



# **The Origin of Nitrogen Isotope Values in Algae**

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A Research Project Conducted by the  
Stable Isotope Laboratory at the:  
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## EXECUTIVE SUMMARY

- The fractionation accompanying assimilation of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  has been measured in two species of macro algae (*Gracilaria sp.* and *Agardhiella sp.*). For *Agardhiella sp.* the values calculated for the assimilation of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  are essentially the same and vary between 1.0045 and 1.008. They show no relationship to nutrient concentration. Data for *Gracilaria* at the low concentrations are equivocal as a result of the difficulty in separating new and old growth. Values obtained at higher concentrations are consistent with the data obtained on *Agardhiella*. These values are significantly higher than reported in the literature.
- As a consequence of these large assimilation factors, significant variations in the stable nitrogen isotopic composition can be produced by uptake of either ammonium or nitrate by algae. As the water becomes progressively lower in DIN, the isotopic composition of the residual DIN become more positive. Such conditions would be expected to most important during the time of year when algae are growing at their maximum rate. There is also expected to be an inverse relationship between the concentration of DIN and the  $\delta^{15}\text{N}$  in the water mass.
- Previous assumptions regarding algal preference in terms of nutrient source (ammonium versus nitrate) may not be universally applicable. Of the two species studied here, only *Agardhiella* demonstrated an obvious preference for ammonium. *Gracilaria* may have a competitive advantage as a generalist in terms of nutrient acquisition. Blanket assumptions about nutrient uptake should be considered with caution as responses are species specific.
- The experiments show that the C:N ratio of the algae is inversely proportional to the concentration of DIN.



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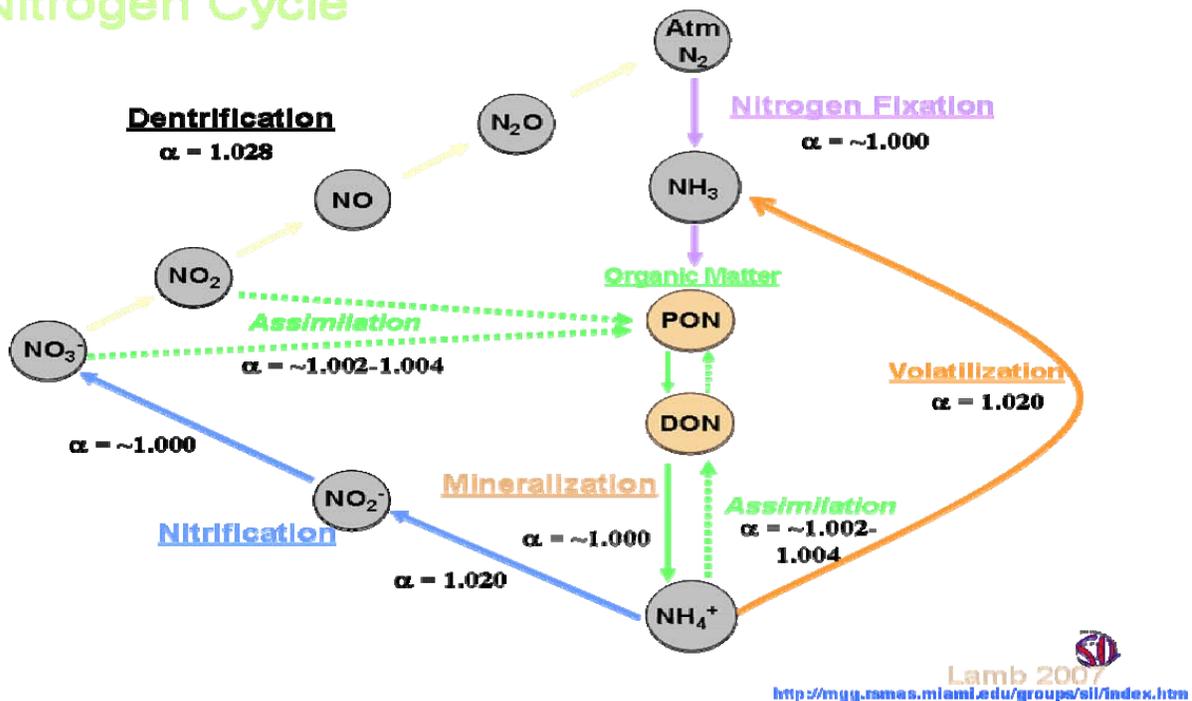
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## INTRODUCTION

Nitrogen availability is an important factor controlling algal growth in marine environments, representing a limiting nutrient throughout much of the global ocean. Anthropogenic inputs to the coastal zone, however, serve to shift the nutrient regime, leading to questions regarding the extent of anthropogenic nutrient impacts in near-shore environments. Recently there have been a

### Nitrogen Cycle



*Figure 1: Biogeochemical processes and associated nitrogen isotopic fractionations factors of the nitrogen cycle. The value  $\alpha$  is defined as the fractionation factor. The term  $\epsilon$  is defined as  $(\alpha - 1) \cdot 1000$  and is equal to the difference in  $\delta^{15}\text{N}$  between two compounds so that is  $\alpha = 1$  which is the case for the fixation of atmospheric nitrogen by certain nitrogen fixing plants, then  $\epsilon = 0$  and there is no difference in isotopic composition between the plant and the atmosphere. If  $\alpha = 1.020$  then there is a 20 ‰ difference.*

significant number of publications on the  $\delta^{15}\text{N}$  of algae, seagrasses, and other benthic organisms in South Florida (Barile 2004; Chasar et al. 2005; Corbett et al. 1999; Fourqurean et al. 2005; Hoare et al. 2003; Lamb and Swart 2008; Lapointe et al. 2004; Swart et al. 2005; Ward-Paige et



al. 2005; Ward-Paige et al. 2005). Some of these studies have attempted to use  $\delta^{15}\text{N}$  values to support the conclusion that there has been significant input of anthropogenic nitrogen into the coastal zone. Previous work by our research group characterizing the  $\delta^{15}\text{N}$  of particulate organic material, algae, and seagrasses in South Florida coral reefs, Biscayne Bay, and associated with waste water discharge points in Dade, Broward, and Palm Beach Counties has suggested that  $\delta^{15}\text{N}$  values alone do not provide unequivocal evidence that significant amounts of anthropogenic nitrogen influence the coastal zone (Lamb 2007; Lamb and Swart 2008). The purpose of the following work was to begin investigating the importance of natural fractionation on isotopic variability in macroalgae, principally during the process of nitrification and preferential assimilation of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  by macroalgae, as opposed to assuming that nitrogen isotopic values are indicative of anthropogenic inputs.

## **Background**

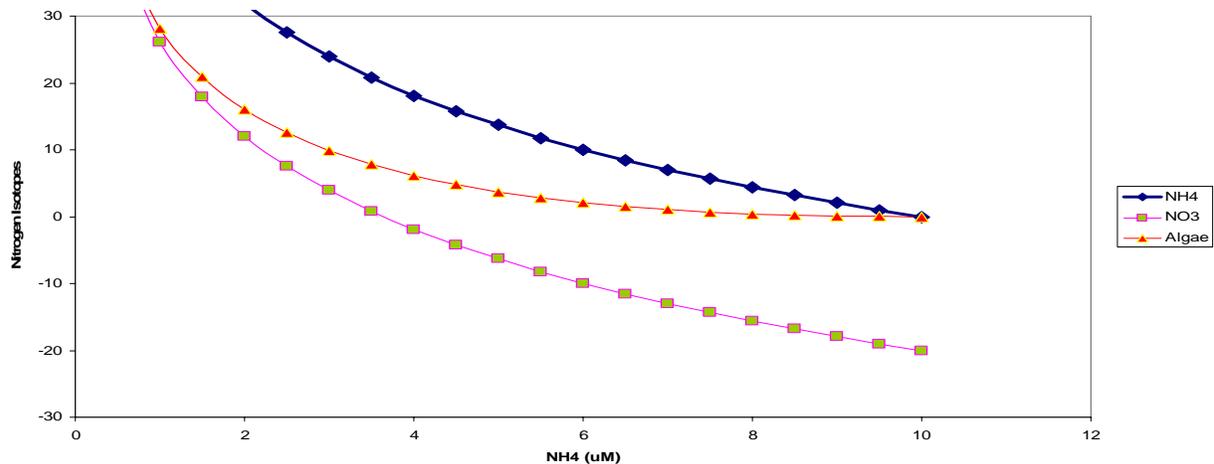
Nitrogen in aquatic settings can be separated into the organic and inorganic phases. The organic nitrogen (total organic nitrogen (TON)) consists of particulate and dissolved organic nitrogen (PON and DON). The inorganic phases (DIN) consist of ammonium, nitrate, and nitrite. In the Florida Keys, the concentration of the TON typically lies between 5 - 25  $\mu\text{M}$ . In contrast the DIN is present at much lower concentrations;  $\text{NO}_3^- = 0.01$  to 1  $\mu\text{M}$ ,  $\text{NO}_2^- = 0.01$  to 0.1  $\mu\text{M}$ , and  $\text{NH}_4^+ = 0.1$  to 1  $\mu\text{M}$ . Measurements of these parameters are made on a quarterly basis by SERC. In the open ocean, the concentration of DON and DIN are much lower (5  $\mu\text{M}$  and  $< 0.1$   $\mu\text{M}$ ). In reefal environments the concentration of nitrite is relative high and probably relates to the conversion of nitrate to nitrite and ammonia under the influence of hydrogen sulfide. Concentrations of nitrite are even greater in Florida Bay.

In the Florida Keys, nitrogen is derived from a variety of sources, although the fluxes involved have not yet clearly been identified. A schematic of the nitrogen cycle is shown in Figure 1. Based on the current measurements, the largest source of nitrogen is organic nitrogen, derived from the decay of *in situ* organics, inputs from land (including anthropogenic sources) and fluxes directly from the sediments. Stable carbon isotopic studies indicate that the largest source of POM results from the contribution from benthic algae and seagrasses (Lamb 2007; Lamb and Swart 2008). The PON and DON undergo mineralization producing ammonium. This in turn



goes through the process of nitrification. The DIN produced along this pathway may be assimilated by algae and seagrasses. Under conditions of low oxygen and hydrogen sulfide, nitrate might be converted back to nitrite and ammonium. Input of nitrate and ammonium from processes other than ammonification and nitrification occurs as input from the atmosphere and from upwelling (nitrate only). It is well known that a large amount of nitrogen is derived from upwelling in the form of nitrate. The impact of upwelled nitrogen is uncertain as there have been no studies which have documented the presence of waters high in nitrate on the reef. It is likely that the nitrate associated with these events is rapidly assimilated, leaving little trace of the upwelling. Anthropogenic sources are unlikely to be significant except locally, such as in canals, or close to coast lines and sewer outfalls.

In order to understand the distribution of the stable isotopes of nitrogen in algae, it is necessary



**Figure 2: The behavior of the  $\delta^{15}\text{N}$  of  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and algae assimilating these forms of nutrients as a function of nitrification of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  using a fractionation factor of 1.020. The algae in this model is assumed to utilize  $\text{NO}_3^-$  and  $\text{NH}_4^+$  in proportions equal to their concentrations in the solution and without any fractionation resulting from assimilation.**

to consider the origin of the natural variability. Nitrogen has two stable isotopes ( $^{15}\text{N}$  and  $^{14}\text{N}$ ), the concentrations of which are measured relative to atmospheric nitrogen ( $^{15}\text{N}/^{14}\text{N} \sim 0.007$ ) in



parts per thousand (‰). Figure 1 illustrates the biogeochemical processes of the nitrogen cycle along with currently accepted isotopic fractionation factors associated with each process.

The fixation of atmospheric nitrogen occurs with minimal fractionation (Hoering and Ford 1960) and hence nitrogen fixing organisms typically have values close to that of the atmosphere (0 ‰). However, changes in the N isotopic composition do occur during the processes of nitrification and denitrification. For example, consider a plant with  $\delta^{15}\text{N}$  close to that of atmospheric nitrogen (0 ‰). During mineralization of the dead plant remains, the organic nitrogen is converted to  $\text{NH}_4^+$  with minimal fractionation and initially the  $\text{NH}_4^+$  will have an isotopic composition similar to the initial plant (i.e.  $\delta^{15}\text{N} = 0$  ‰). During nitrification the  $\text{NH}_4^+$  is converted to  $\text{NO}_2^-$  and then to  $\text{NO}_3^-$ . Each of these processes has a fractionation factor ( $\alpha$ ) of 1.02 (Miyake and Wada 1971), so that the resultant product is about 20‰ more negative than the initial  $\text{NH}_4^+$ . During this process in a closed system, the residual  $\text{NH}_4^+$  becomes enriched in  $^{15}\text{N}$  and the  $\text{NO}_3^-$  depleted in  $^{15}\text{N}$ . If in this example the initial  $\text{NH}_4^+$  has a value of 0‰, then after 50% of the  $\text{NH}_4^+$  is converted to  $\text{NO}_3^-$  (i.e. the concentration of both nitrogen species would be 5  $\mu\text{M}$ ) then the remaining  $\text{NH}_4^+$  will have a N isotopic composition close to  $\sim +14$ ‰. If the algae were taking up only  $\text{NH}_4^+$  and assuming no fractionation during assimilation, then the algae would also have  $\delta^{15}\text{N}$  value of  $\sim +14$ ‰, if the algae were utilizing both  $\text{NO}_3^-$  and  $\text{NH}_4^+$  in quantities proportional to their concentration in the solution then the isotopic composition would be  $\sim +4$ ‰ and if the algae were utilizing only  $\text{NO}_3^-$  the composition would be  $-6.3$ ‰ (See Figure 2). In this example the algae is only assumed to reflect the composition at a specific concentration as the  $\text{NH}_4^+$  is converted  $\text{NO}_3^-$ . If all the  $\text{NH}_4^+$  is converted to  $\text{NO}_3^-$  and no  $\text{NH}_4^+$  or  $\text{NO}_3^-$  is lost to other processes, then the mean isotopic composition of the  $\text{NO}_3^-$  has to be equal to the isotopic composition of the initial  $\text{NH}_4^+$ .

In actual fact, the system can be considered to be dynamic with organic nitrogen continually being mineralized and undergoing nitrification. Very high rates of mineralization would lead to high concentrations of  $\text{NH}_4^+$  and relatively low  $\delta^{15}\text{N}$  values. Very low rates of mineralization would produce low concentrations of  $\text{NH}_4^+$  relative to  $\text{NO}_3^-$  and higher  $\delta^{15}\text{N}$  values. A further variable in this model is the assumption that there is no addition of  $\text{NH}_4^+$  or  $\text{NO}_3^-$  from other sources. This is obviously incorrect and would serve to alter the isotopic composition of the  $\text{NO}_3^-$  and  $\text{NH}_4^+$  and hence the isotopic composition of the plants growing in this environment. These can be added from upwelling (nitrate), atmospheric input (nitrate and ammonium), or



diffusion from the sediments (nitrate and ammonium). The  $\delta^{15}\text{N}$  values of the algae reflect fractionation processes taking place in these sources. For example, the  $\delta^{15}\text{N}$  of upwelled waters is influenced by denitrification (See Figure 1). This process removes isotopically light nitrogen leaving the nitrate enriched in  $^{15}\text{N}$ . Denitrification also takes place in the sediments leading to a flux of nitrate enriched in  $^{15}\text{N}$  from the sediments. Further fractionation in the system occurs during assimilation of  $\text{NO}_3^-$  or  $\text{NH}_4^+$  by plants and algae. For example, if we consider a body of water containing a specified amount of  $\text{NO}_3^-$ , then as it is assimilated by algae or other plants, the residual nitrate becomes fractionated leaving the heavier isotope behind. Hence the  $\delta^{15}\text{N}$  of the residual nitrogen becomes isotopically enriched. A similar situation may occur in an  $\text{NH}_4^+$  dominated system, although the fractionation factors involved are not precisely defined.

In order to attempt to separate the competing controls of source and fractionation upon the  $\delta^{15}\text{N}$  of algae, this project designed a series of preliminary simple experiments which can be separated into two categories. The first set of experiments was designed to measure the fractionation during assimilation. In these experiments algae were grown under varying concentrations with a constant supply of  $\text{NO}_3^-$  or  $\text{NH}_4^+$  with a constant isotopic composition (See Methods for details). Any variations in the  $\delta^{15}\text{N}$  measured in the algae produced during the experiment can be expected to be a result of the assimilation fractionation factor. The second set of experiments was designed to test whether algae preferentially assimilate either  $\text{NO}_3^-$  or  $\text{NH}_4^+$ . It is assumed that they utilize  $\text{NH}_4^+$  rather than  $\text{NO}_3^-$ , but at what concentration of  $\text{NH}_4^+$  do the algae switch to  $\text{NO}_3^-$  or do they always accumulate  $\text{NO}_3^-$  at some rate if  $\text{NO}_3^-$  is present. What is the effect of this upon the eventual  $\delta^{15}\text{N}$  of the algae?



## METHODS AND APPROACH

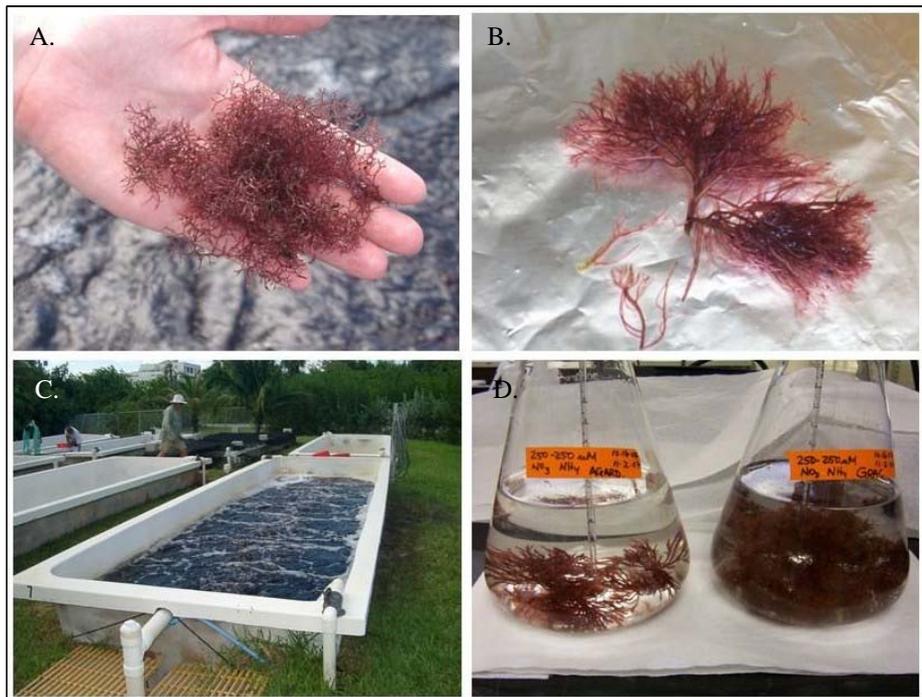
Samples of *Gracilaria sp.* (Fig 3A) and *Agardhiella sp.* (Fig 3B) cultures were collected from the *Aplysia* Mariculture Laboratory's algal aquaculture facility (University of Miami). These species are maintained in a system of seven, 2,400-gallon fiberglass tanks supplied with filtered seawater at a rate of 10 gal/min. Radiant energy and temperature are monitored constantly and algal growth rates are optimized by adjusting nutrient levels weekly. Thalli were rinsed with filtered seawater and

gently scrubbed to remove surface epiphytes. Prior to experimentation, the macroalgae were maintained within 2L flasks at 26°C and approximately

$100\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  for a 14-day acclimation period.

During the acclimation period,

filtered and autoclaved seawater



**Figure 3: Experimental protocols. A. *Gracilaria sp.*; B. *Agardhiella sp.*; C. Flow-through tank system at *Aplysia* Mariculture Facility; D. Culture flasks used in our experiments.**

was changed every 2 days, enriched to  $500\mu\text{M N}$  ( $250\mu\text{M NaNO}_3$  and  $250\mu\text{M NH}_4\text{Cl}$ ) and  $44\mu\text{M KH}_2\text{PO}_4$ , with f/2 medium supplements of B-vitamins (Vitamin B<sub>12</sub>, Biotin, and Thiamine) and trace metals (Fe, Cu, Mo, Zn, Co, Mn) (Guillard 1975). Aeration was provided by bubbling with compressed air.



## Experimental Protocol

### Nutrient concentration experimental protocol

The effect of varied nutrient availability on the nitrogen isotopic composition of new algal growth was investigated through two individual nutrient experiments; one set of incubations was completed with varied  $\text{NO}_3^-$  concentration, one with varied  $\text{NH}_4^+$  concentrations. In both cases, concentrations of 10, 50, 100, and 500  $\mu\text{M N}$  (as  $\text{NaNO}_3$  and  $\text{NH}_4\text{Cl}$ ) were supplied in a medium of autoclaved, filtered (0.2 $\mu\text{m}$  cartridge filter) seawater enriched with the same  $\text{KH}_2\text{PO}_4$ , B-vitamin, and trace metal supplements outlined for the acclimation medium. Subsamples of the rhodophyte algae, *Agardhiella* sp. and *Gracilaria* sp. (0.25-0.5g wet weight; 2.5-3.0 cm) were taken from acclimation flasks, visible epiphytes were removed, and the algae samples were placed in 500 ml flasks of the incubation medium and varied N-concentration. Aeration was provided by bubbling with compressed air. The media was replaced every 24 hours during 7-9 day incubations, at which time each algal sample was weighed to monitor growth and rinsed to prevent epiphyte fouling. Water samples were collected and filtered (GF/F) after each 24 hour period and analyzed for  $\text{NO}_3^-$  or  $\text{NH}_4^+$ . At the conclusion of the incubations, final accumulated biomass was weighed, new growth was trimmed from algal specimens, and samples were dried (40°C 48 hours) then ground with mortar and pestle.



**Figure 4:** Sample of *Agardhiella* sp. Grown in nutrient treatment. Tips of samples were trimmed for isotopic analyses of growth in new treatment.

### Isotopic label experimental protocol

Three experiments were conducted applying enriched isotope tracer techniques. In each experiment 99%  $^{15}\text{N}$  label (Spectra Stable Isotopes) with a  $\delta^{15}\text{N}$  value of 1000‰ was utilized to trace either  $\text{NO}_3^-$  or  $\text{NH}_4^+$  uptake depending on the treatment.

- i. The first isotopic tracer experiment was conducted using 0.25g wet weight *Gracilaria* specimens grown with varied concentrations of non-labeled



$\text{NH}_4\text{Cl}$  and  $\text{Na}^{15}\text{NO}_3$  isotopically enriched spike. Phosphate, vitamin B, and trace mineral supplements were maintained as in algal acclimation flasks. Treatment nutrient concentrations are given in Table 1. The effects of varied  $\text{NH}_4^+$  concentration on uptake  $\text{NO}_3^-$  present at low concentrations and of varied  $\text{NO}_3^-$  concentrations under constant  $\text{NH}_4^+$  availability were examined in comparison with control treatments grown with  $\text{NH}_4^+$  alone. Subsamples of acclimated algae were carefully cleaned and rinsed in filtered seawater before being placed in 500 ml flasks of incubation medium of appropriate nutrient proportions. Nutrient solutions were sampled for nutrient concentration determination and replaced every 24 hours during the 9 day incubation, and algal biomass was weighed. At the end of the growth incubation samples were treated as above and isotopic compositions were measured.

Nutrient Concentrations ( $\mu\text{M}$ )	
$\text{NH}_4\text{Cl}$	$\text{Na}^{15}\text{NO}_3$
500	1
50	1
10	1
50	5
50	25
50	50
500	0
50	0
10	0

***Table 1. Treatment concentrations for isotopic enrichment experiment.***

- ii. The second and third isotopic enrichment studies were conducted using *Agardhiella* specimens, incubated in 2L flasks. 1800 ml of culture medium were used to avoid daily nitrogen limitation induced by uptake depletion of concentrations. In each flask, 250  $\mu\text{M}$  N (either  $\text{NaNO}_3$  or  $\text{NH}_4\text{Cl}$ ) was mixed with 10  $\mu\text{M}$   $^{15}\text{N}$ -tracer of the opposite nutrient composition (i.e. 250  $\mu\text{M}$   $\text{NaNO}_3$  with 10  $\mu\text{M}$   $^{15}\text{NH}_4\text{Cl}$ ). Incubations were run in triplicate and as in the above experiments, were conducted over a period of 9 days with medium sampling and replacement at 24 hour intervals. At the conclusion of the 9 day



incubations, samples were treated as described above and isotopic compositions of new growth were measured. Experiment ii was conducted at 26°C, while Experiment iii was conducted at 21°C to examine the effects of temperature on tracer accumulation within algal tissues.

## **Analytical Protocol**

### **Stable Isotopes**

The organic carbon and nitrogen contents as well as the stable isotopic composition ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of the algae was determined using a modified CN analyzer interfaced with a continuous-



*Figure 5: Elemental analyzer and stable isotope mass spectrometer utilized in this study.*

flow isotope-ratio mass spectrometer (CFIRMS) (20-20, Europa Scientific). Prior to analysis the algae samples were dried and 3 - 6 mg were placed in tin capsules. Data obtained from the 20-20 provides the C/N ratio of the samples in addition to the  $\delta^{13}\text{C}$  content of the organic carbon (measured as  $\text{CO}_2$ ) and the  $\delta^{15}\text{N}$  content of the organic nitrogen (measured as  $\text{N}_2$ ). Samples of the nutrient material were analyzed in a similar manner in order to provide

information on the initial  $\delta^{15}\text{N}$  of the medium. Data are reported to conventional international standards (V-PDB for carbon and atmospheric nitrogen for  $\text{N}_2$ ). External precision is approximately 0.1 ‰ for C and 0.2‰ for N. The ratio of C:N was calculated by comparing the integrated area of the major beams (mass 28 for N and mass 44 for C) to standards with known C:N ratios. The external precision for this method is < 0.1.

### **Nutrient Concentrations**

Concentration of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  were analyzed prior to and after each experiment. Nitrate and nitrite concentrations were determined by diazotization before and after reduction with cadmium



(Grasshoff 1976). Ammonium concentrations were determined with the indophenol-blue method (Koroleff 1970).

## RESULTS

### Nutrient uptake experiments

Results from the nutrient experiments are presented in Table 2. This table includes the growth rate as well as the residual concentration of nitrogen during and after the experiment and the final nitrogen isotopic composition of the algae produced during the incubation. Data from all experiments are presented in Figures 6-14. These graphs document changes in the  $\delta^{15}\text{N}$  of the algae during the growth experiments, the residual DIN concentrations left in each treatment after the 24 hour incubations, and the average growth rates.

**Isotopic results - Nitrate:** The initial  $\delta^{15}\text{N}$  of the *Agardhiella* was  $\sim -0.5$  ‰. The  $\delta^{15}\text{N}$  increased from 1.32 (10  $\mu\text{M}$ ) to 2.43 in the 50  $\mu\text{M}$ , decreased to 2.04 and finally to  $-1.23$  ‰ in the 500  $\mu\text{M}$  experiment (Figure 6 and 7). Similar results were found in the experiments using *Gracilaria* although the highest  $\delta^{15}\text{N}$  value occurred in the 10  $\mu\text{M}$  treatment and the  $\delta^{15}\text{N}$  continually decreased with increasing concentration of nitrate. The initial  $\delta^{15}\text{N}$  of the *Gracilaria* was elevated ( $+3$  ‰) (this will be discussed later). The C:N ratio increased in the low nitrate experiments in both species of algae and steadily decreased with increase nitrate (Figure 12).

**Isotopic results - Ammonium:** The general trend of the  $\delta^{15}\text{N}$  results was similar to those of the nitrate experiments (Figures 8 and 9). The most depleted values occurred in the elevated concentration treatments. In the *Agardhiella* experiments, the maximum  $\delta^{15}\text{N}$  values occurred in the treatments with the lowest concentration, while in the *Gracilaria* experiments the highest values occurred in the 100  $\mu\text{M}$  treatment. The C:N ratio increased in the low ammonium experiments in both species of algae and steadily decreased with increase nitrate (Figure 13).



Algal Species	Nutrient Treatment	Initial Conc.	Initial Biomass	Final Biomass	Growth Rate		Residual N Conc.	$\delta^{15}\text{N}$
		( $\mu\text{M}$ )	(g)	(g)	(g day <sup>-1</sup> (SD))		( $\mu\text{M}$ )	(‰ AIR)
<i>Agardhiella</i> sp.	$\text{NO}_3^-$	10	0.37	0.90	6.56E-02	(0.036)	1	+1.32
		50	0.50	1.52	1.28E-01	(0.061)	2	+2.43
		100	0.41	1.82	1.76E-01	(0.081)	12	+2.04
		500	0.50	1.39	1.11E-01	(0.092)	247	-1.23
	$\text{NH}_4^+$	10	0.16	0.37	3.00E-02	(0.015)	<1	+1.49
		50	0.15	0.39	3.43E-02	(0.016)	10	+0.35
		100	0.20	0.52	4.57E-02	(0.025)	47	-0.96
		500	0.13	0.40	3.86E-02	(0.023)	347	-5.77
<i>Gracilaria</i> sp.	$\text{NO}_3^-$	10	0.23	0.47	3.43E-02	(0.03)	2	+3.46
		50	0.22	0.69	6.71E-02	(0.026)	2	+3.05
		100	0.22	0.49	3.86E-02	(0.023)	12	+2.32
		500	0.26	0.70	6.29E-02	(0.036)	278	+0.88
	$\text{NH}_4^+$	10	0.47	0.87	5.71E-02	(0.020)	<1	-0.38
		50	0.46	0.79	4.71E-02	(0.018)	<1	+0.96
		100	0.42	0.77	5.00E-02	(0.026)	5	+1.70
		500	0.48	1.21	1.04E-01	(0.040)	314	-2.93

**Table 2: Nutrient uptake experiments, summary data** Growth rates are the mean of three measurements measured over 24 hours; the  $\delta^{15}\text{N}$  was measured in replicate on the nitrate experiments and only once on the ammonium experiments (replicate analyses provided values which were equal to or better than the quoted external precision of 0.2 ‰; residual concentrations represent the mean concentration of nutrients remaining after 24 hours as measured on three days during the treatment.

### **Isotopic enrichment with varied proportions of ammonium and $^{15}\text{N}$ -enriched nitrate spike:**

Results from a series of three isotopic enrichment experiments are presented in Tables 3 and 4. These tables include nitrogen isotopic compositions and C:N ratios under various concentration treatments combining isotopically enriched and non-enriched nutrients. Data from these experiments are also presented in Figures 14 and 15, illustrating changes in the  $\delta^{15}\text{N}$  values with varied levels of overall nutrient limitation and isotopically enriched spike availability.

In treatments using *Gracilaria* exposed to only 1  $\mu\text{M}$ , nitrogen isotopic values ranged between -1.5 and +58.6‰, with progressively more enriched values apparent in lower  $\text{NH}_4^+$  treatments. Highest C:N ratios were also evident in the low  $\text{NH}_4^+$  treatment, corresponding to the most enriched isotopic values. When moderately limiting (50  $\mu\text{M}$ )  $\text{NH}_4^+$  conditions were applied with increasing concentrations of  $^{15}\text{NO}_3^-$  spike (5-50  $\mu\text{M}$ ), algal isotopic values were increasingly



enriched to a maximum of +388.7‰, coincident with decreasing C:N ratios. Control treatments in this experiment were not exposed to enriched  $^{15}\text{NO}_3^-$  spike, and showed nitrogen isotopic values ranging between -3.1 and +3.2‰. C:N ratios in algal tissues from these treatments ranged from +5.5 to +28.4. These values are consistent with those found in the previously described nutrient uptake experiments at varied  $\text{NH}_4^+$  concentrations (See Table 2).

**Ammonium with  $^{15}\text{N}$ -enriched nitrate spike:** In this experiment, which used *Agardiella* in contrast to *Gracilaria*, no discernable amount of  $^{15}\text{NO}_3^-$  spike was detected in the algae at the lower temperature of 21°C. At higher temperatures under the same nutrient conditions a small enrichment in the  $\delta^{15}\text{N}$  of the algae was detected. However, lower growth rates of 0.311 g wet weight  $\text{d}^{-1}$  were found at the higher temperature (26°C), compared to 0.368 g wet weight  $\text{d}^{-1}$  at 21°C (See Table 4).

**Nitrate with  $^{15}\text{N}$ -enriched ammonium spike:** In this experiment the new algal growth had a large enrichment in  $\delta^{15}\text{N}$ . This enrichment was more pronounced at the higher temperature of 26°C (See Table 4).



Figure 6

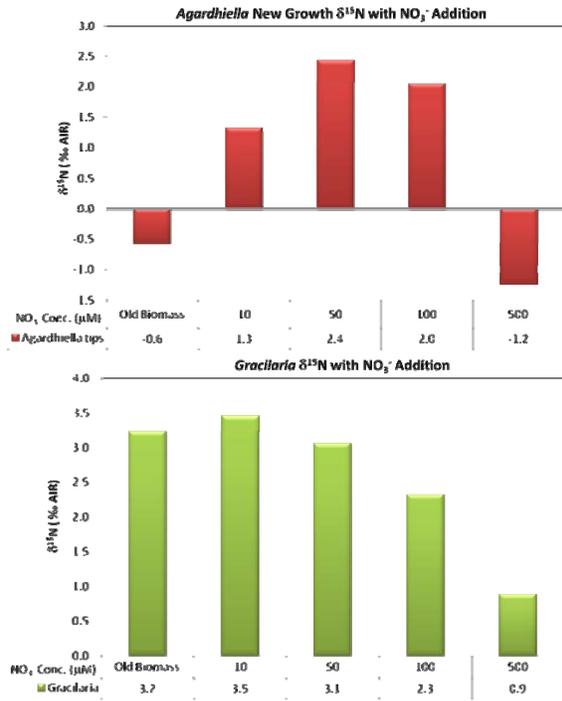


Figure 6. Nitrogen isotopic values of new algal growth following 7-8 day incubations at  $\text{NO}_3^-$  concentrations of 10, 50, 100, 500  $\mu\text{M}$ .

Figure 7

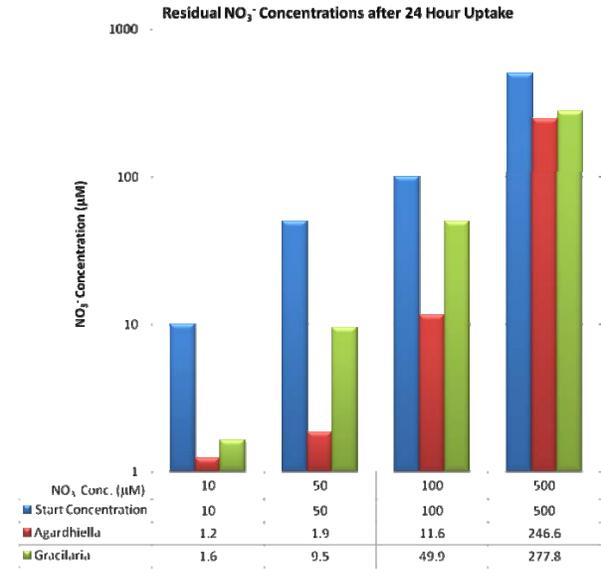


Figure 7. Residual  $\text{NO}_3^-$  concentrations ( $\mu\text{M}$ ) following 24 hours of algal growth in media with initial treatment  $\text{NO}_3^-$  concentrations of 10, 50, 100, 500  $\mu\text{M}$ .

Figure 8

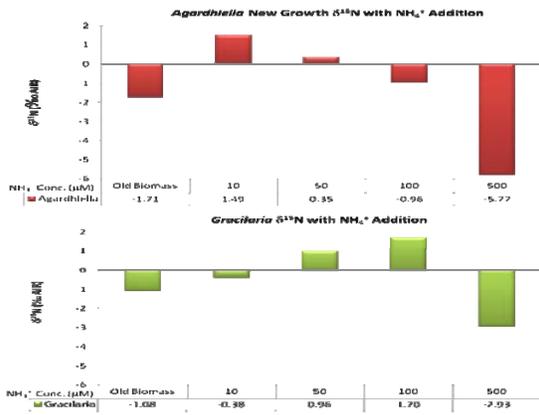


Figure 8. Nitrogen isotopic values of new algal growth following 8 day incubations at  $\text{NH}_4^+$  concentrations of 10, 50, 100, 500  $\mu\text{M}$ .

Figure 9

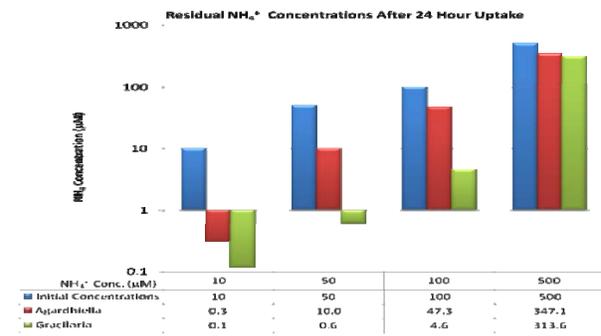


Figure 9. Residual  $\text{NH}_4^+$  concentrations ( $\mu\text{M}$ ) following 24 hours of algal growth in media with initial treatment  $\text{NH}_4^+$  concentrations of 10, 50, 100, 500  $\mu\text{M}$ .



Figure 10

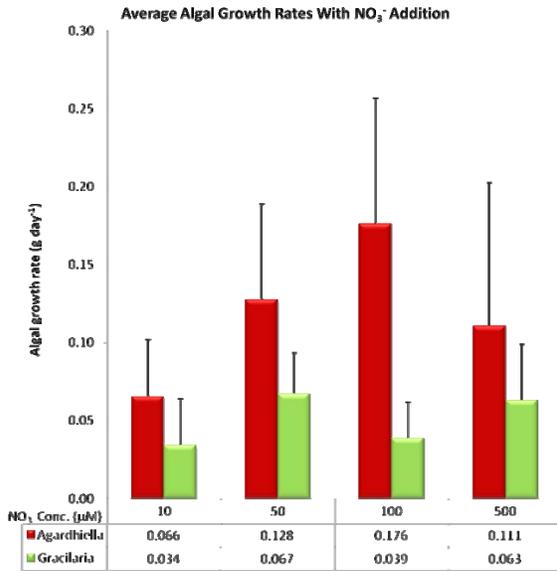


Figure 10. Average algal growth rates (g wet weight day<sup>-1</sup>) during incubations at NO<sub>3</sub><sup>-</sup> concentrations of 10, 50, 100, 500 μM. Error bars represent standard deviation of the mean.

Figure 11

Figure 11

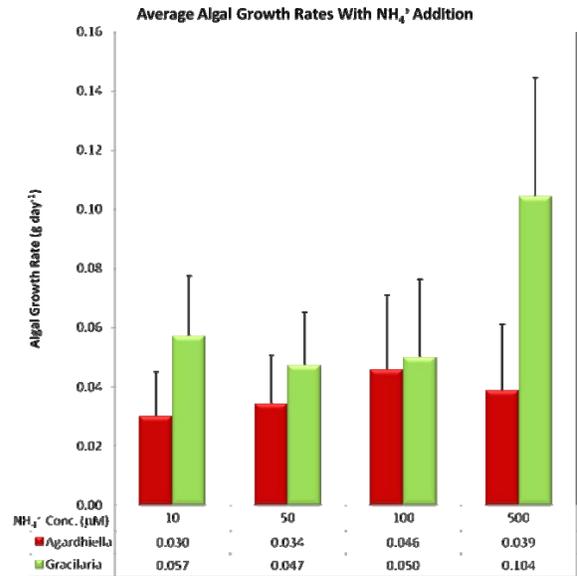


Figure 11. Average algal growth rates (g wet weight day<sup>-1</sup>) during incubations at NH<sub>4</sub><sup>+</sup> concentrations of 10, 50, 100, 500 μM. Error bars represent standard deviation of the mean.

Figure 12

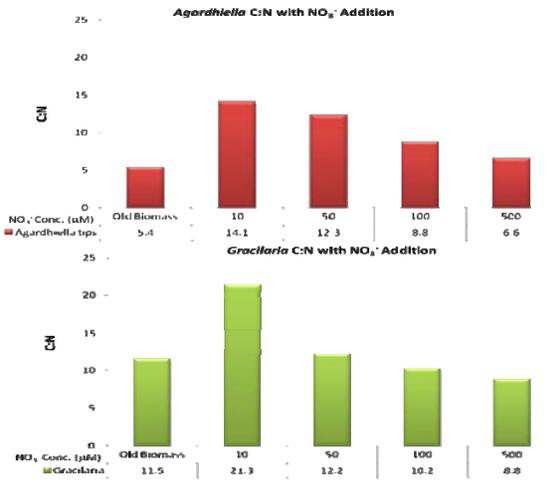


Figure 12. C:N ratios of new algal growth following 7-8 day incubations at NO<sub>3</sub><sup>-</sup> concentrations of 10, 50, 100, 500 μM.

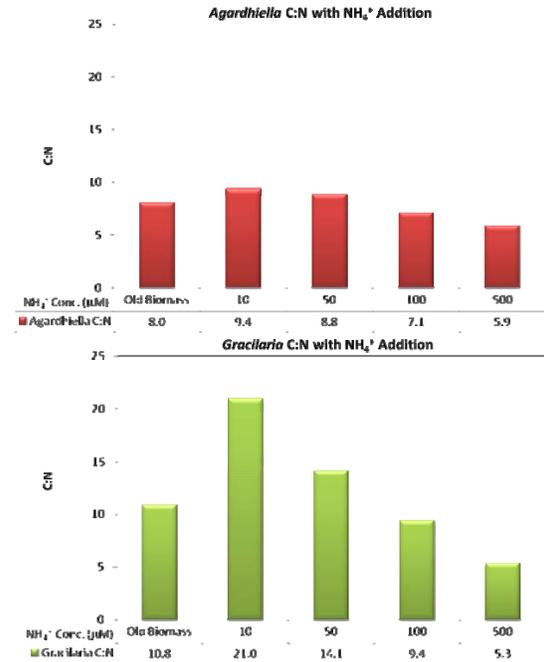


Figure 13. C:N ratios of new algal growth following 8 day incubations at NH<sub>4</sub><sup>+</sup> concentrations of 10, 50, 100, 500 μM.



Figure 14

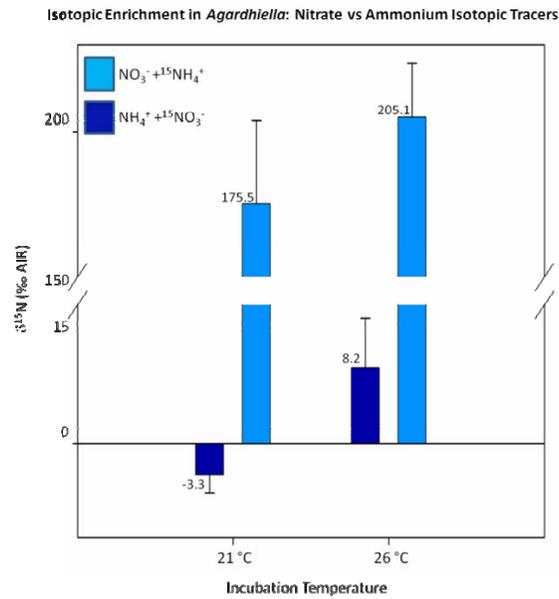


Figure 14.  $^{15}\text{N}$  Tracer Experiment 2-3. Nitrogen isotopic values in *Agardhiella* new growth following 8 day incubations at 2 ambient temperatures, 21 and 26 °C, and under 2 nutrient /isotopic label treatments: [light blue] 250  $\mu\text{M}$   $\text{NO}_3^-$  + 10  $\mu\text{M}$   $^{15}\text{NH}_4^+$ ; [dark blue] 250  $\mu\text{M}$   $\text{NH}_4^+$  + 10  $\mu\text{M}$   $^{15}\text{NO}_3^-$ .

Figure 15

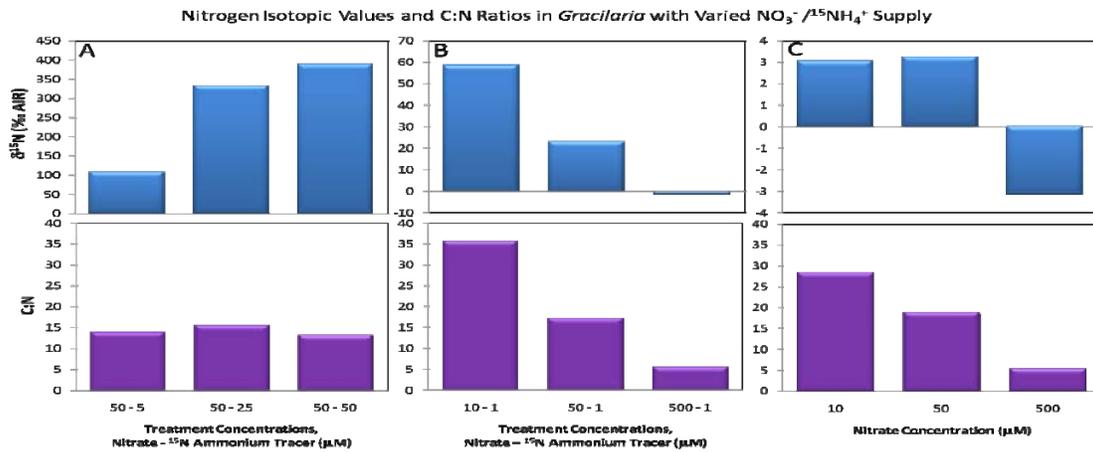


Figure 15.  $^{15}\text{N}$  Tracer Experiment 1. Nitrogen isotopic values and C:N ratios in *Gracilaria* growing with varied  $\text{NO}_3^-$  and  $^{15}\text{NH}_4^+$  isotopic label concentrations. A. 50  $\mu\text{M}$   $\text{NO}_3^-$  with varied concentrations of  $^{15}\text{NH}_4^+$ . B. Varied  $\text{NO}_3^-$  concentrations with 1  $\mu\text{M}$   $^{15}\text{NH}_4^+$ . C. Varied  $\text{NO}_3^-$  concentration, no tracer application.



Nutrient treatment ( $\mu\text{M}$ )		$\delta^{15}\text{N}$ ( $\text{‰ AIR}$ )	C:N	Estimated
$\text{NH}_4\text{Cl}$	$\text{Na}^{15}\text{NO}_3$			
500	1	-1.5 (0.3)	5.5	-1.3
50	1	23.0 (0.3)	17.2	16.5
10	1	58.6 (0.3)	35.6	87.6
50	5	108.5 (0.3)	13.8	87
50	25	332.8 (0.3)	15.4	329
50	50	388.7 (0.3)	13.1	495
500	0	-3.1 (0.3)	5.5	NA
50	0	3.2 (0.3)	18.9	NA
10	0	3.0 (0.3)	28.4	NA

**Table 3: Isotopic Tracer Experiment 1 (*Gracilaria*), summary data;  $\delta^{15}\text{N}$  represent the mean of three analyses.**

Incubation Temperature	<u>250 <math>\mu\text{M}</math> <math>\text{NH}_4\text{Cl}</math> + 10 <math>\mu\text{M}</math> <math>\text{Na}^{15}\text{NO}_3</math></u>			<u>250 <math>\mu\text{M}</math> <math>\text{NaNO}_3</math> + 10 <math>\mu\text{M}</math> <math>^{15}\text{NH}_4\text{Cl}</math></u>		
	$\delta^{15}\text{N}$ ( $\text{‰ AIR}$ ) (SD)	C:N (SD)	Growth Rate	$\delta^{15}\text{N}$ ( $\text{‰ AIR}$ ) (SD)	C:N (SD)	Growth Rate
21 °C	-3.3 (1.9)	6.2 (0.36)	0.368	175.5 (28.3)	7.7 (0.18)	0.376
26 °C	8.2 (5.3)	6.0 (0.15)	0.311	205.1 (18.6)	6.3 (0.15)	0.276

**Table 4: Tracer Experiment 2 & 3, (*Agardhiella*) summary data**



## DISCUSSION

### Nutrient concentration experiments

**Growth Rates:** The growth rates in the ammonium experiment were approximately the same in all treatments (Table 2) for *Agardhiella*. *Gracilaria* showed a growth rate increase in the 500  $\mu\text{M}$  treatment. In the nitrate experiment, *Gracilaria* exhibited slightly lower growth rates than in the ammonium experiment (Figure 10 and 11). *Agardhiella* growth increased with additions of nitrate up to to 100  $\mu\text{M}$ . The absence of difference in growth rates between the various experiments is surprising considering the amount of ammonium actually assimilated. For



Initial biomass    10 $\mu\text{M}$     50 $\mu\text{M}$     100  $\mu\text{M}$     500  $\mu\text{M}$

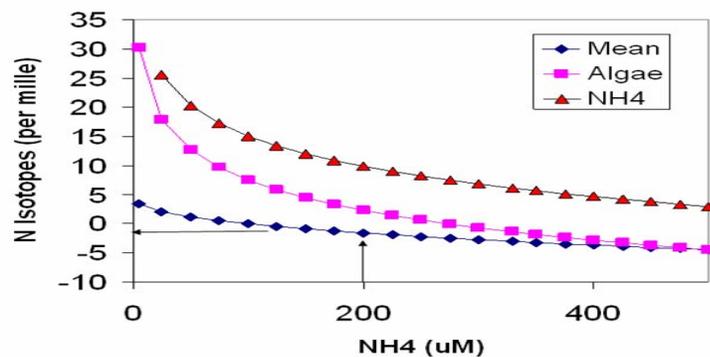
**Figure 16:** Pictures showing samples of *Agardhiella sp.* Grown in different concentrations of  $\text{NH}_4^+$ . From left to right, pictures show initial individual, 10  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100 $\mu\text{M}$ , and 500  $\mu\text{M}$ . All experiments showed approximately similar growth rates, but reduced uptake of N at lower N concentrations. Note differences in color.

example, in the 10  $\mu\text{M}$  experiment the *Agardhiella* assimilated about 9 $\mu\text{M}$  of ammonium in 24 hours, while in the 100  $\mu\text{M}$  experiment 50 $\mu\text{M}$  were incorporated. In spite of this difference the growth rates were approximately the same (Figure 10, 11, and 16). The explanation for this apparent lack of difference can be seen in the C:N ratio. The C:N ratio has been suggested to be an indicator of N availability (Fourqurean et al. 2005) and in fact this can be shown to be the case from the data presented here (Figure 12 and 13). In all cases the C:N ratio decreased from ~15-20 in the 10  $\mu\text{M}$  experiment to ~10 in the 500  $\mu\text{M}$  treatment.



**Isotopic composition:** The pattern of isotopic changes observed in the new growth for both species of algae investigated are essentially the same. The specimens growing in the lowest concentration of  $\text{NH}_4^+$  exhibited the most positive  $\delta^{15}\text{N}$  values, values which approached the  $\delta^{15}\text{N}$  of the original medium ( $\sim +2.8$  and  $+2.7$  ‰ for ammonium and nitrate respectively). In contrast, the  $\delta^{15}\text{N}$  of the specimens grown in solution with highest DIN concentration showed more depleted  $\delta^{15}\text{N}$  values. The explanation for these patterns is that in the low concentration treatments, the algae utilize most of the available nutrients, hence the biomass produced has essentially the same  $\delta^{15}\text{N}$  as the ammonia/nitrate dissolved in the seawater. This is evident in the analyses of the concentration data which shows that within a 24 hour period  $> 95\%$  of the available  $\text{NH}_4^+$  was removed by the *Agardhiella* algae and  $> 99\%$  removed by *Gracilaria*. In the case of *Gracilaria*, this species seemed to be very efficient at  $\text{NH}_4^+$  removal, taking up more than of 95% of all nutrients even in the 100  $\mu\text{M}$  treatment. Interestingly the  $\delta^{15}\text{N}$  of the algae increased with the most positive values being manifested in the 100  $\mu\text{M}$  treatment. In the 500

$\mu\text{M}$  treatment the values decreased to  $\sim -3$  ‰. In the case of *Agardhiella*, the percent of  $\text{NH}_4^+$  removed steadily decreased from about 99% at 10  $\mu\text{M}$  to 90% at 50  $\mu\text{M}$ , 10% at 100  $\mu\text{M}$ , and 5% at 500  $\mu\text{M}$ . This decrease in uptake was accompanied by a decrease in the  $\delta^{15}\text{N}$  from  $\sim +1.5$ ‰ to  $\sim -6$  ‰. The behavior of the  $\delta^{15}\text{N}$  in both of the species and the varying DIN concentrations can be modeled using a Rayleigh distillation model assuming some level of fractionation



*Figure 17: Example of the evolution of the isotopic composition of ammonium during a closed flask experiment. As the ammonium is utilized by the algae, the isotopic composition of the ammonium moves along the line denoted by the triangles, the tissue of the algae formed at any particular time is shown by the squares and the integrated signal by the blue diamonds. In nature the ammonia can be removed from the algae and then be incorporated into another algae specimen at some other location. The result is that there should be an inverse relation between the concentration of ammonia and the  $\delta^{15}\text{N}$ .*



during assimilation. In this model the unknown is the fractionation factor and the amount of  $\text{NH}_4^+$  taken up by the algae is calculated by comparing the concentration of  $\text{NH}_4^+$  in the medium before and after the experiment. An example of this is shown in Figure 17. This shows a hypothetical example of an experiment in which the algae were grown in a solution containing  $500 \mu\text{M}$  of  $\text{NH}_4^+$ . During the experiment the  $\delta^{15}\text{N}$  of the algae evolves along the blue line in Figure 17. The parameter  $\alpha$  is adjusted in equation 1 so that the measured output agrees with the amount of nutrient consumed.

$$R(t) = R(i)\alpha f^{(\alpha-1)} \quad (\text{Equation 1})$$

In this equation  $R(t)$  is equal to the isotopic ratio of the  $\text{NH}_4^+$  after some amount of the  $\text{NH}_4^+$  has been assimilated,  $R(i)$  = the initial ratio of the  $\text{NH}_4^+$ ,  $\alpha$  = fractionation factor, and  $f$  = fraction of the  $\text{NH}_4^+$  assimilated. In this manner the assimilation factor can be calculated for all concentrations of  $\text{NH}_4^+$ . Ideally the fractionation should be independent of concentration, but several experimental issues probably have resulted in errors in the estimation of the precise utilization of nutrients. The main problem arises from the fact that the concentration of nutrients could not held constant, but rather was allowed to drift throughout a 24 hour period. Although the nutrients were replenished to the original concentration at the end of 24 hours, the concentration was only measured 2 to 4 times throughout the 8-9 day experiments. Preferably the concentration in the experiments should have been held constant by adding nutrients as they were utilized using a chemostat principal. Regardless of experimental issues, using the model shown in equation 1 and the data presented in Table 2, the assimilation factor can be estimated for all experiments and both algal species. For *Agardhiella* the assimilation factor is remarkably constant over the entire range of concentrations used varying between  $\sim 1.005$  and  $1.008$  (Table 5). Bearing in mind the various uncertainties, we consider these data to be in good agreement and evidence that the assimilation factor does not vary with respect to nutrient concentration for this species. For *Gracilaria* however the situation is less clear. The data suggest for example



	<i>Agardhiella</i>	<i>Gracilaria</i>
<u>Nitrate</u>		
10	1.0060	?
50	1.0050	?
100	1.0045	1.0010
500	1.0060	1.0060
<u>Ammonium</u>		
10	1.0080	?
50	1.0050	1.0090
100	1.0055	1.0070
500	1.0070	1.0070

*Table 5: Summary of estimated and modeled assimilation factors; data from Gracilaria is difficult to work with as a result of the uncertainty in the inclusion of old growth in the newly measured materials.*

that the  $\delta^{15}\text{N}$  of the algae produced in the 10 $\mu\text{M}$  nitrate experiment were actually more positive than the nitrate used in the experiment. Similarly the data from the 50  $\mu\text{M}$  experiment is difficult to model as a result of its very positive  $\delta^{15}\text{N}$ .

Our preliminary interpretation of these data is that it is difficult to unequivocally separate new from old algal growth in this species and there some of the old growth was incorporated into the measurement. As this old growth reflected growth in tanks in which the  $\delta^{15}\text{N}$  of the nutrients were more positive, then this biased our attempts to calculate the true assimilation factor.

At the present time there appears to be no difference between the fractionation during assimilation of ammonium and nitrate in the case of *Agardhiella* and the fractionation factor appears constant over the range of concentrations examined. The data are inconclusive with regards to *Gracilaria*. The best estimate for *Agardhiella* is that  $\alpha = 1.005875 (+/- 0.001157)$ .

## N-15 Spike Experiments

The  $^{15}\text{N}$  spike experiments provide conflicting results as to the incorporation of ammonium as opposed to nitrate. In the case of *Gracilaria* (Table 3), the expected  $\delta^{15}\text{N}$  of the algae on the algae based on simple mass balance calculations is essentially identical to the values measured. This indicates that the alga was not specific about utilizing ammonium in preference to nitrate.

The second set of isotopic enrichment experiments was designed to examine preferential uptake of  $\text{NH}_4^+$  as opposed to  $\text{NO}_3^-$  in *Agardhiella*. In contrast to the *Gracilaria* experiments, *Agardhiella* did show a marked preference for the incorporation of ammonium relative to nitrate.



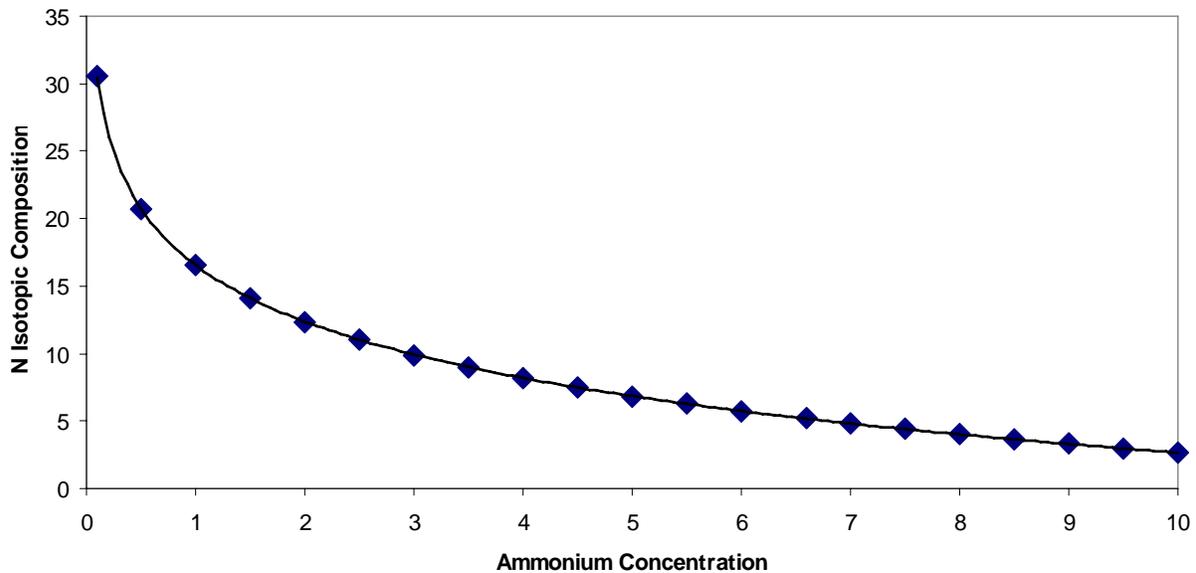
These experiments demonstrate that when high concentrations of  $\text{NH}_4^+$  are available little or no  $\text{NO}_3^-$  is utilized, as no  $^{15}\text{N}$ -label appeared to have influenced the  $\delta^{15}\text{N}$  content of the algal tissue. When ambient  $\text{NO}_3^-$  concentrations are high (250  $\mu\text{M}$ ) however, even relatively low  $\text{NH}_4^+$  (10  $\mu\text{M}$ ) concentrations are exploited to a marked degree, as seen in the enriched  $\delta^{15}\text{N}$  composition of the algae (Figure 14). Interestingly, there was some difference between the algal isotopic compositions invoked through growth at different temperatures, with enriched values at higher ambient growth temperatures. These enrichments would seem to imply increased nutrient limitation presumably related to increased productivity at higher temperatures reminiscent of the summer productivity season in coastal South Florida. However there was no difference in C:N ratios between the temperature treatments and, in fact, growth rates were higher at the cooler temperature. These factors would seem to exclude enrichment due to productivity and nutrient depletion, suggesting a thermodynamic control on either membrane diffusion of the enriched tracer during assimilation, or on reactions involved in assimilatory conversion of DIN to organic biomass.

## IMPLICATIONS

The implications of our data for the utilization of the  $\delta^{15}\text{N}$  of algae as an indicator of the source of nitrogen are profound. First, as may be observed it is possible to produce variations of up to 8‰ in the  $\delta^{15}\text{N}$  of the algae without changing the  $\delta^{15}\text{N}$  of the source, with the  $\delta^{15}\text{N}$  never becoming isotopically more positive than the source. Although not measured in this study, our work mandates that the  $\delta^{15}\text{N}$  of the residual DIN in the culture must become more positive as the nutrients are utilized. For example, this can be seen in a plot (Figure 18) of the concentration of ammonium compared to the  $\delta^{15}\text{N}$  of the ammonium from the modeling result shown in Figure 17. If algae growing in a particular water mass utilizes all the available DIN, then the  $\delta^{15}\text{N}$  of the algae growing in that water mass will be the same as the initial  $\delta^{15}\text{N}$  of the DIN. If however, algae only remove a certain proportion of the nutrients, say for example 50%, before the water mass moves to another location, then the  $\delta^{15}\text{N}$  of that particular water body becomes enriched and this enrichment may be reflected in the isotopic composition of algae growing in the new location. In the case of the example shown in Figure 17, the initial  $\delta^{15}\text{N}$  was +3‰. After 50% was removed, the new value is +12‰. This process continues until all the nutrients are utilized. Naturally the system is much more dynamic than this simple example, with nutrients being



added from many different sources. The relationships shown in this study might explain patterns of heavy stable isotopes reported in the literature (Barile 2004; Lapointe and Barile 2001; Lapointe et al. 2005; Lapointe et al. 2004). These studies reported positive  $\delta^{15}\text{N}$  in algae (+8 to +12‰) in the early portion of the year (March –June) which became more negative during the later portion of the year. These authors attributed these changes to the contribution of anthropogenic nutrients to the marine environment and associated the timing to the wet season. Later multi year studies however failed to find associations between the  $\delta^{15}\text{N}$  of particulate organic material and either wet or dry season (Lamb and Swart 2008). Based on the



**Figure 18: Comparison of the concentration of ammonium with its isotopic composition in a system from which the ammonium is assimilated by algae using a fractionation factor of 1.006. The isotopic composition of the algae would be 6‰ lower than that in the water.**

experimental data presented in this study we suggest that the positive  $\delta^{15}\text{N}$  values relate to the draw down of DIN during the period of maximum algal growth.



## CONCLUSIONS

- The fractionation accompanying assimilation of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  has been measured in two species of macro algae (*Gracilaria sp.* and *Agardhiella sp.*). For *Agardhiella sp.* the values calculated for the assimilation of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  are essentially the same and vary between 1.0045 and 1.008. They show no relationship to nutrient concentration. Data for *Gracilaria* at the low concentrations are equivocal as a result of the difficulty in separating new and old growth. Values obtained at higher concentrations are consistent with the data obtained on *Agardhiella*. These values are significantly higher than reported in the literature.
- As a consequence of these large assimilation factors, significant variations in the stable nitrogen isotopic composition can be produced by uptake of either ammonium or nitrate by algae. As the water becomes progressively lower in DIN, the isotopic composition of the residual DIN become more positive. Such conditions would be expected to most important during the time of year when algae are growing at their maximum rate. There is also expected to be an inverse relationship between the concentration of DIN and the  $\delta^{15}\text{N}$  in the water mass.
- Previous assumptions regarding algal preference in terms of nutrient source (ammonium versus nitrate) may not be universally applicable. Of the two species studied here, only *Agardhiella* demonstrated an obvious preference for ammonium. *Gracilaria* may have a competitive advantage as a generalist in terms of nutrient acquisition. Blanket assumptions about nutrient uptake should be considered with caution as responses are species specific.
- The experiments show that the C:N ratio of the algae is inversely proportional to the concentration of DIN.



## RECOMMENDATIONS FOR FUTURE WORK

Based on our experiments we recommend the following work be carried out as a matter of urgency in order to confirm the observations made in this study.

- Fractionation factors and nutrient uptake affinities need to be determined on a range of other species of benthic algae. We are culturing two additional species of green algae which will be suitable for experimentation.
- Observations appear to suggest that fractionation during assimilation is independent of nutrient concentration. However this needs to be confirmed by performing experiments in which the concentration is held constant or least more constant than was in the case of our experiments. These experiments need to be combined with measurements of the  $\delta^{15}\text{N}$  of the DIN in the water. In particular our lowest concentration was 10  $\mu\text{M}$ , a concentration which is already higher than in most natural environments in South Florida. Future work should test the applicability of the fractionation factor measured at concentrations between 1 and 10  $\mu\text{M}$ . These measurements will help support the model of fractionation presented.
- Preliminary data from our isotopic enrichment studies suggest that fractionation and incorporation may depend upon temperature and light intensity. These parameters need to be further examined.
- In our work the concentration of nutrients was allowed to drift. In other words as the nutrients were assimilated, the concentration decreased. This is particularly critical in the case of the 10mM experiments in which the concentrations were typically  $< 1 \mu\text{M}$  after 24 hours. These experiments need to be repeated using a constant concentration protocol in which nutrients are added during the experiment to compensate for those assimilated.



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