Nutrient Dynamics in the Coral-Algal Symbiosis: Developing Insight from Biogeochemical Techniques

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UNIVERSITY OF MIAMI

NUTRIENT DYNAMICS IN THE CORAL-ALGAL SYMBIOSIS: DEVELOPING INSIGHT FROM BIOGEOCHEMICAL TECHNIQUES

By

Quinn B. Devlin

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NUTRIENT DYNAMICS IN THE CORAL-ALGAL SYMBIOSIS: DEVELOPING
INSIGHT FROM BIOGEOCHEMICAL TECHNIQUES

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This work addresses the threats of nitrogen loading to coral reefs and encompasses many unanswered questions on the topic of dissolved inorganic (DIN) utilization by Scleractinian corals and their endosymbiotic algae. Experimental efforts were made to understand the conditions under which NO$_3^-$ or NH$_4^+$ leads to declines in calcification and identify other physiological changes in the coral-algal symbiosis which may accompany such growth declines. The synergistic influences of NH$_4^+$ and CO$_2$ were investigated in order to gain insight on the potential influences of elevated DIN on corals in a high CO$_2$ world. The study of NO$_3^-$ and NH$_4^+$ uptake and impacts on corals hosting two different endosymbiont clades of *Symbiodinium* was carried out to predict how nutrient enrichments may influence corals which host more thermally tolerant symbiont communities. Finally, in light of the importance of managing nitrogen loading to reefs, the validity of the use of $\delta^{15}$N in coral tissue as a nutrient source indicator was investigated through direct measurements of the $^{15}$N fractionation associated with NO$_3^-$ and NH$_4^+$ uptake.
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Introduction

*The Coral-Algal Symbiosis*

Symbiotic relationships between invertebrates and photosynthetic endosymbionts are widespread throughout the marine environment. The symbiotic relationships between many species of stony coral (Cnidaria: Anthozoa: Scleractinia) and the unicellular dinoflagellate algae of the genus *Symbiodinium* are thought to be the foundation of the high productivity and calcification rates conferring success to shallow tropical reefs. Scleractinian corals can exist with or without a symbiotic algal partner, however, dominant reef builders are typically involved in this symbiotic partnership (Davies 1991, Gattuso et al. 1993). The symbiotic association promotes tight cycling of nutrients (nitrogen, carbon) and thrives in the warm, shallow, clear, oligotrophic waters in which major reef structures are found. While Scleractinian corals first appeared in the Triassic, there are several hypotheses for the timing and drivers of the initiation of the coral-algal symbiosis (Stanley and Swart 1995). The coral-algal symbiosis is generally defined as mutualistic, as it is understood that each partner benefits from the relationship (Trench 1993). It has more recently, however, been suspected that the symbiosis may be dynamic, dependent on environmental conditions and may exist along a continuum in which a more parasitic like relationship could develop under unfavorable conditions (Thrall et al. 2007, Wooldridge 2010).

The unicellular algae (typically 6 – 12 µm in size) reside within the gastrodermal tissue layer of their cnidarian host (Blank and Huss 1989). Distribution of the algal symbionts varies spatially throughout the coral tissues, and the symbiont is located in
what has been termed the symbiosome vacuole inside the gastrodermal host cells (Davy et al. 2012). The primary benefits from cnidarian host to algal symbiont are thought to be shelter and a source of inorganic nitrogen and CO₂ provided through host catabolism (Wang and Douglas 1998, 1999). The major benefit to the coral host is the transfer of photosynthetic products (lipids, carbohydrates and amino acids) across the algal cell membrane to the coral host tissues (Battey and Patton 1987, Wang and Douglas 1997, 1998, 1999, Davy and Cook 2001, Logan et al. 2010). It has been estimated by some studies that the algae translocate more than 90% of synthesized photosynthate to the coral host (Muscatine and Pool 1979, Davies 1991).

The stability of this relationship is undoubtedly key to the persistence of corals into the future. While it is well accepted that severe reductions in algal symbiont density, commonly referred to as coral bleaching, are detrimental to coral health, it has been suggested that unnaturally high algal symbiont densities can have a variety of adverse effects as well (Marubini and Davies 1996, Wooldridge 2010, Cunning and Baker 2012).

**Threats to Stability of Environmentally & Economically Important Habitats**

Coral reefs provide an environmentally and economically important resource to tropical coastal communities in many regions of the world. The world’s shallow coral reefs exist in tropical and subtropical waters along coastlines and surrounding islands in major regions of the Atlantic (Caribbean), Pacific, Middle East (Red Sea & Persian Gulf), Indian Ocean, Southeast Asia, and Australia. Coral reefs have been a resource for local fisheries, tourism, biomedical discoveries, and coastal storm protection. Reefs provide an important habitat for a diverse assemblage of species from bacteria and phytoplankton to providing nesting and foraging habitats for reef sharks and sea turtles (Lesser et al.
The annual economic value of coral reefs in the United States and Territories alone is estimated at over 3.4 billion dollars (NOAA Program 2013). Many small island communities are closely tied to the resources provided by coral reefs and rely on them as a source of livelihood. A loss of this invaluable ecosystem would have devastating impacts on many coastal communities around the world (Burke 2011).

Coral reefs exist in a narrow range of physical, chemical and biological conditions which support the delicate balance between coral and their endosymbiotic algae as well as a diverse community of reef organisms. The environmental factors believed to primarily control the distribution of reefs include temperature, light penetration, salinity, nutrient concentration and aragonite saturation (Kleypas et al. 1999). Corals experience stress and bleaching at both the upper and lower thermal limits (Howells et al. 2013). As corals depend on the availability of bicarbonate to calcify, it is thought that corals are also spatially limited by aragonite saturation state. While mesophotic reefs do exist at greater depths, major reef structures are typically in shallow waters (less than ~45 m) with higher light penetration. Coral reefs thrive under clear, low nutrient waters where concentrations of dissolved inorganic nitrogen (DIN: \(\text{NH}_4^+, \text{NO}_3^-, \text{and NO}_2^-\)) are less than 3.0 \(\mu\text{M}\) and \(\text{PO}_4^{3-}\) is less than 0.5 \(\mu\text{M}\). Reefs generally exist farther from river deltas from which the delivery of high volumes of water associated with low salinity and high loads of sediments, organic and inorganic nutrients alter water chemistry (Kleypas et al. 1999).

With increasing global populations, specifically increasing inhabitance of coastal regions, anthropogenic activities are altering the physical and chemical conditions of coastal marine habitats. Local and global stressors are combining to place 75% of the
World’s reefs under threat. It is estimated that by 2030, this number will increase to 90% and by 2050, 100% of the world’s coral reefs will be at risk (Burke 2011). Understanding the susceptibility of unique and valuable coral reef ecosystems to several natural and anthropogenic disturbances is critical in developing management practices to protect and promote persistence of coral reefs into the future. Global and local stressors challenging reefs include non-sustainable fishing, pollution, nutrient loading, mining and dredging, damaging tourism practices, introduction of invasive species, and increased in temperature and partial pressure of CO₂ (pCO₂) related to climate change (Cesar 2000, Veron et al. 2009, Brander 2012).

As efforts are made to minimize anthropogenically induced climate change by those who recognize the alarming impacts, focus must also be immediately directed to those reefs which are under threats of local stress. Pristine reefs uninfluenced by local stressors (nutrients, overfishing etc.) are thought to display higher resilience, or recovery after a major disturbance such as a mass bleaching event or physical storm destruction (Wooldridge and Done 2009). Pinpointing local, potentially manageable sources of disturbance to coral reefs and taking action to reduce such stressors may allow reefs to persist under the climate induced changes in temperature and pCO₂ (Hughes et al. 2003, Mumby and Steneck 2008).

Direct and Indirect Impacts of Increased Nutrients on Corals

While it is well understood that nutrients degrade coral reefs through fertilization of macroalgae resulting in overgrowth, and stimulate phytoplankton productivity leading to turbid waters (Smith et al. 1981, Fabricius 2005, Littler and Littler 2007, Haas et al. 2009), the direct impacts of nutrients on corals are less clear. A variety of responses in
growth rate have been found to result from elevated levels of nitrogen. Among growth assessments are those studies measuring coral calcification rates (CaCO$_3$ precipitated over a period of time) and those which measure linear extension of the coral skeleton (skeletal extension over a period of time). Studies looking at the effects of elevated inorganic nitrogen levels on coral growth have found positive (Crossland and Barnes 1974; Atkinson et al. 1995), negative (Marubini and Davies 1996, Ferrier-Pages et al. 2001, Fabricius 2005, Stambler 1991) and no significant effects on growth (Koop et al. 2001; Ferrier-Pages et al. 2000). The mechanisms through which nutrients may lead to declines in coral growth rates are not agreed upon. This lack of clarity renders it difficult to predict how nutrient loading will synergistically interact with climatic stressors such as lowered ocean pH and increased sea surface temperatures. Some propose synergistic interactions between elevated nutrients and pCO$_2$ will have additive deleterious impacts (Wooldridge 2012), while others suggest that the disturbances may ameliorate the impacts of each other (Holcomb et al. 2010). Under elevated sea surface temperatures, it is not understood how potential shifts to more thermally tolerant algal endosymbiont communities may influence the response of corals to elevated nutrient levels.

Identifying Nutrient Inputs to Reefs for Management

Identifying local stressors compromising reef resilience is critical in ensuring proper management of coral reefs. As nutrients pose threats to these economically important, fragile ecosystems, especially in the face of changing climatic conditions, it is important to develop proper management to eliminate nutrient inputs as an additional stressor to reefs. Identifying the presence of nitrogen loading on a reef and identifying the source can be complicated as pulses of DIN may be rapidly assimilated by the nitrogen
limited organisms inhabiting a reef (Capone 2008). Collecting a water sample and measuring DIN concentrations holds only a snapshot of information, as higher concentrations of DIN may have been present just days or hours prior to sample collection, but were rapidly assimilated. For this reason, several workers have turned to the use of nitrogen stable isotopes (Heikoop et al. 2000; Raimonet et al. 2013). As an organism incorporates nitrogen into its tissues, it retains the nitrogen isotopic signature ($\delta^{15}N$) of the nitrogen source, dependent on nitrogen turnover in the tissues.

While numerous workers have used the measurement of the $\delta^{15}N$ of cnidarian tissues as indicators of nitrogen source inputs (Dolenec et al. 2005, Risk et al. 2009, Baker et al. 2010) and the $\delta^{15}N$ of skeletal matrix in coral cores to reconstruct past nutrient inputs (Marion et al. 2005, Yamazaki et al. 2011), the isotopic fractionation associated with NO$_3^-$ and NH$_4^+$ uptake by coral and algal symbionts has never been measured. Several photosynthetic organisms have been shown to display variability in magnitude nitrogen isotope discrimination during the uptake of NO$_3^-$ and NH$_4^+$. In comparison, nitrogen acquisition by corals is even more complex as it involves two symbiotic partners. In order to use the $\delta^{15}N$ of coral tissues to interpret the source of nutrient inputs to a reef, a better understanding as to how $\delta^{15}N$ of NO$_3^-$ and NH$_4^+$ are directly related to the $\delta^{15}N$ of coral tissues should be developed.

Dissertation Goals

The work carried out in this dissertation addresses the threats of nitrogen loading to coral reefs and encompasses many unanswered questions on the topic of DIN utilization by Scleractinian corals and their endosymbiotic algae. Experimental efforts were made to identify the conditions under which NO$_3^-$ or NH$_4^+$ leads to declines in
calcification and analyze other physiological responses in the coral-algal symbiosis that accompany such declines. The synergistic influences of \(\text{NH}_4^+\) and \(\text{CO}_2\) were investigated in order to gain insight on the potential influences of elevated DIN on corals in a high \(\text{CO}_2\) world. The study of \(\text{NO}_3^-\) and \(\text{NH}_4^+\) uptake and impacts on corals hosting two different clades of \textit{Symbiodinium} was carried out to predict how nutrient enrichments may influence corals which host more thermally tolerant symbiont communities. Finally, in light of the importance of managing nitrogen loading to reefs, the validity of the use of \(\delta^{15}\text{N}\) in coral tissue as a nutrient source indicator was investigated through direct measurements of the \(^{15}\text{N}\) fractionation associated with \(\text{NO}_3^-\) and \(\text{NH}_4^+\) uptake.
Chapter One: The Uptake of Nitrate and Ammonium by *Pocillopora damicornis*

1.1 Background

While Scleractinian corals thrive in tropical, oligotrophic waters, there are numerous threats of natural and anthropogenic nutrient loading and their response to elevated levels of dissolved inorganic nitrogen (DIN) is not clearly understood. At the ecosystem level, it is generally accepted that increases in DIN will result in competition and overgrowth by macroalgae, leading to a phase shift in reef community composition. The severity of macroalgal dominance has been shown to be influenced by the population of grazers inhabiting a particular reef (Littler and Littler 2007). Some studies suggest that without an external source of DIN, the nitrogen demands for the association of coral and endosymbiotic dinoflagellate algae (*Symbiodinium microadriaticum*) can be met through host heterotrophy and efficient recycling of nutrients from host catabolism. However, degrees of nitrogen limitation may vary from reef to reef, under changing environmental conditions or between coral species. This study investigates the behavior of the coral-algal symbiosis over a range of elevated levels of NO$_3^-$ and NH$_4^+$. While the nitrogen demands may be met under natural low level DIN, this study explores how the symbiotic association utilizes external NO$_3^-$ and NH$_4^+$ when available at elevated concentrations. This study also investigates the long term effects of elevated DIN concentrations on nitrogen cycling within the coral-algal symbiosis.

1.1.1 Natural and Anthropogenic Disturbances to Low Nutrient Reefs

Low levels of dissolved inorganic nutrients in reef waters (typical concentrations: NO$_3^-, NH_4^+: 0.05-0.5 \ \mu M$, NO$_2^- < 1 \ \mu M$, and PO$_4^{3-} < 0.2 \ \mu M$) are thought to confer a competitive advantage on Scleractinian corals. Because of their adaptation to low
nutrient waters, corals are highly susceptible to anthropogenic nutrient loading. A multitude of potential anthropogenic nutrient sources can significantly increase the concentrations of DIN in a reef habitat, highlighting the complexity of the effect of water quality management on coral reefs.

Coral reefs are vulnerable to nutrient loading from a variety of both natural and anthropogenic sources. Anthropogenic nutrient sources which may influence reefs include sewage outfalls and other sewage disposal systems (septic tanks etc.), fertilizer runoff (agricultural/golf courses), atmospheric deposition, submarine groundwater discharge and riverine discharge. Among natural sources of nitrogen to reefs are waste excretion from grazers, upwelling of deep NO$_3^-$ rich waters, and N$_2$ fixation activity by reef dwelling diazotrophs. Additionally, it is suspected that climate change is leading to changing rates of oceanic N$_2$ fixation, which has the potential to increase contributions of newly fixed N to coral reefs (Sherwood et al. 2014).

1.1.2 Response of Corals to Elevated Nitrogen Inputs

In light of several threats to the low nutrient environment of reefs as outlined above, the understanding of how corals are influenced by elevated nitrogen conditions is of great importance. DIN can be acquired by the coral-algal symbiosis from the surrounding seawater as NO$_3^-$, NO$_2^-$ or NH$_4^+$, or recycled from coral host catabolism as NH$_4^+$. Due to the oligotrophic environment, in which this symbiotic association flourishes, the algal symbionts are generally thought to be nitrogen limited under natural conditions and receive their primary nitrogen source from host catabolism. For example, experiments have found that after feeding corals, there is a positive growth increase in
symbionts (as compared with starved corals) suggesting that they are limited by the source of nitrogen available from coral waste products (Cook et al. 1988).

While many studies suggest that coral and algal symbiont nitrogen demands are met under low nutrient environments, several studies have shown the symbiosis to respond to increased environmental concentrations of NO$_3^-$ and NH$_4^+$. The biochemical requirements for the uptake and assimilation of NO$_3^-$ are different than for the uptake of NH$_4^+$, and the significant uptake of both by the coral-algal symbiosis has been measured by many workers (Muscatine and Delia 1978, Delia et al. 1983, Grover et al. 2002a, Grover et al. 2003). Bythell (1990) determined that uptake rates of NO$_3^-$ were twice that of NH$_4^+$ for Acropora palmata, while D Elia and Webb (1977) found the reverse with the uptake rate of NH$_4^+$ being twice that of NO$_3^-$ in Pocillopora sp.

*Mechanisms of Transport into the Cell*

Despite several studies of the influence of inorganic nitrogen on symbiotic corals, the mechanisms through which coral and host acquire inorganic nitrogen from seawater remain unclear. Prior to assimilation by the algal symbionts, inorganic nutrients in the surrounding seawater must pass through the animal gastrodermal cell membrane as well as through the symbiosome vacuole and into the dinoflagellate algal cell. This requires that the host tissue must play some role in uptake of nitrogen (Davy et al. 2012). There are several possible modes of uptake in the coral-algal symbiosis including simple passive diffusion, facilitated diffusion and active transport of molecules across the cell membranes.

The basic kinetic uptake models of these transport mechanisms are represented in Figure 1.1.1. Diffusion is the consequence of random, thermal motion, which will tend to
Figure 1.1.1 The kinetic models of biochemical uptake mechanisms: simple diffusion, facilitated or carrier mediated diffusion and active transport. The relationship between diffusion or transport rate and concentration is linear for simple diffusion and follows Michaelis Menten kinetics for facilitated diffusion and active transport.
elevate the intracellular concentrations of a particular molecule making them equal to extracellular concentrations. For a simple diffusion model of uptake, the diffusion rate depends only on the concentration of the molecule of interest inside and outside the cell, or is directly proportional to the concentration gradient. The uptake kinetics of passive diffusion follow a linear model. Alternatively, the uptake of a particular molecule may be passive, requiring no energy, but facilitated by a carrier protein. The maximum rate of uptake will occur when the carrier proteins become saturated with the solute. The uptake kinetics of facilitated diffusion typically follow a Michaelis Menten type curve. If molecules are transported across a membrane against their electrochemical gradient, the cell must expend some form of energy to transport the molecules. This transport requiring energy is referred to as active transport and can involve ATP-dependent pumps, light driven pumps, symporters and antiporters. The active transport uptake model also follows Michaelis Menten kinetics, reaching the maximum uptake rate when the transporters are saturated. In addition to these mechanisms of uptake, the inhibition of uptake by the presence of a particular molecule can also occur, for example, NH$_4^+$ inhibition of NO$_3^-$ uptake. The resulting kinetics follow an inverse hyperbola model, with uptake rate of the inhibited molecule increasing when the inhibitor has been drawn down to a particular concentration.

The Michaelis Menten equation for biochemical uptake is as follows:

\[ V = V_{max} \frac{S}{K + S} \]  

(1)

Where \( V \) is uptake rate at substrate concentration \( S \), \( V_{max} \) is the maximum uptake rate and \( K \) is the half saturation constant or the concentration of nutrient at which the uptake rate is half \( V_{max} \).
NH\textsubscript{3} is a potential candidate for uptake via simple diffusion, however, at neutral pH, 99% of NH\textsubscript{4}\textsuperscript{+}/NH\textsubscript{3} exists as NH\textsubscript{4}\textsuperscript{+} (Clegg and Whitfield, 1995). At pH of seawater in this study (8.1), approximately 3.8% of NH\textsubscript{4}\textsuperscript{+}/NH\textsubscript{3} exists as NH\textsubscript{3}. The coral and algal symbiont intracellular pH values have been measured to be more acidic than current average seawater pH of reef waters (in the range of 7.0 to 7.8 (Venn et al. 2009, Laurent et al. 2014)), but are thought to vary dependent on a variety of environmental and physiological factors. Previous workers have assumed NH\textsubscript{3} enters the coral tissues through passive diffusion (Miller and Yellowlees 1989b). NH\textsubscript{3} can freely diffuse across cell membranes, but diffusion of NH\textsubscript{4}\textsuperscript{+} across the cell membrane is extremely slow and the transport of NH\textsubscript{4}\textsuperscript{+} across biological membranes must be carrier-mediated. It has been estimated that coral tissue intracellular concentrations of NH\textsubscript{4}\textsuperscript{+} are in the range of 5 to 50 \(\mu\text{M}\), as compared to submicromolar levels in typical oligotrophic reef waters (Crossland and Barnes 1977). Once diffused into the symbiosome most NH\textsubscript{3} will likely be protonated and NH\textsubscript{4}\textsuperscript{+} must be carried across the algal cell membrane and into the algal cell (Miller and Yellowlees 1989b, Davy et al. 2012).

Recent studies have shed some light on the role of active ammonium transport as a mechanism for acquisition of NH\textsubscript{4}\textsuperscript{+} by algal symbionts. Leggat et al. (2007) identified genes coding for NH\textsubscript{4}\textsuperscript{+} transport proteins in an expressed sequence tag study of isolated Symbiodinium microadriaticum. These proteins were determined to be closely related to bacterial NH\textsubscript{4}\textsuperscript{+} transport proteins. In other systems, NH\textsubscript{4}\textsuperscript{+} can be transported through the cell membrane on binding sites for K\textsuperscript{+} because it shares the same ionic radius as hydrated K\textsuperscript{+} (Knepper et al. 1989). Among proposed mechanisms for NH\textsubscript{4}\textsuperscript{+} transport into other animals cells are active transport through Na\textsuperscript{+}/K\textsuperscript{+} ATPase (Post and Jolly 1957),
facilitated diffusion with a $\text{Na}^+\text{K}^+\text{2Cl}^-$ cotransporter (Good et al. 1984, Knepper et al. 1989) or through a $\text{Na}^+/\text{H}^+$ exchanger (Grinstein and Rothstein 1986, Knepper et al. 1989). In considering transfer of $\text{NH}_4^+$ across cell membranes, it should also be noted that $\text{NH}_4^+$ is generated within the animal cells in the mitochondria through glutaminase and glutamate dehydrogenase activity (Kovacevic and McGivan 1983). This catabolic production of $\text{NH}_4^+$ is thought to contribute to elevated levels of $\text{NH}_4^+$ in the coral cytoplasm (Falkowski, 1993).

Intracellular NO$_3^-$ concentrations of coral tissues and algal symbionts are not known. If similar to other marine unicellular algae, the algal intracellular NO$_3^-$ concentrations have the potential to be very high, several orders of magnitude higher than the surrounding seawater. Intracellular NO$_3^-$ concentrations measured for three diatoms, *Thalassiosira pseudonana, T. weisflogii and T. rotula*, exposed to NO$_3^-$ incubations in the laboratory exceeded intracellular concentrations of 200 mM NO$_3^-$. It is accepted that marine unicellular algae use active transporters on the cell surface to transport NO$_3^-$ into the cell (Needoba and Harrison 2004). The transport mechanism of NO$_3^-$ across animal tissues is not clear as animals generally do not assimilate NO$_3^-$, but previous workers have assumed that NO$_3^-$ is passively diffused through coral tissue membranes (Miller and Yellowlees 1989a). Early studies proposed a diffusion-depletion model of nutrient supply where nutrients diffuse along a concentration gradient from the host cell cytoplasm into the symbiont cell in response to the symbionts nutrient demands (Delia et al. 1983). The study of *Symbiodinium microadriaticum* by Leggat et al. (2007) discovered expressed sequence tags for nitrate and nitrite transporter enzymes, supporting the idea that NO$_3^-$ is actively pumped across the algal cell membrane. As the symbionts
deplete NO$_3^-$ in the coral cells, more nitrogen diffuses into coral tissues from the external environment. Miller and Yellowles (1989b) found that the diffusion-depletion model applied for NH$_4^+$ uptake, but not for NO$_3^-$ or PO$_4^{3-}$. Other studies investigating nutrient uptake for intact symbioses have suggested carrier-mediated uptake accompanied by diffusion (Muscatine and Delia 1978, Wilkerson and Trench 1986).

*Mechanisms of Assimilation*

Once NO$_3^-$ and NH$_4^+$ ions have been transported to the site of assimilation, the mechanisms for their assimilation must be considered. The enzymes necessary for NO$_3^-$ assimilation, nitrate and nitrite reductases, have been detected in algal symbionts (Crossland and Barnes 1977) and the gene sequence for nitrite reductase has been measured in type C3 symbionts from *Acropora aspera* (Leggat et al. 2007). While it is accepted that NO$_3^-$ assimilation occurs in the symbiont cells (Kopp et al. 2013), there are different views on NH$_4^+$ assimilation. Some studies suggest that both algal symbionts and coral host directly assimilate NH$_4^+$ with evidence of high levels of NH$_4^+$ assimilation enzymes (NADPH-GDH and GS) detected in host tissues (Catmull et al. 1987, Dudler and Miller 1988, Miller and Yellowles 1989b, Rahav et al. 1989, Wang and Douglas 1998, Grover et al. 2002a, Lipschultz and Cook 2002). It has been observed, however, that NH$_4^+$ assimilation occurs primarily in the symbionts (Miller and Yellowles 1989b, Wang and Douglas 1998, Grover et al. 2002a) with an estimate of algal symbiont cells directly assimilating 14 to 23 times more NH$_4^+$ than coral host cells (Pernice et al. 2012). The assimilation of NH$_4^+$ in coral host tissues proposed to occur is thought to be primarily through the NADPH dependent glutamate dehydrogenase pathway (NADPH-
GDH), catalyzing the synthesis of glutamate from NH$_3$ (Miller and Yellowlees 1989b, Roberts et al. 2001) as follows:

**NADPH-GDH Pathway**

\[ \text{NH}_3 + 2\text{-oxoglutarate} + \text{NADPH} + \text{H}^+ \rightarrow \text{glutamate} + \text{NADP}^+ \]

The algal symbionts are thought to catalyze glutamate from NH$_3$ primarily through the glutamate synthetase/glutamine 2-oxoglutarate amido transferase pathway (GS/GOGAT). GOGAT catalyzes the reductive transfer of the amide-amino group of glutamine to \(\alpha\)-ketoglutarate to produce two glutamates (Yellowlees et al. 1994):

**GS-GOGAT Pathway**

\[ \text{NH}_3 + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi} \]

\[ \text{glutamine} + 2\text{-oxoglutarate} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{ glutamate} + \text{NADP}^+ \]

**Light Requirements for Uptake and Assimilation**

In order to assimilate dissolved inorganic nitrogen and utilize it for synthesis of amino acids, the coral host requires carbon skeletons and reducing power (Miller and Yellowlees 1989b, Turpin et al. 1990, Wang and Douglas 1998). This would suggest that DIN uptake and assimilation may occur only when the symbionts are photosynthetically active, receiving sufficient amounts of light. Studies have, however, measured continued uptake of NO$_3^-$ and NH$_4^+$ in the dark (D Elia and Webb 1977, Muscatine and Delia 1978). It is suggested that this may be possible due to use of photosynthetic energy stores from prior light exposure providing energy necessary for uptake. Conversely, measurements of uptake in the dark after a pre-conditioned dark period were made for a symbiotic clam (*Tridacna gigas*) and the results showed net release of NH$_4^+$ during this period (Wilkerson and Trench 1986). This could suggest that after extended periods without
light, this particular symbiotic association does not have the energy required for uptake of nitrogen. There is limited literature available documenting the behavior of NO$_3^-$ uptake during a dark period. When considering NO$_3^-$ uptake in the dark, it is possible that the enzymes, nitrate and nitrite reductase are not active without a source of reducing energy from photosynthetic products, thus preventing NO$_3^-$ assimilation and further uptake during a dark period.

Coral Mediated Uptake of Nitrogen

It has been proposed that the coral host may actively impose nutrient limitation on the symbionts (Cook et al. 1992, Weis 1993, Cook et al. 1994). This is a suggested mechanism for the coral host to control the density of its algal symbionts but it is unclear as to how this regulation is accomplished. One potential strategy in response to elevated NH$_4^+$ may be to directly increase NH$_4^+$ assimilation in coral host tissues to limit availability to symbionts (Miller and Yellowlees 1989b).

1.1.3 Experimental Objectives

These experiments were carried out in order to determine the uptake kinetics of NO$_3^-$ and NH$_4^+$ under natural and a range of elevated concentrations. The long term influence of elevated NO$_3^-$ and NH$_4^+$ concentrations on uptake rates was investigated as well as the influence of light on uptake rates. While $^{15}$N labeling experiments can be useful to calculate DIN uptake rates by corals and algal symbionts, much useful information concerning kinetics of uptake can also be obtained through DIN concentration measurements made throughout an incubation period. By using a wide range of concentrations between 0 - 50+ $\mu$M DIN, useful information can be acquired on the variety of mechanisms available to the coral-algal symbiosis to take up and assimilate
NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+}, as well as develop insight on the intracellular nitrogen environment of the algal symbiont and coral host tissues.

1.2 Methodology

1.2.1 Experimental Specimens

Specimens of the Pacific branching coral *Pocillopora damicornis* were collected off the coast of Panama in 2007. The corals were analyzed using the quantitative polymerase chain reaction method (qPCR) in order to determine the algal symbiont (*Symbiodinium microadriaticum*) type. The corals were “common gardened” at the University of Miami experimental hatchery indoors for five years prior to experimentation. The corals experienced conditions of relatively constant temperature and light on a 12 hour light/12 hour dark cycle, and were fed an algal protein mixture once weekly. Corals were maintained in flowing seawater pumped in directly from Bear Cut, Miami, FL and experienced natural fluctuations in salinity. For this experiment, two genets of *Pocillopora damicornis* hosting clade C symbionts, and five genets of *P. damicornis* hosting clade D symbionts were chosen. Only corals hosting clade C symbionts are discussed in this chapter. 200 Corals were fragmented from larger parent colonies and fixed to PVC plates specialized for growth and calcification measurements via the scanning optical micrometer method (Langdon Lab (Albright et al. 2010)) and the buoyant weight technique (Jokiel et al. 1978).

1.2.2 Experimental Conditions

Corals were subject to experimental nutrient levels for a period of four weeks. Treatment conditions included Control, 10, 20, and 50 µM NaNO\textsubscript{3} and 10, 20, and 50 µM NH\textsubscript{4}Cl. Controls consisted of 0.2 micron filtered seawater pumped in from Bear Cut
(average NO$_3^-$, NH$_4^+$ <1 μM), while treatments consisted of the same seawater utilized for Control with the addition of NaNO$_3$ or NH$_4$Cl to achieve the desired final concentration. Treatment tanks consisted of 1 gallon glass tanks filled with 2.5 liters of seawater with four tanks assigned to each treatment. Each tank contained six coral fragments for a total of 240 coral fragments distributed among 40 tanks. Seawater in tanks was changed at the beginning of each day with appropriate nutrient additions. The 1 gallon tanks were maintained inside a larger flow thru system to equilibrate temperatures, however, individual tanks were isolated from flowing seawater. Temperature was held constant indoors during the four week experimental period at 26± 0.5⁰ C. Light was supplied by USA Nova Current Extreme lights on a 12 hour light/12 hour dark cycle. Circulation within each tank was provided by a Hydor Koralia Nano mini pump.

1.2.3 Sample Collection

Water samples were collected throughout a 48 hour period on a 12 hour light/12 hour dark cycle at 0, 4, 12, 24, 36 and 48 hours and then during a period of 48 hours of light only at 0, 4, 12, 24 and 48 hours. Results refer to data from the 12 hour light/12 hour dark uptake meant to replicate natural cycles, unless specified as light only incubation data, where light and dark uptake rates are compared. Filtered water samples were collected in 125 ml Nalgene (polyethylene) bottles and acidified to pH 2 with the addition of HCl for preservation (Capone 2008).

1.2.4 NO$_3^-$ and NH$_4^+$ Concentration Analysis and Uptake Calculations

The concentrations of NO$_3^-$ and NH$_4^+$ were determined colorimetrically with a SmartChem200 Autoanalyzer (Westco Scientific). The NO$_3^-$ concentrations were
determined via the vanadium chloride method and the $\text{NH}_4^+$ concentrations were measured through the indophenols-blue method (Berthelot reaction).

Uptake rates are presented as normalized to coral surface area and symbiont density. The values given as “instantaneous” uptake rates are rates calculated between two consecutive sampling times (ex. $T_n$ to $T_{n+1}$) and are meant to represent the “instantaneous” concentration (average concentration of $T_n$ and $T_{n+1}$) at which the uptake rate was obtained.

1.2.5 Determination of Coral Surface Area

Nutrient uptake data is normalized to coral surface area. Coral surface area was determined by modeling individual coral branches as cylinders. The height and diameter of each branch was measured with a digital micrometer and surface area calculations were carried out.

1.2.6 Algal Symbiont Density

Measurements of symbiont density were achieved through manual counts with a Neubauer Bright-Line Hemacytometer. Coral tissues were removed from subsamples of approximately 1 cm branches from each treatment. Complete tissue removal was ensured by dissolving the coral skeleton in 18% HCl. There was no tissue or symbiont loss as a result of the HCl treatment and all tissue remained intact in one piece after dissolution. The tissue mass was homogenized with a Tissue Master Automated Homogenizer in a solution of 1.5 mL 2% buffered glutaraldehyde in seawater and 0.25 mL of Lugols solution (20g KI, 10g I$_2$ in 200 mL deionized water). Six to ten replicate aliquots were counted for each sample. Counts were normalized to surface area obtained with a white light 3D scanner (HDI Advance R2, 3D3 Solutions, see methods of (Enochs et al. 2014)).
1.2.7 Statistical Analyses

Data analysis was carried out with JMP 11.0 Statistical Analysis Software. When determining best fit regression models for uptake kinetics, the corrected Akaike’s Information Criterion (AICc) was calculated. The best fit model results in calculation of the lowest AICc score which considers both fit and parsimony when determining the best fit model to avoid selection of a model which is unnecessarily complex.

1.3 Results

1.3.1 NO$_3^-$ Uptake by Treatment

After four weeks of experimental high NO$_3^-$ conditions, there was a treatment effect on total uptake of NO$_3^-$ after 12, 24, 36 and 48 hours of the 48 hour uptake incubation (ANOVA, 12 hrs: $F = 9.95$, $p = 0.0251$; 24 hrs: $F = 31.52$, $p=0.003$; 36 hrs: $F = 35.81$, $p = 0.0024$; 48 hrs: $F = 30.65$, $p = 0.0032$) (Figure 1.3.1). The total uptake was higher in all elevated NO$_3^-$ treatments than in Controls (Tukey-Kramer method, $p<0.05$). After 24 hours, corals in the 50 $\mu$M NO$_3^-$ treatment had consumed an average of 544.55 ± 84.73 nmoles N cm$^{-2}$ compared to Control corals which had consumed 61.59 ± 16.83 nmoles N cm$^{-2}$. After 48 hours, corals in the 50 $\mu$M NO$_3^-$ treatment had consumed significantly more NO$_3^-$ than those in the 10 $\mu$M treatment, however, the differences
Figure 1.3.1 The average amount of $\text{NO}_3^-$ uptake relative to coral surface area throughout the duration of light/dark cycle incubation in Control, 10, 20 and 50 $\mu$M $\text{NO}_3^-$ treatment tanks. Shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
between 50 µM NO$_3^-$ and 20 µM NO$_3^-$ treatments and the 10 µM NO$_3^-$ and 20 µM NO$_3^-$ treatments were not significant (Tukey-Kramer method, p<0.05).

1.3.2 NO$_3^-$ Uptake in the Presence of Elevated NH$_4^+$

The uptake of ambient levels of NO$_3^-$ (~1.5 µM) in Controls compared to uptake of ambient levels of NO$_3^-$ in elevated NH$_4^+$ treatments (10, 20 and 50 µM NH$_4^+$) is shown in Figures 1.3.2a and 1.3.2b. There was a significant treatment effect of elevated NH$_4^+$ on the uptake of NO$_3^-$ at 4, 12 and 24 hours (ANOVA, 4 hrs: F = 41.93, p=0.0018; 12 hrs: F=10.62, p =0.0224; 24 hrs: F=8.83, p=0.0308). The uptake of NO$_3^-$ by Controls was significantly higher than all elevated NH$_4^+$ treatments at 4 and 12 hours of the incubation (Tukey-Kramer method, p<0.05). At 24 hours, when concentrations of NH$_4^+$ were reduced to an average of 3.3 ± 0.11 µM in the 10 µM NH$_4^+$ treatment tanks, the uptake of NO$_3^-$ increased in the 10 µM NH$_4^+$ and was not significantly less than Controls (Tukey-Kramer method, p>0.05). At 36 hours, when the concentrations of NH$_4^+$ were reduced to an average of 3.35 ± 0.88 µM in the 20 µM NH$_4^+$ treatment tanks, the uptake of NO$_3^-$ increased and was not significantly less than Controls or 10 µM NH$_4^+$ treatment tanks. Throughout the entire 48 hour incubation, the uptake of NO$_3^-$ by corals in the 50 µM NH$_4^+$ treatment tanks was significantly lower than Controls (students t-test, p<0.05).

After 24 hours, the average NO$_3^-$ uptake was more than five times higher in Controls than in the 50 µM NH$_4^+$ treatments, with 61.59 ± 16.83 nmoles N cm$^{-2}$ in Controls and 11.14 ± 5.62 nmoles N cm$^{-2}$ in the 50 µM NH$_4^+$ treatment tanks.

1.3.3 NO$_3^-$ Uptake in Light Only and Light/Dark Cycle Conditions

The uptake of NO$_3^-$ in light only and light/dark conditions is shown in Figure 1.3.3. The time period from 12-24 hours represents the dark period from the light/dark
Figure 1.3.2a The depletion of ambient levels of NO$_3^-$ (~1.5 µM) in Control tanks compared to elevated NH$_4^+$ treatments (10, 20 & 50 µM NH$_4^+$) is shown. Note the raw data is not normalized to coral surface area, thus error bars would have no significance and are not included.
Figure 1.3.2b The uptake of ambient levels of NO$_3^-$ (~1.5 μM) in Control tanks compared to uptake of ambient levels of NO$_3^-$ in elevated NH$_4^+$ treatments (10, 20 & 50 μM) is shown. The amount taken up normalized to coral surface area throughout the incubation is shown. Each error bar is constructed using 1 standard deviation from the mean.
Figure 1.3.3 The comparison of uptake rates of NO$_3^-$ during a light (blue) and dark (red) 12 hour period. Dark measurements were collected during hours 12-24 of the 48 hour light/dark incubation. Light measurements were collected during hours 12-24 of the 48 hour light only incubation. Error bars represent one standard deviation.
incubation and these measurements are compared to measurements made during a corresponding light period from a 48 hour light only incubation. The light only incubation was carried out in order to compare light and dark rates of uptake which occurred at approximately the same initial NO$_3^-$ concentrations, rather than comparing consecutive time periods in one 48 hour incubation, as changing concentration throughout the 48 hour period may influence uptake. The dark uptake rates during this period are significantly lower than those of the light uptake for the 20 and 50 µM NO$_3^-$ treatments (t-test, p<0.05). The average uptake in light at 50 µM NO$_3^-$ during this period was 0.019 µmoles cm$^{-2}$ hr$^{-1}$, while in the dark, the rate of uptake was 0.009 µmoles cm$^{-2}$ hr$^{-1}$. Dark uptake rates were equivalent to 74.7 ± 1.2 % of light uptake for corals grown in 10 µM NO$_3^-$, 26.8 ± 8.6 % of light uptake for corals grown in 20 µM NO$_3^-$ and 46.7 ± 1.2% of light uptake for corals grown in 50 µM NO$_3^-$.

### 1.3.4 Initial and Final NO$_3^-$ Uptake

Initial and final measurements of NO$_3^-$ uptake made at the beginning of the experiment and after four weeks are compared in Figure 1.3.4. There were significant increases in the average NO$_3^-$ uptake in 24 hours between initial and final sampling (µmoles cm$^{-2}$) during the uptake incubation in 10, 20 and 50 µM NO$_3^-$ (t-test, p<0.05). There were no significant differences in the NO$_3^-$ uptake of Controls from initial to final sampling (t-test, p>0.05). The initial uptake of NO$_3^-$ after 24 hours in the 50 µM treatment was 0.20 ± 0.08 µmoles cm$^{-2}$ and after four weeks of experimental conditions, increased to 0.54 ± 0.09 µmoles cm$^{-2}$.
Figure 1.3.4: The uptake of NO$_3$ relative to coral surface area for Controls, 10, 20 & 50 μM NO$_3$ treatments during the light dark incubations. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
1.3.5 NO\textsubscript{3}\textsuperscript{-} Uptake Normalized to Symbiont Density

The uptake of NO\textsubscript{3}\textsuperscript{-} after normalization to coral symbiont density is shown as pmoles NO\textsubscript{3}\textsuperscript{-} consumed per symbiont cell in Figure 1.3.5. The average uptake of NO\textsubscript{3}\textsuperscript{-} per symbiont after 24 hours significantly increased after the four week experimental period for 10 & 20 µM NO\textsubscript{3}\textsuperscript{-} treatments (t-test, p<0.05). While the difference in uptake in the 50 µM NO\textsubscript{3}\textsuperscript{-} treatment from initial to final was not significant at p<0.05, the average NO\textsubscript{3}\textsuperscript{-} uptake in the final incubation (0.30 ± 0.02 pmol NO\textsubscript{3}\textsuperscript{-} per symbiont) was approximately twice that of the initial (0.18 ± 0.12 pmol NO\textsubscript{3}\textsuperscript{-} per symbiont). There were no significant differences in the initial and final uptake of NO\textsubscript{3}\textsuperscript{-} in Controls (t-test, p>0.05).

1.3.6 Modeling NO\textsubscript{3}\textsuperscript{-} Uptake Kinetics

Uptake kinetics of NO\textsubscript{3}\textsuperscript{-} during the final incubation (after 4 weeks) were fit to both a linear regression and the Michaelis Menten model using JMP 11.0 (Figure 1.3.6A). When fitting the final uptake data set, the Michaelis Menten model provided a better fit over a simple linear regression ($R^2_{\text{Michaelis Menten}} = 0.68$, $R^2_{\text{linear}}=0.54$, $\text{AIC}_{\text{Michaelis Menten}} < \text{AIC}_{\text{linear}}$, Figure 1.3.6A). The model calculated a maximum uptake rate ($v_{\text{max}}$) of 0.037 ± 0.003 µmoles cm\textsuperscript{-2} hr\textsuperscript{-1} and a $K_{\text{m}}$ of 5.70 ± 2.09. Uptake kinetics of NO\textsubscript{3}\textsuperscript{-} during the initial incubation were fit to both a linear regression and the Michaelis Menten model using JMP 11.0 (Figure 1.3.6B). While the model was not as well defined as for the final incubation, the Michaelis Menten model provided a better fit than a linear model. The maximum uptake rate ($v_{\text{max}}$) of 0.0080 µmoles cm\textsuperscript{-2} hr\textsuperscript{-1} and the $K_{\text{m}}$ of 0.53.

1.3.7 NH\textsubscript{4}\textsuperscript{+} Uptake by Treatment

After four weeks of experimental conditions, there was a treatment effect of elevated NH\textsubscript{4}\textsuperscript{+} on total uptake of NH\textsubscript{4}\textsuperscript{+} at all sampling times (4, 12, 24, 36 and 48 hours
Figure 1.3.5 The uptake of NO$_3$ relative to symbiont cells for Controls, 10, 20 & 50 μM NO$_3$ treatments. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). Each error bar is constructed using 1 standard deviation from the mean.
Figure 1.3.6 The uptake kinetics of NO$_3^-$ by the coral-algal symbiosis under NO$_3^-$ concentrations from 0 to 50 μM, fit to linear (blue) and Michaelis Menten (green) kinetic models. The data from the initial incubation are on the right, and the data from the final incubation after 4 weeks in elevated NO$_3^-$ are on the left. Data are from all treatment tanks in both light and light/dark condition from the initial incubations. The “instantaneous” rate given is the rate of change between two consecutive samples (e.g., from t$_i$ to t$_{i+1}$), and the “instantaneous” concentration is the average of the NO$_3^-$ concentrations of the two samples from which the rate was calculated. Instantaneous rates were calculated from uptake data collected from 12 to 48 hours of the 48 hour incubations.
of incubation (ANOVA, 4 hrs: F = 20.63, p = 0.0068; 12 hrs: F = 33.92, p = 0.0026; 24 hrs: F = 35.07, p = 0.0025; 36 hrs: F = 40.45, p = 0.0019; 48 hrs: F = 37.94, p = 0.0021).

After 24 hours, the total NH$_4^+$ uptake in 20 and 50 µM NH$_4^+$ treatments were not significantly different, but both treatments had higher uptake than 10 µM NH$_4^+$ treatment. All concentrations of elevated NH$_4^+$ had significantly higher uptake than Controls (Tukey-Kramer method, p<0.05). After 48 hours, corals in the 50 µM NH$_4^+$ treatment had consumed an average of 1.84 µmoles N cm$^{-2}$ as opposed to Control corals which had consumed 0.051 µmoles N cm$^{-2}$ (Figure 1.3.7).

1.3.8 NH$_4^+$ Uptake in Light Only and Light/Dark Cycle Conditions

The uptake of NH$_4^+$ in light only and light/dark conditions is shown in Figure 1.3.8. The time period from 12-24 hours represents the dark period from the light/dark incubation and these measurements are compared to measurements made during a corresponding light period from a 48 hour light only incubation. The light only incubation was carried out in order to compare light and dark rates of uptake which occurred at approximately the same initial NH$_4^+$ concentrations, rather than comparing consecutive time periods one 48 hour incubation, as changing concentration throughout the 48 hour period may influence uptake. There were no significant differences during this period between light and dark uptake rates in Controls, 10 and 20 µM NH$_4^+$ treatments (t-test, p<0.05). The light and dark uptake rates in the 50 µM NH$_4^+$ treatment were significantly different (t-test, p<0.05), with dark uptake rates equivalent to 33.6 ± 3.8 % of light uptake rates.
Figure 1.3.7 The average amount of NH$_4^+$ uptake relative to coral surface area throughout the duration of the light/dark cycle incubation in Control, 10, 20 and 50 μM NH$_3$ treatment tanks. The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 1.3.8 The comparison of uptake rates of NH$_4$ during a light (blue) and dark (red) 12 hour period. Dark measurements were collected during hours 12-24 of the 48 hour light/dark incubation. Light measurements were collected during hours 12-24 of the 48 hour light only incubation.
1.3.9 Initial and Final NH$_4^+$ Uptake

Initial and final measurements of NH$_4^+$ uptake made at the beginning of the elevated NH$_4^+$ experiment and after four weeks are compared in Figure 1.3.9. There were no significant differences in the average uptake of NH$_4^+$ ($\mu$moles cm$^{-2}$) between initial and final sampling during the uptake incubation in Controls, 10 or 20 $\mu$M NH$_4^+$ treatments (t-test, p>0.05). The average uptake by corals in the 50 $\mu$M NH$_4^+$ treatment, however, significantly increased from initial to final sampling at 24, 36 and 48 hour sampling (t-test, p<0.05).

1.3.10 NH$_4^+$ Uptake Normalized to Symbiont Density

When the uptake data is normalized to symbiont density, there are no differences in uptake of NH$_4^+$ per symbiont from initial to final sampling during the uptake incubations in Controls, 10, 20 and 50 $\mu$M NH$_4^+$ treatments (Figure 1.3.10) (t-test, p>0.05). During the initial incubation after 24 hours in the 50 $\mu$M NH$_4^+$ treatment, the uptake normalized to symbiont density was 0.56 ± 0.15 pmoles NH$_4^+$ per symbiont cell. Similarly after four weeks of experimental conditions the uptake normalized to symbiont density was 0.54 ± 0.07 pmoles NH$_4^+$ per cell.

1.3.11 Modeling NH$_4^+$ Uptake Kinetics

When modeling NH$_4^+$ uptake, the simple linear regression and Michaelis Menten model provided a poor fit for the data set as a whole (Figure 1.3.11). This suggested that there were two NH$_4^+$ concentration dependent factors influencing uptake rate. The data were separated into two different data sets, A: instantaneous concentrations less than 42 $\mu$M NH$_4^+$, and B: instantaneous concentrations greater than 42 $\mu$M NH$_4^+$ (Figure 1.3.12). The linear and Michaelis Menten models were then fit to data sets A and B. It was
Figure 1.3.9 The uptake of NH$_4$ relative to coral surface area for Controls, 10, 20 & 50 μM NH$_4$ treatments in the light/dark incubations. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). The shaded areas represent dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 1.3.10 The uptake of $\text{NH}_4^+$ relative to symbiont cells for Controls, 10, 20 & 50 $\mu$M $\text{NH}_4^+$ treatments during the light/dark incubations. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). Shaded areas represent dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 1.3.11 The uptake kinetics of NH$_4^+$ by the coral-algal symbiosis under NH$_4^+$ concentrations from 1.99 to 52.67 μM. Data are from all treatment tanks in both light and light/dark conditions, and data from the initial incubations is shown on the right and data collected after 4 weeks of elevated NH$_4^+$ treatment conditions is on the left. The "instantaneous" rate given is the rate of change between two consecutive samples (i.e., from $t_i$ to $t_{i+1}$), and the "instantaneous" concentration is the average of the NH$_4^+$ concentration of the two samples from which the rate was calculated. Instantaneous rates were calculated from uptake data collected from 4 to 36 hours of the 48 hour incubations. The lines represent a smooth of each data set.
Figure 1.3.12 The uptake kinetics of $\text{NH}_4^+$ by the coral-algal symbiosis under $\text{NH}_4^+$ concentrations from 1.99 to 60 $\mu$M, fit to linear (blue) and Michaelis Menten (green) kinetic models. The data were separated into concentration ranges below and above 42 $\mu$M $\text{NH}_4^+$. Data are from all treatment tanks in both light and light/dark condition from the final incubations after four weeks in elevated $\text{NH}_4^+$ treatments. The “instantaneous” rate given is the rate of change between two consecutive samples (ex. from $t_1$ to $t_2$), and the “instantaneous” concentration is the average of the $\text{NH}_4^+$ concentration of the two samples from which the rate was calculated. Instantaneous rates were calculated from uptake data collected from 4 to 36 hours of the 48 hour incubations.
determined that the Michaelis Menten model provided the best fit at concentrations less than 42 µM NH₄⁺ (Maximum Instantaneous Uptake Rate = 0.129 ± 0.023 μmoles cm⁻² hr⁻¹, Kₘ = 24.37, R² = 0.80, AIC_{Michaelis Menten} < AIC_{Linear}) and the linear model was best fit at concentrations greater than 42 µM NH₄⁺ (Instantaneous Uptake Rate = 0.0062[NH₄⁺] – 0.26, R² = 0.57, AIC_{Linear} < AIC_{Michaelis Menten}). The same approach was taken for the kinetic data from the initial incubation before 4 weeks of elevated NH₄⁺ conditions (Figure 1.3.13). Comparing the kinetics of initial and final uptake at concentrations above 40 µM NH₄⁺, the slope of the linear regression between instantaneous uptake rate and NH₄⁺ concentration changes. The slope increases from 0.0027 at initial sampling (“Instantaneous” Uptake Rate = 0.0027 [NH₄⁺] - 0.05) to 0.0062 after four weeks at final sampling (“Instantaneous” Uptake Rate = 0.0062[NH₄⁺] – 0.26).

1.4 Discussion

1.4.1 Concentration Dependent Uptake of NO₃⁻ and NH₄⁺

This study clearly demonstrated an increased uptake of NO₃⁻ and NH₄⁺ under elevated exposure to these nutrients. These findings are in agreement with previous work (Muscatine and Delia 1978, Wilkerson and Muscatine 1984b, Grover et al. 2002b). It is generally accepted that when nutrient uptake increases with exposure to elevated concentrations, the organism is limited by that nutrient. There are, however, possible exceptions when the uptake of a particulate nutrient may be for the purpose of depleting concentrations within the tissue. An example of such might be to maintain an electrochemical gradient or impose limitation on a symbiotic partner (Miller and Yellowlees 1989b). All elevated NO₃⁻ treatments took up significantly more dissolved inorganic nitrogen than the Controls, with an average increase of 1098% in nitrogen
Figure 1.3.13 The uptake kinetics of NH$_4^+$ by the coral-algal symbiosis under NH$_4^+$ concentrations from 1.99 to 60 μM, fit to linear (blue) and Michaelis-Menten (green) kinetic models. The data were separated into concentration ranges below and above 42 μM NH$_4^+$. Data are from all treatment tanks in both light and light/dark conditions from the initial incubations. The "instantaneous" rate given is the rate of change between two consecutive samples (e.g., from $t_1$ to $t_2$), and the "instantaneous" concentration is the average of the NH$_4^+$ concentration of the two samples from which the rate was calculated. Instantaneous rates were calculated from uptake data collected from 4 to 36 hours of the 48 hour incubations.
acquired from the ambient NO$_3^\text{-}$.

This increase demonstrates the impact that a pulse of elevated NO$_3^\text{-}$ can have on the sources of nitrogen to corals and their endosymbiotic algae.

There are several hypotheses that might explain the increase in NO$_3^\text{-}$ uptake rate with increasing concentration. Increasing seawater NO$_3^\text{-}$ could lead to extracellular concentrations which exceed intracellular coral tissue NO$_3^\text{-}$, altering the concentration gradient to be more favorable for diffusive transport of NO$_3^\text{-}$ into coral tissues. Alternatively, the increase in uptake under elevated external NO$_3^\text{-}$ concentrations could be due to activation of enzymes or carrier proteins which are responsible for the transport of NO$_3^\text{-}$ into the cell, or increased activity of enzymes which are responsible for NO$_3^\text{-}$ assimilation (nitrate and nitrite reductases). Rates of diffusion into coral tissues may increase as a result of increased enzyme activity or as increased transport activity into the algal cells. Alternatively, increased nitrate reductase activity within the algal cells may result in more transport of nitrate across the algal cell membrane and thus more rapid draw down of the coral intracellular NO$_3^\text{-}$ pool maintaining a concentration gradient that promotes higher rates of NO$_3^\text{-}$ diffusion from seawater into coral tissues.

Uptake of NH$_4^+$ in all elevated NH$_4^+$ treatment concentrations was higher than within Control tanks, and all treatments had taken up different amounts of NH$_4^+$ after 48 hours, with uptake amount positively correlating with NH$_4^+$ treatment concentration. The increasing uptake of NH$_4^+$ with increasing concentration could be due to creating a favorable concentration gradient for diffusive transport of NH$_3$ into coral tissues or algal cells or could be a result of the activation of enzymes for active NH$_4^+$ transport or
increased activity of assimilation enzymes. Previous studies demonstrate that both coral and symbionts possess the capability to assimilate NH$_4^+$, so increased uptake with concentration increases could be attributed not only to increased assimilation by symbionts, but also could result from an increase in assimilation rates directly within coral host tissues.

1.4.2 Kinetics of NO$_3^-$ and NH$_4^+$ Uptake

There are limited data describing the nitrogen uptake kinetics by the coral-algal symbiosis and there have been mixed results on the kinetic behavior of NO$_3^-$ and NH$_4^+$ uptake and assimilation by symbiotic corals. Michaelis Menten kinetic behavior is typical of any uptake/assimilation mechanism in which the rate of transport becomes limited by the saturation of a particular enzyme or carrier protein at a maximum concentration, above which the uptake rate will not be increased. For membrane transport, both mechanisms of carrier mediated transport and active transport will follow Michaelis Menten type kinetics. Linear kinetics are typical of simple passive diffusion, where the molecule of interest can freely move across the cell membrane and the rate of membrane transport is dependent only on the concentration gradient between intracellular and extracellular concentrations.

One study demonstrated that NO$_3^-$ uptake by the coral *Pocillopora elegans* followed Michaelis Menten kinetics (D Elia and Webb 1977). Some workers have found linear behavior of NH$_4^+$ uptake kinetics for cnidarian symbioses, with a direct relationship between substrate concentration and uptake rate in the sea anemone *Aiptasia pulchella* over the range of 0.7-10 µM NH$_4^+$. Others have found NH$_4^+$ uptake to follow Michaelis Menten saturation kinetics (D Elia and Webb 1977). This variability in kinetic
models among studies could be due to the different range of nutrient concentrations employed to measure uptake kinetics.

*Nitrate*

In this study, NO$_3^-$ uptake followed a Michaelis Menten model, in which the uptake rate was saturated at 0.036 μmoles N cm$^{-2}$ coral skeleton. Literature data for NO$_3^-$ uptake by unicellular marine algae suggests that active transporters are responsible for moving NO$_3^-$ across the algal cell membrane, because algal intracellular concentrations of NO$_3^-$ are typically orders of magnitude higher than the concentrations in seawater (Needoba and Harrison 2004). Although information concerning NO$_3^-$ transport into animal and specifically coral tissues is limited, previous models have assumed that NO$_3^-$ diffuses into coral tissues. To our knowledge, no known nitrate transporters have been identified in coral host tissues. If this is accepted, then saturation of uptake is likely related to the uptake or assimilation by algal symbionts and potentially due to saturation of algal enzymes. The uptake rates could reach a maximum at saturation of active transporters at the surface of the algal cell membrane. Alternatively, the maximum uptake rate could be saturated as a reflection of nitrate reductase activity inside the algal symbionts.

It is accepted that corals do not have the ability to directly assimilate NO$_3^-$ (Grover et al. 2003), so any assimilation to organic nitrogen must occur within the algal symbionts. The intracellular NO$_3^-$ concentrations of unicellular marine algae are typically in the millimolar range and have been shown to increase in various diatoms as a function of elevated external seawater NO$_3^-$ concentrations (in the micromolar range) (Needoba and Harrison 2004). As the nitrate reductase enzymes become saturated, there may be
greater efflux or leakage of excess NO$_3^-$ from algal cells which is not immediately
reduced to organic nitrogen. This increased efflux of algal symbiont intracellular NO$_3^-$
with increased NO$_3^-$ concentrations would result in an apparent maximum uptake rate at a
saturation where any further increase in uptake rate will be met with an increase in efflux
rate.

*Ammonium*

After an initial review of kinetic data for NH$_4^+$ uptake, neither a linear or
Michaelis Menten kinetic model provided a good fit suggesting there may be two
concentration related components influencing uptake rate. It was determined that the
NH$_4^+$ data should be separated into two models by NH$_4^+$ concentrations before fitting
kinetic uptake models. The Michaelis Menten kinetic behavior was best fit for NH$_4^+$
concentrations less than 42 µM NH$_4^+$, while a linear model was applied for
concentrations greater than 42 µM NH$_4^+$.

While Michaelis Menten kinetics suggest saturation of uptake rate, linear kinetics
indicate simple diffusion may be taking place. A linear relationship describes the kinetic
behavior above 42 µM NH$_4^+$. The estimated range of coral intracellular NH$_4^+$
concentrations is 5-50 µM. The switch from Michaelis Menten behavior to linear
behavior at 42 µM NH$_4^+$ may be indicative of the minimum extracellular NH$_4^+$/NH$_3$
concentration at which there exists a concentration gradient between seawater and
intracellular pools favorable for diffusion into the cells. These data may indicate that the
corals in this particular study have cytoplasmic NH$_4^+$ concentrations of approximately 42
µM. Above 42 µM NH$_4^+$ the uptake rate depends only on the concentration gradient
between seawater and intracellular tissues, with a linear relationship between uptake rate
and NH$_4^+$/NH$_3$ concentration. If indeed the cytoplasmic NH$_4^+$ concentrations are 42 µM, the uptake at seawater NH$_4^+$ <40 must be energy dependent through active transport.

When interpreting kinetic behavior of uptake, the range of concentrations over which data is collected should be considered. For example, a dataset covering a small range in DIN concentrations can be misleading if the linear behavior observed is actually representative of the initial linear behavior of a larger scale Michaelis Menten hyperbola. If the NH$_4^+$ uptake data from this study are reviewed at 0 to 10 µM NH$_4^+$, they are best fit to a linear regression, but when the data analysis is extended to 42 µM, the Michaelis Menten behavior is apparent. Extending the analysis even further above 42 µM revealed yet different kinetic behavior. Kinetic data for uptake of NO$_3^-$ and NH$_4^+$ by cnidarian symbioses should not be extrapolated to understand uptake at concentrations outside the experimental ranges measured. It is possible that the linear behavior that we observe above 40 µM could also be saturated at concentrations above the range encompassed in this study.

1.4.3 Preferential Uptake of NH$_4^+$ and Inhibition of NO$_3^-$ Uptake

As in many previous studies of symbiotic cnidarians, overall higher rates of NH$_4^+$ uptake were observed compared to NO$_3^-$ uptake and NO$_3^-$ uptake was reduced in the presence of elevated NH$_4^+$. The majority of previous studies have found higher NH$_4^+$ uptake rates than NO$_3^-$ (D Elia and Webb 1977, Wilkerson and Trench 1986, Grover et al. 2002b), however, one study did find that NO$_3^-$ uptake rates for the coral Acropora palmata were twice that of NH$_4^+$ uptake (Bythell 1990). In general, across treatments in this study, the uptake rates of NO$_3^-$ were found to be approximately half that of NH$_4^+$ uptake rates. Corals in 50 µM NH$_4^+$ had consumed $0.96 \pm 0.14 \mu$moles N cm$^{-2}$ after 24
hours in incubation and corals in 50 µM NO$_3^-$ had consumed 0.54 ± 0.09 µmoles cm$^{-2}$.

The higher assimilation rates of NH$_4^+$ may simply be because assimilation of NH$_4^+$ is energetically more favorable than for NO$_3^-$, as NH$_4^+$ is in a reduced form. However, NO$_3^-$ must first be reduced by enzymatic activity of nitrate and nitrite reductases before assimilation by GS/GOGAT to organic nitrogen. In addition, there is supporting evidence that the coral host can also contribute to assimilation of NH$_4^+$ potentially also contributing to the higher overall assimilation rates of NH$_4^+$ for the coral-algal symbiosis.

If NO$_3^-$ uptake rates are plotted against NH$_4^+$ concentration the result is an inverse hyperbola which is characteristic of uptake inhibition (Figure 1.4.1). In other photosynthetic marine organisms, this inhibition has been determined to be due to either repression of NO$_3^-$ transport (Tischner and Lorenzen 1979) or the repression of nitrate or nitrite reductase enzymes in the presence of elevated NH$_4^+$ (Eppley et al. 1969).

Crossland and Barnes (1977) noted that the nitrate reductase of the symbiotic algae of the coral *Goniastrea australensis* was depressed when levels of seawater NH$_4^+$ were greater than 10 µM. Our data suggested increased NO$_3^-$ uptake after seawater NH$_4^+$ concentrations fell below ~3.3 µM. It is suspected that the intracellular concentrations of NH$_4^+$ in the host cytoplasm are high due to host catabolism (estimated as 5-50 µM NH$_4^+$ (Crossland and Barnes 1977)); evidence from this study previously discussed in 4.2), thus it is curious why NO$_3^-$ uptake by algal symbionts, which reside in host tissues, is not always inhibited by high intracellular NH$_4^+$. The relationship observed in this study between seawater NH$_4^+$ concentrations and NO$_3^-$ uptake could suggest that NH$_4^+$ inhibition of NO$_3^-$ uptake occurs at the seawater-coral tissue interface and is related to
Figure 1.4.1 The uptake kinetics of ambient levels of NO$_3^-$ by the coral-algal symbiosis under NH$_4^+$ concentrations from 0 to ~58 μM. Data are from all Controls and NH$_4^+$ treatment tanks. The “instantaneous” rate is given by the rate calculated for two consecutive samples (ex. from t$_1$ to t$_{1+1}$), and the “instantaneous” concentration is the average of the NH$_4^+$ concentration of the two samples from which the rate was calculated. Data resembles the inverse hyperbola model characteristic of uptake inhibition kinetics.
transport of \( \text{NO}_3^- \) into the coral tissues. Alternatively, if the seawater and coral intracellular \( \text{NH}_4^+ \) concentrations are directly related, and the algal intracellular \( \text{NH}_4^+ \) concentration also increases in seawater concentrations of greater than 3.3 \( \mu \text{M} \) \( \text{NH}_4^+ \), this may inhibit nitrate or nitrite reductase activity. It is also possible that the uptake of \( \text{NO}_3^- \) could be inhibited by a changing pH gradient between seawater and/or coral tissue and algal symbiont caused by alkalization of vacuoles under high \( \text{NH}_4^+ \) concentrations. It is assumed that primarily \( \text{NH}_3 \) is transported across the cell membrane into tissues and is quickly protonated under more acidic intracellular conditions leading to alkalization of coral tissues.

1.4.4 Increased \( \text{NO}_3^- \) and \( \text{NH}_4^+ \) Uptake with Long Term Exposure

To understand how the coral-algal symbiosis may adapt to elevated \( \text{NO}_3^- \) and \( \text{NH}_4^+ \) levels after long term exposure, initial rates of uptake (when the corals were first introduced to elevated \( \text{NO}_3^- \) and \( \text{NH}_4^+ \) levels) were compared to the final uptake rates (how the coral is responding after long term nutrient exposure). The Control corals exhibited no change in the rate of uptake between the beginning and end of the experiment, suggesting that experimental conditions unrelated to DIN concentration did not affect changes in uptake rate at Control concentrations over the four week period. In all elevated \( \text{NO}_3^- \) treatments, however, the uptake rates were significantly higher after four weeks under elevated \( \text{NO}_3^- \) levels than upon first exposure to elevated \( \text{NO}_3^- \). Uptake rates in 50 \( \mu \text{M} \) \( \text{NH}_4^+ \) also increased after four weeks.

This finding is important, because it sheds some light on the corals ability to control \( \text{NO}_3^- \) and \( \text{NH}_4^+ \) consumption by the algal symbionts. Some studies have suggested that the coral host may have some control over the symbionts under elevated conditions.
nutrient levels, in that corals may be able to enforce some nutrient limitation on their endosymbionts (Davy et al. 2012). It is clear in our data that for this time period of four weeks, the uptake of NO$_3^-$ and NH$_4^+$ has the potential to increase with time in all concentrations of NO$_3^-$ analyzed and in the highest NH$_4^+$ treatment, 50 µM NH$_4^+$. Our data suggest that the change in symbiont density may not be sufficient to account for the increase in NO$_3^-$ uptake after four weeks of experimental exposure, implying that there may be some adaptation occurring within each symbiont, perhaps the activation of assimilatory enzymes after continued exposure. Grover et al. (2003) showed that there was no influence of pre-conditioning with elevated NO$_3^-$ concentrations on NO$_3^-$ uptake for the coral *Stylophora pistillata*, but this was not the case for the corals in this study. These differences could be due to species specific effects, variation in long-term exposure times, or other environmental factors influencing uptake rates during experimentation. It is also possible that the increase in NO$_3^-$ or NH$_4^+$ uptake could be related to changes in coral tissue biomass which was not directly measured in this study. The potential for increases in protein content for both coral tissues and algal symbionts is highly likely as the molar CN ratios of both fractions decreased over the course of the 4 week experiment (Figures 1.4.2 and 1.4.3).

It is interesting that variable response to long term treatment exposure is present between different NH$_4^+$ concentrations. The Control, 10 and 20 µM NH$_4^+$ had almost identical uptake curves as normalized to coral surface area from initial to final uptake measurements and only those corals under 50 µM NH$_4^+$ treatment experienced increased uptake rates after four weeks. This could suggest that the mechanisms for uptake at 50 µM NH$_4^+$ are different than at lower concentrations. At higher concentrations of external
Figure 1.4.2 The organic molar carbon to nitrogen ratio for separated components of the coral-algal symbiosis, coral tissue (orange) and algal symbiont tissue (green) for each elevated NO$_3^-$ treatment and Control tanks. Each error bar represents one standard error.
Figure 1.4.3 The organic molar carbon to nitrogen ratio for separated components of the coral-algal symbiosis, coral tissue (orange) and algal symbiont tissue (green) for each elevated NH$_4^+$ treatment and Control tanks. Each error bar represents one standard error.
NH$_4^+$ (see kinetic discussion), we suggest that diffusion may be responsible for transport at concentrations exceeding 40 µM NH$_4^+$. It is possible that initially symbionts assimilate NH$_4^+$ at lower rates and are unable to deplete intracellular NH$_4^+$ enough to maintain a favorable concentration gradient for high rates of diffusion. Alternatively, the change observed after long term immersion in 50 µM NH$_4^+$ could reflect an increase in direct assimilation by coral host tissues.

The long term results from the 50 µM NH$_4^+$ treatment contrasts with those of Yellowlees (1994), which indicated that the GS activity of algal symbionts was reduced after 2 weeks of elevated NH$_4^+$ exposure for *Pocillopora damicornis*. It is possible that some of the increase in uptake observed in this study at 50 µM NH$_4^+$ is due to increased host assimilation and perhaps increasing GDH enzyme activity in host tissues and unrelated to GS activity of algal symbionts.

1.4.5 Continued, but Reduced Dark Uptake of NO$_3^-$ and NH$_4^+$

Photosynthesis and nitrogen metabolism are known to interact integrally with each other, however, the assimilation of NH$_4^+$ in the dark has been documented for several photosynthetic organisms and continued uptake of NH$_4^+$ by *Pocillopora damicornis* during a 12 hour dark period was measured in this study. The assimilation of inorganic nitrogen into amino acids and proteins requires, ATP, reducing power and carbon skeletons (amino group acceptors). Typically non-symbiotic and aposymbiotic cnidarians do not have the ability to retain inorganic nitrogen and in many cases have been measured releasing NH$_4^+$ (Wilkerson and Muscatine 1984a). Symbiotic corals have the ability to draw down both NO$_3^-$ and NH$_4^+$, but the release of NH$_4^+$ by symbiotic cnidarians has been noted by some workers after a prolonged dark period (after 19 hours
for *Aiptasia pulchella* (Wilkerson and Muscatine 1984b)). There were no significant differences during this period between light and dark uptake rates in Controls, 10 and 20 µM NH$_4^+$ treatments and while dark uptake rates in the 50 µM NH$_4^+$ treatment were reduced, corals still substantially depleted NH$_4^+$ at rates equivalent to 33.6 ± 3.8 % of light uptake rates.

The data from this study show continued, but decreased uptake of NO$_3^-$ in the dark. The 10 µM NO$_3^-$ treatment had the highest dark uptake relative to light with dark uptake equivalent to 75% light uptake, the dark uptake in 20 µM NO$_3^-$ was 27% of light uptake and at 50 µM NO$_3^-$, dark uptake was 47% of light uptake. As only the algal symbionts are known to possess enzymes for nitrate assimilation, it is suggested that these unicellular algae are actively assimilating NO$_3^-$ in the dark. Studies have shown continued dark NO$_3^-$ uptake by other unicellular algae. A free living dinoflagellate algae *Alexandrium tamarense* associated with harmful algal blooms, displayed rates of NO$_3^-$ uptake in the dark equivalent to 67% of the uptake rates for a light period (Leong et al. 2010).

The continued uptake of both NO$_3^-$ and NH$_4^+$ in this study suggests that the coral-algal symbiosis has sufficient resources to assimilate inorganic nitrogen in the dark. Studies of other marine algae have suggested that the carbon skeletons necessary for DIN assimilation can be supplied by respiratory intermediates, thus increased respiratory carbon flow may allow for amino acid synthesis. For the green algae *Selenastrum minutum* cultured in NO$_3^-$ and NH$_4^+$ in the dark, the ratio of CO$_2$ release/N assimilation was 1.25 for NO$_3^-$ and 0.36 for NH$_4^+$. Studies suggest that the assimilation of NO$_3^-$ requires more respiratory carbon release than NH$_4^+$, because the assimilation of NO$_3^-$ not
only requires carbon skeletons from the TCA cycle, but in addition reducing power to reduce NO$_3^-$ (Turpin et al. 1990).

1.5 Conclusions

The significant increase in uptake of NO$_3^-$ and NH$_4^+$ was documented under elevated concentrations for laboratory manipulations of Pocillopora damicornis. Differences in kinetic uptake behavior between NO$_3^-$ and NH$_4^+$ were exhibited at the range of 0-50 µM. While NO$_3^-$ appeared to follow Michaelis Menten kinetics for the entire range of concentrations with a maximum uptake rate of 0.037 µmoles N cm$^{-2}$, NH$_4^+$ appeared to be more complex with multiple mechanisms controlling the rates of uptake and assimilation. Corals retained the ability to take up both NO$_3^-$ and NH$_4^+$ during a dark period, suggesting they had allocated carbon products and reducing power for uptake and assimilation without a continuous source of algal photosynthate.

The data presented here suggest that while coral and algal nitrogen demands may be met under low nutrient conditions, the symbiosis significantly increases uptake of nitrogen when exposed to unnaturally high levels of NO$_3^-$ and NH$_4^+$. When initially exposed, uptake rates in all elevated NO$_3^-$ and NH$_4^+$ treatments are higher than the uptake of ambient nitrogen in the Controls. These uptake rates further increase as the corals are exposed to high NO$_3^-$ and NH$_4^+$ long term, potentially altering the cycling of nitrogen within the coral-algal symbiosis and exacerbating the influence of external inorganic nitrogen.
Chapter Two: Uptake Associated Isotopic Fractionation of Nitrate $^{15}\text{N}$ and $^{18}\text{O}$ by *Pocillopora damicornis*

### 2.1 Background

Scleractinian corals acquire nitrogen through both heterotrophic feeding and autotrophic transfer of nutrients by the endosymbiotic algae, *Symbiodinium microadriaticum*. The $\delta^{15}\text{N}$ signature of corals is often measured to identify sources of nitrogen influencing a reef, however, the interpretation requires an understanding of how $\delta^{15}\text{N}$ of dissolved inorganic nitrogen (DIN) translates to the $\delta^{15}\text{N}$ measured in coral tissue and algal symbionts. In order to address these questions a series of incubations were carried out in elevated concentrations of NO$_3^-$ and the isotopic fractionation of $^{15}\text{N}$ and $^{18}\text{O}$ associated with uptake and assimilation of NO$_3^-$ by the branching coral species *Pocillopora damicornis* was measured. Measurements of the isotopic composition of NO$_3^-$ nitrogen and oxygen in incubation tank water collected throughout an uptake period coupled with isotopic measurements of coral and algal tissues after long term elevated NO$_3^-$ exposure allowed for calculation of the isotopic discrimination factor, $\varepsilon$, associated with NO$_3^-$ uptake and assimilation. The results show that the discrimination factor, $\varepsilon$, was found to vary with concentration of NO$_3^-$ and light cycle conditions.

#### 2.1.1 Isotope Fractionation Background

Kinetic and equilibrium isotope fractionations result from the different reaction rates of molecules containing light and heavy isotopes. Typically during a reaction, the light isotope will preferentially react resulting in a product that is isotopically light and a substrate that is enriched in the heavy isotope. The difference in reaction rates reflects different bond strengths and mobilities. The fractionation factor, $\alpha$, is used to describe the
magnitude of isotope discrimination which occurs and is the ratio of heavy to light isotopes in two substances A and B (equation 1),

$$\alpha_{(A-B)} = \frac{R_A}{R_B}$$  \hspace{1cm} (1)

where $R_A$ and $R_B$ are the ratios of heavy to light isotopes in substances A and B, respectively. The relationship between delta notation (equation 2) and $\alpha$ values is given in equation 3,

$$\delta = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 10^3$$  \hspace{1cm} (2)

$$\delta \approx 1000 \ln \alpha$$  \hspace{1cm} (3)

Where $\delta$ is reported in units per mil (‰) and $R_{\text{sample}}$ and $R_{\text{standard}}$ are the ratio of heavy to light isotopes in the sample and standard, respectively. Isotopic fractionation is also commonly represented by $\varepsilon$, referred to as the isotope effect or the discrimination factor, which will be used to describe fractionation in this study, where:

$$\varepsilon = (\alpha - 1) \times 1000$$  \hspace{1cm} (4)

Following the law of the conservation of mass, in a closed system during a reaction in which a substrate is reacted to form a product, isotope fractionation is only observable under conditions of partial substrate consumption. If all substrate is converted to product, the substrate and product will be isotopically equivalent and no fractionation will be observed.
2.1.2 Nitrogen Isotopes of Marine Organisms as Nutrient Indicators

Reefs thrive in low nutrient waters, and thus it is important to understand nutrient dynamics and potential threats to the water quality of reefs. Measuring the concentration of nutrients such as the various forms of DIN in reef waters is not a definitive method with which to assess nutrient influences on a reef, as pulses of DIN have the potential to be rapidly taken up and synthesized into organic nitrogen. In order to understand contributing nitrogen sources to a reef habitat, several workers have investigated the use of the stable nitrogen isotopic signatures of reef biota (Heikoop et al. 2000, Lapointe et al. 2004, Huang et al. 2013). This is because rather than holding just a snapshot of nutrient information, the δ15N signature of an organism is recorded over a period of time dependent on nitrogen turnover rates of the tissues.

The δ15N signatures of reef biota are of interest because distinct δ15N values are associated with particular reservoirs of nitrogen may hold useful information for investigations of nitrogen sources to the coastal environment. Such differences in δ15N signatures arise as a result of the various transformations of the nitrogen cycle which discriminate differentially against the rare, heavy isotope of nitrogen, 15N. In the case of NO3−, major isotopic fractionations occur during the processes of nitrification, denitrification, and nitrate uptake (Figure 2.1.1). The fractionation of 15N associated with nitrification is estimated to be from 15 to 25 ‰, producing NO3− with relatively more 14N leaving the remaining NH4+ pool with more 15N. The process of denitrification preferentially removes 14N, leaving the remaining NO3− with more 15N, increasing the δ15N with a fractionation effect of 25 to 30 ‰. Nitrogen fixation on the other hand,
Figure 2.1.1 (adapted from Capone (2008)) The various transformations of the marine nitrogen cycle between the atmosphere, ocean and sediments.
converts atmospheric N\textsubscript{2} to combined nitrogen with very little fractionation (Hoering and Ford 1960, Amberger and Schmidt 1987). The estimates of fractionation associated with NO\textsubscript{3}\textsuperscript{-} uptake in the marine environment and laboratory studies by phytoplankton range from 1 to 20 ‰, with the organisms selecting for incorporation of \textsuperscript{14}N rather than \textsuperscript{15}N (Altabet and Francois 1994, Altabet 2001, Needoba et al. 2004).

As a result of the fractionations associated with various transformations of nitrogen, inorganic nitrogen pools can have distinctive isotopic signatures. For example, as NO\textsubscript{3}\textsuperscript{-} is strongly affected by the process of denitrification, it will have a more positive $\delta^{15}$N signature compared to pools unaffected by denitrification. If a reservoir is strongly influenced by inputs of new nitrogen through nitrogen fixation, it will have a $\delta^{15}$N value similar to that of atmospheric N\textsubscript{2} (~0 ‰). Terrestrial sources of nitrogen are often thought to introduce more positive $\delta^{15}$N signatures to the marine environment. This is because the $\delta^{15}$N of DIN associated with sewage, terrestrial runoff and groundwater are heavily influenced by the processes of nitrification, volatilization (NH\textsubscript{4}\textsuperscript{+}) and denitrification (NO\textsubscript{3}\textsuperscript{-}) (Heaton 1986, McClelland and Valiela 1998). The introduction of synthetic fertilizers to coastal habitats through runoff, however, will contribute DIN with $\delta^{15}$N values close to 0‰ since these fertilizers are produced from N\textsubscript{2} through the Haber-Bosch process, which involves very little isotopic fractionation and isotopic change from atmospheric N\textsubscript{2} (Amberger and Schmidt 1987, Widory 2004, Widory et al. 2005).

**Macroalgae**

Several studies have attempted to interpret the $\delta^{15}$N values of reef organisms as nitrogen source indicators. Of these studies, some have related enriched $\delta^{15}$N values of macroalgae to the influence of sewage effluent (Lapointe et al. 2004, Lapointe and
Bedford 2010). The δ¹⁵N signatures of the rhodophyte *Laurencia intricata* in the Florida Keys were found to vary seasonally and with proximity to land. Lapointe et al. (2004) concluded that *L. intricata* growing in an inshore area of the Florida Keys with δ¹⁵N signatures of +4.7 ‰ was recording sewage nitrogen signatures, while the δ¹⁵N sewage signature was diluted in *L. intricata* growing further offshore (+3.1 ‰ and +2.9‰ respectively). In a later study, Lapointe and Bedford (2010) used δ¹⁵N to conclude that growth of the invasive chlorophyte *Caulerpa brachypus* off the coast of West Palm Beach, Florida was fueled by land based nutrient sources reaching the reef during the wet season.

The criteria for determining δ¹⁵N in macroalgae attributable to sewage is unclear, and it appears that many studies interpret modest enrichments in δ¹⁵N as sewage influence. This is troubling considering the wide range in naturally occurring δ¹⁵N signatures of macroalgal tissues. Specifically, in the Florida Keys, workers have shown that the NO₃⁻ pools at the thermocline (2-10 µM) and below (15-25 µM) are relatively enriched in δ¹⁵N (+4.78 ‰ and +5.26 ‰, respectively), and when hydrographic conditions permit, these deeper NO₃⁻ pools can serve as a transiently available source of NO₃⁻ to surface waters (Leichter et al. 2007). Other workers have shown that the δ¹⁵N of macroalgal tissues synthesized from a single NO₃⁻ source can vary greatly depending on ambient NO₃⁻ concentrations (Swart In Review).

Although, the direct relationship between δ¹⁵N of DIN source and macroalgal tissues has not been defined, several further speculations have been made regarding the interpretation of such data. In a study investigating indicators of coral reef health Huang et al. (2013) concluded that “coral conditions” were negatively correlated with
Lobophora variegata δ^{15}N. Because these correlations with δ^{15}N were determined to be stronger than with turbidity, dissolved inorganic nutrient concentrations and water quality index, these workers concluded that macroalgal δ^{15}N is a sound indicator for determining the nutrient stress to a particular reef environment. (Huang et al. 2013)

Seagrasses

The δ^{15}N of seagrasses have also been used to obtain information on nutrient influence. In a coastal ecosystem in the Central Adriatic, Murter Sea, seagrasses growing in close proximity to aquaculture fish cages were analyzed for δ^{15}N. Dolenec et al. (2006) proposed that enriched δ^{15}N values of shoot and leaf tissues (+4.7 ‰) were influenced by the nitrogen content of fish waste. Another study related enriched δ^{15}N values of Thalassia testudinum in the Nicuphyte Lagoon System (off the east coast of the Yucatan Peninsula) to wastewater inputs either directly or through submarine groundwater discharge. The region to the north of Nicuphyte Lagoon, known as the Cancun “Hotel Zone” (Carruthers et al. 2005), is heavily influenced by the local tourism. Other workers (Fourqurean et al. 2005) have discovered, however, a very large range in the natural variability of δ^{15}N (-4.3 ‰ to +9.4 ‰) measured for Thalassia testudinum growing in Biscayne Bay, Florida over a long term study (8 years). The results emphasize that before interpreting δ^{15}N of seagrasses, the controls from local, temporal and spatial variability must be thoroughly understood. These studies suggest that there is not a simple threshold δ^{15}N value which can be used to indicate human sewage pollution.

Sea Fans and Corals

The δ^{15}N values of sea fans and corals have also been used to understand nitrogen impacts on a reef. Heikoop (2000) cited enriched δ^{15}N values for bulk coral tissues on
Indo-Pacific reefs exposed to sewage influence as compared with pristine reefs (Heikoop et al. 2000). A study relating sea fan disease to elevated DIN concentrations. Baker et al. (2007) found that there was no measurable relationship between the prevalence of the fungus Aspergillosis and enriched δ¹⁵N values in the sea fan Gorgonia ventalina along the Florida Keys Reef Tract, and suggested that this could be due to multiple sources of nitrogen in this particular environment contributing to the δ¹⁵N values measured within the sea fan tissues and skeleton. In addition to using coral δ¹⁵N to understand reef environments, some workers have also turned to the preserved skeletal organic matrix in order to reconstruct the nutrient history of a reef (Marion et al. 2005, Yamazaki et al. 2011).

The caveat with the use of coral δ¹⁵N as a nitrogen source indicator is that nutrient cycling within the coral is complex and the controls on coral δ¹⁵N are not understood. In addition to obtaining nitrogen from direct assimilation of inorganic nitrogen by the algal symbionts, corals are also heterotrophic and feed on various sources of particulate organic matter in reef waters. In order to utilize the δ¹⁵N measured in any organism to understand δ¹⁵N of source NO₃⁻, it should be understood how these two are related. The translation of the nitrogen isotopic composition of NO₃⁻, i.e. the isotopic fractionation effect, has not yet been established for coral and algal symbiont tissues.

2.1.3 Potential Contributions to δ¹⁵N in Corals

When measuring isotopic fractionation associated with uptake and assimilation of NO₃⁻ dissolved in seawater by the coral-algal symbiotic association, the Rayleigh Distillation equation can be utilized to calculate fractionation effects. The NO₃⁻ in the surrounding seawater is referred to as the substrate, and the newly formed coral and algal
symbiont tissues as the product. The resultant isotopic composition of the product depends on the original isotopic composition of the substrate and the final amount of substrate remaining in the NO$_3^-$ pool. The apparent fractionation will be governed by concentration of substrate available and the rate of the processes involved in uptake and assimilation, influenced in turn by such things as mechanism of uptake and enzyme activity. If all substrate is consumed during synthesis of product, there will be no gross fractionation and the isotopic composition of the product will be equal to that of the substrate. This is expressed mathematically as equation 5,

$$\delta_{\text{substrate(f)}} = \delta_{\text{substrate(0)}} - \epsilon_{p-s} \cdot \ln(f)$$  \hspace{1cm} (5)

In this equation $\delta_{\text{substrate(f)}}$ is the final isotopic composition of the substrate, $\delta_{\text{substrate(0)}}$ is the initial isotopic composition of the substrate, $\epsilon_{p-s}$ represents the discrimination factor from substrate to product, and $f$ is the fraction of substrate remaining. Figure 2.1.2 depicts the relationship between the fractionation factor and the isotopic composition of the instantaneous product (forming at any given amount of substrate remaining), the cumulative product (average of the new product (tissues) formed as a result of uptake), and the substrate pool during the uptake period.

2.1.4 Previous Laboratory Measurements of $^{15}\varepsilon$ for NO$_3^-$ Uptake and Assimilation

While the fractionation of $^{15}$N and $^{18}$O associated with NO$_3^-$ uptake by coral and their symbiotic dinoflagellate algae, Symbiodinium microadriaticum, has never been studied, comparison to studies of fractionation by unicellular marine algae may be useful. Several laboratory incubations have been carried out to determine $^{15}\varepsilon$ and $^{18}\varepsilon$ by unicellular eukaryotic and prokaryotic plankton (Granger et al. 2004, Needoba and Harrison 2004, Needoba et al. 2004, Granger et al. 2010, Karsh et al. 2012). These
Figure 2.1.2 A hypothetical model of Rayleigh type fractionation for the formation of organic nitrogen (ex. amino acids) from NO$_3$. The initial $\delta^{15}$N of NO$_3$ is 0 $\%_o$, the hypothetical discrimination factor is set at $\varepsilon=10 \%_o$. The isotopic behaviors of the residual substrate, instantaneous product (forming at any given moment) and the cumulative product are shown.
studies have drawn conclusions on the relationship between the rate limiting step of \( \text{NO}_3^- \) uptake and assimilation (transport or assimilation) with the isotope effects which are observed through isotopic analysis of dissolved \( \text{NO}_3^- \) in the water and the organic tissues.

Intracellular \( \text{NO}_3^- \) concentrations of coral tissues and algal symbionts have not been measured. If similar to other marine unicellular algae, the algal intracellular \( \text{NO}_3^- \) concentrations have the potential to be very high, several orders of magnitude higher than the surrounding seawater. It is accepted that marine unicellular algae use active transporters on the cell surface to transport \( \text{NO}_3^- \) into the cell (Needoba and Harrison 2004) and there is some evidence for the presence of nitrate transporters in *Symbiodinium microadriaticum* (Leggat et al. 2007). The transport mechanism of \( \text{NO}_3^- \) across animal tissues is not clear as animals generally do not assimilate \( \text{NO}_3^- \), but previous workers have assumed that \( \text{NO}_3^- \) is passively diffused through coral tissue membranes (Miller and Yellowlees 1989a). Early studies proposed a diffusion-depletion model of nutrient supply where nutrients diffuse along a concentration gradient from the host cell cytoplasm into the symbiont cell in response to the symbiont nutrient demands (Delia et al. 1983).

It is accepted that \( \text{NO}_3^- \) assimilation occurs exclusively within the symbiont cells. Once ions have been transported to the site of assimilation, the mechanisms for their assimilation must be considered. The enzymes necessary for \( \text{NO}_3^- \) assimilation, nitrate and nitrite reductases, have been detected in algal symbionts (Crossland and Barnes 1977) and the gene sequence for nitrite reductase has been measured in type C3 symbionts from *Acropora aspera* (Leggat et al. 2007).
2.1.5 Experimental Objectives

Understanding the incorporation of NO$_3^-$ into the coral-algal system and the extent that uptake of NO$_3^-$ by symbionts may be limited by the coral host, requires a clearer understanding of the mechanisms of uptake and assimilation. The use of $\delta^{15}N$ and $\delta^{18}O$ provides a tool with which to understand and model NO$_3^-$ uptake. Determining the isotopic fractionation of nitrogen from NO$_3^-$ during assimilation at a range of concentrations may provide information regarding the uptake mechanism (passive diffusion, active transport etc.) and the influence of concentration and long term DIN exposure on uptake. In addition measuring the nitrogen isotope fractionation involved in DIN uptake is important when interpreting the $\delta^{15}N$ of coral tissues and symbiotic dinoflagellates, as well as understanding the $\delta^{15}N$ preserved in the skeletal organic matrix of Scleractinian corals and its efficacy in interpreting past nutrient conditions on coral reefs.

2.2 Methodology

2.2.1 Experimental Specimens

Specimens of the Pacific branching coral *Pocillopora damicornis* were collected off the coast of Panama in 2007. The corals were “common gardened” at the University of Miami Experimental Hatchery indoors for five years prior to experimentation. The corals experienced conditions of relatively constant temperature (26 °C) and light regimes on a 12 hour light/12 hour dark cycle, and were fed an algal protein mixture once weekly. Corals were maintained in flowing seawater pumped in directly from Bear Cut, Miami, Florida and experienced natural fluctuations in salinity. The corals were analyzed with the quantitative polymerase chain reaction method (qPCR) in the Baker Coral Conservation
Laboratory in order to determine the algal symbiont type. For this experiment, we chose two genets of *Pocillopora damicornis* hosting clade C symbionts, and five genets of *P. damicornis* hosting clade D symbionts. Only corals hosting clade C symbionts are discussed in this chapter. 300 corals were fragmented from larger parent colonies and fixed to PVC plates specialized for growth and calcification measurements via the scanning optical micrometer method (Langdon Lab - University of Miami (Albright et al. 2010)) and the buoyant weight technique ((Jokiel et al. 1978).

### 2.2.2 Experimental Conditions

Corals were subject to experimental nutrient levels for a period of four weeks. Treatment conditions included Control, 10, 20 and 50 µM NaNO₃. Controls consisted of 0.2 micron filtered seawater pumped in from Bear Cut (NO₃⁻, NH₄⁺ < 1 µM), while treatments consisted of the same seawater utilized for Control with the addition of NaNO₃ or NH₄Cl to achieve the desired final concentration. Treatment tanks consisted of 1 gallon glass tanks filled with 2.5 liters of seawater. Four tanks were assigned to each treatment. Each tank contained six coral fragments for a total of 240 coral fragments distributed among 40 tanks. Tank seawater was changed at the beginning of light exposure each day with the appropriate nutrients added. The 1 gallon tanks were maintained inside a larger flow-through system to maintain temperature control, however, the water within individual tanks was isolated from flowing seawater (Figure 2.2.1). Temperature was held constant indoors during the four week period at 26 ± 0.5⁰ C. Light was supplied by USA Nova Current Extreme lights on a 12 hour light/12 hour dark cycle. Circulation within each tank was provided by a Hydor Koralia Nano mini pump.
Figure 2.2.1 Experimental setup at the University of Miami Experimental Hatchery consists of individual isolated tanks within flow through system for temperature control. Circulation is provided by a Hydor Koralia Nano pump.
2.2.3 Sample Collection

Water samples were collected at the beginning and end of the four week experimental period. Samples were collected throughout a 48 hour period on a 12 hour light/12 hour dark cycle at 0, 4, 12, 24, 36 and 48 hours and then during a period of 48 hours of constant light exposure at 0, 4, 12, 24 and 48 hours. Filtered water samples were collected in 125 ml Nalgeen (polyethylene) bottles and acidified to pH 2 with the addition of hydrochloric acid for preservation (Capone 2008).

2.2.4 NO$_3^-$ and NH$_4^+$ Concentration Analysis

The concentrations of NO$_3^-$ and NH$_4^+$ were determined colorimetrically with a SmartChem200 Autoanalyzer (Westco Scientific). The NO$_3^-$ concentrations were determined via the vanadium chloride method and the NH$_4^+$ concentrations were measured through the indophenols-blue method (Berthelot reaction) (Westco Scientific SmartChem200 Manual). NO$_3^-$ and NO$_2^-$ concentration data are required for sample preparation for NO$_3^-$ isotopic analysis.

2.2.5 NO$_3^-$ Isotopic Analysis

The analysis of NO$_3^-$ $\delta^{15}$N and $\delta^{18}$O was achieved through conversion to N$_2$O gas and subsequent isotopic analysis. NO$_3^-$ is first converted to NO$_2^-$ via the Cd reduction method (McIlvin and Altabet 2005, Ryabenko et al. 2009) and the concentrations of NO$_2^-$ are analyzed to determine percent yield of NO$_3^-$ converted to NO$_2^-$. Samples are then treated with 1:1 acetic acid:azide and buffered with 10 M NaOH to convert NO$_2^-$ to N$_2$O gas. The $\delta^{15}$N and $\delta^{18}$O composition of N$_2$O gas is analyzed on a GV Isoprime isotope ratio mass spectrometer with a purge and trap system. Values are reported in delta notation (equation 6), relative to N$_2$ and VSMOW for nitrogen and oxygen.
Where $R_{sample}$ is the ratio of heavy to light isotopes in the sample and $R_{standard}$ is the ratio of heavy to light isotopes in the standard.

$\delta^{15}N$ and $\delta^{18}O$ sample values for each run are compared to a set of blanks (operational blanks: low nutrient seawater treated with the acetic acid:azide solution, and Cd blanks: low nutrient seawater treated with Cd), a set of three NO$_2^-$ standards and a set of three NO$_3^-$ standards (USGS34, USGS35, and a lab standard). Isotopic data were corrected for the exchange of oxygen between sample and water during conversion to N$_2$O, and the 1:1 addition of azide-N to NO$_2^-$-N in the formation of N$_2$O (McIlvin and Altabet 2005) Average reproducibility for $\delta^{15}N$ was 0.20 ‰ and $\delta^{18}O$ was 0.25 ‰.

2.2.6 Coral Tissue and Algal Symbiont Isotopic Analysis

Coral tissue was removed from skeletons with an airbrush technique using a gentle stream of distilled water. The coral host tissue was immediately separated from algal symbionts using a modified version of the centrifugation and rinsing method with the additional step of filtration twice through 20 µm nitex mesh in order to eliminate contamination by coral nematocysts from the symbiont fraction (Piniak et al. 2003). Coral and algal symbiont tissues were dried at 40 °C for 48 hours and prepared in tin capsules for organic nitrogen isotope analysis. Samples were analyzed on a Thermal Conductivity Elemental Analyzer (Costech) interfaced to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific). Reproducibility for $\delta^{15}N$ was 0.1 ‰.
2.2.7 Determination of Coral Surface Area

Nutrient uptake data were normalized to coral surface area. Coral surface area was determined by modeling individual coral branches as cylinders. The height and diameter of each branch was measured with a digital micrometer and surface area calculations were carried out.

2.2.8 Calculation of Discrimination Factor $\varepsilon$

There are two methods for calculating the discrimination factor. The discrimination factor, $\varepsilon$ can be calculated either by using the $\delta^{15}N$ of the residual NO$_3^-$ or the $\delta^{15}N$ of coral or algal symbiont tissue.

Residual NO$_3^-$

In order to calculate the $\varepsilon$ derived from incubation water, the concentration and isotopic signature of NO$_3^-$ is utilized. Solving for the slope of the Rayleigh Distillation equation (equation 7) gives $\varepsilon$ (equations 8):

$$\delta^{15}N_{NO3(t)} = \delta^{15}N_{NO3(o)} - \varepsilon \ln f$$

$$\varepsilon = \frac{\delta^{15}N_{NO3(o)} - \delta^{15}N_{NO3(t)}}{\ln f}$$

where $\delta^{15}N_{NO3(o)}$ is the isotopic composition of the NO$_3^-$ at the start of the incubation and $\delta^{15}N_{NO3(t)}$ is the isotopic composition of the NO$_3^-$ at time t, when the fraction of original NO$_3^-$ remaining is f. For NO$_3^-$ incubations, the $\varepsilon$ value for $^{18}O$ can be calculated using the same equation, substituting $\delta^{18}O$ for $\delta^{15}N$.

Coral-Algal Tissues

The discrimination factor for nitrogen can also be calculated from the $\delta^{15}N$ value of the coral and algal symbiont tissues. The Rayleigh Distillation equation for
cumulative product can be used to solve for the discrimination factor involved with NO$_3^-$ uptake and assimilation according to equation 9:

$$\delta^{15}N_{\text{product}} = \delta^{15}N_{\text{substrate}} + \left[ \delta(\ln f) / (1-f) \right]$$

(9)

where, $\delta^{15}N_{\text{product}}$ is the isotopic composition of tissue (coral or algal symbiont) after growing in elevated NO$_3^-$, $\delta^{15}N_{\text{substrate}}$ is the initial isotopic composition of the NO$_3^-$ and $f$ is the fraction of original NO$_3^-$ substrate remaining in the tank after 24 hours.

Before using the cumulative product equation to solve for $\delta^{15}\epsilon$ of NO$_3^-$ uptake from coral or algal symbiont tissues, the following factors must be considered in order to correct for the $\delta^{15}N$ value of the tissue that is derived directly from NO$_3^-$ during the experimental period:

1. The algal and coral tissue turnover rate from a $^{15}N$ labeling incubation are used in a mass balance equation 10 to solve for the $\delta^{15}N$ of the tissue formed during the 28 day experimental period:

$$\delta^{15}N_{\text{tissue measured}} = \delta^{15}N_{\text{tissue new}} (X) + \delta^{15}N_{\text{tissue old}} (1-X)$$

(10)

where $\delta^{15}N_{\text{tissue measured}}$ is the cumulative isotopic composition of old and new tissues after the experimental period, $\delta^{15}N_{\text{tissue old}}$ is the isotopic composition of the tissue prior to the experimental period, $X$ is the fraction of total tissue which is composed of new tissues calculated from the $^{15}NO_3^-$ labeling incubation and $\delta^{15}N_{\text{tissue new}}$ is the calculated isotopic composition of the new tissue formed during the experimental period.

2. Feeding rate estimates and total NO$_3^-$ uptake throughout the 28 day experimental period are used to calculate the fraction of coral and algal tissues derived from NO$_3^-$ and from the heterotrophic food source (equation 11). Corals were fed a measured amount of food once weekly for 3 hours. Based on visual examination, it was estimated that 25% of
added food was consumed. The daily uptake of NO$_3^-$ for each treatment condition was used to extrapolate the total NO$_3^-$ consumed over the 28 day period. The fraction of total nitrogen consumed comprised by NO$_3^-$, $Y$, was calculated as follows:

$$Y = \frac{\text{NO}_3^-$N consumed}{(\text{NO}_3^-$N consumed + heterotrophic N consumed)}$$  \hspace{1cm} (11)

3. The isotopic composition of the tissues derived from NO$_3^-$ was then calculated according to equation 12:

$$\delta^{15}N_{\text{tissue new}} = \delta^{15}N_{\text{NO}_3^-$-derived tissues}(Y) + \delta^{15}N_{\text{food}}(1-Y)$$  \hspace{1cm} (12)

where $\delta^{15}N_{\text{tissue new}}$ was calculated in equation 10 and $\delta^{15}N_{\text{food}}$ is the measured isotopic composition of the coral food and $Y$ is the fraction of total nitrogen consumed comprised by NO$_3^-$ calculated in equation 11.

4. The isotopic composition of the tissues derived from NO$_3^-$ was then substituted into equation 10 to solve for the discrimination factor for coral and algal tissues.

$$\varepsilon = \left[ (\delta^{15}N_{\text{NO}_3^-\text{-derived tissues}} - \delta^{15}N_{\text{NO}_3(o)}) \times (1-f) \right] / [f \ln f]$$  \hspace{1cm} (13)

where $\delta^{15}N_{\text{NO}_3^-\text{-derived tissues}}$ was calculated in equation 12, $\delta^{15}N_{\text{NO}_3(o)}$ is the measured initial isotopic composition of the substrate NO$_3^-$ and $f$ is the calculated average $f$ value from each treatment over the 28 day experimental period.

The $\varepsilon$ measured in the water is manifested in both the algal symbiont and coral tissues, thus in order to compare to calculation from the residual NO$_3^-$, the $\varepsilon$ should be a weighted average of the $\varepsilon$ from algal symbiont and coral tissues (equation 14).

$$^{15}\varepsilon_{\text{NO}_3^-} = (Z)(^{15}\varepsilon_{\text{algal}}) + (1-Z)(^{15}\varepsilon_{\text{coral}})$$  \hspace{1cm} (14)

where $^{15}\varepsilon_{\text{NO}_3^-}$ is the fractionation effect measured through analysis of the substrate NO$_3$, $^{15}\varepsilon_{\text{algal}}$ is the fractionation effect by algal symbionts and $^{15}\varepsilon_{\text{coral}}$ from coral tissues. $Z$ is the total fraction of NO$_3^-$ derived nitrogen accumulating in the algal symbiont and (1-
Z) is the fraction accumulating within the coral tissues. Using a literature value for the average measurement of coral to algal protein biomass of 1:1 for *Pocillopora damicornis* (Muscatine and Cernichiari, 1969), the average was calculated to compare to the NO$_3^-$ $^{15}$ε.

### 2.3 Results

#### 2.3.1 δ$^{15}$N of NO$_3^-$ and $^{15}$ε of Uptake and Assimilation

As the proportion of NO$_3^-$ consumed increased, the δ$^{15}$N of the NO$_3^-$ remaining in the incubation medium became more enriched in Controls and all elevated NO$_3^-$ treatments (Figure 2.3.1). Discrimination factors from the calculations of δ$^{15}$N values and concentrations of NO$_3^-$ in seawater during the incubation period were determined according to equation 8. The discrimination factors from both light only and light/dark conditions are represented graphically in Figure 2.3.2, where the absolute value of the slope from the plot of the Δδ$^{15}$N (change in δ$^{15}$N of NO$_3^-$ throughout the incubation) against the natural log of f (the fraction of original NO$_3^-$ remaining in the incubation tank at each sample) yields the discrimination factor. The discrimination factors calculated from pooled data from replicate tanks for each treatment during both light only and 12 hour light/dark cycle incubations are represented in Tables 2.2.1 and 2.2.2. There was a significant treatment effect on the $^{15}$ε measured in both light and light dark conditions (ANCOVA, p<0.05). Individual p-values for the comparisons between treatment $^{15}$ε values are given in Table 2.3.3. The Control tanks had the lowest discrimination factor with an average of $1.96 \pm 0.57 \%$ and the 50 µM NO$_3^-$ tanks had the highest in the light/dark cycle conditions with a discrimination factor of $7.45 \pm 0.83 \%$. 
Figure 2.3.1. The change in $\delta^{15}N$ of NO$_3$ as the fraction of original NO$_3$ remaining ($f$) goes from 1 to 0 for Controls, 10 $\mu$M NO$_3$, 20 $\mu$M NO$_3$, and 50 $\mu$M NO$_3$ treatments. Data are from replicate tanks A and B from each treatment during Light/Dark Incubations.
Figure 2.3.2 The relationship between $\Delta^{15}N$ and $\ln f$ for all treatments during 48 hour Light Only and Light/Dark incubations. The absolute value of the slope of each line gives the discrimination factor, $\varepsilon$. Data from replicate tanks A and B are plotted for each treatment. Note that data for Controls is the same for Light and Light/Dark incubations and is representative of light uptake as NO$_3^-$ was drawn down to concentrations below detection limits for isotopic analysis before the dark period in Control tanks.
Table 2.3.1 From the Light Only Incubations. A summary of the $^{15}\epsilon$ values and the $^{18}\epsilon$:$^{15}\epsilon$ ratios calculated with the Rayleigh Equation for residual substrate NO$_3^-$. The standard error and $R^2$ values for the regression from which each treatment $^{15}\epsilon$ value (slope) was calculated are given. The standard error and $R^2$ values for the regression of $\Delta\delta$O and $\Delta\delta$N are also given.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{15}\epsilon$ (%o)</th>
<th>SE</th>
<th>$R^2$</th>
<th>$^{18}\epsilon$:$^{15}\epsilon$</th>
<th>SE</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.96</td>
<td>0.39</td>
<td>0.91</td>
<td>1.51</td>
<td>0.22</td>
<td>0.96</td>
</tr>
<tr>
<td>10 µM NO$_3^-$</td>
<td>2.50</td>
<td>0.08</td>
<td>0.99</td>
<td>1.51</td>
<td>0.16</td>
<td>0.96</td>
</tr>
<tr>
<td>20 µM NO$_3^-$</td>
<td>3.14</td>
<td>0.27</td>
<td>0.97</td>
<td>1.27</td>
<td>0.06</td>
<td>0.99</td>
</tr>
<tr>
<td>50 µM NO$_3^-$</td>
<td>6.65</td>
<td>0.41</td>
<td>0.97</td>
<td>0.99</td>
<td>0.09</td>
<td>0.97</td>
</tr>
</tbody>
</table>
Table 2.3.2 From the Light Dark Incubations. A summary of the $^{15}$E: values and the $^{16}$E:$^{15}$E ratios calculated with the Rayleigh Equation for residual substrate NO$_3^-$. The standard error and $R^2$ values for the regression from which each treatment $^{15}$E: value (slope) was calculated are given. The standard error and $R^2$ values for the regression of $\Delta\delta$O and $\Delta\delta$N are also given.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{15}$E (‰)</th>
<th>SE</th>
<th>$R^2$</th>
<th>$^{16}$E:$^{15}$E</th>
<th>SE</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.96</td>
<td>0.39</td>
<td>0.91</td>
<td>1.51</td>
<td>0.22</td>
<td>0.96</td>
</tr>
<tr>
<td>10 µM NO$_3^-$</td>
<td>3.43</td>
<td>0.26</td>
<td>0.97</td>
<td>1.21</td>
<td>0.09</td>
<td>0.97</td>
</tr>
<tr>
<td>20 µM NO$_3^-$</td>
<td>5.37</td>
<td>0.55</td>
<td>0.92</td>
<td>1.07</td>
<td>0.04</td>
<td>0.98</td>
</tr>
<tr>
<td>50 µM NO$_3^-$</td>
<td>7.45</td>
<td>0.83</td>
<td>0.89</td>
<td>1.11</td>
<td>0.09</td>
<td>0.93</td>
</tr>
</tbody>
</table>
Table 2.3.3 Statistical analysis of covariance (ANCOVA) determined treatment effects of \( \mathrm{NO}_3^- \) enrichments on \( ^{15}\varepsilon \) calculated in the Light Only incubations \( (F(3,3) = 46.25, p<0.0001) \) and the Light/Dark incubations \( (F(3,3) = 17.88, p<0.0001) \). P values for the multiple comparisons between each treatment \( ^{15}\varepsilon \) value (slope) are given. Treatment comparisons with \( p<0.05 \) are significantly different.

<table>
<thead>
<tr>
<th>LIGHT ONLY INCUBATION</th>
<th>LIGHT/DARK INCUBATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>50 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
<td>50 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
</tr>
<tr>
<td>10 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
<td>10 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
</tr>
<tr>
<td>20 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
<td>20 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>10 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
<td>20 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
</tr>
<tr>
<td>Control</td>
<td>10 ( \mu \mathrm{M} ) ( \mathrm{NO}_3^- )</td>
</tr>
</tbody>
</table>
2.3.2 Relationship with Oxygen

The comparison of the fractionation of $^{18}$O and $^{15}$N during uptake and assimilation provides information upon the mechanisms involved. Cases in which the ratio of $\Delta\delta^{18}$O : $\Delta\delta^{15}$N > 1 indicate a stronger fractionation of $^{18}$O than $^{15}$N during NO$_3^-$ uptake and assimilation. If $\Delta\delta^{18}$O : $\Delta\delta^{15}$N < 1 then there is a stronger fractionation of $^{15}$N.

Graphically, the data from each treatment are compared to a 1:1 and 2:1 relationship in Figure 2.3.3. The Controls had the highest slope of 1.51 and the 20 µM NO$_3^-$ and 50 µM NO$_3^-$ had slopes of 1.26 and 0.92, respectively (Table 3.1). In the light only conditions, the $\Delta\delta^{18}$O : $\Delta\delta^{15}$N slopes of the Control, 10 and 20 µM NO$_3^-$ were significantly greater than 1 (ANCOVA, p<0.05). In the light dark cycle conditions, however, only the $\Delta\delta^{18}$O : $\Delta\delta^{15}$N slopes of the Control and 10 µM NO$_3^-$ were significantly greater than 1 (ANCOVA, p<0.05).

2.3.3 Discrimination Factor and Initial NO$_3^-$ Concentration

A linear relationship was found to exist between the initial concentration of NO$_3^-$ in the incubation medium and the discrimination factor which was calculated from the NO$_3^-$ $\delta^{15}$N isotopic measurements (Figure 2.3.4). As initial concentration of NO$_3^-$ increased, the nitrogen discrimination factor increased. The linear fit was significant (p=0.029) and is given by equation 12.

\[
^{15}\varepsilon = 0.1045[\text{NO}_3^-] + 2.258
\]  

(12)

2.3.4 Discrimination Factor and Light Conditions

The discrimination factor was calculated for a separate incubation period for all treatment tanks which was carried out during 48 hours of exposure to light only. The comparison of the discrimination factors, represented by the slope of the lines, for light
Figure 2.3.3 The relationship between the change in $\delta^{15}N$ and $\delta^{18}O$ of NO$_2^-$ during NO$_2^-$ incubations in Light Only and Light/Dark incubations. Data are from Control, 10 $\mu$M NO$_2^-$, 20 $\mu$M NO$_2^-$, and 50 $\mu$M NO$_2^-$ treatments. Data is compared to lines representing 1:1 and 2:1 relationships. A 1:1 relationship is accepted to describe nitrate reductase activity, while deviation from this relationship may indicate some other process is acting to influence the isotopic fractionations expressed. Membrane transport has been proposed to lead to a 2:1 relationship.
Figure 2.3.4 The relationship between the $^{15}$C measured by analysis of the residual NO$_3^-$ substrate and the initial NO$_3^-$ concentration in the incubation tanks. The linear relationship is significant (ANOVA, F(1,2) = 32.5379, p = 0.029).
only and exposure to twelve hour light/dark cycles are represented in Table 2.3.1 and 2.3.2 and graphically in Figure 2.3.2. While the results are only statistically significant for the 10 μM NO₃⁻ (ANCOVA, p=0.0309), the average fractionation factor obtained during the light/dark cycles is greater than that for the light only period in all treatments. In addition, the average Δδ¹⁸O:Δδ¹⁵N was lower in the light/dark incubation relative to light only incubation for 10 and 20 μM NO₃⁻.

2.3.5 Discrimination Factor Calculated from Coral and Algal Symbiont Tissue

Calculations of the discrimination factor from δ¹⁵N of the coral and algal tissues (Figure 2.3.5b) synthesized during the four week experiment are compared to those values calculated from the residual NO₃⁻ in Table 2.3.4. While the data set for this experiment cannot completely constrain the calculations of ¹⁵ε from algal and symbiont tissues, the purpose of including these calculations is to demonstrate that when reasonable estimates of feeding are assumed (25%), ¹⁵ε values obtained are in the range of those calculated through analysis of residual NO₃⁻. It must be noted that the overall fractionation effect exhibited through analysis of the water will be manifested through the algal symbiont and also the coral tissues as the symbionts are known to translocate amino acids and glyconjugates to the coral host tissues (Sutton and Hoegh-Guldberg, 1990; Markell and Trench, 1993). Thus, assuming no other sinks of NO₃⁻ in the system, the fractionation effect measured on residual NO₃⁻ should be equal to the weighted contributions from the fractionation effects measured in algal symbionts and coral tissues dependent on the proportion of the NO₃⁻ accumulating in each fraction. Calculations from analysis of tissues and residual NO₃⁻ agree with a positive correlation between the initial concentration of NO₃⁻ in the incubation medium and the discrimination factor ¹⁵ε.
Figure 2.3.5  a) The organic nitrogen isotopic composition ($\delta^{15}$N) for separated components of the coral-algal symbiosis, coral tissue (orange) and algal symbiont tissue (green) for each elevated NO$_3^-$ treatment and Control tanks. Each error bar represents one standard error. b) The relationship between the $\delta^{15}$N of the residual NO$_3^-$ substrate and the $\delta^{15}$N calculated from $\delta^{15}$N of the coral and algal symbiont tissues. The coral-symbiont average represents the cumulative fractionation for coral and algal tissues for comparison to that calculated by analysis of the residual NO$_3^-$ substrate. The error bars for the residual NO$_3^-$ represent one standard error. The error bars for the coral-symbiont average represent 10% uncertainty of coral feeding rates used for calculating $\delta^{15}$C.
Table 2.3.4 A summary of the $^{15}$N values calculated from the Rayleigh Equations for cumulative product using coral tissue $\delta^{15}$N and algal symbiont $\delta^{15}$N and the average for coral and algal symbiont $\delta^{15}$N compared to the $^{15}$N calculated by analysis of the residual substrate NO$_{3}^{-}$ ($^{15}$N DIN). Calculations are given for estimates of heterotrophic consumption of 25% of food available to corals during feeding.

<table>
<thead>
<tr>
<th>Method</th>
<th>$^{15}$N (%o) Control</th>
<th>$^{15}$N (%o) 10 µM NO$_{3}^{-}$</th>
<th>$^{15}$N (%o) 20 µM NO$_{3}^{-}$</th>
<th>$^{15}$N (%o) 50 µM NO$_{3}^{-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIN</td>
<td>1.96</td>
<td>3.43</td>
<td>5.37</td>
<td>7.45</td>
</tr>
<tr>
<td>Coral</td>
<td>-----</td>
<td>-1.91</td>
<td>0.64</td>
<td>1.44</td>
</tr>
<tr>
<td>Algal Symbiont</td>
<td>-----</td>
<td>3.85</td>
<td>10.19</td>
<td>13.13</td>
</tr>
<tr>
<td>Coral-Symbiont Avg</td>
<td>-----</td>
<td>0.97</td>
<td>5.42</td>
<td>7.29</td>
</tr>
</tbody>
</table>
2.3.6 Effect of Elevated NH$_4^+$ on $^{15}\varepsilon$ of NO$_3^-$ Uptake and Assimilation

The relationship between the concentration of NH$_4^+$ in the incubation tank and the $^{15}\varepsilon$ associated with NO$_3^-$ uptake and assimilation is shown in Figure 2.3.6. There is a significant linear relationship ($p<0.05$) where $^{15}\varepsilon = 0.63[\text{NH}_4^+]+1.67$.

2.4 Discussion

2.4.1 Range in Discrimination Factors Measured

The range in $^{15}\varepsilon$ (from 1.96 to 7.45 ‰) calculated from $\delta^{15}N$ of NO$_3^-$ in this experiment is consistent with values previously measured in eukaryotic and prokaryotic marine plankton, which have been found to range from 1-20 ‰, but are typically less than 10 ‰ (Granger et al. 2004, Needoba et al. 2004, Granger et al. 2010). Previous measurements of $^{15}\varepsilon$ for cyanobacterial strains have been less than 5 ‰ and the $^{15}\varepsilon$ values measured for a heterotrophic proteobacterium were much lower from to 0.4 to 1 ‰ (Granger et al. 2010). The fractionation expressed for uptake and assimilation of NO$_3^-$ has classically been assumed to be associated with the assimilatory nitrate reductase enzyme during conversion of NO$_3^-$ to NO$_2^-$ prior to assimilation into organic nitrogen. This step has been thought to be the rate limiting step and is associated with a $^{15}\varepsilon$ in eukaryotic assimilatory nitrate reductases of $\sim$26 ‰ (Karsh et al. 2012). Smaller $^{15}\varepsilon$ values, as measured in the heterotrophic bacterium, have been interpreted to result from the step of NO$_3^-$ transport across the cell membrane. The variability in $^{15}\varepsilon$ in this study is correlated with the concentration of NO$_3^-$ in the coral growth medium (Figure 2.3.4).

2.4.2 Potential Causes for Variability in ε for the Coral-Algal Symbiosis

In the coral-algal system, the NO$_3^-$ is first transported across the plasma membrane of the animal tissue. From an intracellular space in the animal tissue, it is then
Figure 2.3.6 The relationship between NH$_4^+$ concentration $\delta^{15}N$ calculated for NO$_3^-$ uptake and assimilation using $\delta^{15}N$ of NO$_3^-$ from Controls, 10, 20 and 50 μM NH$_4^+$ treatment tanks.

$\delta^{15}N = 0.6307[\text{NH}_4^+] + 1.647$

$R^2 = 0.830$
transported across the symbiosome (surrounding the symbiont cell) and the cell membrane of the algal symbiont. Previous workers have assumed that NO$_3^-$ is able to diffuse into the coral tissues from the surrounding seawater, but must be actively transported across the symbiosome and algal cell membrane (D'Elia 1977, Delia et al. 1983). Once transported into the symbiont cell, the NO$_3^-$ has three possible pathways 1) to be converted from NO$_3^-$ to NO$_2^-$ by nitrate reductase and then to NH$_4^+$ by nitrite reductase, 2) to remain in the intracellular NO$_3^-$ pool or 3) to be effluxed or leak out from the symbiont cell back into the coral tissue. This potential for efflux complicates the interpretation of changes in $\delta^{15}$N and $\delta^{18}$O. While no role has been established for NO$_3^-$ efflux in microbial physiology, there are two possible mechanisms proposed by Granger et al. (2010) to describe NO$_3^-$ efflux for unicellular phytoplankton: leakage or passive diffusion through non-specific ion channels, or strictly regulated reverse flow which may be necessary to maintain electrochemical balance or is used to power the movement of other ions across the cell membrane through antiporters. Further evidence by these workers that cell volume, which would influence the amount of passive NO$_3^-$ leakage, had no influence on the isotope effect of several unicellular eukaryotes suggests that the algae must actively efflux NO$_3^-$ to maintain cytosolic NO$_3^-$ homeostasis. This suggests that there may be some maximum intracellular NO$_3^-$ concentration which is regulated within the algal cells.

If all NO$_3^-$ transported across the coral animal cell membrane is retained (never effluxed from the tissue), then the only isotopic fractionation expressed, as measured from analysis of water samples, would be that associated with the initial transport into coral tissues. In contrast, if NO$_3^-$ is effluxed from both symbiont and coral tissue into the
incubation medium, then the NO$_3^-$ of the surrounding water will hold information of not only the fractionation involved in initial transport across the coral tissue membrane and the symbiont membrane, but also the fractionation effect of the nitrate reductase activity converting NO$_3^-$ to NO$_2^-$ within the symbiont.

Two models were developed in Figure 2.4.1 in order to elucidate the controls on the $\delta^{15}$N and $\delta^{18}$O of NO$_3^-$ in the incubation medium and the resulting $\delta^{15}$N of algal symbionts. Hypothetical calculations were carried out to display the influences on $\delta^{15}$N and $\delta^{18}$O of NO$_3^-$ at each step. Note that for purposes of simplicity, each N transformation is compartmentalized, influencing the $\delta^{15}$N and $\delta^{18}$O of NO$_3^-$ in a stepwise manner. In reality, however, these mechanisms are occurring simultaneously thus the NO$_3^-$ $\delta^{15}$N and $\delta^{18}$O are continuously altered by processes adding and removing NO$_3^-$ from each reservoir within the seawater, coral tissue and algal symbiont. Values of $\varepsilon$ and $f$ were set and the resulting $\delta$ values for substrate and product at each step were calculated according to equations 7 and 9. Values were set using estimates for the isotope effects of algal NO$_3^-$ uptake, efflux and NR activity based on previous literature values for unicellular plankton (Karsh et al. 2014). Literature values for *Thalassiosira weisfloggii* for $^{15}\varepsilon$ for uptake and efflux of 2.0 and 1.2 ‰ were used, and values for $^{18}\varepsilon$ uptake and efflux of 2.8 ‰ were used. A mid-range value from the literature was chosen for enzyme activity of nitrate reductase (NR) at 20 ‰. In order to simplify the models, the diffusion-depletion model for NO$_3^-$ uptake by corals was assumed and the isotope effect for diffusion of NO$_3^-$ in and out of coral tissues was assumed to be 0 ‰.

Model A represents a situation under which the transport of NO$_3^-$ across the algal membrane is the rate limiting step responsible for the observed isotope effect. An
arbitrary f value of 0.5 is used for the transport of NO$_3^-$ across the cell membrane, indicating half of the NO$_3^-$ in the incubation medium is transported across the cell membrane. An f value of 0 for the assimilation of NO$_3^-$ by nitrate reductase is used as it can be hypothesized that when transport is the rate limiting step, most of the NO$_3^-$ transported into the algal cell will be assimilated into organic nitrogen. Because all of the NO$_3^-$ transported into the algal cell will be assimilated, this model does not involve efflux of intracellular algal NO$_3^-$ into coral tissues and seawater. The final isotopic composition of NO$_3^-$ measured in the seawater is thus the result of only the first fractionating step of NO$_3^-$ transport across the algal cell membrane.

Model B represents the situation in which the NR activity becomes the rate limiting step of NO$_3^-$ uptake and assimilation. An arbitrary f value of 0.5 is used for the transport of NO$_3^-$ across the cell membrane. Then assuming that the NR is saturated, an arbitrary f value of 0.5 is used considering that NR is able to assimilate half of the intracellular NO$_3^-$.

At the point of saturation of NR activity and/or maximum algal cytosolic NO$_3^-$ concentrations, the residual intracellular NO$_3^-$ is effluxed into coral tissues. The $\delta^{15}$N and $\delta^{18}$O of effluxed NO$_3^-$ propagates the isotope effect of NR activity from the algal cells into the coral tissues where it mixes with the residual NO$_3^-$ remaining in the tissues from the initial transport step. The NO$_3^-$ which diffuses into seawater from coral tissues, and is analytically measured, is thus a combination of the NO$_3^-$ influenced by the isotope effect of cell membrane transport and the NO$_3^-$ influenced by NR activity inside the algal cells.
Figure 2.4.1 Two models are shown for NO₃⁻ uptake and assimilation by the coral-algal symbiosis wherein Model A, the rate limiting step is transport of NO₃⁻ into the algal cell and in Model B, the rate limiting step is during NO₃⁻ reduction by nitrate reductase (NR) and there is consequently significant efflux of algal intracellular NO₃⁻ into coral tissues. Both models start with δ¹⁵N and δ¹⁸O of NO₃⁻ at 0 %o and involve hypothetical 4 and f values for each step from which the resulting δ values for residual NO₃⁻ and formed product were calculated according to equation 3. Model A: 1) diffusion of NO₃⁻ across algal cell membrane, 2) active transport of NO₃⁻ across algal cell membrane, 3) complete assimilation of NO₃⁻ into organic N by NR, nitrite reductase and NH₄⁺ assimilating enzymes and 4) diffusion of coral intracellular NO₃⁻ to external seawater where its is sampled over time. Model B: 1) diffusion of NO₃⁻ across coral cell membrane, 2) active transport of NO₃⁻ across algal cell membrane, 3) partial assimilation of intracellular NO₃⁻ into organic N by NR leaving behind residual NO₃⁻ enriched in ¹⁵N and ¹⁸O and forming organic N depleted in ¹⁵N and ¹⁸O, 4) efflux of the residual NO₃⁻ which has been influenced by NR to coral tissues, leaving behind residual NO₃⁻ influenced by isotope effects of efflux, 5) in the coral tissues, mixing of NO₃⁻ influenced by initial transport across algae membrane and effluxed NO₃⁻ (for this example, we assumed equal contributions from each) and 6) diffusion of mixed NO₃⁻ to seawater where it is sampled over time.
In summary for the two models, it should be noted that Model A results in a relatively small magnitude of $^{15}\epsilon$ and $^{18}\epsilon$ calculated from seawater NO$_3^-$ and algal symbiont $\delta^{15}N$. Model A also has a $\Delta\delta^{18}O:\Delta\delta^{15}N$ ratio much greater than 1 ($\Delta\delta^{18}O:\Delta\delta^{15}N = 1.41$). Model B results in larger magnitude $^{15}\epsilon$ and $^{18}\epsilon$ from both seawater and algal symbiont calculations and a ratio of $\Delta\delta^{18}O:\Delta\delta^{15}N$ which is close to 1 ($\Delta\delta^{18}O:\Delta\delta^{15}N - 0.92$).

2.4.3 Effect of Concentration upon $^{15}\epsilon$ and $^{18}\epsilon$

In this study and similar work on macro algae (Swart In Review), the magnitude of fractionation varied with respect to the concentration of NO$_3^-$. The variability could indicate differing amounts of efflux taking place in each treatment, thus varying degrees of fractionation involved with each of these steps, cell membrane transport or NR activity, are expressed. A model has been proposed by other workers which considers the amount of intracellular NO$_3^-$ efflux when calculating the fractionation factor for unicellular algae (Karsh, 2014).

When the conclusions made from the kinetic data in Chapter One are considered, it is noted that uptake of NO$_3^-$ appears to follow Michaelis Menten saturation kinetics. There are three mechanisms which could be responsible for the saturation of uptake rate: 1) saturation at the active transporters on the plasma membrane of the algal symbionts, 2) saturation of the nitrate reductase enzyme or 3) saturation at a maximum intracellular algal NO$_3^-$ concentration, above which NO$_3^-$ is effluxed. Mechanisms 2 and 3 could potentially be linked, as NR activity saturates, intracellular algal NO$_3^-$ builds up, potentially reaching a maximum concentration at which point efflux would increase as NR is unable to sufficiently draw down intracellular NO$_3^-$. 

The concentration dependent data suggest that the saturation point, observed in the kinetic analysis, likely corresponds to concentrations at which significant efflux from the algal symbiont cells into coral tissue and seawater first occurs. The primary argument for this is the increased isotope effect measured at higher concentrations. The isotope effect associated with nitrate reductase is large and has been measured for a variety of other organisms to range from 15-25 ‰ (Ledgard et al. 1985, Schmidt and Medina 1991, Needoba et al. 2004). In this study, in order to measure the NR isotope effect in a seawater sample, there must be efflux of algal intracellular NO$_3^-$ which has been influenced by preferential removal of $^{14}$N and $^{16}$O during NR activity. With increasing concentrations above the NR saturation point, any potential increase in rate of NO$_3^-$ transport across the algal cell membrane is likely met with an increase in efflux rate. Thus, at higher concentrations, the NR becomes saturated leading to greater efflux of intracellular NO$_3^-$ and a greater observed isotope effect. It is most likely that the corals in this study grown under higher NO$_3^-$ concentrations are following similar uptake mechanisms to those proposed in Model B, Figure 4.1.

At lower concentrations, a much smaller isotope effect was measured for both nitrogen and oxygen. This suggests that most of the NO$_3^-$ transported across the algal cell membrane is actively assimilated into organic nitrogen as the NR activity is not saturated and there is little efflux of algal intracellular NO$_3^-$. This results in a much lower isotope effect measured in both the algal symbiont organic $\delta^{15}$N and the $\delta^{15}$N and $\delta^{18}$O of NO$_3^-$ in the seawater samples collected throughout the incubation. At lower concentrations the rate limiting step responsible for the apparent isotope fractionation must be the transport
of NO$_3^-$ across the algal cell membrane, which has been previously described for other unicellular eukaryotes as very small.

In the case of NO$_3^-$, in addition to the fractionation of $^{15}$N, it is possible to examine the fractionation of $^{18}$O involved in uptake. Additional evidence can be drawn from the $^{18}\varepsilon:^{15}\varepsilon$ ratio from the uptake data. While the isotope effects associated with cell membrane transport are thought to be small for both nitrogen and oxygen, it is suggested that oxygen is more strongly fractionated during transport resulting in a $^{18}\varepsilon:^{15}\varepsilon$ ratio greater than 1. The isotope effects for NR activity are much larger, as previously noted from 15-25‰, and are accepted to be relatively similar for both nitrogen and oxygen resulting in a $^{18}\varepsilon:^{15}\varepsilon$ ratio close to 1 (Granger et al. 2010). The data from the lower NO$_3^-$ concentrations yield a higher $\Delta\delta^{18}$O:$\Delta\delta^{15}$N ratio, suggesting that oxygen is more heavily fractionated with respect to nitrogen in these experiments, providing additional evidence that the rate limiting step is cell membrane transport. The data from the higher concentrations yield a ratio of $\Delta\delta^{18}$O:$\Delta\delta^{15}$N which is closer to 1, suggesting equal fractionating effects for both nitrogen and oxygen, indicative that NR activity is the rate limiting step.

Combining the observations from uptake kinetics and isotope data, it can be concluded that uptake and assimilation of NO$_3^-$ is limited by the rate of transport at lower concentrations, where there are smaller $^{15}\varepsilon$ measurements and a higher $\Delta\delta^{18}$O:$\Delta\delta^{15}$N ratio. At higher concentrations, there is a saturation of uptake rate at 0.026 umoles/cm$^2$ of NO$_3^-$ uptake which likely occurs at saturation of NR activity. The higher $^{15}\varepsilon$ values and $\Delta\delta^{18}$O:$\Delta\delta^{15}$N ratio closer to 1 indicate that at higher NO$_3^-$ concentrations, there is
increased efflux of NO$_3^-$ from algal symbiont cells allowing for the propagation of the isotope effect associated with NR activity.

2.4.4 Other Influences on the Isotope Effect

In addition to increased NO$_3^-$ concentrations in seawater resulting in increased isotope effect, it also appears that darkness and increased NH$_4^+$ concentrations both contribute to an increased isotope effect. The data suggest that the effects of these two factors are related to their influence on NR activity. The inhibition of NO$_3^-$ uptake in the presence of elevated NH$_4^+$ was described in Chapter One. When the NO$_3^-$ isotope data from the NH$_4^+$ inhibition uptake is analyzed, there is a correlation between the NH$_4^+$ concentration in the tanks and the $^{15}\varepsilon$ measured for uptake and assimilation of NO$_3^-$ (Figure 3.11). As the concentration of NH$_4^+$ increases, the isotope effect for NO$_3^-$ uptake and assimilation increases as well. This suggests that under elevated NH$_4^+$, there is more efflux of algal intracellular NO$_3^-$ into coral tissue and seawater, most likely caused by less NR activity inside the algal symbionts. The mechanism by which elevated NH$_4^+$ represses the NR activity is not known, but could either be directly related to NH$_4^+$ presence or increased glutamine synthesis. This finding may have implications regarding the potential for feeding effects on isotope fractionation associated with NO$_3^-$ uptake and assimilation. As increased feeding leads to increased catabolism which produces NH$_4^+$ in the tissues, corals which are feeding more make more NH$_4^+$ available to symbionts and may display a different fractionation during NO$_3^-$ uptake than those corals which are less heterotrophic.

In a comparison of light only exposure uptake period and light/dark exposure cycle, there are slight differences in the fractionations expressed. The light/dark exposure
generally yields a higher isotope effect than the light only exposure period. Darkness has been noted to suppress nitrate reductase activity in several marine photosynthesizers (Young et al. 2007). As light exposure decreases, less photosynthetic energy is available for nitrate reductase activity to reduce NO₃⁻ to NO₂⁻. Light limited NR activity in these experiments may be responsible for the slightly larger isotope effect observed for corals during the light/dark cycle incubations as opposed to the light only incubations. As NR activity is reduced in darkness, the amount of efflux may increase to maintain the intracellular NO₃⁻ homeostasis. This would permit greater propagation of the NR isotope effect as more intracellular NO₃⁻ is effluxed into coral tissues and measured in the water samples collected.

2.5 Conclusions

Synthesis of uptake kinetics and isotope data leads to conclusions that uptake and assimilation of NO₃⁻ is limited by the rate of transport at lower concentrations, where lower $^{15}\varepsilon$ measurements and higher $\Delta\delta^{18}$O:$\Delta\delta^{15}$N ratios are observed. At higher concentrations, we see there is a saturation of uptake rate at 0.037 µmoles/cm² of NO₃⁻ uptake which likely occurs at saturation of NR activity. The higher $^{15}\varepsilon$ values and $\Delta\delta^{18}$O:$\Delta\delta^{15}$N ratio closer to 1 indicate that under higher concentrations, there is increased efflux of NO₃⁻ from algal symbiont cells allowing for the propagation of the isotope effect associated with NR activity. The isotope effect associated with NO₃⁻ uptake and assimilation is also increased in darkness and elevated NH₄⁺ concentrations, which may have implications regarding the influence of feeding on $^{15}\varepsilon$ for NO₃⁻ uptake and assimilation.
The data suggest that intracellular NO$_3^-$ concentrations are not sufficiently high under the low NO$_3^-$ conditions typical of oligotrophic reefs to saturate NR and promote efflux of NO$_3^-$ from algal cells. Thus the large isotope effect of NR is not typically propagated to the reef waters. It is possible that unlike other unicellular marine algae which have been measured to have intracellular NO$_3^-$ in the millimolar range, these endosymbiotic algae could operate under much lower intracellular NO$_3^-$ concentrations. It is alternatively possible that these symbiont cells are able to maintain internal NO$_3^-$ homeostasis at millimolar concentrations by balancing NO$_3^-$ depletion from NR activity and cellular efflux with active uptake. Further experimentation to measure the direct intracellular NO$_3^-$ concentration of *Symbiodinium* under a range of experimental NO$_3^-$ conditions would provide a key piece of information to improve the understanding of the role of NO$_3^-$ in the internal nutrient environment of algal symbionts and overall nitrogen cycling in the coral-algal symbiosis.
Chapter Three – Uptake Associated Isotopic Fractionation of Ammonium $^{15}$N by *Pocillopora damicornis*

3.1 Background

$\delta^{15}$N signatures of marine organisms are commonly used as bioindicators to understand anthropogenic nutrient loads in the marine environment, and the $\delta^{15}$N signatures of coral tissues have been analyzed in attempts to discern nutrient inputs to reef habitats (Heikoop et al. 2000). In addition to understanding how the coral-algal symbiosis fractionates $^{15}$N during NO$_3^-$ uptake and assimilation (Chapter Two) it is important to understand the isotopic fractionation associated with the uptake and assimilation of NH$_4^+$, as corals are known to utilize NH$_4^+$ as a nitrogen source when available. This chapter focuses on the fractionation of $^{15}$N involved with the uptake and assimilation of NH$_4^+$ by the coral-algal symbiosis. There are limited data which document the fractionation of $^{15}$N during uptake and assimilation of NH$_4^+$ by marine organisms, and no studies to date investigating fractionation by the coral-algal symbiosis.

3.1.1 Distinct Isotopic Signatures of NH$_4^+$ Sources

As previously discussed in Chapter Two, the transformations of the nitrogen cycle result in unique isotopic signatures of various pools of dissolved inorganic nitrogen (DIN) (Capone, 2008). Terrestrial inputs of DIN to the coastal environment are typically characterized by enriched $\delta^{15}$N signatures as they are often associated with nutrients derived from such sources as animal waste, septic systems and sewage treatment plants. NH$_4^+$ pools heavily influenced by removal through NH$_3$ volatilization will have very enriched $\delta^{15}$N signatures. One question addressed in this study is how these unique signatures of NH$_4^+$ $\delta^{15}$N are recorded in coral tissues and algal symbionts under variable NH$_4^+$ concentrations. As there has been no previous experimental work on the isotopic
fractionation involved with NH$_4^+$ uptake and assimilation by corals and their symbiotic algae, a review of the mechanisms contributing to the isotopic fractionation during uptake of NH$_4^+$ in other marine organisms is considered. For further discussion on the theory behind isotope fractionation, refer to Chapter Two, Section 2.1.1 Isotope Fractionation Background.

### 3.1.2 Previous Measurements of $^{15}\varepsilon$ Associated with NH$_4^+$ Uptake & Assimilation

**Bacteria**

A range in $^{15}\varepsilon$ values for NH$_4^+$ uptake and assimilation were measured for the marine bacterium, *Vibrio harveyi* (Hoch, 1992). The magnitude of fractionation expressed varied dependent on NH$_4^+$ concentration with concentrations of 23–182 µM resulting in a range of ε from 4 to 27 ‰ and concentrations of 182 µM–23.3 mM resulting in a range of ε from 27 to 14 ‰. The variability in $^{15}\varepsilon$ with concentration was interpreted to be a result of variable influence on the fractionating step from membrane transport enzymes (active transport vs. membrane diffusion) and NH$_4^+$ assimilation enzyme activity (GS vs. GDH) which changed as a function of external NH$_4^+$ concentration. Another study looking at NH$_4^+$ uptake by another bacteria, *Escherichia coli* K12 also measured variability ε values. They found that fractionation at high concentrations of NH$_3$ (≥0.89 µM) was 19.2 ‰ and was associated with equilibrium isotope effect for dissociation of NH$_4^+$ to NH$_3$ + H$^+$ whereas at low concentrations (≤ 0.18 µM) ε was 14.1 ‰ and was determined to be associated with the cell transporter, NH$_4^+$ channel AmtB (Vo et al. 2013).
**Macroalgae**

Data contributing to the understanding of $^{15}$N fractionation during uptake of NH$_4^+$ by macroalgae is scarce. There are a few studies that suggest that there are metabolic effects on the fractionation. Dudley et al. (2010) discovered a greater discrimination against $^{15}$N during uptake by *Ulva pertusa* exhibited by algae that were grown in a shaded treatment as opposed to full light. This difference in discrimination resulted in a 3.7 ‰ difference between the algae grown in shaded versus full light. In a study relating δ$^{15}$N of NO$_3^-$ and NH$_4^+$ to a variety of annual and perennial macroalgae species in proximity to the Charente catchment, Raimonet et al. (2013) suggested that macroalgal δ$^{15}$N may be influenced not only by δ$^{15}$N of NH$_4^+$, but also environmental parameters that may affect macroalgal metabolism (turbidity, temperature and DIN concentrations).

**Phytoplankton**

The ε associated with NH$_4^+$ uptake by the diatom *Phaeodactylum tricornutum* in 3.5 mM NH$_4^+$ was measured to be 2 ‰ when 40% of the NH$_4^+$ substrate was consumed (Wada and Hattori 1978). Another study determined that $^{15}$ε for diatom NH$_4^+$ uptake varied with concentration, and measured a range in $^{15}$ε values from 5 to 29 ‰ for growth of *Skeletonema costatum* in 50-100 µM NH$_4^+$ (Pennock et al. 1996). Waser et al. (1998) measured $^{15}$ε values of 20 to 25 ‰ associated with NH$_4^+$ uptake by the diatoms *Thalassiosira pseudonana* and *Chaetoceros debilis* grown in 166 µM NH$_4^+$. The same study measured $^{15}$ε for the coccolith *Emiliana huxleyi* to range from 16 to 19 ‰. The wide range in $^{15}$ε values (2 to 29 ‰) may suggest variability in the rate limiting step associated with NH$_4^+$ uptake and assimilation related to both species specific effects as well as variable NH$_4^+$ concentration conditions.
**Animals**

While corals possess the machinery to directly assimilate NH$_4^+$ within their tissues and are suggested to do so, there are no literature comparisons available to understand the isotopic fractionation which may occur for such assimilation of NH$_4^+$ directly by animal tissues. There is thought to be very little fractionation associated with heterotrophic nitrogen acquisition by animals, however, there is a general rule that there is approximately a 3 ‰ enrichment with each advancement in trophic level. This is due to the fractionation involved with the process of amino acid degradation during deamination or removal of amine group forming NH$_3$. As animals eliminate NH$_3$, the $^{14}$N bearing NH$_3$ will be preferentially eliminated over $^{15}$N bearing NH$_3$ with a discrimination factor of approximately 3 ‰. This leaves the animal approximately 3 ‰ heavier than the source of nitrogen which it consumed. The magnitude of this “trophic effect”, however, is thought to vary dependent on the nitrogen efficiency of the animal. When a greater amount of waste (isotopically light) is released, the animal will be much heavier isotopically than its nitrogen source. If very little waste is excreted (efficient N use), the animal will be very similar to the isotopic value of its nitrogen source (Capone 2008).

**Corals**

While $^{15}$N fractionation during NH$_4^+$ uptake and assimilation has not been measured directly for corals, workers have made interpretations on environmental factors which may influence the nitrogen isotopic signature of coral tissues (Heikoop et al. 1998). From the analysis of a number of coral samples collected from a variety of depths, workers suggested that corals in high light will experience very low fractionation effects and have isotope values equal to their nitrogen source, while corals in low light may
express higher fractionation effects. The influence of varying degrees of heterotrophy at different depths, however, is also important when interpreting the δ¹⁵N of coral tissues.

3.1.3 Considerations for NH₄⁺ Cycling in the Coral-Algal Symbiosis

As the coral system is a symbiosis, when understanding NH₄⁺ cycling, both components, dinoflagellate algal symbiont (Symbiodinium microadriaticum in this study) and invertebrate host (in this study Pocillopora damicornis) must be considered. It may be suspected that the symbiotic dinoflagellate algae utilizes similar mechanisms during NH₄⁺ uptake and assimilation as other unicellular marine algae for which fractionation has previously been measured, the primary difference being that the unicellular algal symbionts reside within the animal gastrodermal tissue layer and are not in direct contact with external seawater.

Mode of NH₄⁺ Transport into Coral and Algal Cells

Whether or not the host tissue plays a role in the direct assimilation of NH₄⁺ into organic molecules, it must play some role in uptake of nitrogen as algal symbionts reside within the coral gastrodermal cells (Davy et al. 2012). The symbionts are located inside of what has been termed the symbiosome vacuole. In order for the symbiont to receive a source of NH₄⁺ from external seawater, the NH₄⁺ first must pass through the coral gastrodermal tissue layer. Very little is known about the mechanism by which corals and Symbiodinium transport inorganic nitrogen to the site of assimilation. While NH₃ can freely diffuse across the cell membrane, the diffusion of NH₄⁺ is extremely slow and the transport of NH₄⁺ across biological membranes must be carrier-mediated. At typical seawater pH of 8.1, only 3.8% of NH₄⁺ + NH₃ exists as NH₃ (see Figure 3.1.1). The “depletion-diffusion” model of NH₄⁺ uptake for cnidarian – dinoflagellate symbioses was
proposed by Delia et al. (1983). It suggests that the algal symbionts take up NH$_4^+$ at a rate that will deplete concentrations within the coral tissues allowing for continuous diffusion of NH$_3$/NH$_4^+$ from seawater into the animal tissues.

The uptake of NH$_4^+$ could also be through facilitated diffusion, where the maximum rate of uptake will occur when the carrier proteins become saturated. Alternatively, if the intracellular concentrations of NH$_4^+/NH_3$ inside coral tissues are higher than external seawater, then the coral cells must expend some form of energy to transport NH$_4^+$ against the electrochemical gradient and into coral tissues. The potential role of transporters in carrying NH$_4^+$ into cnidarian tissues was recently highlighted in a study comparing gene expression between an aposymbiotic and symbiotic sea anemone, *Aiptasia pallida*. Lehnert et al. (2014) noted differential expression of genes encoding NH$_4^+$ transporters. A “rhesus-like” gene was upregulated 150 fold in the symbiotic vs. aposymbiotic anemone and an “Amt-like” gene was upregulated 5.6 fold. The complications with these interpretations are the role of these NH$_4^+$ transporters. As elevated levels of NH$_4^+$ can be toxic, while the transporters potentially deliver NH$_4^+$ to the coral host from surrounding seawater, it is possible that the role of these transporters is to pump NH$_4^+$ out of the tissues.

The same is true for the concentration gradient between coral tissues and algal symbionts. When concentrations in the algal cell exceed those in the surrounding coral tissues, energy must be expended to actively transport NH$_4^+/NH_3$ into the symbiont cell. The first evidence for NH$_4^+$ transporters within *Symbiodinium* was discovered through a study of expressed sequence tags (EST) by Leggat (2007). The workers discovered genes
Figure 3.1.1 The pH dependent speciation of $\text{NH}_4^+$ and $\text{NH}_3$. Typical pH range of seawater is shown in light blue.
coding for NH$_4^+$ transport proteins that were closely related to the NH$_4^+$ transport proteins of bacteria. If analogous to the AmtB of *E. coli* bacteria, the activity of such transporters are typically dependent on the amount of glutamine within the tissues. Under low extracellular NH$_3$ concentrations, as glutamine levels fall, transporters are activated. It is thought that under such conditions where transporters are activated, there will be very low relative leakage of NH$_3$ from the intracellular NH$_4^+$/NH$_3$ pool.

As there is not a clear understanding of the internal NH$_4^+$ environment of coral tissue and algal symbionts, the mechanisms for transport into and across animal tissues as well as transport across the algal symbiont cell membrane remain unclear. It is hypothesized that coral catabolism leads to elevated intracellular NH$_4^+$ concentrations (Falkowski, 1993). Previous work has estimated the intracellular concentration of NH$_4^+$ to range between 5-50 µM NH$_4^+$ (Crossland and Barnes, 1977), but there are no data concerning the algal intracellular NH$_4^+$ concentrations.

In order to understand the potential for NH$_3$ diffusion in and out of tissues, the speciation of NH$_4^+$/NH$_3$ and the pH of seawater and various compartments must also be considered. Dependent on light and photosynthetic activity, the coral tissue intracellular pH is thought to range from 7.4 to 8.4, likely leading to a diurnal range in NH$_4^+$/NH$_3$ speciation and rates of NH$_3$ diffusion in and out of coral tissues. The pH of the seawater in these elevated NH$_4^+$ experiments is an average of 8.08 pH units. While a study by Kuhl et al. (1995) found that coral tissues exhibited an elevated pH of 8.5 in light, other recent microsensor studies have measured much lower pH values of coral tissues. Venn et al. (2009) found that coral cells hosting symbionts had higher pH in the light (7.41) than in the dark (7.13), while coral cells where symbionts were absent were not significantly
different in light and dark. It was also determined that pH was drastically lowered (pH < 6) surrounding the symbiont cells, potentially within the symbiosome membrane complex. Studies have noted that the area of the subcalicoblastic medium (fluid at the tissue/skeleton interface) is characterized by elevated pH (~ 0.3 units higher than ambient seawater, ~1.0 unit higher than the calcifying cell layer).

Assimilation

While it is generally accepted that nitrate assimilation occurs only in the symbiont cells, studies suggest that along with the algal symbiont, the coral can directly assimilate NH$_4^+$ (Catmull et al. 1987, Dudler and Miller 1988, Miller and Yellowlees 1989b, Wang and Douglas 1998, Grover et al. 2002a, Lipschultz and Cook 2002). It has been noted, however, that assimilation of NH$_4^+$ does occur primarily in the symbionts (Miller and Yellowlees 1989b, Wang and Douglas 1998, Grover et al. 2002a) with an estimate of algal symbiont cells assimilating 14 to 23 times more nitrogen than coral host cells (Pernice et al. 2012). The assimilation of NH$_4^+$ by the coral host is thought to occur primarily through the NADPH-glutamate dehydrogenase pathway (NADPH-GDH) catalyzing the synthesis of glutamate from NH$_4^+$ (Miller and Yellowlees 1989b, Roberts et al. 2001):

**NADPH/GDH Pathway**

\[
\text{NH}_3^+ + 2\text{-oxoglutarate}^+ + \text{NADPH}^+ + \text{H}^+ \rightleftharpoons \text{glutamate}^+ + \text{NADP}^+
\]
The algal symbionts are thought to catalyze glutamate from NH$_4^+$ primarily through the glutamate synthetase/glutamine 2-oxoglutarate amido transferase pathway (GS/GOGAT):

**GS/GOGAT Pathway**

\[
\text{NH}_3 + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi} \\
\text{glutamine} + 2-\text{oxoglutarate} + \text{NADPH} + H^+ \rightarrow 2 \text{glutamate} + \text{NADP}^+ 
\]

Both GS and GDH use NH$_3$ as a substrate, because the bond involves the lone pair of electrons on the N of NH$_3$. This requires that prior to assimilation, there must be deprotonation of NH$_4^+$ to NH$_3$ + H$^+$. (Vo et al. 2013).

**Translocation**

It has been noted that in addition to translocating carbohydrates and lipids to the cnidarian hosts, N-bearing biomolecules are also translocated from algal symbionts. NH$_4^+$ derived nitrogen has been identified in host tissues and studies have determined that algal symbionts translocate important amino acids such as alanine and aspartate and other larger N-bearing glycoconjugates (Markell and Trench 1993, Wang and Douglas 1999, Lipschultz and Cook 2002, Markell and Wood-Charlson 2010).

**Excretion**

While several studies exhibit the ability of algal symbionts to utilize external NH$_4^+$ to contribute to the nitrogen budgets of the coral-algal symbiosis, it is also understood that the algal symbionts recycle coral waste nitrogen in order to conserve nitrogen within the symbiosis. Low to no excretion of NH$_4^+$ has been measured by corals in light and this is thought to be due to the rapid utilization of excreted NH$_4^+$ from corals by their intracellular algal symbionts (Wilkerson and Trench, 1986). This efficient recycling of
nitrogen has been historically thought to contribute to the stability and success of corals in low nutrient environments.

### 3.1.4 Experimental Objectives

The aim of this study was to measure the nitrogen isotopic fractionation associated with uptake and assimilation of $\text{NH}_4^+$ under variable concentrations of elevated $\text{NH}_4^+$. The implications of this work are to gain insights on the mechanisms of $\text{NH}_4^+$ utilization by corals and their algal symbionts as well as to develop an understanding of how the $\delta^{15}\text{N}$ signature of $\text{NH}_4^+$ translates to $\delta^{15}\text{N}$ of coral tissue and algal symbiont tissues and the potential for coral and algal tissue isotope analysis to be used as a means of understanding $\text{NH}_4^+$ sources to a reef habitat.

### 3.2 Methodology

#### 3.2.1 Experimental Specimens

Specimens of the Pacific branching coral *Pocillopora damicornis* were collected off the coast of Panama in 2007. The corals were analyzed with the quantitative polymerase chain reaction method (qPCR) in the Baker Coral Conservation Laboratory in order to determine the algal symbiont type. These corals were “common gardened” at The University of Miami Experimental Hatchery indoors for five years prior to experimentation. The corals experienced conditions of relatively constant temperature ($26^\circ\text{C}$) and light regimes on a 12 hour light/12 hour dark cycle, and were fed an algal protein mixture once weekly. Corals were maintained in flowing seawater pumped in directly from Bear Cut, Miami, FL and experienced natural fluctuations in salinity. For this experiment, two genets of *Pocillopora damicornis* hosting clade C symbionts, and five genets of *P. damicornis* hosting clade D symbionts were studied. Only the corals
hosting clade C symbionts are discussed in this chapter. 300 corals were fragmented from larger parent colonies and fixed to PVC plates specialized for growth and calcification measurements via the scanning optical micrometer method (Langdon Lab - University of Miami (Albright et al. 2010)) and the buoyant weight technique ((Jokiel et al. 1978).

3.2.2 Experimental Conditions

Corals were subject to experimental nutrient levels for a period of four weeks. Treatment conditions included Control, 10, 20 and 50 µM NH₄Cl. Controls consisted of 0.2 micron filtered seawater pumped in from Bear Cut (NH₄⁺, NO₃⁻ <1 µM), while treatments consisted of the same seawater utilized for Controls with the addition of NH₄Cl to achieve the desired final concentration. Treatment tanks consisted of a 1 gallon glass tank which were filled with 2.5 liters of seawater. There were four tanks assigned to each treatment. Each tank contained six coral fragments for a total of 240 coral fragments distributed among 40 tanks. Seawater in tanks was changed at the beginning of light exposure each day with appropriate nutrient additions. The 1 gallon tanks were maintained inside of a larger flow thru system to maintain temperature control, however, individual tanks were isolated from flowing seawater (Figure 2.2.1). Temperature was held constant indoors during the four week period at 26 ± 0.5⁰ C. Light was supplied by USA Nova Current Extreme lights on a 12 hour light/12 hour dark cycle. Circulation within each tank was provided by a Hydor Koralia Nano mini pump.

3.2.3 Sample Collection

At the initiation of the four week experimental period and at again at the termination of the four week period, water samples were collected during 48 hour uptake periods. Water samples were collected throughout a 48 hour period on a 12 hour light/12
hour dark cycle at 0, 4, 12, 24, 36 and 48 hours and then during a period of 48 hours of constant light exposure at 0, 4, 12, 24 and 48 hours. Filtered water samples were collected in 125 ml Nalgeen (polyethylene) bottles and acidified to pH 2 with the addition of hydrochloric acid for preservation (Capone 2008).

3.2.4 NO$_3^-$ and NH$_4^+$ Concentration Analysis

The concentrations of NO$_3^-$ and NH$_4^+$ were determined colorimetrically with a SmartChem200 Autoanalyzer (Westco Scientific). The NO$_3^-$ concentrations were determined via the vanadium chloride method and the NH$_4^+$ concentrations were measured through the indophenols-blue method (Berthelot reaction) (Westco Scientific SmartChem200 Manual). Concentration data are required for sample preparation for NO$_3^-$ and NH$_4^+$ isotopic analysis (described below).

3.2.5 NH$_4^+$ Isotopic Analysis

The ($\delta^{15}$N) isotopic analyses of NH$_4^+$ was carried in the Altabet Biogeochemistry Laboratory at UMass, SMAST in New Bedford, MA. Isotopic analysis was achieved through a method of conversion to N$_2$O gas and subsequent isotopic analysis. NH$_4^+$ is first converted to NO$_2$ with a hypobromite reagent at pH 12, followed by the addition of sodium arsenite to stop the reaction (Zhang et al. 2007). Samples were treated with an 1:1 acetic acid:azide solution and allowed to react for one hour to convert NO$_2$ to N$_2$O gas and then buffered with 10 M NaOH (McIlvin and Altabet 2005). Only NH$_4^+$ samples with greater than 60% yield were considered for analysis. The $\delta^{15}$N composition of N$_2$O gas is analyzed on a GV Isoprime Isotope Ratio Mass Spectrometer. Values are reported in delta notation (equation 1), relative to N$_2$.

$$\delta (\%o) = \frac{(R_{sample} - R_{standard})}{R_{standard}} \times 10^3$$  \hspace{1cm} (1)
Where \( R_{\text{sample}} \) is the ratio of heavy to light isotopes in the sample and \( R_{\text{standard}} \) is the ratio of heavy to light isotopes in the standard.

\[ \delta^{15}\text{N} \] sample values for each run are compared to a set of blanks (operational blanks: low nutrient seawater treated with the acetic acid:azide solution, a set of three \( \text{NO}_2^- \) standards and a set of three \( \text{NH}_4^+ \) standards :USGS25, USGS26, and a lab standard). Isotopic data were corrected for the 1:1 addition of azide-\( \text{N} \) to \( \text{NO}_2^- \)-\( \text{N} \) in the formation of \( \text{N}_2\text{O} \) (McIlvin and Altabet 2005) Average reproducibility for \( \delta^{15}\text{N} \) analysis was 0.17 ‰.

### 3.2.6 Coral Tissue and Algal Symbiont Isotopic Analysis

Coral tissue was removed from skeletons with an airbrush technique using a gentle stream of distilled water. The coral host tissue was immediately separated from algal symbionts using a modified version of the centrifugation and rinsing method with the additional step of filtration twice through 20 \( \mu \text{m} \) nitex mesh in order to eliminate contamination by coral nematocysts from the symbiont fraction (Piniak et al. 2003). Coral tissues and algal symbiont tissues were dried at 40 °C for 48 hours and prepared in tin capsules for organic carbon and nitrogen isotope analysis. Samples were analyzed on a Thermal Conductivity Elemental Analyzer (Costech) interfaced to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific).

### 3.2.7 Determination of Coral Surface Area

Nutrient uptake data was normalized to coral surface area. Coral surface area was determined by modeling individual coral branches as cylinders. The height and diameter
of each branch was measured with a digital micrometer and surface area calculations were carried out.

3.2.8 Calculation of Discrimination Factor $\varepsilon$

There are two methods for calculating the discrimination factor. The discrimination factor, $\varepsilon$ can be calculated either by using the $\delta^{15}$N of the residual NH$_4^+$ or the $\delta^{15}$N of coral or algal symbiont tissue.

**Residual NH$_4^+$**

In order to calculate the $\varepsilon$ derived from incubation water, the concentration and isotopic signature of NH$_4^+$ is utilized. Solving for the slope of the Rayleigh Distillation equation (equation 2) gives $\varepsilon$ (equations 3):

$$\delta^{15}\text{N}_{\text{NH}_4^+(t)} = \delta^{15}\text{N}_{\text{NH}_4^+(o)} - \varepsilon \ln f$$  \hspace{1cm} (2)

$$\varepsilon = \left[ \delta^{15}\text{N}_{\text{NH}_4^+(o)} - \delta^{15}\text{N}_{\text{NH}_4^+(t)} \right] / \ln f$$  \hspace{1cm} (3)

where $\delta^{15}\text{N}_{\text{NH}_4^+(o)}$ is the isotopic composition of the NH$_4^+$ at the start of the incubation and $\delta^{15}\text{N}_{\text{NH}_4^+(t)}$ is the isotopic composition of the NH$_4^+$ at time t, when the fraction of original NH$_4^+$ remaining is f.

**Coral-Algal Tissues**

The discrimination factor for nitrogen can also be calculated from the $\delta^{15}$N value of the coral and algal symbiont tissues. The Rayleigh Distillation equation for cumulative product can be used to solve for the discrimination factor involved with NH$_4^+$ uptake and assimilation according to equation 4:

$$\delta^{15}\text{N}_{\text{product}} = \delta^{15}\text{N}_{\text{substrate}} + \left[ \varepsilon (\ln f) / (1-f) \right]$$  \hspace{1cm} (4)
where, \( \delta^{15}N_{\text{product}} \) is the isotopic composition of tissue (coral or algal symbiont) after growing in elevated \( \text{NH}_4^+ \), \( \delta^{15}N_{\text{substrate}} \) is the initial isotopic composition of the \( \text{NH}_4^+ \) and \( f \) is the fraction of original \( \text{NH}_4^+ \) substrate remaining in the tank after 24 hours.

Before using the cumulative product equation to solve for \( ^{15}\varepsilon \) of \( \text{NH}_4^+ \) uptake from coral or algal symbiont tissues, the following factors must be considered in order to correct for the \( \delta^{15}N \) value of the tissue that is derived directly from \( \text{NH}_4^+ \) during the experimental period:

1. The algal and coral tissue turnover rates from a \( ^{15}N \) labeling incubation are used in a mass balance equation (equation 5) to solve for the \( \delta^{15}N \) of the tissue formed during the 28 day experimental period:

\[
\delta^{15}N_{\text{tissue measured}} = \delta^{15}N_{\text{tissue new}} (X) + \delta^{15}N_{\text{tissue old}} (1-X)
\]  

(5)

where \( \delta^{15}N_{\text{tissue measured}} \) is the cumulative isotopic composition of old and new tissues after the experimental period, \( \delta^{15}N_{\text{tissue old}} \) is the isotopic composition of the tissue prior to the experimental period, \( X \) is the fraction of total tissue which is composed of new tissues calculated from the \( ^{15}\text{NH}_4^+ \) labeling incubation and \( \delta^{15}N_{\text{tissue new}} \) is the calculated isotopic composition of the new tissue formed during the experimental period.

2. Feeding rate estimates and total \( \text{NH}_4^+ \) uptake throughout the 28 day experimental period are used to calculate the fraction of coral and algal tissues derived from \( \text{NH}_4^+ \) and from the heterotrophic food source (equation 6). Corals were fed a measured amount of food once weekly for 3 hours. Based on visual examination, it was estimated that 25% of added food was consumed. The daily uptake of \( \text{NH}_4^+ \) for each treatment condition was
used to extrapolate the total NH$_4^+$ consumed over the 28 day period. The fraction of total nitrogen consumed comprised by NH$_4^+$, Y, was calculated as follows:

$$Y = (\text{NH}_4^+ - \text{N consumed})/(\text{NH}_4^+ - \text{N consumed} + \text{heterotrophic N consumed})$$  \hspace{1cm} (6)

3. The isotopic composition of the tissues derived from NH$_4^+$ was then calculated according to equation 7:

$$\delta^{15}N_{\text{tissue new}} = \delta^{15}N_{\text{NH}_4^+ \text{ derived tissues}}(Y) + \delta^{15}N_{\text{food}}(1-Y)$$  \hspace{1cm} (7)

where $\delta^{15}N_{\text{tissue new}}$ was calculated in equation 10, $\delta^{15}N_{\text{food}}$ is the measured isotopic composition of the coral food and Y is the fraction of total nitrogen consumed comprised by NH$_4^+$ calculated in equation 6.

4. The isotopic composition of the tissues derived from NH$_4^+$ was then substituted into equation 10 to solve for the discrimination factor for coral and algal tissues.

$$\varepsilon = \left[ (\delta^{15}N_{\text{NH}_4^+ \text{ derived tissues}} - \delta^{15}N_{\text{NH}_4^+ (o)}) * (1-f) \right] / [f \ln f]$$  \hspace{1cm} (8)

where $\delta^{15}N_{\text{NH}_4^+ \text{ derived tissues}}$ was calculated in equation 7, $\delta^{15}N_{\text{NH}_4^+ (o)}$ is the measured initial isotopic composition of the substrate NH$_4^+$ and f is the calculated average f value from each treatment over the 28 day experimental period.

The $\varepsilon$ measured in the water is manifested in both the algal symbiont and coral tissues, thus in order to compare to calculation from the residual NH$_4^+$, the $\varepsilon$ should be a weighted average of the $\varepsilon$ from algal symbiont and coral tissues (equation 9).

$$^{15}\varepsilon_{\text{NH}_4^+} = (Z)(^{15}\varepsilon_{\text{algal}}) + (1-Z)(^{15}\varepsilon_{\text{coral}})$$  \hspace{1cm} (9)

where $^{15}\varepsilon_{\text{NH}_4^+}$ is the fractionation effect measured through analysis of the substrate NH$_4^+$, $^{15}\varepsilon_{\text{algal}}$ is the fractionation effect by algal symbionts and $^{15}\varepsilon_{\text{coral}}$ from coral.
tissues. Z is the total fraction of NH$_4^+$ derived nitrogen accumulating in the algal symbiont and (1-Z) is the fraction accumulating within the coral tissues. Using a literature value for the average measurement of coral to algal protein biomass of 1:1 for *Pocillopora damicornis* (Muscatine and Cernichiari, 1969), the average was calculated to compare to the NH$_4^+$ $^{15}\epsilon$.

### 3.3 Results

#### 3.3.1 Calculation of $^{15}\epsilon$ from Residual NH$_4^+$ $\delta^{15}$N

As the 48 hour incubations in both light/dark cycle and light only conditions proceed, the concentration of NH$_4^+$ decreases accompanied by an increase in the $\delta^{15}$N of residual NH$_4^+$ in all treatments. The relationship between the natural log of f (the fraction of the initial NH$_4^+$ remaining in the incubation tank at a given time) and $\delta^{15}$N of residual NH$_4^+$ is represented in Figure 3.3.1 for the pooled data from both light only and the light/dark cycle conditions for 20 and 50 µM NH$_4^+$. While generally, the fractionation effect is calculated as the slope of the linear regression between the natural log of f and $\delta^{15}$N of residual NH$_4^+$, the data were best fit to polynomial models for both treatments (AICc polynomial < AICc linear). In this case, the fractionation effect can be calculated at any given f value by solving for the first derivate. This method was previously used for calculating the fractionation effect of NO$_3^-$ uptake in macroalgae (Swart et al. 2014).
Figure 3.3.1 Data shown are from the both light only and light dark 48 hour incubations for all replicate tanks of 20 and 50 μM NH₄⁺ treatments. The relationship between Δδ¹⁵N of the substrate NH₄⁺ and the natural log of the fraction of original NH₄⁺ remaining in the incubation medium (ln f), is shown. For both treatments, the data were best fit to a quadratic regression, equation given for each treatment condition. The first derivative of the quadratic equation gives the discrimination factor, ½, and can be solved for the corresponding f values.
Although the fractionation at any time during the uptake incubations was best described by the polynomial equations, in order to obtain an estimate of the average daily fractionation expressed throughout the four week experiment, the data from the first 24 hours of the light/dark incubation were fit to simple linear regressions for 20 and 50 µM NH$_4^+$ (Figure 3.3.2). The absolute value of the slope gives the average discrimination factor or isotope effect, $^{15}\varepsilon$. The corresponding data calculated for the 24 hours of uptake in the light/dark incubation are represented in Table 3.3.1. While the $^{15}\varepsilon$ for 20 and 50 µM NH$_4^+$ were not significantly different at p<0.05 (ANCOVA, p = 0.0726), the average $^{15}\varepsilon$ for 50 µM NH$_4^+$ was 7.30 ‰ greater than for 20 µM NH$_4^+$.

3.3.2 Relationship between $^{15}\varepsilon$ and [NH$_4^+$]

The relationships between $^{15}\varepsilon$ calculated and final NH$_4^+$ concentrations, [NH$_4^+$]$t$, is represented for 20 and 50 µM NH$_4^+$ treatments in Figure 3.3.3. The data shown are pooled together from both 48 hour light and light/dark cycle incubations. The NH$_4^+$ concentration on the x-axis is the final concentration for which the plotted $^{15}\varepsilon$ value was calculated, for example calculations were made between from 2 to 6 samples (2 consecutive samples: T$_0$-T$_1$, 3 consecutive samples: T$_0$-T$_2$,..., 6 consecutive samples: T$_0$-T$_5$). The Akaike’s Information Criterion was used to determine the best fit model to describe the relationship between $^{15}\varepsilon$ and [NH$_4^+$]$t$ for each treatment. The 20 µM NH$_4^+$ data shows a positive linear correlation between $^{15}\varepsilon$ and [NH$_4^+$]$t$. While in the 50 µM NH$_4^+$ the $^{15}\varepsilon$ showed an initial linear relationship, increasing $^{15}\varepsilon$ magnitude with increased [NH$_4^+$]$t$, but the $^{15}\varepsilon$ calculated for the highest concentrations were much lower. When all of the data from the 50 µM NH$_4^+$ treatment was modeled together, the best fit relationship between $^{15}\varepsilon$ and [NH$_4^+$]$t$ for was quadratic, with an increase in $^{15}\varepsilon$ as
Figure 3.3.2 Data shown are from the first 24 hours of the Light/Dark incubation for replicate tanks from 20 and 50 μM NH₄⁺ treatments. The relationship between Δδ¹⁵N of the substrate NH₄⁺ and the natural log of the fraction of original NH₄⁺ remaining in the incubation medium (ln f), is shown. The slope of each linear equation gives the discrimination factor, ¹⁵ε.
Table 3.3.1 From the Light Dark Incubations. A summary of the $^{15}\text{C}$ values calculated with the Rayleigh Equation for residual substrate $\text{NH}_4^+$. The standard error and $R^2$ values for the regression from which each treatment $^{15}\text{C}$ value (slope) was calculated are given. The slopes were not significantly different at $p<0.05$ (ANCOVA).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{15}\text{C}$ (%o)</th>
<th>SE</th>
<th>$R^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 $\mu$M $\text{NH}_4^+$</td>
<td>17.55</td>
<td>1.25</td>
<td>0.97</td>
<td>$p&lt;0.0001^*$</td>
</tr>
<tr>
<td>50 $\mu$M $\text{NH}_4^+$</td>
<td>24.85</td>
<td>2.56</td>
<td>0.94</td>
<td>$p&lt;0.0001^*$</td>
</tr>
<tr>
<td>ANCOVA 20 $\mu$M $\text{NH}_4^+$ - 50 $\mu$M $\text{NH}_4^+$</td>
<td></td>
<td></td>
<td></td>
<td>$p=0.0726$</td>
</tr>
</tbody>
</table>
Figure 13.3 Data shown are from the light only and light dark incubations for replicates from the 20 and 50 μM NH₄⁺ treatments. The relationship between ¹⁵N calculated from the analysis of the substrate NH₄⁺ and the final concentration of NH₄⁺ remaining in the tank at which the ¹⁵N was calculated, is shown. The regression equations describe the concentration dependence of ¹⁵N within 20 and 50 μM NH₄⁺ treatments. The data points within the ellipse from the μM NH₄⁺ at the highest concentration do not follow the same linear trend.
concentrations approach 50 µM NH$_4^+$ and then a decrease in $^{15}\varepsilon$ at higher concentrations (Figure 3.3.4).

3.3.3 $^{15}\delta$N of Coral and Symbiont Tissues

After 28 days under experimental conditions, the nitrogen isotopic composition of coral and algal symbiont tissues in elevated NH$_4^+$ treatments were less enriched in $^{15}$N than those corals in Control tanks (Figure 3.3.5a). The relationship between $^{15}\delta$N of coral tissues, algal symbiont tissues and the initial $^{15}\delta$N NH$_4^+$ are shown in Figure 3.3.5a. The 50 µM NH$_4^+$ treatment had the lowest average $^{15}\delta$N values for both coral tissues (0.96 ‰) and symbiont tissues (-4.08 ‰) among all treatments.

3.3.4 Calculation of Discrimination Factor from Coral Tissues and Algal Symbionts

Calculations of the discrimination factor from the $^{15}\delta$N analysis of the coral algal symbiont organic material synthesized during the four week experiment are compared to those values calculated from the residual NH$_4^+$ in Table 3.3.2 and Figure 3.3.5b. While the data set for this experiment is unable to completely constrain the calculations of $^{15}\varepsilon$ from algal and symbiont tissues, the purpose of including these calculations is to demonstrate that when reasonable estimates of feeding are accounted for (25% of food available to corals consumed), $^{15}\varepsilon$ values obtained are in the range of those calculated through analysis of the substrate NH$_4^+$. Depending on the allocation of NH$_4^+$ derived N between algal symbiont and coral tissues, the weighted average of the fractionation measured for each of these components should be comparable to the $^{15}\varepsilon$ measured in the residual NH$_4^+$ from the water. While scenarios for 25% feeding provided fractionation values that were similar for algal symbionts in the 20 and 50 µM treatments, the coral tissues had higher overall fractionation values in the 50 µM NH$_4^+$ treatments.
Figure 3.3.4 Data shown are from the light only and light dark incubations for replicates from the 50 μM NH₄⁺ treatment. The relationship between $^{15}\%$ calculated from the analysis of the substrate NH₄⁺ and the final concentration of NH₄⁺ remaining in the tank at which the $^{15}\%$ was calculated, is shown. The polynomial equation describes the concentration dependence of $^{15}\%$ within the 50 μM NH₄⁺ treatment. The fit to a polynomial model suggests highest fractionation occurring at mid concentrations with fractionation measured being lower both at the beginning of the incubation (at highest concentrations) and as the NH₄⁺ is drawn down.
Figure 3.3.5  a) The δ¹⁵N of algal symbiont tissues (green) and coral tissues (orange) after four weeks of experimental elevated NH₄⁺ conditions for Control, 10, 20 and 50 μM NH₄⁺ treatments. Error bars represent one standard deviation from the mean. b) The relationship between the δ¹⁵N measured by analysis of the residual NH₄⁺ substrate and the δ¹⁵N calculated from isotopic analysis of the coral and algal symbiont tissues. The coral-symbiont average represents the cumulative fractionation for coral and algal tissues for comparison to that calculated by analysis of the residual NH₄⁺. The error bars for the residual NH₄⁺ represent one standard error. The error bars for the coral-symbiont average represent 10% uncertainty of coral feeding rates used for calculating δ¹⁵N.
Table 3.3.2 A summary of the $^{15}\varepsilon$ values calculated from the Rayleigh Equations for cumulative product using coral tissue $\delta^{15}$N and algal symbiont $\delta^{15}$N and the average for coral and algal symbiont $\delta^{15}$N compared to the $^{15}\varepsilon$ calculated by analysis of the residual substrate $\text{NH}_4^+$ ($^{15}\varepsilon$: DIN). Calculations are given for estimates of heterotrophic consumption of 25% of food available to corals during feeding.

<table>
<thead>
<tr>
<th>Method</th>
<th>$^{15}\varepsilon$ (%)</th>
<th>$^{15}\varepsilon$ (%)</th>
<th>$^{15}\varepsilon$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 $\mu$M $\text{NH}_4^+$</td>
<td>20 $\mu$M $\text{NH}_4^+$</td>
<td>50 $\mu$M $\text{NH}_4^+$</td>
</tr>
<tr>
<td>DIN</td>
<td>-----</td>
<td>17.45</td>
<td>24.85</td>
</tr>
<tr>
<td>Coral</td>
<td>8.41</td>
<td>8.52</td>
<td>16.21</td>
</tr>
<tr>
<td>Algal Symbiont</td>
<td>23.50</td>
<td>23.85</td>
<td>22.19</td>
</tr>
<tr>
<td>Coral-Symbiont Avg</td>
<td>15.96</td>
<td>16.19</td>
<td>19.20</td>
</tr>
</tbody>
</table>
3.4 Discussion

3.4.1 Range in $^{15}\varepsilon$ Calculated from $\delta^{15}$N-NH$_4^+$

The average $^{15}\varepsilon$ for 20 µM NH$_4^+$ during 24 hours of light/dark uptake was 17.55 ‰ and the average $^{15}\varepsilon$ for 50 µM NH$_4^+$ was 24.85 ‰. When the $^{15}\varepsilon$ was calculated from several end points (from data over 4, 12, 24, 36 or 48 hours) there was a wide range in $^{15}\varepsilon$ from 12.34 to 41.42 ‰. The average values fall in the range of $^{15}\varepsilon$ for NH$_4^+$ uptake previously measured for marine phytoplankton with literature values range from 2 to 29 ‰ (Pennock et al. 1996; Wada & Hattori, 1978, Waser et al. 1998) and within the range measured for bacteria from 4 to 27 ‰ (Hoch et al., 1992, Vo et al., 2013), however the highest “instantaneous” fractionation effects measured are among higher literature values for fractionation associated with uptake and assimilation of NH$_4^+$.

3.4.2 Concentration Dependent Variability in $^{15}\varepsilon$

The variability in $^{15}\varepsilon$ with concentration may suggest variability in the rate limiting steps associated with uptake and assimilation of NH$_4^+$ by coral and their algal symbionts which are contributing to the isotope effect measured. The linear relationship between $^{15}\varepsilon$ and concentration in the 20 µM NH$_4^+$ treatment suggests that as NH$_4^+$ concentration increases there is greater influence from a step that is associated with a large isotope effect. As concentrations are drawn down, the isotope effect measured decreases.

In the 50 µM NH$_4^+$ treatments, the quadratic relationship between $^{15}\varepsilon$ and NH$_4^+$ indicates that initially at high concentrations, the fractionation propagated to the tank seawater is small, increases at intermediate concentrations and then decreases as NH$_4^+$ is drawn down. This suggests that throughout the uptake period in 50 µM NH$_4^+$ the rate
limiting step involved in uptake and assimilation is changing or there are variable amounts of NH₃ being effluxed from coral and/or algal tissues into the seawater.

3.4.3 Comparison of $^{15}\varepsilon$ from Residual NH₄⁺ and $^{15}\varepsilon$ from Coral and Algal Tissues

The $\delta^{15}N$ of the coral tissues and algal symbiont provide a cumulative signature of uptake and assimilation processes over the duration of the experiment, as water is changed daily, the total fractionation recorded from NH₄⁺ is representative of the average fractionation effect exhibited over 24 hours in incubation. While the the tissues represent a cumulative effect, the water data allow for the analysis of individual time slices of fractionation over a range of external NH₄⁺ concentrations as the NH₄⁺ in each tank is drawn down during the 24 hour period. The $^{15}\varepsilon$ values calculated for the fractionation for algal symbionts are much larger than the $^{15}\varepsilon$ values calculated for coral tissues in all treatments. While assumptions have been made that corals in all treatments are consuming the same amount of heterotrophic nitrogen, calculations of $^{15}\varepsilon$ from algal symbiont showed very little variability between 20 and 50 µM NH₄⁺, whereas, the $^{15}\varepsilon$ calculated from coral tissues in the 20 µM NH₄⁺ were smaller than the $^{15}\varepsilon$ values calculated from coral tissues in the 50 µM NH₄⁺ treatment (Table 3.3.2).

3.4.4 Developing Models to Understand $^{15}N$ Fractionation

In the range of concentrations studied, there appeared to be no concentration dependent influences on $^{15}\varepsilon$ calculated for algal symbionts, but concentration dependent effects on $^{15}\varepsilon$ calculated from both coral tissue and residual $\delta^{15}N$ of the seawater. Models for the steps involved in the uptake and assimilation of NH₄⁺ which lead to variable $\delta^{15}N$ of water, coral tissue and algal symbiont tissues are proposed considering the possible mechanisms involved in uptake and assimilation.
**Uptake**

The models for $^{15}$N fractionation during NH$_4^+$ uptake and assimilation must consider uptake across coral tissue membrane as well as across the algal symbiont cell membrane. The kinetic models for NH$_4^+$ uptake for this study developed in Chapter One are represented in Figure 1.3.12. While uptake below 42 µM follows Michaelis Menten kinetics, above 42 µM, uptake rate increases linearly with increasing NH$_4^+$ concentration. This suggests that the mechanism involved in uptake may be concentration dependent with saturation at around 40 µM and with some mechanism allowing for increased uptake rates above 42 µM NH$_4^+$. Considering the NH$_4^+$/NH$_3$ speciation at seawater pH of ~ 8.1, small amounts of NH$_3$ diffusion may contribute to uptake when the concentration gradients are favorable from seawater to coral tissues and/or from coral tissues to algal symbionts. The depletion-diffusion model proposed by D’Elia (1983) suggests that algal symbionts actively transport NH$_4^+$ across the algal cell membrane at a rate sufficient to deplete NH$_4^+$ in coral tissues to promote continuous diffusion from seawater.

Additionally, the evidence of NH$_4^+$ transporters within *Symbiodinium* (Leggat, 2007) supports the idea that NH$_4^+$ is actively transported into algal cells from the coral tissues. It is likely that under low tissue NH$_3$ concentrations, as glutamine levels fall, transporters are activated on the algal cell surface and have more influence on the uptake of NH$_4^+$. The active transport from seawater into coral tissues should also be considered as Lehnert et al. (2014) highlighted the potential role of NH$_4^+$ transporters in the symbiotic anemone *Aiptasia pallida*. 
Assimilation

The presence of the high affinity enzyme glutamine synthetase (GS) and low affinity glutamate dehydrogenase (GDH) have been discovered within both coral host tissues and *Symbiodinium* (Catmull et al. 1987, Rahav et al. 1989, Yellowlees et al. 1994). Although coral host tissues possess the machinery capable of assimilating NH$_4^+$, studies estimate that the rate of NH$_4^+$ incorporation into algal tissues is more than ten times higher than incorporation into coral host tissues (Grover et al. 2002b, Pernice et al. 2012). It is thought that the symbionts primarily utilize the GS/GOGAT pathways for NH$_4^+$/NH$_3$ assimilation while the coral host assimilates NH$_4^+$ through the NADPH dependent GDH and/or GS (Pernice et al. 2012). Both GS and GDH use NH$_3$ as a substrate, because the bond involves the lone pair of electrons on the N of NH$_3$. This confirms that prior to assimilation, there must be deprotonation of NH$_4^+$ to NH$_3^+H^+$ (Vo et al. 2013).

Efflux from Algae and Coral Tissues

As NH$_3$ can freely diffuse across cell membranes, the potential for NH$_3$ leakage from both algal symbiont into coral tissues and from coral tissues into seawater exists. Typically when NH$_4^+$/NH$_3$ levels are low, glutamine levels fall and NH$_4^+$ transporters are activated. It is thought that under such conditions where transporters are activated, there will be very low relative leakage of NH$_3$ from the intracellular NH$_4^+$/NH$_3$ pool (Vo et al. 2013).

Potential Steps Associated with $^{15}$N Fractionation

Previously measured $^{15}$ε values for the various steps of uptake and assimilation of NH$_4^+$ by *E.Coli* are summarized in Table 3.4.1. In the case where pH dependent data
Table 3.4.1 A summary of the literature $^{15}$ε values for each process at a given pH associated with uptake and assimilation of NH$_4^+$ with standard error and the values used for the models in this study. It is indicated whether the value is the observed $^{15}$ε value (obs) or if it has been corrected for deprotonation (corr). Where available, the pH data from the literature were used to calculate the $^{15}$ε for the assumed pH of 7.4 for coral tissues and algal symbionts, yielding $^{15}$ε Coral Observed. Calculations were made assuming a linear relationship between $^{15}$ε and pH. The $^{15}$ε Coral Observed is then corrected for the deprotonation fractionation (19.2 %o) in order to isolate the $^{15}$ε for each process, giving $^{15}$ε Coral Corrected. Literature values come from a summary by Hoch et al. (1992) and values obtained by Vo et al. (2013) for bacteria.

<table>
<thead>
<tr>
<th>Process/Enzyme</th>
<th>pH</th>
<th>$^{15}$ε Literature</th>
<th>$^{15}$ε Coral (obs.)</th>
<th>$^{15}$ε Coral (corr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deprotonation</td>
<td>7.0</td>
<td>19.2 %o</td>
<td>19.2 %o</td>
<td>19.2 %o</td>
</tr>
<tr>
<td>NH$_4^+$ Diffusion</td>
<td>-----</td>
<td>30.1 %o (obs.)</td>
<td>30.1 %o</td>
<td>10.9 %o</td>
</tr>
<tr>
<td>AmtB transporter</td>
<td>7.4</td>
<td>14.1 %o (corr.)</td>
<td></td>
<td>14.1 %o</td>
</tr>
<tr>
<td>GDH</td>
<td>5.8</td>
<td>18.0 ± 1.0 %o (obs.)</td>
<td>12.49 %o</td>
<td>-6.7 %o</td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>-9.8 ± 0.1 %o (obs.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS</td>
<td>7.1</td>
<td>8.0 ± 0.2 %o (obs.)</td>
<td>8.9 %o</td>
<td>-10.3 %o</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>12.5 ± 0.3 %o (obs.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
are available, the estimated $^{15}\varepsilon$ was calculated for the pH appropriate in this study using a linear relationship between pH and $^{15}\varepsilon$. Figure 3.4.1 displays a cell model with all possible steps involved in uptake and assimilation of $\text{NH}_4^+$. The steps involved in $\text{NH}_4^+/\text{NH}_3$ uptake and assimilation that can lead to fractionation observed in water, coral tissues and algal symbiont and coral tissues are as follows:

1. the equilibrium fractionation involved with protonation/deprotonation ($\text{NH}_4^+ \rightarrow \text{NH}_3 + \text{H}^+$) estimated to be 19.2 ‰, within seawater, tissues or algal symbiont

2. the diffusion of $\text{NH}_3$ across both coral tissue membrane and the algal cell membrane (measured for $\text{E.Coli}$ 10.9 ‰),

3. active transport of $\text{NH}_4^+$ across coral cell membrane or algal cell membrane (measured for $\text{E.Coli}$ 14.1 ‰),

4. assimilation by the low affinity enzyme glutamate dehydrogenase (GDH) within coral tissues or algal symbiont cells (in $\text{E.Coli}$ calculated for appropriate pH -6.7 ‰),

5. assimilation of $\text{NH}_3$ by glutamine synthetase (GS) in coral tissues or algal symbiont cells (in $\text{E.Coli}$ calculated for appropriate pH, -10.3 ‰).

Modeling Isotopic Fractionation during $\text{NH}_4^+$ Uptake and Assimilation

Model equations were developed based on all potential inputs and outputs of $\text{NH}_4^+/\text{NH}_3$ within external seawater, coral intracellular pool, algal intracellular pool and organic N of coral and algal tissues outlined in Table 3.4.2 and the model for multiple kinetic fractionations developed by Anderson et al (1998). The goals of the model are to
Figure 3.4: Potential Steps of NH₄⁺ Uptake and Assimilation and Literature Values for Associated Isotope Fractionation.
Table 3.4.2 Potential inputs and outputs to each NH$_4^+$/NH$_3$ pool within the seawater, coral host and algal symbiont.

<table>
<thead>
<tr>
<th>Nitrogen Pool</th>
<th>Inputs</th>
<th>Outputs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Seawater</strong></td>
<td>added NH$_4$Cl</td>
<td>diffusion of NH$_3$ into coral tissues</td>
</tr>
<tr>
<td></td>
<td>efluxed NH$_3$ from coral tissues</td>
<td>transport of NH$_4^+$ into coral tissues</td>
</tr>
<tr>
<td><strong>Coral Intracellular</strong></td>
<td>transport of NH$_4^+$ from SW</td>
<td>assimilation by GS</td>
</tr>
<tr>
<td></td>
<td>diffusion of NH$_3$ from SW</td>
<td>assimilation by GDH</td>
</tr>
<tr>
<td></td>
<td>efluxed NH$_3$ from symbiont</td>
<td>diffusion of NH$_3$ into symbiont</td>
</tr>
<tr>
<td></td>
<td></td>
<td>transport of NH$_4^+$ into symbiont</td>
</tr>
<tr>
<td></td>
<td></td>
<td>eflux of NH$_3$ into SW</td>
</tr>
<tr>
<td><strong>Algal Intracellular</strong></td>
<td>transport of NH$_4^+$ from coral tissues</td>
<td>assimilation by GS</td>
</tr>
<tr>
<td></td>
<td>diffusion of NH$_3$ from coral tissues</td>
<td>eflux of NH$_3$ into tissues</td>
</tr>
<tr>
<td><strong>Coral Tissue</strong></td>
<td>assimilation of NH$_3$ by GS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>assimilation of NH$_4$ by GDH</td>
<td></td>
</tr>
<tr>
<td><strong>Algal Symbiont</strong></td>
<td>assimilation of NH$_3$ by GS</td>
<td>translocation of amino acids to host</td>
</tr>
</tbody>
</table>
develop an understanding of the fractionating steps which contribute to the δ\(^{15}\)N measured in external seawater NH\(_4\)\(^+\), coral tissue and algal symbiont tissues. Literature values for estimates of fractionation with each step (Table 3.4.1) were used to solve the model equations, to test whether the output was in agreement with the results of this study.

The following basic equations were developed in order to build models:

**Residual pool w/multiple sinks:**

\[
\delta_{\text{substrate}(f)} = \delta_{(o)} - [(s_1\epsilon_1 + s_2\epsilon_2 + s_3\epsilon_3)\ln f] \tag{7}
\]

where \(\delta_{\text{substrate}(f)}\) is the isotopic composition of the substrate when the fraction, \(f\) of original substrate remains, \(\delta_{(o)}\) is the initial isotopic composition of the substrate, \(s_1, s_2\) and \(s_3\) are the relative contributions from sinks 1, 2 and 3 to total draw down, and \(s_1 + s_2 + s_3 = 1\). \(\epsilon_1, \epsilon_2\) and \(\epsilon_3\) are the isotope effects associated with sinks 1, 2 and 3.

**Product forming w/multiple processes from same starting source:**

\[
\delta_{\text{product}(f)} = \delta_{(o)} + [f^* (q_1\epsilon_1 + q_2\epsilon_2 + q_3\epsilon_3)\ln f]/(1-f) \tag{8}
\]

where \(\delta_{\text{product}(f)}\) is the isotopic composition of the cumulative product when the fraction, \(f\) of original substrate remains, \(\delta_{(o)}\) is the initial isotopic composition of the substrate, \(q_1, q_2\) and \(q_3\) are the relative contributions from process 1, 2 and 3 to form product and \(q_1 + q_2 + q_3 = 1\). \(\epsilon_1, \epsilon_2\) and \(\epsilon_3\) are the isotope effects associated with processes 1, 2 and 3.
Product forming w/multiple processes from different starting sources:

\[
\delta_{\text{product}(f)} = a\{\delta_{(a-o)} + [f_a^* (q_1\varepsilon_1 + q_2\varepsilon_2 + q_3\varepsilon_3)\ln f]/(1-f_a)\} + \\
b\{\delta_{(b-o)} + [f_b^* (q_4\varepsilon_4 + q_5\varepsilon_5 + q_6\varepsilon_6)\ln f_b]/(1-f_b)\}
\]

(9)

where \(\delta_{\text{product}(f)}\) is the isotopic composition of the cumulative product when the fraction, \(f_a\) of original substrate a remains, and \(f_b\) of original substrate b remains, \(\delta_{(a-o)}\) and \(\delta_{(b-o)}\) are the initial isotopic composition of the substrates a and b, respectively. \(q_1, q_2\) and \(q_3\) are the relative contributions from process 1, 2 and 3 to form product from substrate a and \(q_1+q_2+q_3 = 1\), and \(\varepsilon_1, \varepsilon_2\) and \(\varepsilon_3\) are the isotope effects associated with processes 1, 2 and 3. \(q_4, q_5\) and \(q_6\) are the relative contributions from process 4, 5 and 6 to form product from substrate b and \(q_4+q_5+q_6 = 1\). \(\varepsilon_4, \varepsilon_5\) and \(\varepsilon_6\) are the isotope effects associated with processes 4, 5 and 6.

While there are many possible combinations of uptake and assimilation mechanisms that should be considered, for the simplicity of this work we will overview three likely scenarios and the resulting fractionation effects.

**Model 1 – Slow Diffusion into Coral Tissues/Active Transport into Symbiont/No Efflux**

This model would represent what has been termed the “depletion-diffusion” model. While the diffusion rate of \(\text{NH}_3\) into tissues is slow, the coral intracellular concentrations remain low enough to activate the active transporters on the algal cell surface. The majority of \(\text{NH}_4^+\) will enter the algal symbionts through active transport, the \(\text{NH}_3\) will diffuse into coral tissues limited by the rate that the symbiont can transport from tissues into algal cell. All \(\text{NH}_3\) taken into algal cell will be assimilated by GS in the algal symbiont, and no efflux will occur.
The model for diffusive transport into coral tissues and active uptake by algal symbionts with no efflux from coral tissues would result in a measured fractionation effect of 30.1‰ (the additive isotope effects of deprotonation and NH₃ diffusion uptake). As the process of active uptake by the algal cell would fractionate the coral intracellular NH₄⁺/NH₃ pool, leaving it enriched in ¹⁵N, the symbionts would be isotopically lighter than the coral tissues.

**Model 2 – Active Transport into Coral Tissues/Active Transport into Symbiont/No Efflux**

Under low external concentrations, we consider that the active transporters will be activated and the majority of NH₄⁺ will enter both coral tissues and algal symbionts through active transport, the diffusion of NH₃ into coral tissues will be extremely slow and contribute negligible amounts to the total NH₄⁺/NH₃ taken up. All NH₄⁺/NH₃ taken up taken into algal cell will be assimilated by GS in the algal symbiont, and no efflux will occur from either symbiont or coral tissues.

The model for active transport only and no diffusion or efflux would result in a measured fractionation of ~14.1‰ in the surrounding seawater as a result of initial transport of NH₄⁺ across the coral tissue membrane. If not all coral intracellular NH₄⁺ is moved into the algal cell the additional fractionation resulting from active transport across the algal cell membrane will result in algal symbionts which are less enriched in ¹⁵N than coral tissues.
Model 3 – High Rates of Diffusion into Coral Tissues – Diffusion and Active Transport into Algal Symbiont – Diffusion/Efflux Out

Under high concentrations of NH$_4^+$/NH$_3$ transport into the coral tissue and algal cells there will likely be higher rates of diffusion from seawater into coral tissues. The active transporters in coral tissues will not be active. Significant amounts of efflux will occur as active transporters are not controlling uptake. When diffusion is rapid, there may be equilibration across the coral tissue cell membrane and the large fractionation effect associated with diffusion would thus not be expressed, leading to an overall lower fractionation effect measured in the seawater (Hoch et al. 1992). This is in contrast to when rates of diffusion are slow and the fractionation effects of deprotonation and diffusion will be expressed as the intracellular and extracellular concentrations will not be in equilibrium. In this case involving potentially high amounts of efflux, the fractionation effects of the NH$_3$ assimilating enzymes within the coral tissues (GDH) or within the algal symbionts (GS or GDH), may be propagated to external seawater.

*In the case that diffusion rates are rapid, the isotope effect measured in the water may be a combination of the intracellular processes of GS (combined deprotonation effect: 19.2 ‰ and GS -10.30 ‰), GDH (combined deprotonation effect: 19.2 ‰ and GDH: -6.7 ‰) and active transport into the algal cell (14.1 ‰).*

3.5 Conclusions

These data suggest that in the range of 0-55 μM NH$_4^+$, the coral algal symbiosis takes up NH$_4^+$ by two different pathways. The kinetic data suggested a concentration at which NH$_4^+$ uptake rate was saturated, but then show an increase in uptake rate above 42 μM NH$_4^+$. At this point, we see a decrease in the $^{15}ε$ of 50 μM NH$_4^+$ uptake and
assimilation. We interpret these results as changing pathways of NH$_4^+$/NH$_3$ uptake by the coral-algal symbiosis. Under the highest concentrations, the concentration gradient is favorable for higher rates diffusion of NH$_3$ into coral tissues, suggesting that the coral intracellular NH$_4^+$ is below 50 µM. When diffusion is rapid, the large isotopic fractionation associated with membrane diffusion (the combined effects of deprotonation and diffusion - estimates of 30.1 ‰ and 39 ‰ from O’leary, 1978; Hoch et al. 1992 and Vo et al. 2013) is not fully expressed as intracellular and extracellular NH$_4^+$ will likely reach some degree of isotopic equilibrium. This could explain the smaller fractionation effects which we measured at the highest concentrations of NH$_4^+$ (above 50 µM). At these highest concentrations, there may also be influence of the intracellular fractionating processes as it is thought that more of the intracellular NH$_3$ will be effluxed to the surrounding seawater. These processes could include assimilation directly in coral tissues by GS or GDH, the active transport effect of NH$_4^+$ into the algal symbiot cell and if NH$_3$ is also effluxed from the algal cells then it may propagate the assimilation effects of GS in the algal symbiont.

As concentrations fall to 40 µM, the fractionation expressed increases. This could be due to lower rates of diffusion occurring at this concentration, thus a greater expression of the diffusion isotope effect as intracellular and extracellular pools of NH$_4^+$ do not reach isotopic equilibrium. As concentrations continue to fall to lower concentrations (ex. below 20 µM), it is possible that active transporters on the coral tissues or algal cell membranes become active and contribute to the transport of NH$_4^+$ across cell membranes. The isotope effect cited for active membrane transport is 14.1 ‰ for bacteria (Vo et al.
2013), and thus has the potential to contribute to an overall lower isotope effect than was expressed for diffusion.

It is probably more useful to investigate the behavior of $^{15}\varepsilon$ and concentration for the two treatments (20 and 50 µM NH$_4^+$) separately as they may have different intracellular NH$_4^+$ environments after four weeks of exposure to 20 and 50 µM extracellular NH$_4^+$ concentrations. For example, Hoch (1992) showed that there was a direct relationship between external NH$_4^+$ concentrations and intracellular NH$_4^+$ concentrations, thus corals in the 50 µM NH$_4^+$ treatments may be maintaining higher intracellular concentrations than those in the 20 µM NH$_4^+$ treatments. Measurements of the intracellular NH$_4^+$ concentrations would provide a useful piece of information in making further interpretations for these data.

While high levels of GS/GDH have been detected within coral tissues, the role of these NH$_4^+$ assimilating enzymes is not clear. The ability for corals to assimilate NH$_4^+$ directly has been documented, but is thought to be more than ten times lower than the assimilation rates by their algal symbionts. These data may suggest that above 50 µM NH$_4^+$, the coral may increase its uptake of NH$_4^+$. Perhaps at these higher concentrations, the coral will take up NH$_4^+$ to avoid toxic NH$_4^+$ levels within the tissues, to maintain an electrochemical gradient within tissues or to limit symbionts from increased levels of NH$_4^+$. This would allow for the increased diffusion rates seen in the kinetic data above 42 µM NH$_4^+$. It is also possible that the “rhesus-like” protein identified in coral tissues (Leggat 2007), is responsible for transporting NH$_4^+$ out of the coral tissues actively when rates of diffusion are high. This would result in delivery of the depleted intracellular NH$_4^+$
pool to the external seawater leading in decreases in $\delta^{15}$N and an overall lower calculation of the fractionation effect.

Our data set suggests that the fractionation at lower concentrations is the result of one of the initial steps, either active transport or diffusive transport, while at higher concentrations, when diffusion rates increase, there may be propagation of the fractionating steps affecting the intracellular $\text{NH}_4^+$/NH$_3$ pools to the external seawater $\text{NH}_4^+$ isotopic signature.

**Comparison with $^{15}_\varepsilon$ of NO$_3^-$ uptake/assimilation**

The overall magnitudes of the fractionation effects associated with uptake and assimilation of $\text{NH}_4^+$ are much larger than those observed for uptake and assimilation of NO$_3^-$ by the coral algal symbiosis. We suspect that this is the case because the large fractionation effect associated with NO$_3^-$ uptake (NR activity) occurs after the initial transport into the coral and algal tissues and is only expressed as a function of the amount of efflux which occurs. Nitrate reduction activity inside the algal symbiont is primarily responsible for the large fractionation during NO$_3^-$ uptake and there is a clear concentration dependence on $^{15}_\varepsilon$. Higher $^{15}_\varepsilon$ values are indicative of saturation at the algal cell processes of either transport or nitrate reductase activity and allow for propagation of the isotopic information in the intracellular NO$_3^-$ to the external seawater. At lower NO$_3^-$ concentrations, the fractionation is being dictated by the initial step of NO$_3^-$ transport across the algal cell membrane (rate limiting step at lower concentrations). We suspect that higher fractionation is expressed for $\text{NH}_4^+$ uptake and assimilation as a result of the large fractionation associated with the initial steps (up to 39 %o for deprotonation and membrane diffusion), therefore, regardless of efflux, the large isotope effect can be
detected. For NH$_4^+$ there was not a simple relationship between fractionation and concentration, as it appeared that fractionation initially increased as concentration increased, but showed a decrease at highest concentrations dependent on the pathway for uptake. The potential for increased direct assimilation of NH$_4^+$ in coral tissues at extremely high concentrations could also allow for variations in the expressed isotope effect. This work suggests that mechanisms controlling the $^{15}$N fractionation during incorporation of NO$_3^-$ and NH$_4^+$ into coral-algal symbiosis are very different, while NO$_3^-$ uptake is limited by enzyme activity inside the symbiont, the uptake of NH$_4^+$ is likely limited by a transport step.

Previous work relating $\delta^{15}$N of coral tissues to anthropogenic nutrient loading has been based to the relationship made between modest enrichments ($\sim 1$‰) in coral tissue $\delta^{15}$N on reefs that are thought to be influenced by sewage as compared to tissues from reference sites (unaffected) (Heikoop et al., 2000), and direct measurements of isotopic fractionation associated with uptake of NH$_4^+$ and NO$_3^-$ have not been made. It is clear in this work that variable concentrations of NH$_4^+$ with the same initial $\delta^{15}$N can manifest in a wide range of coral and algal symbiont $\delta^{15}$N values.
Chapter Four – Physiological Responses of *Pocillopora damicornis* and *Acropora cervicornis* to Variable Elevated Nutrient Concentrations

4.1 Background

Developing a clear understanding of how the coral-algal symbiosis responds to elevated nutrients is critical in predicting how nutrient stress may interact with future environmental and climatic stressors influencing coral reefs. Experimental nutrient enrichment incubations were carried out in order to gain a better understanding of the mechanisms through which dissolved inorganic nitrogen may alter coral growth rates and other physiological aspects of the coral-algal symbiosis. Three different types of experiments were carried out and are discussed in this chapter: 1) NO$_3^-$ and NH$_4^+$ enrichments with *Pocillopora damicornis*, 2) NO$_3^-$ and PO$_4^{3-}$ enrichments with *Acropora cervicornis*, and 3) NH$_4^+$ and CO$_2$ enrichments with *Pocillopora damicornis*. Nutrient enrichment experiments were carried out coupled with analysis of coral growth rates, symbiont cell densities, indicators of photosynthesis rates and quantifications of carbon cycling within the symbiosis.

4.1.1 Coral-Algal Response to Elevated Dissolved Inorganic Nitrogen

The ability to conserve and tightly cycle nutrients contributes to the high growth rates of Scleractinian corals in low nutrient waters (Szmant et al. 1990). The adaptation for success in an oligotrophic environment, however, renders corals highly susceptible to nutrient loading. Studies investigating the influences of NO$_3^-$ and NH$_4^+$ on coral nutrient cycling and other physiological parameters have been carried out by numerous researchers, with highly variable results leaving several knowledge gaps (Fabricius 2005).
Algal Symbiont Density and Photosynthesis Rates

While the parameter limiting symbiont growth may vary seasonally, or from one reef to another, most studies have found that elevated levels of NH$_4^+$ result in near doubling of symbiont densities (Muscatine et al. 1989, Stambler 1991, Stimson and Kinzie 1991), and increases in chlorophyll and algal proteins (Muller-Parker 1994). Increased symbiont densities have been also been measured to result from the long term influence of elevated NO$_3^-$ (Marubini and Davies 1996). This increase in zooxanthellae density after nitrogen exposure suggests that *Symbiodinium* in hospite (living in a host cell) are nitrogen limited (Marubini and Davies 1996) and the increased availability of nitrogen promotes replication. Some studies, however, have found no effect of modest enrichments of NO$_3^-$ [2 µM NO$_3^-$: (Ferrier-Pagès et al. 2001), 15 µM NO$_3^-$: (Nordemar et al. 2003)] on symbiont densities. Symbiont numbers increase in response to nitrogen if nitrogen has been the limiting factor in their growth. If biosynthesis in symbiont cells is limited by nitrogen (sufficient light and carbon available), releasing this nitrogen limitation will result in increased biosynthesis of algal tissues. It has been suggested that *Symbiodinium* may only be nitrogen limited at high irradiances (Dubinsky & Jokiel, 1994; Falkowski, 1984). A recent model on heterotrophic and autotrophic budgets of a coral-symbiosis considered symbiont growth and cell division. It was proposed that once a symbiont cell contains 80pg of N and reaches 10 µm in diameter it will begin to divide (Gustafsson et al. 2013).

Increases in rates of algal photosynthesis will result from an increase in photosynthetic units or thylakoid membranes. Thus, photosynthetic increases measured after elevated nitrogen exposure could indicate an increase in photosynthetic units per
algal cell, an increase in algal cells or both. While photosynthesis rates have been found to increase relative to surface area of the coral under elevated NO$_3^-$ (1, 5 and 20 µM), there is often no change in rate per algal cell when rates are normalized to symbiont density (Marubini and Davies 1996) suggesting the increased photosynthetic activity after exposure to elevated nitrogen is the result of an increased number of algal symbionts (Marubini and Davies 1996). In one study, NO$_3^-$ exposure lead to decreased rates of photosynthesis when normalized to algal symbiont cell (Nordemar et al. 2003).

*Growth Rate Changes*

A variety of responses in coral skeletal growth, or skeletogenesis, have been found to result from elevated levels of nitrogen. Among growth assessments are those studies measuring coral calcification rates (mg CaCO$_3$/surface area x time) and those which measure linear extension of the coral skeleton (skeletal extension/time). Studies investigating the effects of elevated inorganic nitrogen levels on coral growth have found positive, negative and no significant effects on growth. These conflicting findings could be due to large error involved with measurements of growth, species specific responses or other confounding factors such as variable environmental conditions which were not considered. A summary of the studies with varied responses to elevated NO$_3^-$ and NH$_4^+$ treatments is provided in Table 4.1.1.

There are some studies which have found overall positive influences of elevated DIN on coral growth. A study carried out with moderately high levels of nitrogen (5 µM) found several species to thrive under these nutrient conditions (Atkinson et al. 1995). The outcome of this experiment, however, may have been influenced by abnormally high concentrations of DIC in the seawater. In another study, *Acropora acuminata* displayed
Table 4.1.1 A literature review of studies citing increased skeletogenesis, no change in skeletogenesis or decreased skeletogenesis in response to elevated levels of dissolved inorganic nitrogen.

<table>
<thead>
<tr>
<th>Increased Skeletogenesis</th>
<th>No Change in Skeletogenesis</th>
<th>Decreased Skeletogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koop et al. 2001</td>
<td></td>
<td>Marubini and Davies, 1996</td>
</tr>
<tr>
<td>Meyer and Schultz, 1985</td>
<td></td>
<td>Snidvongs and Kinzie, 1994</td>
</tr>
<tr>
<td>Lough and Barnes, 1992</td>
<td></td>
<td>Wellington and Glynn, 1983</td>
</tr>
</tbody>
</table>
enhanced calcification during a one hour incubation in 100 µM NH₄⁺ (Crossland and Barnes 1974), however, this was a short term incubation experiment and did not investigate long term NH₄⁺ influence on coral calcification. Enhanced rates of linear extension were also found as a result of increases in particulate and dissolved nutrients from fish excretions (Meyer and Schultz 1985). A variety of studies have documented no positive or negative influence of elevated nutrient conditions on coral growth including 10 µM NH₄⁺ treatment of Stylophora pistillata (Ferrier-Pages et al. 2000). In another experiment with enrichments of 11.5 and 36.2 µM NH₄⁺, it was found that the higher dose resulted in more deleterious effects on a variety of coral species, however, inconsistent growth rates were measured with decreased, no change or increased linear extension rates resulting from the treatment (Koop et al. 2001).

While a variety of responses of coral linear extension and calcification have been measured under the influence of unnaturally high DIN, decreased growth results from a majority of the studies measuring the effects of elevated nutrient levels. Under elevated NO₃⁻ levels above 1 µM, reduced calcification rates for P.damicornis were measured (Marubini and Davies 1996) and Stylophora pistillata showed reduced calcification after three weeks in 2 µM NO₃⁻ (Ferrier-Pagès et al. 2001). Reduced calcification in Stylophora pistillata was also measured after elevated 20 µM NH₄⁺ exposure (Ferrier-Pages et al. 2000). Decreased rates of linear extension were measured for Pocillopora damicornis under 15 µM NH₄⁺ (Stambler 1991).

### 4.1.2 Response to Phosphate Enrichments

PO₄³⁻ concentrations in reef waters are generally very low (<0.1 µM), but may be higher on terrestrially influenced reefs (Kleypas et al. 1999). Concentrations greater than
2 µM PO$_4^{3-}$ have been measured in reef waters in the Southern Gulf of Mexico (Cruz-Pinon et al., 2003). It is generally accepted that symbionts respond with increased productivity and density to elevated PO$_4^{3-}$ levels, but scattered results have been obtained from experimental and field measurements of the response in symbiont densities, growth rates and other physiological parameters (Ferrier-Pages et al. 2000; Kinsey and Davies, 1979; Koop et al. 2001; Rasmussen, 1986; Rasmussen et al. 1993; Renegar and Riegl, 2005; Stambler et al. 1991). A more recent study in Acropora muricata measured both increased linear extension and calcification rates in PO$_4^{3-}$ concentrations above 5 µM (Dunn et al. 2012). Dunn (2012) found that while overall calcification increased, the skeletal density was actually decreased and suggested that as a result the brittle skeleton may be more susceptible to physical damage.

4.1.3 Response to Combined Effects of Elevated CO$_2$ and NH$_4^+$

Both dissolved inorganic carbon (DIC) (CO$_2$, HCO$_3^-$ & CO$_3^{2-}$) and dissolved inorganic nitrogen (DIN) (NO$_3^-$, NO$_2^-$ and NH$_4^+$) are important nutrients for the coral-algal symbiosis. DIC is utilized by the coral host to build a calcium carbonate skeleton and is also photosynthetically fixed by the algal symbionts. Algal symbionts are generally thought to be nitrogen limited (Muscatine and Porter, 1977; Cook and D’Elia, 1987; Kinsey, 1991) and require nitrogen for amino acid synthesis. There are variable hypotheses on the effects of each of these nutrients on the coral-algal symbiosis when they are introduced at unnaturally high concentrations. Even more complex is the interactive effect that elevated levels of both may have. As concerns for increasing atmospheric pCO$_2$ levels, thus dissolved CO$_2$ and increased acidity in oceanic waters are growing, the understanding of how corals will respond to rising CO$_2$ levels is critical.
Experiments have documented that additions of bicarbonate alone (2 mM) resulted in doubling of calcification rates in *Porites porites*. This was thought to result from the abundance of bicarbonate ions available for calcification (Marubini and Davies, 1999). Along with future projected increases in dissolved CO$_2$, however, the average pH of seawater is predicted to fall. A drop in pH alters the speciation of DIC, leading to lower abundance of carbonate ions available for calcification. In the Marubini and Davies (1999) experiment, when corals treated with 20 µM NH$_4^+$ were spiked with 2 mM bicarbonate, the deleterious effects of NH$_4^+$ on calcification were alleviated. These workers suggested that the negative effects of NH$_4^+$ enrichments result from DIC limitation. As NH$_4^+$ fertilizes the photosynthetic activity of the endosymbiotic algal community, the availability of DIC to the coral for calcification is reduced and thus additions of DIC relieve the limitation imposed by fertilization with NH$_4^+$. Alternatively, Holcomb et al. (2010) hypothesized that elevated nutrients may ameliorate the effects of ocean acidification on coral calcification. This was based on the theory that with elevated levels of nitrogen, symbionts increase their relative rates of photosynthesis and thus would be able to draw down more CO$_2$ which was acting to decrease saturation state of the calcifying medium. They measured higher rates of skeletal extension in corals under high pCO$_2$ and increased nitrogen than in high pCO$_2$ alone.

4.1.4 Experimental Goals

4.1.4.1 Influence of Elevated NO$_3^-$ and NH$_4^+$ on *Pocillopora damicornis*

*Pocillopora damicornis* is a Pacific branching coral species, which is typically known to be weedy. It is thought, however, that because *Pocillopora* is relatively faster growing than other corals, it may be more susceptible to environmental change. *Pocillopora* was
exposed to a range of NO$_3^-$ or NH$_4^+$ concentrations (1-52 µM) for four weeks. Responses in cell density, photosynthetic parameters, carbon cycling and growth were assessed.

**4.1.4.2 Influence of Elevated NO$_3^-$ and PO$_4^{3-}$ on Acropora cervicornis**

*Acropora cervicornis* is a critically important species in the Caribbean and understanding the influences of elevated DIN on the health of this endangered coral are important for developing management practices. *Acropora cervicornis* was subject to a long term experiment with elevated levels of NO$_3^-$ and PO$_4^{3-}$ to assess influences on cell density, photosynthetic parameters, carbon cycling and growth.

**4.1.4.3 Effects of Combined Elevated pCO$_2$ and NH$_4^+$ on Pocillopora damicornis**

Future projections for elevated levels of atmospheric pCO$_2$ have implications for the altered chemistry of reef waters. In order to assess the interactive effects of elevated CO$_2$ and NH$_4^+$, incubations were carried out at a target pCO$_2$ of 790 µatm and elevated NH$_4^+$ at 20 µM. Responses of symbiont cell density, photosynthetic parameters and growth were assessed.

**4.2 Methodology**

**4.2.1 Experimental Design**

All experimental incubations were held indoors at the University of Miami Experimental Hatchery. Treatment tanks consisted of 1 gallon glass tanks which were filled with 2.5 liters of seawater daily. The 1 gallon tanks were maintained inside of a larger flow thru system to maintain temperature control, however, individual tanks were isolated from flowing seawater and circulation within each tank was provided by a Hydor Koralia Nano mini pump. Temperature was held constant indoors at 26 ± 0.5$^\circ$ C. Light was supplied by USA Nova Current Extreme lights on a 12 hour light/12 hour dark cycle.
Control tanks for each experiment consisted of 0.2 micron filtered seawater pumped in from Bear Cut, while treatments consisted of the same seawater utilized for Controls with the appropriate addition of respective nutrients as specified for each experiment below. Seawater in tanks was changed at the beginning of each day with appropriate nutrient additions. Corals were subject to experimental nutrient levels for a period of three to eighteen weeks as specified for each separate experiment.

4.2.1.1 *Pocillopora damicornis Exposed to Variable NO$_3^-$ and NH$_4^+$ Enrichments*

a. January 2010 - *Pocillopora damicornis* strain PD-PAN-10 common gardened indoors at The University of Miami Experimental Hatchery were used for experimental incubations. Treatment conditions included Control, 10, 20, and 50 µM NaNO$_3$ and 10, 20, and 50 µM NH$_4$Cl. There were two replicate tanks assigned to each treatment. Each tank contained six coral fragments for a total of 90 coral fragments distributed among 14 tanks. The experimental period was 30 days. Data available for this experiment include algal cell density and linear extension.

b. May 2012 - *Pocillopora damicornis* strain PD-PAN-10 common gardened indoors at The University of Miami Experimental Hatchery were used for experimental incubations. Treatment conditions included Control, 5 and 20 µM NaNO$_3$ and 5 and 20 µM NH$_4$Cl. There were three replicate tanks assigned to each treatment. Each tank contained 6 coral fragments for a total of 90 coral fragments distributed among 15 tanks. The experimental period was 32 days. Data available from this experiment includes algal cell density.
c. November 2012 – *Pocillopora damicornis* from two strains PD-PAN-8 and PD-PAN-10 common gardened indoors at the University of Miami Experimental Hatchery were used for experimental incubations. Treatment conditions included Control, 10, 20, and 50 μM NaNO₃ and 10, 20, and 50 μM NH₄Cl. There were four replicate tanks assigned to each treatment. Each tank contained 6 coral fragments for a total of 240 coral fragments distributed among 40 tanks. The experimental period was 32 days. A comprehensive data set is available from this experiment.

### 4.2.1.2 *Acropora cervicornis* Exposed to Variable NO₃⁻ and PO₄³⁻ Enrichments

*Acropora cervicornis* from strain AC Smithsonian common gardened at the University of Miami Experimental Hatchery were used for experimental incubations. Treatment conditions included Control, Low Nutrient, 20 μM NaNO₃, 2 μM KH₂PO₄, and combined 20 μM NaNO₃ and 2 μM KH₂PO₄. Low nutrient seawater served as the base for all treatments except for Ambient (Controls) and was made new daily by incubating seawater for 24 hours in the light with *Chaetomorpha* algae which depleted NO₃⁻ and PO₄³⁻ levels down to ~0 μM. There were four replicate tanks assigned to each treatment. Each tank contained two coral fragments for a total of 40 coral fragments distributed among 20 tanks. The experimental period was 18 weeks.

### 4.2.1.3 *Pocillopora damicornis* Exposed to NH₄⁺ and CO₂ Enrichments

June 2012 - *Pocillopora damicornis* strain PD-PAN-10 common gardened indoors at The University of Miami Experimental Hatchery were used for experimental incubations. Treatment conditions included Control, 20 μM NH₄Cl,
Elevated CO₂ (target pCO₂ 792 µatm) and combined 20 µM NH₄Cl and Elevated CO₂. CO₂ enrichments were achieved through the addition of bicarbonate and hydrochloric acid. Alkalinity and pH measurements determined the actual levels met for CO₂ enrichments (average pH: 7.83, average alkalinity: 2.406, average DIC: 2230 µmol L⁻¹). The total DIC was slightly higher than the target level (2191 µmol L⁻¹) likely due to the higher than expected baseline DIC in the seawater utilized. There were two replicate tanks assigned to each treatment. Each tank contained 6 coral fragments for a total of 60 coral fragments distributed among 10 tanks. The experimental period was 21 days.

4.2.2 Surface Area Measurements

Coral surface area was determined by modeling individual coral branches as cylinders for *Pocillopora damicornis* and modelling coral nubbins as cones for *Acropora cervicornis*. The height and diameter of each branch was measured with a digital micrometer and surface area calculations were carried out. For the measurements of symbiont density, in order to provide more precise surface area measurements for small fragments symbiont density counts were normalized to surface area obtained with the foil method (Marsh 1970), except for the November 2012 *Pocillopora damicornis* experiment in which surface area was determined with a white light 3D scanner (HDI Advance R2, 3D3 Solutions, see methods of (Enochs et al. 2014)).

4.2.3 Algal Symbiont Density Measurements

Measurements of symbiont density were achieved through manual counts with a Neubauer Bright-Line Hemacytometer. Coral tissues were removed from subsamples of approximately 1 cm branches from each treatment. Complete tissue removal was ensured
by dissolving the coral skeleton in 18% HCl. There was no tissue or symbiont loss as a result of the HCl treatment and all tissue remained intact in one piece after dissolution. The tissue mass was homogenized with a Tissue Master Automated Homogenizer in a solution of 1.5 mL 2% buffered glutaraldehyde in seawater and 0.25 mL of Lugols solution (20g KI, 10g I₂ in 200 mL deionized water). Six to ten replicate aliquots were counted for each sample. Counts were normalized to surface area.

4.2.4 Calcification Measurements

4.2.4.1 Buoyant Weight Technique

The buoyant weight technique was utilized to obtain calcification rates (Davies 1989). It was ensured that salinity and temperature of seawater were recorded in order to determine seawater density and that density was the same for each measurement obtained throughout an experimental period. Measurements were carried out in a temperature controlled room to provide consistency. In addition, a standard mass was repeatedly measured between every three coral buoyant weight measurements during buoyant weighing. When calculating calcification rates, literature density values of 2.781 and 1.958 for Pocillopora sp. (Davies 1989) and Acropora cervicornis (Hughes 1987), respectively, were utilized to convert to micromoles of CaCO₃ precipitated according to the following Equation 1:

$$ Actual \ Weight = \frac{\text{Wet Weight}}{1 - \frac{\text{SW density}}{\text{Skeletal density}}} $$ (1)

The total mass changes were then converted into hourly rates as µmoles CaCO₃ cm⁻² hr⁻¹ in order to compare to rates obtained from the alkalinity anomaly technique.
4.2.4.2 Alkalinity Anomaly Technique

The alkalinity anomaly technique is based on the theory that the change in total alkalinity of seawater accompanies the precipitation of CaCO$_3$ (Smith and Key 1975): for every mole of CaCO$_3$ which is precipitated, the total alkalinity is reduced by two molar equivalents. In considering this method for measuring the calcification rate in corals, it is useful because neither the drawdown of CO$_2$ during algal photosynthesis nor the addition of CO$_2$ during algal or coral respiration influence the total alkalinity of the seawater. For the various experiments, short term incubations (1.3 – 6.5 hours) were carried out in light only and dark only conditions in order to calculate rates for light and dark calcification separately. The calcification rates were calculated in µmoles CaCO$_3$ cm$^{-2}$ hr$^{-1}$, according to Equation 2:

$$\text{Calcification rate (µmoles CaCO}_3/\text{cm}^2 \text{ hr}) = \frac{0.5(TA_o - TA_f) \times V}{SA \times t}$$ (2)

Where, TAo and TAf are the initial and final measurements of total alkalinity (mM), V is the volume (liters) of the incubation chamber, SA is the total surface area of the corals in the incubation chamber (cm$^2$), and t is time (hrs).

4.2.5 Linear Extension Measurements

Linear extension rates were measured with a scanning optical micrometer developed by Dr. Chris Langdon for use with corals (Albright et al. 2010). The sensitivity of the measurements provides the ability to obtain rates of skeletal extension in microns/day.

4.2.6 Estimating Photosynthesis and Respiration

The alkalinity and pH of seawater measured at the start and end of closed short term incubations were utilized to calculate initial and final total dissolved inorganic
carbon concentrations, [DIC]₀ and [DIC]ᵢ. These calculations were carried out with use of the equations and tables for calculating seawater carbon chemistry parameters provided by Strickland (1972). The total change in [DIC] during each incubation was calculated. The change in [DIC] which was attributed to result from calcification (determined by the alkalinity anomaly technique for the same incubation samples) was subtracted from the total [DIC] change in order to obtain the change in [DIC] which resulted from the net contributions of algal photosynthetic fixation (removal of DIC) and coral and algal respiration (additions of DIC).

4.2.7 Isotopic Analyses

4.2.7.1 δ^{13}C and δ^{15}N of Coral and Algal Tissues

Coral tissue and algal symbionts were separated for δ^{13}C and δ^{15}N isotopic analysis and determination of molar CN ratios. The method for separation is a modified version of the series of centrifugation and rinsing with the additional step of filtration twice through 20 micron nitex mesh in order to eliminate contamination of the symbiont fraction by coral nematocysts (Piniak et al. 2003). Coral and algal symbiont tissues were dried at 40 °C for 48 hours and prepared in tin capsules for organic carbon and nitrogen stable isotope analysis. Samples were analyzed on an Elemental Analyzer (Costech) interfaced to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific). Replicate analyses of standards and samples resulted in standard deviations for δ^{13}C and δ^{15}N of <0.05 ‰ and <0.1 ‰, respectively.
4.2.7.2 Inorganic $\delta^{13}C$ and $\delta^{18}O$ of Carbonate

Skeletal carbonate $\delta^{13}C$ and $\delta^{18}O$ was analyzed on Finnigan Delta plus with an attached Kiel device. Delta values are reported in reference to Vienna Pee Dee Belemnite (V-PDB). Standard deviation for replicate samples and standards was <0.1 ‰.

4.2.7.3 $\delta^{13}C$ of Dissolved Inorganic Carbon

Water samples for $\delta^{13}C$ analysis of DIC were filtered and preserved with mercuric chloride prior to analysis. Samples were analyzed on a Gas Bench interfaced to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific). Average standard deviation for replicate analyses was 0.05 ‰.

4.2.8 Calculation of Skeletal Enrichment

To compare the relative enrichment in $\delta^{13}C$ of the skeletal carbonate that may result from increases in productivity between treatments, an enrichment factor was calculated to normalize for slight changes in total coral surface area within each tank. The enrichment from the minimum skeletal $\delta^{13}C$ value was weighted to surface area, to calculate the “skeletal enrichment factor”. The weighted surface area was equal to the total surface area of coral in the tank from which the sample was taken divided by the average of total coral surface area from all tanks (equation 3).

\[
\text{Skeletal Enrichment} = (\delta^{13}C_{\text{skeletal measured}} - \delta^{13}C_{\text{skeletal minimum}}) \times \frac{\text{Tank Surface Area}}{\text{Average Surface Area}}
\]

(3)

4.2.9 Quantifying Carbon Cycling in Coral-Algal Symbiosis

To assess the relative amounts of photosynthetic carbon which are allocated to algal symbiont and coral host, an isotope tracer method was employed. At the end of each experimental period (i.e. after 4 weeks of nutrient enrichment), corals were incubated in labeled seawater spiked with NaH$^{13}$CO$_3$ at ~1000 ‰ for 24 hours. Similarly, NH$_4^+$ or
NO$_3^-$ was added at the appropriate treatment concentrations. The corals were rinsed in filtered seawater immediately following label incubations and coral host tissue was separated from algal symbionts. The method for separation is a modified version of the series of centrifugation and rinsing with the additional step of filtration twice through 20 micron nitex mesh in order to eliminate contamination the symbiont fraction by coral nematocysts (Piniak et al. 2003). Coral and algal symbiont tissues were dried at 40 °C for 48 hours and prepared in tin capsules for organic carbon and nitrogen stable isotope analysis (See 4.2.7.1). The amount of photosynthetic carbon allocated to coral host and retained in algal symbionts was calculated utilizing the isotopic composition of tissues prior to labeling, the isotopic composition of the DIC and the resulting isotopic composition of the tissues after labeling. The amounts of photosynthetically derived carbon after 24 hours were calculated for coral and algal tissues.

4.2.10 Statistical Analyses

JMP 11.0 Statistical Analysis Software was used to perform statistical analysis. Data which did not satisfy the Shapiro-Wilk Goodness of Fit test for normality were analyzed with non-parametric methods (Kruskal Wallis and Wilcoxin tests). Normally distributed data were analyzed with One-Way ANOVA and Tukey HSD post hoc methods. Two-Way ANOVA tests were used to detect interactive treatment effects. In the data sets with unequal variances, data were analyzed using an unequal variance t-test or Welch’s ANOVA and a Dunnet post hoc test.
4.3 Results

4.3.1 Pocillopora damicornis Exposed to Elevated NO$_3^-$ and NH$_4^+$

4.3.1.1 Symbiont Density

A summary of the algal symbiont cell density data normalized to coral surface area for the *Pocillopora damicornis* NO$_3^-$ enrichment experiments (January 2010, May 2012 and November 2012) is shown in Figure 4.3.1.1a. There was a significant treatment effect of elevated NO$_3^-$ on symbiont density (ANOVA, F(4,32) = 13.90, p <0.0001). The symbiont density was higher for corals in all elevated NO$_3^-$ treatments (5, 10, 20, and 50 µM NO$_3^-$) than corals in Control tanks (Tukey-Kramer method, p<0.05). Multiple ANOVAs determined that there were no significant differences between symbiont densities among different replicate experiments within the same treatment conditions.

A summary of the algal NH$_4^+$ symbiont cell density data normalized to coral surface area for the *Pocillopora damicornis* NH$_4^+$ enrichment experiments (January 2010, May 2012 and November 2012) are shown in Figure 4.3.1.1b. There was a significant treatment effect of elevated NH$_4^+$ on algal symbiont density (ANOVA, F(4,37) = 25.11, p<0.0001). The symbiont density was higher for corals in all elevated NH$_4^+$ treatments (5, 10, 20 and 50 µM NH$_4^+$) than corals in Control tanks, and the symbiont densities in 50 and 20 µM NH$_4^+$ were also significantly higher than those from corals grown in 5 µM NH$_4^+$ (Tukey-Kramer method, p<0.05). Multiple ANOVAs determined that there were no significant differences between symbiont densities among different replicate experiments within the same treatment conditions.
Figure 4.3.1.1a Algal symbiont cell density normalized to coral skeleton surface area for *Pocillopora damicornis* after 4 weeks in variable NO$_3^-$ concentrations. Data are from three replicate experiments carried out in January 2010, May 2012 and November 2012. There was no significant difference in density for same treatments between replicate experiments. The 5 μM NO$_3^-$ data are from only the May 2012 experiment. Treatments not connected by the same letter are significantly different (Tukey-Kramer method, p<0.05). Error bars represent 1 standard deviation from the mean.
Figure 4.3.1.1b Algal symbiont cell density normalized to coral skeleton surface area for *Pocillopora damicornis* after 4 weeks in variable NH$_4^+$ concentrations. Data are from three replicate experiments carried out in January 2010, May 2012 and November 2012. There was no significant difference in density for same treatments between replicate experiments. The 5 µM NH$_4^+$ data are from only the May 2012 experiment. Treatments not connected by the same letter are significantly different (Tukey-Kramer, p<0.05). Error bars represent 1 standard deviation from the mean.
4.3.1.2 Indicators of Photosynthesis and Respiration Rates

$\delta^{13}C$ Summary Pocillopora damicornis Elevated NO$_3^-$ (Coral, Algal, Skeletal, DIC)

The $\delta^{13}C$ values for the coral tissues, algal symbiont tissues and skeletal carbonate measured at the end of the November 2012 *Pocillopora damicornis* experiment are given in Figure 4.3.1.2a. The $\delta^{13}C$ of the DIC of incubation water is also shown. The average $\delta^{13}C$ of coral tissues from each treatment ranged from -22.27 ‰ to -22.39 ‰. There was no significant treatment effect of elevated NO$_3^-$ on the $\delta^{13}C$ of coral tissues (ANOVA F(3,12) = 0.23, p=0.8738). There was no significant treatment effect of elevated NH$_4^+$ on coral tissue $\delta^{13}C$ (ANOVA F(3,12)=0.35, p=0.7894).

The $\delta^{13}C$ of algal tissues ranged from -23.89 to -21.61 ‰ and the tissues of the 10 µM NO$_3^-$ treatment were significantly more depleted than the Control tissues (Tukey-Kramer method, p=0.0191). There was no significant treatment effect of elevated NH$_4^+$ on the $\delta^{13}C$ of algal tissues (ANOVA, F(3,12)=0.94, p=0.4536).

To correct for slight variations in biomass within each tank, the enrichment in skeletal $\delta^{13}C$ from the lowest $\delta^{13}C$ of all samples measured was calculated. The enrichment was normalized to coral surface area in each tank and is shown in Figure 4.3.1.2b as enrichment from the minimum $\delta^{13}C$ measured. There was no effect of elevated NO$_3^-$ treatments on the skeletal enrichment factor (ANOVA, F(3,10) = 1.49, p=0.2754). There was a significant treatment effect of elevated NH$_4^+$ conditions on the skeletal enrichment factor (ANOVA, F(3,12) = 8.07, p=0.0033), and the skeletal enrichment was significantly higher in 50 µM NH$_4^+$ than for Controls (Tukey-Kramer method, p=0.0075), 10 µM NH$_4^+$ (p=0.006) and 20 µM NH$_4^+$ (p=0.0125).
Figure 4.3.1.2a A summary from the November 2012 *Porites lobata* experiment of the carbon isotopic signatures of daily initial seawater DIC (blue), skeletal growth (red), algal symbiont tissues (green) and coral tissues (orange) after the 4 week experimental period.
Figure 4.3.1.2b Data from the November 2012 Pocillopora damicornis experiment representing the amount of natural carbon isotopic enrichment in the new skeletal material from the minimum skeletal δ¹³C value obtained from all samples. Enrichment is normalized to the total coral surface area for the tank in which the corals were grown for 4 weeks. Error bars represent one standard deviation from the mean.
ΔDIC Summary from Pocillopora damicornis in Elevated NO₃⁻ Incubations

The DIC drawdown rate was similar between, Control corals and elevated NO₃⁻ treatments at an average of 0.78 μmoles cm⁻² hr⁻¹. There were slight variations in the contributions from the processes responsible for DIC drawdown (Figure 4.3.1.2c). An average of 52.7 % of drawdown in Controls was attributed to the process of calcification and 47.3 % from the net effect of photosynthesis and respiration, while in the 10 and 20 μM NO₃⁻, 55.9 % and 54.7 % of drawdown, respectively, was attributed to net effect of photosynthesis and respiration.

Δδ¹³CDIC from Pocillopora damicornis in Elevated NO₃⁻ Incubations

The δ¹³C[DIC] from initial and final samples collected during the short term light incubations are represented in Figure 4.3.1.2d. In all tanks, the final δ¹³C value measured is more enriched that the initial samples indicating that the combined processes of photosynthesis, respiration and calcification have the cumulative effect of a positive isotopic fractionation, selecting for the light isotope of carbon (¹²C).

ΔDIC Summary Pocillopora damicornis elevated NH₄⁺ Incubations

The DIC drawdown rate was similar between, Control corals and elevated NH₄⁺ treatments at an average of 0.78 μmoles cm⁻² hr⁻¹ (slightly lower average in 20 μM NH₄⁺ 0.6 μmoles cm⁻² hr⁻¹) , however, there were slight differences in the contributions from the processes responsible for DIC drawdown (Figure 4.3.1.2c). An average of 52.7 % of drawdown was attributed to the process of calcification and 47.3 % from the net effect of photosynthesis and respiration for Controls, while in the 20 and 50 μM NH₄⁺, 66.0% and 58.7% of drawdown was attributed to photosynthesis and respiration.
Figure 4.3.1.2c The rate of drawdown of the total DIC during a closed light incubation for the November 2012 *Pocillopora damicornis* experiment under various conditions of elevated NO₃. The pie charts display the percentages of contribution to the total DIC change from the processes of calcification (orange) and the net effect of photosynthesis and respiration (green). Error bars represent one standard error.
Figure 4.3.1.2d The raw data for the average $\delta^{13}C_{\text{DIC}}$ of the initial sample and ($T_0$) and final sample ($T_f$) after a closed two hour light incubation from the November 2012 Pocillopora damicornis experiment under various conditions of elevated NO$_3^-$. Error bars represent one standard error.
Figure 4.3.1.2e The rate of drawdown of the total DIC during a closed light incubation for the November 2012 _Pocillopora damicornis_ experiment under various conditions of elevated NH$_4^+$ concentration. The pie charts display the percentage of contribution to the total DIC change from the processes of calcification (orange) and the net effect of photosynthesis and respiration (green). Error bars represent one standard error.
$\Delta^{13}C_{[DIC]}$ \textit{Pocillopora damicornis} Elevated $NH_4^+$ Incubations

The $\delta^{13}C_{[DIC]}$ from initial and final samples collected during the short term light incubations from the elevated $NH_4^+$ experiments are represented in Figure 4.3.1.2f. In all tanks, the final $\delta^{13}C$ value measured is more enriched that the initial samples indicating that in these treatments, the combined processes of photosynthesis, respiration and calcification have the cumulative effect of a positive isotopic fractionation, selecting for the light isotope of carbon ($^{12}C$).

4.3.3.1 Carbon Cycling Between Algal Symbiont and Coral Tissue

\textit{Percent New Carbon in Coral vs. Algal Symbiont High $NO_3^-$ Treatments}

The enrichment in $^{13}C$ during the labeling incubation of the corals exposed to four weeks of various elevated $NO_3^-$ concentrations is shown in Figure 4.3.1.3a and the enrichment in a set of control corals with no prior exposure to elevated $NO_3^-$ in Figure 4.3.1.3b. In the four week treated corals, there was a significant effect of elevated $NO_3^-$ on the percentage of new carbon accumulating in algal symbionts (ANOVA $F(3,25) = 7.0765$, $p = 0.0013$). The percentage of new carbon in algal symbionts for all elevated $NO_3^-$ treatments was reduced compared to Controls (Tukey HSD, $p<0.05$). There was not a significant effect of elevated $NO_3^-$ on the percentage of new carbon in the coral tissues (ANOVA $F(3,27) = 1.879$, $p = 0.157$). In the control set of corals (Figure 4.3.1.3b) there was also a treatment effect on the percentage of new carbon accumulating in the algal symbionts (ANOVA $F(3,27) = 11.16$, $p<0.0001$), with elevated $NO_3^-$ treatments reduced in comparison to Controls (Tukey HSD, $p<0.05$).
Figure 4.3.1.2f The raw data for the average $\delta^{13}C_{\text{DIC}}$ of the initial sample and ($T_0$) and final sample ($T_f$) after a closed two hour light incubation from the November 2012 *Pocillopora damicornis* experiment under various conditions of elevated NH$_4^+$. Error bars represent one standard error.
Figure 4.3.1.3a Data from the November 2012 Pocillopora damicornis experiment after four weeks of high NO₃ treatments, showing percent new photosynthetically derived carbon composing algal symbiont tissues (green) and coral tissues (orange) after the 24 hour H¹³CO₂ labelling incubation. Percent new carbon was calculated according to isotope mass balance equations. Error bars represent one standard error.

Figure 4.3.1.3b Data from the November 2012 Pocillopora damicornis experiment for a control set of corals with no prior NO₃ treatments, showing percent new photosynthetically derived carbon composing algal symbiont tissues (green) and coral tissues (orange) after the 24 hour H¹³CO₂ labelling incubation. Percent new carbon was calculated according to isotope mass balance equations. Error bars represent one standard error.
Percent New Carbon in Coral vs. Algal Symbiont High NH$_4^+$ Treatments

The enrichment in $^{13}$C during the labeling incubation of the corals exposed to four weeks of various elevated NH$_4^+$ concentrations is shown in Figure 4.3.1.3c and the enrichment in a set of control corals with no prior exposure to elevated NH$_4^+$ in Figure 4.3.1.3d. In the four week treated corals, there was no effect of elevated NH$_4^+$ on the percentage of new carbon accumulating in algal symbionts (ANOVA F(3,26) = 0.43, p = 0.7352). There was, however, a significant effect of elevated NH$_4^+$ on the percentage of new carbon in the coral tissues (ANOVA F(3,26) = 5.64, p = 0.0041). In the control set of corals (Figure 3.1.3d) there was a treatment effect on the percentage of new carbon accumulating in the algal symbionts (ANOVA F(3,26) = 7.98, p= 0.0006), with 50 µM NH$_4^+$ treatments accumulating less new carbon than Controls and 20 µM NH$_4^+$ (Tukey HSD, p<0.05). In the control set, the Control coral tissues accumulated significantly less new carbon than the elevated 20 µM NH$_4^+$ tanks (Tukey HSD, p<0.05).

4.3.1.4 Growth Rates

Linear Extension (January 2010 Experiment)

The linear extension rates for Pocillopora damicornis grown in 10, 20 and 50 µM NO$_3^-$ or NH$_4^+$ from the January 2010 experiment are shown in Figure 4.3.1.4a. There was no significant effect of elevated NO$_3^-$ on linear extension rates (Welch’s ANOVA F(3,3) = 2.6517, p = 0.3087). There was a significant effect of elevated NH$_4^+$ on linear extension rates (Welch’s ANOVA, F(3,4) = 39.73, p=0.0267). The linear extension rates of Controls (0.075 ± 0.003 mm/day) were significantly higher than 10 µM NH$_4^+$ (0.049 ± 0.008 mm/day; p=0.0462), 20 µM NH$_4^+$ (0.044 mm/day; p=0.0239) and 50 µM NH$_4^+$ (0.036 ± 0.004 mm/day; p=0.0102) treatments (Dunnet Test, p<0.05).
Figure 4.3.1.3c Data from the November 2012 *Pocillopora damicornis* experiment after four weeks of high NH$_4^+$ treatments, showing percent new photosynthetically derived carbon composing algal symbiont tissues (green) and coral tissues (orange) after the 24 hour H$^{13}$CO$_3^-$ labeling incubation. Percent new carbon was calculated according to isotope mass balance equations. Error bars represent one standard error.

Figure 4.3.1.3d Data from the November 2012 *Pocillopora damicornis* experiment for a control set of corals with no prior NH$_4^+$ treatments, showing percent new photosynthetically derived carbon composing algal symbiont tissues (green) and coral tissues (orange) after the 24 hour H$^{13}$CO$_3^-$ labeling incubation. Percent new carbon was calculated according to isotope mass balance equations. Error bars represent one standard error.
Figure 4.3.1:4a The linear extension rates measured by the optical micrometer technique for *Porites porites* from the January 2010 experiment for Control, 10 µM NO₃⁻, 20 µM NO₃⁻, 50 µM NO₃⁻, 10 µM NH₄⁺, 20 µM NH₄⁺, and 50 µM NH₄⁺ treatments. Error bars represent one standard deviation from the mean.
Calcification - Buoyant Weight Technique

The calcification rate as obtained from the buoyant weight technique for corals in the November 2012 *Pocillopora damicornis* experiment is given in Figure 4.3.1.4b. There were no effects of elevated NO$_3^-$ detected on calcification rates (ANOVA F(3,16) = 0.84, p = 0.49). There was a significant effect of elevated NH$_4^+$ on calcification rate (ANOVA F(2,18) = 8.11, p = 0.0013), with rates of calcification in Controls being significantly higher than in 50 µM NH$_4^+$ (Tukey-Kramer method, p = 0.0044). Rates of calcification in 10 µM NH$_4^+$ were also higher than those in 50 µM NH$_4^+$ (Tukey-Kramer method, p = 0.0044).

Calcification – Alkalinity Anomaly Technique

Calcification rates obtained from the alkalinity anomaly technique for the November 2012 *Pocillopora damicornis* experiment are given in Figure 4.3.1.4c. While the calcification rates obtained from the alkalinity anomaly technique were higher in the light than the rates obtained during the dark incubation, dark calcification rates were still significant and made up on average 76 % of light calcification rates for NO$_3^-$ treatments. There were no significant differences in the calcification rates between Controls and elevated NO$_3^-$ treatments during light (ANOVA, F(3,12) = 0.7128, p = 0.91) or dark incubations (ANOVA, F(3,12), p>0.05). The average light calcification rates were 0.37 ± 0.15 µmoles CaCO$_3$ cm$^{-2}$ hr$^{-1}$ for Controls, 0.39 ± 0.22 µmoles CaCO$_3$ cm$^{-2}$ hr$^{-1}$ for 10 µM NO$_3^-$, 0.31 ± 0.15 µmoles CaCO$_3$ cm$^{-2}$ hr$^{-1}$ for 20 µM NO$_3^-$ and 0.33 ± 0.13 µmoles CaCO$_3$ cm$^{-2}$ hr$^{-1}$ for 50 µM NO$_3^-$. For the elevated NH$_4^+$ treatments, dark calcification rates were similar to light calcification rates. The average light calcification rates were
Figure 4.3.1.4b The average of light and dark calcification rates as measured by the buoyant weight technique for *Pocillopora damicornis* from the November 2012 Experiment for Control, 10 μM NO$_3^-$, 20 μM NO$_3^-$, 50 μM NO$_3^-$, 10 μM NH$_4^+$, 20 μM NH$_4^+$ and 50 μM NH$_4^+$ treatments. Error bars represent one standard deviation from the mean.
Figure 4.3.1.4c: The light (blue) and dark (red) calcification rates as measured by the alkalinity anomaly technique for *Porites lobata* from the November 2012 Experiment for Control, 10 μM NO₃⁻, 20 μM NO₃⁻, 50 μM NO₃⁻, 10 μM NH₄⁺, 20 μM NH₄⁺, and 50 μM NH₄⁺ treatments. Error bars represent one standard deviation from the mean.
0.37 ± 0.15 μmoles CaCO₃ cm⁻² hr⁻¹ for Controls, 0.24 ± 0.08 for 10 μM NH₄⁺, 0.30 ± 0.25 μmoles CaCO₃ cm⁻² hr⁻¹ for 20 NH₄⁺ and 0.27 ± 0.17 μmoles CaCO₃ cm⁻² hr⁻¹ for 50 μM NH₄⁺. There were no significant effects detected of elevated NH₄⁺ on calcification rate during light (ANOVA F(3,10) = 0.51, p = 0.687) or dark incubations (ANOVA F(3,12) = 0.21, p = 0.89). The calcification rates obtained from the light and dark incubations were averaged together to represent an average hourly rate (Figure 4.3.1.4d).

4.3.2 Acropora cervicornis Response to Elevated NO₃⁻ and PO₄³⁻

4.3.2.1 Symbiont Density

The algal symbiont density data from the Acropora cervicornis experiment is shown in Figure 4.3.2.1. There was a significant effect of treatment on symbiont density (Welch’s ANOVA, F = 27.3113, p = 0.0055). Corals in the 20 μM NO₃⁻ and combined 20 μM NO₃⁻ and PO₄³⁻ had significantly higher symbiont densities than Low Nutrient treatments (Dunnet’s Test p = 0.0039, p = 0.0009) and Controls (Dunnet’s Test, p = 0.0282, p = 0.0072).

4.3.2.2 Indicators of Photosynthesis and Respiration Rates

δ¹³C Summary from Acropora cervicornis (Coral, Algal, Skeletal, DIC)

The δ¹³C values for the coral tissues, algal symbiont tissues and skeletal carbonate measured at the end of the July 2011 Acropora cervicornis experiment are given in Figure 4.3.2.2a. The δ¹³C of the DIC of incubation water is also shown. The average δ¹³C of coral tissues ranged from -23.83 ‰ to -23.09 ‰. There was a treatment effect on the δ¹³C of coral tissues (ANOVA F(4,13) = 8.22, p = 0.0016), with the coral tissues grown under Control and combined NO₃⁻ and PO₄³⁻ conditions being significantly lower than
Figure 4.3.1.4d The average of light and dark calcification rates as measured by the alkalinity anomaly technique for *Pocillopora damicornis* from the November 2012 Experiment for Control, 10 μM NO$_3^-$, 20 μM NO$_3^-$, 50 μM NO$_3^-$, 10 μM NH$_4^+$, 20 μM NH$_4^+$ and 50 μM NH$_4^+$ treatments. Error bars represent one standard deviation from the mean.
Figure 4.3.2.1 Algal symbiont cell density normalized to coral skeleton surface area for *Acropora cervicornis* after 15 weeks in variable NO$_3$ and PO$_4^3-$ conditions. Error bars represent 1 standard deviation from the mean.
Figure 12.2. A summary from the Acropora cervicornis experiment of the carbon isotopic signatures of daily initial seawater DIC (blue), new skeletal material (red), algal symbiont tissues (green) and coral tissues (orange) after the experimental period.
elevated tissues from the low nutrient and elevated NO$_3^-$ treatments (Tukey-Kramer method, p<0.05). There was also a treatment effect on the $\delta^{13}$C of algal symbiont tissues (ANOVA, F(4,14)=13.01, p=0.0002). The average algal symbiont $\delta^{13}$C values ranged from -23.68 ‰ to -22.66 ‰, with the algal symbionts from corals grown in Control conditions being significantly lower than those in high NO$_3^-$, high PO$_4^{3-}$ and low nutrient conditions (Tukey-Kramer method, p<0.05).

To correct for slight variations in biomass within each tank, the enrichment in skeletal $\delta^{13}$C from the lowest $\delta^{13}$C of all samples measured was calculated. The enrichment was normalized to coral surface area in each tank and is shown in Figure 4.3.2.2b. The skeletal enrichments in _Acropora cervicornis_ grown in both 20 µM NO$_3^-$ and combined 20 µM NO$_3^-$ and 2 µM PO$_4^{3-}$ were higher than those grown in Control conditions, however, small sample sizes were used for these preliminary analyses and the differences were not significant (Kruskal Wallis, p>0.05). Additional analyses must be carried out to determine the presence of an effect of elevated NO$_3^-$ and PO$_4^{3-}$ on skeletal enrichment.

$\Delta$[DIC] Summary for _Acropora cervicornis_ NO$_3^-$ and PO$_4^{3-}$ Incubations

The DIC drawdown rate was similar between, Control, Low Nutrient and 20 µM NO$_3^-$ treatments at an average of 1.0 µmoles cm$^{-2}$ hr$^{-1}$, and the DIC drawdown in the PO$_4^{3-}$ treatment was the highest at 1.7 µmoles cm$^{-2}$ hr$^{-1}$. There were variations in the contributions from the processes responsible for DIC drawdown (Figure 4.3.2.2c). An average of 46.4% and 49.7 % of drawdown was attributed to the process of calcification for Control and Low Nutrient treatments, while in the 20 µM NO$_3^-$, and combined 20 µM NO$_3^-$ and 2 µM PO$_4^{3-}$, there was no measured calcification and thus 100 % of
Figure 4.3.2.2b Data from the Acropora cervicornis experiment representing the amount of carbon isotopic enrichment in the new skeletal material normalized to the total coral surface area for the tank in which the corals were grown for 4 weeks. Error bars represent one standard deviation from the mean.
Figure 4.3.2.2c The rate of drawdown of the total DIC during a closed light incubation for the Acropora cervicoris experiment under various conditions of elevated NO$_3^-$ and/or PO$_4^{3-}$. The pie charts display the percentage of contribution to the total DIC change from the processes of calcification (orange) and the net effect of photosynthesis and respiration (green). Error bars represent one standard error.
drawdown was attributed to photosynthesis and respiration. In the \( \text{PO}_4^{3-} \) treatment 63.9% of drawdown was attributed to the net effect of respiration and photosynthesis and 36.1% to the process of calcification.

\[ \Delta \delta^{13}C_{\text{DIC}} \text{ for Acropora cervicornis } \text{NO}_3^- \text{ and } \text{PO}_4^{3-} \text{ Incubations} \]

The \( \delta^{13}C_{\text{DIC}} \) from initial and final samples collected during the short term light incubations are represented in Figure 4.3.2.2d. In all tanks, the final \( \delta^{13}C \) value measured is more enriched than the initial samples indicating that in these treatments, the combined processes of photosynthesis, respiration and calcification have the cumulative effect of a positive isotopic fractionation, selecting for the light isotope of carbon (\( ^{12}C \)).

4.3.2.3 Percent New Carbon in Coral vs. Algal Symbiont

The percentages of new carbon synthesized during a 24 hour incubation were calculated from a \( ^{13}C \) labeling technique (Figure 4.3.2.3). The percentage of new carbon composing algal tissues was higher than the percent new carbon composing coral tissues in all treatments and Controls (t-tests, \( p<0.05 \)). The percent of new carbon in both fractions appeared to be increased in elevated \( \text{NO}_3^- \), elevated \( \text{PO}_4^{3-} \) and combined elevated \( \text{NO}_3^- \) and \( \text{PO}_4^{3-} \) treatments. There was a significant treatment effect on the percentage of new carbon accumulating in the coral tissues ((ANOVA F(4,14) = 5.15, \( p = 0.0092 \)) with elevated \( \text{PO}_4^{3-} \) and combined elevated \( \text{NO}_3^- \) and \( \text{PO}_4^{3-} \) having significantly higher percent new carbon than Controls (Tukey HSD, \( p<0.05 \)). There was not a significant treatment effect on the percent new carbon accumulating in algal symbionts (ANOVA F(4,14) = 1.9013, \( p = 0.162 \)).
Figure 4.3.2.2d The raw data for the average $\delta^{13}C_{\text{DIC}}$ of the initial sample and ($T_0$) and final sample ($T_f$) after a closed five hour light incubation from the *Acropora cervicornis* experiment under various conditions of elevated $\text{NO}_3^-$ and/or $\text{PO}_4^{3-}$. Error bars represent one standard error.
Figure 4.3.2.3 Data from the *Acropora cervicornis* experiment showing the percent new photosynthetically derived carbon composing algal symbiont tissues (green) and coral tissues (orange) after the 24 hour H¹³CO₃⁻ labeling incubation. Percent new carbon was calculated according to isotope mass balance equations. Error bars represent one standard error.
4.3.2.4 Growth Rates

Calcification - Buoyant Weight Technique

The calcification rates as obtained from the buoyant weight technique for corals in the July 2011 Acropora cervicornis experiment are given in Figure 4.3.2.4a. An ANOVA detected significant effect of treatment condition on calcification rate (F(4,31) = 2.76, p = 0.0451). The Controls had significantly higher calcification rates at 0.088 µmoles cm$^{-2}$ hr$^{-1}$ than the combined NO$_3^-$ and PO$_4^{3-}$ treatment at 0.031 µmoles CaCO$_3$ cm$^{-2}$ hr$^{-1}$ (Tukey-Kramer method, p=0.0324). While not significantly different, the average calcification rate for 20 µM NO$_3^-$ was lower than Controls at 0.046 µmoles cm$^{-2}$ hr$^{-1}$. There were no detected interactive effects of NO$_3^-$ and PO$_4^{3-}$ (Two Way ANOVA, p>0.05).

Calcification - Alkalinity Anomaly Technique

The calcification rates as obtained by the alkalinity anomaly technique for the Acropora cervicornis experiment during a light and dark incubation are represented in Figure 4.3.4.2b. The calcification rates were higher in the light period for Control, Low Nutrient and elevated PO$_4^{3-}$ than in the dark period. The rates of calcification in the NO$_3^-$ and combined NO$_3^-$ and PO$_4^{3-}$, however, were higher in the dark period than the light. There was a significant treatment effect measured for the light incubation (Welch’s ANOVA, F(4,5) = 1209.2245, p = 0.0008). The calcification rates in Controls, Low Nutrient, and elevated PO$_4^{3-}$ treatments were significantly higher than the calcification rates of the elevated NO$_3^-$ and combined elevated NO$_3^-$ and PO$_4^{3-}$ (Dunnet test, p<0.05). There was a significant treatment effect on dark calcification rates detected among treatments (Welch’s ANOVA, F(4,3) = 66.0782, p = 0.0292), and the Controls had the
Figure 4.3.2.4a The average calcification rates for replicate coral nubbins during the experimental period as measured by the buoyant weight technique (Davies et al. 1989) for Acropora cervicornis in Control, Low Nutrient, High NO₃⁻, High PO₄³⁻ + NO₃⁻ and High PO₄³⁻ treatments. Error bars represent one standard deviation from the mean.
Figure 4.3.2.4b The light (blue) and dark (red) calcification rates as measured by the alkalinity anomaly technique for Acropora cervicornis in Control, Low Nutrient, High NO$_3^-$, High PO$_4^{3-}$ + NO$_3^-$ and High PO$_4^{3-}$ treatments. Error bars represent one standard deviation from the mean.
highest average calcification rates 0.28 µmoles cm⁻² hr⁻¹ while the PO₄³⁻ had the lowest at 0.06 µmoles cm⁻² hr⁻¹. There were no interactive effects of NO₃⁻ and PO₄³⁻ detected for the light or dark calcification rates obtained by the alkalinity anomaly technique (Two Way ANOVA, p>0.05). The average of light and dark calcification rates from the alkalinity anomaly technique meant to represent the average hourly rate are represented in Figure 4.3.2.4c. There was a significant treatment effect on the average calcification rate (Welch’s ANOVA, F = 68.803, p = 0.0144), with elevated NO₃⁻ and combined elevated NO₃⁻ and PO₄³⁻ having significantly lower average calcification rates than Control, Low Nutrient and elevated PO₄³⁻ (Dunnet’s test, p<0.05).

*Linear Extension*

The linear extension rates for the *Acropora cervicornis* experiment are represented in Figure 4.3.2.4d. There was not a significant treatment effect on linear extension rate (ANOVA F (4,23) = 0.84, p = 0.516). The highest average extension rate was measured for Controls at 0.0044 ± 0.0011 mm day⁻¹ while the lowest rates were measured for the combined NO₃⁻ and PO₄³⁻ (0.0039 ± 0.0007 mm day⁻¹) and the elevated PO₄³⁻ treatment (0.0038 ± 0.0008 mm day⁻¹).

### 4.3.3 *Pocillopora damicornis* response to elevated NH₄⁺ and CO₂

#### 4.3.3.1 Symbiont Density

The algal symbiont density from the *Pocillopora damicornis* NH₄⁺ and CO₂ enrichment experiment is shown in Figure 4.3.3.1. There was a significant treatment effect on symbiont cell density (ANOVA F(3,11) = 49.5565, p<0.0001). The combined NH₄⁺ and CO₂ was significantly higher than all other treatments (Tukey-Kramer method, p<0.00001), while the CO₂ and 20 µM NH₄⁺ treatments were higher than Controls.
Figure 4.3.2.4c The light and dark calcification rates averaged to represent an hourly rate measured by the alkalinity anomaly technique which can be compared to the hourly rate calculated from the buoyant weight technique for *Acropora cervicornis* in Control, Low Nutrient, High NO₃, High PO₄²⁻ + NO₃, and High PO₄²⁻ treatments. Error bars represent one standard deviation from the mean.
Figure 4.3.2.4d Linear extension rates obtained through scanning measurements with an optical micrometer throughout the duration of the experimental period for *Acropora cervicornis* grown under Control, Low Nutrient, High NO$_3$, High PO$_4^3$ + NO$_3$, and High PO$_4^3$ treatments. Error bars represent one standard deviation from the mean.
Figure 4.3.3.1 Algal symbiont cell density normalized to coral skeleton surface area for *Pocillopora damicornis* after 3 weeks in variable CO$_2$ and NH$_4^+$ conditions: Control, 20 $\mu$M NH$_4^+$, Elevated CO$_2$ (target $p$CO$_2$ = 793 $\mu$atm), and 20 $\mu$M NH$_4^+$ + Elevated CO$_2$. Error bars represent 1 standard deviation from the mean.
(Tukey-Kramer method, \( p = 0.0043 \) and \( p = 0.0146 \), respectively). There was a significant interactive effect of \( \text{CO}_2 \) and \( \text{NH}_4^+ \) on algal symbiont cell density (Two Way ANOVA, \( F = 10.3237, p = 0.0083 \)).

**4.3.3.2 Indicators of Photosynthesis and Respiration Rates**

\( \delta^{13}C \) Summary of *Pocillopora damicornis* Elevated \( \text{NH}_4^+ \) and \( \text{CO}_2 \) (Coral, Algal, DIC)

The \( \delta^{13}C \) values for the coral tissues, algal symbiont tissues and skeletal carbonate measured at the end of the \( \text{NH}_4^+ \) and \( \text{CO}_2 \) *Pocillopora damicornis* experiment are given in Figure 4.3.3.2a. The \( \delta^{13}C \) of the DIC of incubation water is also shown. The \( \delta^{13}C \) for coral tissues ranged from \(-23.15 \text{‰} \) to \(-22.28 \text{‰} \). An ANOVA detected a treatment effect on \( \delta^{13}C \) of tissues (ANOVA, \( F(3,12) = 10.71, p = 0.001 \)) and the tissues of the elevated \( \text{CO}_2 \) treatment were significantly more negative than Control and 20 \( \mu \text{M} \) \( \text{NH}_4^+ \) tissues (Tukey-Kramer method, \( p<0.05 \)). There was an interactive effect of elevated \( \text{NH}_4^+ \) and \( \text{CO}_2 \) on tissues (Two Way ANOVA, \( p = 0.0471 \)), with tissues in combined \( \text{NH}_4^+ \) and \( \text{CO}_2 \) (-22.66 ‰) being more enriched than \( \text{CO}_2 \) alone (-23.15 ‰), but depleted compared to 20 \( \mu \text{M} \) \( \text{NH}_4^+ \) only (-22.33 ‰). The \( \delta^{13}C \) for algal tissues ranged from -23.52 ‰ to -22.12 ‰ and the tissues of the algal symbionts grown in elevated \( \text{CO}_2 \) were significantly more depleted than those grown in Control conditions, elevated \( \text{NH}_4^+ \) and combined elevated \( \text{CO}_2 \) and \( \text{NH}_4^+ \) (Tukey-Kramer method, \( p<0.05 \)). When considering the source of variability of \( \delta^{13}C \) of tissues and algal symbionts after the treatment period, however, the variability in the \( \delta^{13}C_{[\text{DIC}]} \) should be taken into account. The average starting \( \delta^{13}C_{[\text{DIC}]} \) in the elevated \( \text{CO}_2 \) and combined \( \text{CO}_2 \) and 20 \( \mu \text{M} \) \( \text{NH}_4^+ \) treatments (-1.48 ‰) was 0.68 ‰ lower than the \( \delta^{13}C_{[\text{DIC}]} \) in Controls and 20 \( \mu \text{M} \) \( \text{NH}_4^+ \) (-0.80 ‰) as a result of the DIC composition of the bicarbonate additions carried out to achieve the desired \( \text{CO}_2 \)
Figure 4.3.3.2a A summary from the *Porites damicornis* elevated NH$_4^+$ and CO$_2$ experiment of the carbon isotopic signatures of daily initial seawater DIC (blue), algal symbiont tissues (green) and coral tissues (orange) after the experimental period.
concentrations. Comparisons concerning the $\delta^{13}$C of tissues should only be made between elevated CO$_2$ and combined CO$_2$ and 20 µM NH$_4^+$ treatments (same initial DIC) and Control and 20 µM NH$_4^+$ treatments (same initial DIC).

$\Delta[DIC]$ Summary from *Pocillopora damicornis* Elevated NH$_4^+$ and CO$_2$

The DIC drawdown rate was similar between Control corals and the 20 µM NH$_4^+$ and combined 20 µM NH$_4^+$ and elevated CO$_2$ treatments with an average of 0.70 µmoles cm$^{-2}$ hr$^{-1}$ (Figure 4.3.3.2b). Highest drawdown rates were in the elevated CO$_2$ only treatment at 1.25 µmoles cm$^{-2}$ hr$^{-1}$. There were variations in the contributions from the processes responsible for DIC drawdown. An average of 54.5 % of drawdown in Controls was attributed to the process of calcification and 45.5 % from photosynthesis and respiration, while in the 20 µM NH$_4^+$ and combined 20 µM NH$_4^+$ and elevated CO$_2$, averages of 58.3 % and 60.3 % of drawdown, respectively, were attributed to the net effects of photosynthesis and respiration. In the elevated CO$_2$ tank, an average of 70.9 % of the drawdown was attributed to the net effect of photosynthesis and respiration.

$\Delta\delta^{13}$C$_{[DIC]}$ from *Pocillopora damicornis* Elevated NH$_4^+$ and CO$_2$

The $\delta^{13}$C$_{[DIC]}$ from initial and final samples collected during the short term light incubations are represented in Figure 4.3.3.2c. In all tanks, the final $\delta^{13}$C value measured is more enriched that the initial samples indicating that in these treatments, the combined processes of photosynthesis, respiration and calcification have the cumulative effect of a positive isotopic fractionation, selecting for the light isotope of carbon ($^{12}$C).
Figure 4.3.3.2b The rate of drawdown of the total DIC during a closed light incubation for the *Pocillopora damicornis* experiment under various conditions of elevated NH$_4^+$ and/or elevated CO$_2$. The pie charts display the percentages of contribution to the total DIC change from the processes of calcification (orange) and the net effect of photosynthesis and respiration (green). Error bars represent one standard error.
Figure 4.3.3.2c The raw data for the average $\delta^{13}C_{\text{DIC}}$ of the initial sample and $T_0$ and final sample $T_f$ after a closed three hour light incubation from the *Pocillopora damicornis* experiment under various conditions of elevated NH$_4^+$ and/or elevated CO$_2$. Error bars represent one standard error.
4.3.3 Growth Rates

*Calcification - Buoyant Weight Technique*

The calcification rates obtained from the buoyant weight technique for the *Pocillopora damicornis* elevated NH$_4^+$ and CO$_2$ experiment are represented in Figure 4.3.3.3a. The rates were slightly lower in the 20 µM NH$_4^+$ treatment (0.18 ± 0.03 µmoles cm$^{-2}$ hr$^{-1}$) than Controls (0.25 ± 0.14 µmoles cm$^{-2}$ hr$^{-1}$), however there were no significant treatment effects on calcification measured by buoyant weight (ANOVA F(3,11) = 0.33, p = 0.8014). There were no interactive effects of elevated NH$_4^+$ and CO$_2$ on calcification rate determined by buoyant weight (Two Way ANOVA, p>0.05).

*Calcification - Alkalinity Anomaly Technique*

The calcification rates obtained from the alkalinity anomaly technique were higher in the light than the dark for all treatments with the exception of the elevated CO$_2$ having similar average calcification rates during the light and dark periods (Figure 4.3.3.3b). There were no significant differences in the calcification rates between Controls and elevated CO$_2$ and NH$_4^+$ treatments during the light (Welch’s ANOVA F(3,4) = 10.2031, p = 0.1192) or dark incubations (Welch’s ANOVA F(3,3) = 35.045, p = 0.0645), however, there was a significant interactive effect of elevated CO$_2$ and 20 µM NH$_4^+$ on dark calcification rates (Two Way ANOVA F=11.76, p = 0.0415). The calcification rates from light and dark incubations were averaged together to represent an average rate hourly rate (Figure 4.3.3.3c). While Controls had the highest average rate of 0.340 ± 0.084 µmoles cm$^{-2}$ hr$^{-1}$ and combined elevated NH$_4^+$ and CO$_2$ had the lowest average rate (0.136 ± 0.008 µmoles cm$^{-2}$ hr$^{-1}$), there was no significant effect of treatment on the average calcification rate (Welch’s ANOVA F(3,3) = 4.708, p = 0.21).
Figure 4.3.3.3a The average calcification rates as measured by the buoyant weight technique for *Pocillopora damicornis* in Control, 20 μM NH$_4^+$, Elevated CO$_2$ (target pCO$_2$ = 793 μatm), and combined 20 μM NH$_4^+$ and Elevated CO$_2$. Error bars represent one standard deviation from the mean. R
Figure 4.3.3.3b The light (blue) and dark (red) calcification rates as measured by the alkalinity anomaly technique for *Pocillopora damicornis* in control, 20 μM NH₄⁺, Elevated CO₂ (target pCO₂ = 793 μatm), and combined 20 μM NH₄⁺ and Elevated CO₂. Error bars represent one standard deviation from the mean.
Figure 4.3.3.3c The average of light and dark calcification rates as measured by the alkalinity anomaly technique for *Pocillopora damicornis* in Control, 20 μM NH$_4^+$, Elevated CO$_2$ (target pCO$_2$ = 793 μatm), and combined 20 μM NH$_4^+$ and Elevated CO$_2$. Error bars represent one standard deviation from the mean.
**Linear Extension**

The linear extension rates for the *Pocillopora damicornis* in the elevated CO$_2$ and NH$_4^{+}$ experiment are represented in Figure 4.3.3.3d. The linear extension rates ranged from 0.039 mm/day to 0.55 mm/day. While the rates in the elevated CO$_2$ treatment were highest, there were no significant differences detected between treatments and Controls (ANOVA, F(3,11) = 1.39, p = 0.29775).

**4.4 Discussion**

**4.4.1 Pocillopora damicornis Exposed to Elevated NO$_3^{-}$ and NH$_4^{+}$**

While both forms of inorganic nitrogen had significant effects on algal symbiont cell densities, the results of this experiment suggest that elevated NH$_4^{+}$ may have a more deleterious influence on growth of *Pocillopora damicornis* and more intense response in the algal symbiont density. The highest concentrations of NH$_4^{+}$ had significantly lower rates of linear extension and calcification as measured by the buoyant weight technique.

The rates of DIC drawdown appeared to be similar in Controls, NO$_3^{-}$ and NH$_4^{+}$ treatments, however, in Controls more than 50% of the total DIC drawdown was attributed to calcification, whereas generally in the NH$_4^{+}$ treatments more than 50% of drawdown was the result of the net effect of photosynthesis and respiration (66% in 20 µM NH$_4^{+}$) suggesting more of the DIC pool in the elevated NH$_4^{+}$ treatments is allocated to the photosynthetic uptake by algal symbionts than calcification by the coral host tissues. As DIC becomes limited under increased rates of productivity, this can often be manifested as an enrichment in the $\delta^{13}$C signature of various biogenic materials. From measurements of the initial and final $\delta^{13}$C DIC from a short term incubation, it is clear that the net fractionation effects of the processes of calcification, photosynthesis and
Figure 4.3.3.3d Linear extension rates obtained through scanning measurements with an optical micrometer throughout the duration of the experimental period for *Pocillopora damicornis* grown under Control, 20 μM NH₄⁺, Elevated CO₂ (target pCO₂ = 793 μatm), and 20 μM NH₄⁺ + Elevated CO₂. Error bars represent one standard deviation from the mean.
respiration are working to enrich the residual DIC pool in $^{13}$C. The primary driver for this enrichment is thought to be the process of photosynthesis as it is associated with a large fractionation effect (28 ‰, O’Leary, 1978). After removing the variable influence of coral biomass on the DIC utilization in each tank, the calculated skeletal enrichment factor allowed for the discernment of relative enrichments in the coral skeletal carbonate of the 50 µM NH$_4^+$ treatments. This skeletal enrichment combined with the DIC data suggests that there is more influence of photosynthesis enriching the DIC pool in the 50 µM NH$_4^+$ treatments.

In order to assess changes in carbon cycling between the algal symbionts and the coral host tissues, a labeling incubation traced the fate of photosynthetically derived carbon in the coral-algal symbiosis. After four weeks of elevated NO$_3^-$ exposure, the algal symbionts of the NO$_3^-$ treated corals had significantly lower percent new carbon than the control algal tissues, while there was no change in the percentage of carbon in the coral tissues. In the NH$_4^+$ treated corals, there was no change in the percentage of new C in the algal symbionts between Controls and treatments, but there was an increase in the percentage of new carbon accumulating in the coral tissues.

The responses of *Pocillopora damicornis* to elevated levels of NO$_3^-$ are in agreement with previous work that found significant increases in symbiont cell density with NO$_3^-$ additions. The effects of NO$_3^-$ on coral growth have varied among studies. While Marubini (1996) measured a 50 % reduction in skeletogenesis under elevated NO$_3^-$ for *Montastraea annularis* and *Porites porites*, other studies have measured no effect of NO$_3^-$ on growth rate. In this study, it appears that NO$_3^-$ does not have a clear effect on the calcification or linear extension of *Pocillopora damicornis* after four weeks at elevations
of 10, 20 and 50 µM NO$_3^-$\_. This study suggests that NH$_4^+$ influences the calcification of *Pocillopora damicornis* only at the highest concentration of additions (50 µM NH$_4^+$), but may influence linear extension rates at lower enrichments as well.

### 4.4.2 Acropora cervicornis exposed to elevated NO$_3^-$ and PO$_4^{3-}$

The symbiont cell densities were higher in the 20 µM NO$_3^-$ and combined 20 µM NO$_3^-$ and 2 µM PO$_4^{3-}$ than in the corals in Control and Low Nutrient treatments, and there were several indications that elevated NO$_3^-$ and PO$_4^{3-}$ resulted in increased photosynthetic activity in *Acropora cervicornis*. The change in the δ$_{13}$C of the DIC over the short term incubation again indicated that the combined fractionation effects of calcification, photosynthesis and respiration act to enrich the DIC pool in $^{13}$C, likely the biggest influence coming from the large fractionation effect involved in photosynthesis (28 ‰). In the combined 20 µM NO$_3^-$ and 2 µM PO$_4^{3-}$ treatment, there were indications of $^{13}$C enrichment in both coral tissues and skeletal carbonate, suggesting these corals had overall higher photosynthetic activity. In addition, while the DIC drawdown rate was not changed in comparison to the other treatments, there was no calcification measured during the light incubation thus 100% of the change in DIC was attributed to photosynthetic drawdown.

The $^{13}$C labeling incubation indicated changes in carbon cycling as a result of elevated NO$_3^-$ and PO$_4^{3-}$ treatments. The coral tissues in elevated NO$_3^-$, PO$_4^{3-}$ and combined NO$_3^-$ and PO$_4^{3-}$ had higher percentage of new carbon than those in Low Nutrient and Control conditions. This provides additional support for the increased photosynthetic activity within *Acropora cervicornis* exposed to high NO$_3^-$ and PO$_4^{3-}$.
When examining the influence of NO$_3^-$ and PO$_4^{3-}$ on growth of *Acropora cervicornis*, there was no measurable significant decline in linear extension rates, however there were significant decreases in the rates of calcification. While density was not measured, this could potentially be due to the corals secreting a less dense skeleton under elevated nutrient conditions which has previously been found for corals grown under elevated PO$_4^{3-}$ conditions (Dunn et al. 2012). While the elevated NO$_3^-$ and combined NO$_3^-$ and PO$_4^{3-}$ showed no calcification during the light period, the calcification rates were slightly increased in the dark. This suggests that the factor that was inhibiting calcification in the light has less influence on the process of calcification in the dark allowing for these corals to secrete some skeletal material in the dark.

**4.4.3 Pocillopora damicornis exposed to elevated CO$_2$ and NH$_4^+$**

The algal symbiont cell density increased both in response to elevated NH$_4^+$ and elevated CO$_2$. In addition, there was an interactive effect, with combined NH$_4^+$ and CO$_2$ having the highest overall algal cell densities. These results suggest that both NH$_4^+$ and CO$_2$ can be limiting factors to algal symbiont biosynthesis and replication.

There were isotopic indications of increased photosynthetic activity in the combined CO$_2$ and NH$_4^+$ treatment as compared with the CO$_2$ alone. Both coral and algal tissues were more enriched in $^{13}$C when NH$_4^+$ was added as compared to CO$_2$ alone. This suggests that under the elevated CO$_2$ treatment, these conditions have created nitrogen limitation. From analysis of the DIC drawdown, it can be inferred that rates of photosynthesis were stimulated in all treatments, CO$_2$, NH$_4^+$ and combined CO$_2$ and NH$_4^+$ as compared to rates in Controls.
While there have been mixed results on the influence of conditions of ocean acidification on calcification among different species of corals, this study is in agreement with a recent study on the responses to acidification displayed by *Pocillopora damicornis* (Huang et al. 2014). There were no indications that elevated CO$_2$ alone resulted in decreases in either linear extension or calcification. In agreement with our previous experiment with NH$_4^+$ enrichments in *Pocillopora damicornis*, there were no measurable effects on calcification at 20 µM NH$_4^+$ enrichments. This experiment, did however suggest that there may be a decline in calcification in response to the combined effects of elevated NH$_4^+$ and CO$_2$. This is contradictory to some of the previous theories which have proposed that the effects of elevated NH$_4^+$ and CO$_2$ will ameliorate each other (Holcomb et al. 2010).

4.5 Conclusions

*Previously Proposed Mechanisms for Decreased Growth Rates in High NO$_3^-$ or NH$_4^+$*

When synthesizing the results of the three experiments in this study, we can consider the previously proposed theories for deleterious effects of elevated DIN on skeletogenesis. Three theories have been proposed by various workers to explain direct deleterious effects of nitrogen enrichment on coral skeletogenesis. The first suggests that nutrients lead to increased photosynthesis rates, and a diffusion limited amount of CO$_2$ is preferentially utilized in the process of photosynthesis, resulting in reduced availability of dissolved inorganic carbon for calcification. This is known as the competitive interaction between the processes of photosynthesis and calcification (Marubini and Davies 1996, Marubini and Atkinson 1999, Marubini and Thake 1999, Langdon and Atkinson 2005). Although increased photosynthetic activity may draw down the DIC pool, it can also
create water chemistry which may be more favorable for calcification, relatively higher amounts of CO$_3^{2-}$ favorable for precipitation of CaCO$_3$ (Goreau 1963, Gattuso et al. 1999). Positive effects on skeletogenesis from light enhanced drawdown of CO$_2$ have also been measured (Moya et al. 2006).

Another theory previously provided to explain decreased calcification is specific to NH$_4^+$ and suggests that the increase in NH$_4^+$ alters the chemistry of the calcifying fluid, with a source of protons creating conditions less favorable for the precipitation of calcium carbonate (Crossland and Barnes 1974, McGuire 1997). NH$_4^+$ is presumed to move through the membrane as NH$_3$ + H$^+$. This hypothesis, however, does not explain deleterious effects of NO$_3^-$ on coral growth previously measured by many workers and in Acropora cervicornis in this study.

The third theory proposed to explain the decrease in skeletogenesis under elevated nutrient levels involves the idea that photosynthetic compounds serve as energy supply important for the energetically expensive process of calcification or that photosynthetic compounds may be precursors to calcification (ex. organic matrix). It is suggested that in previously nitrogen limited algal symbionts, tissue synthesis will no longer be limited by nitrogen, thus symbionts will utilize more carbon to promote cell growth and division and less carbon will be excreted as waste to the coral host. This would result in an overall reduction of energetically important photosynthetic materials translocated to the coral host (Davy and Cook 2001). A similar alternative explanation may also be that the bulk amount of translocate is not significantly reduced, but the type of compounds which are translocated are different, having a negative impact on calcification. Alternatively, photosynthetically derived amino acids and proteins have been found to be important in
skeletal organic matrix, thus it could be suggested that enhanced uptake of nitrogen may have some positive response on coral skeletogenesis (Falkowski et al. 1993, Wang and Douglas 1999).

*Proposed Mechanism for Declined Growth under Elevated Nutrients in This Study*

The data collected from these experiments suggest that while effects vary among species, both enrichments in NO$_3^-$ and NH$_4^+$ can lead to declines in coral skeletogenesis. In every elevated nutrient treatment in which declines in linear extension or calcification rate were measured, the corals in these treatments had experienced significant increases in algal symbiont cell density. In addition to increased symbiont densities, these corals also exhibited several indications of increased rates of photosynthesis. While it cannot be confirmed that there were no changes in the types of biomolecules translocated from algal symbiont to coral host under elevated nutrient conditions, the presence of elevated NO$_3^-$ and NH$_4^+$ generally did not result in lower accumulation of photosynthetic carbon within coral tissues as compared to Controls. In most cases, the presence of elevated NO$_3^-$ or NH$_4^+$ actually resulted in an increase the percent of photosynthetic carbon within the coral tissues. This suggests that a decrease in energy supply from algal symbionts is likely not responsible for measured declines in calcification.

The mechanism that can best explain the decreased calcification rates measured in this study under high NO$_3^-$ or NH$_4^+$ is the resulting competition for DIC between the processes of coral calcification and algal symbiont photosynthesis. Several studies suggest that coral and algal symbionts can be limited by carbon availability. Evidence for such limitation is supported by studies of carbonic anhydrase (Weis et al. 1989),
stimulation of calcification after additions of HCO$_3^-$ (Burris et al. 1983; Marubini & Davies, 1999) and evidence from increased $^{13}$C fractionation (Muscatine et al. 1989).

While the overall DIC drawdown rates appear to be unaffected by high nutrient conditions, the allocation of more of the DIC pool to photosynthesis rather than calcification may be responsible for measured declines in calcification rate. It has been suggested that when it comes to competition for DIC, the algal symbionts may have an advantage in scavenging DIC as they are in closer proximity to the DIC source (seawater) than the coral cells responsible for calcification in the calicoblastic epithelial cell layer (Marubini and Thake 1999).

An interesting advantage of the alkalinity anomaly technique in this study is that measurements of dark calcification could be obtained. While some carbon fixation could occur in the dark by algal symbionts, the symbionts are less photosynthetically active in the dark and thus likely will not be able to deplete the inorganic carbon pools as in the light. If rates of calcification decline in response to DIC competition, then it should follow that the dark calcification rates will not be as affected as the light calcification rates. This would be because without photosynthetic drawdown, there should be higher availability of inorganic carbon at the site of calcification. This was seen in the elevated NO$_3^-$ and PO$_4^{3-}$ experiment in *Acropora cervicornis*, where no calcification was measured in the light for corals in the elevated NO$_3^-$ and combined NO$_3^-$ and PO$_4^{3-}$ treatments, but there was some measurable calcification that occurred during the dark period.

While the calcification rates were not declined in all elevated nutrient treatments, it is possible that there is an imbalance between changes in rates of algal biomass synthesis and changes in skeletogenesis rates. Tanaka et al. suggested that moderate
NO$_3^-$ enrichments stimulated photosynthesis rates 2.8 fold over Controls, but only resulted in 1.3 fold increase in the amount of calcification (Tanaka et al. 2007).

It is unclear what may be contributing to overall decline in calcification measured for the combined NH$_4^+$ and CO$_2$ treatments. It was noted that this treatment resulted in the highest overall algal symbiont cell density, so it is likely that the decline is related to the extreme increase in algal cell density. It is difficult to suggest that DIC could be limiting under these conditions, as these treatments had higher overall DIC. It could, however, be possible that while the overall DIC is higher, the relative availability of CO$_3^{2-}$ ions is lower, resulting in the decrease in rates which were observed.
Chapter Five – The Response of *Pocillopora damicornis* Hosting Two Different Clades of Algal Symbiont to Elevated Nitrate and Ammonium

5.1 Background

Important findings that corals hosting genetically distinct algal symbionts exhibit differences in thermal tolerance may provide hope for the persistence of corals through rising sea surface temperatures. In addition to understanding how these different coral-algal associations fare in thermal stress, understanding their response to variable environmental conditions is critical. This chapter investigates how these classified thermally tolerant coral-algal associations with different symbiont clades may respond to elevated nutrient levels. Specimens of *Pocillopora damicornis* hosting either clade C or clade D symbionts were grown in elevated levels of NO$_3^-$ and NH$_4^+$. Responses in symbiont density, photosynthesis rates and calcification rates were measured. The recycling of carbon between host and symbiont was quantified with use of stable isotope tracers. Analysis of isotopic fractionation involved with uptake and assimilation of NO$_3^-$ and NH$_4^+$ provides important information regarding the utilization of external DIN by these two symbiont clades.

5.1.1 The Coral-Algal Symbiosis and Thermal Stress

Recent focus for the future of coral reefs has been on their potential to persist under increasing sea surface temperatures. Coral bleaching, or reduction in the density of algal symbionts, is often the result of thermal stress but can also be caused by increased light intensity, severely decreased temperatures and other environmental stressors. Coral bleaching involves a disassociation of the algal symbionts from the coral host. There are several mechanisms through which the coral – algal system can break down including symbiont expulsion, degradation or cell cycle control (Davy et al. 2012). It is accepted
that thermal bleaching is preceded by an accumulation of reactive oxygen species (ROS) and when the rate of protein photodegradation exceeds the rate of protein synthesis, photoinhibition induced bleaching may occur (Baird et al. 2009). Increases in temperature increase the rate of photoinhibition through inhibition of the repair process, preventing the processes that normally keep ROS levels under control.

In addition to differences in thermal tolerance exhibited among coral species (Baird et al. 2009), it has been noted that genetically distinct Symbiodinium may display differences in thermal tolerance (Baker 2003, Rowan 2004). This finding has provided a glimmer of hope with regards to the future of symbiotic corals in warming seas, and some associations, such as that of Pocillopora sp. and clade D1 symbionts, may be more tolerant to rising sea surface temperatures (Cunning et al. 2013). It has been noted that a wide variety of symbiont – host taxa associations exist (Silverstein et al. 2012). The corals hosting those “thermally tolerant” symbionts may be more resistant to coral bleaching than corals hosting symbionts that are less “thermally tolerant”. Studies, however, have found that there may be “trade-offs” to this thermal tolerance which is costly for the coral host. Corals hosting the thermally tolerant clades have been found to have lower growth rates than those with clades found to be more susceptible to thermal bleaching (Jones and Berkelmans 2010). Jones and Berkelmans (2010) found under controlled conditions that corals hosting clade D symbionts had 29% slower growth than those hosting clade C2, and in the natural environment 38% slower growth. The mechanism for this is not clear, but several theories have been proposed to explain this “trade-off” phenomenon.
Further studies have posed the question as to whether community shifts to slower growing corals hosting these thermally tolerant symbionts will allow for the success of reefs under future increases in temperature (Ortiz et al. 2013). It has been suggested that due to overall reduced calcification rates contributed by these corals, less diverse reefs such as those in the Caribbean may experience a loss of resilience as result of shifting to thermally tolerant symbiont communities. Other more diverse reefs, such as some of those located in the Pacific may not be as susceptible.

While the adaptation of corals under increased temperatures may not be rapid enough to keep up with global warming, generation times of *Symbiodinium* are much shorter and may provide a mechanism for adaptation. If the future of Scleractinian corals is shifting towards a dominance of corals hosting thermally tolerant symbionts, it is important to understand how these symbioses will fare under other environmental stressors which threaten the health of coral reefs. Nutrient loading threatens the oligotrophic conditions of reefs through vectors such as agricultural runoff and sewage outfalls. While the direct effects of elevated nutrient levels on symbiotic corals are somewhat unclear, the differences in the nutrient interactions among coral symbioses involving different clades of *Symbiodinium* have not been studied. It is possible that thermally tolerant symbionts may respond differently to elevated nutrient levels than symbionts which display lower thermal tolerance, but whose symbiotic partnerships result in higher growth for their coral host.

### 5.1.2 Experimental Goals

In a time where the future of coral reefs is uncertain, it is critical that in our attempts to predict the future response of corals we consider all environmental factors at
play. While the future of Scleractinian corals may involve shifting to hosting a thermally
tolerant clade of *Symbiodinium*, we aim to understand how these coral-algal associations
will respond to elevated nutrient stress. This chapter investigates the variability of
response to elevated nutrient levels between corals with symbionts known to be thermally
tolerant and those hosting symbionts which have been found to be more susceptible to
bleaching. Experiments were carried out with the Pacific branching coral *Pocillopora
damicornis*, using colonies which have been confirmed to host primarily clade C or clade
D (thermally tolerant) symbionts. This chapter addresses variable responses to nutrients
among parameters such as nutrient uptake, symbiont density, photosynthesis rates, carbon
translocation rates and calcification rates.

5.2 Methodology

5.2.1 Experimental Design

All experimental incubations were held indoors at the University of Miami
Experimental Hatchery. Treatment tanks consisted of 1 gallon glass tanks which were
filled with 2.5 liters of seawater daily. The 1 gallon tanks were maintained inside of a
larger flow thru system to maintain temperature control, however, individual tanks were
isolated from flowing seawater, and circulation within each tank was provided by a
Hydor Koralia Nano mini pump. Temperature was held constant indoors at 26 ± 0.5° C.
Light was supplied by USA Nova Current Extreme lights on a 12 hour light/12 hour dark
cycle. Control tanks for each experiment consisted of 0.2 micron filtered seawater
pumped in from Bear Cut, while treatments consisted of the same seawater utilized for
Controls with the appropriate addition of nutrients to achieve 20 μM NaNO₃ and 20μM
NH₄Cl.
*Pocillopora damicornis* from two strains PD-PAN-8 and PD-PAN-10 hosting clade C symbionts and *Pocillopora damicornis* from eight strains hosting clade D symbionts confirmed by qPCR analysis were common gardened indoors at the University of Miami Experimental Hatchery prior to use for experimental incubations. Treatment conditions included Control, 20 μM NaNO₃ and 20μM NH₄Cl. There were four replicate tanks assigned to each treatment. Each tank contained 6 coral fragments for a total of 144 coral fragments distributed among 24 tanks. Seawater in tanks was changed at the beginning of each day with appropriate nutrient additions. Corals were subject to experimental nutrient levels for 32 days.

5.2.2 Water Sample Collection for [DIN] and δ¹⁵N_DIN

Water samples were collected at the beginning and end of the four week experimental period. Samples were collected throughout a 48 hour period on a 12 hour light/12 hour dark cycle at 0, 4, 12, 24, 36 and 48 hours and then during a period of 48 hours of constant light exposure at 0, 4, 12, 24 and 48 hours. Filtered water samples were collected in 125 ml Nalgeen (polyethylene) bottles and acidified to pH 2 with the addition of hydrochloric acid for preservation (Capone 2008).

5.2.3 NO₃⁻ and NH₄⁺ Concentration Analysis

The concentrations of NO₃⁻ and NH₄⁺ were determined colorimetrically with a SmartChem200 Autoanalyzer (Westco Scientific). The NO₃⁻ concentrations were determined via the vanadium chloride method and the NH₄⁺ concentrations were measured through the indophenols-blue method (Berthelot reaction) (Westco Scientific SmartChem200 Manual). NO₃⁻ and NO₂⁻ concentration data are required for sample preparation for NO₃⁻ isotopic analysis.
5.2.4 NO$_3^-$ and NH$_4^+$ Isotopic Analysis

The analysis of NH$_4^+$ and NO$_3^-$ $\delta^{15}$N and NO$_3^-$ $\delta^{18}$O was achieved through conversion to N$_2$O gas and subsequent isotopic analysis in the Altabet Biogeochemistry Laboratory at UMass, SMAST in New Bedford, MA. NO$_3^-$ is first converted to NO$_2^-$ via the Cd reduction method (McIlvin and Altabet 2005, Ryabenko et al. 2009) and the concentrations of NO$_2^-$ are analyzed to determine percent yield of NO$_3^-$ converted to NO$_2^-$. NH$_4^+$ is first converted to NO$_2$ with a hypobromite reagent at pH 12, followed by the addition of sodium arsenite to stop the reaction (Zhang et al. 2007). All samples are then treated with 1:1 acetic acid:azide and buffered with 10 M NaOH to convert NO$_2^-$ to N$_2$O gas. The $\delta^{15}$N and $\delta^{18}$O composition of N$_2$O gas is analyzed on a GV Isoprime isotope ratio mass spectrometer with a purge and trap system. Values are reported in delta notation (equation 1), relative to N$_2$ and VSMOW for nitrogen and oxygen.

$$\delta (\%) = \frac{(R_{\text{Sample}} - R_{\text{Standard}})}{(R_{\text{Standard}})} \times 10^3$$  

(1)

Where $R_{\text{Sample}}$ is the ratio of heavy to light isotopes in the sample and $R_{\text{Standard}}$ is the ratio of heavy to light isotopes in the standard.

$\delta^{15}$N and $\delta^{18}$O sample values for each run are compared to a set of blanks (operational blanks: low nutrient seawater treated with the acetic acid:azide solution, and Cd blanks: low nutrient seawater treated with Cd), a set of three NO$_2^-$ standards and a set of three NO$_3^-$ standards (USGS34, USGS35, and a lab standard). $\delta^{15}$N of NH$_4^+$ is also compared to a set of three NH$_4^+$ standards (USGS25, USGS26 and a lab standard). Isotopic data were corrected for the exchange of oxygen between sample and water during conversion to N$_2$O, and the 1:1 addition of azide-N to NO$_2^-$-N in the formation of
N₂O (McIlvin and Altabet 2005) Average reproducibility for δ¹⁵N was 0.20 ‰ and δ¹⁸O was 0.25 ‰.

5.2.5 Calculation of Discrimination Factor ε

The discrimination factor, ε can be calculated by using the δ¹⁵N of the residual NO₃⁻ or NH₄⁺ or δ¹⁵N of the synthesized tissues. The method using the δ¹⁵N of the residual NO₃⁻ or NH₄⁺ is used in this chapter.

Calculations of ε from Residual NO₃⁻ or NH₄⁺

In order to calculate the ε derived from incubation water, the concentration and isotopic signature of NO₃⁻ is utilized. Solving for the slope of the Rayleigh Distillation equation (equation 2) gives ε (equations 3):

\[ \delta^{15}N_{NO_3(t)} = \delta^{15}N_{NO_3(o)} - \varepsilon \ln f \] (2)

\[ \varepsilon = \frac{\delta^{15}N_{NO_3(o)} - \delta^{15}N_{NO_3(t)}}{\ln f} \] (3)

where \( \delta^{15}N_{NO_3(o)} \) is the isotopic composition of the NO₃⁻ at the start of the incubation and \( \delta^{15}N_{NO_3(t)} \) is the isotopic composition of the NO₃⁻ at time t, when the fraction of original NO₃⁻ remaining is f. ε for NH₄⁺ uptake can be similarly calculated. For NO₃⁻ uptake, the ε for ⁰¹⁸O can also be calculated using the same equation, substituting δ¹⁸O for δ¹⁵N.

5.2.6 Surface Area Measurements

Coral surface area was determined by modeling individual coral branches as cylinders. The height and diameter of each branch was measured with a digital micrometer and surface area calculations were carried out. For the measurements of symbiont density, in order to provide more precise surface area measurements for small fragments, surface area was determined with a white light 3D scanner (HDI Advance R2, 3D3 Solutions, see methods of (Enochs et al. 2014)).
5.2.7 Symbiont Density Measurements

Measurements of symbiont density were achieved through manual counts with a Neubauer Bright-Line Hemacytometer. Coral tissues were removed from subsamples of approximately 1 cm branches from each treatment. Complete tissue removal was ensured by dissolving the coral skeleton in 18% HCl. There was no tissue or symbiont loss as a result of the HCl treatment and all tissue remained intact in one piece after dissolution. The tissue mass was homogenized with a Tissue Master Automated Homogenizer in a solution of 1.5 mL 2% buffered glutaraldehyde in seawater and 0.25 mL of Lugols solution (20g KI, 10g I$_2$ in 200 mL deionized water). Six to ten replicate aliquots were counted for each sample. Counts were normalized to surface area.

5.2.8 Calcification Measurements

5.2.8.1 Buoyant Weight Technique

The buoyant weight technique was utilized to obtain calcification rates (Davies 1989). It was ensured that salinity and temperature of seawater were recorded in order to determine seawater density and that density was the same for each measurement obtained throughout an experimental period. Measurements were carried out in a temperature controlled room to provide consistency. In addition, a standard mass was repeatedly measured between every three coral buoyant weight measurements during buoyant weighing. When calculating calcification rates, the coral skeletal density must be accounted for to correct buoyant weight. A literature density values of 2.781 for *Pocillopora sp.* (Davies 1989) was utilized to convert to mg and then micromoles of CaCO$_3$ precipitated according to the following Equation 4:
\[ Actual \ Weight = \frac{Wet \ Weight}{\text{SW \ density}} \times \frac{1}{\text{Skeletal \ density}} \] (4)

The total mass changes were then converted into hourly rates as \( \mu \)moles \( \text{CaCO}_3 \) \( \text{cm}^{-2} \) \( \text{hr}^{-1} \) in order to compare to rates obtained from the alkalinity anomaly technique.

### 5.2.8.2 Alkalinity Anomaly Technique

The alkalinity anomaly technique is based on the theory that the change in total alkalinity of seawater accompanies the precipitation of \( \text{CaCO}_3 \) (Smith and Key 1975): for every mole of \( \text{CaCO}_3 \) which is precipitated, the total alkalinity is reduced by two molar equivalents. In considering this method for measuring the calcification rate in corals, it is useful because neither the drawdown of \( \text{CO}_2 \) during algal photosynthesis or the addition of \( \text{CO}_2 \) during algal or coral respiration influence the total alkalinity of the seawater. For the various experiments, short term incubations (1.3 – 6.5 hours) were carried out in light only and dark only conditions in order to calculate rates for light and dark calcification separately. The calcification rates were calculated in \( \mu \)moles \( \text{CaCO}_3 \) \( \text{cm}^{-2} \) \( \text{hr}^{-1} \), according to Equation 5:

\[
\text{Calcification rate (}\mu\text{moles CaCO}_3 /\text{cm}^2 \text{hr}) = \frac{0.5(\text{TA}_0 - \text{TA}_f) \times V}{\text{SA} \times t} \] (5)

Where, \( \text{TA}_0 \) and \( \text{TA}_f \) are the initial and final measurements of total alkalinity (mM), \( V \) is the volume (l) of the incubation chamber, \( \text{SA} \) is the total surface area of the corals in the incubation chamber (cm\(^2\)), and \( t \) is time (hrs).

### 5.2.9 Estimating Photosynthesis and Respiration

The alkalinity and pH of seawater measured at the start and end of closed short term incubations were utilized to calculate initial and final total dissolved inorganic carbon concentrations, \([\text{DIC}]_o\) and \([\text{DIC}]_f\). These calculations were carried out with use of the equations and tables for calculating seawater carbon chemistry parameters.
provided by Strickland (1972). The total change in [DIC] during each incubation was calculated. The change in [DIC] which was attributed to result from calcification (determined by the alkalinity anomaly technique for the same incubation samples) was subtracted from the total [DIC] change in order to obtain the change in [DIC] which resulted from the net contributions of algal photosynthetic fixation (removal of DIC) and coral and algal respiration (additions of DIC).

5.2.10 Isotopic Analyses – Stable Isotope Laboratory RSMAS

5.2.10.1 δ¹³C and δ¹⁵N of Coral and Algal Tissues

Coral tissue and algal symbionts were separated for δ¹³C and δ¹⁵N isotopic analysis and determination of CN ratios. The method for separation is a modified version of the series of centrifugation and rinsing with the additional step of filtration twice through 20 micron nitex mesh in order to eliminate contamination of the symbiont fraction by coral nematocysts (Piniak et al. 2003). Coral and algal symbiont tissues were dried at 40 °C for 48 hours and prepared in tin capsules for organic carbon and nitrogen stable isotope analysis. Samples were analyzed on an Elemental Analyzer (Costech) interfaced to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific). Replicate analyses of standards and samples resulted in standard deviations for δ¹³C and δ¹⁵N of <0.05 and <0.1 ‰.

5.2.10.2 Inorganic δ¹³C and δ¹⁸O of Carbonate

Skeletal carbonate δ¹³C and δ¹⁸O was analyzed on Finnigan Delta plus with an attached Kiel device. Delta values are reported in reference to Vienna Pee Dee Belemnite (V-PDB). Standard deviation for replicate samples and standards was <0.1 ‰.
5.2.10.3 $\delta^{13}$C of Dissolved Inorganic Carbon

Water samples for $\delta^{13}$C analysis of DIC were filtered and preserved with mercuric chloride prior to analysis. Samples were analyzed on a Gas Bench interfaced to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific). Average standard deviation for replicate analyses was 0.05 ‰.

5.2.11 Calculation of Skeletal Enrichment

To compare the relative enrichment in $\delta^{13}$C of the skeletal carbonate that may result from increases in productivity between treatments, an enrichment factor was calculated to normalize for slight changes in total coral surface area within each tank. The enrichment from the minimum skeletal $\delta^{13}$C value was weighted to surface area, to calculate the “skeletal enrichment factor”. The weighted surface area was equal to the total surface area of coral in the tank from which the sample was taken divided by the average of total coral surface area from all tanks (equation 6).

\[
\text{Skeletal Enrichment} = (\delta^{13}\text{C}_{\text{skeletal measured}} - \delta^{13}\text{C}_{\text{skeletal minimum}}) \times \frac{\text{Tank Surface Area}}{\text{Average Surface Area}}
\]  

5.2.12 Quantifying Carbon Cycling in Coral-Algal Symbiosis

To assess the relative amounts of photosynthetic carbon which are allocated to algal symbiont and coral host, an isotope tracer method was employed. At the end of each experimental period (i.e. after 4 weeks of nutrient enrichment), corals were incubated in labeled seawater spiked with NaH$^{13}$CO$_3$ at ~1000 ‰ for 24 hours. NH$_4$ or NO$_3$ was added at the appropriate treatment concentrations. The corals were rinsed in filtered seawater immediately following label incubations and coral host tissue was separated from algal symbionts and analyzed for isotopic composition (See 2.10.1). The amount of photosynthetic carbon allocated to coral host and retained in algal symbionts was
calculated utilizing the isotopic composition of tissues prior to labeling, the isotopic composition of the DIC and the resulting isotopic composition of the tissues after labeling to solve a mass balance equation. The amounts of photosynthetically derived carbon composing the tissues after 24 hours were calculated for coral and algal tissues.

5.2.13 Statistical Analyses

JMP 11.0 Statistical Analysis Software was used to perform statistical analysis. Data which did not satisfy the Shapiro-Wilk Goodness of Fit test for normality were analyzed with non-parametric methods (Kruskal Wallis and Wilcoxin tests). Normally distributed data were analyzed with t-tests or one-way ANOVA and Tukey HSD post hoc methods. When normally distributed data were determined to have unequal variances, a t-test for unequal variances or a Welch’s ANOVA and a Dunnet post hoc test were used. When significant differences were detected in any parameter between corals hosting clade C and D symbionts an ANOVA or t-test was used to test for any differences arising from coral host strain.

5.3 Results

5.3.1 NO$_3^-$ Uptake by _Pocillopora damicornis_ Hosting Clade C and D Symbionts

The uptake of NO$_3^-$ by clade C and clade D symbionts in 20 μM NO$_3^-$ is shown in Figure 5.3.1a. There were no significant differences in the uptake over the 24 hour period between corals hosting clade C and clade D symbionts in Control or 20 μM NO$_3^-$ treatments (t-test, p>0.05). The comparison of uptake rates during a light and dark period is shown in Figure 5.3.1b for corals hosting clade C or clade D symbionts in 20 μM NO$_3^-$.
Figure 5.3.1a The average amount of $\text{NO}_3^-$ uptake relative to coral surface area throughout the duration of the light/dark cycle incubation in corals hosting clade C or clade D symbionts in Control or 20 $\mu$M $\text{NO}_3^-$ treatment tanks. The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 5.3.1b The comparison of uptake rates of NO$_3^-$ during a light (blue) and dark (red) 12 hour period for corals hosting clade C or clade D symbionts under 20 µM NO$_3^-$ treatment conditions. Dark measurements were collected during hours 12-24 of the 48 hour light/dark incubation. Light measurements were collected during hours 12-24 of the 48 hour light only incubation. Error bars represent one standard deviation from the mean.
While the dark uptake is similar for both, the dark uptake is equivalent to 25% of light uptake in clade C corals and more than 50% of light uptake in clade D corals.

The initial uptake of 20 µM NO₃⁻ at the initiation of the four week experimental period and the final uptake of NO₃⁻ are compared in Figure 5.3.1c. Uptake from initial to final significantly increased for both clade C and clade D corals (t-test, p<0.05) and both more than doubled their uptake of NO₃⁻ after four weeks in elevated NO₃⁻ conditions. When the data was normalized to symbiont cell density (Figure 5.3.1d), the final uptake was still higher than initial uptake for both clades, but was not significantly different (t-test, p>0.05). The uptake rates of NO₃⁻ during the final light and light/dark incubations in Control and 20 µM NO₃⁻ were best fit to a Michaelis Menten kinetics model for both corals hosting clade C and clade D symbionts (AICc_{Michaelis Menten} < AICc_{Linear}). The calculated maximum uptake rate was higher for clade D, at 0.029 ± 0.006 µmoles cm⁻² hr⁻¹, than clade C (0.021 ± 0.002 µmoles cm⁻² hr⁻¹).

5.3.2 NO₃⁻ Uptake Associated ¹⁵ε and ¹⁸ε Fractionation

The ¹⁵N fractionation associated with the uptake of NO₃⁻ by corals hosting clade C and clade D symbionts is given in Figure 5.3.2a and Tables 5.3.1 and 5.3.2, where the slope of the linear relationship between the natural log of the fraction of original NO₃⁻ remaining in the incubation medium (ln f), plotted against the change in the δ¹⁵N of the NO₃⁻ yields the discrimination factor, ¹⁵ε. The discrimination factor for corals in 20 µM NO₃⁻ hosting clade C symbionts was significantly higher in both light only (3.21 ± 0.21) and light/dark (5.37 ± 0.55 ‰) incubations than the discrimination factor for corals
Figure 5.3.1c. The uptake of NO$_3^-$ relative to coral surface area for corals hosting clade C or clade D symbionts in 20 $\mu$M NO$_3^-$ treatments. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 5.3.1d. The uptake of NO$_3^-$ relative to symbiont density for corals hosting clade C or clade D symbionts in 20 μM NO$_3^-$ treatments. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 5.3.2a The relationship between Δδ¹⁵N and ln f for corals hosting clade C or clade D symbionts in Control and 20 μM NO₃ treatments during 48 hour Light Only and 48 hour 12 hour light/12 hour dark cycle incubations. The absolute value of the slope of each line gives the discrimination factor, ε. Data from replicate tanks are pooled for each treatment. Note that data for Controls is the same for Light and Light/Dark incubations and is representative of light uptake as NO₃ was drawn down to concentrations below detection limits for isotopic analysis before the dark period in Control tanks.
Table 5.3.1 From the Light Dark Incubations. A summary of the $^{15}$\(\varepsilon\) values and the $^{16}$\(\varepsilon\):$^{15}$\(\varepsilon\) ratios calculated with the Rayleigh Equation for residual substrate NO$_3$ for corals hosting clade C or clade D symbionts in Control and 20 \(\mu\)M NO$_3$ conditions. The standard error and $R^2$ values for the regression from which each treatment $^{15}$\(\varepsilon\) value (slope) was calculated are given. The standard error and $R^2$ values for the regression of $\Delta^{18}$O and $\Delta^{15}$N are also given.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{15}$(\varepsilon) (%o)</th>
<th>SE</th>
<th>$R^2$</th>
<th>$^{16}$(\varepsilon):$^{15}$(\varepsilon)</th>
<th>SE</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control C</td>
<td>1.96</td>
<td>0.39</td>
<td>0.91</td>
<td>1.51</td>
<td>0.22</td>
<td>0.96</td>
</tr>
<tr>
<td>Control D</td>
<td>2.55</td>
<td>0.05</td>
<td>0.99</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20 (\mu)M NO$_3$ C</td>
<td>5.37</td>
<td>0.55</td>
<td>0.92</td>
<td>1.04</td>
<td>0.05</td>
<td>0.98</td>
</tr>
<tr>
<td>20 (\mu)M NO$_3$ D</td>
<td>1.66</td>
<td>0.35</td>
<td>0.73</td>
<td>1.26</td>
<td>0.17</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Table 5.3.2 From the Light Incubations. A summary of the $^{15}$\(\varepsilon\) values and the $^{16}$\(\varepsilon\):$^{15}$\(\varepsilon\) ratios calculated with the Rayleigh Equation for residual substrate NO$_3$ for corals hosting clade C or clade D symbionts in Control and 20 \(\mu\)M NO$_3$ conditions. The standard error and $R^2$ values for the regression from which each treatment $^{15}$\(\varepsilon\) value (slope) was calculated are given. The standard error and $R^2$ values for the regression of $\Delta^{18}$O and $\Delta^{15}$N are also given.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{15}$(\varepsilon) (%o)</th>
<th>SE</th>
<th>$R^2$</th>
<th>$^{16}$(\varepsilon):$^{15}$(\varepsilon)</th>
<th>SE</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control C</td>
<td>1.96</td>
<td>0.39</td>
<td>0.91</td>
<td>1.51</td>
<td>0.22</td>
<td>0.96</td>
</tr>
<tr>
<td>Control D</td>
<td>2.55</td>
<td>0.05</td>
<td>0.99</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20 (\mu)M NO$_3$ C</td>
<td>3.21</td>
<td>0.21</td>
<td>0.98</td>
<td>1.30</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>20 (\mu)M NO$_3$ D</td>
<td>1.62</td>
<td>0.07</td>
<td>0.99</td>
<td>1.72</td>
<td>0.13</td>
<td>0.97</td>
</tr>
</tbody>
</table>
hosting clade D symbionts (light: 1.62 ± 0.07; light/dark: 1.66 ± 0.35) (ANCOVA light: F = 67.8582, p<0.0001; light/dark: F = 33.8183, p<0.0001). In Controls, however, the discrimination factor for clade D corals was not significantly different.

The relationship between the enrichment in δ¹⁸O and δ¹⁵N throughout the incubation is represented in Figure 5.3.2b. In the light only incubation, the Δδ¹⁸O:Δδ¹⁵N ratio was significantly greater than 1 for corals hosting both clade C and clade D symbionts in 20 µM NO₃⁻ treatments (ANCOVA, p<0.05). While not significantly different than the 1:1 ratio, in the light/dark incubation, the Δδ¹⁸O:Δδ¹⁵N ratio for corals hosting clade D symbionts was greater (1.26 ± 0.17) than for corals hosting clade C symbionts (1.04 ± 0.05). When comparing the ¹⁵ε measured for corals hosting clade D symbionts to the previously established linear relationship for Pocillopora damicornis hosting clade C symbionts (Chapter Two) between concentration of NO₃⁻ and discrimination factor (Figure 5.3.2c), the data do not fall within the relationship, but have a much lower ¹⁵ε than would be predicted by the model.

5.3.3 NH₄⁺ Uptake by Pocillopora damicornis Hosting Clade C and D Symbionts

The uptake of NH₄⁺ over 48 hours was very similar for clade C and clade D corals with an average of 1.00 µmoles cm⁻² after 48 hours (Figure 5.3.3a). There were no significant differences between the uptake rates of NH₄⁺ during a light and a dark period for both corals hosting clade C and clade D symbionts (t-test, p<0.05) (Figure 5.3.3b). The initial uptake measured at the initiation of the four week experimental period was very similar to the final uptake after four weeks for both clade C and clade D corals in 20 µM NH₄⁺ (Figure 5.3.3c). When the data were normalized to symbiont density, however, the initial uptake for corals hosting clade C symbionts was higher than the final uptake,
Figure 5.3.2b The relationship between the change in δ¹⁵N and δ¹⁸O of NO₃⁻ during NO₃⁻ incubations in Light Only and Light/Dark incubations for corals hosting clade C or clade D symbionts in Control and 20 μM NO₃⁻ treatments. Data is compared to lines representing 1:1 and 2:1 relationships. A 1:1 relationship is accepted to describe nitrate reductase activity, while deviation from this relationship may indicate some other process is acting to influence the isotopic fractionations expressed. Membrane transport has been proposed to lead to a 2:1 relationship.
Figure 5.3.2c The previously established linear relationship between the $^{15}$C measured by analysis of the residual NO$_3^-$ substrate and the initial NO$_3^-$ concentration in the incubation tanks for corals hosting clade C symbionts in variable high NO$_3^-$ conditions. The linear relationship is significant. The data point in red shows where the clade D 20 μM NO$_3^-$ $^{15}$C does not fall within this relationship.
Figure 5.3.3a The average amount of NH$_4^+$ uptake relative to coral surface area throughout the duration of the light/dark cycle incubation in corals hosting clade C or clade D symbionts in Control or 20 μM NH$_3$ treatment tanks. The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 5.3.3b The comparison of uptake rates of $\text{NH}_4^+$ during a light (blue) and dark (red) 12 hour period for corals hosting clade C or clade D symbionts under 20 $\mu$M $\text{NH}_4^+$ treatment conditions. Dark measurements were collected during hours 12-24 of the 48 hour light/dark incubation. Light measurements were collected during hours 12-24 of the 48 hour light only incubation. Error bars represent one standard deviation from the mean.
Figure 5.3.3c The uptake of NH$_4^+$ relative to coral surface area for corals hosting clade C or clade D symbionts in 20 μM NH$_4$ treatments. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
and there were no differences in uptake per symbiont cell from initial to final for corals hosting clade D symbionts (Figure 5.3.3d). The uptake rates of $\text{NH}_4^+$ during the final light and light dark incubations in Control and 20 $\mu$M $\text{NH}_4^+$ were best fit to a Michaelis Menten kinetics model for both corals hosting clade C and clade D symbionts ($\text{AICc}_{\text{Michaelis Menten}} < \text{AICc}_{\text{Linear}}$). The maximum uptake rate was higher for clade C, at $0.080 \pm 0.040 \mu$moles cm$^{-2}$ hr$^{-1}$, than clade D ($0.057 \pm 0.016 \mu$moles cm$^{-2}$ hr$^{-1}$).

### 5.3.4 $\text{NH}_4^+\text{^{15}N}$ Fractionation Pocillopora damicornis Hosting Clade C and D Symbionts

The relationship between $\delta^{15}$N of the residual $\text{NH}_4^+$ substrate during uptake relative to the log of the fraction of original $\text{NH}_4^+$ remaining in the tank is shown in Figure 5.3.4a for corals hosting clade C or clade D symbionts in 20 $\mu$M $\text{NH}_4^+$ treatments. The data are pooled from replicate tanks from the light and light/dark incubations. A quadratic model provided the best fit relationship for the data. The discrimination factor can be calculated by taking the first derivative for any given $f$ value throughout the incubation. To represent the average daily discrimination, data from the first 24 hours of the light/dark incubation were fit to a linear regression model in Figure 5.3.4b. The average daily discrimination factor, $\epsilon$, is given as the absolute value of the slope of each regression. For corals hosting clade C symbionts in 20 $\mu$M $\text{NH}_4^+$, the $\epsilon$ of uptake was significantly smaller ($17.55 \pm 1.25$ ‰) than for corals hosting clade D symbionts ($24.35 \pm 2.58$ ‰) (ANCOVA, $p = 0.026$). The relationship between the measured discrimination factor and the final concentration of $\text{NH}_4^+$ in the tank for which the discrimination factor was calculated ($[\text{NH}_4^+]_\epsilon$) was fit to a linear model for both corals hosting clade C and clade D symbionts, and provided a better fit for corals hosting clade C symbionts (clade C: $R^2 = 0.91$; clade D: $R^2 = 0.22$) (Figure 5.3.4c).
Figure 5.3.3d. The uptake of NH$_4^+$ relative to symbiont cell density for corals hosting clade C or clade D symbionts in 20 μM NH$_4^+$ treatments. Comparisons are made between initial measurements made at the beginning of the 4 week treatment period (red) and final measurements made after 4 weeks (blue). The shaded areas represent the dark periods. Each error bar is constructed using 1 standard deviation from the mean.
Figure 5.3.4a Data shown are from the NH₄⁺ incubations pooled for replicate tanks in light and light/dark cycle conditions for corals hosting clade C (blue) or clade D (red) symbionts in 20 μM NH₄⁺ treatments. The relationship between the change in δ¹⁵N of NH₄⁺ and the natural log of the fraction of original NH₄⁺ remaining in the incubation medium, f, is shown. The data were best fit to a quadratic relationship.
Figure S3.4b Data shown are from the NH$_4^+$ incubations pooled for replicate tanks for the first 24 hours of the light/dark cycle incubations for corals hosting clade C (blue) or clade D (red) symbionts in 20 μM NH$_4^+$ treatments. The relationship between the change in δ$^{15}$N of NH$_4^+$ and the natural log of the fraction of original NH$_4^+$ remaining in the incubation medium, f, is shown. The absolute value of the slope of the linear relationship represents the average δ$^{15}$N for the 24 hour period.
Figure 5.3.4c Data shown are from the 48 hour incubation under both light only and light/dark cycle conditions for corals hosting clade C (blue) or clade D (red) symbionts in 20 \( \mu M \) \( NH_4^+ \) treatments. The relationship between \( ^{15}E \) and the final concentration of \( NH_4^+ \) for which \( ^{15}E \) was calculated is shown (\([NH_4^+]E\)). The data are from two replicate tanks for each treatment. The data are fit separately for clade C and D to a linear regression.
5.3.5 Symbiont Cell Density *Pocillopora damicornis* Hosting Clade C and D

**Symbionts**

The algal symbiont cell densities for the clade C and clade D *Pocillopora damicornis* grown in Control, 20 µM NO$_3^-$ and 20 µM NH$_4^+$ are shown in Figure 5.3.5. The average densities for clade C were $0.89 \pm 0.18 \times 10^6$ cells cm$^{-2}$ for Control, $1.37 \pm 0.29 \times 10^6$ cells cm$^{-2}$ for 20 NO$_3^-$ and $1.30 \pm 0.50 \times 10^6$ cells cm$^{-2}$ for 20 NH$_4^+$. The average densities for clade D were $1.20 \pm 0.26 \times 10^6$ cells cm$^{-2}$ for Controls, $1.58 \pm 0.47 \times 10^6$ cells cm$^{-2}$ for 20 NO$_3^-$ and $1.70 \pm 0.49 \times 10^6$ cells cm$^{-2}$ for 20 NH$_4^+$. The average densities for clade D were higher overall than for clade C (t-test, p<0.05). For clade C, the cell densities in 20 µM NO$_3^-$ and 20 µM NH$_4^+$ were significantly higher than Control corals (Tukey-Kramer method, p = 0.029, p = 0.0473).

5.3.6 Indicators of Increased Photosynthetic Activity

The carbon isotopic signature of coral tissues, algal symbiont tissues, coral skeletal material and DIC of seawater are summarized in Figure 5.3.6a. The clade C algal symbiont tissues ranged from -22.93 to -22.28 ‰, while the clade D symbionts ranged from -21.70 to -21.35 ‰. The clade D symbionts were significantly more enriched in $^{13}$C than clade C symbionts under elevated NO$_3^-$ or NH$_4^+$ conditions (t-test, p<0.05), and while the average $\delta^{13}$C of clade D symbionts under Control conditions (-21.70 ‰) was more enriched than the $\delta^{13}$C of clade C symbionts (-22.28 ‰), the difference was not significant at the p<0.05 level (t-test, p=0.0786). There was no significant effect detected of treatment on the $\delta^{13}$C of clade C algal symbionts (ANOVA, F(2,8) = 3.82, p = 0.0686)) or clade D symbionts (ANOVA, F(2,8)=1.19, p = 0.3519).
Figure 5.3.5 Data are from the November 2012 *Pocillopora damicornis* experiment in which corals hosting clade C or clade D symbionts were exposed to elevated nutrient conditions. Algal symbiont cell density is normalized to coral skeleton surface area after 4 weeks in Control, 20 μM NO$_3^-$, and 20 μM NH$_4^+$ treatments. There was no interaction between clade and symbiont density (2 Way ANOVA, $F = 0.16$, $p=0.85$), but average clade D densities were higher than clade C (t-test, $p<0.05$). Error bars represent 1 standard deviation from the mean.
Figure 5.3.6a From the November 2012 experiment with *Pocillopora damicornis* hosting clade C or clade D symbionts. The carbon isotopic signatures of daily initial seawater DIC (blue), new skeletal growth (red), algal symbiont tissues (green) and coral tissues (orange) after the 4 week experimental period.
The coral tissues of clade C hosting corals ranged from -22.38 to -22.19 ‰, while the coral tissues of clade D hosting corals ranged from -21.52 to -21.18 ‰. There were no significant effects of treatment condition on δ^{13}C of coral tissues from corals hosting clade C symbionts (ANOVA, F(2,9) = 0.42, p=0.6667) or corals hosting clade D symbionts (ANOVA, F(2,9)=1.06, p=0.3865). In Controls and both treatment conditions, the δ^{13}C of clade D coral tissues were significantly more enriched in ^{13}C than the tissues of corals hosting clade C symbionts (t-test, p<0.05).

The enrichment in δ^{13}C relative to the lowest δ^{13}C value obtained for all coral skeleton samples is represented in Figure 5.3.6b. The enrichment was normalized to the total coral surface area in each tank in order to correct for the slight variations in coral biomass between tanks. There were no effects of treatment on the skeletal enrichment for both clade C hosting corals (ANOVA, F(2,8)=0.86, p =0.459) or clade D hosting corals (ANOVA, F(2,7)=2.01, p=0.2045), however for corals hosting clade C symbionts, it was determined that this enrichment was significantly higher when NH_4^+ was increased to 50 µM (See Chapter Four – Figure 4.3.2.1b ). For corals in the Control treatments, the skeletal enrichment was significantly higher in corals hosting clade D symbionts than for corals hosting clade C symbionts (t-test, p<0.05).

The DIC drawdown rate is shown for the short term light incubations for corals hosting clade C or D symbionts in Control, 20 µM NO_3^- and 20 µM NH_4^+ treatments in Figure 5.3.6c. The rates of drawdown for clade C and clade D Controls were similar at ~0.80 µmoles cm^{-2} hr^{-1}, and were lowest in 20 µM NH_4^+ treatments for both clade C and clade D corals. While 52.7% and 46.2% of DIC drawdown for clade C and clade D
Figure 5.3.6b Data from the November 2012 experiment with *Pocillopora damicornis* hosting clade C or clade D symbionts. Data represents the amount of carbon isotopic enrichment in the new skeletal material from the minimum skeletal δ¹³C value obtained from all samples. Enrichment is normalized to the total coral surface area for the tank in which the corals were grown for 4 weeks. Error bars represent one standard deviation from the mean.
Figure 5.3.6c The rate of drawdown of the total DIC during a closed light incubation for the November 2012 *Pocillopora damicornis* experiment with corals hosting clade C or clade D symbionts under various conditions of elevated 20 μM NO₃⁻ or 20 μM NH₄⁺. The pie charts display the percentage of contribution to the total DIC change from the processes of calcification (orange) and the net effect of photosynthesis and respiration (green). Error bars represent one standard error.
Controls, respectively, was attributed to calcification, in 20 µM NH₄⁺ treatments only
34% and 40.4% of DIC drawdown for clade C and clade D corals, respectively, was
attributed to calcification.

The $\delta^{13}C_{[DIC]}$ from initial and final samples collected during the short term light
incubations from the clade C and clade D corals in Control, 20 µM NO₃⁻ and 20 µM NH₄⁺
experiments are represented in Figure 5.3.6d. In all tanks, the final $\delta^{13}C$ value measured
is more enriched that the initial samples indicating that in these treatments, the combined
processes of photosynthesis, respiration and calcification have the cumulative effect of a
positive isotopic fractionation, selecting for the light isotope of carbon ($^{12}C$).

5.3.7 Carbon Cycling between Algal Symbiont and Coral Tissue

The percent of new photosynthetically derived carbon within coral tissues and
algal symbionts after 24 hours is shown for corals hosting clade D symbionts in Figure
5.3.7a. The data in Figure 5.3.7a were collected after 4 weeks of elevated NO₃⁻ or NH₄⁺
exposure and are representative of the post-treatment rates of photosynthetic carbon
accumulation within each fraction (coral or symbiont). There was a significant effect of
treatment on both the percentage of carbon accumulating within the algal symbionts
(Welch’s ANOVA F(2,21)=18.14, p=0.0004) and within the coral tissues (Welch’s
ANOVA, p(2,21)=26.60, p<0.0001). Algal symbionts in the 20 µM NH₄⁺ treatment had
higher accumulations of new carbon than both the symbionts grown in 20 µM NO₃⁻ and
Control conditions (Dunnet’s test, p<0.05). The coral tissues in 20 µM NH₄⁺ had higher
accumulation of new photosynthetically derived carbon than 20 µM NO₃⁻ and Controls
(Dunnet’s test, p<0.05).
Figure 5.3.6d The raw data for the average $\delta^{13}C_{\text{DIC}}$ of the initial sample ($T_0$) and final sample ($T_f$) after a closed two hour light incubation from the November 2012 *Porites damicornis* with experiment with corals hosting clade C or clade D symbionts under 20 $\mu$M NO$_3^-$ or NH$_4^+$. Error bars represent one standard error.
Figure S3.7a Data from *Pocillopora damicornis* hosting clade C algal symbionts after four weeks of 20 μM NO₃⁻ or 20 μM NH₄⁺ treatments, showing percent new photosynthetically derived carbon composing algal symbiont tissues (green) and coral tissues (orange) after the 24 hour H¹³CO₂ labeling incubation. Percent new carbon was calculated according to isotope mass balance equations. Error bars represent one standard error.

Figure S3.7b Data from *Pocillopora damicornis* hosting clade D algal symbionts after four weeks of 20 μM NO₃⁻ or 20 μM NH₄⁺ treatments, showing percent new photosynthetically derived carbon composing algal symbiont tissues (green) and coral tissues (orange) after the 24 hour H¹³CO₂ labeling incubation. Percent new carbon was calculated according to isotope mass balance equations. Error bars represent one standard error.
For comparison, the percentage of new photosynthetically derived carbon within coral tissues and algal symbionts after 24 hours is shown for corals hosting clade C symbionts in Figure 5.3.7b. The data in Figure 5.3.7b were also collected after 4 weeks of elevated NO$_3^-$ or NH$_4^+$ exposure and are representative of the post-treatment rates of photosynthetic carbon accumulation within each fraction (coral or symbiont). There was a significant effect of treatment on both the percentage of carbon accumulating within the algal symbionts (Welch’s ANOVA F(2,17)=3.7768, p=0.0439) and within the coral tissues (ANOVA, p(2,19)=9.50, p<0.0014). There were no differences between carbon accumulated in the Control symbionts and the 20 µM NH$_4^+$ algal symbionts, however, the percentage of new carbon in algal symbionts was significantly lower in 20 µM NO$_3^-$ than Controls (Dunnet’s test, p<0.05). The percentage of new carbon in coral tissues was significantly higher in 20 µM NH$_4^+$ treatment than in Controls (Tukey HSD, p<0.05).

**5.3.8 Growth Rates**

The calcification rates obtained from the buoyant weight technique are shown in Figure 5.3.8a. While the average calcification rates for Controls were higher than those obtained for 20 µM NO$_3^-$ or 20 µM NH$_4^+$, at p<0.05 there was not a treatment effect on calcification rates for corals hosting clade C symbionts (Kruskal Wallis, p=0.0624) or clade D symbionts (Kruskal Wallis, p=0.65). In Controls and both treatments, the calcification rates for corals hosting clade C symbionts were significantly higher than the calcification rates for corals hosting clade D symbionts (Wilcoxon tests, p<0.05).

The light and dark calcification rates obtained from the alkalinity anomaly technique are shown in Figure 5.3.8b for corals hosting clade C symbionts (blue) and corals hosting clade D symbionts (red). There were no significant treatment effects on
Figure 5.3.8a The average calcification rates as measured by the buoyant weight technique for the November 2012 experiment for *Pocillopora damicornis* hosting clade C or clade D symbionts exposed to Control, 20 μM NO$_3^-$ or 20 μM NH$_4^+$ conditions for 4 weeks. Error bars represent one standard deviation from the mean. There were no significant differences among treatments for both clade C (ANOVA, F(2,14) = 2.7298, p = 0.0998) and clade D (ANOVA, F(2,20) = 0.44, p = 0.6495), however calcification rates for corals hosting clade C symbionts were significantly higher than those hosting clade D in Controls and both treatment conditions (t-test, p<0.05).
Figure 5.3.8b The calcification rates as measured by the alkalinity anomaly technique after the November 2012 experiment for *Pocillopora damicornis* hosting clade C or clade D symbionts exposed to Control, 20 μM NO$_3^-$ or 20 μM NH$_4^+$ conditions for 4 weeks. Error bars represent one standard deviation from the mean. There were no significant differences in calcification rate among treatments for clade C in both light (ANOVA, F(2,7) = 0.1641, p = 0.8518) and dark incubations (ANOVA, F(2,9) = 0.0635, p = 0.9389), however there was a significant treatment effect in light calcification rates for corals hosting clade D symbionts (ANOVA, F(2,9) = 4.7676, p=0.0387). The light calcification rate in Controls hosting clade D was significantly higher than the light calcification rate in corals hosting clade D in the 20 μM NH$_4^+$ treatment.
dark calcification rates for both clade C hosting (ANOVA F(2,9)=0.06, p=0.9389) and clade D hosting corals (ANOVA, F(2,9)= 3.49, p=0.0754). There was, however, a significant effect of treatment measured on light calcification rates in corals hosting clade D symbionts (ANOVA, F(2,9)=4.77, p=0.0387), with the Control calcification rates being significantly higher than for corals in the 20 µM NH₄⁺ treatment (Tukey-Kramer method, p=0.0331). The average of light and dark rates was calculated in order to give an average hourly rate in Figure 5.3.8c. There was a significant treatment effect on the hourly average calcification rate in corals hosting clade D symbionts (ANOVA, F(2,9)=13.47, p=0.002), with Controls having higher average calcification rates than the corals in the 20 µM NO₃⁻ (p=0.0022) and 20 µM NH₄⁺ (p=0.0087) treatments (Tukey HSD).

5.4 Discussion

5.4.1 Uptake and Isotopic Fractionation of Dissolved Inorganic Nitrogen

The total uptake of NO₃⁻ was similar for corals hosting clade C and D symbionts, with both significantly increasing the uptake after four weeks of exposure to 20 µM NO₃⁻. Both clade C and clade D uptake rates were best fit to Michaelis Menten saturation kinetics, suggesting that the maximum uptake rate occurs at saturation of either active membrane transporters or at the nitrate reductase enzyme in the algal symbiont. The maximum uptake rate calculated by the Michaelis Menten kinetics model was higher for clade D than for clade C corals.

The fractionation effect measured for both corals hosting clade C and clade D symbionts under Control conditions (~1.5 µM NO₃⁻) was small (C: 1.96 ‰, D: 2.55 ‰) and was likely related to the initial step of transport across the algal cell membrane (See
Figure 5.3.8c The average of light and dark hourly calcification rates as measured by the alkalinity anomaly technique for the November 2012 experiment for *Pocillopora damicornis* hosting clade C or clade D symbionts exposed to Control, 20 μM NO$_3^-$ or 20 μM NH$_4^+$ conditions for 4 weeks. Error bars represent one standard deviation from the mean. There were no significant differences among treatments for clade C (ANOVA, F(2,9) = 0.0991, p = 0.9066). There was a significant treatment effect for clade D (ANOVA, F(2,9) = 13.4725, p = 0.002), and Controls had higher average calcification rates than both 20 μM NO$_3^-$ or 20 μM NH$_4^+$ (Tukey-Kramer method, p<0.05). Overall calcification rates for corals hosting clade C symbionts were significantly higher than for corals hosting clade D (t-test, p<0.05).
Chapter Two Discussion for NO$_3^-$ Isotope Fractionation Background). The fractionation effect was very different, however, between corals hosting clade C and clade D symbionts in 20 µM NO$_3^-$. The fractionation effect expressed by corals with clade D symbionts was again very small (1.66 ‰) and indicative of influence from the initial membrane transport step. The much larger fractionation effect expressed in corals hosting clade C symbionts (5.37 ‰), likely indicated influence from the large fractionating step of nitrate reductase activity, suggesting significant amounts of algal intracellular NO$_3^-$ was effluxed from algal cells into coral tissues and seawater. The small fractionation measured for clade D corals, on the other hand, indicates that most or all of the NO$_3^-$ taken up is retained and assimilated by the algal symbionts and very little efflux of algal intracellular NO$_3^-$ occurs. This idea is also supported by the different $\Delta \delta^{18}O: \Delta \delta^{15}N$ ratios measured for each clade, with the ratio measured in clade C corals falling closer to 1:1, while in clade D corals this ratio measured during the light incubation was 1.7:1 suggesting a stronger fractionation effect on $^{18}O$ than $^{15}N$ in the corals hosting clade D symbionts.

The finding that clade D symbionts may be more efficient in the utilization of NO$_3^-$ is contrasting to the conclusions made in a study of $^{15}N$ label uptake by Acropora tenuis hosting clade C and D symbionts (Baker et al. 2013). This study suggested that clade C symbionts were able to assimilate 22% more nitrogen than clade D symbionts and suggested clade C symbionts were more efficient in their NO$_3^-$ utilization. Our study, however, shows that a significantly higher amount of efflux occurs during uptake by clade C symbionts than clade D suggesting that clade C may not be as efficient in
depleting intracellular nitrate, allowing for efflux into the water and expression of the large isotopic fractionation effect.

The total uptake of $\text{NH}_4^+$ was similar for corals hosting clade D and clade C symbionts, however there were differences in the fractionation expressed under 20 $\mu$M $\text{NH}_4^+$. Corals hosting clade C symbionts had a fractionation effect of 17.55 ‰, while the fractionation expressed by clade D symbionts was significantly higher at 24.35 ‰. While interpretations of the $^{15}\text{N}$ fractionation associated with $\text{NH}_4^+$ uptake are quite complex (See Chapter Three Discussion), there appears to be variability in the rate limiting (fractionating) step of $\text{NH}_4^+$ uptake and assimilation for the two clades. The large fractionation (~39 ‰) associated with uptake is thought to be associated with the combined fractionation effects of deprotonation of $\text{NH}_4^+$ to $\text{NH}_3 + \text{H}^+$ and the diffusion across the cell membrane. This fractionation will only be measured when the intracellular and extracellular $\text{NH}_4^+$ pools do not reach isotopic equilibrium or when diffusion rates are slow. Influence from both active transport effects (~14.1 ‰) and the fractionating effects of assimilation by glutamine synthetase (GS) and glutamate dehydrogenase (GDH) would contribute smaller fractionation effects to the total observed fractionation.

5.4.2 Variations in Photosynthetic Activity

There were several indications that the *Pocillopora damicornis* hosting clade D corals were more photosynthetically active than those hosting clade C symbionts. The corals hosting clade D symbionts had overall higher background symbiont densities. The clade D corals also showed carbon isotope $^{13}\text{C}$ enrichments in their tissues (both coral and algal) and skeletal material, which is typically indicative of higher photosynthetic activity and the presence of some level of carbon limitation. While the $^{13}\text{C}$ labeling
incubation suggested that under Control conditions, the clade C symbionts accumulated a higher percentage of new photosynthetically fixed carbon in 24 hours than clade D symbionts, exposure to 20 µM NH₄⁺ altered this relationship. After four weeks of exposure to elevated NH₄⁺, there was no significant change in the percent of new carbon accumulating in clade C symbionts, but the percent of new carbon accumulating in clade D symbionts significantly increased reaching the same level of new carbon accumulation as clade C Controls. This suggests that exposure to NH₄⁺ may provide a competitive advantage for clade D algal symbionts enabling them to increase either the rate of carbon fixation, or the retention of photosynthetic carbon or a combination of both.

5.4.3 Responses of Coral Growth to Elevated NH₄⁺

While there were no measureable effects of 20 µM NO₃⁻ or 20 µM NH₄⁺ on calcification rates in Pocillopora damicornis hosting clade C symbionts, the alkalinity anomaly technique detected significant declines in the hourly calcification rates for corals hosting clade D symbionts exposed to 20 µM NO₃⁻ and 20 µM NH₄⁺. This measured decline in calcification could indicate that corals hosting clade D symbionts may be more susceptible to nitrogen loading than coral hosting clade C symbionts.

5.5 Conclusions

Recent work suggests that the future of corals may involve a shift in the community of endosymbiotic algae which reside in the gastrodermal tissues of reef building corals. These algal symbionts play a key role in the success of corals, and understanding their response to environmental variability is critical in determining how corals may fare in response to future stressors accompanying climate change. This work suggests that there may be clade specific differences in the utilization of inorganic
nitrogen. The data suggest that clade D symbionts are able to more efficiently drawdown and retain NO$_3^-$ when available at elevated concentrations. The results of this work also suggests variability in the effects of nutrients upon calcification in corals hosting clade C and clade D symbionts. While it is accepted that clade D symbionts provide a degree of thermal tolerance to the coral host, corals hosting these thermally tolerant symbionts may be more susceptible to nutrient loading. Stressors which contribute further to the already lowered calcification rates of corals hosting thermally tolerant symbionts may be detrimental to the persistence of those coral communities which have shifted to host primarily thermally tolerant symbionts.
Conclusions

The symbiotic relationships between Scleractinian corals and unicellular dinoflagellate algae *Symbiodinium* allow for high rates of productivity and calcification in a low nutrient environment. The efficiency of the symbiosis under stable conditions contributes to the ecological success of coral reefs. As global populations increase and coastal areas become more densely populated, threats from anthropogenic nutrient loading to the coastal environment rise. This work contributes to a more clear understanding of the response of coral-algal symbiosis and calcification to elevated levels of DIN. This is critical in directing management to address local stressors, especially in the face of deteriorating climatic conditions of temperature and pCO$_2$. Determining the efficacy of tools used for the identification of nitrogen sources influencing fragile reef habitats is important in ensuring proper management practices and is addressed in this work.

*The Utilization of DIN by Pocillopora damicornis*

Much of the early work on NO$_3$ and NH$_4^+$ uptake by Scleractinian corals was carried out at very low concentrations in order to develop nitrogen budgets and understand how coral nitrogen demands can be met in an oligotrophic environment. This study expanded the range of concentrations to understand the influence of nutrient loading on the uptake and assimilation behavior of the coral-algal symbiosis (Chapter One). The kinetics of NO$_3^-$ uptake are in agreement with previous work on NO$_3^-$ uptake by symbiotic corals that saturation occurs at a maximum rate of uptake (D Elia and Webb 1977, Grover et al. 2003, Badgley et al. 2006). Maximum NO$_3^-$ uptake rates were slightly higher than previous measurements, although species specific effects should be
considered (0.037 µmoles cm\(^{-2}\) hr\(^{-1}\)) \textit{Pocillopora damicornis} this study, compared to 0.027 µmoles cm\(^{-2}\) hr\(^{-1}\) for \textit{Diploria strigosa} (Badgley et al. 2006).

Previous studies have scattered results for the uptake kinetics of NH\(_4^+\) by the coral-algal symbiosis (Miller and Yellowlees 1989a). This is likely due to both a diffusive and active component involved in uptake. The results of this study show that two different dominant modes of uptake dependent on concentration range may explain the previous disparities in NH\(_4^+\) uptake kinetics. At the highest concentrations of NH\(_4^+\), diffusive uptake dominates with increasing concentrations leading to further increases in uptake rate. It is logical to suspect that diffusive uptake will become dominant when extracellular concentrations exceed intracellular tissue NH\(_4^+\) concentrations, thus this study suggests in our high NH\(_4^+\) treated corals, the intracellular NH\(_4^+\) concentration is around 40 µM.

Under such elevated external concentrations of NH\(_4^+\), there is likely an increase in the amount of NH\(_4^+\)/NH\(_3\) accumulating within the coral tissues becoming available to the algal symbionts. The coral may not be able to control the intracellular NH\(_4^+\) concentrations unless it begins actively pumping NH\(_4^+\) out of tissues or directly assimilating NH\(_4^+\) (Grover et al. 2002a). It is possible that the low affinity enzyme, GDH, thought primarily responsible for any direct NH\(_4^+\) assimilation occurring in the coral tissues (Pernice et al. 2012) could become more active under such high concentrations of NH\(_4^+\). While further work should be done to investigate the role of GDH activity in coral tissues at higher NH\(_4^+\) concentrations, it could be a mechanism for either preventing toxic levels of NH\(_4^+\) from accumulating in the coral tissues or as a means to keep NH\(_4^+\) from reaching the algal symbionts.
The increase in uptake of $\text{NO}_3^-$ and $\text{NH}_4^+$ by *Pocillopora damicornis* after long term exposure demonstrates that the mechanisms for uptake and physiological effects of long term DIN may be quite different than the effects previously interpreted from some studies which carried out short term incubation experiments. Previous experiments carried out for only hours finding no negative influence of 100 $\mu$M $\text{NH}_4^+$ on coral calcification may not provide an accurate measure of long term influence (Crossland and Barnes 1974). In addition, more recent studies investigating DIN uptake through use of advanced tools such as NanoSIMS should consider carrying out these experiments on corals which have been previously exposed to elevated nutrients in order to determine the long term effects (Pernice et al. 2012, Kopp et al. 2013). 

The increases in uptake over the duration of the experiments could be attributed to increased algal cell density, however, may involve other changes which have occurred within the coral or algae such as increased cell size, protein content (supported by decreased tissue CN ratios), increased activity of enzymes involved with the transport mechanisms of uptake or increased activity of assimilatory enzymes.

*Physiological Responses to Elevated Nutrient Levels ($\text{NO}_3^-, \text{NH}_4^+, \text{PO}_4^{3-}$)*

The high calcification rates of symbiotic corals as compared to non-symbiotic corals are proposed to be directly related to photosynthetic activity (Gattuso et al. 1999). The link between algal symbionts and increased calcification could be the result of photosynthesis contributing to increased pH favorable for the process of calcification or through a less direct manner such as translocation of biomolecules which support the coral host and the energetically expensive process of calcification. The results of these nutrient enrichment experiments, however, suggest a point at which the increased
photosynthetic levels may reduce calcification. Influences of elevated nutrient levels on symbiont densities of *Pocillopora damicornis* hosting clade C symbionts were apparent at all enrichments of NO$_3^-$ and NH$_4^+$. Effects on calcification rate were only measurable at the highest enrichments of NH$_4^+$ indicating that modest enrichments in NO$_3^-$ and NH$_4^+$ may not have an influence on calcification, at least on the timescale of this experiment. Decreased calcification rates were associated with the highest algal symbiont cell densities and evidence of increased photosynthetic activity through analysis of the DIC drawdown as well as the $^{13}$C isotopic enrichments measured within the coral skeletons. These findings are in line with the theory proposed by Marubini and Davies (1996) that nutrients lead to declines of coral growth through limiting DIC availability for the process of calcification. Reductions in calcification occur when symbionts reach density and productivity levels that scavenge the limited DIC pool for photosynthetic fixation at a rate that out-competes calcification.

The response of *Acropora cervicornis* to NO$_3^-$ was similar to the response measured in the highest NH$_4^+$ enrichments for *Pocillopora damicornis*. Symbiont density increased and calcification decreased in response to 20 µM NO$_3^-$ additions with and without 2 µM PO$_4^{3-}$. This suggests that algal symbionts are N limited, and additions of NO$_3^-$ release N limitation on algal symbionts resulting in increased photosynthetic activity and DIC consumption. It is not clear whether or not the addition of NO$_3^-$ created any PO$_4^{3-}$ limitation, but there were no interactive effects of NO$_3^-$ and PO$_4^{3-}$ detected on symbiont cell density or calcification rates. Total DIC drawdown was not different between treatments, but calcification almost completely ceased in high NO$_3^-$, strongly supporting the DIC limitation theory (Marubini and Davies 1996) in which symbionts are
able to competitively scavenge $\text{HCO}_3^-$ and $\text{CO}_2$ for the process of photosynthesis, inhibiting calcification.

*Synergistic Interactions between Nutrients and CO$_2$*

Both nitrogen enrichment and elevations of CO$_2$ have been shown to stimulate rates of photosynthesis, thus the interaction of the two may have additive effects on photosynthesis and coral calcification. It could be hypothesized that through stimulation of photosynthesis, CO$_2$ would have a positive influence on calcification rates. In the case of elevated pCO$_2$ accompanied by decreases in seawater pH, however, the effects of saturation state on precipitation of skeletal material must also be considered. Previous work on the effects of CO$_2$ on corals have found mixed results, with different species of corals exhibiting positive and negative growth responses (Langdon et al. 2000, Langdon and Atkinson 2005, Huang et al. 2014). Previous work on the synergistic interactions between elevated NH$_4^+$ and CO$_2$ have also found mixed results, some suggesting that the two enrichments will have additive deleterious effects while others suggesting they will ameliorate each other (Renegar and Riegl 2005). While there was determined to be no influence of elevated CO$_2$ alone on coral calcification, this study showed a synergistic deleterious effect of NH$_4^+$ and CO$_2$. This leads us to believe that the deleterious effects of CO$_2$ are related to the proliferation of the algal symbiont population. Under normal conditions (Controls), it is likely that the algal symbionts are nitrogen limited. As the nitrogen limitation is released by the addition of 20 $\mu$M NH$_4^+$, the algae then become DIC limited. In the synergistic study of NH$_4^+$ and CO$_2$, the addition of CO$_2$ releases the DIC limitation allowing algal symbiont populations to further increase. Indeed, the highest measurements of symbiont density were obtained for the combined NH$_4^+$ and CO$_2$.
treatment. The results of this study suggest that the deleterious effects may be directly related to the density of algal symbionts and may not in this case be related to DIC limitation. These findings are in line with the theories proposed by Wooldridge (Wooldridge 2009, 2010, 2012).

Response of Clade C and D Symbionts to NO$_3^-$ and NH$_4^+$

Recent studies have revealed the wide diversity in the dinoflagellate algae which live in symbiosis with corals. Findings that some of these algae display characteristics of thermal tolerance has given hope for the persistence of symbiotic corals exposed to rising sea surface temperatures. Some workers suggest that future ocean warming may lead to a shift in the community of endosymbiotic algae to those which are able to tolerate elevated temperatures (Baker 2003, Rowan 2004, Sampayo et al. 2008, Cunning et al. 2013, Stat et al. 2013). The success of corals is highly dependent on the stability of their relationship with their endosymbiotic algae. Understanding the response of the more thermally tolerant algal symbionts to environmental variability is critical in determining how corals may fare in response to future stressors involved with climate change. This work suggests that clade C and D symbionts differ in their utilization of inorganic nitrogen. The data suggest that clade D symbionts may more efficiently drawdown and retain NO$_3^-$ when unnaturally high concentrations are available in the surrounding seawater. This study also finds that there is variability in the effects of nutrients upon calcification in corals hosting clade C and clade D symbionts. Although clade D symbionts provide a degree of thermal tolerance to the coral host and may persist through periods of elevated temperatures, corals hosting these thermally tolerant symbionts may be more susceptible to nitrogen loading, suffering reduced calcification. As corals hosting the thermally tolerant
symbionts have generally been classified to have lower rates of calcification, stressors which act to further depress calcification (Jones and Berkelmans 2010) may be a major concern for the future success of these corals and the reefs which they inhabit.

*Measurements of* $^{15}\epsilon$ *and Coral and Algal* $\delta^{15}N$

In considering the declines in coral calcification that result from nutrient enrichments, sound methods for assessment of nutrient influence on reef habitats are critical to ensure proper management. The measurements of $^{15}\epsilon$ made in this study reveal not only information regarding the mechanism for uptake of NO$_3^-$ by *Pocillopora damicornis*, but also highlight the variability in $\delta^{15}N$ of coral and symbiont tissues which can result from a single starting source of NO$_3^-$ at varying concentrations. The fractionation of $^{15}N$ associated with the uptake of NO$_3^-$ by *Pocillopora damicornis* was found to increase with increasing NO$_3^-$ concentration in corals hosting clade C symbionts as a result of variable efflux and expression of the nitrate reductase fractionation step. This suggests that if loads of NO$_3^-$ are high, the large fractionation effect may mask the signature of the source NO$_3^-$ within the coral tissues. Under low NO$_3^-$ conditions which are expected for reefs, however, the fractionation effect will be very small and thus very little NO$_3^-$ efflux will occur, resulting in a $\delta^{15}N$ of coral tissues which are similar to those of the source NO$_3^-$ $\delta^{15}N$.

For NH$_4^+$ uptake, on the other hand, it appears that the very large $^{15}N$ fractionation measured in this study (~25 %o) involved with uptake and assimilation is a result of the initial step, the diffusion or transport into tissues. Therefore, it is not required that the intracellular NH$_4^+/NH_3$ is effluxed in order for this large fractionation to be expressed in tissues and seawater. This suggests that even under low concentrations, the
fractionation may be quite large and thus the analysis of tissues is likely not a good indicator of any original $\delta^{15}\text{N} \text{NH}_4^+$ source signature. Further, there was variability in $^{15}\varepsilon$ as concentrations of NH$_4^+$ were drawn down in the tanks likely related to the switch in uptake pathways from diffusion to active transport, complicating the interpretation of the resulting $\delta^{15}\text{N}$ of tissues.

The deleterious effects of DIN on coral calcification and the potential for synergistic interactions which lead to further declines in calcification highlight the need to manage coastal nutrient inputs. This study emphasizes the uncertainties involved with use of coral tissue $\delta^{15}\text{N}$ in assessing nutrient inputs. It is critical to develop more constrained methods for identifying sources of nitrogen loading to delicate coral reef ecosystems in order to ensure proper management practices.
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