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Comparative examination of processes affecting the marine Nitrogen cycle of the ETNP

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NONTECHNICAL SUMMARY

Nutrients in the ocean are used by plankton at the bottom of the food web, which act as food for progressively larger organisms. In particular, nitrogen based nutrients are often in short supply, limiting the growth of the plankton. The process of denitrification converts these nutrients into nitrogen gas which escapes into the air, preventing them from being used. Denitrification can be broken into two processes, known as heterotrophic denitrification and the anammox reaction, which are both driven by bacteria. This study focused on these two processes in attempt to show which of them removes more nitrogen from the water. To do so, each nutrient was tagged with an isotope tracer and injected into bags of seawater. As the nutrients incubate in the bag, the bacteria inside converted the tagged nutrients into tagged nitrogen gas, which was measured using a mass spectrometer. The amount of nitrogen gas produced from each process is an indicator of the rates of heterotrophic denitrification and the anammox reaction. This information allows for an interpretation of each process' contribution to nitrogen removal in the ocean.

ABSTRACT

Rates of heterotrophic denitrification and anaerobic ammonium oxidation (anammox) differ from one another, especially under varying oceanic conditions and locations within an oxygen minimum zone (OMZ). Accordingly, there is some debate concerning which process is more dominant in a given mass of water, and why is removes more nitrogen. To measure the rates of these two processes, a ^{15}N stable isotope incubation method was used, measuring the amount of $^{15}N_2$ produced from samples incubated with $^{15}NO_2^-$ to observe rates of denitrification and $^{15}NH_4^+$ to observe anammox. Mass spectrometer analysis of the incubated samples recorded anammox occurring at two different locations, at a rate of 1.02 nmol L^{-1} d^{-1} at station 1 and 0.762 nmol L^{-1} d^{-1} at station 2. No heterotrophic denitrification was observed at the two sampling locations within the Eastern Tropical North Pacific (ETNP), making anammox the primary denitrifying process in the area of study. This data contributes to the knowledge behind the marine nitrogen cycle- an important process that ultimately affects nutrient availability, productivity, and carbon uptake.

INTRODUCTION

The marine nitrogen cycle is incompletely understood. Several processes affect the nitrogen compounds present in the ocean, making its study difficult. Discoveries are being made, but many features of the cycle are still unknown. In particular, the process of denitrification is a topic of interest. Denitrification begins to occur when oxygen levels reach below 20 μmol kg^{-1} in an oxygen minimum zone (OMZ) (Lam and Kuypers 2010). Without sufficient O_2, nitrate (NO_3^-) becomes the preferred electron acceptor for some bacterial metabolisms. Ammonium (NH_4^+) acts as an electron donor for nitrite (NO_2^-). The

conversion of these forms of nitrogen to N_2 gas causes removal of nitrogen from the ocean. This process, broadly defined as "denitrification," occurs primarily through the processes known as "heterotrophic denitrification" and anaerobic ammonium oxidation, simplified as "anammox". These processes are driven by anaerobic bacteria in oxygen deficient conditions.

Heterotrophic denitrification was believed to be the only nitrogen loss pathway until the discovery of anammox. It is a sequential reduction of nitrogen species, beginning with nitrate and terminating with nitrogen gas (equation 1).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

In this process, organic matter is respired to produce nitrogen gas from NO_3^- (Lam and Kuypers 2011) (equation 2).

$$\text{Organic matter} + NO_3^- \rightarrow CO_2 + N_2 + H_2O \quad (2)$$

Due to varying enzyme efficiencies in denitrifying organisms, intermediate chemicals such as NO_2^- and nitrous oxide tend to build up in the water column, creating spikes in concentration. The buildup of NO_2^- creates a "secondary nitrite maxima," which is an indicator of heterotrophic denitrification (Naqvi 1987).

Anaerobic ammonium oxidation with nitrite is a more recent discovery in the nitrogen cycle. The process involves oxidizing nitrite with ammonium to produce nitrogen gas.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (3)$$

Some anammox bacteria can conduct dissimilatory nitrate reduction to ammonium (DNRA). Combined with nitrite, it can create nitrogen gas (Lam and Kuypers 2011). Because of this process, ammonium is seldom observed in OMZ conditions, as anammox bacteria utilize any that becomes available. Anammox has been thought to occur at rates lower than that of denitrification in oxygen minimum zones due to the slow growth of the bacteria. However, the exact details behind this pathway are not completely understood, and anammox rates generally have a positive correlation with anammox bacterial abundance. Therefore, if integrated over the entire oxygen minimum zone, their overall contribution to nitrogen removal may exceed that of heterotrophic denitrification.

In various oxygen minimum zones, measured rates of these two processes have varied. There is contradictory data on the relative contribution to N-removal of each one; in the Arabian sea oxygen minimum zone, anammox was detected as the dominant process (Jensen et al. 2011) while another study in roughly the same area observed denitrification to be the main N-removing process (Ward et al. 2009). These differences suggest the possibility of denitrification events occurring at different spatial/temporal scales. It is possible that certain conditions influence the rates of each process and have thus far gone undetected.

Due to constraints on the study transect and amount of time available, direct exploration of broad scales of time and space were compensated by sampling water with different properties from two different stations. Samples collected near the Baja Peninsula contained higher amounts of organic matter, which simulated the temporal effects such as seasonal changes in sinking particulate organic carbon (POC) rates. This effect may influence rates of denitrification, as enhanced denitrification is believed to be associated with increased POC (Ward et al. 2009). Potential spatial differences in denitrification rates were observed by collecting water at different depths, as different types of bacteria have been found to be more abundant at specific depths. (Pitcher et al. 2011). However, these observations may not hold true for all locations and situations. This project was designed to explore the possibility of these spatial and temporal effects despite limited sampling. In this regard, traveling to this oxygen deficient zone was a unique chance to get a better look at the marine nitrogen cycle and consider why previous data have not been consistent.

Mass spectrometer analysis revealed that heterotrophic denitrification was not occurring at either station, while small amounts of anammox were detected at 850 m at station 1 and 600 m at station 2. Observing nutrient concentrations, there is not a secondary nitrite maximum, indicating that heterotrophic denitrification may not be occurring.

METHODS

^{15}N stable isotope incubation is the standard method for measuring rates of heterotrophic denitrification and anammox. By injecting water samples with ^{15}N tagged NO_2^- to measure heterotrophic denitrification and ^{15}N tagged $^{15}NH_4^+$ to measure anammox, bacteria within the sample will metabolize the compounds. This process generates nitrogen gas that preserves the ^{15}N label, distinguishing it from gas already present in the water. This can act as an indication of each processes' contribution to nitrogen removal by comparing bags incubated with NO_2^- and NH_4^+.

Nitrogen gas normally has an atomic weight of 28, so stable isotope incubation will allow the amount of gas produced by heterotrophic denitrification and anammox to be measured. This is done through observations of the amount of $^{29}N_2$ and $^{30}N_2$ produced. Denitrification produces both $^{29}N_2$ and $^{30}N_2$. Only $^{29}N_2$ results from incubation of $^{15}NH_4^+$, a component of anammox (Lam and Kuypers 2010). Rates are obtained by the measurement of the del (δ) amount of ^{15}N tagged nitrogen gas present in the sample (equation 4).

$$\delta^{15}N = \left(\frac{\text{R sample}}{\text{R standard}} - 1\right) * 1000 \quad (4)$$

Where R sample is the ratio of $^{15}N/^{14}N$ in the ^{15}N labeled sample and R standard is the $^{15}N/^{14}N$ in a standard mixture of air.

The amount of ^{15}N produced is determined by calculating the total amount of nitrogen in the sample and multiplying by the ratio of $^{15}N/^{14}N$. Rates of heterotrophic denitrification and anammox are obtained through a comparison of this amount across a time series. Increasing ^{15}N over time indicates positive rate of nitrogen production from the denitrification processes.

Samples were collected aboard the *R/V Thomas G. Thompson*, in the oxygen deficient zone (ODZ) of the ETNP. Two stations were sampled, with 5 depths at each (Figure 1). Water column sampling was done at the transition to the ODZ, 3 spots within the ODZ, and the transition back to oxygenation below the ODZ. Station 1 was closer to the coast and had higher particulate matter, while station 2 was in the open ocean. Water was collected via 12 L niskin bottles. Oxygen levels were determined on the CTD downcast.

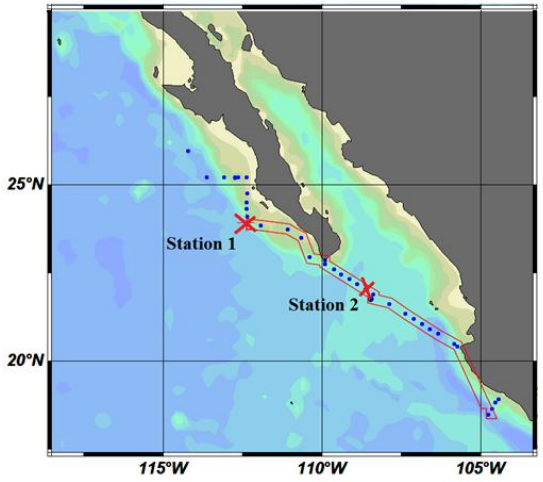

Fig 1. The transect along the Baja Peninsula in the ETNP, with the location of stations 1 and 2 labeled. The cruise involved measurement of several oceanic parameters including nutrient concentrations, and dissolved oxygen. Samples for ^{15}N incubations were collected at station 1 and station 2. The red outline represents the area shown in figures 2 and 3.

Water samples of approximately 4 L were transferred into ten 8 L incubation bags, each purged with helium and completely evacuated prior to filling to prevent atmospheric contamination. The transferring process required air-tight tygon and nylon tubing and 3-way valves. To fill the bag, tubing was attached to the niskin and the valve to the bag was closed, letting the water flow out of the empty side. After removing bubbles from the tubes and filling them with

water, the valve to the bag was opened, allowing uncontaminated water to flow into it.

Two bags were allocated to each depth, with one bag injected with 10 µM/L $^{15}NO_2^-$ to measure heterotrophic denitrification. The other was injected with 10 µM/L $^{15}NH_4^+$ for anammox measurements. After filling, the bags were weighed to determine the amount of water that they contained. Each had roughly 4 L, allowing for a consistent concentration of the isotope solution between samples.

Sub-samples were collected from the incubation bags at 0, 6, 12, 24, and 48 hours after sampling. To prevent contamination, water was drawn as it flowed through the tubing with a gas tight syringe. 6 mL were transferred into a helium purged 12 mL glass exetainer vial. The sample was injected through the exetainer septa from the syringe, with an additional vent needle to prevent pressure from building up. Once the sub-sample was collected, each exetainer was injected with 0.15 mL sulfuric acid to terminate any biological activity. After sub-sampling, bags were returned to a refrigerator to continue incubating. These samples were returned to UW for analysis. Measurements of $^{29}N_2$ and $^{30}N_2$ were taken with the Thermo Finnigan DELTA Plus mass spectrometer in Paul Quay's Stable Isotope Lab.

Overall, this stable isotope method is effective for identifying relative rates of denitrification and anammox. However, there are several places for contamination with atmospheric gases to occur. As a result, exetainer sub-samples or entire incubation bags can become contaminated with oxygen, which would halt the denitrification process, or atmospheric nitrogen, skewing the results. Attention to precision and proper materials is therefore crucial to obtaining accurate results with this method.

RESULTS

Dissolved oxygen concentrations at each of the sampled sites reach below the level where anaerobic respiration begins. Where anammox was detected at station 1, O_2 concentration reached 8.67 umol kg^{-1} (Fig 2). At station 2, anammox was detected at an O_2 concentration of 1.01 umol kg^{-1}. However, oxygen did not reach an undetectable level. NO_2^- concentrations were virtually zero at both locations, with no secondary nitrite maxima from heterotrophic denitrification (Fig 3).

Ammonium levels were low, with an average value of 0.023 umol L^{-1} in the ODZ of station 1 and 0.01 umol L^{-1} in the ODZ of station 2. South of the study site is a discernible nitrite maximum with lower oxygen concentrations. Neither of the two sampling stations was located within this area. N* values indicated that the concentration of N-based nutrients are lower than the expected Redfield ratios (equation 5), indicating that denitrification is occurring within the ODZ (Gruber and Sarmiento 1997).

$$N^* = 16P - DIN + 2.9 \quad (5)$$

Where DIN stands for "dissolved organic nitrogen," which is $[NO_3^-] + [NO_2^-] + [NH_4^+]$.

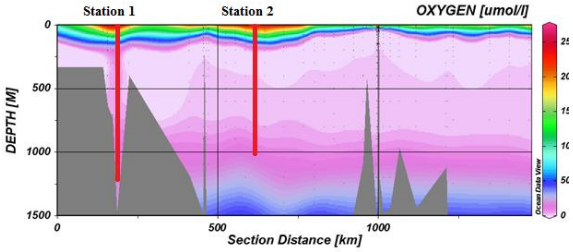

Fig 2. Dissolved oxygen concentrations at various depths across the study transect (Fig 1). O_2 concentrations at stations 1 and 2 (red bars) are generally higher than the area that corresponds with the nitrite maximum. The presence of oxygen makes denitrification a less favorable metabolic process, effectively shutting it down.

Of the 10 water samples taken, slight evidence of anammox was found at 850 m at

station 1 and convincing evidence of anammox at 600 m at station 2. The observed rate at station 1 was 1.02 nmol L^{-1} d^{-1} while the rate at station 2 was 0.762 nmol L^{-1} d^{-1}, which are relatively low rates. No heterotrophic denitrification was detected at either station, at any of the depths sampled. DNA analysis at station 1 determined that neither anammox nor heterotrophic denitrifying bacteria were present in the water above 350 m depth.

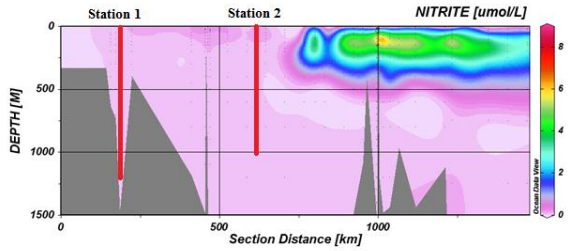

Fig 3. Nitrite concentrations at various depths across the study transect (Fig 1) for both sampling stations. Neither station was within the maximum nitrite zone. A secondary nitrite maximum is generally formed from active heterotrophic denitrification.

Over time, the $\delta\,^{29}N_2/^{28}N_2$ at 850 m at station 1 tends to increase, though correlation between points is not very significant (p=0.3202, R^2=0.4622) (Fig 4). The observed trend may be due to active anammox occurring at this location. However, due to the high amount of variation between data points, it may also be attributed to contamination in the sub-samples, making this rate calculation questionable. At station 2, the increase in $\delta\,^{29}N_2/^{28}N_2$ from $^{15}NH_4^+$ is more significant (p=0.0047, R^2=0.9508) (Fig 4), indicating strong evidence that anammox is occurring. The spread from the $\delta\,^{30}N_2/^{28}N_2$ shows no obvious outliers, suggesting little to no contamination in the sample. As expected, $\delta\,^{30}N_2/^{28}N_2$ does not rise with time, as anammox does not produce $^{30}N_2$.

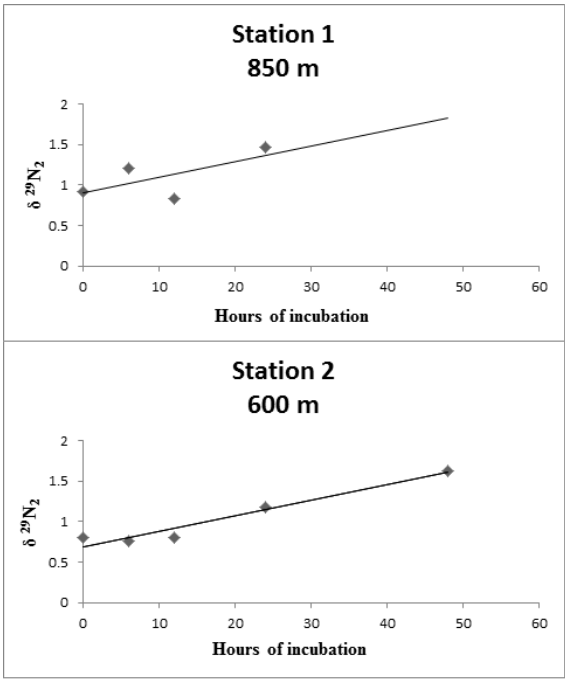

Fig 4. Plots of $\delta\,^{29}N_2$ over time at station 1 and station 2. An increasing $\delta\,^{29}N_2$ indicates a contribution of ^{15}N generated via heterotrophic denitrification or anammox. These results are from bags tagged with $^{15}NH_4^+$, which corresponds with anammox.

DISCUSSION

The lack of a secondary nitrite maximum indicated that heterotrophic denitrification was not actively occurring. This observation is consistent with previous denitrification studies (Naqvi 1987) and the results from the ^{15}N bag incubations. However, rates of heterotrophic denitrification have been observed outside of secondary NO_2^- maxima (Jensen et al. 2011).

In the study section, consistently low NO_2^- concentrations indicate the possibility that neither anammox nor denitrification are occurring, or are functioning at very low rates. This may be due to slightly higher O_2 concentrations in the area; compared to the NO_2^- maximum observed south of the sampling stations, O_2 concentrations at these stations may reach a level where some amounts of aerobic respiration can occur. Oxidation of organic matter with oxygen is more energetically favorable, effectively shutting down the denitrification process (Lam and Kuypers 2011).

Accordingly, with oxygen present in the water column, oxidation of NH_4^+ to NO_2^- and subsequently NO_2^- to NO_3^- would be prioritized above heterotrophic denitrification and anammox (Froelich et al. 1979). Low concentrations of NO_2^- and NH_4^+, along with the presence of NO_3^- in the water column support this possibility.

Anammox detection is congruent with low concentrations of NO_2^- and NH_4^+, as the anammox bacteria utilize the two compounds. It may explain the NH_4^+ deficit. Low NH_4^+ concentrations throughout the entire water column suggest that it may be occurring at more locations than just the observed depths; if anammox were occurring at only two depths, then NH_4^+ concentrations would likely reflect those locations. However, concentrations of NH_4^+ are low throughout the water column. This suggests that something is actively utilizing the NH_4^+ as it becomes available.

If the presence of some O_2 is interfering with denitrification rates, and bag incubation yields only small amounts of anammox, then it is possible that significant amounts of denitrification do not occur until certain conditions are met. O_2 concentration undergoes a noticeable drop after entering the NO_2^- maximum area to the south, where nutrient concentrations suggest that denitrification is actively occurring. Therefore it appears that, at the two stations sampled, oxygen levels may be shutting down denitrification in lieu of aerobic forms of respiration. Alternatively, oxygen contamination in the bag experiment may have inhibited or shut down both denitrifying processes.

Anammox has been observed to continue operating at O_2 concentrations of up to 13.5 umol L^{-1} (Jensen et al. 2008) indicating only partial inhibition from the presence of O_2. Because slight amounts of anammox were found at the two stations and no heterotrophic denitrification was detected, it appears that anammox may be the dominant denitrifying process under suboxic conditions. Heterotrophic denitrification may therefore be more affected by the presence of O_2, shutting down when concentrations are too high. When O_2 levels drop, the NO_2^- maximum forms south of the sampling stations, perhaps indicating that heterotrophic denitrification is beginning to occur. This is further evidence that O_2 is shutting down denitrification until very low concentrations, while anammox is still able to continue at a reduced rate.

Certain results in the data suggest that atmospheric contamination may have occurred. This may be due to the 3-way valves that were used to attach tubing to the incubation bags; subsequent to the cruise it was found that the valves were not gas tight. Some leaking may have occurred as a result. The introduction of O_2 into the sample may have shut down the denitrification process. Considerable spread observed in many sample's data points is either an indication of air contamination in the water samples, as varying amounts of exposure to atmospheric nitrogen will mask the effect of denitrification, or evidence that no denitrification in either form is occurring. Because of this, it is difficult to claim that anammox is the only denitrifying process at the sampling stations.

Much more than the recorded rates of denitrification are likely to occur in an area with a secondary nitrite maximum. Low NH_4^+ concentrations in the secondary nitrite maximum suggest that anammox is active at a higher depth range than observed. Low NO_2^- concentrations that do not reach a secondary spike indicate that heterotrophic denitrification is not very active in the study site. However, further south in the ODZ, the wide NO_2^- maximum indicates that denitrification is occurring there. Extremely low O_2 concentrations throughout that area facilitate the anaerobic respiration process. It is unlikely that only small amounts of anammox are occurring, and the nutrient concentrations support that. Because the ODZ has a section with virtually no NO_2^- and a large section that contains a NO_2^- maximum, it is possible that anammox is the dominant N-removing process in one area, while rates of heterotrophic denitrification increase in the other. Journeying to these areas of known nutrient concentrations at different seasons would therefore be a useful study in determining which denitrifying process in dominant in the ETNP. Doing so would facilitate tracking the crucial conditions that affect the rates of both heterotrophic denitrification and anammox, providing a deeper view into the marine nitrogen cycle.

CONCLUSION

The two sampling locations were in an area where nutrient concentrations did not correspond with positive rates of heterotrophic denitrification. Accordingly, O_2 concentrations may have been too high for heterotrophic denitrification. Small amounts of anammox indicate that, when O_2 levels are above a certain threshold, heterotrophic denitrification ceases while anammox continues at reduced rates. Therefore, I conclude that the O_2 concentration in the study area prevented significant amounts of denitrification from occurring, with only minimal amounts of anammox being detected. Possible oxygen contamination within the bags may also be responsible for the low amount of denitrification detected in the study.

It is unclear why heterotrophic denitrification appears to increase further south in the ETNP other than the possible effects of O_2 regulation. Additional studies on the effects that regulate denitrification would be beneficial for extending the knowledge of the marine nitrogen cycle. While it is known that oxygen levels must be low for both heterotrophic denitrification and anammox to occur, concentrations and environmental conditions affect and regulate rates of the denitrifying processes. A comprehensive study of several oceanic conditions and processes would therefore be extremely beneficial for a deeper understanding of denitrification. Obtaining this knowledge can be crucial for increased understanding of nutrient dynamics in the ocean, which have driving effects on atmospheric carbon uptake and productivity of higher trophic levels.

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