Expression and Activity of the Enzyme Nitrate Reductase in the Marine Diatom *Thalassiosira pseudonana:* Light and Nutrient Effects

by

Marcos G. Lagunas
B. Sc., Universidad Nacional de la Patagonia (Argentina), 2009

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

Supervisory Committee

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Abstract

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The main goal of this study was to assess the impact that nitrate and light have on the relationship between the gene expression of the enzyme nitrate reductase and the incorporation of nitrate in the cosmopolitan diatom *Thalassiosira pseudonana*, both in laboratory experiments and in natural environments. Continuous cultures were grown at different nitrate (NO_3^-) concentrations (*i.e.*, 60, 120, and 400 μM) to evaluate their effects on the expression levels of different genes of the nitrogen metabolic pathway (*i.e.*, nitrate and ammonium transporters, nitrate and nitrite reductases, glutamine synthetases II and III). Semi-continuous cultures were grown under different irradiances (*i.e.*, 50, 110, 200, and 320 μmol photon cm^{-2} s^{-1}) to assess the influence of light intensity (irradiance) on the relationship between the expression of those genes, uptake, and assimilation of nitrate.

The expression of all of the genes that were tested decreased significantly ($p < 0.05$) at the highest concentration of NO_3^- (*i.e.,* 400 μM), with nitrate transporters showing the most pronounced change from 27.97 to 0.59 fold change $cell^{-1}$ x 10^{-6}, at 60 and 400 μM NO_3^- concentrations respectively. Ammonium transporters were detected at all concentrations of NO_3^-, suggesting that cells are always ready to metabolize

ammonium. Growth was limited (μ = 0.99 d^{-1}) by the low irradiance treatment, was maximum (μ = 2.04 d^{-1}) at 200 µmol photon cm^{-2} s^{-1} and was inhibited (μ = 1.54 d^{-1}) at the highest irradiance. These trends were reflected in gene expression and uptake rates, with minimum values at the lowest and highest irradiance levels. However, results from the enzymatic assay did not show any significant differences between treatments ($p > 0.05$). The trends observed in the enzymatic rates could be explained by the gene expression of NO_3^- reductase and the uptake and growth rates in a multiple regression analysis ($R^2 = 0.66$, $p < 0.05$).

The results of this study show that uptake is independent of gene expression, probably because of a decoupling between transcription and protein synthesis. Not all of the newly synthesized transcripts will inevitably be translated into proteins. And even if they were, there could be post-translational mechanisms preventing the enzymes to become active. This indicates that uptake can be independent of the expression.

It was attempted to measure the expression of *T. pseudonana* genes involved in the metabolism of NO_3^- in natural diatom assemblages. The use of gene expression as a proxy for metabolic processes carried out by a phytoplankton assemblage in the field is limited and depends on environmental factors, since the current methods of assessing expression rely on genomic sequences that are particularly variable in phytoplankton. The assessment of gene expression provides a useful insight into physiological studies of phytoplankton, and it should be complemented with other measurements, such as the biomass and taxonomic composition of the assemblage for a more complete picture of marine ecosystem nutrient dynamics.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACT: β-actin
BLAST: Basic Local Alignment Search Tool
C: carbon
CCGS: Canadian Coast Guard Ship
cDNA: complementary deoxyribonucleic acid
CO_2: carbon dioxide
Cq: quantification cycle
CTD: conductivity-temperature-density
d: day
DNA: deoxyribonucleic acid
DOC: dissolved organic carbon
DON: dissolved organic nitrogen
DTT: dithiothreitol
ESAW: artificial seawater media
F: flow
Fe: iron
GAP: glyceraldehyde-3-phosphate dehydrogenase
GSII: glutamine synthetase II
GSIII: glutamine synthetase III
h: hour
H_2: dihydrogen
HCl: hydrochloric acid
H_2S: hydrogen sulfide
KNO_3: potassium nitrate
MFS: major facilitator super family of proteins
mRNA: messenger ribonucleic acid
N: nitrogen
NADH: nicotinamide adenine dinucleotide
$NaNO_3$: sodium nitrate
NaOH: sodium hydroxide
NAT: nitrate transporters
NH_4^+: ammonium
NiR: nitrite reductase
NNP: nitrate and nitrite porter family of proteins
NO_3^-: nitrate
NO_2^-: nitrite
N_2O: nitrous oxide
NR: nitrate reductase
P: phosphorus
PAR: photosynthetically active radiation
PMS: phenazine methosulphate
PNP: purine-nucleoside phosphorylase

PO_4^{3-}: orthophosphate
POC: particulate organic carbon
POM: particulate organic matter
PON: particulate organic nitrogen
POT: proton-dependent oligopeptide transport family of proteins
PVP: polyvinyl pyrrolidone
PyNP: pyrimidine-nucleoside phosphorylase
qPCR: quantitative polymerase chain reaction
RNA: ribonucleic acid
rRNA: ribosomal ribonucleic acid
Si: silicon
$Si(OH)_4$: silicic acid
tRNA: transfer ribonucleic acid
TUB: β-tubulin
V: volume

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I am very grateful to my supervisor, Dr. Diana Varela, for her immense patience and constant support. It has been a real pleasure and honour to be under the supervision of such an outstanding professor, and more importantly, an exceptional person. This thesis would have never been completed without her invaluable help.

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Chapter 1: Introduction

1.1 The Role of Phytoplankton in the Oceans

Primary production is the rate at which organic carbon (C) is fixed by autotrophic organisms that utilize carbon dioxide (CO_2) as a C source. In the oceans, photosynthesis is responsible for > 99% of primary production, by a process in which light energy is converted into chemical energy and stored in the bonds of organic molecules (*i.e.,* photo-autotrophy) (Falkowski, 2003). In contrast, < 1% of marine primary production is the product of chemosynthesis, where chemical energy derived from the oxidation of inorganic molecules (*e.g.*, H_2, H_2S) is responsible for the reduction of CO_2 into organic C compounds (*i.e.,* chemo-autotrophy) (Falkowski, 2003). When the C consumed by other metabolic processes is subtracted, the remaining C that is available for upper trophic levels is called net primary production. Marine primary producers account for almost half of the world's annual net primary production (~46%) (Field *et al.* 1998; Chavez *et al.* 2011).

The fixation of CO_2 into organic matter by photosynthesis in the oceans is mainly regulated by the availability of nutrients such as nitrogen (N), phosphorus (P), silicon (Si) (for diatoms and silicoflagellates), trace elements (such as iron, Fe), and light. The depth at which sunlight levels are ~0.1% of surface light intensity (or irradiance) is the bottom of the euphotic zone, and represents the lower limit of the fraction of the water column where photosynthesis occurs. If all gross primary production were consumed within the euphotic zone by heterotrophy, all organic matter produced by

photosynthesis would be respired and inorganic nutrients would be recycled within the upper water column to further support photo-autotrophy (*i.e.,* 'regenerated' production). However, a fraction of the organic matter produced in surface waters escapes remineralization and is exported to deep waters (Eppley and Peterson, 1979). Those nutrients lost to deep waters are eventually replaced by nutrients from other sources. The re-supply of inorganic nutrients to the euphotic zone is mainly from deep waters (*e.g.*, upwelling and mixing), the atmosphere (*e.g.*, N fixation), and continental runoff. The resulting primary production is called 'new production' and, under steady-state conditions, should be approximately equal to the export flux of organic matter from the surface to the deep ocean (Falkowski, 2003). The fixation of CO_2 and the settling of the resulting particulate organic matter (POM) to deep waters are referred to as to the 'biological carbon pump' (Ducklow *et al.* 2001).

Phytoplankton are the autotrophic components of the pelagic community, and are responsible for the vast majority of marine primary production, which affect the C cycle, as well as those of other nutrients (*e.g.*, N, Si and P) through the biological carbon pump (Duarte and Cebrian, 1996). Phytoplankton represents a link between several biogeochemical processes, and their role in the ocean is comparable to that of plants in terrestrial ecosystems (Falkowski, 2003). Phytoplankton are composed of organisms from at least eight different phyla (with ~2 x 10^4 species). In contrast, only one phylum is responsible for autotrophy on land (*i.e.*, Embryophyta, comprising ~2.5 x 10^5 species) (Falkowski *et al.* 2004). Thus, the phylogenetic diversity of primary producers is much greater in marine ecosystems than terrestrial ecosystems.

One of the most important marine phytoplankton phyla is Heterokontophyta, and the class Bacillariophyceae (*i.e.*, diatoms) stands out for the large number of species it contains, with about 5000 marine species and 5000 freshwater species. Diatoms play a significant role in marine energy fluxes by controlling the particulate organic carbon (POC) flux to deep waters due to their large size and fast sinking rates (Sarthou *et al.* 2005; Ragueneau *et al.* 2006; Armbrust, 2009). Diatoms have unique characteristics, such as the presence of vacuoles that occupy ~35% of the cell volume (Falkowski, 2003). These cellular structures give them the ability of storing nutrients in high concentrations, representing an advantage over other taxonomic groups when the supply of nutrients is in short pulses, such as wind driven upwelling (Falkowski, 2003).

1.2 The Marine Nitrogen Cycle

The cycle of N in the ocean is complex, because N is present in many chemical forms, *i.e.,* molecular nitrogen (N_2), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), and dissolved organic nitrogen (DON), such as urea and amino acids. Molecular nitrogen represents ~94% of the oceanic N inventory, and all of the other forms account for the remaining ~6%. The bulk of this smaller fraction (~88%) is represented by NO_3^- and almost 12% by DON. Ammonium, particulate organic nitrogen (PON), NO_2^- and nitrous oxide (N_2O) account for less than ~0.3% and the transfer of N from one pool to another is mainly biologically mediated (Gruber, 2008).

Nitrogen is a key component in living organisms, being part of nucleic acids and amino acids that compose proteins. All of the N chemical forms present in the ocean (except N_2) are referred to as 'fixed nitrogen' and can be used by phytoplankton to

produce organic matter (Dugdale and Goering, 1967; McCarthy, 1972). Eppley and Peterson (1979) proposed the *f*-ratio parameter to estimate the export efficiency of an ecosystem. This was defined as the ratio between NO_3^- uptake and the sum of NH_4^+ and NO_3^- uptake (*i.e.*, the ratio between new to total production). However, many factors have been found to introduce great complexity to the model since its formulation. Before the recent realization of the importance of N_2-fixation (Capone *et al.* 2005), upwelling of oxidized forms of nitrogen (*i.e.*, NO_3^-) was thought to be the only source of N for new production in open waters (Dugdale and Goering, 1967). However, studies have also shown that NO_3^- could be regenerated within the water column (*i.e.*, nitrification) (Zehr and Ward, 2002). Similarly, NH_4^+ was thought to be the only N form responsible for regenerated production (Eppley and Peterson, 1979), but other compounds that make up DON (urea and others) can also support phytoplankton growth (Mulholland and Lomas, 2008). Considering this, measurements of urea uptake need to be taken into account when estimating the 'regenerated' component of the *f*-ratio. In addition, phytoplankton can release NH_4^+ and NO_2^- (Bronk *et al.* 1994), and bacteria can also take up NO_3^- and NH_4^+ (Kirchman and Wheeler, 1998). None of the processes previously mentioned should be neglected when estimating new production.

Nitrogen is the major limiting nutrient in the ocean and the ratio between N_2-fixation and the dissimilatory removal of NO_3^- from the water column (*i.e.*, denitrification) controls the inventory of marine N and in turn regulates CO_2 fixation in the ocean (Mulholland and Lomas, 2008). On a global scale, the N cycle is subject to perturbations that might alter its equilibrium, affecting the amount of fixed N available for phytoplankton. The anthropogenic fixation of N_2 in the production of fertilizers and

the runoff of N from the continent has contributed to the eutrophication of coastal areas (Nixon, 1995; Gruber, 2004; Gruber and Galloway, 2008). A decrease in nitrification caused by a decrease in the oxygen concentration in the interior ocean, predicted to occur as one of the consequences of global warming (Gruber *et al.* 2004) would also affect the fixed N stock. Understanding the factors that regulate the uptake of the different chemical forms is critical for evaluating the response of the marine system to natural or anthropogenic perturbations.

1.3 Using Molecular Tools in the Study of Marine Phytoplankton Physiology of Nitrogen

Deoxyribonucleic acid (DNA) holds the genetic information necessary to synthesize proteins in a process called translation. However, before this can take place an intermediate step must occur. Transcription (*i.e.*, the synthesis of ribonucleic acid, RNA, using a DNA molecule as a template) is a process highly regulated by DNA-binding proteins (transcription factors) that determine the rate, repression and initiation of transcription. There are different types of RNA, each one performing different functions in the cell, but three of them are involved in protein synthesis (*i.e.*, translation). Ribosomal RNA (rRNA) is a structural component of the protein synthesis machinery (*i.e.*, ribosomes), messenger RNA (mRNA) serves as a template for peptide synthesis, and transfer RNA (tRNA) delivers amino acids to the ribosomes. Since mRNA is the intermediate step in the flow of genetic information from the genome to the synthesis of a protein, the expression of a functional gene (*i.e.*, a gene responsible for either enzymes or regulatory proteins) can be determined by measuring mRNA levels (Alberts *et al.* 2013).

The enzymes that carry out uptake, reduction and assimilation of N compounds in phytoplankton play key roles in the cellular metabolism of this element (Figure 1). Transporter proteins are responsible for the uptake of NO_3^-, NO_2^-, and NH_4^+. Nitrate and nitrite assimilative reductases catalyze the reduction of NO_3^- into NO_2^-, and NO_2^- into NH_4^+, respectively, and glutamine synthetases catalyze the assimilation of NH_4^+ into amino acids (Berges and Mulholland, 2008). Enzymatic activity does not always correlate with the expression of a gene. Some genes such as NO_3^- transporters (NAT) are inducible by the concentration of the substrate and their expression reaches a peak a few minutes after induction (*i.e.,* initiation or enhancement of the gene expression). However, high levels of gene expression do not necessarily reflect high concentrations of the proteins which ultimately carry out metabolic processes. High levels of mRNA from the NAT gene were observed when cells are transferred into different media (*e.g.*, from a low substrate concentration to a higher concentration) but once the rate of uptake reaches a maximum, the mRNA concentration decreases (Song and Ward, 2007). Some proteins are kept at basal levels in the cell and reach a peak when they are induced (*e.g.*, NAT in the marine diatom *Cylindrotheca fusiformis* (Hildebrand and Dahlin, 2000)), whereas other proteins are not present until transcription is activated. Therefore, the relationship between levels of mRNA and the concentration of the protein varies depending on the gene.

The amount of a protein in a cell is the result of the balance between its synthesis and degradation. Synthesis is usually regulated at its beginning, the transcription of the gene sequence. Therefore, by studying the synthesis of RNA one can determine when genes are turned on and what conditions triggered the process.

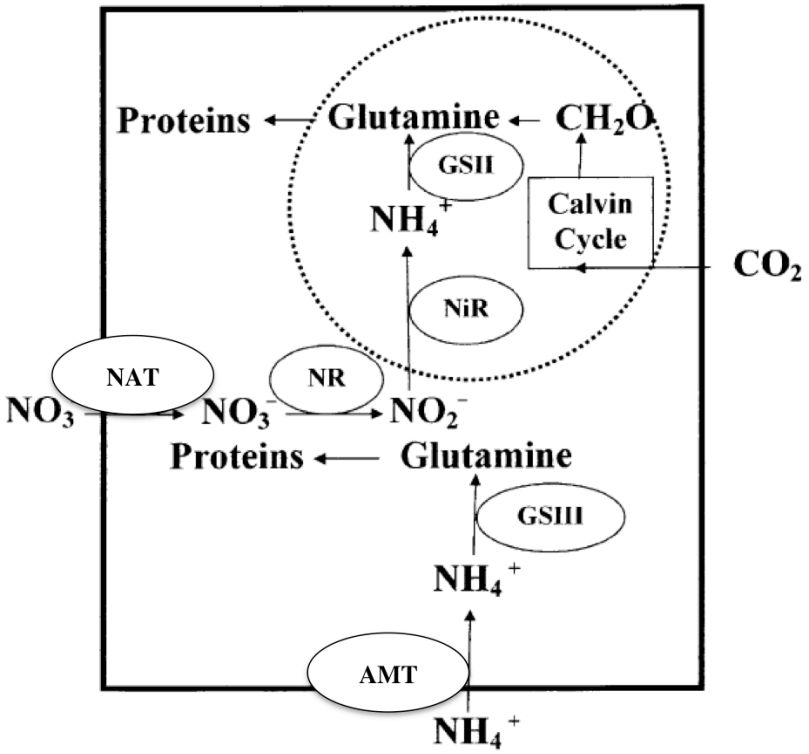

Figure 1: Summary of the major metabolic pathways of nitrogen in a diatom cell. The large rectangle represents the plasma membrane, while the broken circular line represents the chloroplast membrane. Nitrate is taken up by the cell by means of nitrate (NO_3^-) transporters (NAT), where it is reduced to nitrite (NO_2^-) by the enzyme nitrate reductase (NR). Nitrite is then translocated inside the chloroplast, where it is further reduced to ammonium (NH_4^+) by the enzyme nitrite reductase (NiR). The enzyme glutamine synthetase II (GSII) incorporates this NH_4^+ into amino acids. Ammonium is also taken up across the plasma membrane by ammonium transporters (AMT), and is incorporated into amino acids by the enzyme glutamine synthetase III (GSIII). Modified from Takabayashi *et al.* (2005).

Nitrate transporters have been classified into two families: *nrt1* and *nrt2* (Forde, 2000). The *nrt1* genes belong to the Proton-dependent Oligopeptide Transport (POT) family of proteins and are dissimilar to the *nrt2* family, as *nrt1* genes have completely different sequences and are mostly related to low-affinity transporters and *nrt2* to high-

affinity ones. The n*rt2* genes belong to the NO_3^- and NO_2^- porter (NNP) family of proteins, and this family belongs in turn to the Major Facilitator Super-family (MFS) (Galvan and Fernandez, 2001). Nitrate transporters from both families can be classified into constitutive and inducible, and in high affinity or low affinity depending on the concentration of the substrate where they become active (Galvan and Fernandez, 2001). Both families have been identified in marine diatoms (Hildebrand and Dahlin, 2000; Song and Ward, 2007), picoeukaryotes (McDonald *et al.* 2010) and also in cyanobacteria (Herrero *et al.* 2001; Scanlan and West, 2002). However, not all cyanobacteria contain NAT, since members of the genus *Prochlorococcus* that lack this gene cannot metabolize NO_3^- and only grow when NH_4^+ is present (Scanlan and West, 2002). In contrast, *Synechococcus* sp. is capable of utilizing NO_3^- as a source of N. They do not possess genes related to NO_3^- metabolism and that might be a consequence of the niche that *Prochlorococcus* sp. occupies, mainly in surface waters with low NO_3^- concentrations.

The expression of NAT differs in different phytoplankton species, even within the same genus. For example, the transcription of *nrt2* was found to increase under N-starvation and the presence of NO_3^- for the diatoms *Thalassiosira wesisflogii* and *Chaetoceros muelleri*, but only under the presence of NO_3^- for the chlorophyceae *Dunaliella tertiolecta* (Song and Ward, 2007). Ammonium was found to inhibit NAT transcription in the diatom *C. fusiformis* though basal levels of expression have also been found (Hildebrand and Dahlin, 2000). In general, phytoplankton prefer NH_4^+ over NO_3^-, and the presence of NH_4^+ inhibits the uptake of NO_3^- (Dortch, 1990). This is because NH_4^+ can be quickly assimilated and cells do not have to spend energy on its reduction.

Similarly to NAT, there are two major families of ammonium transporter genes (AMT), *amt1* and *amt2*. The expression of both families of genes increased when cells of the diatom *C. fusiformis* were grown in a NO_3^--rich medium and under N-starvation (Hildebrand, 2005). However, members of the *amt2* family might function as an N sensor rather than as an efficient transporter, given the low efficiency and low levels of mRNA associated with its expression (Hildebrand, 2005). Ammonium transporters have been found in marine diatoms (Hildebrand, 2005), picoeukaryotes (McDonald *et al.* 2010) and cyanobacteria (Herrero *et al.* 2001; Scanlan and West, 2002).

Nitrate reductase (NR) was one of the first enzymes studied in marine phytoplankton (Eppley *et al.* 1969). The activity of NR was found to be regulated by its rate of synthesis and degradation (Berges, 1997), and highly correlated with NO_3^- uptake (Berges and Harrison, 1995). The regulation of NR seems to be highly coupled with the activity of NiR (Berges and Mulholland, 2008) and the activity of NiR is generally greater than NR (Berges and Mulholland, 2008). Both glutamine synthetase enzymes (GSII and GSIII) are highly regulated by feedback mechanisms (*i.e.,* the action of the enzyme is inhibited by excessive levels of the end product of the metabolic pathway) (Stadtman, 2001) and regulatory circuit controls (*i.e.,* when the binding of specific proteins to the DNA sequences triggers the gene expression such as the transcriptional regulator *ntcA* in cyanobacteria) (Herrero *et al.* 1981; Berges and Mulholland and Lomas, 2008). Although these enzymes play key roles in the conversion of different forms of fixed N within the phytoplankton cell, there are still many unknown aspects of their regulation.

Molecular tools can be used to evaluate how phytoplankton growth and distribution are controlled in natural environments (Jenkins and Zehr, 2008). The expression of functional genes can be used to determine what organisms are responsible for specific biogeochemical reactions in oceanic ecosystems (Zehr and Capone, 1996; Ward, 2005; Ward, 2008), which represents an advantage especially when studying the small cells of phytoplankton assemblages (Moon-van der Staay *et al.* 2001; Moreira and Lopez-Garcia, 2002). Different genes have been used to study the active fraction of the phytoplankton community, such as the large subunit of the ribulose-1,5-biphosphate carboxylase/oxygenase enzyme (*i.e.,* Rubisco) (Pichard *et al.* 1997; Wawrik *et al.* 2002; Corredor *et al.* 2004; John *et al.* 2007), dinitroreductase (Church *et al.* 2005) and NR (Ward, 2008). The quantification of mRNA levels could also be used to determine the degree of nutrient deficiency (Kang *et al.* 2009). The expression of functional genes varies under different physico-chemical conditions in the ocean. For example, Wawrik *et al.* (2003) showed that the expression of the Rubisco enzyme gene varied with depth, and nutrient availability, but particularly with light intensity in a low salinity-high chlorophyll plume of the Mississippi River in the Gulf of Mexico. Although NAT, AMT and NR have been identified in many phytoplankton species (~20 species), little is known about their regulation and spatial distribution under the physico-chemical conditions characteristic of coastal and oceanic waters.

Light plays a key role in the regulation of the uptake of nutrients, and the relationship between NO_3^- uptake and light intensity can be described by a hyperbolic equation (MacIsaac and Dugdale, 1972). In some areas of the ocean, light can be a limiting factor for photosynthesis and the degree of its influence on uptake of N seems to

depend on the chemical N species and the acclimation of phytoplankton to the environmental light levels (Kudela *et al.* 1997; Maguer *et al.* 2011). Phytoplankton can also receive light in excess. And they can avoid damage to the photosystem by redirecting excess energy to the uptake of NO_3^- and release the N in the form of DON (Lomas and Glibert, 1999). An increase in the expression of NR has been measured in the diatom *T. pseudonana* under high light conditions (Schnitzler Parker and Armbrust, 2005). Thus, light has been shown to be a factor influencing NR gene expression, at least indirectly in this species.

Most studies have focused on the effects of the presence or absence of nitrate on the expression patterns of transporter proteins and the enzyme NR, with special emphasis in the latter because the reduction of NO_3^- is considered to be the limiting step in the metabolism of NO_3^- (Solomonson and Barber, 1990). However, there is no information available on the effects that different levels of light intensity (irradiance) and different concentrations of NO_3^- have on the gene expression.

1.4 Question that Motivated this M.Sc. Project

The goal of my M.Sc. project was to answer the following questions:

How do irradiance levels and NO_3^- concentrations affect the expression of different genes involved in the metabolism of NO_3^-?

Can NR gene expression in T. pseudonana be used as a proxy of NO_3^- uptake rates in marine natural assemblages?

To address these questions, I compared the relative expression of four genes involved in the metabolism of NO_3^- (*i.e.*, NAT, NR, NiR, and GSII) and two genes involved in the metabolism of NH_4^+ (*i.e.*, AMT and GSIII) in continuous cultures of the cosmopolitan marine diatom *Thalassiosira pseudonana* growing at three different nitrate concentrations. This species was chosen because its genome was completely sequenced (Armbrust *et al.* 2004), and therefore there are sequences available from the functional genes of interest and also from genes that could be used as normalizers. In a separate set of experiments, I assessed the expression of the NR gene, the activity of the enzyme, and the uptake rates using semi-continuous cultures of *T. pseudonana* exposed to different irradiance treatments. I also took samples from natural phytoplankton assemblages in coastal and oceanic stations in the NE Pacific Ocean to assess the NR gene expression of *T. pseudonana* and related it to the environmental conditions present at the time of sampling and NO_3^- uptake rates.

The ultimate aim of this work was to find the relationship between NR gene expression and the incorporation of NO_3^- in *T. pseudonana* and compare its expression to that of other functional genes related to the metabolism of NO_3^-. If the expression of the NR gene is a good predictor of the activity of the NR enzyme and the uptake of NO_3^-, then that knowledge could be used to forecast these metabolic processes without actually measuring them. This would allow the assessment of these processes in the lab instead of carrying out time consuming incubations *in situ*.

Chapter 2: Materials and Methods

2.1 Effect of Nitrate Concentrations on Gene Expression in *T. pseudonana*

2.1.1 Culture conditions

Continuous cultures of the diatom *Thalassiosira pseudonana* (Hustedt) were used in these experiments. The strain employed was CCMP 1335 from the Bigelow National Center for Marine Algae and Microbiota collection; originally isolated from Moriches Bay, Forge River, Long Island, New York, USA. Cultures were grown in 4 L glass flasks in sterile artificial seawater media (ESAW, Berges *et al.* 2001) at 18ºC under continuous photosynthetically active radiation (PAR) (*i.e.*, wavelengths from 400 to 700 nm). The culture volume (V = ~1.5 l) was stirred continuously and bubbled with pre-filtered air through a sterile 0.2 µm polycarbonate filter. The flow rate (F) of fresh medium addition and culture removal was 2 l d^{-1}, giving a theoretical growth rate µ of 1.33 d^{-1} (*i.e.*, $\mu = F/V = 2\ l\ d^{-1}/1.5\ l$). The experiment was run by triplicate at each of three NO_3^- concentrations. The different treatments were: 60, 120, and 400 µM NO_3^-. The highest concentration (*i.e.,* 400 µM) was chosen as it is in excess compared to the concentration of other nutrients present in the artificial seawater (*i.e.,* ESAW). The two lowest concentrations (*i.e.,* 60 and 120 µM) represent common values in highly eutrophic waters (Yao *et al.* 2008).

Growth was monitored through measurements of cell densities using a Z2 Coulter Counter particle counter and size analyzer (Beckman-Coulter). After acclimation of the cultures to the experimental conditions (~10 generations, about 12 days), sampling took

place. Experiments lasted about 18 days including acclimation and sampling.

2.1.2 Sampling

For each triplicate set of experiments (*i.e.,* for each treatment), aliquots were taken for cell counting and gene expression analysis three consecutive times every 24 hours. The results from the 3 consecutive days were used to calculate an average value for that particular replicate in each treatment (Figure 2). Cell numbers were monitored at all times to ensure that the biomass was in balance at the moment of sampling (*i.e.,* to ensure that the growth rate was constant).

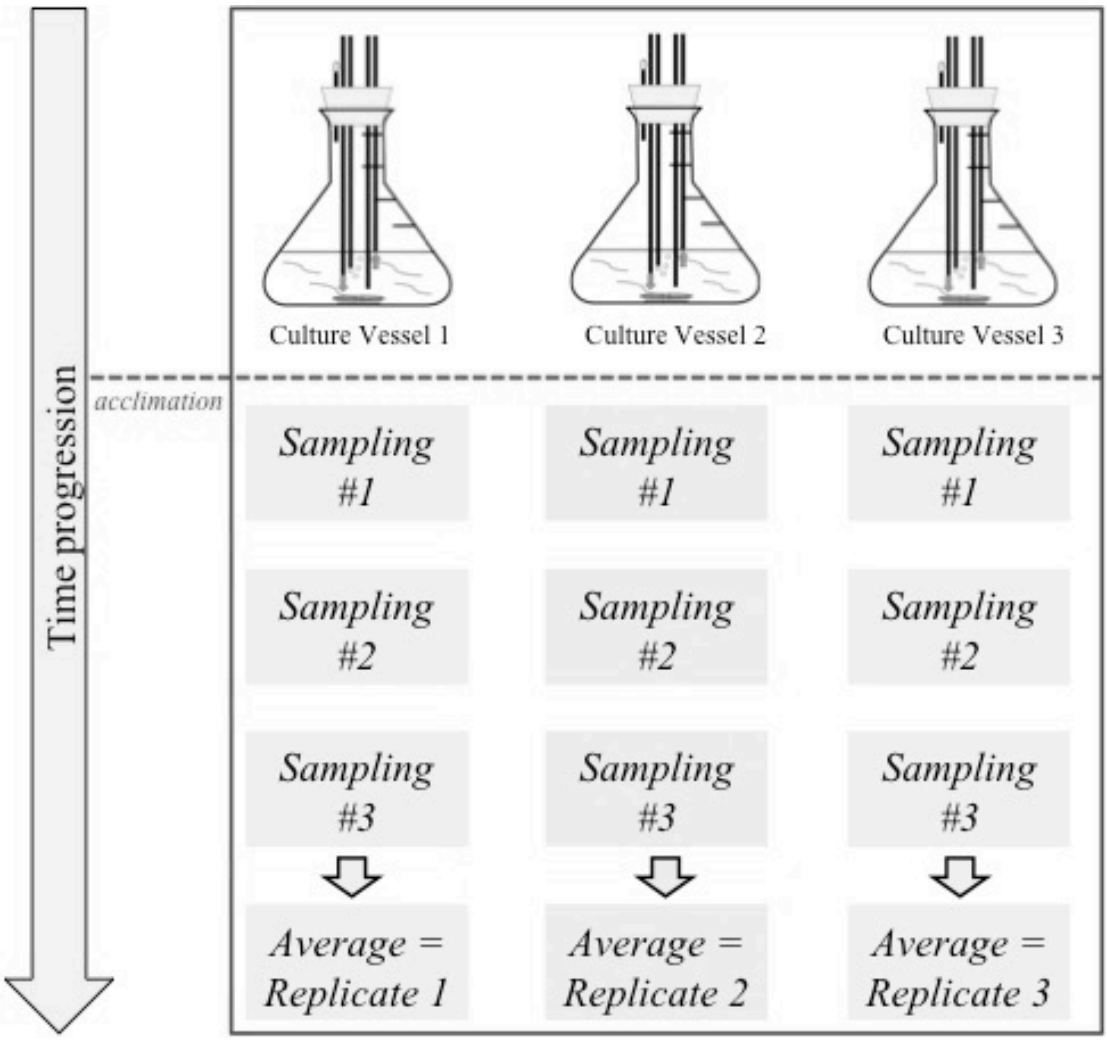

Figure 2: Experimental design of continuous cultures used for the determination of the effect of NO_3^- concentrations on gene expression in *T. pseudonana*. For each treatment, acclimated cultures were sampled 3 consecutive times every 24 hours and an average value was calculated per replicate.

2.1.3 Cell counting

Aliquots (15 ml) were taken from each culture vessel for the determination of *T. pseudonana* cell concentrations using a Z2 Coulter Counter particle counter and size analyzer (Beckman-Coulter). This instrument measures changes in the impedance of electrodes caused by the passing of particles, and these changes can be translated into size and volume estimations, as well as the concentration of particles. The measurements were performed within 30 min of the sampling for the other parameters.

2.1.4 Gene Expression Measurements

The relative expression of the NR gene from *T. pseudonana* was assessed by performing quantitative polymerase chain reaction (qPCR) assays on cDNA created from RNA samples taken from cultures growing at the experimental conditions. These samples had a similar cell concentration (see section 2.1.4.2). The ΔΔCq method (Livak and Schmittgen, 2001) was used to determine relative levels of gene expression. In this method, the expression of a target gene is normalized with the expression of a mean value obtained from constitutive genes. In this experiment, three genes were used as normalizers (see Appendix B). Cq values were normalized by the number of cells present in the sample at each sampling time.

2.1.4.1 RNA sampling

Samples for RNA (100 ml) were filtered through sterile 2 μm pore size polycarbonate filters (AMD Manufacturing Inc.) and placed into 2 ml polypropylene vials, where 600 μl of RLT buffer containing 1% β-mercaptoethanol (which is used to

cleave disulfide bonds of ribonuclease proteins) also were added. RLT buffer allows the binding of RNA to the silica membranes used in the extraction kits (Qiagen Inc., Valencia, CA). The vials were immediately immersed into liquid N_2 for 1 h and stored at -80°C until extraction.

2.1.4.2 RNA extraction and generation of cDNA

Three to four sterile silica-zirconium beads (BioSpec Products) were added to the vials containing the filters and buffer. The tubes were vigorously shaken using a Mini-Bead Beater-8 (BioSpec Products) at max speed for 1.5 min. The RNA was extracted using an RNeasy® kit (Qiagen Inc., Valencia, CA) following the instructions from the manufacturer and digested with DNase to eliminate genomic DNA (Thermo Fisher Scientific Inc.). After assessing the RNA concentration using a Nanodrop spectrophotometer (Fischer Scientific, Toronto, ON), the RNA was converted to cDNA using a SuperScript® II Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA) and random hexamer primers. The reverse transcription was performed at 42°C for 60 min and the reaction was stopped by heating to 85°C for 5 min. The cDNA was stored at -20°C until the qPCR analysis.

2.1.4.3 Primers

Primers were obtained from the literature (Schnitzler Parker and Armbrust, 2005; Brown *et al.* 2009) or designed using the software Primer Premier (Premier Biosoft). See Table 1 for a detailed list of primer sequences. A thorough validation process was followed to ensure the correct amplification of the target sequences and normalization

genes (*i.e.,* β-actin, purine-nucleoside phosphorylase, pyrimidine-nucleoside phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, and β-tubulin) (Bustin *et al.* 2009). After establishing the optimal thermocycling parameters by observing the products on electrophoresis gels, the fragments were sequenced to confirm the identity of the amplicons (see Figure A1 in Appendix A). Finally, an analysis of the efficiency of the amplification of both target and normalizer genes was performed (*i.e.,* the slope of the line of log2 dilutions against the ΔCq values (between the gene of interest and the normalizer β-actin) was checked to be between -0.1 and 0.1). The suitability of the normalizer genes was also checked using the online tool RefFinder (http://www.leonxie.com/referencegene.php?type=reference). Only β-actin, purine-nucleoside phosphorylase, and glyceraldehyde-3-phosphate dehydrogenase proved to have similar amplification efficiencies throughout the experimental conditions and were therefore used as normalizer genes (see Appendix B).

Table 1: Name of the target gene for qPCR reactions, their National Center for Biotechnology Information (NCBI) reference sequence used during primer design, their actual sequence and the size of the amplicon generated for *T. pseudonana*.

Target gene	**NCBI Ref. Seq.**	**Primer**	**Sequence (5' → 3')**	**Amplicon Size**
β-actin *(normalizer)*	XM_002286075.1	Forward Reverse	AGCCCAACCTTACTGGATTGGAGA TGTGAACAATCGAAGGTCCCGACT	327 bp
Purine-nucleoside phosphorylase *(normalizer)*	XM_002292901.1	Forward Reverse	GTGGCGAAGGAGCTACAGTT CGGAACAGTCGACATCCCAA	144 bp
Pyrimidine-nucleoside phosphorylase *(normalizer)*	XM_002286045.1	Forward Reverse	TCTCACCGGGAGCTACTTCA GGGACGCTCTGCAATCTTCT	173 bp
Glyceraldehyde-3-phosphate dehydrogenase *(normalizer)*	XM_002291813.1	Forward Reverse	AGTCTGATCTCCGTCGTGCT TCCTCGCTAGCCTTTTTCAA	232 bp
β-tubulin *(normalizer)*	XM_002286326.1	Forward Reverse	GCCTTTGATGCCAAGAACAT GATGGATGCCTTGAGGTTGT	183 bp
Nitrate reductase	XM_002294374.1	Forward Reverse	TGAGGAAGCATAACAAGGAGG AGCATCAGAAACAACCGCCA	233 bp
Nitrate transporter (*nrt2*)	XM_002295868.1	Forward Reverse	TGGAGGAAATGTTGGAGCCG GTCCAGTGAAGAGACCAGCG	152 bp
Ammonium transporter (*amt2*)	XM_002287349.1	Forward Reverse	AACTGAATGGAGAATGGACCG AAGTCAATAACGCCCGAACCC	260 bp
Ferredoxin dependent-Nitrite reductase	XM_002289229.1	Forward Reverse	ATCAGCAAAGGAGTGCCGTG CAGTCCAGTGAATACGAATCG	322 bp
Glutamine synthetase II	XM_002294909.1	Forward Reverse	GTACCGTGCCTGTCTCTACG CCACAATAGGCTTGGGGTGT	197 bp
Glutamine synthetase III	XM_002295238.1	Forward Reverse	GACCGTGGAGCAAACACCTA CCATGCTTATTCAAGGCGGC	362 bp

2.1.4.4 qPCR and fold change calculations

Samples of cDNA generated as specified in section 2.1.4.2 were amplified on a C1000TM thermal cycler with Chromo 4 Real time Detector (Bio-Rad, Hercules, CA) in 96-well opaque qPCR plates with adhesive seals (Bio-Rad) using a SsoFast EvaGreen Supermix® (Bio-Rad). Reactions were performed in 20 µl mixtures that consisted of 10 µl of SsoFast Evagreen Supermix, 1 µl of each forward and reverse primer, 7.25 µl of RNase/DNase free water and 0.75 µl of cDNA template. The qPCR consisted of a denaturing step of 2 min at 94°C, and 35 cycles of 94°C for 10 sec, 65°C for 30 sec, and 72°C for 1 min. The ΔΔCq method was applied to determine fold changes in the gene expression of different samples (Litvak and Schmittgen, 2001). The fold change was calculated as the power of the ΔΔCq values (*i.e.*, the difference of Cq values (the threshold cycle values, an indirect measure of expression) from the genes of interest minus the geometric mean of Cq values obtained from the normalized genes, using the 400 µM treatment as the control). These values were normalized by the number of cells present in each sample at each sampling time.

2.2 Effect of Irradiance Levels on NR Expression and Activity in *T. pseudonana*

2.2.1 Culture conditions

Semi-continuous cultures of the diatom *T. pseudonana* (strain CCMP 1335) were grown in sterile artificial seawater media (ESAW, Berges *et al.* 2001) at 18ºC under continuous light. Cells were acclimated to four different irradiance levels of PAR (*i.e.,* 50, 110, 200, 320 µmol photon cm^{-2} s^{-1}) in 50 ml glass tubes (in triplicate). Growth was

monitored with *in vivo* fluorescence to calculate growth rates using a Turner Designs 10-AU fluorometer (Sunnyvale, CA, USA). After ~10 generations of acclimation to culture conditions, cells were scaled up and transferred to three 500 ml polycarbonate bottles (Nalgene) containing fresh media. After a couple of generations growing under the same acclimation conditions, sampling took place when the cultures were in exponential phase.

2.2.2 Sampling

Aliquots were taken for cell counting, gene expression analysis, NR activity measurements, and the determination of NO_3^- concentrations twice, 24 hours apart. Refer to sections 2.1.3 and 2.1.4 above for details on cell counting and gene expression analyses, respectively. The fold change of relative expression was calculated using the ΔΔCq method using the 200 μmol cm^{-2} s^{-1} treatment as control because this irradiance was found to be the optimal for *T. pseudonana* growing at 18ºC in a preliminary experiment (see Appendix C).

2.2.3 NR activity measurements

The enzymatic assay was performed as explained in Berges and Harrison (1995). The enzyme NR reduces nitrate to nitrite while oxidizing NADH into NAD^+ in a 1:1 ratio. The disappearance of NADH from the solution can be followed through spectrophotometry and used as a proxy for the oxidation of NO_3^-. Cells were filtered on 2 μm polycarbonate filters (AMD) and put in 2 ml microcentrifuge vials, which were immediately frozen in liquid N until further analysis. One ml of extraction buffer containing 200 mM phosphate buffer pH 7.9, 1 mM dithiothreitol (DTT), 0.3 % (wt/vol)

polyvinyl pyrrolidone (PVP), 0.1 % Triton X-100 and, 3% bovine serum albumin (BSA) was added to each sample. Cells were disrupted using a glass-teflon homogenizer while keeping the tubes containing the filter in an ice-water slurry to prevent overheating. Samples were centrifuged at 750 x *g* at 4ºC for 5 min and the supernatant was removed for use in the NR enzymatic assays. One hundred to 400 µl of the supernatant were added to a total reaction volume of 2 ml containing 200 mM phosphate buffer, pH 7.9, and 0.2 mM NADH. The reaction was initiated by adding KNO_3 to a final concentration of 10 mM. The oxidation of NADH over time was followed for 10-15 min with a temperature-controlled spectrophotometer (Beckman-Coulter, Pasadena, CA). The absorbance at 340 nm (absorbance maximum of NADH) was converted to a rate of oxidation by using a millimolar extinction coefficient of 6.22 according to the Beer-Lambert law (Berges and Harrison, 1995) and normalized with the cell concentration of the cultures at the moment of sampling.

The reaction was stopped by adding 2 ml of 550 µM zinc acetate and the excess NADH was oxidized by adding 20 µl of 125 µM phenazine methosulphate (PMS). The NO_2^- produced was measured colorimetrically as explained below in section 2.2.4 to check for a successful reaction.

2.2.4 Determination of NO_2^- concentrations

The concentration of NO_2^- was determined manually by performing the colorimetric method described in Strickland and Parsons (1972). A sulphanilamide solution was added to the samples and after ~10 min, 2 ml of N-(1-naphthyl)-ethylendiamine solution was also added. The absorbance of the resulting compound was

measured immediately at 543 nm using a Beckman DU 530 (Beckman-Coulter) spectrophotometer.

2.2.5 Determination of NO_3^- concentrations and uptake rates

Culture samples (~50-60 ml) were filtered onto 0.7 μm pore size glass fiber filters (AMD Manufacturing Co.) and collected in acid-washed polycarbonate bottles. They were frozen at -20ºC until measurement of NO_3^- concentrations using an Astoria II autoanalyzer (Astoria-Pacific, Clackamas, OR). Uptake rates were calculated by measuring the disappearance of NO_3^- from the media during a 24 h incubation. The rate of uptake was normalized by the average cell concentration for the 24 h, measured at the start and end of the incubation period.

2.3 Expression of Nitrate Reductase in Natural Assemblages of Diatoms

2.3.1 Seawater sampling

Seawater samples were collected during the month of July of 2010 aboard the CCGS John P. Tully from a coastal and an oceanic station at each of 6 transects along the west coast of Canada (see Figure 3).

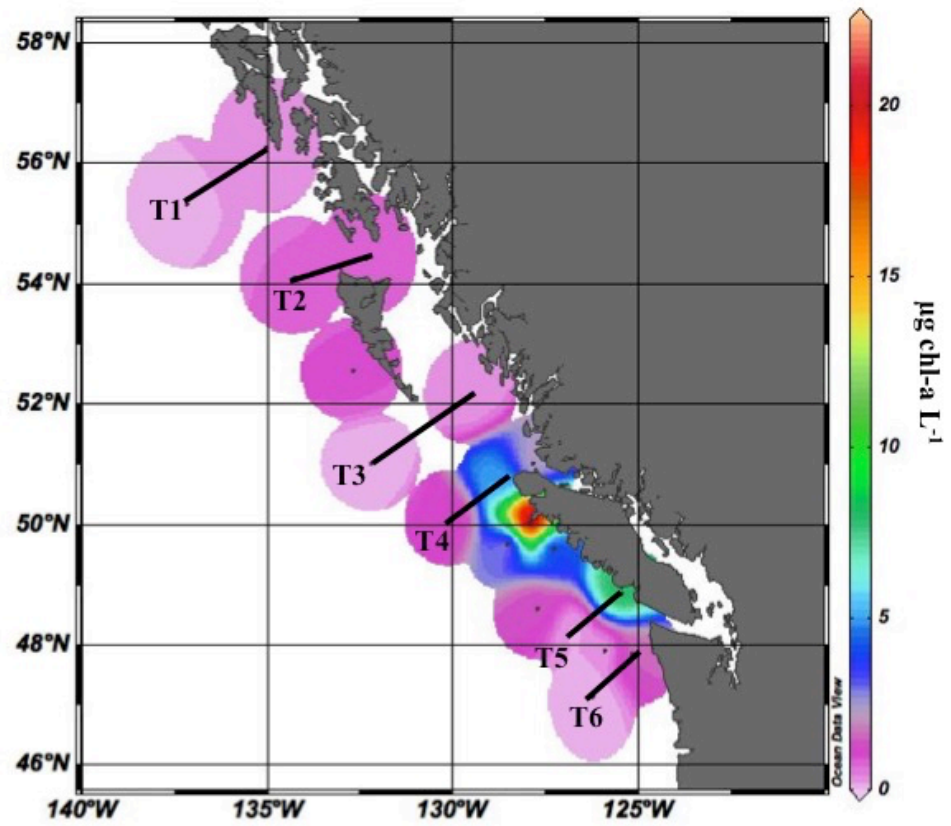

Figure 3: Location of the sampling transects (T1 to T6) and ambient chlorophyll-a concentration (from the depth of chlorophyll-a maximum) off the west coast of Canada in the NE Pacific Ocean.

Water was collected using 10 L Niskin bottles that were attached to a sampling rosette equipped with a CTD (*i.e.*, conductivity, temperature, density) package, which allowed for the identification of the depth of the chlorophyll-a maximum fluorescence. This variable depth was chosen for sampling. The seawater obtained was used for the measurements of dissolved nutrient concentrations (*i.e.,* NO_3^-, phosphate (PO_4^{3-}) and silicic acid ($Si(OH)_4$)), cholorophyll-a concentrations, uptake rates of NO_3^- (through incubations on deck), biogenic silica, and RNA.

2.3.2 Dissolved nutrient concentrations

Samples for dissolved nutrient concentrations were collected in acid washed 15 ml polycarbonate tubes, frozen at -20 ºC and analyzed on board of the ship at the end of the cruise using a Technicon autoanalyzer as described in Barwell-Clarke and Whitney (1996).

2.3.3 Chlorophyll-a concentrations

Samples (400 ml) for chlorophyll-a analysis were filtered onto 25 mm diameter, 0.7 µm pore size glass fiber filters (AMD) and frozen immediately at -20ºC until later analysis ashore. Chlorophyll-a was extracted in 90% acetone and analyzed using a Turner Designs 10AU field fluorometer (Sunnyvale, CA, USA) following the procedure explained in Parsons *et al.* (1984). Interference by phaeopigments was corrected by acidification with 1.2 N HCl.

2.3.4 Nitrate uptake rates

Seawater samples (1000 ml) were placed in 1 L polycarbonate bottles and inoculated with a variable volume of a solution of $Na^{15}NO_3$ (Cambridge Isotope Laboratories, +98% purity) in order to achieve ~10% of the ambient concentration of NO_3^-. The samples were then placed in a Plexiglas tank under the same irradiance levels from the chlorophyll-a max depth (achieved with the use of a neutral density mesh) and at constant temperature (maintained with surface seawater being pumped continuously). After ~8 h the samples were filtered onto precombusted (4 h at 450 ºC) glass fiber filters (0.7 µm pore size) which were dried at 60ºC. The N content and the isotopic composition

of N in the samples (^{14}N:^{15}N) was measured at the Stable Isotope Facility at the University of California Davis with a PDZ Europa ANCA-GSL elemental analyzer and a PDZ Europa 20-20 isotope ratio mass spectrometer. Nitrogen uptake rates were calculated using equations 1 to 3 of the method outlined by Dugdale and Wilkerson (1986).

2.3.5 Biogenic silica

A variable of seawater (1.5-2 L) was filtered onto a 47 mm diameter, 0.6 μm pore size polycarbonate filter. The filter was placed in a 15 ml centrifuge tube, dried at 60ºC and stored in a desiccator until analysis. Samples were treated with 0.2 N NaOH and incubated at 95ºC for 1 h to digest the particulate biogenic silica into dissolved $Si(OH)_4$ (Ragueneau *et al.* 2005). The pH was later neutralized with 1 N HCl. The resulting $Si(OH)_4$ was measured spectrophotometrically according to Strickland and Parsons (1972) using a Beckman DU 530 UV/Vis spectrophotometer.

2.3.6 Gene expression measurements

Sampling of phytoplankton cells for later RNA analysis was performed on board minimizing as much as possible the time between water collection and the freezing of the filter containing the cells in liquid N_2 (within 1 h). Samples were transferred to a -80ºC freezer upon return to shore and kept at -80ºC until the extraction of samples (performed within 3 months).

Refer to section 2.1.4 for a detailed explanation of the methodology.

2.3.7 Phytoplankton diversity identification through microscopy

Samples for taxonomic identification of phytoplankton were collected in 125 ml amber glass bottles and fixed with Lugol's solution. Back in the laboratory, a 50 ml aliquot was allowed to settle in an Utermöhl chamber for 24 h. Phytoplankton groups were enumerated (*i.e.,* diatoms, dinoflagellates, and flagellated cells < 10 μm in diameter) according to Villafañe and Reid (1995). Measurements were performed using an inverted epifluorescence microscope Olympus IX71 (Tokyo, Japan) at 400X total magnification.

2.4 Statistical analyses

Non-parametric analyses (*i.e.*, Kruskal-Wallis test (Sokal and Rohlf, 1995)) were used to establish differences between the growth rates, gene expression, nitrate uptake, and NR activity, at every irradiance and NO_3^- concentration treatment with a confidence level of 95%. A Tukey range test was used to perform *post hoc* comparisons and identify what treatments were significantly different (Sokal and Rohlf, 1995). These tests were performed under the assumption that the data collected were not normally distributed. For the regression models, an ANOVA test was run to determine statistically significant regression parameters. The programs Igor Pro version 6.35A5 (WaveMetrics Inc.) and R Statistical software (R Development Core Team, 2008) were used to carry out all statistical analyses.

Chapter 3: Results

3.1 Effect of Nitrate Concentrations on Gene Expression

The number of cells in the continuous cultures growing at the three different concentrations started out differently, but as time progressed values stabilized at about ~ 1 x 10^6 cells ml^{-1} (Figure 4). Cell volume (~45 μm^3) was also monitored and did not show any significant variations among the three treatments (see Figure 5).

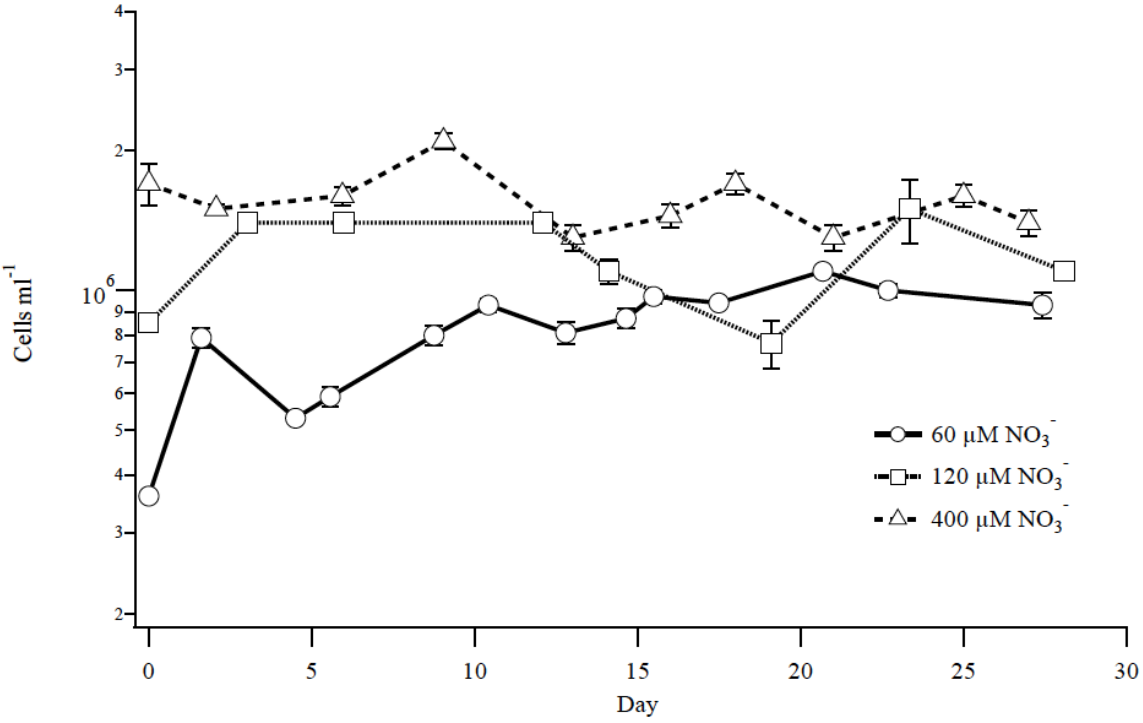

Figure 4: Cell densities in the continuous cultures of *T. pseudonana* at the three different NO_3^- concentrations over the duration of the experiments. Sampling took place towards the end of the incubations, once the cells were acclimated (*i.e.,* after day 12). Vertical bars indicate standard error, and when not visible, they are smaller than the symbols.

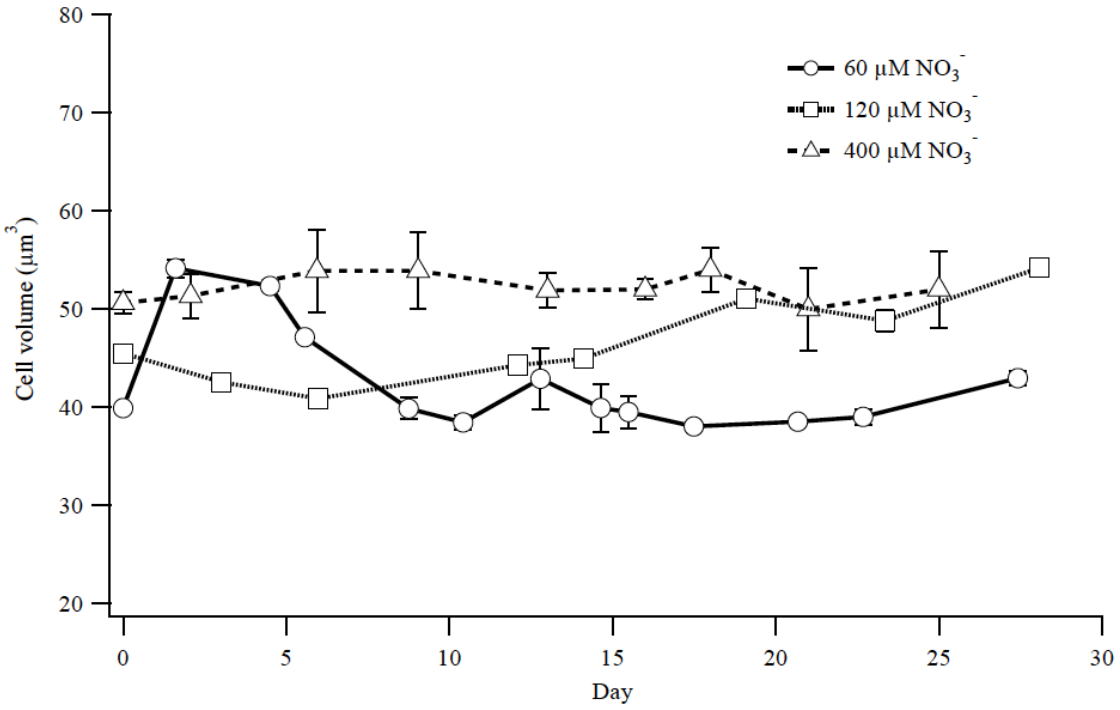

Figure 5: Cell volume in the continuous cultures of *T. pseudonana* at the three different NO_3^- concentrations over the duration of the experiments. Sampling took place towards the end of the incubations, once the cells were acclimated (*i.e.,* after day 12). Vertical bars indicate standard error, and when not visible, they are smaller than the symbols.

All of the genes tested showed lower levels of expression than those of the normalizer genes (*i.e.*, they presented higher Cq values than the normalizer genes). The genes involved in the NO_3^- metabolism (*i.e.,* NAT, NR, NiR, GSII) showed a significantly lower fold difference at 400 µM of NO_3^- compared to the other concentrations ($p < 0.05$). When moving from 60 to 400 µM, the expression of NAT significantly changed from 27.97 to 0.59 fold change $cell^{-1}$ x 10^{-6}, NR from 27.29 to 0.53

fold change cell^{-1} x 10^{-6}, NiR from 8.47 0.69 fold change cell^{-1} x 10^{-6} GSII from 1.05 to 0.38 fold change cell^{-1} x 10^{-6} (Figure 6).

In regards to the expression of the genes related to the metabolism of NH_4^+, AMT showed a significantly higher expression at 400 μM than at the other two concentrations (*i.e.,* 0.43 fold change cell^{-1} x 10^{-6}) ($p < 0.05$). Glutamine synthetase III, on the other hand, presented the lowest relative expression at this concentration (*i.e.,* 0.2 fold change cell^{-1} x 10^{-6}) ($p < 0.05$) (Figure 6).

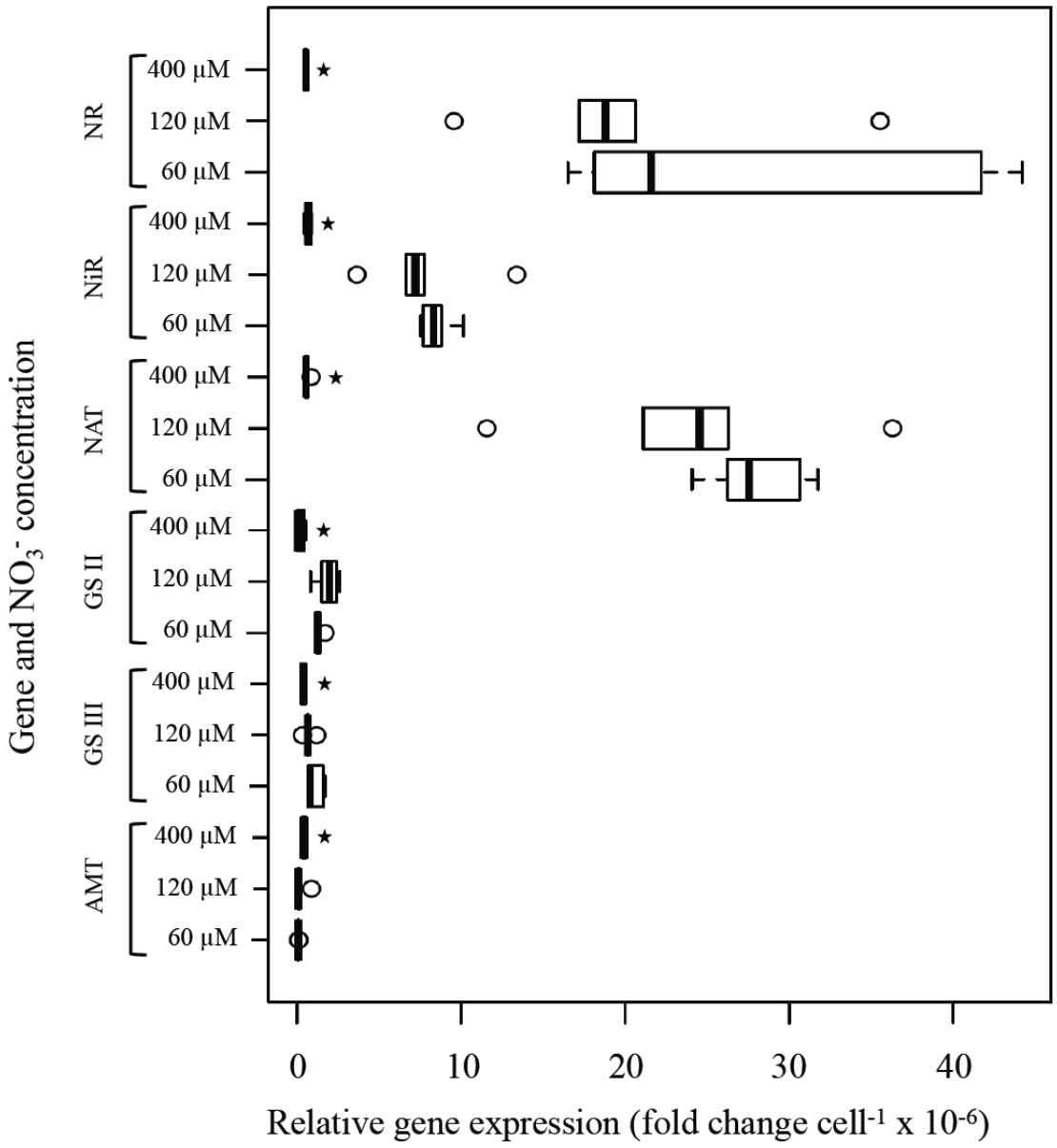

Figure 6: Relative gene expression in *T. pseudonana* cultures measured as fold change per cell concentration of all six genes studied at the different concentrations of NO_3^- (*i.e.*, 60, 120, and 400 µM). These values were normalized by the cellular density of the cultures. Stars indicate significant differences ($p < 0.05$) between treatments. The middle line in the box plots represents the median, and the whiskers show the lower and upper quartiles (*i.e.*, 25% and 75%). Outlier points (*i.e.,* points outside 1.5 times the interquartile range above and below the upper and lower quartiles, respectively) shown as circles. Error bars represent the standard error calculated from 3 replicate samples.

3.2 Effect of Irradiance Levels on NR Expression and Activity

Cultures of *T. pseudonana* showed a significantly lower growth rate at 50 µmol photon cm^{-2} s^{-1} ($p < 0.05$) ($\mu = 0.99$ d^{-1}), and a significantly higher rate at 200 µmol photon cm^{-2} s^{-1} ($p < 0.05$) ($\mu = 2.04$ d^{-1}) (Figure 7A). The highest expression of the enzyme NR gene was measured at 200 µmol photon cm^{-2} s^{-1} ($p < 0.05$) (a 7-fold change $cell^{-1}$ x 10^{-6}) and the lowest at 320 µmol photon cm^{-2} s^{-1} ($p < 0.05$) (a 1.07-fold change $cell^{-1}$ x 10^{-6}) (Figure 7B). The uptake rates measured at 320 µmol photon cm^{-2} s^{-1} presented significant differences ($p < 0.05$) when compared to the rates measured at the 110 and 200 µmol cm^{-2} s^{-1} treatments. However, they were not significantly different from those measured at 50 µmol cm^{-2} s^{-1} ($p > 0.05$) (Figure 7C). The cultures did not show significantly different nitrate activity rates when exposed to the different irradiance levels (Figure 7D).

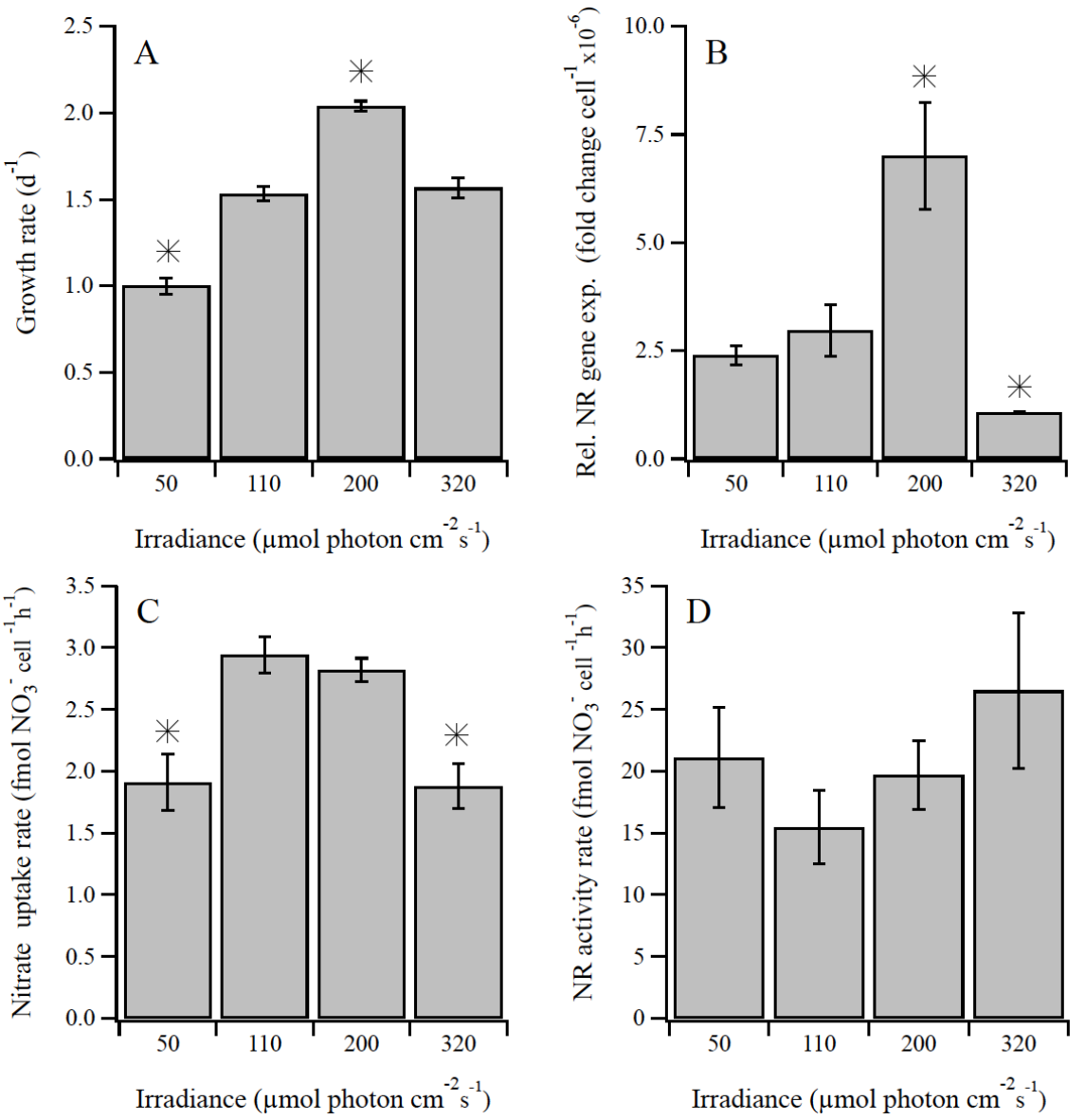

Figure 7: Effects of irradiance on growth rates (A), relative gene expression of NR (B), NO_3^- uptake rates (C), and NR activity rates (D) in cultures of *T. pseudonana*. Stars indicate significant differences with at least one other treatment ($p < 0.05$). Error bars represent the standard error calculated with 3 replicate samples.

A series of regressions were run to determine what parameter better explained the gene expression and enzymatic activity of NR and the uptake rates for the different irradiance treatments. The stronger relationships were observed between the growth rate and the NO_3^- uptake, between the growth rate and the gene expression, and between the gene expression and the NO_3^- uptake (see Table 2). The analyses that showed significance were used in Figure 8. The first two models were linear, where an increase of NR activity and NO_3^- uptake would result in a proportional increase of the growth rates (although only the latter was statistically significant). The third model was a second order polynomial equation with growth rate as the independent variable and relative expression as the dependent one. The fourth and fifth models were logarithmic, with relative expression as independent variable and NR activity and NO_3^- uptake as the dependent variables. In these models, the dependent variables reach a plateau in their response at higher values of the independent variable. Only the latter model was statistically significant (Table 2).

Table 2: Parameters of regression models between growth rate vs. NR activity, NO_3^- uptake and NR expression, and relative expression vs. NR activity and NO_3^- uptake rates in *T. pseudonana*. Regression models with significant p values were graphed in Figure 6 (indicated here with an asterisk).

Model	**Parameters**	**R^2**	**F statistic**	**p value**
Linear	Growth rate vs NR activity	0.002	0.021	0.887
Linear	Growth rate vs Nitrate uptake	0.344	5.239	0.045*
Polynomial	Growth rate vs Relative expression	0.630	7.645	0.011*
Logarithmic	Relative expression vs NR activity	0.177	2.147	0.094
Logarithmic	Relative expression vs Nitrate uptake	0.423	7.321	0.022*

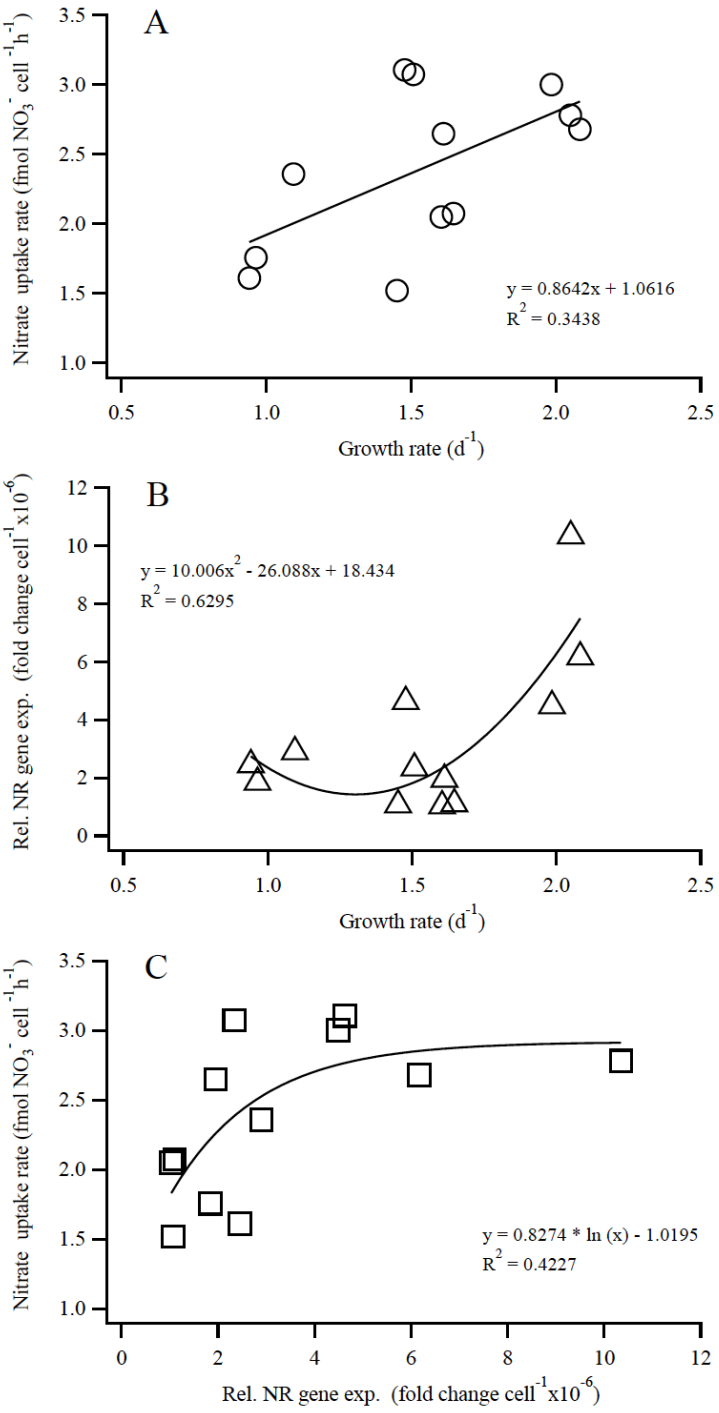

Figure 8: Regression models for growth rates vs. NO_3^- uptake rates in *T. pseudonana* (A), growth rates vs. NR relative gene expression (B), and NR gene expression vs. NO_3^- uptake rates (C) calculated with all of the data from all irradiance treatments.

A linear regression model was the best fit between the growth rates and the uptake rates of nitrate, although this model explained only ~34 % of the variance. The model that explained most of the dispersion in the data, as shown by its coefficient of determination (*i.e.,* a value that indicates how close the data are to the fitted regression line, R^2), was the polynomial model for the relationship between the growth rates and the NR expression (*i.e.*, $R^2 = 0.63$). The relationship between the NR expression and the NO_3^- uptake rates was better explained with a logarithmic model (*i.e.*, $R^2 = 0.42$).

Since both growth rates and NR expression seemed to better explain the uptake rates, a multiple regression was attempted. However, when using only these two variables the R^2 value obtained was 0.52. When the parameter corresponding to the NR activity is added to the equation, the R^2 value increases to 0.66 (see Table 3 for the ANOVA results).

Table 3: ANOVA results for a multiple regression including the variables that better explained the uptake rates separately (*i.e.*, logarithm of the relative expression of NR, and growth rates) and the NR activity rates for *T. pseudonana*. The log of NR expression was the only variable to be statistically significant variable ($p < 0.05$). Df: degrees of freedom; Sum Sq: sum of squares; Mean Sq: mean square.

Variable	Df	Sum Sq	Mean Sq	F ratio	p value
Log of Relative Expression	1	1.54	1.54	10.05	0.013
Growth rates	1	0.37	0.37	2.41	0.156
NR activity rates	1	0.51	0.51	3.32	0.105
Residuals	8	1.22	0.15		

3.3 Expression of Nitrate Reductase in Natural Assemblages of Diatoms

The concentration of all nutrients and chlorophyll-a was higher in all coastal stations from the 6 transects, except for the PO_4^{3-} in transect T6 and the chlorophyll-a concentration in transects T2 and T4, where oceanic stations showed higher values (Table 4).

Table 4: Nutrient and chlorophyll-a concentrations for the coastal and oceanic stations of each of the 6 transects in the NE Pacific Ocean. Transect numbers increase from North to South (*i.e.,* T1 is the northernmost transect and T6 the southernmost transect).

Transect	Station	NO_3^- (µM)	PO_4^{3-} (µM)	$Si(OH)_4$ (µM)	Chlorophyll-a (µg l^{-1})
T1	Coastal	8.90	1.05	18.8	0.38
	Oceanic	4.20	0.72	10.2	0.28
T2	Coastal	8.60	0.93	16.1	0.94
	Oceanic	0.90	0.54	12.5	1.29
T3	Coastal	0.80	0.50	7.10	0.72
	Oceanic	0.00	0.48	0.20	0.09
T4	Coastal	14.9	1.25	26.8	0.19
	Oceanic	0.00	0.65	8.20	0.30
T5	Coastal	6.60	0.81	25.6	1.02
	Oceanic	0.80	0.58	1.40	0.74
T6	Coastal	1.50	0.48	10.2	0.87
	Oceanic	0.70	0.54	1.20	0.56

The uptake rates of NO_3^- were significantly higher in the coastal stations of transects T1, T2, T4, and T6 (Figure 9A). The biogenic silica was higher in the coastal stations of transects T2, T4, T5, and T6, suggesting a higher abundance of diatoms (Figure 9B). This was confirmed with the microscopy analyses, although at those stations the diatom abundance was below 50%. This indicates that other phytoplankton groups (mainly phytoplankton cells < 10 μm) were responsible for the uptake rates measured (Figure 10).

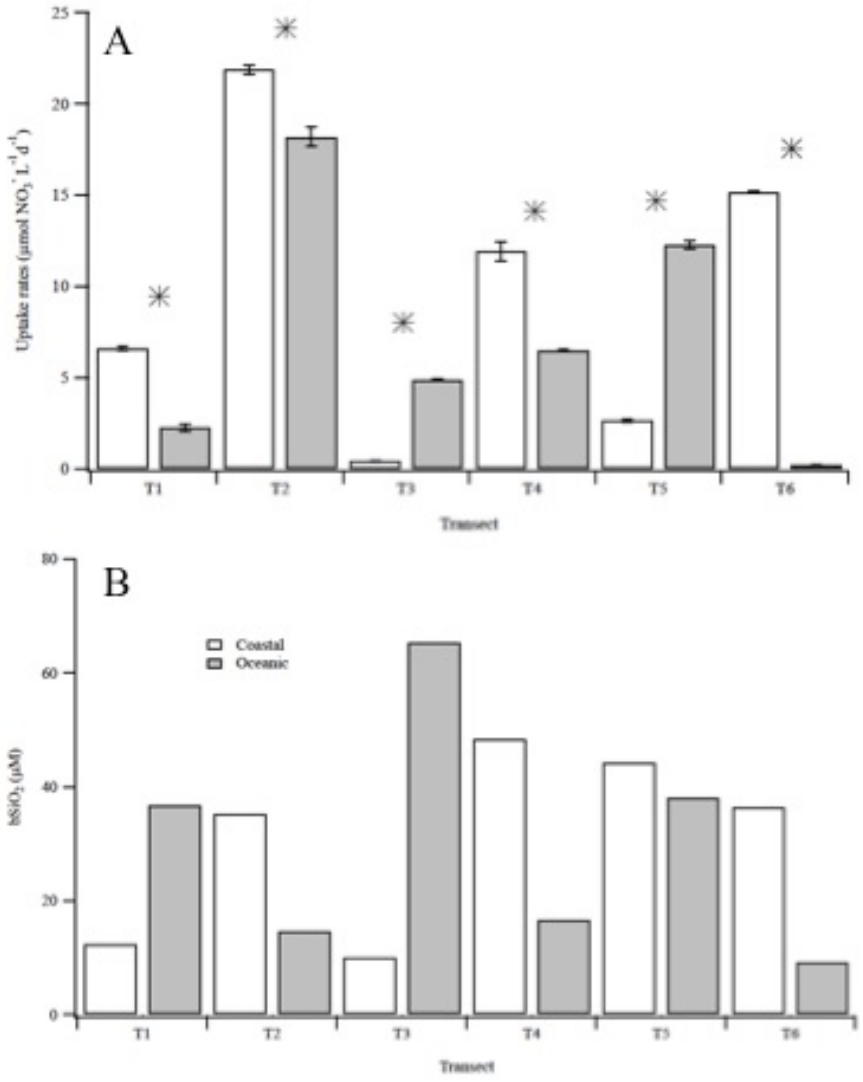

Figure 9: Nitrate uptake rates (A) and biogenic silica concentrations (B) measured at both ends of the transects (*i.e.*, coastal and oceanic stations) in the NE Pacific Ocean. White bars indicate coastal stations, grey bars oceanic ones. Stars indicate significant differences between stations the two stations in each transect ($p < 0.05$). Transect numbers increase from North to South.

In general, small phytoplankton cells < 10 µm dominated the assemblage in both coastal and oceanic stations in all transects. The exceptions were the coastal stations of transects T1 (with only dinoflagellates), T4 and T5 (with diatoms) and the oceanic stations of transect T3 (with mostly diatoms) (see Figure 10). Individuals from the genus

Thalassiosira were present in the two coastal stations in transects T4 and T5 representing ~23% and ~16% of the diatoms present, respectively. However, it was not possible to determine the presence of the species *T. pseudonana* under the light microscope.

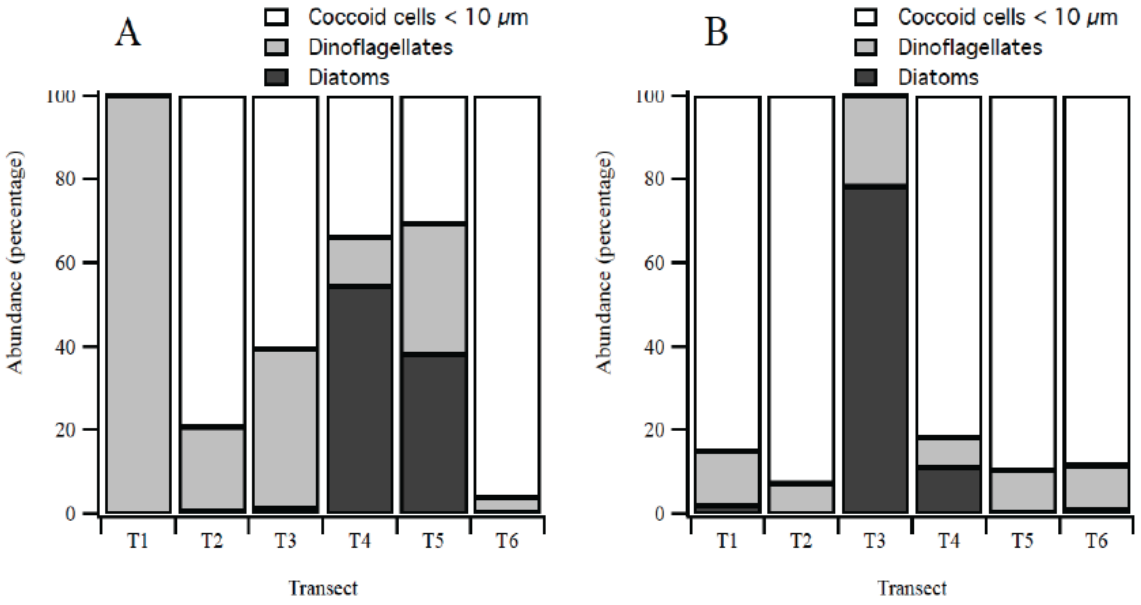

Figure 10: Phytoplankton groups identification using light microscopy measured at coastal stations (A) and oceanic stations (B). The numeric order of the transects indicates an increasing southward position in the location of the transects.

It was not possible to amplify any *T. pseudonana* genes from RNA samples taken at the different stations. The Cq values were > 38 (data not shown). This indicates that there were probably not enough copies of gene transcript sequences from *T. pseudonana*, if any.

Chapter 4: Discussion

The main objective of this study was to assess the effects of different concentrations of nitrate and irradiance levels on the expression and activity of the enzyme nitrate reductase (NR) in *T. pseudonana* and to test the possible application of gene expression as a proxy for NO_3^- uptake. The ultimate goal was to determine whether the expression of the NR gene could be used as a predictor of the activity of the enzyme and/or the uptake of NO_3^- in cultures and in natural assemblages.

Effect of Nitrate Concentrations on Gene Expression in T. pseudonana

The expression of the four genes involved in the metabolism of NO_3^- (*i.e.,* NAT, NR, NiR, and GSII) did not show differences when the cultures were growing at 60 µM and 120 µM. However, at 400 µM the expression decreased significantly to levels very similar to those of the normalizer genes. The expression of NAT showed the most pronounced change at 400 µM NO_3^- when compared to the other NO_3^- concentrations. The decrease in the expression at the highest NO_3^- concentration was more pronounced for NAT, followed by the reductase enzymes NR and NiR and less pronounced for GSII, which showed the least difference between treatments. This change in the expression suggests a lower requirement for these proteins at high concentrations of NO_3^-, perhaps due to a saturation effect and the fact that the enzymes that are already present are enough for the metabolic requirements of the cells.

In general, the uptake of nutrients follows a Michaelis-Menten relationship with uptake rates reaching a plateau after a saturation point in the concentration of the

substrate is reached (Aksnes and Egge, 1991). However, it was found that many phytoplankton species are capable of acclimating to higher concentrations of NO_3^- as shown by higher Ks values (*i.e.*, the half saturation parameter in the Michaelis-Menten equation). Furthermore, concentrations of $NO_3^- > 20$ μM stimulated higher uptake rates in many diatom species (Collos *et al.* 2005), yet this was not reflected in higher expression levels.

The AMT transcripts were still measurable when the concentration of NO_3^- reached 400 μM, which demonstrates that perhaps the cells are always ready to utilize NH_4^+, since it involves a lower investment of energy as opposed to NO_3^-, which has to be reduced first (Hildebrand, 2005). Glutamine synthetase III, on the other hand, showed a decrease in its expression in cells growing at 400 μM. This gene is thought to be exclusively involved in the metabolic pathway of cytosolic NH_4^+ in both vascular plants and phytoplankton (Lam *et al.* 1996; Takabayashi *et al.* 2005). However, Robertson and Alberte (1996) also found that this gene was expressed when cultures of the diatom *Skeletonema costatum* were grown in media containing NH_4^+ and NO_3^- as sole N sources. The results of the present study corroborate the concept that glutamine synthetase III is not necessary for the assimilation of NH_4^+ that is coming from the reduction of NO_3^- in the diatom *T. pseudonana* since it is being incorporated into aminoacids inside the chloroplast, while GSIII is located in the cytosol.

Effect of Irradiance Levels on NR Expression and Activity in T. pseudonana

The maximum growth rate of *T. pseudonana* was observed at 200 μmol photon cm^{-2} s^{-1} ($p < 0.05$) but the growth rate was lower at 320 μmol photon cm^{-2} s^{-1} (Figure 7),

suggesting photoinhibition of growth as this process has been observed previously in other phytoplankton species (Powles, 1984; Falkowski, 1984; Long *et al.* 1994).

The gene expression of NR showed a significantly higher expression at 200 photon cm^{-2} s^{-1} indicating that at this irradiance the cells were in need of more of this enzyme to meet the higher demand of NO_3^- when the growth was maximum. The expression was not significantly different between 50 and 120 photon cm^{-2} s^{-1}, but the values were nonetheless higher than at 320 photon cm^{-2} s^{-1}, where the expression showed the lowest level. Nitrate reductase has been shown to convert lower quantities of NO_3^- into NO_2^- at low irradiances (Muggli and Smith, 1993; Maguer *et al.* 2011), but in this experiment the expression did not show a higher level as it would have been expected when the cells are in need of more enzyme. At the highest irradiance, it is likely that the cells were experiencing photoinhibition of growth, and therefore had a lower demand of nutrients. However, as it was suggested by Lomas and Glibert (1999), the reduction of NO_3^- could be a possible way of dissipating the excess of energy entering the photosystem. If that was the case, the expression of this gene should have been higher at 320 µmol photon cm^{-2} s^{-1}. The fact that the expression was actually significantly lower at this light treatment suggests that reduction of NO_3^- was not a coping mechanism used by *T. pseudonana* against excess irradiance.

The uptake rates of NO_3^- were highest at 110 and 200 µmol photon cm^{-2} s^{-1}, with no significant differences among those treatments. It presented the lowest values at 50 and 320 µmol photon cm^{-2} s^{-1}, reflecting what was observed with the growth rates. The growth rate and uptake rate of NO_3^- are equivalent under steady state conditions since both processes can be explained with an hyperbolic function (Eppley and Thomas, 1969).

However, despite being the best fitting model, a linear regression between the growth rate and the uptake rates only explained ~34% of the variance. If the points corresponding to the inhibiting irradiance are removed, the R^2 value climbs to 54% (data not shown). However, this is not the case for the other relationships tested (*i.e.*, growth rate vs. NR gene expression, and NR gene expression vs. NO_3^- uptake). This indicates that when there is photoinhibition of growth, the uptake rates of NO_3^- will not be equivalent to the growth rates.

The growth rate, however, explained much better the variation in the relative expression of NR. Even though the relationship was not linear, at higher growth rates the expression was higher. However, at the lowest growth rates the relative expression did not show the lowest values. And although this may appear counter intuitive, this higher expression may indicate a higher demand of this enzyme at low growth rates due to a higher degradation rate of RNA, which has been shown to occur in slow-growing prokaryotic phytoplankton (Steglich *et al.* 2010).

However, one must consider that the concentration of mRNA in one cell at any given time (which is what is being measured with qPCR) is the balance between its synthesis and degradation. The first control of gene expression is the initiation of transcription. There are some regulatory proteins that bind to specific sequences of DNA called promoter sequences, usually located close to the gene sequence, that activate transcription. Also, the binding of certain proteins to other DNA sequences, called enhancers, can affect the structure of the chromatin and either promote or inhibit transcription. The processing of the transcripts is another way to control their concentration. In eukaryotes, transcripts can be modified before they leave the nucleus.

The splicing of intervening sequences that will not be part of the mature RNA (*i.e.,* introns), the addition of a 7-methylguanosine cap to the 5' end of the transcripts and the polyadenylation of the 3' end of the sequences are some of the regulatory mechanisms that can affect their stability (Philips, 2008; Nelson and Cox*,* 2012).

Transcripts can also be degraded by specific enzymes called ribonucleases that usually require a prior destabilization of the transcript sequences (*e.g.,* removal of the 5' cap or a deadenylation) by other enzymes (Nelson and Cox, 2012). All of these processes are very common in eukaryotic cells, and although the information on phytoplanktonic cells is scarce, they should be taken into consideration when analyzing gene expression.

In regards to the NO_3^- uptake rates, they were explained by a logarithmic function of the expression of the NR gene, and this was better observed in a multiple regression analysis ($p < 0.013$). With an increase in the expression, the NO_3^- uptake rates reached a plateau, and this could be explained by a decoupling between the transcription of the gene and the synthesis of the enzyme, as suggested by Smith *et al.* (1992). Not necessarily all of the newly synthesized transcripts will be translated into proteins. And even if they were, there could be post-translational mechanisms preventing the enzymes to become active. This indicates that uptake can be independent of expression.

An enzymatic *in vitro* assay requires maintaining variables such as temperature and pH at constant levels, and when this is achieved this type of assay can be used as a proxy for enzyme quantification (Rossomando, 1990). Higher concentrations of enzyme would yield higher activity values, and thus the lack of significant differences in enzymatic activity for the different irradiance treatments could be interpreted as a similar

concentration of the enzyme NR in all of the samples. However, it is also possible that the activation by phosphorilation, if existent, may have been lost during extraction (Berges, 1997) and thus the observed activity of NR may have been lower than what would be measured *in vivo* (*i.e.,* when the enzyme is at an optimal environment inside the cell), losing the significant differences between treatments.

Expression of Nitrate Reductase in Natural Assemblages of Diatoms

The expression of functional genes can be used to assess the metabolically active fraction of the phytoplankton assemblage. Traditional techniques used to measure uptake of NO_3^- are made in bulk volumes of water, and the estimation of the rates correspond to the whole phytoplankton assemblage, without identifying the metabolic active organisms. The application of molecular biology can provide the means to assess metabolic functions at the taxonomic group level, or even the species level (Zehr and Voytek, 1999).

As shown in this study, the expression of the NR gene in *T. pseudonana* varies depending on the NO_3^- concentration and the irradiance at which the cells are exposed. The light levels of the experiments performed with *T. pseudonana* are similar to what phytoplankton experiences in natural conditions. The maximum growth rate irradiance (*i.e.,* 200 µmol photon cm^{-2} s^{-1}) is actually very close to what Smith *et al.* (1992) used in their simulations of upwelling events (*i.e.,* 170 µmol photon cm^{-2} s^{-1}), where they measured a delay between the expression of the NR gene and an increase in the concentration of the enzyme. Thus, one of the possible applications of this study is to employ the primers developed in these lab experiments in samples coming from the field comparing similar environmental variables, such as light. This would allow the

assessment of gene expression of NR in *T. pseudonana* (and possibly other diatoms, provided that the primers are not species specific). Measurements of expression could be coupled with complementary measurements of diatom presence and primary production to obtain a complete picture of diatom utilization of NO_3^-. This was actually attempted in this study, but unfortunately it was not possible to amplify genes from *T. pseudonana* in natural samples since this species was probably absent. The measurement of the expression of functional genes in natural environments depends on the specificity of the primers used, and the presence of the targeted species in the phytoplankton assemblage. Current online databases are limited in the number of species and sequences available, and even using the number of sequences at hand would be a challenge, because many sets of primers would have to be used, or degenerate primers developed, which would have to be tested in cultures in the laboratory (Ward, 2008). The coupling of gene expression with uptake rate measurements is very challenging and this thesis attempted to bridge the quantification of both.

Although many precautions were taken to minimize RNA degradation (*e.g.,* wiping working surfaces and tools with a ribonuclease decontamination solution, minimizing the time between water collection and freezing of the sample) there is the possibility that RNA decay influenced the results obtained in this study. It was not possible to determine whether this species was involved in the NO_3^- uptake rates measured at the coastal station of two of the transects (*i.e.*, T4 and T5) (see Figure 9), although there were many individuals from the same genus *Thalassiosira* that comprised ~23% and ~16% of the diatoms present, respectively (detected through light microscopy).

The application in the field of species-specific primers that were successfully tested in the lab is very challenging and the measurement of any signal is dependent on the presence of sequences from the targeted species. In future research it would be advisable to develop primers that are capable of amplifying more than one sequence, perhaps "degenerate" primers. This would allow the measurement of gene expression from more than one species and would help identify more metabolically active members of the phytoplankton assemblage. The implementation of more sensitive techniques to measure the diversity of the eukaryotic phytoplankton assemblage, such as the sequencing and analysis of DNA sequences corresponding to the ribosomal subunit 18S RNA could aid in the detection of the species of interest. This would allow in a better interpretation of the results, especially in the cases where there is no amplification of transcript sequences.

From genes to metabolic function

Genes hold the information required to synthesize proteins that carry out vital functions for the cells and there are many control mechanisms that regulate the whole flow of information. The expression of different genes has two levels of control: the amount of mRNA produced from a particular gene and a series of events that affect the translation of that mRNA into proteins (*i.e.*, post-transcriptional regulation) (Phillips, 2008). The first level is usually the result of the binding of specific proteins to genes called promoters that affect the action of the RNA polymerase and in eukaryotes is said to be combinatorial, because it requires many of these proteins (Pulverer, 2005). The second level usually involves the modification of certain bases in the nucleotide sequencing, which affects its conformation. The addition of a 7-methylguanosine cap to

the 5' end and the polyadenylation of the 3' end of the transcripts constitute some examples. An example is the blocking of certain bases through the addition of methyl groups (Helm, 2006). However, once the proteins have been synthesized, there are many ways to regulate their activity. Among the most common ways to achieve this is the addition of functional groups such as acetate and phosphate and the change in the conformation of peptides by creating bonds between them (*e.g.*, disulfide bridges) (Mann and Jensen, 2003).

The only known regulation of the enzyme NR is by its synthesis and degradation, processes that depend on the presence or absence of NO_3^-, respectively (Berges, 1997). Vergara *et al.* (1998) proved this transcriptional regulation of enzymatic activity, but the actual mechanisms by which the presence of NO_3^- induces the expression are still unknown. In cyanobacteria, however, there is a transcription activator, the DNA binding protein *ntcA*, which regulates the expression of many genes involved in the metabolism of both NO_3^- and NH_4^+ (Suzuki *et al.* 1995; Lindell *et al.* 1998; Ohashi *et al.* 2011); there is also indication of post-translational regulation mechanisms (Omata, 1995). But in eukaryotic phytoplankton there is still a big gap in the information available, and the number of species studied is very limited.

There are other phytoplankton groups that play a significant role in primary production. Picophytoplankton are mainly composed of cyanobacteria and photosynthetic eukaryotic organisms that mostly belong to the phylum Prasinophyta, typically < 2 μm in size. They are responsible for the majority of the primary production in many oceanic regions, especially oligotrophic areas (Zubkov *et al.* 2003; Shi *et al.* 2009). They are thought to contribute in a smaller extent to C flux to deep waters because of their small

size, low sinking rates and fast conversion of their associated organic matter into inorganic nutrients within the microbial loop (Guidi *et al.* 2009). The microbial loop describes the uptake of dissolved organic carbon (DOC) by heterotrophic bacteria, and its subsequent bioavailability to higher trophic levels (*i.e.,* protists) (Hagström *et al.* 1988; Herndl *et al.* 2008). However, picoplanktonic contribution to the biological pump could be greater than previously estimated. Alternative sinking paths such as aggregation of cells into larger particles in the water column and into mesozooplankton fecal pellets are potential venues for rapid sinking rates (Richardson and Jackson, 2007). Understanding the mechanisms that regulate nutrient uptake in the smaller fraction of phytoplankton, especially the eukaryotic component, may prove useful when assessing the contribution of these organisms to the primary production of the whole phytoplankton assemblage.

For the reasons explained above, I intended to compare the results obtained from *T. pseudonana* cultures with those obtained from cultures of the picoeukaryote *Micromonas pusilla* (see Appendix D). *M. pusilla* is 2-3 μm in diameter and its whole genome has been sequenced (Worden *et al.* 2009), providing the opportunity to test the expression of functional genes related to the metabolism of N. Small cells have a high surface area to volume ratio, and that allows them to grow under nutrient-limiting conditions (Azam *et al*. 1983).

Chapter 5: Conclusions

The application of molecular biology tools in oceanographic studies presents many challenges but can prove to be a useful way of complementing existing techniques to measure metabolic processes. *Thalassiosira pseudonana* is a cosmopolitan diatom whose genome has been fully sequenced, thus providing with an opportunity to assess the expression of genes involved in different metabolic processes. In this study, the enzyme NR from *T. pseudonana* was selected to attempt a comparison of measurements of gene expression, its enzymatic activity, and the uptake of NO_3^-, which must occur prior to its reduction and has been shown to be influenced by the enzymatic steps down the metabolic pathway (*i.e.,* reduction into NO_2^- first, and NH_4^+ later). The expression of other genes involved in the metabolism of NO_3^- and NH_4^+ was also measured to address the effects of different concentrations of NO_3^-.

The expression of NR showed a decrease with increasing concentrations of NO_3^-, which indicates that the cells might not require more of the enzyme to process more of the available nutrient. The fact that the expression reached a plateau with increasing uptake indicates that the uptake is independent of the expression, probably due to a decoupling between transcription and protein synthesis. With increasing irradiance, the uptake of NO_3^- increased significantly due to an increase in the growth rates, but the NR enzymatic activity did not show differences among the different irradiance treatments (*i.e.*, 50, 110, 200, and 320 μmol photon cm^{-2} s^{-1}).

The measurement of enzymatic activity of NR was intended as a proxy for the enzyme concentration. The lack of a correlation between expression of NR and enzymatic activity shows that the relationship between these parameters is very complex and post-translational activation mechanisms such as phosphorylation may be influencing the results. A proper isolation, purification, and quantification of the enzyme and establishing the relationship between enzymatic activity with the gene expression is a key piece of information needed to better understand the NO_3^- metabolism in *T. pseudonana* in particular, and diatoms in general.

The use of molecular tools that target particular functional genes in field samples is challenging with many issues to be addressed before its application. The reduced number of gene sequences in online databases, and the labor-intensive and time-consuming design of specific primers and their test in the lab are factors that constraint the number of species that can be studied. The presence or absence of these species in the phytoplankton assemblage may render the measurement efforts futile.

One way to ensure that the organisms of interest are present in the assemblage would be the sequencing and analysis of the phytoplankton DNA existent in the water samples used for gene expression measurements. Although the detection of DNA sequences from the same organism would not be conclusive (DNA has been found to persist in the water after the death of the cells), its absence would prove that the species of interest was not in the water at the time of sampling.

The traditional techniques of measuring physiological processes of phytoplankton in natural environments (such as nutrient uptake rates) involve incubations of water that

yield data corresponding to the whole phytoplankton assemblage, but do not indicate the identity of the organisms actually responsible for those processes. Coupling these techniques with gene expression measurement could be a way to provide evidence of the species that are metabolically active, and this study was carried out towards that goal.

Future Research Directions

The relationship between gene expression, concentration of the enzyme encoded in the transcripts, and enzymatic activity is very complex. There are many factors that could be regulating these processes and affecting the comparisons. However, one way to determine the actual link between NR gene expression and NO_3^- uptake would be the direct quantification of the NR enzyme instead of using enzymatic activity as an estimation of enzyme concentration. The application of quantitative proteomics techniques such as mass spectrometry and two-dimensional gel electrophoresis would allow for an accurate comparison between amount of protein and gene expression (whether relative to normalizer genes or assessed with the measurement of transcript copy numbers).

As for the assessment of gene expression in the field, if the goal is to look into the metabolic functions of one particular species, then transcript measurements should be complemented with measurements of conserved DNA sequences to determine the presence of the species of interest. If the goal is to observe the activity of more than one species from a taxonomic group, then degenerate primers could be used in natural samples after testing their amplification efficiencies with complex mixtures of transcripts in the lab.

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Appendices

Appendix A: Primers used in the qPCR reactions

After optimization of the thermocycle parameters for the qPCR reactions, the amplicons were run in a 1.5% agarose gel. The bands were excised, the DNA was extracted from the gel core, further amplified, and sequenced to confirm the identity of the amplicons (see Figure A1). The sequences obtained were checked using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology (http://blast.ncbi.nlm.nih.gov/).

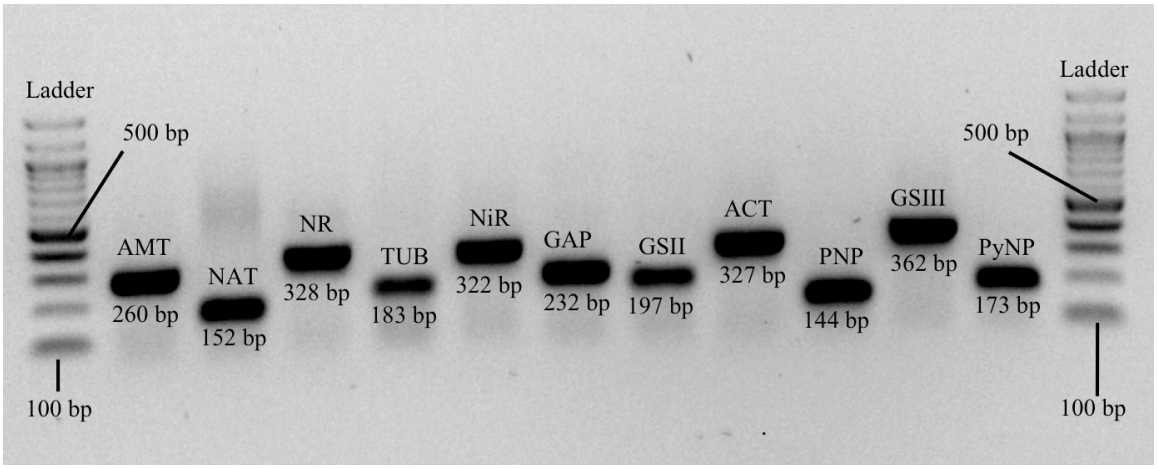

Figure A1: Gel electrophoresis of the amplicons used in qPCR reactions in the experiments with *T. pseudonana*. AMT: ammonium transporter; NAT: nitrate transporter; NR: nitrate reductase; TUB: β-tubulin; NiR: nitrite reductase; GAP: glyceraldehyde-3-phosphate dehydrogenase; GSII: glutamine synthetase II; ACT: β-actin; PNP: purine-nucleoside phosphorylase; GSIII: glutamine synthetase III; PyNP: pyrimidine-nucleoside phosphorylase. After excising the bands, they were purified and sequenced to confirm the identity of the amplicons.

Appendix B: Expression of normalizer genes at different experimental conditions

The three different genes used in the measurements of gene expression of *T. pseudonana* (*i.e.,* β-actin, purine-nucleoside phosphorylase, and glyceraldehyde-3-phosphate dehydrogenase) showed similar levels of expression at the three NO_3^- concentrations and the four irradiance levels used in the experiments.

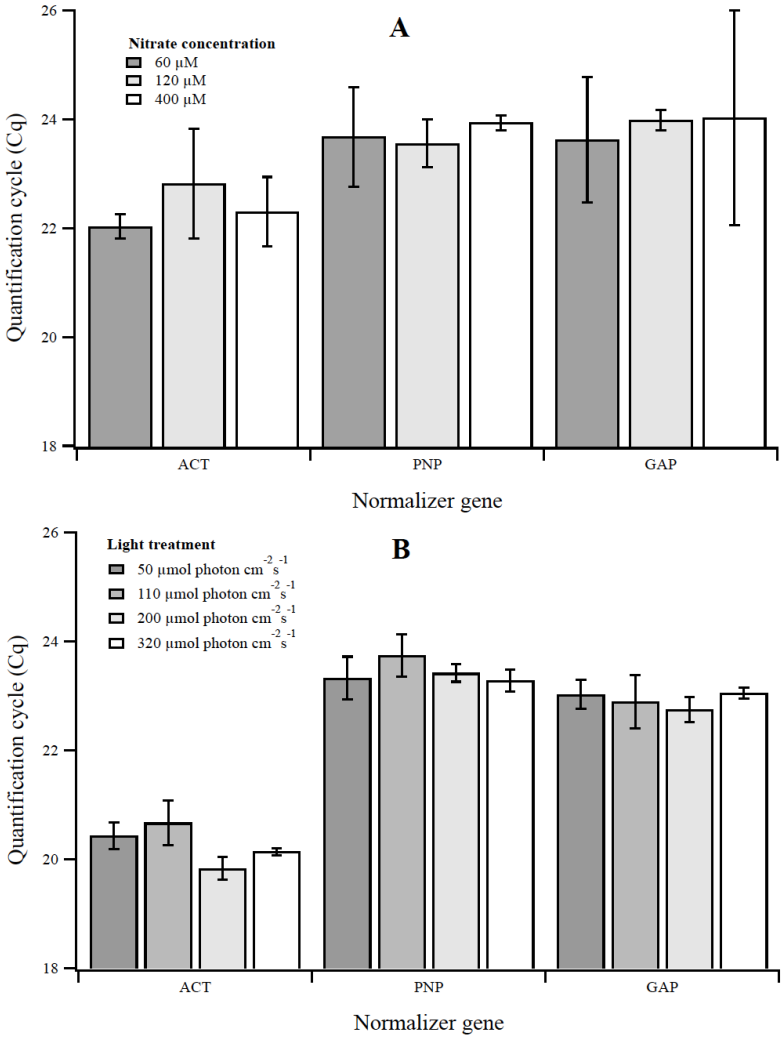

Figure B1: Gene expression of the three normalizer genes used in the experiments with *T. pseudonana* (*i.e.,* β-actin (ACT), purine-nucleoside phosphorylase (PNP), and glyceraldehyde-3-phosphate dehydrogenase (GAP)) for the three NO_3^- concentrations (A) and the four irradiance treatments (B). Error bars represent standard error.

Appendix C: Growth of *T. pseudonana* at different irradiances

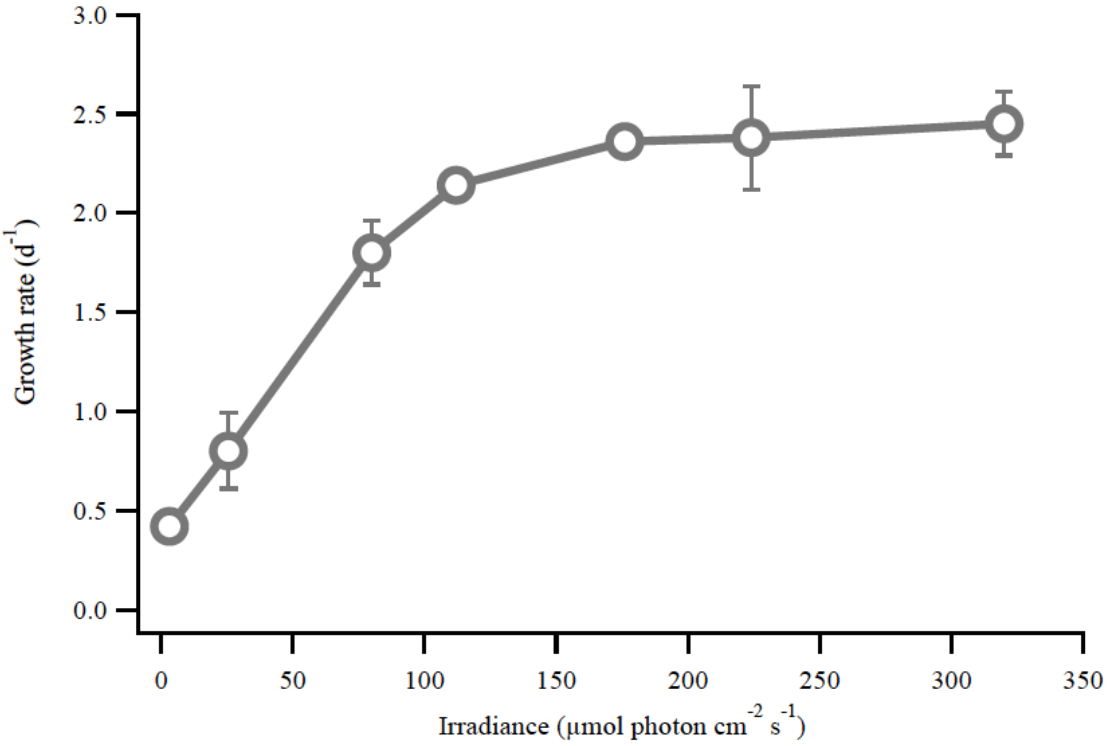

Figure C1: Growth curve of *T. pseudonana* cultures growing at 7 different irradiances. The growth rates were calculated using *in vivo* fluorescence measurements of triplicate cultures. Error bars show standard error.

Appendix D: Measurements of growth, NR expression and activity in the picoeukaryote *Micromonas pusilla*

Introduction

Micromonas pusilla is a small eukaryotic phytoplankton species that belongs to the taxonomic group Prasinophytes, of wide distribution in the world oceans. *M. pusilla* is part of picophytoplankton given its small size (*i.e.*, 2-3 μm in diameter) and its whole genome has been sequenced (Worden *et al.* 2009). The availability of sequences of genes related to the metabolism of N allowed the design of specific primers that were used in gene expression analyses.

The objective of this study was to compare the gene expression and enzymatic activity of NR between *M. pusilla* and *T. pseudonana*.

Materials and Methods

Refer to point 2.2 in Materials and Methods for a detailed explanation of the experimental design, which was exactly the same than the design used for *T. pseudonana.*

Results and Discussion

The growth rates were lowest at 50 μmol photon cm^{-2} s^{-1} and did not show any significant differences at 110, 220, and 320 μmol photon cm^{-2} s^{-1} ($p > 0.05$) (Figure D1A). Growth was limited at low irradiance (*i.e.*, 50 μmol photon cm^{-2} s^{-1}), but surprisingly it was not inhibited when cells were exposed to 320 μmol photon cm^{-2} s^{-1} as

it occurred with *T. pseudonana*.

The uptake rates were variable among replicates, but at 50 μmol photon cm^{-2} s^{-1} they were significantly lower ($p < 0.05$) (Figure D1B), reflecting what was occurring with the growth rates.

The NR activity rates did not show significant differences between treatments ($p > 0.05$) (Figure D1C). However, it is possible that this enzymatic assay needed an optimization for this species. For instance, the temperature used in the experiments of this study was 18ºC as it was the optimum for *T. pseudonana* and the growth of *M. pusilla* was high as well. However, it was shown that for some species of phytoplankton, particularly those with a higher content of chlorophyll-b (*e.g.*, green algae) (Gao *et al.* 2000), the optimum temperature for NR assays was around 30ºC. Although not part of the green algae, the order to which *M. pusilla* belongs, the Mamiellales, is phylogenetically closer to them than diatoms (McDonald *et al.* 2010).

Unfortunately it was not possible to successfully amplify in *M. pusilla* the functional genes that were used in the experiment with *T. pseudonana* after many trials with different primers, rendering impossible the intended comparison. One of the possible reasons for this lack of quantification is the fact that the culture strain used in this study (*i.e.,* NEPCC 29) was different from the one used to obtain the sequences that are available at the NCBI Gen Bank (*i.e.,* CCMP 1545). The designed primers may have not been specific enough since different strains may have genetic variations. Future research could focus on sequencing of genomic DNA from this particular strain. This would allow for the development of specific primers or the application of other

techniques designed to increase the specificity of qPCR (such as Taqman probes) in order to perform gene expression studies on *M. pusilla*.

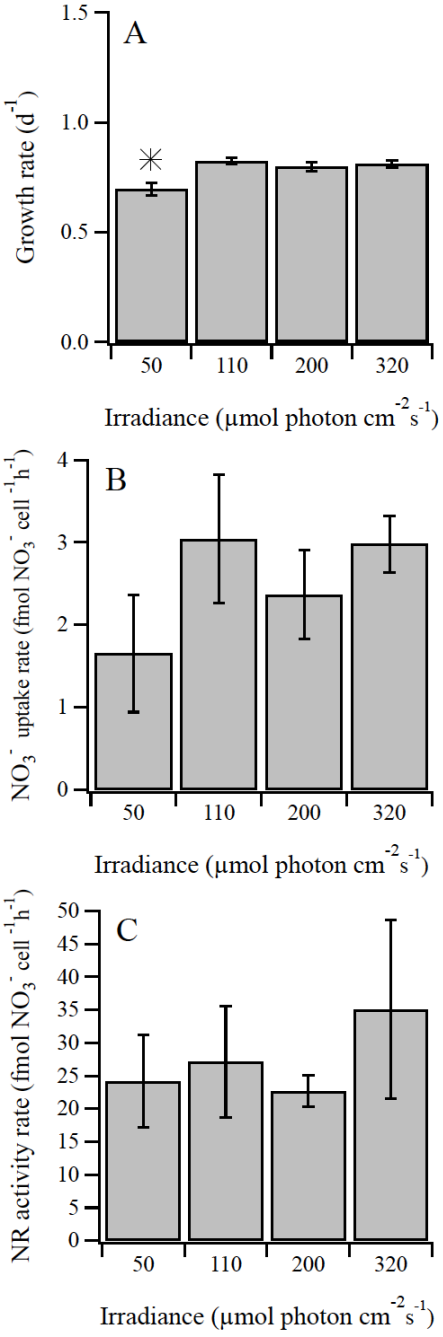

Figure D1: Effects of irradiance on growth rates (A), nitrate uptake rates (B), and NR activity rates (C) in *M. pusilla*. Stars indicate significant differences with at least one other treatment ($p < 0.05$). Error bars represent the standard error of the mean of triplicate cultures.