EXPRESSION AND ACTIVITY OF NOVEL NITRATE REDUCTASE ENZYMES IN *CHATTONELLA SUBSALSA* AND IMPLICATIONS FOR COMPETITIVE DYNAMICS IN MARINE ENVIRONMENTS

by

Yanfei Wang

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by

Yanfei Wang

Approved: ____________________________________________
Kathryn J. Coyne, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: ____________________________________________
Mark A. Moline, Ph.D.
Director of the School of Marine Science and Policy

Approved: ____________________________________________
Mohsen Badiey, Ph.D.
Acting Dean of the College of Earth, Ocean, and Environment

Approved: ____________________________________________
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education
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ABSTRACT

*Chattonella subsalsa* is a harmful alga that can form fish-killing blooms and cause severe damage to the ecosystem. In Delaware Inland Bays, *C. subsalsa* has formed mixed blooms with other species in recent years. The reason for the persistence of these blooms and the capacity for these species to avoid competitive exclusion remains unknown. Nitrogen is a limiting source in the aquatic environment, and its input may stimulate blooms dominated by *C. subsalsa*. Therefore, competing for nitrogen source may contribute to the success and survival of this species. For organisms to use nitrate as a nitrogen source, nitrate reductase catalyzes the first and also rate limiting step in nitrate assimilation. Algal nitrate reductase is responsive to nitrogen source, temperature, light intensity and its endogenous diel rhythm. In plants, it is also regulated by reversible phosphorylation of a conserved serine residue in the hinge 1 region, and sequential binding of 14-3-3 proteins at the post-translational level. However, 14-3-3 binding motifs within nitrate reductase were only found in plants, but not in algae. Previous research found a novel nitrate reductase, NR2-2/2HbN (NR2), in *C. subsalsa*, and this enzyme has a 2/2 hemoglobin domain within its hinge 2 region. In this research, another novel nitrate reductase, NR3, was found in this alga, and its sequence indicates the presence of a 14-3-3 binding motif in the hinge 1 region. To date, this is the first report for the presence of the 14-3-3 binding motif in algal nitrate reductase.

In Chapter 2, the sequence of NR3 was analyzed and compared with nitrate reductase sequences in algae and plants. The presence of a putative 14-3-3 binding
motif in this enzyme was discussed. The expression and activity of nitrate reductase in *C. subsalsa* were measured in response to light, nitrogen source, and temperature. The results indicate that, at the gene expression level, both NR2 and NR3 were regulated by light and nitrogen source, while only NR2 was regulated by temperature. At the protein translational level, evidence was provided that NR activity was regulated by nitrogen and temperature by reversible phosphorylation and binding of 14-3-3 proteins, while NR activity in response to light may be regulated by alternative mechanisms.

In Chapter 3, natural *C. subsalsa* blooms were stimulated by different nitrogen sources in two mesocosm experiments. One pulse of nitrogen was added to the first mesocosm experiment, while repeated pulses of nitrogen along with phosphate were added to the second mesocosm experiment. The growth rate of *C. subsalsa* and the entire assemblage, as well as NR2 and NR3 expression, were tested in order to investigate the implications of NR expression to the competition and survival of *C. subsalsa* in a dynamic environment. The results indicate that *C. subsalsa* out-competed other species with a low nitrogen concentration. Several strategies for the survival and success of *C. subsalsa* in the low nitrogen-loading environment were proposed based on the results: 1). *C. subsalsa* performed surge uptake and could store nitrogen; 2). *C. subsalsa* was capable of utilizing nitrate produced by nitrogen fixers or released by dead cells; 3). *C. subsalsa* regulates NR2 and NR3 expression differentially in response to different nitrogen conditions, such that NR2 may benefit *C. subsalsa* in favorable environments with a high concentration of nitrate, while NR3 may benefit *C. subsalsa* in a more dynamic and unfavorable environment with ammonium as the dominant nitrogen source.
Chapter 1

INTRODUCTION

1.1 Harmful Algal Blooms (HABs)

Phytoplankton are extremely important to primary production of the ocean (Mann 1999; Werner 1977; Granéli and Turner 2006). Phytoplankton blooms happen when chlorophyll a (as a measure of biomass) increases significantly to exceed a normal seasonal cycle (Carstensen, Henriksen, and Heiskanen 2007). In general, phytoplankton blooms can benefit the community by reducing the losses caused by viruses, sedimentation and grazing, while harmful algal blooms (HABs) can have negative impacts on humans, marine mammals, birds, fish and marine ecosystems (Granéli and Turner 2006). These impacts include bottom-water anoxia, water quality reduction, and biotoxin production (Anderson 2009). The toxins produced by HABs can affect the cellular process of varieties of organisms from plankton, fish and birds, to mammals including human beings. These toxins can be transferred and accumulate through the food chain, and impact the respiration and digestion systems of the organisms (Anderson 2009; Sellner et al. 2003). Non-toxic algal blooms can also cause mortalities of marine organisms. When the high biomass of algae decays at the terminal stage, oxygen is consumed, leading to anoxia and mortalities of marine organisms. High biomass of algae also reduces the light penetration to the bottom of the water column, causing the death of bottom aquatic vegetation and damages to marine ecosystems (Anderson 2009). Additionally, HABs have huge negative impacts on the economy. The average annual economic loss because of HABs in the US is
about $75 million from 1987 to 2000, and impacts resulting from individual blooms may exceed this annual average (Hoagland and Scatasta 2006).

Raphidophytes are unicellular eukaryotic flagellates that can form toxic blooms and cause severe damage to the ecosystem (Imai and Yamaguchi 2012). These blooms can be toxic to a variety of marine organisms by producing reactive oxygen species (ROS), nitric oxide (NO), free fatty acids (FFA), brevetoxin/ brevetoxin-like compounds (PbTx-like), hemolytic compounds, and other unidentified toxins (Khan et al., 1996; Bourdelais et al., 2002; Tanaka et al., 1994; Hiroishi et al., 2005; Band-Schmidt et al., 2012; Kim et al., 2006, 2008; Marshall et al., 2002, 2003; Fu et al., 2004; Kuroda et al., 2005; Shen et al., 2010; Astuya et al. 2015). Heterosigma and Chattonella genera are representative notorious fish-killing species of the Raphidophyceae. Noxious blooms of H. akashiwo and Chattonella spp. (C. antiqua, C. marina and C. subsalsa) have been associated with mortalities of cultured and wild fish and shellfish, and reported to cause massive fish kills in Japan, Korea, China, India, USA, and Australia (Kim et al., 2007; Tseng et al., 1993; Jugnu and Kripa 2009Tomas, 1998; Lewitus et al., 2008; Hallegraeff et al., 1998; Oda et al., 1997; Hard et al., 2000; Imai et al., 2001; Landsberg, 2002; Hiroishi et al., 2005; Matsubara et al., 2007; Shen et al., 2011).

1.2 Nitrogen Assimilation in Microalgae

Nitrogen (N) is essential for synthesis of nucleotides and amino acids of organisms, and therefore algae uptake nitrogen as a key nutrient to maintain their metabolism and growth (Terrado et al. 2015). Phytoplankton can take up dissolved organic nitrogen (DON), including urea, amino acids, and DNA; and also dissolved inorganic nitrogen (DIN), such as nitrate, nitrite, and ammonium (Twomey, Piehler,
and Paerl 2005). Among these different nitrogen sources, nitrate and ammonium are the most important and common ones; approximately 40% of the global primary production is the result of assimilation of these two nitrogen sources by phytoplankton (Malerba, Connolly, and Heimann 2015 and references therein).

In the natural environment, nitrogen is considered as one of the limiting nutrients for phytoplankton (Tremblay and Gagnon 2009). The chemical form, concentration and ratios of different nitrogen sources not only affect the abundance, frequency, and toxicity of HABs, but also play a role in selection of the species that dominate HABs (Davidson et al. 2012). Generally, algae prefer ammonium over nitrate because ammonium is in the same oxidation state as most amino acids, while nitrate has to be reduced to ammonium before assimilation (Malerba, Connolly, and Heimann 2015). In addition, uptake of nitrate can be suppressed or inhibited by ammonium, even at low concentrations (Dugdale et al. 2007). Nevertheless, published studies revealed differences in the nitrogenous preference among algal species. *Pseudo-nitzschia australis*, for example, preferred nitrate over ammonium or glutamine, and the substrate- saturated rate of nitrate uptake by this species was the largest compared to the other two sources (Cochlan, Herndon, and Kudela 2008). In contrast, different nitrogen source (nitrate, ammonium, and urea) and ratios of these nitrogen sources had no effect on the growth of *Pseudo-nitzschia seriata* (Fehling 2004; Thessen, Bowers, and Stoecker 2009); while *Skeletonema* grew significantly faster on ammonium compared to nitrate (Yamada, Tadad and Harrison 2009; Tada et al. 2009).

The nitrogenous nutrition preference of raphidophytes remains unclear. The published studies indicate the nitrogenous preference of these algae can be different
among strains of the same species (Table 1.1) (Herndon and Cochlan 2007a; Fredrickson et al. 2011; Zhang et al. 2006; Chang and Page 1995; Yamaguchi, Sakamoto, and Yamaguchi 2008). For instance, *H. akashiwo* (CCMP 3150) preferred organic nitrogen sources, including alanine, glutamine, and urea; while strain CCMP2808 and CCMP2809 of the same species preferred inorganic nitrogen sources, such as nitrate and ammonium, and had no growth on organic nitrogen sources (Fredrickson et al. 2011). Additionally, the nitrogenous preference of the same algal strain may also be different when the light irradiance changes. *Heterosigma carterae*, for example, had the most rapid growth when growing with nitrate at high light irradiance (160 µmol photons m\(^{-2}\) s\(^{-1}\)), and when the light intensity decreased, the growth rate when cultured with nitrate decreased significantly, with a change of their nitrogenous preference from nitrate to ammonium and urea (Chang and Page 1995). In order to understand, predict, and reduce the damage of these HABs, determining their preference of nitrogen sources and the rationale behind this are extremely important (Herndon and Cochlan 2007).

### 1.3 Nitrate Reductase of Microalgae

To utilize nitrate as a nitrogen source, algae reduce nitrate to ammonium through two sequential reactions that are catalyzed by nitrate reductase (NR) and nitrite reductase, respectively (Flores et al. 2005). The ammonium reduced from nitrate is then assimilated through glutamate dehydrogenase or the glutamine synthetase/glutamate synthase cycle (Flores et al. 2005). In this process, the reaction catalyzed by NR is the first and also the rate-limiting step, and therefore, the capacity
and efficiency of nitrate assimilation by algae is affected by their NR activity (Campbell 1999; Berges and Harrison 1995).

NR is a flavoprotein containing five structurally distinct domains (Fig.1.1 a) (Campbell, 1999): (a) a nitrate-reducing active site with molybdenum-molybdopterin cofactor (Mo-MPT); (b) a dimer interface domain (DI); (c) cytochrome b5 domain with Heme-Fe (Cb); (d) flavin adenine dinucleotide (FAD) domain; and (e) an NAD(P)H domain at C-terminus. The FAD and NADH are combined to form the cytochrome b reductase fragment (CbR), and the cytochrome c reductase fragment (CcR) can be formed when the Cb domain is combined with CbR. Mo-MPT and cyb5-binding domains are highly conserved (Zhou and Kleinhofs 1996). NR is also characterized by three different sequence regions: (a) N-terminal “acidic” segment which has the function to regulate and stabilize the enzyme; (b) a hinge 1 region containing a site of reversible protein phosphorylation at a serine (Ser) residue in plants and a trypsin proteolytic site; and (c) a hinge 2 region containing another proteinase site (Campbell, 1999). The phospho-Ser within hinge 1 region provides the unique binding site for 14-3-3 proteins in NR of plants (Chi et al. 2015). 14-3-3 binding proteins are key regulators involved in varieties of cellular process, affecting sugar synthesis and storage, as well as nutrient uptake and metabolism (Denison et al. 2011a). In plants, 14-3-3 binding proteins inhibit NR at post-translational level under the dark condition to prevent the accumulation of nitrites that are potentially toxic and cannot be metabolized in the dark (Denison et al. 2011a; Markus Bachmann et al. 1996). Although the phosphorylated Ser residue that provides site for 14-3-3 binding is conserved in NR of higher plants, it has not been identified in NR of mosses or algae (Medina-Andrés and Lira-Ruan 2012; Sanz-Luque et al. 2015). So far, there is
no evidence to support a post-translational regulation for NR in algae (Nemie-Feyissa et al. 2013; Sanz-Luque et al. 2015).

Previous research identified NR1 in *H. akashiwo*, as well as NR2-2/2HbN (NR2) in *H. akashiwo* and *C. subsalsa* (Coyne 2010; Stewart and Coyne 2011). The sequence of NR1 in *H. akashiwo* presents the conserved structure of NR, including five domains and three regions, but shared relatively low identity with diatoms and green algal species (Coyne 2010). *Hs*NR2 and *Cs*NR2 have a novel 2/2 hemoglobin (2/2Hb) domain inserted in the hinge2 region (Stewart and Coyne 2011) (Fig. 1.1 b). These 2/2Hbs share high identity with 2/2Hbs of genus *Mycobacterium*, which work to detoxify nitric oxide (Lama, Pawaria, and Dikshit 2006; Giordano and Raven 2014). Due to the presence of 2/2Hbs in NR2, this enzyme was proposed to couple nitric oxide dioxygenase (NOD) and NR activities that would reduce nitric oxide to nitrate, with sequential reduction of nitrate to ammonium (Stewart and Coyne 2011). The factors related to regulation of expression and activity of NR2 remain unknown.

Recently, another NR was sequenced and identified from *C. subsalsa*, designated NR3. This NR is different from NR1 identified from raphidophytes in previous research work (Fig. 1.1 c). The deduced amino acid sequence of this enzyme has the conserved domains of NR, except that it lacks the heme-Fe (cytochrome b5) domain between the DI and FAD domains. The enzyme also differs from NR2 in that it lacks the 2/2HbN domain in the hinge 2 region. In addition, a putative 14-3-3 protein binding motif was identified in the hinge 1 region of NR3. This is the first evidence to support the existence of a 14-3-3 binding site and the potential for post-translational regulation by 14-3-3 binding of this enzyme in algal NR.
In general, NR expression and activity are regulated by nitrogen source, temperature, and light conditions (Lomas and Glibert 2000; Giordano and Raven 2014; Sanz-Luque et al. 2015; Stewart and Coyne 2011). However, the environmental factors related to NR2 and NR3 regulation remain unknown. In Chapter 2 of this study, I measured the expression of NR2 and NR3 in *C. subsalsa* cultured with different nitrogen sources, temperature and light conditions, to investigate the regulation of these enzymes at the transcriptional level. NR enzyme activity was also measured under conditions to evaluate the potential for inhibition by 14-3-3 binding proteins. In Chapter 3, two mesocosm experiments were performed to evaluate the regulation of NR2 and NR3 expression in *C. subsalsa* by different nitrogen source input during natural blooms, and the role of NR regulation in the competitive dynamics of *C. subsalsa* in the marine environment.
Table 1.1 The Nitrogenous Preference of Species of \textit{Heterosigma} and \textit{Chattonella} spp.

The preference of nitrogen source by species is indicated by symbols (>, <, and =).

<table>
<thead>
<tr>
<th>Species</th>
<th>Nitrogenous preference</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Heterosigma akashiwo} (CCMP1912)</td>
<td>( \text{NH}_4^+ &gt; \text{NO}_3^- &gt; \text{urea} )</td>
<td>Herndon and Cochlan 2007a</td>
</tr>
<tr>
<td>\textit{Heterosigma akashiwo} (CCMP2808)</td>
<td>( \text{NH}_4^+ = \text{NO}_3^- = \text{urea} ), and no growth on glutamine, alanine, hypoxanthine, ethanolamine, and acetamide.</td>
<td>Fredrickson et al. 2011</td>
</tr>
<tr>
<td>\textit{Heterosigma akashiwo} (CCMP3149)</td>
<td>( \text{NH}_4^+ = \text{NO}_3^- = \text{urea} = \text{glutamine} = \text{alanine} ), and no growth on hypoxanthine, ethanolamine, and acetamide</td>
<td>Fredrickson et al. 2011</td>
</tr>
<tr>
<td>\textit{Heterosigma akashiwo} (CCMP3150)</td>
<td>Urea = glutamine = alanine = nitrate = ammonium, and no growth on hypoxanthine, ethanolamine, and acetamide</td>
<td>Fredrickson et al. 2011</td>
</tr>
<tr>
<td>\textit{Heterosigma akashiwo} (CCMP2393)</td>
<td>( \text{NO}_3^- &gt; \text{NH}_4^+ )</td>
<td>Zhang et al. 2006</td>
</tr>
<tr>
<td>\textit{Heterosigma carterae} (NZOI/HCBGB06)</td>
<td>( \text{NO}_3^- ) at high irradiance (160 ( \mu \text{mol} \text{ photons m}^{-2} \text{ s}^{-1} )); ( \text{NH}_4^+ ) and urea at lower irradiance.</td>
<td>Chang and Page 1995</td>
</tr>
<tr>
<td>\textit{Chattonella subsalsa} (CCMP2191)</td>
<td>( \text{NH}_4^+ &gt; \text{NO}_3^- )</td>
<td>Zhang et al. 2006</td>
</tr>
<tr>
<td>\textit{Chattonella ovata}</td>
<td>( \text{NH}_4^+ = \text{NO}_3^- = \text{NO}_2^- ), and no growth on organic nitrogen source</td>
<td>Yamaguchi, Sakamoto, and Yamaguchi 2008</td>
</tr>
</tbody>
</table>
a. Conserved structure of NR.

b. Structure of NR2 with a 2/2HbN domain inserted in its hinge 2 region.

c. Structure of NR3 with a 14-3-3 binding motif at a phosphor-serine residue (P-S) in the hinge 1 region.

Figure 1.1. a. Nitrate reductase of algae consists of five domains (MO-MPT, DI, Heme, FAD, and NADH domains) and 3 regions (N-terminal, hinge1 and 2 regions); b. structure of NR2 with a 2/2HbN domain inserted in its hinge 2 region; c. structure of NR3 with a 14-3-3 binding motif at a phosphor-serine residue (P-S) in the hinge 1 region.
Chapter 2

EXPRESSION AND ACTIVITY OF NOVEL NITRATE REDUCTASE ENZYMES (NR2 AND NR3) IN CHATTONELLA SUBSALSA

2.1 Abstract

*Chattonella subsalsa* is a toxic alga that has been associated with massive fish kills worldwide and may be stimulated by nitrogen input. Nitrate reductase (NR) catalyzes the first enzymatic and also rate-limiting step in nitrate assimilation. In plants, NR activity is regulated by reversible phosphorylation and the subsequent binding of 14-3-3 proteins at a conserved Ser residue. However, 14-3-3 binding motifs have not been identified in algal NRs. Previous research in our laboratory indicates that *C. subsalsa* possesses a novel nitrate reductase enzyme, NR2-2/2HbN (NR2), which incorporates a 2/2 hemoglobin domain. In this research, a second nitrate reductase, designated NR3, was found in this alga. Evidence from this research indicates that NR3 may lack the cytochrome b5-binding domain, which is a conserved functional domain in nitrate reductase. What is more, a putative binding motif for 14-3-3 proteins was found in this alga. This is the first report of a 14-3-3 binding motif in algal nitrate reductase. Here, I measured expression and activity of nitrate reductase in *C. subsalsa* in response to light, nitrogen source, and temperature. Results indicate that both NR2 and NR3 expression were regulated by light and nitrogen source, while NR2 expression was also regulated by temperature. At the post-translational level, I provide evidence that NR activity was regulated by nitrogen source and temperature through reversible phosphorylation and putative binding of 14-3-3 proteins, while regulation of NR activity by light was through other regulatory mechanisms. These results suggest that *C. subsalsa* responses to environmental factors rapidly by regulating its NR at
both transcriptional and translational levels, which may contribute to the survival of *C. subsalsa* in the environment.

### 2.2 Introduction

Nitrogen is one of the limiting nutrients for phytoplankton in the natural environment; it is not only a key factor to stimulate harmful algal blooms (HABs), but also plays an important role in the selection of the species that dominate in these HABs (Tremblay and Gagnon 2009; Davidson et al. 2012). Nitrate and ammonium are the most important and common nitrogen sources that phytoplankton utilize for their growth and metabolism and approximately 40% of the global primary production is the result of assimilation of these two nitrogenous nutrients by phytoplankton (Malerba, Connolly, and Heimann 2015 and references therein).

In order to utilize nitrate as a nitrogen source, phytoplankton convert nitrate to ammonium by two sequential reactions that are catalyzed by nitrate reductase (NR) and nitrite reductase, respectively, with electrons donated by NAD(P)H (reviewed by Stewart and Coyne 2011; Campbell 1999). NR catalyzes the first enzymatic and also the rate-limiting step in nitrate assimilation, and NR activity plays a key role in coordinating nitrogen and carbon assimilation in higher plants, algae, and fungi (Campbell 1999; Parker and Armbrust 2005). The conserved structure of NR includes five domains (Fig.1.1) (Campbell 1999): (a) a molybdenum-molybdopterin cofactor (Mo-MPT); (b) a dimer interface domain (DI); (c) a cytochrome b5 domain with Heme-Fe (Heme); (d) a flavin adenine dinucleotide domain (FAD); (e) a NAD(P)H domain. NR is also characterized by three regions: (a) an N-terminal region that has the function to regulate and stabilize the enzyme; (b) a hinge 1 region that is located between the Mo-MPT and heme domains, and contains a phospho-serine residue that
provides the binding site for 14-3-3 proteins in plants; and (c) a hinge2 region between FAD and heme domains.

NR is regulated by environmental factors (Meyer et al. 2005; Lillo et al. 2008; Nemie-Feyissa et al. 2013; Lomas and Glibert 2000; Davison and Stewart 1984; Ramalho, Hastings, and Colepicolo 1995; Lopes et al. 1997). The tight regulation of NR not only plays an important role for organisms to optimize nitrogen assimilation and adjust different metabolic pathways, but also prevents the accumulation of potentially toxic nitrite and formation of reactive oxygen and nitrogen species as side products (Meyer et al. 2005; Lillo 2008; Nemie-Feyissa et al. 2013).

In general, NR is responsive to nitrogen source. NR expression is often induced by the presence of nitrate, and strongly repressed or inhibited completely by the presence of ammonium (Llamas, Igeño, et al. 2002; Fernandez and Galvan 2008; Imamura et al. 2010; Glibert et al. 2016). Nitrate uptake and NR activity can be inhibited in seconds and minutes, respectively, after adding ammonium to the nitrate induced cells of algae (reviewed by Sanz-Luque et al. 2015). Temperature is another factor that regulates NR, and the optimal temperature for NR is different among species. The NR in the diatom Skeletonema costatum has a temperature optimum of about 15 °C, for example, while some dinoflagellate and chlorophyte species have the optimal temperature of 22 to 28 °C (reviewed by Lomas and Glibert 2000).

NR is also controlled by light and the endogenous diel rhythm of algae; their NR activity fluctuates during the light- dark cycle but may also fluctuate during constant light in a species-specific manner (Davison and Stewart, 1984; Ramalho et al., 1995, Lopes et al., 1997). NR activity of the red alga Gracilaria chilensis, for example, was 4 times higher during the light phase than in the dark, and had no
circadian fluctuation during continuous light (Chow, De Oliveira, and Pedersén 2004). Another red alga, *Kappaphycus alvarezii*, exhibited diurnal rhythm of NR activity under either a light-dark cycle or constant light with the maximal activity during the midday phase (Granbom et al. 2004). Regulation of NR activity by light has been studied extensively for higher plants but there are few studies on the effects of light on NR activity of microalgae, and even less known about the effects of light on NR gene expression in algal species (reviewed by Tischner 2000; Giordano and Raven 2014; Falcão, Oliveira, and Colepicolo 2010).

In higher plants, the regulation of NR is, in part, mediated at the post-translational level by reversible phosphorylation of a conserved Ser residue in the hinge 1 region, permitting binding of 14-3-3 proteins (Tischner 2000; Christensen et al. 2004; Pozuelo et al. 2001). Binding of 14-3-3 proteins inhibits NR activity via obstruction of intra-molecular electron flow (Lambeck et al. 2012; Bachmann et al. 1996; Denison et al. 2011b). It should be noted that binding of 14-3-3 proteins to phosphorylated NR requires divalent cations, such as Mg$^{2+}$, at millimolar concentrations (Athwal and Huber 2002). The requirement for divalent cations provides a means to evaluate the differential regulation of NR activity by 14-3-3 binding in cellular homogenates (Fig. 2.1) (Mackintosh, Douglas, and Lillo 1995; Nemie-Feyissa et al. 2013). Total NR activity can be measured by removing Mg$^{2+}$ through chelation with EDTA, while the addition of excess Mg$^{2+}$ would allow the formation of NR-Ser-P/14-3-3 binding protein complex, resulting in reduced NR activity representing unphosphorylated NR. The difference in activity in the presence and absence of Mg$^{2+}$, then, provides a measure of NR activity that is inhibited by 14-3-3 binding to phosphorylated NR (Mackintosh, Douglas, and Lillo 1995; Nemie-
According to the published studies, the phospho-Ser residue that is necessary for the binding of 14-3-3 proteins is conserved in NR of higher plants. NR of mosses and algae are thought to lack this residue (Medina-Andrés and Lira-Ruan 2012; Sanz-Luque et al. 2015; Nemie-Feyissa et al. 2013; Sanz-Luque et al. 2015).

HABs caused by the genus *Chattonella* (*C. antiqua*, *C. marina*, *C. subsalsa*, and *C. ovata*) have been responsible for massive fish kills in Japan, Korea, India, China, USA, and South Australia, with severe damages to fishery industries and economy (Kim et al. 2007; Tseng et al. 1993; Jugnu and Kripa 2009; Tomas 1998; Lewitus et al. 2008; Hallegraeff et al. 1998; Songhui and Hodgkiss 2001; Guarado et al. 2004; Takatsui et al. 2005; Hiroshi et al. 2005; Yamaguchi et al. 2008). The mechanisms of fish-killing by *Chattonella* spp. are still not clear, but suffocation of fish is considered as the ultimate cause of death (Matsusato and Kobayashi 1974; Imai and Yamaguchi 2011). Brevetoxin (neurotoxin), polyunsaturated fatty acids, and reactive oxygen species (ROS, e.g. superoxide) are among the major bioactive compounds that are produced by *Chattonella* spp. and possibly related to fish death (Okaichi 1980; Shimada et al. 1983; Onoue and Nozawa 1989; Endo et al. 1992; Khan et al. 1996; Keppler et al. 2006; Tanaka et al. 1994; Ishimatsu et al. 1996; Marshall et al. 2005). In nature, *Chattonella* spp. produce cysts to survive in unfavorable environments, and may exist as background flora with low cell densities between bloom events (Imai and Itoh 1986, 1988; Yamaguchi et al. 2008; Smaida 2002; Imai et al. 2006; Imai and Yamaguchi 2011). Previous research has identified temperature, salinity, irradiance, and nutrients as the most important factors to stimulate the growth and bloom formation of *Chattonella* spp. (Imai and Yamaguchi 2011). Nitrate is one of the favorable nitrogen source for *Chattonella* spp. (Zhang et al. 2006; Parker and
Armbrust 2005), suggesting that environmental factors that regulate NR play a key role in nitrogen assimilation by these species.

A novel NR, NR2-2/2HbN (NR2), was recently identified in the raphidophytes *H. akashiwo* and *C. subsalsa* (Stewart and Coyne 2011) with a 2/2 hemoglobin (2/2Hb) domain inserted in the hinge2 region. As with other algae, a 14-3-3 binding site was not identified in the translated amino acid sequence of this enzyme (Stewart and Coyne 2011), and the factors related to regulation of this enzyme in raphidophytes remain unclear (Stewart and Coyne 2011). Recently, the transcriptome of *C. subsalsa* was sequenced (Keeling et al. 2014), revealing the presence of a unique NR in this species, designated NR3. The deduced amino acid sequence of NR3 lacks both the heme-Fe and 2/2HbN domains, but instead includes a potential phosphorylation site and a canonical 14-3-3 binding protein motif in the hinge1 region. Here, I investigated the regulation of NR2 and NR3 in *C. subsalsa*, at both transcriptional and translational levels, in response to different nitrogen sources and ratios, as well as temperature and light conditions, and propose a mechanism by which NR3 may retain function without the heme-Fe domain.

2.3 Material and Methods

2.3.1 Identification of NR3 of *C. subsalsa* and putative 14-3-3 binding protein motif

The transcriptome of *C. subsalsa* (CCMP2191) was sequenced by Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP; Keeling et al. 2014). The transcriptome (Sample Name: MMETSP0947-0950; PI: Kathryn Coyne) can be found on the Moore Foundation sequencing site (iMicrobe; Gordon and Betty Moore Foundation and University of Arizona). Within the transcriptome, NR2
(MMETSP0948_20120912_5896_1) and a unique NR (designated NR3; MMETSP0948_20120912_5262_1) sequence were identified via BLASTX search (Altschul et al. 1997; http://blast.ncbi.nlm.nih.gov). The alignment of NR3 was compared with NR2 from NCBI database using MAFFT (format: Clustal, sorted; strategy: G-INS-i; software version: v7.273) (Altschul et al. 1997; Katoh and Standley 2013). The translated amino acid sequence of NR3 was submitted to Scansite 3 (http://scansite3.mit.edu) to identify potential 14-3-3 binding motifs in this sequence (Yaffe et al. 2001; Obenauer, Cantley, and Yaffe 2003). Scansite is a computational tool to predict interactions between proteins and sites of phosphorylation. The parameters used in this search are listed here: group: phosphoserine/threonine binding; motif: 14-3-3 mode 1; and stringency: high (Obenauer, Cantley, and Yaffe 2003).

2.3.2 Culture conditions

2.3.2.1 Stock Culture

Stock cultures of *C. subsalsa* (National Center for Marine Algae and Microbiota, CCMP2191) were maintained in f/2 medium (-Si) with either f/2 nitrate or 100 µM ammonium as nitrogen source, at salinity of 20 psu and temperature of 25 °C (Guillard and Ryther 1962). Cultures were maintained at 100 µE m⁻² s⁻¹ irradiance on a 12:12 h light: dark cycle.

2.3.2.2 Diel expression and activity of NR2 and NR3 under different light conditions

*C. subsalsa* was cultured at 25 °C with f/2 medium and nitrate as the nitrogen source. Cell density was determined in triplicate by microscopy and resuspended in fresh f/2 medium at a cell density of 11,400 cells mL⁻¹. Cultures (N=3) were exposed
to a 12:12 h light: dark cycle. Replicate cultures (N=3) were subjected to constant light at 100 µmol photons m⁻² s⁻¹ for 24 hours before sampling and remained under constant light for the entire sampling period. Samples were collected at five time points: 1 hour before lights on (7:00), 1 hour after lights on (9:00), 6 hours after lights on (14:00), 1 hour after dark (21:00), and 6 hours after dark (2:00). At each time point, samples of 50 ml were gently filtered through 3 µm pore size polycarbonate filters for RNA extraction, described below. An additional 50 ml sample was collected to determine NR activity as described below. This sample was centrifuged, the supernatant was discarded, and the cell pellet was frozen in liquid nitrogen immediately and stored at -80 °C until analysis.

2.3.2.3 Expression and activity of NR2 and NR3 with different nitrogen sources

Algae were cultured with 100 µM NO₃⁻, 100 µM NH₄⁺, or three different ratios of NO₃⁻ to NH₄⁺ (5:95, 50:50, 95:5 µM), with a light intensity of 100 µmol photons m⁻² s⁻¹, and temperature of 25 °C (N=3). Cultures were acclimated to growth on each nitrogen condition, and harvested at exponential phase at 6 hours after the start of the light phase. Samples were collected as described above for RNA extraction and NR activity analysis. Cell density was determined by microscopy. Dissolved nutrients (ammonium, nitrate+nitrite, and phosphate) were collected and duplicate samples from each culture analyzed using a segmented-flow autoanalyzer (Seal Analytical, Mequon, WI).

2.3.2.4 Expression and activity of NR2 and NR3 under different temperatures

*C. subsalsa* was cultured with f/2 medium and nitrate as the nitrogen source at 18 °, 25 °, and 28 °C, with a light intensity of 100 µmol photons m⁻² s⁻¹ (N=3). All
cultures were acclimated to each condition before harvesting at exponential phase 6 hours after the start of the light phase. The samples were collected as described above for RNA extraction and NR activity analysis.

2.3.3 Analysis of transcript abundance

2.3.3.1 RNA extraction

RNA was extracted from filtered cells using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). RNA concentration was measured by UV-Vis spectrophotometer (NanoDrop Technologies). The integrity was evaluated by electrophoresis before cDNA synthesis.

2.3.3.2 Complementary DNA (cDNA) synthesis

Contaminating DNA was digested in 10 µl reaction volumes containing 8 µl (300 to 1000 ng) RNA, 1 µl 10X DNase I Reaction Buffer (Invitrogen, Carlsbad, CA) and 1 µl DNase (100 units, Amplification Grade, Invitrogen) for 15 minutes at room temperature. One microliter of 25 mM EDTA (pH 8.0, Invitrogen) was added to the reaction, the reaction was then incubated at 65 °C for 15 min to degrade the DNase. The purified RNA was stored at -80 °C until use.

First strand cDNA was synthesized using the Superscript III First Strand Supermix Kit (Invitrogen) according to manufacturer’s directions. Six microliters of DNase-digested RNA and random hexamers were used in this process. The reaction was incubated at 25 °C for 10 min to maximize the binding of primer and RNA template; followed by 50 °C for 50 min for reverse transcription to proceed, and 85 °C for 5 min to stop the reaction. The cDNA was stored at -80 °C until PCR analysis.
2.3.3.3 Plasmid Preparation

Complementary DNA (cDNA) was diluted 1:20 with LOTE [3 mM Tris-HCl (pH 7.5), 0.2 mM EDTA] and used as template in PCR reactions with primers targeting *C. subsalsa* NR2 and NR3. Each PCR reaction of 20 µl included 0.2 mM dNTPs, 2.5 mM MgCl₂, 1X Taq polymerase buffer (Sigma Chem. Co., St. Louis, MO, USA), 0.25 units Jump-Start Taq Polymerase (Sigma Chem. Co.), 0.075 µM forward primer, 0.075 µM reverse primer, and 1.0 µL diluted template. Primer sequences are listed in Table 1. The PCR cycle consisted of 37 cycles of 30 s at 94 °C, 30 s at 53 °C, and 1 min at 72 °C. PCR products diluted to 1:20 with LOTE were cloned into pCR4 TOPO plasmid vector (Invitrogen). Plasmid was then prepared using QuickClean 5M Miniprep Kit (GenScript Corporation, Piscataway, NJ) from liquid media following the manufacturer’s protocol. The plasmid for NR3, which included the hinge 1 region, was sequenced for comparison to the sequence provided by the Marine Microbial Eukaryote Transcriptome Sequencing Project.

To calculate the copy number of plasmid, single strand plasmid molecular weight (MW) was first calculated by adding the MW of pCR4-TOPO vector to the MW of PCR product inserted into the vector. The mass per copy of the plasmid was then determined by dividing the MW of double stranded plasmid by the Avogadro constant (6.022 x 10²³ per mole). The plasmid concentrations of 10 replicate dilutions were measured by UV-Vis spectrophotometer (NanoDrop Technologies) and the average concentration of the plasmid in gram per microliter was then divided by mass per copy of plasmid to calculate the copy number per microliter of the plasmid dilution.
2.3.3.4 Fragment length analysis to evaluate missing Heme-Fe domain

Another PCR reaction using CsNR3 1443F and CsNR3 1515R as the primers, and cDNA of *C. subsalsa* as the template, was conducted using the same protocol as above to span the region from hinge 1 to the beginning of FAD domain in NR3. The amplified products were examined by electrophoresis on 2% agarose gels. The length of the expanded region was compared with the expected fragment length as sequenced by Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP; Keeling et al. 2014).

2.3.3.5 Determination of NR expression

Expression of NR2, NR3, and actin (as a normalizing gene) were determined by quantitative real-time PCR (qPCR) using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). Diluted cDNA (1:20) was used as template in triplicate 10 µL reactions. Each reaction included 5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.3 µM forward primer, 0.3 µM reverse primer, 2 µl sterile water, and 1 µl diluted template. Primers sequences are listed in Table 1. Ten-fold dilutions of NR2 and NR3 plasmids were used to make a standard curve of known copy numbers for each plate. Negative controls (without template) were run simultaneously in the same plates. The cycling for each set of reactions consisted of 50 °C for 2 min, and 95 °C for 10 min, then followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s, and 60 °C for 1 min. To identify potential primer-dimer formation, a denaturation step was performed. The denaturation step included 1 cycle of 95 °C for 15 s, 60 °C for 1 min, followed by 95 °C for 15 s, and 60 °C for 15 s. The copy numbers of NR2 and NR3 for each sample were determined by linear regression.
analysis, and then normalized to actin expression to produce a relative expression value for each gene.

2.3.4 NR activity Assay

The NR activity assay was optimized for _C.subsalsa_ (Appendix A). The frozen cell pellet was resuspended in 1 ml extraction buffer (200 mM KPi buffer, pH 7.9) on ice, homogenized by sonication, and centrifuged to clarify at 4 °C. The supernatants were removed and assayed immediately (Fig. 2.2).

The homogenate was divided to evaluate total NR activity (with EDTA) or NR activity in the presence of excess Mg$^{2+}$ representing unphosphorylated NR (Berges and Harrison, 1995; Nemie-Feyissa, 2013). The assay consisted of extract, an equal volume of assay buffer (198 mM KPi, pH 7.9, with either 2 mM EDTA or 6 mM MgCl$_2$), 0.2 mM NAD(P)H (Sigma Chem. Co., St. Louis, MO) and 10 mM KNO$_3$. Sterile water was added to the negative control reaction in place of KNO$_3$. The reaction was incubated for 30 min at room temperature in the dark. Zinc Acetate (47.62 mM) was added to stop the reaction. The samples were centrifuged and the supernatants were used below.

The concentration of nitrite produced was then measured colorimetrically. Ten microliters of 750 µM N-Methylphenazonium methyl sulfate (Sigma–Aldrich Fluka, Buchs SG, Switzerland) was added to 200 µl supernatants from above. The reaction was incubated in the dark for 20 min, and 12.5 µl 5 M HCl, 125 µl 58 mM Sulfanilimide (Sigma Chem. Co.) were added to the reaction. After 5 min incubation in the dark, 125 µl 4 mM N-(1-Naphthyl)ethylenediamine Hydrochloride (Sigma Chem. Co.) was added to the reaction. The samples were incubated for 10 minutes and
then were aliquoted into 96 well plates. The absorbance was measured at 540 nm (Berges and Harrison 1995; Nemie-Feyissa 2013). NR activity was normalized to protein content within the cellular homogenate and measured using the Pierce BCA Protein Assay Kit as directed (Pierce, Rockford, IL, USA; MacKintosh et al., 1995).

2.3.5 Statistical Analysis

Repeated measures ANOVA was used to test if there was a significant effect of light on the expression and activity of each NR (NR2 and NR3 in expression study; total NR and non-phosphorylated NR in activity study). If the effect was significant (p<0.05), then paired t-test was conducted to analyze the significance of the difference between the values at adjacent time points. Two sample t-test was used to test the significance of the difference between NR expression or activity in the light to dark cycle compared to the ones in constant light at each time point. Additionally, paired t-test was used to analyze the difference between NR2 and NR3 expression at each time point.

One-way ANOVA was used to test if different nitrate to ammonium ratios and temperatures had a significant effect on NR expression and activity. If the effect was significant, then Tukey’s HSD test was used to analyze the difference between values in all possible pairs of groups of different nitrate to ammonium ratios and temperatures in each experiment. One-way ANOVA was also used to analyze the difference between expression or activity of NR at each nitrate to ammonium ratio and temperature. Additionally, paired t-test was used to test if NR activity was inhibited significantly by Mg$^{2+}$ for each experiment. All statistical analyses were performed in R (V.3.2.4 Revised, R Core Team 2016).
2.4 Results

2.4.1 Identification of *C. subsalsa* NR3 and putative 14-3-3 binding protein

The comparison of alignment of NR3 and NR2 confirms the lack of both the 2/2 HbN domain as well as the cytb-5 binding domain in NR3 (Appendix C). However, PCR analysis of NR3 from the hinge 1 region to the beginning of FAD domain indicated that this region in NR3 of *C. subsalsa* was longer than 400 bp (Fig. 2.4), exceeding the expected 372 bp length of the same region in the sequence of NR3 of *C. subsalsa* from the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP; Keeling et al. 2014).

The hinge 1 region of NR3 in *C. subsalsa* was compared with the same regions of NR2 of *C. subsalsa* and *H. akashiwo*, as well as NR of two higher plants, *Arabidopsis thaliana* (Accession: NP_177899.1 GI:15218104) and *Camelina sativa* (Accession: XP_010428861.1 GI: 727505528) (Altschul et al. 1997; Katoh and Standley 2013). The 14-3-3 proteins bind to a phosphorylated Ser residue within the motifs RXXpSXP as originally described by Muslin et al. (1996), or KSXpSXP in angiosperms (Halford et al. 2003; Lillo 2008; Johnson et al. 2010; Muslin et al. 1996; Athwal and Huber 2002). The X in the sequence means any of the 20 types of amino acids, and pS is the phosphor-Ser residue (Li et al. 2016). The existence of the motif RRLSAP in NR3 suggests the potential for regulation of NR activity through reversible phosphorylation of the Ser residue and 14-3-3 binding in this species.

The binding motif of 14-3-3 proteins was predicted by Scansite within the larger sequence SNRSRRlSAPALPLA at the Ser430 site in the hinge 1 region of NR3. Additionally, the search resulted in a Scansite score of 0.308. This score is generated from a scoring matrix to quantitatively evaluate the divergence of a given
sequence from the optimal motif sequence (Obenauer, Cantley, and Yaffe 2003), where the score increases from zero (a perfect match). The score of 0.308 falls at the top 0.147% of sequence scores when the interested motif matrix was applied to the vertebrate subset of SWISS-PROT, indicating the high stringency of the prediction (Obenauer, Cantley, and Yaffe 2003). However, the predicted surface accessibility for this site within NR3 was 0.7828, revealing that this site may be buried in the protein, which may negatively affect the interactions between NR3 and the 14-3-3 binding proteins (Obenauer, Cantley, and Yaffe 2003).

2.4.2 Diel expression of NR2 and NR3 under different light conditions

Treatments were sampled at five time points over a 19 h period from C. subsalsa growing in a 12:12h light: dark cycle or constant light (Fig. 2.5). NR2 expression was significantly (3-30 fold) higher than NR3 expression at all time points in each light condition (p<0.05). For C. subsalsa cultured in a light: dark cycle, there was no significant change in NR2 expression from the first time point, 1 hour before lights on (07:00), to the second time point, 1 hour after lights on (09:00; Fig. 2.5; Table 2.3). NR2 expression then significantly decreased from the first two time points to the third time point at 6 hours after light on (14:00); NR2 expression was 4.5-fold higher at the first two time points than the third time point. From the third to the fifth time point, there was no significant change in NR2 expression. In addition, there was no significant difference in NR2 expression between any of two time points in C. subsalsa cultured in constant light (p>0.05).

When comparing NR2 expression in C. subsalsa cultured with the light: dark cycle to cultures exposed to constant light, the lowest occurred at the same time point, 6 hours after lights on (14:00). Additionally, there were significant differences in NR2
expression (p<0.05) of *C. subsalsa* cultured in a light to dark cycle compared with the ones cultured in constant light, with 2-fold higher expression in the 12:12 h light: dark cultures at 1 hour before and 1 hour after lights on (7:00 and 9:00), as well as a 2-fold higher expression in the constant light cultures at 1 hour after dark (21:00). There were no significant differences in NR2 expression between treatments at 6 hours after lights on (14:00) or at 6 hours after dark (2:00, p>0.05).

In contrast to NR2, expression of NR3 in *C. subsalsa* cultured under a 12:12 h light: dark cycle was significantly (1.8-fold) higher at 1 hour before lights on (7:00) than the expression at 1 hour after lights on (9:00, Fig. 2.5, Table 2.3). No significant difference of NR3 expression was detected between 1 hour after lights on and 6 hours after lights on (14:00). NR3 expression increased from 6 hours after lights on (14:00) to 6 hours after dark (2:00), although there was no significant difference between the expression at 6 hours after lights on (14:00) and 1 hour after dark (21:00). From 1 hour after dark (21:00) to 6 hours after dark (2:00), however, NR3 expression significantly increased 1.6-fold. Additionally, for *C. subsalsa* cultured in constant light, there was no significant difference in NR3 expression between any of two time points.

Comparing NR3 expression of *C. subsalsa* cultured in the light: dark cycle and constant light, the expression was higher during lights on (09:00 and 14:00) in cultures kept under constant light compared to those under 12:12 h light: dark cycle, although the difference was not significant, and lower during dark (07:00, 17:00 and 02:00), with significantly higher expression (by 2-fold) in the 12:12 h light: dark cycle cultures at 6 hours after dark (02:00) compared to constant light (p<0.05).
2.4.3 Diel activity of NR under different light conditions

When *C. subsalsa* was cultured in a light: dark cycle, there was no significant change of total NR activity from 1 hour before lights on (7:00) to 1 hour after lights on (9:00), or from 1 hour after lights on to 6 hours after lights on (14:00, Fig. 2.6, Table 2.3). From the mid-day to night, total NR activity was significantly 3 fold higher at 6 hours after lights on (14:00) than 1 hour after dark (21:00); and there was no significant difference of total NR activity between 1 hour after dark, and 6 hours after dark (2:00). Total NR activity in *C. subsalsa* cultured in the light: dark cycle was 5-fold higher at the highest time point, 6 hours after light (14:00), than its lowest point, 6 hours after dark (2:00). In contrast to the treatments cultured with a light: dark cycle, there was no significant difference of total NR activity between any of two time points in *C. subsalsa* cultured in constant light. In addition, total NR activity was significantly 2-fold higher in treatments cultured in the light: dark cycle at 6 hours after lights on (14:00), as well as 2 and 5 fold higher in the ones cultured in constant light at 1 hour after dark (21:00) and 6 hours after dark (2:00), respectively. There was no significant difference between total NR activities comparing treatments cultured in these light conditions at the other 2 time points (p>0.05).

For non-phosphorylated NR in *C. subsalsa* cultured in the light: dark cycle, there was no significant difference of its activity between any two time points except between 1 hour before lights on (7:00) and 1 hour after dark (21:00, p<0.05). (Fig. 2.6, Table 2.3) Here, non-phosphorylated NR activity was 3 fold higher at 1 hour before lights on than 1 hour after dark. The activity of non-phosphorylated NR in *C. subsalsa* cultured in the light: dark cycle was 2.8-fold higher at the highest two time points at 1 hour before lights on and 6 hours after lights on (7:00 and 14:00) than the lowest two points at 1 hour after dark and 6 hours after dark (21:00 and 2:00).
In contrast to *C. subsalsa* cultured in the light: dark cycle, there was no significant difference of non-phosphorylated NR activity between any two time points in treatments cultured with constant light (Fig. 2.6, Table 2.3). Comparing non-phosphorylated NR activity in treatments cultured under a light to dark cycle and constant light at each time point, there was significant difference between non-phosphorylated NR activity at 1 hour after dark (21:00), and the activity was 2.3-fold higher in treatments cultured in constant light compared to those cultured in the light:dark cycle (p<0.05). There was no significant difference in non-phosphorylated NR activity between treatments cultured in these light conditions between other time points (p>0.05).

Additionally, there was no significant difference between the activity of total NR and non-phosphorylated NR in each light condition at any time point, meaning that there was no evidence of regulation by 14-3-3 protein binding under diel light:dark or constant light conditions at the time points tested.

### 2.4.4 NR2 and NR3 expression during growth with different nitrogen sources

Cells in each culture were counted by microscopy on the day of sampling (Table B.1). Nutrients data were examined in each group (Table 2.2). The data exhibited different ratios of nitrate to ammonium compared to the ratios of these nitrogen sources added to the cultures in the experiments. Interestingly, in the cultures with 100:0, 95:5, and 50:50 μM NO$_3^-$ : NH$_4^+$, the actual ratios of nitrate to ammonium were all lower than the ratios added to the cultures; while the actual ratios of nitrate to ammonium in the cultures added 5:95 and 0:100 μM NO$_3^-$ : NH$_4^+$ were higher than the ratios of nitrogen source added. What is more, the ammonium concentration in the 100:0 μM NO$_3^-$ : NH$_4^+$ group, as well as the nitrate concentration in the 0:100 μM
NO$_3^-$ : NH$_4^+$ were larger than 0, in spite of the fact that no ammonium or nitrate was added to the 100:0 and 0:100 µM NO$_3^-$ : NH$_4^+$ groups, respectively.

Expression of NR2 and NR3 were evaluated in treatments cultured with different ratios of NO$_3^-$ to NH$_4^+$: 100:0, 95:5, 50:50, 5:95, and 0:100 µM (Fig. 2.7). NR2 expression significantly increased with a decrease of NO$_3^-$ : NH$_4^+$ ratios from 100:0 to 50:50 µM (p<0.05). NR2 expression increased 2.5-fold from treatments cultured with 100:0 to 95:5 µM NO$_3^-$ : NH$_4^+$, as well as 11.7 and 4.6 fold between treatments cultured with 100:0 and 95:5 µM NO$_3^-$ : NH$_4^+$ and those cultured with 50:50 µM NO$_3^-$ : NH$_4^+$, respectively. Additionally, NR2 expression significantly decreased 9.7 and 6.5 fold from cultures in 50:50 µM NO$_3^-$ : NH$_4^+$ to the treatments cultured with 5:95 and 0:100 µM NO$_3^-$ : NH$_4^+$, respectively (p<0.05); and there was no significant difference in NR2 expression between the treatments cultured with 5:95 and 0:100 µM NO$_3^-$ : NH$_4^+$ (p>0.05).

The only significant difference of NR3 expression was found in treatments cultured with 50:50 µM NO$_3^-$ : NH$_4^+$ compared to each other group (p<0.05), but no significant difference among other groups (p>0.05) (Fig. 2.7). NR3 expression increased 9.1 and 6.3 fold from treatments cultured with 100:0 and 95:5 µM NO$_3^-$ : NH$_4^+$ those cultured with 50:50 µM NO$_3^-$ : NH$_4^+$, respectively. Additionally, NR3 expression was 6.3 and 4.2 fold higher in the 50:50 µM NO$_3^-$ : NH$_4^+$ cultures compared to those cultured with 5:95 and 0:100 µM NO$_3^-$ : NH$_4^+$, respectively. The expression of NR3 relative to NR2 was not significantly affected by nitrogen source (p>0.05).
2.4.5 NR activity during growth with different nitrogen sources

Only the treatments cultured with 100:0, 95:5 and 50:50 µM NO$_3^-$ : NH$_4^+$ exhibited NR activity (Fig. 2.8), while the ones cultured with 5:95 and 0:100 µM NO$_3^-$ : NH$_4^+$ were below detection. For the treatments that exhibited NR activity, the activity of total NR was significantly 3.3 and 23.3 fold higher in treatments cultured with 100:0 µM NO$_3^-$ : NH$_4^+$ than those cultured with 95:5 and 50:50 µM NO$_3^-$ : NH$_4^+$, respectively (p<0.05). There was no significant difference of total NR activity between cultures with 95:5 and 50:50 µM NO$_3^-$ : NH$_4^+$ (p>0.05).

The activity of non-phosphorylated NR was significantly 5.8 and 27.4 fold higher in the cultures treated with 100:0 µM NO$_3^-$ : NH$_4^+$ compared to those treated with 95:5 and 50:50 µM NO$_3^-$ : NH$_4^+$, respectively (p<0.05). There was no significant difference between activities of non-phosphorylated NR in cultures treated with these two different nitrogen ratios (p>0.05). NR activity was inhibited by Mg$^{2+}$ significantly (by 1.8 fold) in cultures treated with 95:5 µM NO$_3^-$ : NH$_4^+$ (p<0.05), but not in other groups (p>0.05), suggesting that 14-3-3 protein binding plays a role in regulating NR3 activity under different N-sources and ratios.

2.4.6 NR2 and NR3 expression when grown at different temperatures

The effect of temperature (18 °C, 25 °C, and 28 °C) on the expression of NR2 and NR3 was investigated (Fig. 2.9). Cells in each culture were counted by microscopy on the day of sampling (Table B.1). NR2 expression was significantly (3.0 and 2.6 fold) higher in the cultures treated in 18 °C than those acclimated to growth in 25° and 28 °C, respectively. Additionally, there was no significant difference in NR2 expression between C. subsalsal cultured in 25 ° and 28 °C (p>0.05). NR3 expression was not significantly affected by temperature (p>0.05). Furthermore, NR2 expression values
were significantly higher (10.2, 4.7, and 4.5 fold), than NR3 expression in treatments cultured at 18 °C, 25 °C, and 28 °C, respectively (p<0.05).

### 2.4.7 NR activity when growth at different temperatures

Total NR activity decreased with the increase of temperature from 18 °C to 28 °C. Total NR activity was significantly (2.2 fold) higher in cultures acclimated to 18 °C than those at 28 °C (p<0.05), although there was no significant difference between total NR activities in treatments cultured at 18 °C and 25 °C, or between 25 °C and 28 °C (p>0.05) (Fig. 2.10). There was no significant difference of non-phosphorylated NR activities between any of two temperatures (p>0.05). The addition of Mg$^{2+}$ significantly inhibited NR by 1.5-fold in cultures acclimated to 18 °C (p<0.05), but not at other two temperatures (p>0.05), suggesting that 14-3-3 protein binding may play a role in regulating NR3 activity at different temperatures.

### 2.5 Discussion

The amino acid sequence of NR3 in harmful alga *C. subsalsa* indicates this enzyme is distinct from CsNR2, as well as *Hs*NR2 and *Hs*NR1 identified from raphidophytes previously (Coyne 2010; Stewart and Coyne 2011). Sequence analysis shows that NR3 lacks the conserved heme-Fe domain (cytochrome b5) between the DI and FAD domains, although it was considered as one of the three (Heme-Fe, FAD, and Molybdenum cofactor) highly conserved functional domains in eukaryotic NRs (Sanz-Luque et al. 2015; Zhou and Kleinhofs 1996). PCR analysis of the fragment spanning the hinge 1 region to the beginning of FAD domain in NR3 of *C. subsalsa* was longer than the expected 372 bp length of the same region in NR3 of *C. subsalsa* sequenced by Marine Microbial Eukaryote Transcriptome Sequencing Project.
(MMETSP; Keeling et al. 2014), indicating the possibility of sequencing error. The length of the heme-Fe domain in NR2 of *C. subsalsa* (MMETSP0948_20120912_5896_1) and *H. akashiwo* (Accession: JN831656.1 GI: 355398356), as well as NR of two higher plants, *Arabidopsis thaliana* (Accession: NP_177899.1 GI:15218104) and *Camelina sativa* (Accession: XP_010428861.1 GI: 727505528) is approximately 225 bp. If the heme-Fe domain of the same size exists in NR3, then the length of the PCR fragment should be greater than 500 bp. However, in fact, the length of the PCR product spanning this region of the transcript was between 400 and 500 bp (Fig. 2.4), indicating the possibility that either a shorter functional heme-Fe domain exists in the NR3, or NR3 does not have a functional heme-Fe domain.

Eukaryotic NRs are members of sulfite oxidase family that belongs to mononuclear molybdenum enzymes (reviewed by Stolz and Basu 2002). SO catalyzes the oxidation of sulfite (SO$_3^{2-}$) to sulfate (SO$_4^{2-}$) (reviewed by Schwarz, Mendel, and Ribbe 2009). Similar to eukaryotic NRs, sulfite oxidase (SO) in animals contains three conserved domains, heme-Fe domain, molybdenum cofactor (Moco), and the dimerization domain (DI), sequentially from the N to C terminal (reviewed by Schrader et al. 2003). As a part of the electron transfer chain in both NR and animal SO, the heme-Fe domain either donates electrons to the molybdenum sites (NR) or accepts electrons from the molybdenum center (SO) (Schwarz, Mendel, and Ribbe 2009). Intriguingly, plant SO lacks the additional redox center, heme-Fe domain (Schrader et al. 2003), as does the deduced amino acid sequence of NR3. In animal SO, the reaction occurs at its Mo(VI) center, and the reducing equivalents are passed through the heme-Fe domain, where cytochrome c works as the physiological electron
acceptor (reviewed by Hänsch et al. 2007). In this process, this enzyme has multiple forms, including Mo(VI)/Fe(III) (the fully oxidized state), Mo(IV)/Fe(III), Mo(V)/Fe(II), and Mo(V)/Fe(III); and Fe is essential in this process to reoxidize Mo(IV) to Mo(V) (Feng, Tollin, and Enemark 2007). For plant SO, which lacks the heme-Fe domain, one-half equivalent of ferricyanide has to be added to this process in order to generate the Mo(V) form (Astashkin et al. 2005; Hemann et al. 2005).

The absence of heme-Fe domain in NR3 suggests the potential for a different electron transport pathway compared to other eukaryotic NRs, such that the tertiary structure of NR3 may make it possible for this enzyme to function without this domain. Alternatively, the absence of heme-Fe may indicate the requirement for an extraneous reductant to enable this enzyme’s catalytic process, similar to the requirement of extra ferricyanide for the function of plant SO. In addition, lacking of one of the redox centers may lead to a reduction in efficiency of NR3 compared to other NRs found in the same species. Overall, the ability of NR3 to function without the conserved heme-Fe domain requires more study of the functional domains and their roles in the catalytic activity of this enzyme. What is more, the significance of NR3 for the survival and evolution of C. subsalsa also becomes an interesting question.

The analysis of amino acids sequence of NR3 also revealed a putative 14-3-3 binding motif in the hinge 1 region (Fig. 2.3). This motif was considered to be highly conserved in plant NRs, but not in NRs of mosses nor algae (Lambeck et al. 2012; Sanz-Luque et al. 2015). The 14-3-3 proteins bind to the phosphorylated serine residue of plant NR and block the electron flow from its NADH domain at the C-terminus to the Mo-MPT domain at the N-terminus in order to inhibit NR activity under certain
conditions (Denison et al. 2011). Although 14-3-3 proteins also work to regulate nitrogen assimilation in the green alga *Chlamydomonas*, the regulatory mechanism is different since the targets for these 14-3-3 proteins are glutamine synthetase I instead of NR (reviewed by de Montaigu et al. 2010; Pozuelo et al., 2001). The binding of 14-3-3 proteins regulates widespread cellular processes in eukaryotes, including intracellular signaling, control of cell cycles, as well as the physiology of metabolism and ion channels (Carol Mackintosh 2004). The binding of these proteins often triggers events to promote cell survival, including protecting target proteins from dephosphorylation and proteolysis, avoiding metabolic imbalances in leaves due to sudden darkness, and promoting the survival of mammalian cells related to growth factors (Hutchins, Dikovskaya, and Clarke 2002; Cotelle et al. 2000; Carol Mackintosh 2004). In plants, nitrite produced by nitrate reduction is further reduced to ammonium in the chloroplast, and ammonium is then incorporated into amino acids (MacKintosh and Meek 2001). When photosynthesis is interrupted due to environmental stressors, such as sudden darkness, the chloroplast loses the ability to process nitrite due to depletion of ATP. To avoid the build-up of potentially toxic nitrite, signals are passed to the cytosol, where NR is phosphorylated, and 14-3-3 proteins bind to the phosphorylated site of NR to inhibit its activity (Denison et al. 2011a; Carol Mackintosh 2004).

In the study of the effect of light on NR gene expression in *C. subsalsa*, both NR2 and NR3 exhibited significant changes over time in the light to dark cycle but not in constant light, indicating that both NR2 and NR3 were regulated by light at the transcriptional level, but not by their endogenous diel rhythm (Fig. 2.5). In addition, NR2 expression was at its highest value at the onset of the light phase, and then
decreased through the day. In contrast, NR3 expression had the lowest value at the beginning of the light phase, and increased over time until mid-night. The changing pattern of NR2 expression is consistent with the regulation of NR gene expression by light in the diatom, *Thalassiosira pseudonana*; its NR gene transcript abundance also decreased through the light phase from the highest point at the beginning of the phase (Brown, Twing, and Robertson 2009). Similar to *C. subsalsa*, this diel cycle of NR gene expression in *T. pseudonana* was not present when the treatments were cultured under constant light (Brown, Twing, and Robertson 2009). Additionally, although *C. subsalsa* and *H. akashiwo* are both raphidophytes, NR gene expression in these species is differentially regulated: in *C. subsalsa*, expression patterns suggested it was not regulated by its endogenous diel rhythm, while preliminary experiments suggested that NR1 transcript abundance in *H. akashiwo* may be under the circadian control (Coyne 2010). Interestingly, both NR2 and NR3 in *C. subsalsa* had low expression in light especially at mid-day. This is opposite to the induced NR gene expression by light in plants, due to the higher supply of energy in light than dark for nitrate reduction and assimilation of ammonium to amino acids (Yanagisawa 2014). Overall, these results suggest that there may be multiple mechanisms regulating NR transcription within and among algal species, as well as alternative regulatory mechanisms of NRs in algae compared to in plants.

Results of this investigation also indicated that activity of NR was regulated by light but not in response to an endogenous diel rhythm (Fig. 2.6). In addition, the activity of total and non-phosphorylated NR had high values in light phase, and the lowest values in dark. This was also observed in red alga *Gracilaria chilensis*, whose NR activity was also regulated by light but not its endogenous rhythm, and had the
highest value in light and lowest value in the dark (Chow, De Oliveira, and Pedersén 2004). The similar rhythm of photosynthesis and nitrate reduction is consistent with the idea that nitrate reduction requires ATP and reduced ferredoxin generated by photosynthesis. At the same time, nitrate assimilation serves as a crucial sink for excess reductant produced by photosynthesis (Sanz-Luque et al. 2015; Denison et al. 2011a; Carol Mackintosh 2004; Chow, De Oliveira, and Pedersén 2004; Glibert et al. 2016). What is more, studies of NR activity in H. akashiwo also revealed reduced NR activity in dark (reviewed by Coyne 2010; Tomas 1979), indicating the mechanisms regulating NR may be shared by these two species at the activity level. Interestingly, NR in C. subsalsa had the largest activity in light following the peaks of NR expression in dark or early morning, indicating that NR transcript and enzyme levels may serve as down-regulating signals to each other to prevent NR from over expression or activation. This was also observed in red alga G. chilensis, as well as many plants, such as Nicotiana tabacum and Lycopersicon esculentum (Chow, De Oliveira, and Pedersén 2004; Galangau et al. 1988).

There was no evidence for NR inhibition by 14-3-3 protein binding at any time points during the light experiment (Fig. 2.6). This may suggest a distinct regulatory mechanism of this alga compared to plant NRs at the post-translational level, although they shared the similar 14-3-3 binding motif. Alternatively, inhibition of NRs by 14-3-3 proteins may have occurred very quickly after the change in light regime, introducing the possibility that the best time point for evaluating NR inhibition was missed in this experiment. In plants, NRs are rapidly activated and inactivated in responding to a change of light intensity and binding of 14-3-3 proteins, and this process is always completed within 1 hour (Nemie-Feyissa et al. 2013; Riens and
Heldt 1992; Lea et al. 2006). For instance, when *Selaginella kraussiana* was transferred from light to dark, its NR activity decreased 67% in 45 min, and increased rapidly when it was transferred back to light again (Nemie-Feyissa et al. 2013).

NR gene expression and activity in *C. subsalsa* were investigated in treatments cultured with different ratios of NO$_3^-$ to NH$_4^+$: 100:0, 95:5, 50:50, 5:95, and 0:100 µM (Fig. 2.7-2.8). Nutrients from each group were collected and analyzed (Table 2.2). The nutrients data exhibited different ratios of NO$_3^-$ to NH$_4^+$ in cultures compared to the ratios added to each group in the process of acclimation and before sample collection. It should be noted that the cultures were acclimated with each nitrogen ratio for more than 2 months, and the requirement and take-up rates of nitrate and ammonium by the alga in each condition was different. Therefore, it is not surprising that there was a difference between the ratios of nitrate to ammonium added and the ratios observed.

Interestingly, the observed nitrate to ammonium ratios were lower than the ratios added into the cultures with 100:0, 95:5, and 50:50 µM NO$_3^-$: NH$_4^+$, while the observed ratios were higher than the ratios added in other groups (5:95 and 0:100 µM NO$_3^-$: NH$_4^+$). This may indicate a difference in the preferred nitrogen source of these treatments; nitrate might be taken up more quickly than ammonium by the cultures grown with 100:0, 95:5, and 50:50 µM NO$_3^-$: NH$_4^+$, while ammonium might be preferred by the treatments cultured with 5:95 and 0:100 µM NO$_3^-$: NH$_4^+$. This is consistent with NR activity observed in these groups (Fig. 2.8), when nitrate was preferred, NR activity decreased with increase of ammonium concentration in cultures; while when ammonium concentration was high enough and became the preferred nitrogen source, NR activity was not observed even though nitrate existed in cultures.
The concentration of NH$_4^+$ was positive although no ammonium was added to the cultures grown with 100:0 µM NO$_3^-$:NH$_4^+$ (Table 2.2), indicating the ability to release excess ammonium to the medium by *C. subsalsa*. This was also observed in other algal species, the NH$_4^+$ release rates of nitrogen-replete diatoms *Chaetoceros* sp., *Skeletomena costatum* and *Thalassiosira weissflogii* for instance, could be as high as 84% of their nitrate take-up rates. What is more, nitrate was also found in the treatments cultured with 0:100 µM NO$_3^-$:NH$_4^+$. *C. subsalsa* was cultured in f/2 medium with nitrate as the sole nitrogen source before acclimated to the condition with ammonium as the only nitrogen source, therefore, the nitrate in the medium indicates that *C. subsalsa* may have an ability to store nitrate and release nitrate into the medium in the process of death and lysis of cells. The storage of nitrate was well known as a strategy for benthic microalgae assemblage and diatoms to survive dark and anoxic conditions (Kamp et al. 2011; García-Robledo et al. 2010). Rather intriguingly, in a recent research, *H. akashiwo* was also found to have the ability to store nitrogen nutrients, including nitrate, ammonium, and organic nitrogen; the authors suggested it may be important for *H. akashiwo* to accumulate intracellular nitrogen to sustain their cell productivity (Wai et al. 2015).

NR gene expression is induced by the presence of nitrate, and strongly repressed by ammonium in varieties of algal species, such as *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, and *Cyanidioschyzon merolae* (Loppes et al., 1999; Cannons and Shiflett, 2001; Llamas et al., 2002; Imamura et al., 2010, reviewed by Sanz-Luque et al. 2015). In this study, NR2 and NR3 genes in *C. subsalsa* were constitutively expressed under all nitrogen conditions tested, even when ammonium was the sole nitrogen source (Fig. 2.7). This is consistent with the effect of ammonium
on NR1 expression in *H. akashiwo*, which was constitutively expressed even in the medium with ammonium as the only nitrogen source and during nitrogen starvation (Coyne 2010). In contrast, NR gene expression in some algal species, *Cylindrotheca fusiformis* and *Dunaliella tertiolecta* for instance, is fully inhibited by ammonium, even in the presence of nitrate in the medium (Poulsen and Kröger 2005; Song and Ward 2004). Interestingly, NR2 and NR3 expression increased significantly with the increase of ammonium concentration in the medium, from nitrate to ammonium ratios of 100:0 to 50:50 µM, and then decreased significantly with nitrate to ammonium ratios of 5:95 and 0:100 µM. This suggests that NR expression in *C. subsalsa* was not strictly inhibited by the presence of ammonium, but regulated quantitatively by the nitrate to ammonium balance. This was also found in studies related to NR expression in *Chlamydomonas*; its NR gene was expressed in the presence of ammonium, and the transcript abundance changed with the shift of nitrate to ammonium balance, although NR gene expression in *Chlamydomonas* was induced when the nitrate to ammonium balance shifted toward nitrate (Llamas, Igeno, et al. 2002; de Montaigu et al. 2011; Sanz-Luque et al. 2015). NR gene expression in *C. subsalsa* was induced by a small amount of ammonium, and the inhibition of NR gene by ammonium initiated when the nitrate to ammonium balance passed the point of 50:50 µM, which might be the break point of nitrate to ammonium ratios that leads to induce or inhibit NR expression in this alga. To be noted, the samples in different nitrogen groups were collected on different days after acclimation, and cell densities in cultures were different, so that these may also contribute to the difference in NR expression among groups.

The activity of total NR and non-phosphorylated NR in *C. subsalsa* was repressed by ammonium quantitatively, and fully inhibited by higher concentrations of
ammonium, at nitrate to ammonium ratios of 5:95 and 0:100 µM (Fig. 2.8). In algal species, *Chlamydomonas reinhardtii*, the full inactivation of NR activity by high concentrations of ammonium, as well as its induction with the shifting of nitrate to ammonium balance toward nitrate, has been well established for at least thirty years (Sanz-Luque et al. 2015 and reference therein). This is in agreement with the hypothesis that algal cells generally are not able to transport nitrate unless the supply of ammonium is not sufficient for their high internal N needs, due to the less energy cost by utilizing ammonium directly (reviewed by Sanz-Luque et al. 2015). In this study, an uncoupling between NR activity and its expression in *C. subsalsa* was also observed (Fig. 2.7-2.8). In treatments cultured with nitrate to ammonium ratios of 5:95 and 0:100 µM, NR2 and NR3 were still expressed while NR activity was not detected. This has also been observed in other algal species. For example, de Montaigu et al. (2010) reported NR activity in *Chlamydomonas* was fully inhibited by high concentration of ammonium (2mM NH$_4^+$ in this study), while the NR gene was still highly expressed under the same condition.

Despite the well-established post-translational control of algal NR by nitrogen source, the mechanisms involved remain unclear (reviewed by Sanz-Luque et al. 2015). NR in *C. subsalsa* was significantly inhibited when Mg$^{2+}$ was added to treatments cultured with nitrate to ammonium of 95:5 µM, indicating the potential binding of 14-3-3 proteins to the phosphorylated site of this NR, as well as inhibition of this enzyme. In *Arabidopsis*, phosphorylation levels of NR were higher in cultures supplied with ammonium than with nitrate, when NR activity of cultures with ammonium was low; and the phosphorylation levels of NR decreased with increasing duration after resupplied N-starved cultures with nitrate (Engelsberger and Schulze
2012). Altogether, results imply that the reversible phosphorylation and the sequential binding of 14-3-3 proteins may play a role in regulation of NR by nitrogen source in this *C. subsalsa*; and the inhibition of nitrate reduction by 14-3-3 proteins may fall within a narrow range of ammonium concentrations that are enough to induce NR phosphorylation but not sufficient to inhibit total NR activity altogether. As mentioned above, the timing of sampling may have also affected the ability to detect inhibition due to 14-3-3 binding (Engelsberger and Schulze 2012).

Analysis of NR2 transcript abundance in response to temperatures ranging from 18 °C to 28 °C revealed a higher level of NR2 gene expression in colder temperatures (Fig. 2.9). This is in agreement with several other studies about algal NR expression related to temperatures. *Thalassiosira pseudonana*, for instance, had higher NR expression in treatments cultured under 12 °C than 22 °C (Parker and Armbrust 2005). This may be due to the energy dissipation capability by nitrate reduction under high light, low temperature condition (Lomas and Glibert 1999a,b, Parker and Armbrust 2005, reviewed by Brown, Twing, and Robertson 2009). In contrast, NR3 expression was not regulated by temperature (Fig. 2.9). Interestingly, this is consistent with the expression of NR1 in *H. akashiwo*; its expression was also not significantly affected by the temperatures ranging from 18 °C to 28 °C (Coyne 2010).

Analysis of NR activity in *C. subsalsa* revealed that total NR activity was highest at low temperature, while non-phosphorylated NR activity was not significantly affected by temperature (Fig. 2.10). This observation is different from the results of studies related to NR activity and temperatures in green algae. NR activities in two strains of *Stichococcus*, HJ-10 and NJ-17, for instance, also increased with increasing temperature from 5 °C to 20 °C, but were inhibited by increasing temperature
from 20 ° to 40 °C (Chen, He, and Hu 2012). NR activity in *C. subsalsa* was significantly inhibited by Mg\(^{2+}\) at 18 °C (Fig. 2.10), suggesting the post-translational regulation of NR by 14-3-3 proteins under different temperatures. The regulation of NR by temperature and 14-3-3 binding proteins remains unknown. However, studies suggest that temperature may be a stressor that affects the regulation of hormone signaling pathways in plants, such as abscisic acid signaling pathway (ABA), through 14-3-3 proteins (reviewed by Denison et al. 2011).

In conclusion, this work reveals the presence of a novel nitrate reductase, NR3, in *C. subsalsa*. The sequence of this enzyme indicates the existence of a 14-3-3 binding motif in the hinge 1 region of this enzyme. The analysis of NR3 sequence also indicates that a conserved domain, the heme-Fe (cytochrome b5) domain, may not be present in this enzyme. The absence of the heme-Fe domain may lead to a different electron transport pathway in NR3 compared to other NRs, or a reduction of efficiency of this enzyme. What is more, the research exhibits different responses to environmental factors by NRs in *C. subsalsa* at the transcriptional and translational levels. Results indicate that both NR2 and NR3 were regulated by nitrogen and light, while NR2 was also regulated by temperature, at the transcriptional level. At the translational level, evidence was found that NRs in *C. subsalsa* were regulated by nitrogen source and temperature through the reversible phosphorylation and sequential binding of 14-3-3 proteins, while the regulation of NRs by light may through other mechanisms. To date, this is the first report of the presence of a 14-3-3 binding motif in algal NRs, as well as the regulation of algal NRs by the reversible phosphorylation and putative 14-3-3 binding proteins.
Table 2.1. Sequence of *C. subsalsa*- specific primers for nitrate reductase [NR2 (CsNR Glob) and NR3 (CsNR3)], and Actin (Cs Actin). F, forward primer; R, reverse primer.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Gene Targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsNR Glob 1005F</td>
<td>GAAGCTGCAATCCAAAGCTGTA</td>
<td>NR2</td>
</tr>
<tr>
<td>CsNR Glob 1240R</td>
<td>CCAACTCAGTCAACGTGTTCA</td>
<td>NR2</td>
</tr>
<tr>
<td>CsNR3 1143F</td>
<td>CTGGACACACGCTGAGTTACA</td>
<td>NR3</td>
</tr>
<tr>
<td>CsNR3 1228R</td>
<td>GCAATGCGATGAGTTGGAG</td>
<td>NR3</td>
</tr>
<tr>
<td>CsNR3 1515R</td>
<td>CTGTAGTGCAAATCGCAGTC</td>
<td>NR3</td>
</tr>
<tr>
<td>Cs Actin163F</td>
<td>GTGGGAGATGAGGCAC</td>
<td>Actin</td>
</tr>
<tr>
<td>Cs Actin 294R</td>
<td>TGCCACTCGAAGCTCAT</td>
<td>Actin</td>
</tr>
</tbody>
</table>
Table 2.2 Nitrate and ammonium concentration in each group of nitrate to ammonium ratios ($\text{NO}_3^{-}:\text{NH}_4^+$, 100:0, 95:5, 50:50, 5:95, and 0:100 µM). Asterisk indicates the groups that the actual ratios of nitrate to ammonium was lower than the ratios of nitrogen source added.

<table>
<thead>
<tr>
<th>Group (NO₃⁻:NH₄⁺, µM)</th>
<th>Concentration of NO₃⁻ (µM)</th>
<th>Concentration of NH₄⁺ (µM)</th>
<th>Actual ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>*100:0</td>
<td>7.46</td>
<td>6.87</td>
<td>1:1</td>
</tr>
<tr>
<td>*95:5</td>
<td>108.53</td>
<td>28.47</td>
<td>4:1 (20:5)</td>
</tr>
<tr>
<td>*50:50</td>
<td>66.60</td>
<td>117.69</td>
<td>1:2 (25:50)</td>
</tr>
<tr>
<td>5:95</td>
<td>21.81</td>
<td>206.62</td>
<td>1:9 (5:45)</td>
</tr>
<tr>
<td>0:100</td>
<td>6.56</td>
<td>236.95</td>
<td>1:36</td>
</tr>
</tbody>
</table>
Table 2.3. p values generated by paired t-test to compare the difference of expression or activity of NR in *C. subsalsa* at adjacent time points under a light to dark cycle. According to repeated measurements ANOVA, there was no significant difference of NR expression or activity between any pair of time points when treatments were cultured under constant light (not shown in table). T1, 1 hour before lights on; T2, 1 hour after lights on; T3, 6 hours after lights on; T4, 1 hour after dark; T5, 6 hours after dark. The bold and italic numbers are the p values that are less than 0.05, indicating the significance.

<table>
<thead>
<tr>
<th>Pair of Time</th>
<th>NR Expression</th>
<th>NR Activity</th>
<th>non-phosphorylated NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2</td>
<td>NR3</td>
<td>Total NR</td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>0.04602</td>
<td><strong>0.008054</strong></td>
<td>0.1381</td>
</tr>
<tr>
<td>T2-T3</td>
<td><strong>0.03154</strong></td>
<td>0.8494</td>
<td>0.08915</td>
</tr>
<tr>
<td>T3-T4</td>
<td>0.7728</td>
<td>0.2018</td>
<td><strong>0.009553</strong></td>
</tr>
<tr>
<td>T4-T5</td>
<td>0.1484</td>
<td><strong>0.02794</strong></td>
<td>0.3555</td>
</tr>
</tbody>
</table>
Figure 2.1. Evaluating of activity of total NR and non-phosphorylated NR. (a) 14-3-3 requires Mg$^{2+}$ to bind to phosphorylated serine residue of NR (P-S), and inhibits NR activity by blocking the electron flow, so that only activity of non-phosphorylated NR can be detected under this condition; (b) Adding EDTA chelates Mg$^{2+}$ and prevents 14-3-3 binding, so that the activity of total (phosphorylated and non-phosphorylated) NR can be measured.
© Step 1: Nitrate converted to Nitrite catalyzed by NR

![Diagram of Step 1]

© Step 2: Measure the concentration of nitrite produced by step 1.

![Diagram of Step 2]

Figure 2.2. Assay to measure the activity of total NR by adding EDTA or non-phosphorylated NR by adding excess Mg$^{2+}$ to the cell homogenates. Nitrate was converted to nitrite catalyzed by NR in the first step; a chromogenic reaction was conducted in the second step to measure the concentration of nitrite produced in the first step.
Figure 2.3. The 14-3-3 binding motif in hinge 1 region of NR3 and adjacent regions were aligned with the same regions of NR2 for *C. subsalsa* and *H. akashiwo*, as well as NR in two higher plants, *Arabidopsis thaliana* and *Camelina sativa*. The underlined sequences indicate the motif containing the serine phosphorylation site for 14-3-3 protein binding in NR3 and NR of higher plants. The identical residues in all sequences are marked by “*”, the highly and weakly conserved ones are marked by “:” and “.”, respectively (Chenna 2003; Larkin et al. 2007).
Figure 2.4. PCR amplification of cDNA of NR3 in *C. subsalsal*, spanning the region from hinge 1 to the beginning of FAD domain of this enzyme. Lane 1, DNA marker; Lane 2 and 3, amplification of two cDNA samples of NR3 in *C. subsalsal*. 
Figure 2.5. Expression of NR2, and NR3 in \textit{C. subsalsa} cultured in a 12:12h light:dark cycle (dashed lines, L/D) and constant light (solid lines, L). The portion with shade indicates the time that was in dark for algae cultured with the light: dark cycle. Asterisk (*) reveals the significant difference in NR expression between the treatments cultured in constant light and the light: dark cycle at the indicated time point. Statistical differences within a treatment are presented in Table 2. Error bars are standard deviation of three biological replicates.
Figure 2.6. Activity (pmol nitrite/µg protein/min) of total NR (non-phosphorylated and phosphorylated NR) and non-phosphorylated NR in *C. subsalsa* cultured in a 12:12h light: dark cycle (dashed lines, L/D) and constant light (solid lines, L). The portion with shade indicates the time that was in dark for algae cultured with the light: dark cycle. Asterisk (*) reveals the significant difference of NR activity between the treatment cultured in constant light and the light: dark cycle at the indicated time point. Statistical differences within a treatment are presented in Table 2. Error bars are standard deviation of three biological replicates.
Figure 2.7. NR2 and NR3 expression in response to different NO$_3^-$: NH$_4^-$ ratios: 100:0, 95:5, 50:50, 5:95, and 0:100 µM. Different letters above the bars indicate significant differences of NR expression in treatments cultured with different NO$_3^-$: NH$_4^-$ ratios (p<0.05). Error bars are standard deviation of three biological replicates.
Figure 2.8. NR activity (pmol nitrate/µg protein/min) of total NR (non-phosphorylated and phosphorylated NR), non-phosphorylated NR, and phosphorylated NR in response to different NO$_3$\(^+\):NH$_4$\(^-\) ratios: 100:0, 95:5, and 50:50 µM. No NR activity was detected in NO$_3$\(^+\):NH$_4$\(^-\) ratios of 5:95 and 0:100 groups, and activity of phosphorylated NR was not significant in NO$_3$\(^+\):NH$_4$\(^-\) ratios of 100:0, 50:50 µM, or other three groups (not shown in figure). Different letters above the bars indicate significant differences of NR activity in treatments cultured with different NO$_3$\(^+\):NH$_4$\(^-\) ratios (p<0.05). Error bars are standard deviation of three biological replicates.
Figure 2.9. NR2 and NR3 expression at different temperatures: 18°C, 25°C, and 28°C. Different letters above the bars indicate significant differences of NR expression in treatments cultured at different temperatures (p<0.05). Error bars are standard deviation of three biological replicates.
Figure 2.10. Activity (pmol nitrite/ µg protein/ min) of total NR (non-phosphorylated and phosphorylated NR), non-phosphorylated NR, and phosphorylated NR in response to different temperatures: 18 °C, 25 °C, and 28 °C. The activity of phosphorylated NR was not significant in treatments cultured at 25 °C, and 28 °C (not shown in figure). Different letters above the bars indicate significant differences of NR activity in treatments cultured at different temperatures (p<0.05). Error bars are standard deviation of three biological replicates.
Chapter 3

EFFECTS OF NITROGEN SOURCE ON NITRATE REDUCTASE EXPRESSION IN CHATTONELLA SUBSALSA AND IMPLICATIONS FOR COMPETITIVE DYNAMICS IN MARINE ENVIRONMENTS: A MESOCOSM STUDY

3.1 Abstract

*Chattonella subsalsa* is a harmful alga that has been associated with fish mortality events around the world. In recent years, mixed blooms of *C. subsalsa* and other algal species have been observed in Delaware’s Inland Bays. The reason for the persistence of these blooms and these species’ ability to avoid competitive exclusion remain a puzzle. Nitrate is one of the dominant nitrogen sources in Delaware Inland Bays, and the ability of *C. subsalsa* to compete for nitrate may play a part for the survival of this alga. For organisms to use nitrate as a nitrogen source, nitrate reductase catalyzes the first and also rate limiting step in nitrogen assimilation.

Previous work identified two novel nitrate reductase enzymes in this alga, NR2-2/2HbN (NR2), which has a 2/2 hemoglobin domain inserted in its hinge 2 region; and NR3, which has a putative 14-3-3 binding motif in its hinge 1 region. In this study, mesocosm experiments were conducted in which a single or multiple pulses of nitrogen (nitrate, ammonium or both) were added to the natural community during a *C. subsalsa* bloom. The effect of nitrogen source on the growth rate of *C. subsalsa* and the entire assemblage was investigated. NR2 and NR3 expression in respond to different nitrogen sources were also measured, in order to identify the contribution of NR expression to the competitive dynamics of *C. subsalsa* in the environment. The
potential role of phosphate addition in nitrogen assimilation and NR expression was also discussed. Results showed that *C. subsalsa* exhibited no nitrogenous preference, while growth rates of the entire assemblage were higher with ammonium as a nitrogen source. The results of this study indicate that *C. subsalsa* was able to out-compete other species in the assemblage when nitrogen concentrations were low. Evidence for surge uptake of nitrogen was also found in this alga, which may benefit *C. subsalsa* in the environment with low nitrogen concentration. NR expression in *C. subsalsa* under different nitrogen conditions implies that expression of NR2 may benefit this alga in environments with a high concentration of nitrate, and NR3 may benefit this alga in a more dynamic and unfavorable environment where ammonium was the dominant nitrogen source.

### 3.2 Introduction

Raphidophycean species produce a range of bioactive compounds, including reactive oxygen species (ROS), nitric oxide (NO), free fatty acids (FFA), brevetoxin/brevetoxin-like compounds (PbTx-like), hemolytic compounds, and other unidentified toxins that are harmful to fish, shellfish, marine mammals and even human beings (Khan et al. 1996; Bourdelais et al. 2002; Hiroishi et al. 2005; Kim, Yamaguchi, and Oda 2006; Kim et al. 2008; Marshall et al. 2002; Marshall et al. 2003; Fu et al. 2004; Kuroda et al. 2005; Shen et al. 2010; Astuya et al. 2015; Tanaka, Muto, and Shimada 1994; Okaichi 1980; Shimada et al. 1983; Onoue and Nozawa 1989; Endo et al. 1992; Keppler et al. 2006; Ishimatsu et al. 1996; Marshall et al. 2005). The harmful algal blooms dominated by these species have caused massive fish kills and severe economic damages to fishery industries in around the world, including Japan, Korea, China, India, USA, and Australia (Kim et al. 2007; Tseng et al. 1993; Jugnu and Kripa

In Delaware Inland Bays (DIB), the raphidophyte *Chattonella subsalsa* forms mixed blooms with other raphidophytes, including *Chattonella cf. verruculosa*, *Heterosigma akashiwo*, and *Fibrocapsa japonica* (Zhang et al. 2006). The reason behind the persistence of these blooms and these species’ ability to avoid competitive exclusion remains a puzzle (Zhang et al. 2006). Previous research related to *C. subsalsa* isolated from DIB demonstrated that this species is able to use multiple different nitrogen sources, including nitrate, ammonium, and glutamic acid, with a preference for ammonium; and they are able to grow over a wide range of temperature and salinities (Zhang et al. 2006). In general, nitrate enrichment is one of the most common and important factors affecting phytoplankton growth and bloom initiation, especially for shallow and greatly eutrophied ecosystems such as the DIB (reviewed by Coyne 2010).

For phytoplankton to use nitrate as a nitrogen source, nitrate is reduced to ammonium by two sequential reactions, the reduction of nitrate to nitrite, followed by reduction of nitrite to ammonium. In this process, nitrate reductase (NR) catalyzes the first and also the rate-limiting step (Flores et al. 2005). NR is often regulated by nitrogen source, light, and temperature at the transcriptional level in plants, algae, and fungi (Tischner 2000; Christensen et al., 2004; Coyne, 2010; Falcao et al., 2010; Krouk et al., 2010; de Montaigu et al., 2010; Konishi and Yanagisawa, 2011; Nemie-Feyissa et al. 2013; Sanz-Luque et al. 2015; Glibert et al. 2016). Investigations conducted related to NR gene expression in algae revealed different patterns of gene
expression among species, which indicated the complexity of the regulatory mechanisms (Parker and Armbrust 2005; Poulsen and Kröger 2005; Brown, Twing, and Robertson 2009; Prieto et al. 1996; Giordano, Chen, and Koblizek 2005; Fernandez and Galvan 2008; Cannons and Shiflett 2001; Song and Ward 2004; Coyne 2010; Imamura et al. 2010; Sanz-Luque et al. 2015; Glibert et al. 2016)). Additionally, the regulation of NR gene expression is even different within the same species. For instance, membrane-bound and the cytosolic forms of NR in *Thalassiosira weisflogi* responded differentially to ammonium (reviewed by Glibert et al. 2016; Jones and Morel 1988). Previous research investigating transcriptional regulation of CsNR2-2/2HbN (NR2) and NR3 in *C. subsalsa* indicates that NR2 gene expression was regulated by light, nitrogen source, and temperature; while NR3 expression was only regulated by light and nitrogen source (Chapter 2).

To date, no research has attempted to compare the impact of nitrogen source on the expression of homologous NR inside one species during nitrogen-stimulated blooms, or investigated the implications of NR regulation to this species’ competitive dynamic in the marine environment. In this research, I conducted two mesocosm experiments using water samples collected from DIB during *C. subsalsa* blooms in July and September, 2015. I stimulated the natural blooms by a single or multiple pulses of different nitrogen sources (nitrate, ammonium, or both); measured NR2 and NR3 gene expression in *C. subsalsa* under each condition; and investigated the growth rate of *C. subsalsa* and the whole assemblages of phytoplankton during the blooms under each condition to study relationships between NR transcriptional regulation and the success of this species during natural blooms.
3.3 Material and Methods

3.3.1 Field sampling and mesocosm preparation

Water samples were collected in July and September from IR32 in Inland River Bay (38°34’14.5’’N, 75°05’04.2’’W) during blooms of *C. subsalsa*. Temperature and salinity for each sample were measured.

Water samples were transported back to the lab and divided into 1 L polyethylene bottles for 4 treatments with 4 replicates in each treatment, 700 ml samples in each bottle, no nutrients other than nitrogen (in both experiments) and phosphate (only in the second experiment, designated Me-multiple) were added (Fig. 3.1). In both of the experiments, nutrients were amended as follows: 100 µM nitrate (the NO3 group), 100 µM ammonium (the NH4 group), or 50 µM nitrate and 50 µM ammonium (the BOTH group). Nutrients were dissolved in milli-Q water, and added as 1 ‰ of the final volume to each bottle. Control samples did not receive additional nitrogen (the No addition group). In the first experiment (designated Me-single), no nutrients were added except the first nitrogen pulse after taking samples at the initial time point (T0). In the second experiment (Me-multiple), f/2 concentrations of phosphate (36.2 µM) was added to all groups in addition to the nitrogen between T0 and T1hr. An additional pulse of nutrients at the same concentrations was added to each treatment in Me-multiple at 24 hours after setting the experiments (T24hr).

Dissolved nutrients (ammonium, nitrate, and phosphate) were collected and analyzed using a segmented-flow autoanalyzer (Seal Analytical, Mequon, WI) at T0 and T24hr for Me-single and at T0, T1hr, T24hr and T48hr for Me-multiple. Chlorophyll *a* was collected at those same time points and extracted with 90% acetone in the dark overnight, and measured fluorometrically (Welschmeyer 1994). Growth
rate ($\mu$) of the entire community was calculated as follows (N1 and N2 are chlorophyll fluorescence of the whole community at times T1 and T2, Guillard, 1973):

$$\mu = \frac{\ln(N2/N1)}{T2-T1}$$

The cell density of *C. subsalsa* was determined by microscopy, and the growth rate of *C. subsalsa* was also calculated as above, where N1 and N2 are cell densities of *C. subsalsa* at times T1 and T2.

Fifty milliliters of each sample was gently filtered through 3µm polycarbonate filters for RNA extraction at T0, T1hr, and T24hr for Me-single, and at T0, T1hr, T24hr, and T48hr for Me-multiple.

### 3.3.2 Analysis of transcript abundance

#### 3.3.2.1 RNA extraction

RNA was extracted from filtered cells using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). RNA concentration was measured by UV-Vis spectrophotometer (NanoDrop Technologies). The integrity was evaluated by electrophoresis before cDNA synthesis.

#### 3.3.2.2 Complementary DNA (cDNA) synthesis

Contaminating DNA was digested and cDNA was synthesized as in Chapter 2.

#### 3.3.2.3 Determination of NR expression

Expression of NR2, NR3, and actin (as a normalizing gene) were determined by quantitative real-time PCR (qPCR) using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). Diluted cDNA was used as template in triplicate 10 µL
reactions as described in Chapter 2. The sequence of primers used in this research was shown in Table 3.1.

3.3.3 **Statistical Analysis**

In both experiments, one-way ANOVA was used to test if different nitrogen source had a significant effect on NR expression. If the effect was significant, then Tukey’s HSD test was used to analyze the difference between values of all possible pairs of groups (Tukey 1949). Paired t-test was used to analyze the difference between NR2 and NR3 expression at each time point in each experiment. Additionally, paired t-test was also performed to compare NR expression at different time points in the same groups. All statistical analyses were performed in R (V.3.2.4 Revised, R Core Team 2016).

3.4 **Results**

3.4.1 **Growth rate of C. subsalsa and the entire community**

A mixed bloom consisting of *C. subsalsa*, *H. akashiwo*, *Chloromorum toxicum*, *Gyrodinium instriatum*, *Heterocapsa rotundata*, *Peridinium sp.*, *Prorocentrum triestinum*, *Protoperidinium quinquecorne* and *Karlodinium veneficum* (dominated by *C. subsalsa* and *C. toxicum*) was observed 24 hours prior to collection of samples used for Me-single (Whereat 2015). On the day of collection (T0), the cell density of *C. subsalsa* was 4.3x10^6 cells L^-1. At T24hr, cell densities were 6.08 x10^6, 7.0 x10^6, 6.5 x10^6, and 5.6 x10^6 cells L^-1 in the NO3, NH4, BOTH and No addition group, respectively, showing positive growth for all treatments.

Nutrients were collected and analyzed for Me-single at T0 and T24hr. A hypothetical time point T0+ was used to represent concentrations of nutrients after
addition of nitrogen to samples in Figure 3.2 and Table 3.2. Nitrate and ammonium added to each group were taken up rapidly as noted by comparing concentrations between T0+ and T24hr (Fig. 3.2). Nitrate concentration was 6 fold higher in the NO3 treatment at T0+ than T24hr, while the ammonium concentration was 13 fold higher in the NH4 treatment comparing the same time points. Nitrate and ammonium concentrations were 9 and 11 fold higher at T0+ than at T24hr in the BOTH group. The rapid utilization of phosphate was also observed as phosphate concentrations were 2-25 fold higher in each group at T0 than at T24hr.

In Me-single, C. subsalsa had a positive growth rate over the 24 hours’ experiment in all groups. The growth rate of the entire community, as measured by changes in chlorophyll a, was positive for nitrogen-addition treatments but negative in the No addition group (Fig. 3.3, Table 3.3). There was no significant difference in C. subsalsa growth rate between treatments. In addition, there was no significant difference between the growth rate of C. subsalsa and the entire community in the NO3 group, while the growth rate of the entire community was significantly 1.5 and 1.7 fold higher than the growth rate of C. subsalsa in the NH4 and BOTH group, respectively. In contrast, the growth rate of the entire community was significantly lower than the growth of C. subsalsa in the No addition group, with negative growth of the entire community compared to positive growth of C. subsalsa. The growth rates of the entire community in the NH4 and BOTH treatments were significantly 2 fold higher than in the NO3 treatment, and the growth rate of the entire community in the No addition group was significantly lower than each of the other three treatments.

A mixed bloom consisting of C. subsalsa, H. akashiwo, C. toxicum, K. veneficum, and Kryptoperidinium foliaceum (dominated by C. subsalsa, C. toxicum, K.
*veneficum,* and *K. foliaceum*) was observed 24 hours prior to sampling for Me-
multiple (Whereat 2015). On the day of collection, the cell density of *C. subsalsa* was
6.23x10^6 cells L^-1 at T0. Consistent with Me-single, cell densities increased to 8.20 x10^6, 9.21 x10^6, 8.03 x10^6, and 6.45 x10^6 cells L^-1 in the NO3, NH4, BOTH and No
addition treatments, respectively, at T24hr; and 9.39 x10^6, 1.02 x10^7, 8.65 x10^6, and
7.85 x10^6 in the NO3, NH4, BOTH and No addition treatments, respectively, at T48hr.

Nutrients data for Me-multiple are shown in Fig. 3.4 and Table 3.2 for T0,
T1hr, T24hr and T48hr, with hypothetical T0+ and T24+ concentrations representing
the nutrient concentrations after addition. The rapid utilization of nutrients was also
observed in this experiment. Nitrate and ammonium concentrations in the nitrogen
added groups at T0+ was 1.3 to 2.3 fold higher than at T1hr, while their phosphate
concentration was 37 to 44 fold higher at T0+ than T1hr. The rapid drop down of
nutrients was also observed in the No addition group from T0+ to T1hr. Here, nitrate,
ammonium, and phosphate concentrations were 14, 3, and 51 fold higher in this group
at T0+ than T1hr. What is more, nitrate and ammonium concentrations were 1.6 to 14
fold higher, respectively, at T0+ than T24hr, and phosphate concentrations were 37 to
86 fold higher at T0+ compared at T24hr. Comparing T24+ and T48hr, the
concentration of phosphate did not change dramatically as compared to T0+ and T1hr,
or T0+ and T24hr; it was 1.7 to 4.1 fold higher at T24hr+ than T48hr in each group.
Nitrate concentration was 3.2 fold higher in the NO3 treatment, while ammonium
concentration was 23 fold higher in the NH4 group; nitrate and ammonium
concentrations were 3.4 and 11.6 fold higher, respectively, in the BOTH group, at
T24hr+ than T48hr.
Similar to Me-single, the growth rate of *C. subsalsa* was positive in each treatment group at all time points in Me-multiple. The growth rate of the entire community was positive except for the No addition group, which was negative at each time period (Fig. 3.5, Table 3.3). From T0 to T24hr, there was no significant difference between the growth rate of *C. subsalsa* in the NO3, BOTH, and NH4 group; while the growth rates of *C. subsalsa* in nutrient amended treatments were significantly higher than in the No addition group. In the same time period, the growth rate of the entire community in the NH4 and BOTH group was significantly 2 fold higher than it was in the NO3 group; and the growth rate of the entire community in the No addition group was significantly lower than in the other three groups. From T24hr to T48hr, there was no significant difference in growth rate of *C. subsalsa* among treatment groups. In the same time period, the growth rate of the entire community was not significantly different among the NO3, NH4, and BOTH treatment groups; while the growth rate of the entire community was, again, significantly lower in the No addition group than in other three groups.

Comparing the growth rate of *C. subsalsa* and the entire community in each group, there was no significant difference between them in the NO3 and NH4 treatment from T0 to T24hr (Fig. 3.5, Table 3.3). However, the growth rate of the entire community was significantly 1.8 fold higher than *C. subsalsa* in the BOTH group, and significantly lower than *C. subsalsa* in the No addition group. From T24hr to T48hr, the only significant difference between the growth rate of the entire community and *C. subsalsa* was exhibited in the NO3 treatment, where the growth rate of the entire community was 2.2 fold higher than that of *C. subsalsa*. Over time, there was no significant difference in the growth rate of the entire community.
comparing T0 to T24hr time period to the T24hr to T48hr time period. The growth rate of *C. subsalsa* in the BOTH group decreased significantly from T0 to T24hr compared to T24hr to T48hr, while the growth rate of *C. subsalsa* in the No addition group was significantly 4.5 fold higher in the T24hr to T48hr time period compared to growth during the T0 to T24hr period.

### 3.4.2 NR2 and NR3 expression in the mesocosm experiments

In Me-single, samples were taken at T0, T1hr and T24hr to measure NR2 and NR3 expression (Fig. 3.6, Table 3.4). From T0 to T1hr, NR2 expression significantly increased 5-fold in the NO3 treatment, while it decreased significantly in the NH4 group and the BOTH treatment. There was no significance difference in NR2 expression at T1hr compared to T0 in the No addition group. After the initial increase, NR2 expression at T1hr significantly decreased in the NO3 treatment as well as No addition group. In contrast, NR2 expression significantly increased 17 fold in the BOTH treatment while there was no significance difference in NR2 expression in the NH4 group. Comparing NR2 expression in each group at T1hr, NR2 expression in the NO3 group was significantly higher (4- to 34-fold higher) than the other three treatment groups, while there was no significant difference between NR2 expression in the NH4, BOTH, and No addition groups. At T24hr, NR2 expression was significantly (4- to 13-fold higher) in the NO3 and BOTH group than it in the NH4 and No addition groups. There was no significant difference between NR2 expression in the NO3 and BOTH treatment groups at T24hr, or between the NH4 and No addition groups.

In contrast to NR2, there was no significant difference in NR3 expression at T0 compared to T1hr in Me-single for the NO3, NH4, and BOTH treatment groups, or
between the T1hr and T24hr samples in the same groups (Fig. 3.6, Table 3.4). NR3 expression in the No addition group significantly increased 3 fold from T0 to T1hr, followed by a significant decrease from T1hr to T24hr. At T1hr, NR3 expression in No addition group was significantly higher (2.2 and 1.7-fold) than it was in the BOTH and NH4 groups, but not significantly different from the NO3 group. By T24hr, however, NR3 expression in No addition group was still significantly higher than it was in the NH4 treatment group, but there was no significance difference between any other pairs of these four groups.

Comparing NR2 and NR3 expression in Me-single, NR2 expression was significantly higher than NR3 expression at all time points in the NO3 group (Fig. 3.6, Table 3.4). At T1hr, NR2 expression was also significantly higher than NR3 expression in the No addition group, while NR3 expression was 2.7 and 2.0 fold higher than NR2 expression in the NH4 and BOTH treatments. At T24hr, there was no significant difference between NR2 and NR3 expression in the NH4 and No addition group, while NR2 expression was 6.2 fold higher than NR3 expression in the BOTH treatment.

In Me-multiple, samples were taken at T0, T1hr, T24hr, and T48hr (Fig. 3.7, Table 3.5). NR2 expression significantly increased 15- and 4-fold from T0 to T1hr in the NO3 and BOTH treatments, respectively; while it significantly decreased in the NH4 treatment. There was no significant difference in NR2 expression in No addition group between these two time points. Although there was no significant difference in NR2 expression in the NH4, BOTH, and No addition groups at T24hr compared to T1hr, it decreased significantly over the same time frame in the NO3 treatment. For the T24hr to T48hr time frame, there was no significant difference in NR2 expression
for any treatment group. Comparisons between treatments revealed that at T1hr, NR2 expression in the NO3 treatment was significantly 3- to 35-fold higher than it was in the NH4, BOTH, and No addition treatments. In addition, NR2 expression was significantly higher (10-fold) in the BOTH treatment group than in the NH4 group. At T24hr, there was no significant difference in NR2 expression between the NO3 and BOTH group, or the NH4 and No addition treatment; while NR2 expression in the NO3 and BOTH treatments were significantly higher (4.3- to 4.8-fold) than in the NH4 and No addition treatments. At T48hr, however, NR2 expression was significantly higher in the NO3 group than in the NH4 and BOTH group, while there was no significant difference in NR2 expression between the NO3 group and No addition group, or among the NH4, BOTH, and No addition group.

NR3 expression significantly increased 6 fold in the NO3 group from T0 to T1hr in Me-multiple, and it increased 3 fold in the BOTH and No addition group, while there was no significant difference in NR3 expression in the NH4 group over this same time period (Fig. 3.7, Table 3.5). From T1hr to T24hr, there was no significant change in NR3 expression for the NO3, NH4, and BOTH treatment groups, while in the No addition group, NR3 expression decreased significantly from T1hr to T24hr. Additionally, there was no significant change in NR3 expression within any treatment groups from T24hr to T48hr. When comparing NR3 expression between treatments, NR3 expression in the NO3 group was significantly 2 fold higher at T1hr, than it was in other three treatment groups, while at T24hr, there was no significant difference in NR3 expression between any pairs of groups. At T48hr, NR3 expression in the No addition group was 3 fold higher than in the BOTH group, but there was no significant difference in NR3 expression in any other pairs of groups.
NR2 and NR3 expression in Me-multiple were compared within each group at each time point (Fig. 3.7, Table 3.5). NR2 expression was significantly higher (2.9-fold) than NR3 expression at T0, before addition of nutrients. At T1hr and T24hr, NR2 expression was significantly higher (3.9- to 7.4-fold) than NR3 expression in the NO3 and BOTH group, respectively, while there was no significant difference between NR2 and NR3 expression in the NH4 and No addition group at these same time points. By T48hr, there was no significant difference between NR2 and NR3 expression in any treatment.

3.5 Discussion

In Me-single and T0 to T24hr of Me-multiple, the entire community had significantly higher growth in the NH4 and BOTH group compared to the NO3 group, suggesting the preference of ammonium as a nitrogen source by both assemblages (Fig. 3.3, Fig. 3.5). However, there was no significant difference in C. subsalsa growth among these three groups from T0 to T24hr in both experiments, or from T24hr to T48hr in Me-multiple; suggesting no preference of nitrogen source by C. subsalsa. Therefore, although C. subsalsa was one of the dominant species in the blooms, other species in the assemblages grew more quickly with NH$_4^+$ addition compared to C. subsalsa. In both experiments, the blooms were dominated by C. subsalsa and C. toxicum, while the bloom in Me-multiple was also dominated by K. veneficum and K. foliaceum. The preference of ammonium by C. toxicum and K. veneficum was also reported by Tomas et. al (2005) and Solomon and Glibert (2008), respectively, while K. foliaceum had the preference of nitrate (Domingues, Barbosa, et al. 2011).

Therefore, it is likely that the species preferred ammonium, such as C. toxicum and K. veneficum, had higher growth rate than the species preferred nitrate or had no
nitrogenous preference, such as *K. foliaceum* and *C. subsalsa*, in the ammonium added groups. What is more, the no nitrogenous preference of *C. subsalsa* was not consistent with the results from the research conducted by Zhang et al. (2006), who demonstrated that NH$_4^+$ was the preferred nitrogen source by *C. subsalsa* in unialgal laboratory culture. However, it should be noticed that *C. subsalsa* was cultured in f/2 medium with nitrate or ammonium as the sole nitrogen source in the research conducted by Zhang et. al (2006), and *C. subsalsa* had been acclimated to the lab condition before the experiment. In contrast, the effect of *C. subsalsa* growth by nitrogen source in this research was tested within a mixed phytoplankton assemblage, so that the potential effect of the environment on the nitrogenous preference of *C. subsalsa* may be altered in a more dynamic environment. This is consistent with other studies. For example, nitrogenous preference was also affected by environmental conditions in *Heterosigma carterae*, which preferred nitrate at high light irradiance, and ammonium and urea at low light irradiance (Chang and Page 1995).

The growth rate of *C. subsalsa* was less than or equal to the growth rate of the entire community in each treatment for both experiments, except for the No addition group (Fig. 3.3, Fig. 3.5). Here, *C. subsalsa* had positive growth and the entire community had negative growth, suggesting that *C. subsalsa* out-competed other species in the assemblage under lower nitrogen concentrations. Previous research also suggested the ability of *Chattonella* spp. to utilize nitrogen source at low levels. For instance, Zhang et. al (2006) indicated that the half- saturation constants (K$_s$) for growth of *C. subsalsa* were 9 and 1.5 µM on nitrate and ammonium, respectively; and Nakamura et al. (1988) suggested that the Ks for growth of *C. antiqua* on nitrate and ammonium was 1 and 0.23 µM, respectively. What is more, *Chattonella* spp. are
reported to be able to maintain low cell densities as background flora and become dominant with reloading of nutrients (Smayda 2002; Imai et al. 2006; Imai and Yamaguchi 2012). Therefore, maintaining a positive growth but low cell density could be part of the strategy for *Chattonella* spp. to survive in environments with low concentrations of nutrients and be able to out-compete other phytoplankton when nutrients become available.

Interestingly, in Me-multiple, the growth rate of *C. subsalsa* of the No addition group increased significantly in the period of T24hr to T48hr compared to in the period of T0 to T24hr, while there was no increase in the growth rate of *C. subsalsa* in other groups, or the growth rate of the whole assemblage in any groups (Fig. 3.5). The nutrients data for the No addition treatment indicates that nitrate concentration increased 5.3 and 5.7 fold at T48hr and T24hr compared to at T1hr (Table. 3.2), respectively, indicating the potential presence of nitrogen-fixing organisms in the assemblage, or the releasing of nitrate by other organisms in the process of cell death and lysis. Overall, these imply that *C. subsalsa* was able to survive and out-compete other organisms in the assemblage by utilizing the recycled nitrogen when there were no extra nutrients inputs.

Nitrogen is one of the limiting nutrients in the ocean, and plays an important role in stimulating HABs dominated by dinoflagellates, raphidophytes, and cyanobacteria (Doucette and Harrison 1991; Glibert et. al 2008; Gobler et al. 2007; Davis et al. 2010; reviewed by Imai and Yamaguchi 2012). As the dominant form of nitrogen that contributes to eutrophication in aquatic environments (reviewed by Glibert et al. 2016), nitrate stimulates the growth and proliferation of these HABs. In the process to utilize nitrate, NR catalyzes the first and also rate limiting step,
converting nitrate to nitrite in cells (Campbell 1999). Therefore, it is extremely important to investigate NRs in HAB species and their potential roles in the interaction and competition among these species. In two mesocosm experiments of this study, the expression of NR2 and NR3 of *C. subsalsa* in natural blooms, as well as their implication for the growth and survival of this alga were investigated.

NR2 expression increased upon addition of nitrate in the NO3 group and was repressed within the first hour after addition of high concentrations of ammonium in both Me-single and Me-multiple. NR2 also decreased with the decrease of nitrate concentrations from T1hr to T24hr in both experiments (Fig. 3.6-3.7), providing evidence that NR2 expression is regulated by nitrate concentrations. This is consistent with the induction and repression of NR transcription abundance by nitrate and ammonium, respectively, in a wide range of algal species, including *Heterosigma akashiwo*, *Cyanidioschyzon merolae*, and *Dunaliella tertiolecta* (Imamura et al. 2010; Song and Ward 2004; de Montaigu et al. 2011; Coyne 2010). For example, NR transcription abundance in the diatom, *Dunaliella tertiolecta*, was induced and repressed by nitrate and ammonium, respectively (Song and Ward 2004). What is more, NR expression in the nitrogen starved *Dunaliella tertiolecta* was also induced by nitrate addition immediately and reached its maximum at 1 hour after adding nitrate, and then decreased after 1-hour incubation (Song and Ward 2004).

In Me-single, NR2 expression in the BOTH treatment group, where nitrate and ammonium were provided at equal concentrations, decreased from T0 to T1hr, while it increased in the same group of Me-multiple (Fig. 3.6-3.7). This difference may due to the distinct nutrient status at the initial time point and after adding the nutrients in these two experiments (Fig. 3.2, Fig. 3.4, Table 3.2). In the BOTH group, the nitrate
and ammonium concentration of Me-single was 2.38 and 4.72 µM (NO₃⁻:NH₄⁺=1:2), respectively, at T0; as well as 50.85 and 54.72 µM (NO₃⁻:NH₄⁺=1:1) at the hypothetical time point T0+. The nitrate and ammonium concentration in the same group at T0 in Me-multiple was 36.77 and 13.62 µM (NO₃⁻:NH₄⁺=2.7:1), respectively; as well as 86.77 and 63.62 µM (NO₃⁻:NH₄⁺=1.3:1), respectively, at T0+. When nitrate and ammonium are present together, the repression of NR by ammonium may depend on the relative abundance of nitrate and ammonium, as well as absolute concentrations (reviewed by Glibert et al. 2016). This is in agreement with the effect of nitrate:ammonium balance on NR expression in Chlamydomonas, where higher nitrate:ammonium ratios resulted in lower repression by ammonium to its NR gene (Sanz-Luque et al. 2015; de Montaigu et al. 2011; Llamas et al., 2002).

However, the reduced NR2 gene expression in BOTH group of Me-single, as well as the increased NR2 expression in the same group of Me-multiple, from T0 to T1hr, were not consistent with the results of Chapter 2, where NR expression increased with decreased nitrate to ammonium ratios from 100:0 µM to 50:50 µM, and decreased when nitrate to ammonium ratios further decreased from 50:50 µM to 5:95 and 0:100 µM. To be noted, the rapid utilization of phosphate in each group of these two experiments indicate that C. subsalsa might be phosphorus-limited in both experiments, and f/2 concentration of phosphate was added to each group in Me-multiple but not Me-single. Phosphorus plays a key role in energy metabolism of algae (reviewed by Cai, Park, and Li 2013). As the preferred phosphorus source, phosphate was assimilated by algae through phosphorylation, and much of the incorporated phosphorus was used to generate ATP (Martínez, Jiménez, and El Yousfi 1999; reviewed by Cai, Park, and Li 2013). It is well established that ATP is necessary for
nitrite reduction, as well as ammonium incorporation in the nitrate assimilation process (Denison et al. 2011a; Carol Mackintosh 2004). Therefore, phosphorus deficiency may negatively affect nitrate assimilation and NR expression indirectly. What is more, phosphorus deficiency may have a negative impact on Rubisco activation and ribulose 1,5-bisphosphate recycling (reviewed by Touchette and Burkholder 2000; Brooks 1986; Rao, Arulanantham, Terry 1989), which can lead to a decrease of photosynthesis rates as much as 68% in plants (reviewed by Touchette and Burkholder 2000; Dietz and Foyer 1986; Rao, Arulanantham, Terry 1989; Usuda and Shimogawara 1991). The interruption of photosynthesis could result in a decrease of ATP and reductant required by nitrite reductase, resulting in a negative impact on NR. Therefore, it is likely that the decrease and increase of NR2 gene expression from T0 to T1hr in the BOTH group of Me-single and Me-multiple were affected more by phosphorus limitation and addition, than the nitrogen status here.

NR3 expression increased in the No addition group from T0 to T1hr in both Me-single and in Me-multiple, although the change in Me-multiple was not significant (Fig. 3.6-3.7). The increased NR3 expression in the No addition group suggests the sensitivity of NR3 to certain environmental conditions changed after setting up the experiment at T1hr compared to T0. The higher sensitivity to some environmental factors in NR3 than NR2 was also observed in the lab experiments conducted in Chapter 2, where NR3 expression dropped immediately after lights on, while there was no change in NR2 expression until after 1 hour of light. It should be noted that, however, NR3 exhibited less sensitivity to temperature compared to NR2 in the lab experiment, where NR2 gene expression decreased when temperature increased, and NR3 was not affected by temperatures. This indicates the sensitivity or insensitivity of
NR3 depends on the environmental factors, and the mechanism behind this requires further studies. NR3 expression in the BOTH and NH4 treatment groups was lower than the No addition group at T1hr in Me-single, indicating the repression of NR3 expression by ammonium. However, the lower expression of NR3 in the BOTH and NH4 treatment groups compared to No addition group was not observed in Me-multiple at T1hr. This is consistent with the difference in NR2 expression in the BOTH group at T1hr for Me-single compared to Me-multiple, which may be impacted by phosphorus status in these two experiments, as noted above.

NR3 expression in the NO3 group of Me-multiple at T1hr was significantly 2 fold higher than it in the No addition group, indicating the effect of nitrate addition in regulating NR3 expression (Fig. 3.7). However, this was not observed in NR3 expression at the same time point in Me-single. As noted above, phosphate limitation can have a negative impact on nitrogen assimilation and NR expression indirectly. Therefore, it is possible that because of the phosphorus limitation, NR3 expression did not increase with the addition of nitrate in the Me-single, while NR3 expression increased with nitrate addition in Me-multiple due to the phosphorus addition in this experiment. What is more, at T1hr, NR2 expression was induced by the addition of nitrate in the NO3 group of both experiments, and exhibited significantly higher values than NR3 expression, suggesting NR2 may benefit *C. subsalsa* in the environment with high concentration of nitrate; while from T1hr to T24hr, NR2 in the NO3 group of both experiments decreased significantly, while NR3 did not change, suggesting that NR3 may benefit *C. subsalsa* in an environment when nitrate concentration changes rapidly.
In the NH4 treatment group, there was no indication that NR3 expression was repressed by ammonium in either mesocosm experiment, while nitrate to ammonium ratios had an effect on NR2 expression. In Me-single, NR3 expression was significantly higher than NR2 expression at T1hr in the NH4 treatment. Likewise, in Me-multiple, NR3 gene expression was not repressed by ammonium at T1hr in the NH4 group, while NR2 expression was significantly repressed. These results suggest that NR3 was less repressible by ammonium compared to NR2. Although NR in \textit{Chlamydomonas}, \textit{Chlorella vulgaris}, and \textit{Cyanidioschyzon merolae} for instance, was inhibited by the presence of ammonium, especially in high concentrations (Fernandez and Galvan 2008; Loppes et al., 1999; Cannons and Shiflett, 2001; Llamas et al., 2002; Imamura et al., 2010, reviewed by Sanz-Luque et al. 2015), the repression of NR by ammonium is not universal. For example, after culturing with enriched ammonium (50 \(\mu\)mol. L\(^{-1}\) pulses) for 4 days, no significant change was observed in NR activity of brown alga, \textit{Fucus serratus}, while NR activity of \textit{Laminaria digitate} decreased by 80\% under the same condition (Young, Dring, and Berges 2007). In addition, NR enzymes in dinoflagellates, \textit{Gonyaulax polyedra} and \textit{Peridinium cinctum}, were not inhibited by ammonium; and the author noted that this might be related to the location in the chloroplast of these NRs, in contrast to cytoplasmic NRs in other species (reviewed by Glibert et al. 2016; Berges 1997).

Interestingly, the high NR2 expression of \textit{C. subsalsa} in medium with high concentrations of nitrate, and lower repression of NR3 expression by ammonium compared to NR2 was also observed in lab experiments with unialgal cultures of \textit{C. subsalsa} (Chapter 2). When nitrate was the sole nitrogen source, NR2 expression was up to 30-fold higher than NR3 expression in experiments examining the effect of light.
intensity and temperature on NR expression. In the experiments evaluating NR expression affected by different nitrogen sources, however, there was no significant difference between NR2 and NR3 expression under high ammonium concentrations (5:95 \( \text{NO}_3^- : \text{NH}_4^+ \), or 100% \( \text{NH}_4^+ \)). Overall, these results suggest that NR2 may benefit \( C. \ subsalsal \) in the environment with high concentrations of nitrate; while NR3 may benefit this species in a more dynamic environment when the nitrate to ammonium balance moves toward ammonium.

Although another pulse of nitrogen was added at T24hr in Me-multiple, NR expression was not further increased by nitrate addition nor repressed by ammonium from T24hr to T48hr (Fig. 3.7). It is notable that phosphate utilization from T24hr+ to T48hr was also not as rapid and dramatic as from T0+ to T24hr, suggesting that the intracellular phosphate status of \( C. \ subsalsal \) may be different comparing T0 to T24hr and T24hr to T48hr. As mentioned above, adding phosphate may have an effect on NR expression in phosphate-limited cultures, hence, the potentially distinct intracellular phosphate status may contribute to the different response of NRs to nitrogen source comparing these two time periods. The different effect by nitrogen pulse on NR of \( C. \ subsalsal \) comparing the two time periods may also due to the distinct intercellular nitrogen status of \( C. \ subsalsal \) in each period. It is possible that surge uptakes of nitrogen occurred with nutrient input and cells assimilated enough nitrogen through the first nutrient input, but then took up nitrogen for storage with the input of the second nutrient pulse. In this case, NR expression may have been more affected by nitrogen source after the first pulse of nitrogen, but remained at a low level after the second pulse of nitrogen. Surge uptake and storage of nitrogen was also observed in diatoms, flagellates, and the Raphidophycean species, \( H. \ akashiwo \)
Phytoplankton can perform surge uptake to utilize and store nutrients inside of the cells when nutrients are available in the environment, and when the external nutrient supply is depleted, phytoplankton can use these intracellular nutrients for growth and survival (Domingues, Anselmo, et al. 2011; Sommer, 1985, 1989). What is more, the uncoupling of function and expression of NR was also observed in laboratory experiments described in Chapter 2. When *C. subsalsa* was acclimated to high ammonium concentrations, NR activity was lost completely, while low expression of both NR2 and NR3 were still maintained. Although this hypothesis still needs more evidence to support, maintaining the transcript abundance in unfavorable environments could benefit the survival of *C. subsalsa*.

In conclusion, this research suggests the ability of *C. subsalsa* to survive and out-compete other species in the assemblage with a low nitrogen concentration, especially when there was no nitrogen input. Several potential strategies for *C. subsalsa* to survive in a low-nitrogen environment were also suggested in this research: 1) *C. subsalsa* may be able to maintain a low cell density but positive growth rate when the ambient nitrogen concentration is low; 2) *C. subsalsa* is capable of using nitrate produced by nitrogen-fixing organisms or released by dead cells; 3) *C. subsalsa* is able to uptake nutrients massively and rapidly, and it is capable of storing these excess nutrients inside of the cells; 4) *C. subsalsa* regulates NR2 and NR3 expression differentially in response to different nitrogen conditions such that NR2 may benefit *C. subsalsa* in favorable environments with high concentration of nitrate, while expression of NR3 may benefit *C. subsalsa* in a more dynamic and unfavorable environment with ammonium as the dominant nitrogen source.
Table 3.1. Sequence of *C. subsalsa*- specific primers for nitrate reductase [NR2 (CsNR Glob) and NR3 (CsNR3)], and Actin (Cs Actin). F, forward primer; R, reverse primer.

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<tr>
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Table 3.2. Nitrate, ammonium, and phosphate concentrations in each group at T0, T0+ (the hypothetical time point after adding nutrients between T0 and T1hr), and T24hr in Me-single; T0, T0+, T1hr, T24hr, T24hr+(the hypothetical time point after adding nutrients between T24hr and T48hr) and T48hr in Me-multiple.

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<td>37.54</td>
<td>0.91</td>
<td>0.51</td>
<td>36.71</td>
</tr>
<tr>
<td>No addition</td>
<td>NO₃⁻</td>
<td>36.77</td>
<td>36.77</td>
<td>2.54</td>
<td>14.47</td>
<td>14.47</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>13.62</td>
<td>13.62</td>
<td>5.08</td>
<td>7.25</td>
<td>7.25</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1.34</td>
<td>37.54</td>
<td>0.74</td>
<td>1.01</td>
<td>37.21</td>
</tr>
</tbody>
</table>
Table 3.3. Comparison of the growth rate of the entire community (community) or *C. subsalsa* between groups in the period of T0 to T24hr (T0T24), as well as T24hr to T48hr (T24T48, Me-multiple only) in Me-single and Me-multiple (A); comparing the growth rate of the entire community and *C. subsalsa* in each group and time period in Me-single and Me-multiple (B); comparing growth rate of the entire community or *C. subsalsa* between T0 to T24hr and T24hr to T48hr in Me-multiple (C). The bold and italic numbers are the p values that are less than 0.05, indicating the significance.

A. Comparison of the growth rate of the entire community (community) or *C. subsalsa* between groups in the period of T0 to T24hr (T0T24), as well as T24hr to T48hr (T24T48, Me-multiple only) in Me-single and Me-multiple. There was no significant difference in growth rate of *C. subsalsa* from T0 to T24hr between groups in Me-single, or T24hr to T48hr between groups in Me-multiple.

<table>
<thead>
<tr>
<th>Pairs of group</th>
<th>Me-single</th>
<th>Me-multiple</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. subsalsa</em></td>
<td>community</td>
<td><em>C. subsalsa</em></td>
</tr>
<tr>
<td>T0T24</td>
<td>T0T24</td>
<td>T24T48</td>
</tr>
<tr>
<td>No addition-</td>
<td>0</td>
<td>0.010</td>
</tr>
<tr>
<td>BOTH NH4-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BOTH NO3-</td>
<td>0.857</td>
<td>0.092</td>
</tr>
<tr>
<td>BOTH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO3-</td>
<td>0.001</td>
<td>0.981</td>
</tr>
<tr>
<td>NH4-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>0</td>
<td>0.005</td>
</tr>
<tr>
<td>NO3-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH4-</td>
<td>0</td>
<td>0.169</td>
</tr>
</tbody>
</table>

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B. Comparing the growth rate of the entire community and *C. subsalsa* in each group and time period in Me-single and Me-multiple.

<table>
<thead>
<tr>
<th></th>
<th>Me-single</th>
<th>Me-multiple</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td><strong>T0T24</strong></td>
<td><strong>T0T24</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>T24T48</strong></td>
</tr>
<tr>
<td>NO3</td>
<td>0.345</td>
<td>0.150</td>
</tr>
<tr>
<td>NH4</td>
<td>0.014</td>
<td>0.355</td>
</tr>
<tr>
<td>BOTH</td>
<td>0.048</td>
<td>0.022</td>
</tr>
<tr>
<td>No addition</td>
<td>0.003</td>
<td>0.029</td>
</tr>
</tbody>
</table>

C. Comparing growth rate of the entire community or *C. subsalsa* between T0 to T24hr and T24hr to T48hr in Me-multiple.

<table>
<thead>
<tr>
<th></th>
<th>C. subsalsa</th>
<th>Community</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td><strong>T0T24~T24T48</strong></td>
<td><strong>T0T24~T24T48</strong></td>
</tr>
<tr>
<td>NO3</td>
<td>0.326</td>
<td>0.552</td>
</tr>
<tr>
<td>NH4</td>
<td>0.095</td>
<td>0.431</td>
</tr>
<tr>
<td>BOTH</td>
<td>0.035</td>
<td>0.097</td>
</tr>
<tr>
<td>No addition</td>
<td><strong>0.001</strong></td>
<td>0.476</td>
</tr>
</tbody>
</table>
Table 3.4. P values generated by TukeyHSD test (A) and paired t-test (B and C) comparing NR2 or NR3 expression between groups at T1hr and T24hr (A) in Me-single; NR2 or NR3 expression in each group between T0 and T1hr, as well as T1hr and T24hr (B); NR2 and NR3 in each group at T0, T1hr, and T24hr (C). The bold and italic numbers are the p values that are less than 0.05, indicating the significance.

A. Comparing NR2 or NR3 expression between groups at T1hr and T24hr.

<table>
<thead>
<tr>
<th>Group pairs</th>
<th>T1hr.NR2</th>
<th>T24hr.NR2</th>
<th>T1hr.NR3</th>
<th>T24hr.NR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition-BOTH</td>
<td>0.089</td>
<td>0</td>
<td><strong>0.010</strong></td>
<td>0.497</td>
</tr>
<tr>
<td>NH4-BOTH</td>
<td>1.000</td>
<td>0</td>
<td>0.836</td>
<td>0.147</td>
</tr>
<tr>
<td>NO3-BOTH</td>
<td>0</td>
<td>0.520</td>
<td>0.268</td>
<td>0.998</td>
</tr>
<tr>
<td>NH4-No addition</td>
<td>0.086</td>
<td>0.837</td>
<td><strong>0.042</strong></td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>NO3-No addition</td>
<td>0</td>
<td><strong>0.002</strong></td>
<td>0.360</td>
<td>0.408</td>
</tr>
<tr>
<td>NO3-NH4</td>
<td>0</td>
<td>0</td>
<td>0.661</td>
<td>0.191</td>
</tr>
</tbody>
</table>

B. Comparing NR2 or NR3 expression in each group between T0 and T1hr, as well as T1hr and T24hr.

<table>
<thead>
<tr>
<th>Pairs of time point</th>
<th>NO3</th>
<th>NH4</th>
<th>BOTH</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0-T1hr</td>
<td><strong>0.041</strong></td>
<td>0.008</td>
<td><strong>0.003</strong></td>
<td>0.083</td>
</tr>
<tr>
<td>T1hr-T24hr</td>
<td><strong>0.041</strong></td>
<td>0.107</td>
<td><strong>0.011</strong></td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td></td>
<td>NR3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0-T1hr</td>
<td>0.210</td>
<td>0.099</td>
<td>0.143</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>T1hr-T24hr</td>
<td>0.395</td>
<td>0.461</td>
<td>0.100</td>
<td><strong>0.016</strong></td>
</tr>
</tbody>
</table>

C. Comparing NR2 and NR3 in each group at T0, T1hr, and T24hr.

| T0                     |          |          |          | **0.002**|
|                        | NO3      | NH4      | BOTH     | N        |
|                        |          |          |          |          |
| T1hr                  | **0.021**| **0.004**| **0.007**| **0.003**|
| T24hr                 | **0.001**| 0.067    | **0.012**| 0.781    |
Table 3.5. P values generated by TukeyHSD test (A) and paired t-test (B and C) comparing NR2 or NR3 expression in Me-multiple between groups at T1hr, T24hr, and T48hr (A); NR2 or NR3 expression in each group between T0 and T1hr, T1hr and T24hr, as well as T24hr and T48hr (B); NR2 and NR3 in each group at T0, T1hr, T24hr, and T48hr (C). The bold and italic numbers are the p values that are less than 0.05, indicating the significance.

A. Comparing NR2 or NR3 expression between groups at T1hr, T24hr, and T48hr.

<table>
<thead>
<tr>
<th>Group pairs</th>
<th>T1hr NR2</th>
<th>T24hr NR2</th>
<th>T48hr NR2</th>
<th>T1hr NR3</th>
<th>T24hr NR3</th>
<th>T48hr NR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition-BOTH</td>
<td>0.052</td>
<td><strong>0.013</strong></td>
<td>0.989</td>
<td>0.999</td>
<td>0.998</td>
<td><strong>0.018</strong></td>
</tr>
<tr>
<td>NH4-BOTH</td>
<td><strong>0.011</strong></td>
<td><strong>0.010</strong></td>
<td>0.998</td>
<td>0.984</td>
<td>0.740</td>
<td>0.660</td>
</tr>
<tr>
<td>NO3-BOTH</td>
<td><strong>0.000</strong></td>
<td>1.000</td>
<td><strong>0.047</strong></td>
<td><strong>0.030</strong></td>
<td>0.974</td>
<td>0.265</td>
</tr>
<tr>
<td>NH4-No addition</td>
<td>0.800</td>
<td>0.998</td>
<td>0.998</td>
<td>0.996</td>
<td>0.827</td>
<td>0.075</td>
</tr>
<tr>
<td>NO3-No addition</td>
<td><strong>0.000</strong></td>
<td><strong>0.024</strong></td>
<td>0.079</td>
<td><strong>0.024</strong></td>
<td>0.993</td>
<td>0.248</td>
</tr>
<tr>
<td>NO3-NH4</td>
<td><strong>0.000</strong></td>
<td><strong>0.018</strong></td>
<td><strong>0.042</strong></td>
<td><strong>0.016</strong></td>
<td>0.951</td>
<td>0.827</td>
</tr>
</tbody>
</table>

B. Comparing NR2 or NR3 expression in each group between T0 and T1hr, T1hr and T24hr, as well as T24hr and T48hr.

<table>
<thead>
<tr>
<th></th>
<th>NO3</th>
<th>NH4</th>
<th>BOTH</th>
<th>N</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0-T1hr</td>
<td><strong>0.002</strong></td>
<td>0.027</td>
<td><strong>0.009</strong></td>
<td>0.053</td>
<td></td>
<td></td>
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<tr>
<td>T1hr-T24hr</td>
<td><strong>0.024</strong></td>
<td>0.354</td>
<td>0.809</td>
<td>0.272</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T24hr-T48hr</td>
<td>0.140</td>
<td>0.793</td>
<td>0.074</td>
<td>0.326</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0-T1hr</td>
<td><strong>0.008</strong></td>
<td>0.058</td>
<td><strong>0.022</strong></td>
<td><strong>0.002</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1hr-T24hr</td>
<td>0.067</td>
<td>0.938</td>
<td>0.160</td>
<td><strong>0.005</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T24hr-T48hr</td>
<td>0.429</td>
<td>0.392</td>
<td>0.060</td>
<td>0.450</td>
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<td></td>
</tr>
</tbody>
</table>

C. Comparing NR2 and NR3 in each group at T0, T1hr, T24hr, and T48hr.

<table>
<thead>
<tr>
<th>T0</th>
<th><strong>0.005</strong></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>NO3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH4</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BOTH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1hr</td>
<td><strong>0.002</strong></td>
<td>0.110</td>
<td><strong>0.002</strong></td>
<td>0.192</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T24hr</td>
<td><strong>0.010</strong></td>
<td>0.716</td>
<td><strong>0.042</strong></td>
<td>0.461</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T48hr</td>
<td>0.064</td>
<td>0.643</td>
<td>0.395</td>
<td>0.233</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1. Experiments set-up of two mesocosm experiments. One pulse of nitrate, ammonium or both nitrate and ammonium were added as nitrogen sources to experiment Me-single, while multiple pulses of the same nitrogen source along with f/2 phosphate were added to experiment Me-multiple.
Figure 3.2. Nitrate (NO$_3$), ammonium (NH$_4$), and phosphate (P) concentrations at T0 (the initial time point) before addition of nutrients, and T24hr (24 hours after adding nutrients and setting the experiments) in Me-single. Concentration of nitrate and ammonium added to each treatment at T0+ was indicated by dots. One pulse of nitrogen was added after taking samples at T0 (100 µM NO$_3$, 100 µM NH$_4^+$, 50 µM NO$_3^-$ and 50 µM NH$_4^+$, and no nutrients were added to NO3, NH4, BOTH and No addition groups, respectively).
Figure 3.3. Growth rate (hr⁻¹) of *C. subsalsa* and the entire community in groups added nitrate (NO₃), ammonium (NH₄), both of nitrate and ammonium (BOTH), or no nutrients (No addition) in experiment Me-single. Error bars are standard deviation of four biological replicates. Asterisks above the bars indicate a significant difference in growth rates between *C. subsalsa* and the entire community in the same treatment group.
Figure 3.4. Nitrate (NO3), ammonium (NH4), and phosphate (P) concentration at T0 (the initial time point), T1hr, T24hr, and T48hr (1, 24 and 48 hour(s) after setting the experiments, respectively) in Me-multiple. Concentration of nitrate, ammonium and phosphate in each group at T0+ and T24hr+ was indicated by white and black dots, respectively. Two pulses of nutrients with f/2 phosphate (36.2 µM) were added after taking samples at T0 and T24hr; 100 µM NO3\(^-\), 100 µM NH4\(^+\), 50 µM NO3\(^-\) and 50 µM NH4\(^+\), and no nitrogen source were added to NO3, NH4, BOTH and No addition groups, respectively.
Figure 3.5. Growth rate (hr$^{-1}$) of *C. subsalsa* and the entire community in groups added nitrate (NO3), ammonium (NH4), both of nitrate and ammonium (BOTH), or no nutrients (No addition) in experiment Me-multiple. Error bars are standard deviation of four biological replicates. Asterisks above the bars indicate the significant difference in growth rate between *C. subsalsa* and the entire community in the same treatment group and time period.
Figure 3.6. NR2 and NR3 expression during Me-single for treatments added nitrate (NO3), ammonium (NH4), or both nitrate and ammonium (BOTH), and controls (No addition). T0, the initial time point, before adding any nutrients; T1hr and T24hr are 1 hour and 24 hours after starting the experiments, respectively. Nutrients were added after taking samples at T0, and 1 hour before sample collection for T1hr. Error bars are standard deviation of four biological replicates. Asterisks besides the symbols indicate the significant difference in NR expression between the indicated group and the No addition group at the same time point.
Figure 3.7. NR2 and NR3 expression in groups added nitrate (NO3), ammonium (NH4), both of nitrate and ammonium (BOTH), or no nutrients (No addition) in experiment Me-multiple. f/2 concentration of phosphate was added to each group at the time points when nitrogen was added. T0, the initial time point, before adding any nutrients; T1hr, T24hr, and T48hr are samples collected at 1, 24 and 48 hour(s) after starting the experiment, respectively. Nutrients were added after taking samples at T0, as well as after sample collection at 24 hours. Error bars are standard deviation of four biological replicates. Asterisks besides the symbols indicate the significant difference in NR expression between the indicated group and the No addition group at the same time point.
Chapter 4

CONCLUDING REMARKS

The work in Chapter 2 reveals the presence of a novel nitrate reductase, NR3, in *C. subsalsa*. The sequence of NR3 indicates the existence of a 14-3-3 binding motif in the hinge 1 region of this enzyme. The analysis of NR3 sequence also indicates that a conserved domain, the heme-Fe (cytochrome b5) domain, may not be present in this enzyme. The absence of the heme-Fe domain may lead to a different electron transport pathway in NR3 compared to other NRs, or a reduction of efficiency of this enzyme.

In the study related to the effect on NR expression and activity by light conditions, results indicate that NRs in *C. subsalsa* were regulated by light but not the endogenous rhythm at both transcriptional and translational levels. NRs had high activities in light and low activities in dark, while NR expression exhibited the opposite changing patterns, indicating the transcription and enzyme levels may serve as down-regulating signals for each other to prevent NRs from over expression or activation.

When *C. subsalsa* was cultured with different nitrate to ammonium ratios, NR2 and NR3 were expressed constitutively in all nitrogen conditions, even when ammonium was the sole nitrogen source. NR gene was induced by small amount of ammonium, but repressed when nitrate to ammonium ratios passed 50:50 µM. NR activity was repressed by ammonium quantitatively, and inhibited fully when nitrate to ammonium ratios were 5:95 and 0:100 µM. The ability for *C. subsalsa* to release excess ammonium into the medium, as well as its capacity to store nitrate into the cells were also observed. Temperature also contributes to the regulation of NRs in *C. subsalsa*. NR2 expression decreased when temperature increased, while NR3 gene
expression was not regulated by temperature. NR activity of *C. subsalsa* also decreased with the increase of temperature. Evidence was found that NRs in *C. subsalsa* were regulated by nitrogen source and temperature through the reversible phosphorylation and sequential binding of 14-3-3 proteins, while the regulation of NRs by light may through other mechanisms. To date, this is the first report of the presence of a 14-3-3 binding motif in algal NRs, as well as the regulation of algal NRs by the reversible phosphorylation and putative 14-3-3 binding proteins.

In Chapter 3, two mesocosm experiments were conducted to stimulate *C. subsalsa* natural blooms with different nitrogen sources (nitrate, ammonium, both nitrate and ammonium, or no nitrogen source to the control). In Me-single, only one pulse of nitrogen was added at the beginning of the experiment; while in Me-multiple, two pulses of nitrogen with f/2 concentration of phosphate were added at the initial time point and after 24 hours of the experiment. NR2 and NR3 expression in *C. subsalsa* in response to different nitrogen sources were tested, in order to investigate the implications of NR expression to the competitive dynamics of *C. subsalsa* in the environment. *C. subsalsa* exhibited no nitrogenous preference, while the assemblage preferred ammonium in this research. The effect of phosphate limitation on nitrogen assimilation and NR expression in *C. subsalsa* was observed.

The results indicate that *C. subsalsa* was able to out-compete other species in the assemblage when no nitrogen was added, but not in the nitrogen-added groups. The results imply the ability of *C. subsalsa* to utilize the nitrate produced by nitrogen fixers or released by dead cells, which may advantage *C. subsalsa* to out-compete other species in the environment with low nitrogen concentrations. What is more, evidence for the surge uptake and storage of nitrogen of *C. subsalsa* was also found in
this research, which may also advantage the competition and survival of this alga in an unfavorable environment. Different NR regulation responding to nitrogen source was observed in the experiments, indicating that NR2 may benefit *C. subsalsa* in a favored environment with a high concentration of nitrate, while NR3 may benefit *C. subsalsa* in a more dynamic and unfavorable environment where ammonium was the dominant nitrogen source.

Here, I suggest that the future work related to algal NRs may concentrate on these following aspects: 1). searching for 14-3-3 binding motifs in other algal NRs; 2). investigating the contribution by its functional domains to the regulation of novel phosphorylated algal NR; 3). studying the interactive regulation on the novel NRs by 14-3-3 binding proteins and environmental factors; and 4). investigating the implications of NR regulation on algal survival and competition in a more dynamic and complex ecosystem.
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Appendix A

NR ACTIVITY ASSAY OPTIMIZATION

A.1 Material and Methods

A.1.1 Culture conditions and collection

Replicate cultures of *C. subsalsa* (N=3 for each set of experiment) were maintained in f/2 medium (-Si) with f/2 nitrate as nitrogen source, at salinity of 20 psu and temperature of 25 °C (Guillard and Ryther, 1962). Cultures were maintained at 100 µEm⁻²s⁻¹ irradiance on a 12:12 h light: dark cycle.

Fifty milliliters of each culture was collected to determine NR activity as described below. The sample was centrifuged, the supernatant was discarded, and the cell pellet was frozen in liquid nitrogen immediately and stored at -80 °C until analysis.

A.1.2 Optimize NR assay with different extraction buffers

Four different extraction buffers were tested in this experiment: 200 mM phosphate buffer (KPi buffer, pH 7.9), 200 mM 3-(N-Morpoli-no)propanesulfonic acid (MOPS) buffer (pH 7.9), 50 mM MOPS buffer (pH 7.9), and 50 mM Tris(hydroxyl-methyl)aminomethane (Tris) buffer (pH 7.9) (Berges and Harrison 1995).

The frozen cell pellet was resuspended in 1 ml extraction buffer (200 mM KPi, 200 mM MOPS, 50 mM MOPS, or 50 mM Tris buffer, pH 7.9) on ice, homogenized
by sonication, and centrifuged to clarify at 4 °C. The supernatants were removed and assayed immediately (Fig. 2.2).

The assay consisted of extract, an equal volume of assay buffer (200 mM KPi, pH 7.9), 0.2 mM NAD(P)H (Sigma Chem. Co., St. Louis, MO) and 10 mM KNO₃. Sterile water was added to the negative control reaction in place of KNO₃. The reaction was incubated for 30 min at room temperature in the dark. Zinc Acetate (47.62 mM) was added to stop the reaction. The samples were centrifuged and the supernatants were used in the following steps described in Chapter 2 (2.2.4).

A.1.1 Optimize NR assay at different temperatures

The frozen cell pellet was resuspended in 1 ml extraction buffer (200 mM KPi, pH 7.9) on ice, homogenized by sonication, and centrifuged to clarify at 4 °C. The supernatants were removed and assayed immediately (Fig. 2.2).

The reagents used in the following step were maintained at temperatures of 18 °C, 25 °C, and 28 °C before the reaction except NAD(P)H and PMS. The assay consisted of extract, an equal volume of assay buffer (200 mM KPi, pH 7.9), 0.2 mM NAD(P)H (Sigma Chem. Co., St. Louis, MO) and 10 mM KNO₃. Sterile water was added to the negative control reaction in place of KNO₃. The reaction was incubated for 30 min at 18 °C, 25 °C, and 28 °C in the dark. Zinc Acetate (47.62 mM) was added to stop the reaction. The samples were centrifuged and the supernatants were used in the following steps described in Chapter 2 (2.2.4).

One-way ANOVA was used to analyze if there was a difference between NR activity in samples assayed in different temperatures.
A.1.3 Optimize NR assay in different pH

The extraction buffer (200mM KPi) and assay buffer (500mM KPi) were adjusted to pH 7.4, 7.9, and 8.4 before the experiment. The frozen cell pellet was resuspended in 1 ml extraction buffer (200 mM KPi, pH 7.4, 7.9, and 8.4) on ice, homogenized by sonication, and centrifuged to clarify at 4 °C. The supernatants were removed and assayed immediately (Fig. 2.2).

The assay consisted of extract, an equal volume of assay buffer (200 mM KPi, pH 7.4, 7.9, and 8.4), 0.2 mM NAD(P)H (Sigma Chem. Co., St. Louis, MO) and 10 mM KNO₃. Sterile water was added to the negative control reaction in place of KNO₃. The reaction was incubated for 30 min at room temperature in the dark. Zinc Acetate (47.62 mM) was added to stop the reaction. The samples were centrifuged and the supernatants were used in the following steps described in Chapter 2 (2.2.4).

One-way ANOVA was used to analyze the effect of pH on NR activity.

A.1.4 Results

NR activity in C. subsalsa was tested using different extraction buffers (200 mM KPi, 200 mM MOPS, 50 mM MOPS, and 50 mM Tris buffer). In the four extraction buffer tested, only the samples resuspended in 200 mM KPi exhibited activity. Therefore, 200 mM KPi was used as the extraction buffer in the following experiments.

NR activity was tested in different temperatures: 18 °C, 25 °C, and 28 °C (Fig. 4.1). Although there was no significant difference in NR activity between samples tested in different temperatures (p>0.05), NR activity tested in room temperature (25 °C) was 2.1 and 1.3 fold higher than tested in 18 °C and 28 °C, respectively. Therefore, 25 °C was considered as the optimized temperature for NR activity assay.
In the experiment testing the effect of pH on NR activity, there was no significant difference between NR activity in samples assayed in extraction and assay buffers of pH 7.4, 7.9, and 8.4 (p>0.05). NR activity of samples assayed in pH 7.9 was 1.6 fold higher than the samples assayed in other pH. Therefore, pH 7.9 was used as the optimized pH for NR activity assay in this study.
FIGURES

Figure A.1 NR activity in *C. subsalsa* tested in different temperatures. Error bars are standard deviation of three biological replicates.
Figure A.2 NR activity in C. subsalsa tested in different pH. Error bars are standard deviation of three biological replicates.
Appendix B

CELL DENSITY OF *C. SUBSALSA* IN LAB EXPERIMENTS (CHAPTER 2)

Table B.1 Cell density in cultures of nutrient and temperature experiments. Cells were counted under microscope in triplicates at the time of sampling.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Cell density (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO3:NH4 (µM)</td>
<td></td>
</tr>
<tr>
<td>100:0</td>
<td>20467</td>
</tr>
<tr>
<td>95:5</td>
<td>16850</td>
</tr>
<tr>
<td>50:50</td>
<td>25383</td>
</tr>
<tr>
<td>5:95</td>
<td>11100</td>
</tr>
<tr>
<td>0:100</td>
<td>19750</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>8333</td>
</tr>
<tr>
<td>25</td>
<td>15633</td>
</tr>
<tr>
<td>28</td>
<td>38666</td>
</tr>
</tbody>
</table>
Appendix C

SEQUENCE OF NR3 IN C. SUBSALSA, NR2 IN C. SUBSALSA AND H. AKASHIWO, AND NR IN TWO HIGHER PLANTS

C. subsalsa NR3

C. subsalsa NR3

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR

C. subsalsa NR3

C. subsalsa NR2

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR

C. subsalsa NR3

C. subsalsa NR2

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR

C. subsalsa NR3

C. subsalsa NR2

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR

C. subsalsa NR3

C. subsalsa NR2

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR

C. subsalsa NR3

C. subsalsa NR2

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR

C. subsalsa NR3

C. subsalsa NR2

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR

C. subsalsa NR3

C. subsalsa NR2

H. akashiwo NR2

H. akashiwo NR1

A. thaliana NR

C. sativa NR
Figure C.1 Alignment of amino acid sequence of NR3 in *C. subsalsa*, NR2 in *C. subsalsa* and *H. akashiwo*, NR in two higher plants: *Arabidopsis thaliana* and *Camelina sativa*. Domains within the alignment are underlined. The red sequences indicate the motif containing the serine phosphorylation site for 14-3-3 protein binding in NR3 and NR of higher plants. The identical residues in all sequences are marked by “*”, the highly and weakly conserved ones are marked by “:” and “.”, respectively (Chenna 2003; Larkin et al. 2007).