii*, urease activity was expressed constitutively regardless of the nitrogen source and showed no upregulation. Nitrogen starvation did not induce activity in either species.

Introduction

Dissolved nitrogen in the ocean exists in several different forms, primarily NO_3^-, NO_2^-, NH_4^+, and dissolved organic nitrogen (DON) such as urea. The occurrence and uptake of these four forms of nitrogen have been well characterized in the open ocean (e.g. Eppley et al. 1971, Antia et al. 1991). McCarthy (1972 a,b) demonstrated the ability of a natural phytoplankton assemblage to grow on urea as the sole nitrogen source. Several laboratory studies of unialgal cultures have also shown that the majority of phytoplankton are able to grow on urea (Carpenter et al. 1972, Anita et al. 1975, Bekheet & Syrett 1977, Oliveira & Antia 1986). Representatives from all major phytoplankton taxa have been shown to use urea as a source of assimilatory nitrogen.

Urea ($CO(NH_2)_2$) is metabolized to ammonium (NH_4^+) and carbon dioxide (CO_2) via one of two pathways in marine algae: urease or ATP:urea amidolyase. Activity of the latter has been shown to exist only in several classes of the Chlorophyceae (Leftley & Syrett 1973; Bekheet & Syrett 1977). It is believed to be a two step process involving the ATP dependent catalysis of urea to allophanate by urea carboxylase followed by allophanate hydroxylase mediated breakdown to NH_3 and CO_2.

All other algal classes tested contain a urease enzyme for urea hydrolysis (Leftley & Syrett 1973, Oliviera & Antia 1986) which is shown in the following equation:

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} NH_3 + CO_2 \qquad (1)$$

Urease is unique in that it contains a nickel (Ni) atom at its catalytic site (Mobley & Hausinger 1989). Algal ureases appear to be no exception and nickel deficiencies have shown reduced growth on urea for *Phaeodactylum tricornutum, Tetraselmis subcordiformis* (Rees & Bekheet 1982), *Thalassiosira weissflogii* (Price & Morel 1991) and many other algae (Oliviera & Antia 1986).

Much of current knowledge regarding urease expression in marine diatoms has been inferred from urea uptake. For instance, measurements of urea uptake using thiourea, a non-metabolizable sulfur analogue of urea, by the marine diatom *Phaeodactylum tricornutum* shows low uptake (8 nmol 10^8 cells^{-1} h^{-1}) in NH_4^+-grown cultures and high uptake (31.5 nmol 10^8 cells^{-1} h^{-1}) in NO_3^- and urea-grown cultures (Rees & Syrett 1979a). Urea uptake is also enhanced within 24 h of nitrogen starvation and declines after 48 h of starvation in the same organism (Rees & Syrett 1979a,b). Inhibition of urea uptake by NH_4^+ has been observed for *Thalassiosira pseudonana* and *Skeletonema costatum* as well (Horrigan & McCarthy 1982). In *Thalassiosira pseudonana* urea uptake occurs simultaneously with NO_3^- uptake and is enhanced following 24 h of NO_3^- starvation (Price & Harrison 1988b). Based on these studies and assuming urea uptake and assimilation are coordinated, one would predict that in marine diatoms NH_4^+ represses urease activity and nitrogen starvation induces urease.

Past measurements of urease activity have not attempted to account for the assimilated nitrogen of the test organism (e.g. Rees & Bekheet 1982). In addition, assays performed using the same method and organism give widely different activities. Rees & Bekheet (1982) measured urease activity 200 times greater than activities measured by Leftley & Syrett (1973) for the diatom *Phaeodactylum tricornutum*. Price & Morel (1991) were unable to account for the

assimilation rate of nitrogen in urease assays performed on the marine diatom *Thalassiosira weissflogii* and attributed this to unfavorable assay conditions.

The objective of this research was to develop an assay for urease that spectrophotometrically measured production of NH_4^+ from the cleavage of urea in crude cell homogenates. A novel assay buffer was developed to allow for maximal urease activities to be measured. The method is the first to account for the assimilation rate of N in a phytoplankton culture growing on urea. The assay is able to distinguish between upregulated and constitutive activities in both *T. pseudonana* and *T. weissflogii* grown on urea, NO_3^- and NH_4^+.

Materials and Methods

Unialgal cultures of *Thalassiosira pseudonana* (clone 3H, NEPCC #58) and *Thalassiosira weissflogii* (clone Actin, NEPCC #741) were obtained from the Northeast Pacific Culture Collection (NEPCC), Department of Botany, University of British Columbia. Axenic cultures of *Thalassiosira pseudonana* (clone 3H, CCMP #1335) and *Thalassiosira weissflogii* (clone Actin, CCMP #1336) were obtained from the Provasoli - Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, USA. Cells were grown in ESAW artificial seawater (Harrison et al. 1980) with the following modifications. Selenium and nickel were added at final concentrations of 10 nM and 63 nM respectively (Price et al. 1987, Price & Harrison 1988). For enzyme optimization studies, urea (150 μM) was supplied as the sole nitrogen source. For activity studies, single nitrogen additions of urea (150 μM) NO_3^- (300 μM) and NH_4^+ (300 μM) were made to each of three replicate cultures. The medium was sterilized either by filtering (bacterized cultures) using autoclaved nitrocellulose membranes (pore size 0.8, 0.45 and 0.2 μm)

or by autoclaving (axenic cultures). Cultures were grown in 1 L flat-bottom borosilicate boiling flasks at 18.5 ± 0.5°C under a constant photon flux density of 180 μmol m^{-2} s^{-1} (Vitalite) measured with a QSL - 100 meter (4π collector, Biophysical Instruments Inc.). To ensure mixing and an adequate supply of CO_2, cultures were bubbled with air that was scrubbed through 5% sulfuric acid, distilled water and a 0.8/0.2 μm filter set. Axenic cultures were not bubbled. Instead $NaHCO_3$ was added when dissolved inorganic carbon (DIC) levels fell below 1.7 mM. To ensure carbon saturation, total dissolved inorganic carbon was measured daily using an infrared gas analyzer (Analytical Development Co. 225-MK3) calibrated against a 449 ppm CO_2 gas standard.

Cell counts were obtained using a Coulter particle counter (Model TA II). Growth rates were calculated from cell counts using the formula:

$$\mu = \ln (C_1/C_0)/(t_1-t_0) \quad (2)$$

where μ is the intrinsic rate of growth in units of per day, C_0 is the cell count at day zero (t_0) and C_1 is the cell count at day one (t_1). Cellular carbon and nitrogen quotas were obtained by filtering 5 ml aliquots of culture onto pre-combusted (460°C for 4 h) 13 mm AE (Gelman) glass fiber filters and analyzed with a Carlo Erba NCS analyzer NA 1500. Carbon and nitrogen were calculated against a standard curve generated from sulfanilamide standards. The particulate nitrogen growth rate was calculated using the formula:

$$\mu PN = \mu \times (N\ cell^{-1}) \quad (3)$$

where μPN is the intrinsic rate of increase of particulate nitrogen in units of pg d^{-1} and N is cellular nitrogen in grams.

Bacterial cell counts were performed on cells harvested using 0.2 μm, 25 mm polycarbonate filters (Poretics) and counted visually using acridine orange stain and epifluorescence microscopy.

Dissolved external NH_4^+ concentrations were determined colorimetrically according to Parsons *et al.* (1984). Dissolved external NO_3^- was determined using the spongy cadmium method of Jones (1984). Dissolved external urea concentrations were determined colorimetrically using the method of Price & Harrison (1987).

Enzyme Assay

Aliquots of culture (40 ml) were harvested in log phase by gentle vacuum (<15 mm) filtration onto pre-combusted (4 h at 460°C) 25 mm diameter A/E glass fiber filters (Gelman), folded into 1.5 ml microcentrifuge tubes, immediately frozen and stored in liquid nitrogen for no longer than 1 week. Frozen samples were homogenized with a motorized Teflon pestle and mortar in 1 ml 50 mM HEPES, 200 mM phosphate (potassium phosphate) buffer, or 150 mM potassium phosphate buffer, at a range of pH (5-12), along with various combinations of buffer additives: 3.0% w/v bovine serum albumin (BSA), 5 mM ethylenediaminetetracetic acid (EDTA) 0.3% w/v polyvinyl pyrolidone (PVP) and 0.03% w/v dithiothreitol (DTT). All buffers contained 0.1% v/v Triton X-100, to aid in protein solublization. The cell homogenate was spun for 5 s at 15,000 rpm in an Eppendorf 5414 centrifuge to remove glass particulates. Clarified homogenate (200 μl) was added to 750 μl of buffer solution minus additives in 1.5 ml clear microcentrifuge tubes (Fisher). Incubations (15 min) were started with the addition of 50 μl of 150 mM urea in

the buffer solution minus additives and run in the same incubator used for cultures. Time zero samples (t_0) and incubations were "killed" by heat (60 s, 100°C), or the addition of 20 µl of 4N HCL prior to addition of urea. Incubation periods were stopped by heat (60 s, 100°C) or the addition of 20 µl of 4N HCl with and without subsequent neutralization with 20 µl of 4N NaOH. Ammonium production was measured using the indophenol method (Solorzano 1969) where phenol and nitroprusside reagents were mixed 1:1 as recommended by Chaney & Marback (1962). Samples were reacted with ammonium reagents in the proportion and order of 300 µl sample : 200 µl phenol-nitroprusside solution : 400 µl alkaline solution in 1.5 ml disposable cuvettes (Fisher). Following addition of each reagent, samples were mixed by vortexing. Absorbance at 640 nm was read on a LKB Ultraspec II against a blank of buffer and ammonium reagents.

Results

The indophenol method for NH_4^+ detection was found to be linear for the range of NH_4^+ concentrations typically found in incubated enzyme assays (Fig. 1). Absorbance in the majority of assays fell between 0.1 and 0.3 absorbance units (640 nm).

Urease activity had a pH optimum of 7.9 for *Thalassiosira weissflogii* and a broad pH optimum for *Thalassiosira pseudonana* using potassium phosphate buffer (Fig. 2). Urease activity had a pH optimum of 8 when using Hepes buffer at a range of pH from 5-12 (See Fig. 2.3B)

Urease activities using 50 mM HEPES buffer were found to be roughly equivalent to the N assimilation rate in urea-grown *Thalassiosira pseudonana* (buffer 1, Fig. 3). However, a buffer comprised of 200 mM potassium phosphate (buffer 2, Fig. 3) gave values above the assimilation

rate, albeit with high variance. Additions of 0.3% PVP and 5 mM EDTA in 200 mM potassium phosphate (buffers 3 and 4) gave similar activities of 1.65 and 1.74 pg N $cell^{-1}d^{-1}$ respectively, and improved the reproducibility but underestimated the N assimilation rate (Fig. 3).

Addition of 3% w/v bovine serum albumen (BSA) to 150 mM potassium phosphate buffer (buffer 5) gave activities above those for 150 mM potassium phosphate buffer (buffer 2) alone (Fig. 3), but with unacceptably high variance. Addition of 3% w/v BSA and 5 mM EDTA to 150mM potassium phosphate buffer (buffer 6) reduced activity and also yielded high variance. Analysis of BSA alone showed high concentrations of contaminating NH_4^+, which most likely contributed to the observed variance. Separate additions of DTT and 2-mercaptoethanol in 150 mM potassium phosphate buffer resulted in low, highly variable activities (Data not shown). Reducing the phosphate concentration in the buffer from 200 mM (buffer 3) to 150 mM (buffer 7), resulted in a three-fold increase of activity to 4.4 pg N $cell^{-1}$ $d^{-1}$and the highest activity was obtained using 150 mM PO_4 buffer with additions of 5 mM EDTA and 0.3% w/v PVP (buffer 8, 8.67 pg N $cell^{-1}$ d^{-1}).

A number of different "kill" methods were employed to ascertain the easiest and most precise method. Heat (60 s, 100°C), addition of zinc acetate, addition of 20 µl 4N HCl with subsequent neutralization with 4N NaOH and 4N HCl without neutralization were employed. Zinc acetate produced highly variable t_0 NH_4^+ concentrations, while the other 3 methods were found to give reproducibly low NH_4^+ concentrations for t_0 samples. The HCl kill without neutralization gave poor color development using the indophenol method and neutralization with NaOH was required. The addition of 20 µl of 4N HCl and neutralization with 20 µl of 4N NaOH was chosen over a heat "kill" because of the ease of processing large numbers of samples.

Urease activity within crude homogenates, stored on ice, was found to be stable for up to 1 h (Fig. 4). By 2 h the activity of urease declined to approximately 70% of its original value. Subsequent assays were all processed within 1 h of the grinding step.

Urease activity in Thalassiosira pseudonana

The urease activity of axenic cultures of *T. pseudonana* depended on nitrogen supply. Urea-grown cultures had the highest activities which were roughly two times the nitrogen assimilation rate (μPN = 3.91; SD = 0.32) (Fig. 5). Nitrate-grown cultures expressed urease at levels roughly equivalent to the assimilation rate (μPN = 3.33; SD = 0.35) while NH_4^+-grown cultures expressed a basal level of roughly 25% of the assimilation rate (μPN = 3.55; SD = 0.37) (Fig. 5). Following nitrogen depletion, urease activities dropped in all cultures, but were maintained at the highest levels in urea-grown cultures.

In bacterized cultures of *T. pseudonana*, activities of nitrate replete cultures were similar to axenic cultures (t = 0, Fig. 5), while NH_4^+ replete cultures expressed activities approximately four times higher (Fig. 6A). Urease activities as well as variance around the mean increased dramatically in bacterized cultures following nitrogen depletion of the medium (Fig. 6B).

Urease activity in Thalassiosira weissflogii

Axenic cultures of *T. weissflogii* expressed urease at similar levels regardless of nitrogen supply (Fig. 7A). Activities in all nitrogen sources were roughly equivalent to assimilation rates (μPN = 36.4; SD = 0.4). Following two days of nitrogen exhaustion, urease activities increased

for urea-grown cultures only, while both NO_3^- and NH_4^+-grown cultures maintained similar activities to nitrogen replete conditions (Fig. 7B). On the third day of nitrogen starvation both urea and NO_3^--grown cultures had increased urease activities while NH_4^+-grown cultures maintained levels of activity similar to NH_4^+-replete conditions. During the starvation stage axenic cultures became contaminated with bacteria. Increased activities in axenic cultures corresponded to increases in the abundance of contaminating bacteria, although the correlation between bacterial numbers and urease activity was poor ($p = 0.15$).

In nitrogen-replete bacterized cultures, urease activities behaved similarly. No differences in activity were observed among the three nitrogen sources (Fig. 8). Activities for urea-grown cultures significantly decreased following nitrogen exhaustion, while NH_4^+ starved cultures remained similar to NH_4^+ replete cultures, although variance increased dramatically. Both activities and variance increased as nitrate starvation increased which corresponded to an increase in bacterial biomass.

Discussion

Urease enzyme assay

Urease was successfully measured in two marine diatoms using a novel homogenization buffer and simple colorimetric measurement for the production of NH_4^+ in crude homogenates. This study is the first report of a method which successfully accounts for the rate of nitrogen assimilation in marine algae grown on urea.

The common method previously used to measure urease in marine algae utilized the method of Leftley & Syrett (1973) which was modified from a method by Adams (1971). This method utilizes radioactive urea ($^{14}CO(NH_2)_2$) and tris (tris(hydroxymethyl) amino ethane) - HCl buffer (pH 8.4) in sealed bottles in which the hydrolysis of urea liberates $^{14}CO_2$ and is trapped using a solution of KOH (Leftley & Syrett 1973, Price & Morel 1991). The method employed in our study avoids the complications of using radioactive isotopes and allows manipulation of the pH to values which are physiologically relevant, but where liberation of CO_2 is problematic. The buffer pH of 7.9 was found to be appropriate for use with both species of diatoms.

Although an indophenol assay has not been used for phytoplankton, it has been applied to prokaryotes. In a review of urease assay techniques, Mobley & Hausinger (1989) recommended the indophenol method for its ease and accuracy. The indophenol assay is linear and accurate despite the complicating factors of algal internal NH_4^+ pools and reactive amines.

Mobely & Hausinger (1989) recommended using a 50 mM HEPES, 5 mM EDTA grinding buffer and warned against the use of phosphate buffer, as the acid of the phosphate anion is known to interfere with microbial enzyme activity. The HEPES buffer was found to give low

activities when applied to *T. pseudonana* and the use of PO_4 buffers gave the maximum observed activities. There may be some inhibition of the enzyme by phosphate, as a reduction in PO_4 concentrations from 200 to 150 mM resulted in a 3-fold increase in observed urease activities. Leftley & Syrett (1973), Bekheet & Syrett (1977), Rees & Bekheet (1982), Singh (1990 &1992), and Price & Morel (1991) all employed assay buffers based on Tris (tris(hydroxymethyl)aminoethane)-HCl buffer. Tris is commonly used in higher plant urease assay buffers as well (Zonia et al. 1995). Tris was not explored as an optional buffer in our study as it produces high and variable NH_4^+ blanks using the indophenol method (Suttle pers. comm.).

Bovine serum albumin (BSA) was added to act as a protease substrate (Berges 1993). Free proteases in a cell homogenate have the ability to digest urease in addition to other cell constituents throughout the course of the incubation period. The addition of BSA could reduce this process. However, contaminating ammonium within the BSA preparation produced highly variable values with standard deviations greater than the average activity. Despite the benefits that BSA might have for preservation of urease, it should not be considered further because of its contaminating properties.

Polyvinyl pyrolidone, PVP, was shown to reduce the variance in urease activities in this work. PVP acts as an adsorbent for phenolic compounds in the buffer homogenate that might otherwise inhibit enzyme activity (Loomis & Battaile 1966). It is recommended that 0.3% w/v PVP be used in all grinding buffer preparations.

Nickel is an integral part of all algal urease enzymes studied thus far (Oliviera & Antia 1986, Price & Morel 1991), and lack of this metal causes a loss in function. Use of the chelator EDTA in the grinding buffer should bind any divalent metal ions that could interfere with the activity by displacing Ni in the active site. Addition of 5 mM EDTA was found to increase

activity and reduce the error between measurements when used in concert with PVP in a 150 mM potassium phosphate buffer. Therefore it is recommended that this be included in the grinding buffer.

Price & Morel (1991) found that urease activity in *T. weissflogii* was <50% of their calculated N assimilation rate. They attributed this low activity to unfavorable enzyme assay conditions. Their assay buffer was modeled after that of Leftley & Syrett (1973) and therefore contained 1.4 μM dithiothreitol (DTT). Mobley & Hausinger (1989) stated that thiols, such as DTT or 2-mercaptoethanol, should be omitted from urease assay buffers as they competitively inhibit the enzyme. Thiols appear to bind to the active site of jack bean and bacterial ureases, rendering the enzyme inactive. It is possible that the low activities observed by Price & Morel (1991) are due to the inhibition of urease by DTT. When DTT was included in this study, poor activities were also observed.

Enzyme activities were normalized to cell number in this study rather than protein, which is used predominantly in the literature (e.g. Singh 1990 & 1992, Leftley & Syrett 1973). In addition, many techniques employ the measurement of $^{14}CO_2$ liberated from radiolabeled urea. This makes it difficult to directly compare results between studies conducted with different groups of phytoplankton in the past, but rough comparisons can be made. Using a protein content of 4.5 pg cell^{-1} (Berges 1993) for *T. pseudonana* and assuming that cells grown on urea do not alter their protein constituents significantly from those grown on NO_3^-, enzyme activity was normalized to protein and converted to carbon units. The converted activity measured in this study, for *T. pseudonana* grown on urea, is 2.7×10^3 nmol C mg protein^{-1} h^{-1} with a converted assimilation rate of 1.38×10^3 nmol C mg protein^{-1} h^{-1}. Leftley & Syrett (1973) measured urease activities of

2.22 nmol C mg protein^{-1} h^{-1} for the diatom *Phaeodactylum tricornutum.* However, using the same method, Rees & Bekheet (1982) were able to measure activities of up to 450 nmol C mg protein^{-1} h^{-1} in the same species grown on NO_3^-. Singh (1990) measured a urease activity of 399.2 nmol C mg protein^{-1} h^{-1} in the freshwater cyanobacterium *Anabaena doliolium.*

Price & Harrison (1988b) found that the uptake of urea by *T. pseudonana* resulted in the rapid efflux of NH_4^+ into the medium, presumably due to high rates of urea hydrolysis and excretion of ammonia which could not be assimilated. The high activity of urease (twice the assimilation rate) for *T. pseudonana* grown on urea measured in this study supports this observation.

Urease activity under nitrogen-replete conditions

Urease was found to be expressed regardless of the nitrogen source in the two diatoms studied, although activities showed distinctly different patterns according to the form of nitrogen supplied. Under nitrogen-replete conditions, urease activity in *T. pseudonana* was expressed constitutively when grown on NH_4^+ growth and upregulated when grown on NO_3^- or urea. In *T. weissflogii,* urease activity was expressed constitutively regardless of the nitrogen source and showed no upregulation.

Similar variability in urease activity according to nitrogen supply has been observed by others. In the freshwater cyanobacteria *Anabaena doliolum, Anacystis nidulans* and *Nostoc muscorum,* Singh (1990 & 1992) found that urease activity remained the same when grown on NO_3^- and urea as the sole N source, but was reduced by the presence of NH_4^+. Ge et al. (1990)

found that urease remained at a constitutive level in the freshwater cyanobacterium *Anabaena variabilis*, regardless of N source.

Variations in urease activity which do not agree with findings of this study have also been observed. For instance, in the marine cyanobacterium *Synechococcus,* Collier & Palenik (1996) found that urease activity remained similar when grown on NH_4^+ or urea, but growth on NO_3^- showed increased urease activity. In two different studies, the marine diatom *Phaeodactylum tricornutum* (clone 1052/6) expressed higher levels of urease when grown on NO_3^- as the sole nitrogen source (Rees & Bekheet 1982) than when grown on urea (Leftley & Syrett 1973).

Urease activity in axenic and bacterized cultures *T. pseudonana* showed no difference for nitrate-grown cultures, however, bacterized NH_4^+-replete cultures had higher urease activities than axenic cultures due to bacterial urease activity (Mobley & Hausinger 1989). Clearly the contribution of bacterial urease activity was high enough to make inferences about nitrogen source regulation of urease impossible even though bacterial biomass was a minor fraction of total biomass of the culture.

Urease activity under nitrogen starvation

When axenic *T. pseudonana* cultures entered nitrogen starvation, urease activity declined but activities remained similar relative to each nitrogen source indicating at least a 3 day influence from growth on each nitrogen source. Bacterized cultures showed a dramatic upregulation of urease with high variability between replicates indicating the variability in bacterial activity in each flask. Algal cell death and an increase in dissolved organic compounds most likely contributed to the enhanced bacterial urease activity.

Axenic cultures of *T. weissflogii* showed no change in urease activity under nitrogen starvation with the exception of urea-grown cultures which developed bacterial contamination and had increased activity. Bacterized cultures of *T. weissflogii* showed a similar pattern with the exception of NO_3^--grown cultures which developed bacterial contamination.

In both species tested, urease activity was not upregulated by nitrogen starvation. This observation appears contrary to the observation that several marine algal species induce high uptake rates of urea following nitrogen starvation, however, urease expression and uptake are likely separately regulated systems in marine diatoms. For instance, uptake but not assimilation of urea is inhibited by the presence of NH_4^+ in *Phaeodactylum tricornutum,* a marine diatom (Syrett & Leftley 1976, Rees & Syrett 1979a). With the exception of NH_4^+-grown *T. pseudonana* urease activity remains at levels high enough to satisfy the nitrogen demands of nitrogen starved cells. Steady state nitrogen limited growth was not tested in this work and could potentially give different results. Our cultures experienced complete exhaustion of nitrogen (N starvation) which can result in remobilization of proteins such as RUBISCO and pigment proteins (Geider et al. 1993). If limiting nitrogen was made available under steady state conditions using a continuous culture apparatus, urease may be upregulated.

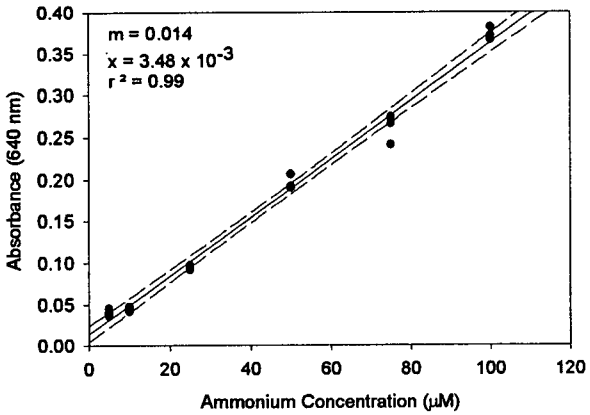

Figure 1. Ammonium measurement in algal homogenates using the indophenol method. A linear regression with 95% confidence limits is shown. Homogenates were spiked with NH_4Cl.

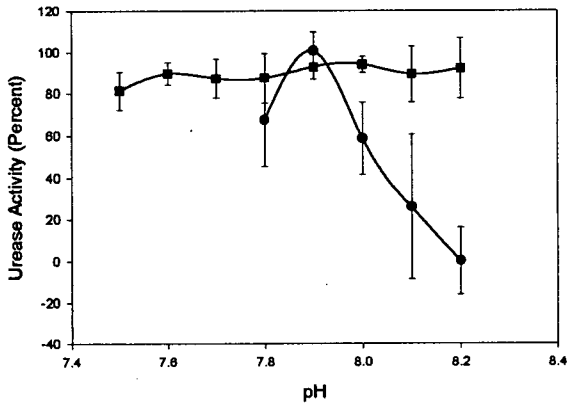

Figure 2. Urease pH optimum for A) *Thalassiosira weissflogii* (●) B) *Thalassiosira pseudonana* (■). Error bars are ± 1 SD (n= 3).

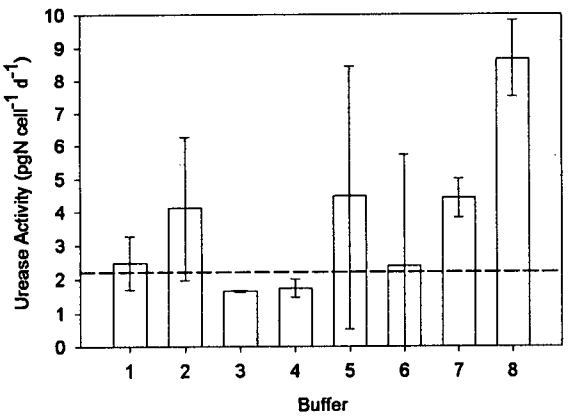

Figure 3. Comparison of urease activity of *Thalassiosira pseudonana* using: 1) 50 mM HEPES (n=6), 2) 200 mM potassium phosphate buffer (n=4), 3) 200 mM potassium phosphate buffer, 0.3% PVP (n=3), 4) 200 mM potassium phosphate, 5 mM EDTA, (n=3), 5) 150 mM potassium phosphate, 3% BSA (n=4), 6) 150 mM potassium phosphate, 3% BSA 5 mM EDTA, (n=4), 7) 150 mM potassium phosphate, 0.3% PVP, (n=4), 8) 150mM potassium phosphate 0.3% PVP, 5mM EDTA, (n=5). Nitrogen assimilation rate for growth on urea was 2.2 pg N $cell^{-1}$ d^{-1} (see dashed horizontal line. Error bars represent ± 1 STD.

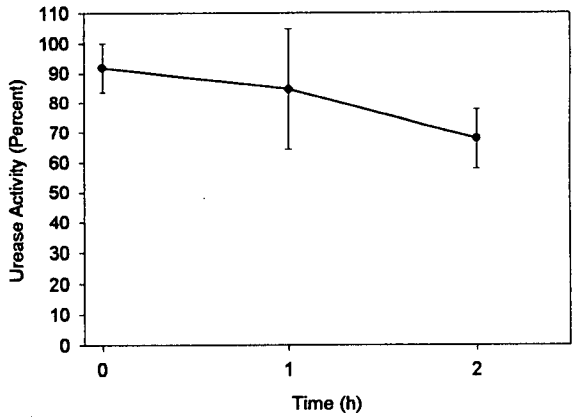

Figure 4. Urease stability following extraction in homogenization buffer for *Thalassiosira pseudonana*. Homogenization buffer contained potassium phosphate buffer (pH 7.9) with 0.1% PVP and 5 mM EDTA. Error bars are ±1 SD (n=3).

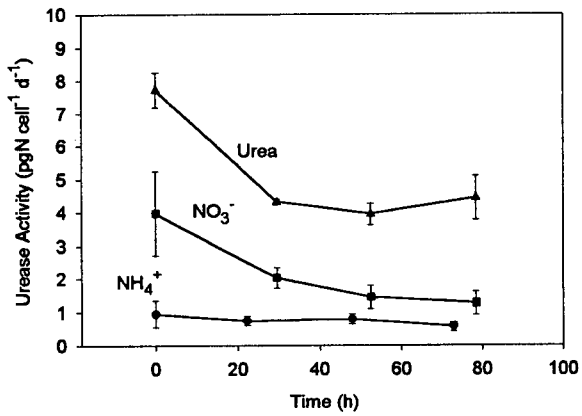

Figure 5. Urease activity in axenically-grown *Thalassiosira pseudonana* under three nitrogen sources (NH_4^+ (●), NO_3^- (■), and urea (▲)). Nitrogen concentrations reached zero at approximately 20 h. Error bars are ±1 SD (n=3).

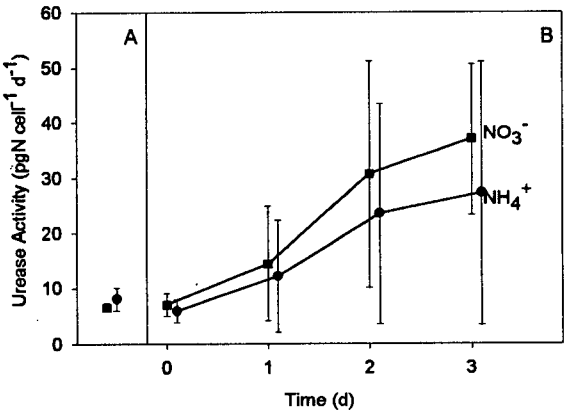

Figure 6. Urease activity in *Thalassiosira pseudonana* cultures containing bacteria under two nitrogen sources (NH_4^+ (●), NO_3^- (■)). A) Nitrogen replete cultures, and B) Separate experiment with cultures entering nitrogen limitation. Nitrogen concentrations reached zero between day one and two. Error bars are ±1 SD (n=3).

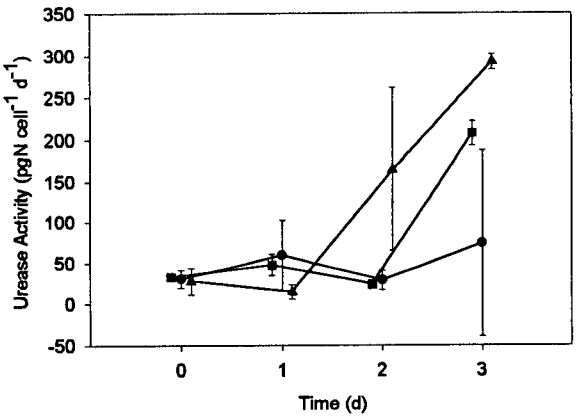

Figure 7. Urease activity in axenically-grown *Thalassiosira weissflogii* under three nitrogen sources (NH_4^+ (●), NO_3^- (■), and urea (▲)). Nitrogen concentrations reached zero between day zero and one. A) Nitrogen replete cultures, and B) Cultures entering nitrogen limitation. Error bars are ±1 SD (n=3).

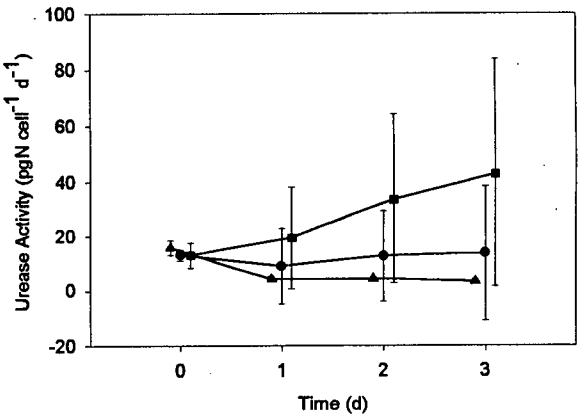

Figure 8. Urease activity in *Thalassiosira weissflogii* cultures containing bacteria, grown with three nitrogen sources (NH_4^+ (●), NO_3^- (■), and urea (▲)). Error bars are ±1 SD (n=3).

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