The Origin of Nitrogen Isotope Values in Algae

Saturday, May 07, 2011

A Research Project Conducted by the
Stable Isotope Laboratory at the:
The University of Miami

PETER K. SWART & SAMANTHA EVANS
TOM CAPO

For:
The U.S. Environmental Protection Agency

EPA

United States
Environmental Protection Agency

Contact Information:

Peter K. Swart
Professor and Chair Marine Geology and Geophysics
MGG/RSMAS
University of Miami
4600 Rickenbacker Causeway
Miami Fl 33149
Pswart@rsmas.miami.edu
305 421 4103

Samantha Evans*
Post Doctoral Research Associate
MGG/RSMAS
University of Miami
4600 Rickenbacker Causeway
Miami Fl 33149
Sevans@rsmas.miami.edu
305 421 4903

*** Present Address: Department of Geosciences, Boise State University, 1910 University Drive, Boise, Idaho 83725-1535 (samevans@boisestate.edu)**

Tom Capo
Manager Aplysia Facility
MBF/RSMAS
University of Miami
4600 Rickenbacker Causeway
Miami Fl 33149
tcapo@rsmas.miami.edu
305 421 4946

Any comments regarding this report should be referred to Dr. Peter K. Swart

Acknowledgments: We would like to thank the staff of the Aplysia Facility and the Stable Isotope Laboratory at the University of Miami. Additional funding for this project was provided by the Stable Isotope Laboratory at the University of Miami.

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EXECUTIVE SUMMARY

- The fractionation factor (α) accompanying assimilation of NO_3^- and NH_4^+ has been measured in two species of macro algae (*Ulva sp*. and *Agardhiella sp*) utilizing two different types of experiments. In the first, a free drift approach was used in which cultures were initially maintained at a starting concentration of between 10 to 500 μM and the solutions were replaced every 24 hours with fresh water with similar nutrient concentrations. In the second set of experiments a syringe pump continually added nutrients to the algal cultures in order to maintain a constant low nutrient concentration (< 10 μm). In addition separate experiments were carried out to measure the change in $\delta^{15}N$ of the DIN during the assimilation. Data obtained from both approaches were modeled in order to calculate the fractionation during assimilation.

- For *Agardhiella sp.* the values calculated for the assimilation of NO_3^- appear to vary depending upon the concentration of NO_3^- in the solution. At high concentrations (100 and 500 μM), a larger fractionation factor fitted the data (α=1.006) while at low concentrations the data were better fitted with a value of 1.0025. Modeling the solid algae data produced values which did not fit the Rayleigh distillation model as well. This is probably because the growth of the algae varies as a function of nutrient concentration.

- For *Ulva sp.,* the nitrate data shows excellent fit with a model suggesting an a value of 1.0025. The solid data did not show as good a fit with the nitrate data.

- In the case of NH_4^+ fractionation factors as large as 1.010 was modeled using both the algae and the DIN data. For both species.

- The syringe experiments in which the concentration of nitrate and ammonia was kept low, exhibited only minor amounts of fractionation for ammonia , but a similar amount of fractionation for nitrate. The discrepancy in the ammonia experiments is believed to result from nitrification in the free drift experiments, producing depleted NO_3^- and enriched NH_4^+.

- The experiments show that the C:N ratio of the algae is inversely proportional to the concentration of DIN.

The Determination of the Fractionation Factors of Stable Nitrogen Isotopes During Assimilation of Nitrate and Ammonium by Macro-Algae

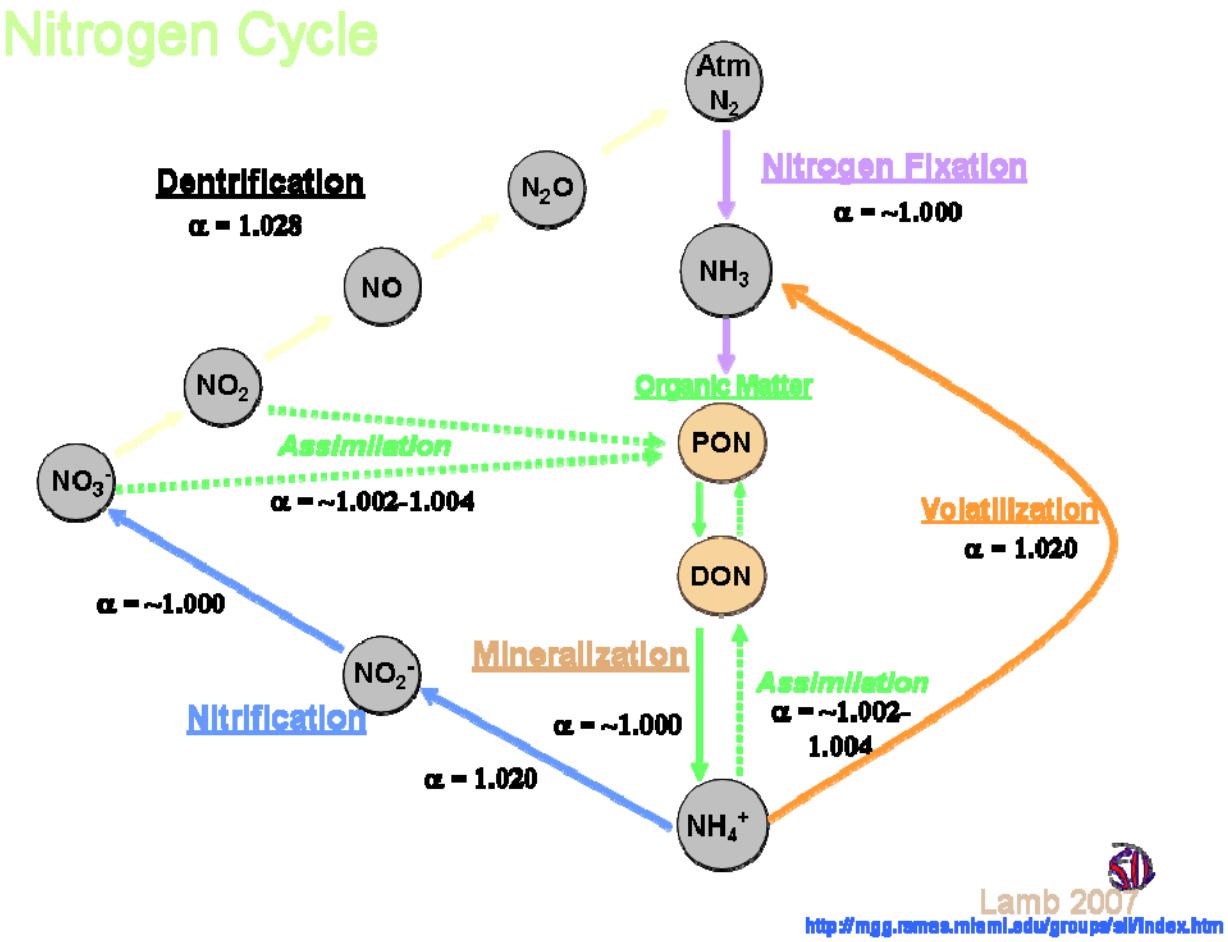

Figure 1: Biogeochemical processes and associated nitrogen isotopic fractionation factors of the nitrogen cycle. The value α is defined as the fractionation factor. The term ε is defined as $(\alpha-1)*1000$ and is equal to the difference in $\delta^{15}N$ between two compounds so that is $\alpha = 1$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

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 significant number of publications on the $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 NH_4^+ and 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 μM. In contrast the DIN is present at much lower concentrations; NO_3^- = 0.01 to 1 μM, NO_2^- = 0.01 to 0.1 μM, and NH_4^+ = 0.1 to 1 μM. 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 μM and < 0.1 μM). 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 to consider the origin of the natural variability. Nitrogen has two stable isotopes (^{15}N and ^{14}N), the concentrations of which are measured relative to atmospheric nitrogen ($^{15}N/^{14}N \sim 0.007$) 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 $\delta^{15}N$ close to that of atmospheric nitrogen (0 ‰). During mineralization of the dead plant remains, the organic nitrogen is converted to NH_4^+ with minimal fractionation and initially the NH_4^+ will have an isotopic composition similar to the initial plant (i.e. $\delta^{15}N$ = 0 ‰). During nitrification the NH_4^+ is converted to NO_2^- and then to NO_3^-. Each of these processes has a fractionation factor (α) of 1.02 (Miyake and Wada 1971), so that the resultant product is about 20‰ more negative than the initial NH_4^+. During this process in a closed system, the residual NH_4^+ becomes enriched in ^{15}N and the NO_3^- depleted in ^{15}N.

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 NH_4^+ and relatively low $\delta^{15}N$ values. Very low rates of mineralization would produce low concentrations of NH_4^+ relative to NO_3^- and higher $\delta^{15}N$ values. A further variable in this model is the assumption that there is no addition of NH_4^+ or NO_3^- from other sources. This is obviously incorrect and would serve to alter the isotopic composition of the NO_3^- and 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 $\delta^{15}N$ values of the algae reflect fractionation processes taking place in these sources. For example, the $\delta^{15}N$ of upwelled waters is influenced by denitrification (See Figure 1). This process removes isotopically light nitrogen leaving the nitrate enriched in ^{15}N. Denitrification also takes place in the sediments leading to a flux of nitrate enriched in ^{15}N from the sediments. Further

fractionation in the system occurs during assimilation of NO_3^- or NH_4^+ by plants and algae. For example, if we consider a body of water containing a specified amount of NO_3^- , then as it is assimilated by algae or other plants, the residual nitrate becomes fractionated leaving the heavier isotope behind. Hence the $\delta^{15}N$ of the residual nitrogen becomes isotopically enriched. A similar situation may occur in an NH_4^+ dominated system, although the fractionation factors involved are not precisely defined.

Figure 2: Experimental protocols. A. Ulva sp.; B. Agardhiella sp.; C. Flow-through tank system at Aplysia Mariculture Facility; D. Agardhiella sp grown under differing concentrations of nitrate.

In this work we have examined the influence of nutrient concentration upon the assimilation of ^{15}N into benthic macroalgae. This work was based on the results of a previously funded EPA project (2006-2007) in which we established that macro-algae have much larger isotopic fractionation factors (4-9 ‰) during the assimilation of nitrate and ammonium than previously reported. The implication of these large fractionation factors is that as the algae assimilate these nutrients, they enrich the isotopic composition of the remaining pool of dissolved inorganic nitrogen (DIN). This enrichment was unrelated to the source of either the nitrate or the ammonium and casted doubt on previous conclusions in many studies attributing isotopic enrichment in algal tissue to anthropogenic nutrient inputs (sewage) to coastal waters. In the work carried out between 2009-2010 we built on the results from the first study, carrying out further

experiments that will unequivocally establish values of macroalgal isotopic assimilation factors. Extending the original experiments included additional algal species, the measurement of the fractionation factors at different nutrient concentrations, and the measurement of the $\delta^{15}N$ of the nitrate and ammonium in the water during the experiments.

<u>*Experiment Set 1*</u>: The series of experiments were conducted which replicated previous work varying the concentrations of either NO_3^- or NH_4^+ in the medium. Concentrations ranged from 0.5 μM to 100 μM. These incubations were applied to two species (*Ulva sp.*, and *Agardhiella sp.)*. The uptake rates of NO_3^- and NH_4^+ was measured in each of the experiments and based on these measurements nutrients were added at the appropriate rate using a syringe pump in order to maintain the DIN concentration at an appropriate level. In order to ensure that the rate of addition is correct we continued to monitor concentration and adjust the rate as appropriate. This method contrasted to our previous experiments which were essentially free drift experiments, with the concentration of DIN being augmented every 24 hours. In addition, this method accurately allowed us to determine the assimilation factor at solutions with low DIN concentrations. At the end of the experiment the new growth was removed and analyzed (Figure 3).

D.

Figure 3: Sample of <u>Agardhiella sp</u>. Grown in nutrient treatment. Tips of samples were trimmed for nitrogen isotopic analyses of growth in new treatment.

<u>*Experiment Set 2*</u>: We also repeated some of the free drift experiments so we can determine the changes in the $\delta^{15}N$ of the DIN. These experiments allowed us to replicate the previous experiments, to add the green alga, *Ulva sp*. In contrast to the previous experiments we analyzed water samples for the $\delta^{15}N$ of dissolved N species allowing calculation of assimilation fractionation, nitrification rate, and organic N exudation from the algal thalli. This was not done during the previous free drift experiments.

METHODS AND APPROACH

Samples of *Ulva sp.*, (Fig 2A) and *Agardhiella sp*. (Fig 2B) cultures were collected from the *Aplysia* Mariculture Laboratory's algal aquaculture facility (University of Miami). Samples of the algae *Gracillaria sp.* were analyzed in a previous EPA funded study. These were not examined in this study as it is difficult to separate new from old growth. 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 100µmol photons m^{-2} s^{-1} for a 14-day acclimation period. During the acclimation period, filtered and autoclaved seawater was changed every 2 days, enriched to 500µM N (250 µM $NaNO_3$ and 250 µM NH_4Cl) and 44 µM KH_2PO_4, with f/2 medium supplements of B-vitamins (Vitamin B_{12}, Biotin, and Thiamine) and trace metals (Fe, Cu, Mo, Zn, Co, Mn) (Guillard 1975). Aeration was provided by bubbling with compressed air.

In the case of the experiments carried out to check changes in the $\delta^{15}N$ of the DIN, two different protocols were used. In the case of *Agardhiella sp.,* the algae were grown in solutions of between 10-500 µM DIN and the water sampled after 24 hours. In the case of the 500 µM treatment the water was sampled after 48 hours as well. In the case of *Ulva*, water was sampled at intervals of 12, 24, and 48 hours.

Experimental Protocol

Nutrient concentration experimental protocol

Figure 4: Elemental analyzer and stable isotope mass spectrometer utilized in this study.

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 NO_3^- concentration, one with varied NH_4^+ concentrations. In both cases, concentrations of 10, 50, 100, and 500 µM N (as $NaNO_3$ and NH_4Cl) were supplied in a medium of autoclaved, filtered (0.2µm cartridge filter) seawater enriched with the same KH_2PO_4, B-vitamin, and trace metal supplements outlined for the acclimation medium. Subsamples of the green

algae *Ulva* and rhodophyte algae and *Agardhiella 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 2000 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 concentrations of both NO_3^- or 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 for subsequent N and C isotopic analyses.

Analytical Protocol

Stable Isotopes

Solids: The organic carbon and nitrogen contents as well as the stable nitrogen isotopic composition ($\delta^{15}N$) of the algae was determined using a modified CN analyzer interfaced with a continuous-flow isotope-ratio mass spectrometer (CFIRMS) (20-20, Europa Scientific) (Figure 4). 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 $\delta^{15}N$ content of the organic nitrogen (measured as N_2). Carbon isotopic values were also determined but these are not reported here. Samples of the nutrient material were analyzed in a similar manner in order to provide information on the initial $\delta^{15}N$ of the medium. Data are reported relative to conventional international standards (atmospheric nitrogen for N_2). External precision is approximately 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.

Waters: The $\delta^{15}N$ and $\delta^{18}O$ composition of the samples were determined on a Finnigan MAT 251 with an external automated purge-and-trap system at the University of Massachusetts, Dartmouth, SMAST campus. Data are reported relative to atmospheric N_2 and VSMOW for nitrogen and oxygen, respectively. Each run of $NO_2^- + NO_3^-$ samples consisted of one operational blank (low nutrient seawater treated with azide), three NO_2^- standards, a cadmium blank (low nutrient seawater treated with cadmium), and three NO_3^- standards (USGS 34, 35, and KNO_3), followed by the prepared samples. Three randomly selected samples were also prepared in triplicate to check for method and machine reproducibility. The run ended with three more NO_2^- standards, three NO_3^- standards, a cadmium blank, and an operational blank. Each run of DON + NO_3^- +NO_2^- consisted of all the above mentioned standards and blanks, plus an additional persulfate blank (low nutrient seawater treated with $K_2S_2O_8$) and an amino acid standard at the beginning and end of each run. Analytical precision measured from multiple determinations on standards was approximately ±0.2 ‰ for $\delta^{15}N$ and ±0.7 ‰ for $\delta^{18}O$.

Isotopic data produced from each run were scrutinized for standard precision throughout individual runs. Samples were corrected for the oxygen exchange that occurs between the sample and water during the conversion to nitrous oxide, and fractionation due to oxygen removal (see McIlvin and Altabet (2005) for an in depth discussion of

$\delta^{15}N$ and $\delta^{18}O$ corrections). The results of the isotopic measurements also needed to be transformed one step further. Since this method involved the stepwise reduction of N species, the actual samples analyzed were not just NO_3^- or DON individually; they were the measured $NO_2^- + NO_3^-$ and $NO_2^- + NO_3^-$ + DON, respectively. Consequently, the extraneous species needed to be accounted for, in order to ascertain the "real" isotopic composition of the targeted N species. If the $[NO_2^-]$ were below 0.25μM, then the $NO_2^- + NO_3^-$ results treated as a measurement of NO_3^-. For the DON measurements, however, the original NO_3^- from the sample would contribute to the signal, and therefore needed to be accounted for with the following equation:

$$\delta DON = \frac{([TN]*\delta TN) - ([NO_3^-]*\delta NO_3^-)}{([TN] - [NO_3^-])} \quad (1)$$

where [TN] is the measured concentration of the $NO_2^- + NO_3^-$ + DON, the δTN is the measured isotopic composition of the $NO_2^- + NO_3^-$ + DON, the $[NO_3^-]$ is the measured NO_3^- concentration, and δNO_3^- is the measured isotopic composition of the NO_3^-.

Nutrient Concentrations

Concentration of NO_3^- and NH_4^+ were analyzed prior to, during, 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.

RESULTS

Nutrient uptake experiments

Results from the free drift nutrient experiments from *Ulva* sp. and *Agardhiella sp.* are presented in Table 1 & 2. Data from all experiments are presented in Figures 5 to 8. These graphs document changes in the $\delta^{15}N$ 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.

Solid Isotopic results - Nitrate: The initial $\delta^{15}N$ of the *Agardhiella sp.* was ~ -0.5 ‰. The $\delta^{15}N$ decreased from 1.8 ‰ (10 μM) to 1.6 ‰ in the 50 μM treatment, decreased to 0.7 in the 100 μM treatment and finally to -3.00 ‰ in the 500 μM experiment (Figure 6). Similar results were found in the experiments using *Ulva sp.* although the $\delta^{15}N$ values were all higher. The C:N ratio increased in the low nitrate experiments in both species of algae and steadily decreased with increasing nitrate .

Solid Isotopic results - Ammonium: The general trend of the $\delta^{15}N$ results was similar to those of the nitrate experiments (Figure 5). The most depleted values occurred in the elevated concentration treatments. The C:N ratio increased in the low ammonium experiments in both species of algae and steadily decreased with increase nitrate .

Solid Carbon and Nitrogen Ratios: The C:N ratios in both the nitrate and ammonium experiments exhibited very strong relationship to the concentration of DIN in the culture (Table 1 and 2).

Isotopic analysis of waters: Every 24 hours the free drift experiment water medium was replaced and the nutrient concentration restored to the original starting value. In order to examine the affect of the assimilation on the fractionation, special experiments were performed in which the same water was kept in the algal cultures for periods of up to 48 hours. After 12, 24, and 48 hours water samples were taken and the $\delta^{15}N$ of the NO_3^- and NH_4^+ measured. In addition the $\delta^{18}O$ of the nitrate was analyzed. This report presents data from the NO_3 analyses only. These data are presented in Table 3 and 4 and graphically in Figures 8 and 9. The trend in the $\delta^{15}N$ mirrored that of the solid algae. As the nitrate was consumed, the residual nitrate became isotopically enriched (Figure 7). During this process the $\delta^{18}O$ shows an approximate 1:1 ratio with the $\delta^{15}N$ (Figure 8). The NH_4^+ analyses are given in Table 5 and 6. These data show a significantly larger change in the $\delta^{15}N$ as a function NH_4^+ utilization.

Syringe Experiment Results

Specimens of *Ulva* and *Agardiella* were maintained in solutions with less than 10 μM NO_3^- and NH_4^+. The concentrations were measured continually and eventually concentrations were maintained at less than 3 μM NO_3^- and approximately 1 μM NH_4^+ . The $\delta^{15}N$ of the NH_4^+ and NO_3^- was not measured during the course of the experiments as these nutrients were continually being added to the solution. The $\delta^{15}N$ of the algae measured at the end of the experiment was +1.77 (+/- 1.2) ‰ for the algae maintained in the NO_3^- solutions and +2.44 (+/- 0.33) ‰ for the NH_4^+ experiment. The C:N ratio in these experiments was the lowest of any produced (~30) indicating the general low concentrations of DIN.

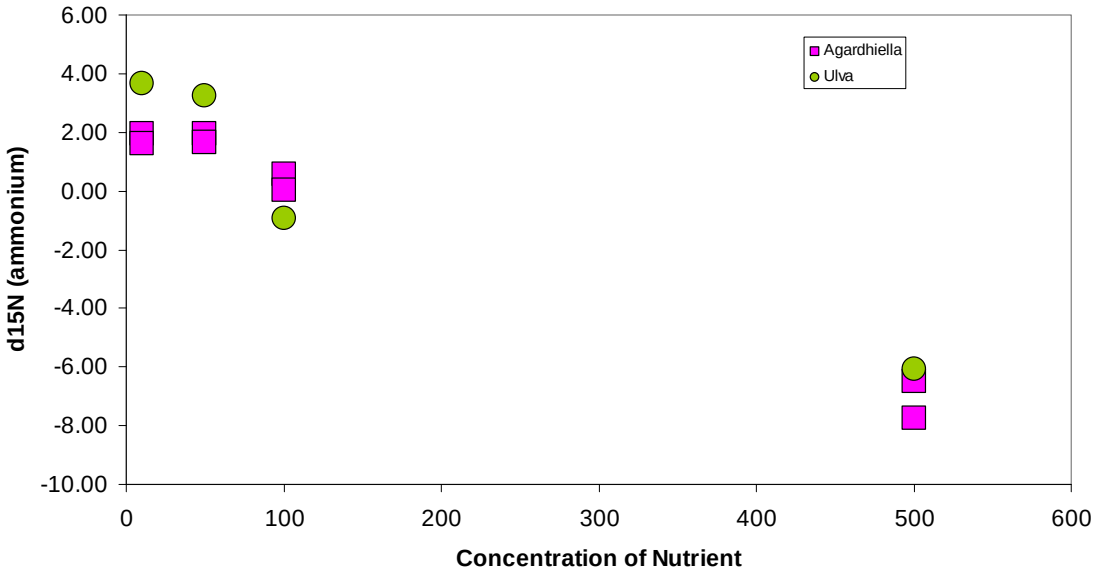

Figure 5: Change in the $\delta^{15}N$ of the algae in the free drift experiments using the data presented in Tables 1 and 2 for NH_4+.

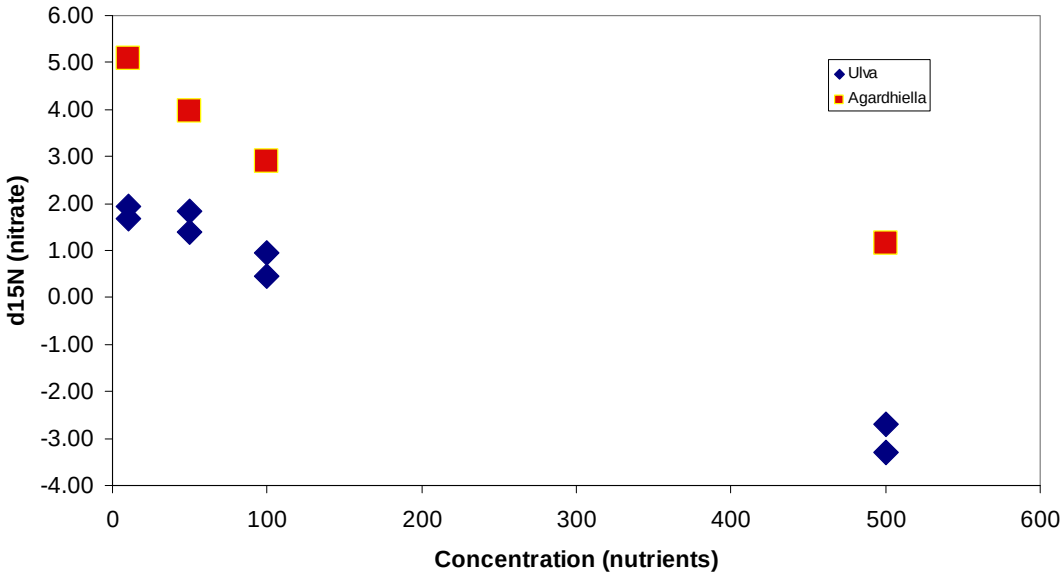

Figure 6: Change in the $\delta^{15}N$ of the algae in the free drift experiments using the data presented in Tables 1 and 2 for NO_3-.

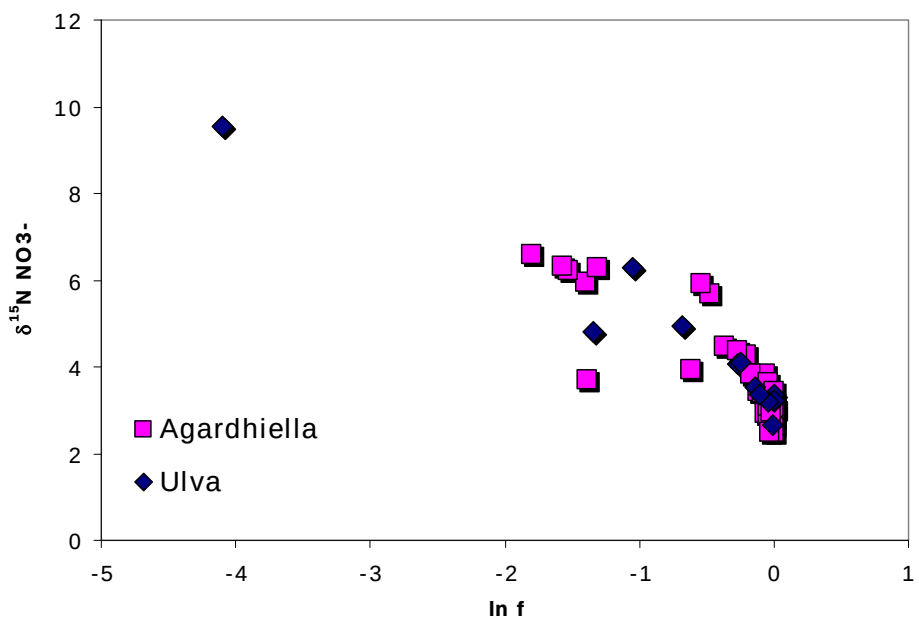

Figure 7: Plot of the fraction of nitrate utilized in each experiment (ln f) against the measured $\delta^{15}N$ of the residual nitrate.

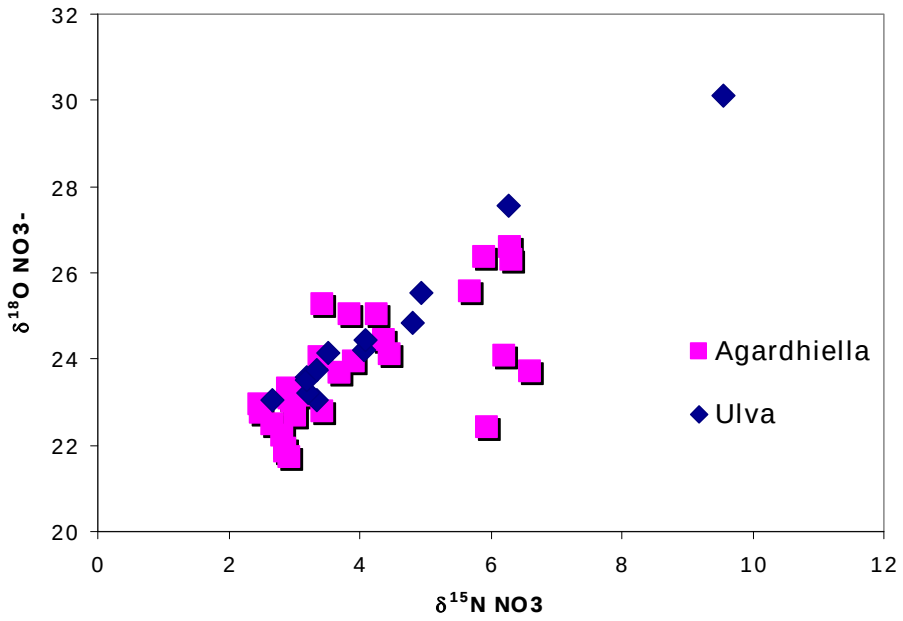

Figure 8: Plot of the $\delta^{15}N$ and $\delta^{18}O$ of the residual nitrate.

DISCUSSION

Free Drift Experiments: Nitrate

Agardhiella: The change in the $\delta^{15}N$ of the nitrate and the algae can be modeled using a Rayleigh distillation model in order to calculate the fractionation during assimilation. Ideally the results of these calculations should provide similar fractionation factors. Best fit models to both set of data are shown in Figure 9. The value of 1.0025 has been

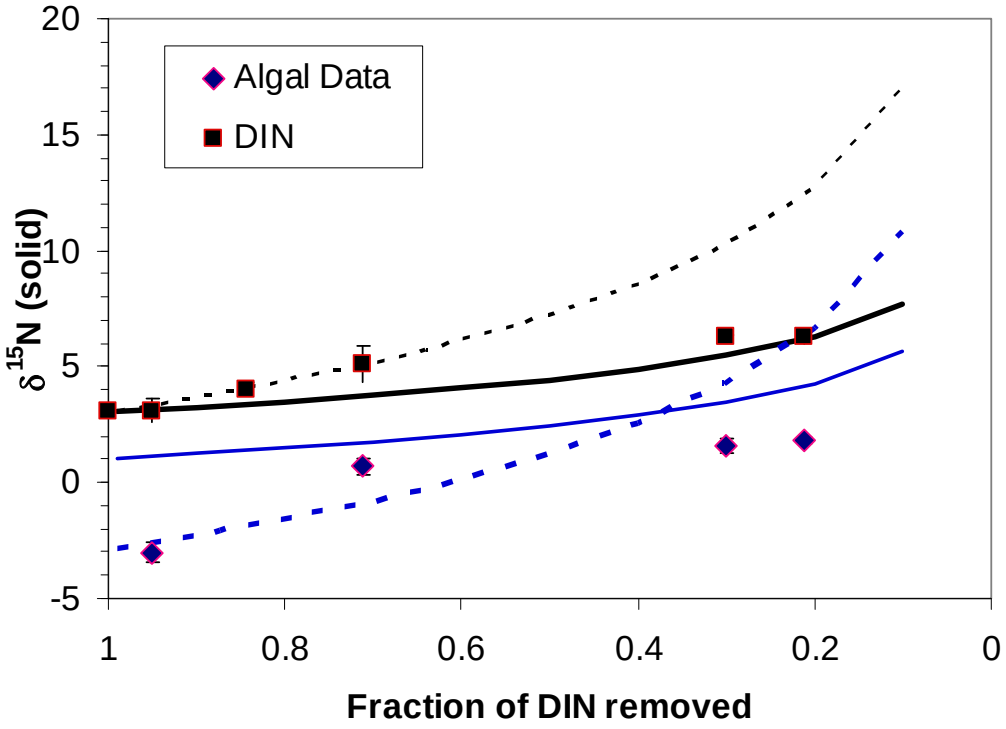

Figure 9: Rayleigh distillation model fits to the data from the *Agardhiella sp.* and the nitrate (DIN). The model (solid line) uses a fractionation factor of 1.0025 and 1.006. The value of 1.0025 has been optimized for the DIN to give the lowest difference between the model and the actual data. The data suggests that there may be two types of fractionation process in operation, one at low concentrations and one at high concentrations (dashed line). The high concentration data are bested fitted with a fractionation factor of 1.006.

optimized for the DIN to give the lowest difference between the model and the actual data. The data suggests that there maybe two types of fractionation process in operation, one at low concentrations and one at high concentrations (dashed line). The high concentration data are best fitted with a fractionation factor of 1.006. The problem with utilizing the algal data to calculate precise fractionation factors is that the $\delta^{15}N$ of the algal biomass produced in the experiment is a function of the $\delta^{15}N$ of the water, which is continually changing during the course of the experiment as the concentration is reduced and the nitrate is fractionated. There may be a growth rate dependency upon the concentration and therefore the growth may be higher at the start of the incubation and

lower near the end. Without knowing this relationship it is not possible to use these data in a quantitative manner to estimate fractionation.

Ulva: In contrast to the *Agardhiella sp.* data the fractionation factor of 1.0025 seems to fit all the data extremely well. Once again this value has been chosen to minimize the difference between the measured and model data (Figure 10).

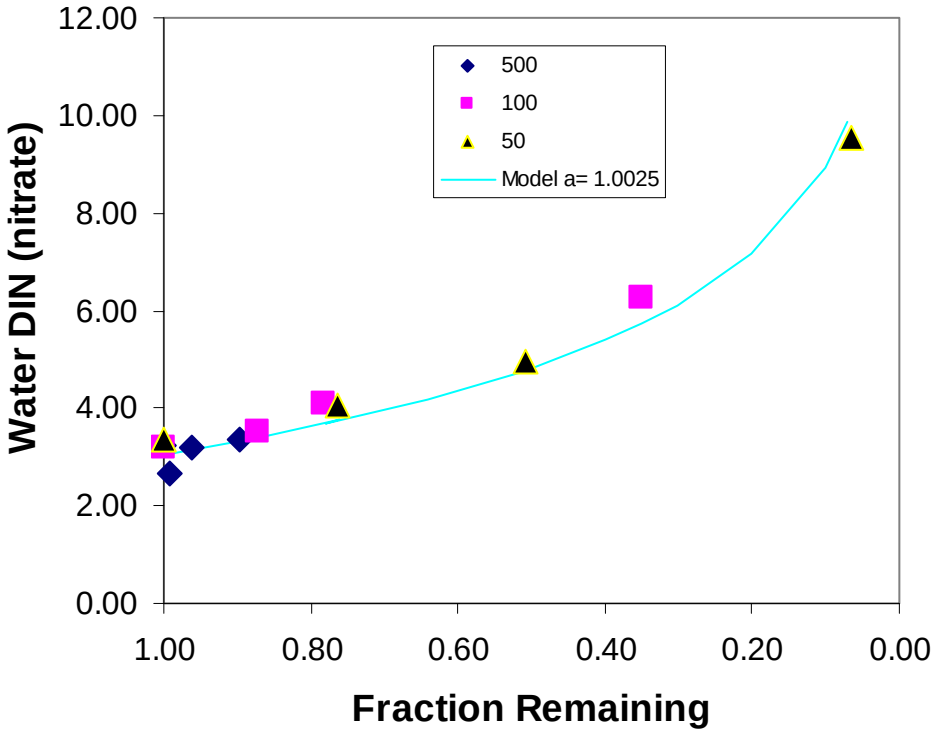

Figure 10: The $\delta^{15}N$ of the nitrate in the *Ulva sp.* free drift experiment. Data from the 10 µM experiment were lost. The other experiments all contain three data points in addition to the initial value. These were from 12, 24, and 48 hours. The model line was calculated using a fractionation factor of 1.0025. This is the same value which fitted the *Agardhiella sp.* data.

The solid data (Figure 11) do not seem to fit the model data as well as the DIN data (as in the case of *Agardhiella sp.*). This is probably for the same reasons postulated to explain the absence of fit in *Agardheilla sp*.

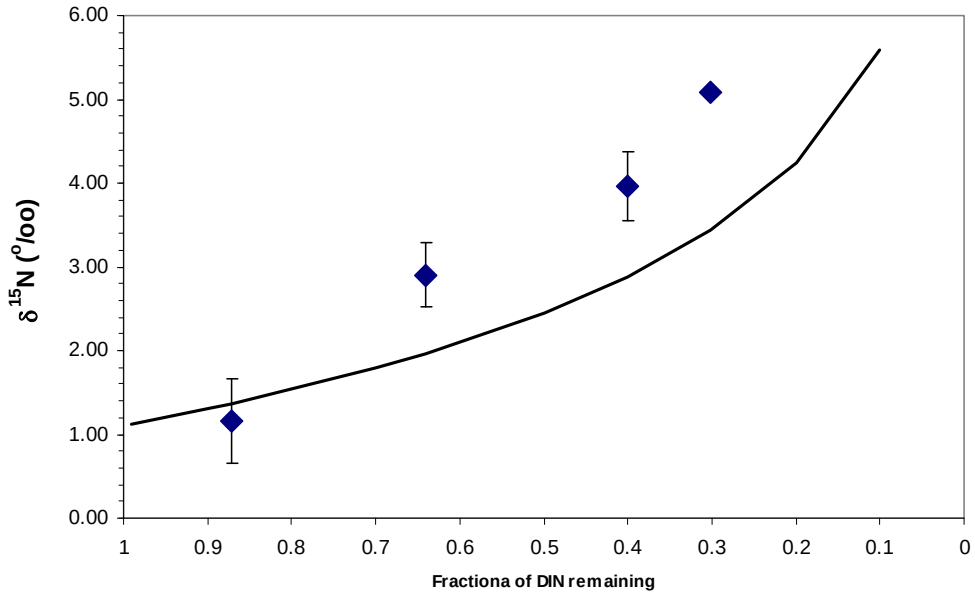

Figure 11: Data from the solid Ulva sp. samples. The solid line represents a model with a fractionation factor of 1.0025 as estimated using the DIN data.

Free Drift Experiments: Ammonia

Agardhiella: The change in the $\delta^{15}N$ of the ammonia and the algae can be modeled using a Rayleigh distillation model in order to calculate the fractionation during assimilation. The data and the modeling results are shown in Figures 12 and 13. Both the modeling of the DIN and the algae provide a best fit solution using an α value of 1.010.

Ulva sp: The data for *Ulva sp.* are shown in Figures 14 and 15. These show similar fractionation factors when compared to *Agardhiella sp*.

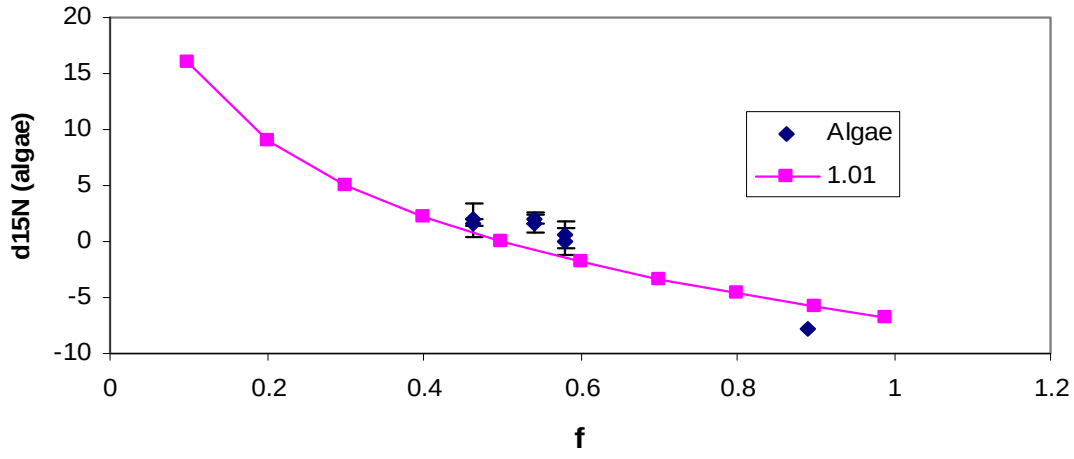

Figure 12: Changes in the $\delta^{15}N$ of Agardiella sp. grown in 10, 50, 100, and 500 μM NH_4^+. The best fit is provided by an α value 1.010. The error bars show the range of value measured on replicates.

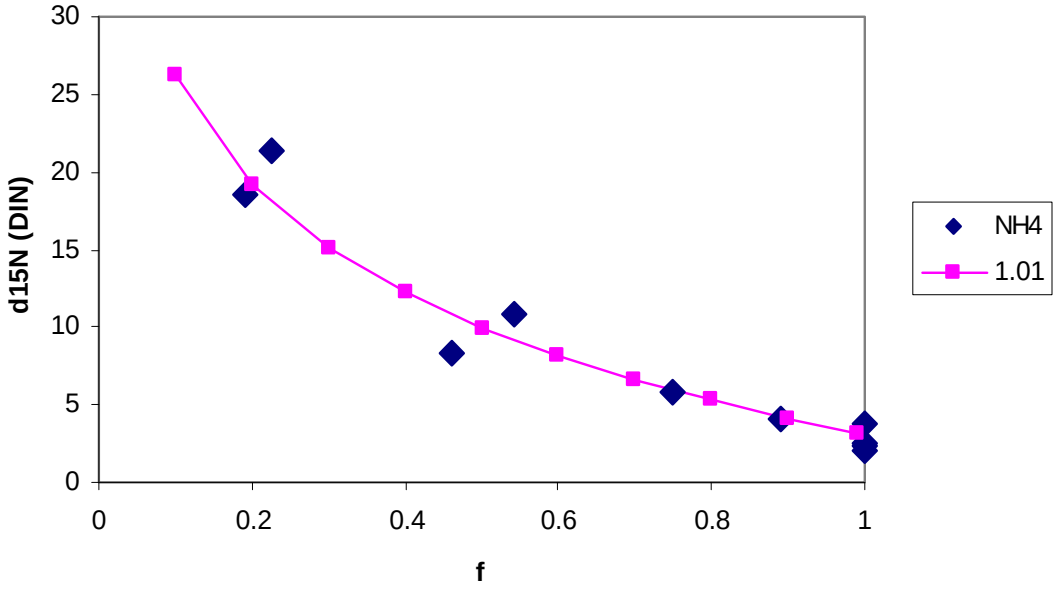

Figure 13: Changes in the $\delta^{15}N$ of NH_4^+ during assimilation in 10, 50, 100, and 500 μM NH_4^+ free drift experiment with Agardiella sp. The best fit is provided by an α value 1.010. This value is similar to that calculated using the solid samples.

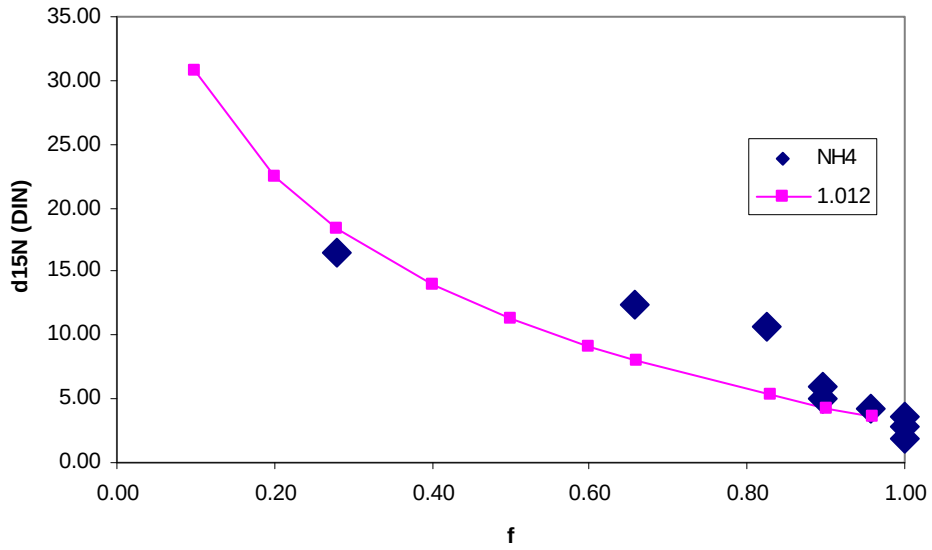

Figure 14: Changes in the $\delta^{15}N$ of NH_4^+ during assimilation in 10, 50, 100, and 500 μM NH_4^+ with Ulva sp. The best fit is provided by an α value 1.010. This value is similar to that calculated using the solid samples.

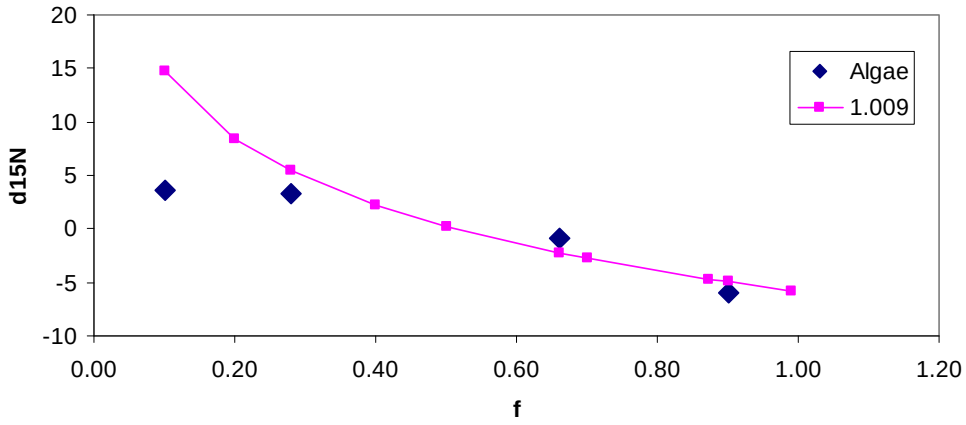

Figure 15: Changes in the $\delta^{15}N$ of Ulva sp. grown in 10, 50, 100, and 500 μM NH_4^+ . The best fit is provided by an α value 1.012. The error bars show the range of value measured on replicates.

Syringe Experiments

The data from the syringe experiments illustrate the influence of fractionation from a solution of constant, but essentially unlimited low nutrient concentration. In these experiments nearly all the nutrients added were utilized by the algae. The DIN was rapidly being utilized, but by adding DIN we maintained the concentration between 2-4 μM, similar to concentrations found under natural conditions (Figure 16). In free drift experiments for NH_4^+ there was clearly quite a large fractionation factor (α= 1.010 to 1.009) for the assimilation of NH_4^+. This fractionation factor was manifested to a reduced degree in the syringe experiments. The algae grown in NH_4^+ had a $\delta^{15}N$ of +2.44, a reduction of 0.7 ‰ compared to the initial DIN $\delta^{15}N$ value. This reduced the effective fractionation to 1.0007 for the incorporation of NH_4^+ into *Ulva sp*. In the case of NO_3^- the value of the *Ulva sp.* was 0.84 ‰. The difference between the original algae and that produced is 2.25 giving an α value of 1.0025, a value identical to that using the free drift experiments.

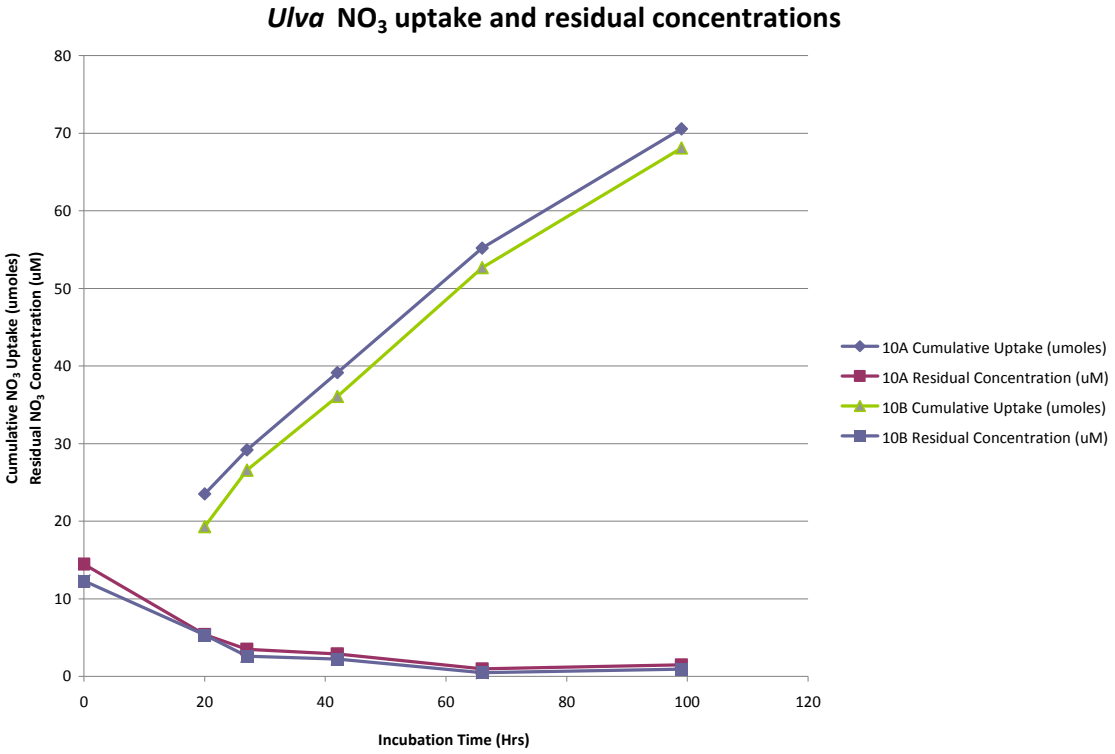

Figure 16: Change in the concentration of NO_3^- in the Ulva sp. syringe experiment. Note that the experiment started with 10 μM and was maintained at between 1-3 μM NO_3^-.

The explanation for the difference between the fractionation factor calculated from the free drift experiments and syringe experiments for NH_4^+ is probably a result of fractionation during nitrification during the experiments. There is a well known fractionation approximating 1.020 during nitrification. This would have the effect of producing residual NH_4^+ enriched in $\delta^{15}N$ and depleted NO_3^-. Nitrate was not detected in

these samples but it is likely that the nitrate would have been assimilated as soon as it was produced. Nitrite would have also been produced and this was detected in the higher concentration free drift experiments.

Carbon to Nitrogen Ratios

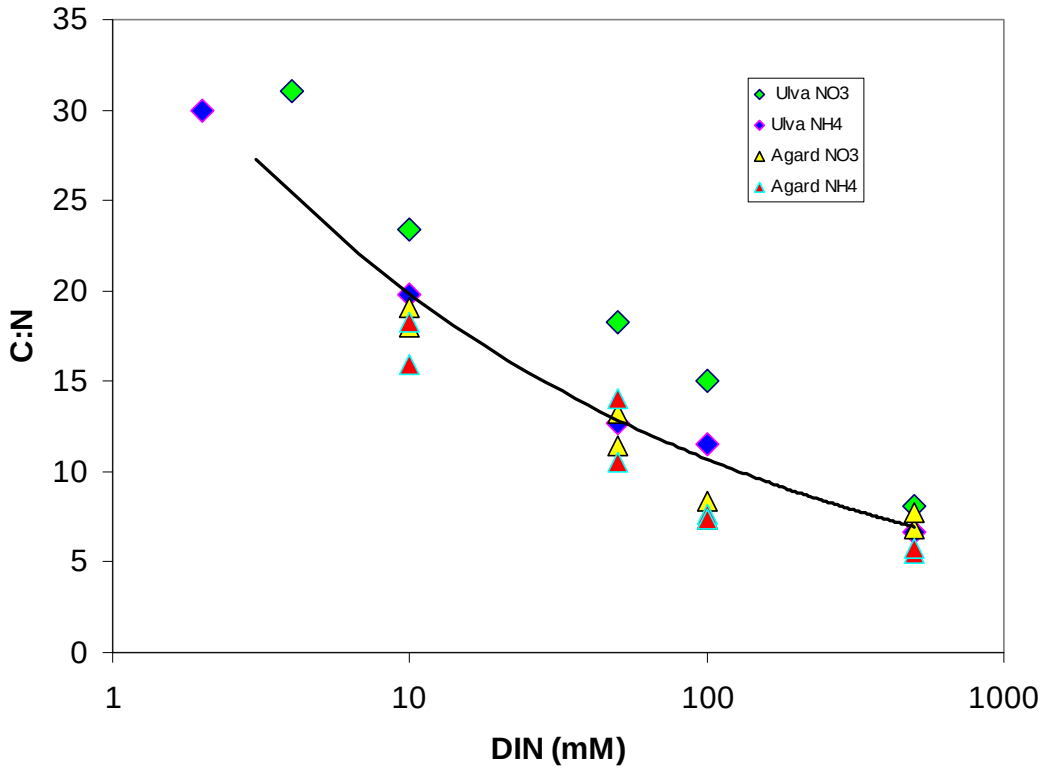

Figure 17: The change in the C:N ratio relative to the concentration of DIN in the environment including the free drift and the syringe experiments.

The C:N ratio is strongly dependent upon the concentration of DIN in the growth solution and follows an exponential relationship (Figure 17).

CONCLUSIONS

- The fractionation factor (α) accompanying assimilation of NO_3^- and NH_4^+ has been measured in two species of macro algae (*Ulva sp*. and *Agardhiella sp*) utilizing two different types of experiments. In the first, a free drift approach was used in which cultures were initially maintained at a starting concentration of between 10 to 500 μM and the solutions were replaced every 24 hours with fresh water with similar nutrient concentrations. In the second set of experiments a syringe pump continually added nutrients to the algal cultures in order to maintain a constant low nutrient concentration (< 10 μm). In addition separate experiments were carried out to measure the change in $\delta^{15}N$ of the DIN during the assimilation. Data obtained from both approaches were modeled in order to calculate the fractionation during assimilation.

- For *Agardhiella sp.* the values calculated for the assimilation of NO_3^- appear to vary depending upon the concentration of NO_3^- in the solution. At high concentrations (100 and 500 μM), a larger fractionation factor fitted the data (α=1.006) while at low concentrations the data were better fitted with a value of 1.0025. Modeling the solid algae data produced values which did not fit the Rayleigh distillation model as well. This is probably because the growth of the algae varies as a function of nutrient concentration.

- For *Ulva sp.,* the nitrate data shows excellent fit with a model suggesting an a value of 1.0025. The solid data did not show as good a fit with the nitrate data.

- In the case of NH_4^+ fractionation factors as large as 1.010 were modeled using both the algae and the DIN data. For both species.

- The syringe experiments in which the concentration of nitrate and ammonia was kept low, exhibited only minor amounts of fractionation for ammonia , but a similar amount of fractionation for nitrate. The discrepancy in the ammonia experiments is believed to result from nitrification in the free drift experiments, producing depleted NO_3^- and enriched NH_4^+.

- The experiments show that the C:N ratio of the algae is inversely proportional to the concentration of DIN.

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	ID	$\delta^{15}N$	σ	$\delta^{13}C$	σ	CN	σ
NO3							
	10	1.94	0.05	-23.60	0.01	19.06	0.57
	10	1.69	0.16	-24.10	0.24	18.01	1.73
	50	1.82	0.10	-22.31	0.23	11.40	0.90
	50	1.39	0.31	-19.64	0.00	13.19	0.98
	100	0.46	0.17	-20.30	0.12	8.33	0.05
	100	0.95	0.20	-24.63	0.34	7.41	0.49
	500	-3.30	0.11	-23.09	0.51	6.82	0.24
	500	-2.70	0.68	-22.88	0.13	7.73	0.42
NH4							
	10	1.94	1.49	-24.94	0.10	15.97	0.67
	10	1.63	1.48	-25.19	1.21	18.25	2.11
	50	1.97	0.30	-23.86	0.35	14.03	5.94
	50	1.67	0.40	-23.24	0.31	10.52	1.16
	100	0.56	0.84	-25.52	0.39	7.67	0.73
	100	0.05	1.19	-23.29	1.59	7.38	0.28
	500	-7.76	1.16	-21.64	0.06	5.51	0.34
	500	-6.50	1.35	-22.83	0.75	5.75	0.09

Table 1: Results of the C and N isotopic analyses of the free drift experiment for *Agardhiella* sp. Each row represents the mean of two values. Experiments were performed in duplicate.

NO3	ID	$\delta^{15}N$	σ	$\delta^{13}C$	σ	CN	σ
	10	5.08	0.04	-19.67	0.26	23.41	1.85
	50	3.96	0.40	-19.65	0.40	18.26	0.58
	100	2.91	0.38	-19.58	0.38	15.00	0.07
	500	1.16	0.51	-21.67	0.51	8.08	0.54
NH4							
	10	3.67	0.04	-17.51	0.11	19.75	1.02
	50	3.24	0.44	-17.85	0.39	12.71	0.37
	100	-0.93	0.29	-16.90	0.63	11.56	0.29
	500	-6.06	0.21	-16.54	0.14	6.63	0.10

Table 2: Result from free drift experiment for *Ulva* sp. Each row represents the mean of three values.

Sample ID	Conc.	Replicate	Time	NO3	$\delta^{15}N$	$\delta^{18}O$	ln f
NO3-1	10	A	0	14.75	3.16	16.83	0.00
NO3-2	10	B	0	13.82	3.84	16.36	-0.07
NO3-17	10	A	12	2.44	6.60	23.71	-1.80
NO3-18	10	B	12	3.66	5.93	22.43	-1.40
NO3-33	10	A	24	3.22	6.21	24.08	-1.53
NO3-34	10	B	24	3.71	3.69	23.67	-1.38
NO3-42	10	B	36	0.33			0.00
NO3-3	50	A	0	56.50	2.94	21.72	0.00
NO3-4	50	B	0	54.45	2.85	21.85	-0.04
NO3-35	50	A	24	30.34	3.92	23.95	-0.62
NO3-36	50	B	24	3.46			-2.79
NO3-43	50	A	36	0.33			-5.14
NO3-5	100	A	0	109.78	2.67	22.48	0.00
NO3-6	100	B	0	98.31	3.43	25.26	-0.11
NO3-37	100	A	24	68.62	5.68	25.58	-0.47
NO3-38	100	B	24	64.23	5.91	26.37	-0.54
NO3-7	500	A	0	532.89	2.48	22.94	0.01
NO3-8	500	B	0	509.72	2.90	23.32	-0.04
NO3-39	500	A	24	485.29	3.39	24.06	-0.09
NO3-40	500	B	24	495.32	2.92	23.08	-0.07
NO3-55	500	A	48	432.99	4.25	25.03	-0.20
NO3-56	500	B	48	445.04	3.85	25.03	-0.17
NO3-57	10	A	0	13.88	3.19	17.77	0.00
NO3-58	10	B	0	13.28	3.61	18.14	-0.05
NO3-65	10	A	24	2.10	6.38	21.32	-1.89
NO3-66	10	B	24	2.63	6.67	21.34	-1.66
NO3-59	50	A	0	55.77	2.82	22.22	0.00
NO3-60	50	B	0	53.43	2.87	21.93	-0.04
NO3-67	50	A	24	14.97	6.29	26.60	-1.32
NO3-68	50	B	24	11.56	6.32	26.28	-1.57
NO3-61	100	A	0	104.26	3.44	22.77	0.00
NO3-62	100	B	0	105.13	3.02	22.68	0.01
NO3-69	100	A	24	72.45	4.46	24.12	-0.36

Sample ID	Conc.	Replicate	Time	NO3	$\delta^{15}N$	$\delta^{18}O$	ln f
NO3-70	100	B	24	79.77	4.36	24.44	-0.27
NO3-63	500	A	0	514.05	3.05	23.23	0.00
NO3-64	500	B	0	499.63	2.49	22.75	-0.03
NO3-71	500	A	24	493.89	3.09	23.16	-0.04
NO3-72	500	B	24	503.95	2.97	23.03	-0.02

Table 3: Analysis of nitrate for O and N isotopes in the *Agardhiella sp.* experiments (See Table 1). Ln f represents the fraction of the nitrate assimilated.

Sample ID	Conc.	Time	NO3	$\delta^{15}N$	$\delta^{18}O$	ln f
			(uM)	ppt	ppt	
UM SE U-NO3 1	10	0	14.37	3.56	21.80	0.00
UM SE U-NO3 2	10	12	8.66	4.48	22.41	-0.51
UM SE U-NO3 3	10	24	3.76	4.81	24.85	-1.34
UM SE U-NO3 4	10	48	0.43	9.47	20.92	-3.52
UM SE U-NO3 5	50	0	60.31	3.35	23.04	0.00
UM SE U-NO3 6	50	12	46.05	4.06	24.20	-0.27
UM SE U-NO3 7	50	24	30.66	4.94	25.52	-0.68
UM SE U-NO3 8	50	48	1.00	9.54	30.12	-4.10
UM SE U-NO3 9	100	0	103.10	3.19	23.56	0.00
UM SE U-NO3 10	100	12	89.94	3.52	24.14	-0.14
UM SE U-NO3 11	100	24	80.68	4.10	24.44	-0.25
UM SE U-NO3 12	100	48	36.18	6.28	27.57	-1.05
UM SE U-NO3 13	500	0	485.29	3.22	23.22	0.00
UM SE U-NO3 14	500	12	466.74	3.19	23.53	-0.04
UM SE U-NO3 15	500	24	480.84	2.66	23.05	-0.01
UM SE U-NO3 16	500	48	434.59	3.35	23.74	-0.11

Table 4: Analysis of nitrate for O and N isotopes in the *Ulva sp*. experiments (See Table 1). Ln f represents the fraction of the nitrate assimilated.

ID	Treatment		Time	NH_4^+	F	$\delta^{15}N$
				μM		‰
NH4-1	10	A	0	15.00	0.99	1.38
NH4-2	10	B	0	15.10	1.00	1.28
NH4-57	10	A	0	14.52	0.96	2.78
NH4-58	10	B	0	12.14	0.80	2.63
NH4-17	10	A	12	10.48	0.69	2.19
NH4-18	10	B	12	8.25	0.55	8.69
NH4-34	10	B	24	8.28	0.55	3.98
NH4-65	10	A	24	3.51	0.23	
NH4-66	10	B	24	7.86	0.52	12.64
NH4-42	10	B	36	17.35	0.00	9.04
NH4-49	10	A	48	4.96	0.33	15.29
NH4-50	10	B	48	4.17	0.28	21.78
NH4-73	10	A	48	3.14	0.21	
NH4-74	10	B	48	0.87	0.00	4.62
NH4-3	50	A	0	61.09	1.00	2.52
NH4-4	50	B	0	58.06	0.95	3.24
NH4-59	50	A	0	57.51	0.94	2.73
NH4-60	50	B	0	49.85	0.82	1.57
NH4-35	50	A	24	36.77	0.60	11.08
NH4-36	50	B	24	54.06	0.88	8.48
NH4-67	50	A	24	21.11	0.35	13.09
NH4-68	50	B	24	10.69	0.17	
NH4-51	50	A	48	16.06	0.26	9.67
NH4-52	50	B	48	13.07	0.21	13.14
NH4-75	50	A	48	3.22	0.05	
NH4-76	50	B	48	2.88	0.05	23.03
NH4-5	100	A	0	130.83	1.10	2.48
NH4-6	100	B	0	118.54	1.00	2.38
NH4-61	100	A	0	110.31	0.93	1.98
NH4-62	100	B	0	102.93	0.87	2.79
NH4-37	100	A	24	75.66	0.64	11.96
NH4-38	100	B	24	76.08	0.64	8.54
NH4-69	100	A	24	52.72	0.44	21.55
NH4-70	100	B	24	64.58	0.54	11.53
NH4-53	100	A	48	27.48	0.23	22.91
NH4-54	100	B	48	39.20	0.33	18.54

ID	Treatment		Time	NH_4^+	F	$\delta^{15}N$
				µm		‰
NH4-77	100	A	48	9.30	0.08	23.08
NH4-78	100	B	48	28.38	0.24	21.19
NH4-8	500	B	0	654.40	1.00	2.72
NH4-64	500	B	0	431.66	0.66	4.82
NH4-39	500	A	24	588.53	0.90	3.38
NH4-40	500	B	24	574.27	0.88	4.60
NH4-72	500	B	24	584.90	0.89	3.48
NH4-55	500	A	48	420.39	0.64	5.22
NH4-56	500	B	48	410.68	0.63	6.66
NH4-79	500	A	48	560.52	0.86	5.60
NH4-80	500	B	48	571.93	0.87	5.46

Table 5: Concentration and $\delta^{15}N$ of NH_4^+ in *Agardhiella sp.* experiments

ID	Treatment	Period	NH_4^+	F	$\delta^{15}N$
			µm		‰
UM SE U-NH4 1	10	0	15.38	1.00	0.47
UM SE U-NH4 2	10	12	4.64	0.38	
UM SE U-NH4 3	10	24	6.20	0.10	6.49
UM SE U-NH4 4	50	0	60.85	1.00	2.86
UM SE U-NH4 5	50	12	61.16	0.83	10.66
UM SE U-NH4 6	50	24	24.89	0.28	16.51
UM SE U-NH4 7	100	0	115.99	1.00	1.94
UM SE U-NH4 8	100	12	100.07	0.90	5.02
UM SE U-NH4 9	100	24	68.94	0.66	12.42
UM SE U-NH4 10	500	0	577.90	1.00	3.61
UM SE U-NH4 11	500	12	525.00	0.96	4.17
UM SE U-NH4 12	500	24	578.94	0.90	5.95

Table 6: Concentration and $\delta^{15}N$ of NH_4^+ in *Ulva sp.* experiments