



# Niche occupation of anammox bacteria in the eastern tropical North Pacific

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## NONTECHNICAL SUMMARY

Nitrogen is a limiting nutrient controlling oceanic primary production and important to the global carbon cycle. As an Oxygen Minimum Zone (OMZ), the eastern tropical North Pacific is an area of the ocean characterized by low dissolved oxygen levels and as an area with high rates of nitrogen removal. OMZs are expected to grow in the future, possibly increasing nitrogen removal from the ocean. Because nitrogen is required for primary productivity, the growth of OMZs has potential to slow the biological pump. Anaerobic ammonium oxidizing (anammox) bacteria are of particular interest in OMZs because their ability to autotrophically convert fixed nitrogen (ammonium and nitrite) into the form of  $N_2$  gas, which is biologically unavailable. To gain understanding of the niches that anammox bacteria occupy in the ETNP, anammox presence was examined in relationship to dissolved oxygen (DO), nitrite, and ammonia as well as with association to particle attachment. Presence of anammox bacteria was constructed through DNA extraction, Polymerase Chain Reaction and gel electrophoresis. Bacteria abundances were generated with epifluorescence microscopy using a DAPI stain to gain insight to the general bacterial population. Anammox bacteria were present in the OMZ of the ETNP in less than  $10 \mu\text{mol L}^{-1}$  of oxygen with low concentrations of  $\text{NH}_4^+$  and  $\text{NO}_2^-$ . Anammox bacteria were found on  $0.2\mu\text{m}$  and  $30\mu\text{m}$  filters, suggesting the ability of anammox bacteria to live within anoxic microniches of particles and also as free floating bacteria in the ETNP

## ABSTRACT

Nitrogen is a limiting nutrient controlling oceanic primary production and important to the global carbon cycle. Consequently the nitrogen cycle is important to understanding how the oceans will react to climate change in respect to the biological pump. Oxygen Minimum Zones (OMZs) are areas of the ocean characterized by low oxygen levels and areas with high rates of nitrogen removal. OMZs are expected to grow in the future, possibly increasing nitrogen removal from the ocean (Paulmier and Ruiz-Pino. 2008). The OMZ in the Eastern Tropical North Pacific (ETNP) is the largest OMZ in the world, running from  $0^\circ$ - $25^\circ$  N with a core ranging in depths from 280m to 850m (Paulmier and Ruiz-Pino. 2008). Anaerobic ammonium oxidizing (anammox) bacteria are of particular interest in the OMZs because of their ability to autotrophically convert fixed nitrogen in the form of ammonia ( $\text{NH}_4^+$ ) and nitrite ( $\text{NO}_2^-$ ) into  $N_2$  gas, which is a state unavailable to most biology. To gain understanding of the niches that anammox bacteria occupy in the ETNP, anammox presence was examined in relationship to dissolved oxygen (DO),  $\text{NO}_2^-$  and  $\text{NH}_4^+$  as well as with association to particle attachment. Presence of anammox bacteria was constructed through DNA extraction, Polymerase Chain Reaction and gel electrophoresis. Bacteria abundances were generated with epifluorescence microscopy using a DAPI stain to gain insight to the general bacterial population. Anammox bacteria were present in the OMZ in the ETNP in less than  $10 \mu\text{mol L}^{-1}$  of oxygen with low concentrations of  $\text{NH}_4^+$  and  $\text{NO}_2^-$ . Anammox bacteria were found on  $0.2\mu\text{m}$  and  $30\mu\text{m}$  filters, suggesting the ability of anammox bacteria to live within anoxic microniches of particles and also as free floating bacteria in the ETNP.

Nitrogen is a limiting nutrient, controlling oceanic primary production and important to the global carbon cycle. The only source of fixed nitrogen to marine environments is through nitrogen fixation by cyanobacteria resulting in the remineralization of organic nitrogen into ammonium ( $\text{NH}_4^+$ ), which is then oxidized into other fixed nitrogen species (Capone et al. 2008). Upwelling of fixed nitrogen from the deep ocean is an additional source of nitrogen to the euphotic zone, but only contributes a fraction of the total amount fixed nitrogen in the ocean compared to nitrogen fixation (Arrigo, 2005). Once nitrogen has been fixed, it may have many pathways before it sinks to the deep ocean in particles or is removed by denitrification into  $\text{N}_2$  gas which escapes to the atmosphere. Oxygen Minimum Zones (OMZs) are areas of the ocean where a large portions of the water column drops below  $20\mu\text{mol L}^{-1}$  and are thought to be a location where much of the nitrogen is removed from the oceans because nitrate ( $\text{NO}_3^-$ ) is the next most efficient electron acceptor in the absence of oxygen (Capone et al. 2008). OMZs are important to determining the balance of the sources and sinks of nitrogen in the ocean. They are also important to understanding the future of the ocean because they are predicted to grow due to changes in ventilation predicted from climate change (Paulmier and Ruiz-Pino. 2008).

Denitrification is any process that removes fixed nitrogen into biologically unavailable  $\text{N}_2$ . Denitrification was thought to be a sequence of biological reductions of fixed nitrogen using organic matter as the electron donor. This pathway is called “canonical denitrification” and until recently was the only known pathway where fixed nitrogen was removed from marine environments (Capone et al. 2008). Remineralization of organic nitrogen suggested there should be a buildup of  $\text{NH}_4^+$  as a result of denitrification (Arrigo. 2005). The discovery of anammox helped answer the previous questions about  $\text{NH}_4^+$  providing a solution as a sink of  $\text{NH}_4^+$  in marine environments. Anammox bacteria use  $\text{NO}_2^-$  as the electron acceptor to autotrophically oxidize  $\text{NH}_4^+$ . The significance of anammox bacteria as denitrifiers of the marine environment is currently being debated. One estimate suggest that anammox bacteria produce 30 to 50% of all  $\text{N}_2$  in the ocean (Arrigo, 2005).

Both processes, heterotrophic denitrification and anammox, need low dissolved oxygen (DO) concentrations to proceed. Anammox bacteria are obligate anaerobes, but it is unclear what is the maximum DO concentration in which the anammox reaction can proceed. Some experiments in bioreactors have shown that the anammox reaction may not occur in water with more than  $1\mu\text{mol L}^{-1}$  of DO (Woebken et al. 2007). However anammox rates have been found in water with DO concentrations up to  $9\mu\text{mol L}^{-1}$  (Woebken et al. 2007). A study in the Namibian Sea found anammox rates active in water with DO concentration of  $25\mu\text{mol L}^{-1}$  (Woebken et al. 2007). This study found that anammox bacteria were able to survive with high DO by living in micro niches within particles with locally low DO concentration (Woebken et al. 2007).

Anammox bacteria have a unique structure called the anammoxosome which facilitates denitrification (Gala'n et al. 2008). The anammoxosome is constructed of ladderane lipids, a material thought to be a building block only for anammox bacteria (Gala'n et al. 2008). The ladderane lipids act as a barrier to the rest of the cell to protect from toxic intermediates (Gala'n et al. 2008). Past research suggest that the anammox pathway has four genes coding for enzymes that convert  $\text{NO}_2^-$  and  $\text{NH}_4^+$  to  $\text{N}_2$  (Klotz and Stei. 2011). The nirS gene is responsible for the change of  $\text{NO}_2^-$  to Nitric oxide (NO), the Hh gene is responsible for the transformation from NO to hydrazine and the Hzo gene is responsible for the production of  $\text{N}_2$  by the oxidation of hydrazine ( $\text{N}_2\text{H}_4$ ) by the enzymes produced by the Hao and the Hzo genes (Klotz and Stei. 2011). This oxidation of  $\text{N}_2\text{H}_4$  is the step that anammox bacteria use to produce energy (Meng et al. 2010). NirS and Hzo are genes that have been used to examine anammox bacteria in the past (Klotz and Stei. 2011).

A recent study in the ETNP used ladderane fatty acids as a biomarker for evidence of anammox bacteria (Rush et al. 2011). Two different  $\text{NO}_2^-$  maximums were present at every station. One occurred in the euphotic zone from remineralization and the other larger maximum corresponded to the peak in anammox bacteria at depths varying from 300m to 600m (Rush et al. 2011). The OMZ in the ETNP was found on the

shelf, showing an anammox bacteria peak present depth 81-115m (Rush et al. 2011). Anammox bacteria were detected in water with less than 10  $\mu\text{mol L}^{-1}$  of oxygen (Rush et al. 2011).  $\text{NO}_2^-$  was thought to be the limiting nutrient for anammox in this study in the ETNP at all except one station, at least 0.2  $\mu\text{mol L}^{-1}$  of  $\text{NO}_2^-$  was necessary for ladderane fatty acid presence (Rush et al. 2011). Anammox bacteria abundances have been examined in the OMZ off of Peru in the Eastern Tropical South Pacific (ETSP). In the study anammox bacteria had peaks in abundance in the upper part of the OMZ in the area just below the oxycline (Gala'n et al. 2009). In the ETSP, anammox activity decreased in abundance with depth and  $\text{NH}_4^+$  concentration was thought to control the vertical distribution of anammox (Gala'n et al. 2009). Past studies provided a background for locations to examine anammox bacteria in relation to particle association in the ETNP.

To gain understanding of the niches that anammox bacteria occupy in the ETNP, anammox presence was examined in relationship to dissolved oxygen (DO),  $\text{NO}_2^-$  and  $\text{NH}_4^+$  as well as with association to particle attachment. To answer these questions presence/ absence data of anammox bacteria has been constructed using PCR with primers that target the NirS gene. In addition to presence of these specific denitrifying bacteria, bacterial abundances were constructed with an epifluorescent microscope using DAPI-citifluor stain. Presence of denitrifying bacteria was also examined at corresponding depths. Based on the study in the ETNP by Rush et al. 2011, I hypothesized that anammox bacteria will be present in the water column from just below the oxycline all the way throughout the OMZ. To gain insight to particle association a double filter technique was used. This technique assumed that bacteria associated with particle attachment would end up on a 30 $\mu\text{m}$  and free living bacteria in the water column will be found on a 0.2 $\mu\text{m}$  filter (Fuchsman et al. 2011). Anammox bacteria were expected to present on 0.2 $\mu\text{m}$  filters because they are autotrophic.

## METHODS

The *R.V. Thompson* departed from San Diego on March 16th, 2012 and arrived in Manzanillo, Mexico on March 27<sup>th</sup>, 2012. Water sample were collected at seven stations (Figure 1). The CTD was deployed at every station and the Niskin bottles were fired at similar points along the oxygen profile. Understanding where anammox reside along the oxycline, in out of the OMZ, was important to this project. A bottle was fired above the OMZ at the chlorophyll max, on the way into the OMZ, two evenly spaced through the OMZ, in the oxycline on the way out of the OMZ, and one bottle fired below the OMZ. These positions in the OMZ were targeted because they offer a range of oxygen concentrations to examine for the existence of anammox bacteria.

At each depth 2 liters of water were filtered with a 0.2  $\mu\text{m}$  filter. At stations 6 and 24 water was filtered with two different filter sizes (0.2  $\mu\text{m}$  and 30  $\mu\text{m}$ ) to distinguish between bacteria that were attached and unattached to particles (Fuchsman et al. 2011). Samples were stored in a -80 °C freezer. Bacterial counts were carried out for every depth. One mL of sample was fixed with paraformaldehyde. The sample was filtered with 0.2  $\mu\text{m}$  polycarbonate membrane. The filter was mounted on a slide with the 10 $\mu\text{L}$  of DAPI-citifluor stain. An epifluorescent microscope used ultra violet light to excite the DAPI stain which bound to the DNA in the bacteria. 10 counts in different fields of view were done for every sample.

DNA extraction, PCR and gel electrophoresis were completed back at the University of Washington. A phenol:chloroform DNA extraction method was used from Thurber et al, 2009. Extracted DNA samples were evaluated for purity as well as the concentration of the DNA spectrophotometrically using a Nannodrop. PCR amplifications occurred in a total volume of 25  $\mu\text{L}$  with of 2.5  $\mu\text{L}$  10X PCR buffer, 2.5  $\mu\text{L}$  forward primer, 2.5  $\mu\text{L}$  reverse primer, 1.25  $\mu\text{L}$  dNTPs, 1.25  $\mu\text{L}$   $\text{MgCl}_2$ , 0.2 Taq polymerase, 5  $\mu\text{L}$  DNA, and 9.8  $\mu\text{L}$  deionized water. PCR was carried out with primers (Scnir272F and Scnir845R) using PCR conditions provided by Lam et al, 2009. Condition for PCR: 95 °C for 2 min, 30 cycles x

(94° C for 30s, 51 °C for 1 min, 72 °C for 1 min), 72 °C for 10 min. PCR products were visualized using gel electrophoresis in a 1.5% agarose gel. The gel was stained with SYBR green and examined under ultra violet light. A fragment size of 435 base pairs was used for the identification of the anammox bacteria fragments.

## RESULTS

Station 6, 13, 24 and 32 were examined for the presence of anammox bacteria and heterotrophic denitrifying bacteria. Anammox bacteria and heterotrophic denitrifying bacteria were present at station 6 and 24. Anammox bacteria were found in a DO range of 1.14 to 9.37  $\mu\text{mol L}^{-1}$ . Anammox bacteria were present in concentrations of  $\text{NO}_2^-$  ranging from undetectable to 0.04  $\mu\text{mol L}^{-1}$  and were present in concentrations of  $\text{NH}_4^+$  ranging from undetectable to 0.02  $\mu\text{mol L}^{-1}$  (Table 1).

### *Station 6*

Station 6 was located in the Soledad Basin. The CTD ran the full length of the water column to 540ft. Anammox were present at depths of 160m and 300m on 0.2  $\mu\text{m}$  filters. Denitrifying bacteria were present at 160m on a 30 $\mu\text{m}$  filter (Figure 2). Denitrifying bacteria were also present at 200m and 300m on a 0.2 $\mu\text{m}$  filter (Figure 2). The DO declined to less than 10  $\mu\text{mol L}^{-1}$  by 200m and less than 3  $\mu\text{mol L}^{-1}$  by 250m. DO decreased with depth and reached a minimum of 1.93  $\mu\text{mol L}^{-1}$  at 540m. A small increase in  $\text{NH}_4^+$  of 0.05  $\mu\text{mol L}^{-1}$  occurred at 60m then dropped to undetectable amounts through the remainder for the water column. A  $\text{NO}_2^-$  peak of 0.17  $\mu\text{mol L}^{-1}$  also occurred at 60m then dropped to an undetectable amount by 100m.  $\text{NO}_2^-$  stayed at undetectable levels until 540m where a  $\text{NO}_2^-$  increased to 0.06  $\mu\text{mol L}^{-1}$ .  $\text{NO}_3^-$  concentrations were undetectable at the surface jumped to 15.35  $\mu\text{mol L}^{-1}$  at 60m and increased until the seafloor up to 25.03  $\mu\text{mol L}^{-1}$  at 540m. Bacterial abundance decreases from 60m to 120m from  $9.3 \times 10^5$  cells  $\times \text{mL}^{-1}$  to  $1.6 \times 10^5$ . Bacteria abundance was in the range of  $2.3 \times 10^5$  to  $4.9 \times 10^5$  from 160m to 300m. Bacterial

abundance increased to 7.

### *Station 13*

Anammox bacteria and denitrifying bacteria were not detected at station 13 at 50m, 250m or 350m. The DO dropped to 2.75  $\mu\text{mol L}^{-1}$  and only held a minimum oxygen concentration for one depth at 600m. At this station  $\text{NO}_2^-$  was undetectable below 100m.  $\text{NH}_4^+$  was present throughout the water column with a concentration of 0.17  $\mu\text{mol L}^{-1}$  at the surface, 0.02  $\mu\text{mol L}^{-1}$  from 100m to 850m and 0.07  $\mu\text{mol L}^{-1}$  at 1000m.

### *Station 24*

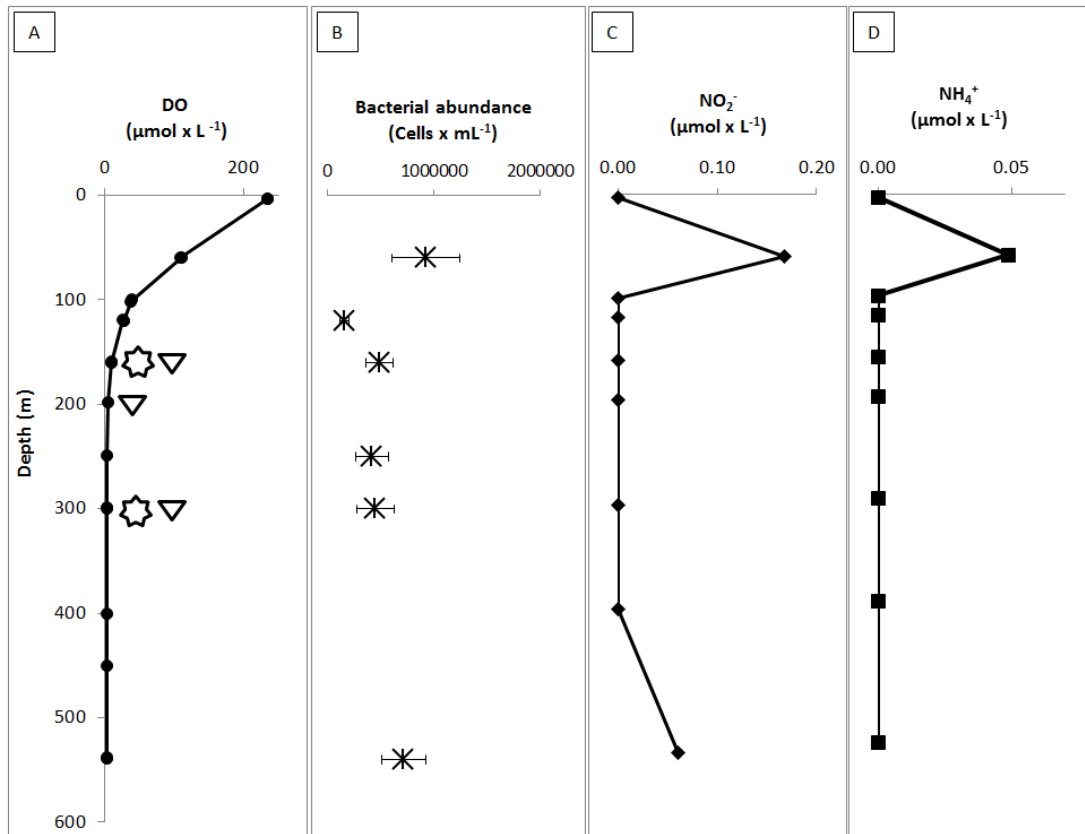
Anammox bacteria were present at station 24 at 600m on a 30 $\mu\text{m}$  filter (Figure 3). Denitrifying bacteria were present at 50m, 150m and 300m on 30 $\mu\text{m}$  filters (Figure 3). Oxygen levels dropped to below 10  $\mu\text{mol L}^{-1}$  at 150m and decreased until 0.97  $\mu\text{mol L}^{-1}$  at 450m then increased to 12  $\mu\text{mol L}^{-1}$  by 1000m. Anammox bacteria were present at 600m in water with DO of 1.14  $\mu\text{mol L}^{-1}$ .  $\text{NO}_2^-$  was present throughout the water column. A peak in  $\text{NO}_2^-$  of 0.16  $\mu\text{mol L}^{-1}$  was observed at 60m.  $\text{NO}_2^-$  decreased with a less dramatic secondary peak of 0.04  $\mu\text{mol L}^{-1}$  at 600m.  $\text{NH}_4^+$  was detected throughout the water column in concentration ranging from 0.01  $\mu\text{mol L}^{-1}$  to 0.05  $\mu\text{mol L}^{-1}$  except at 60m where concentrations were undetectable.  $\text{NO}_3^-$  increased from undetectable at surface to 41.9  $\mu\text{mol L}^{-1}$  at 1000m. Bacterial abundance decreases with surface value of  $1.7 \times 10^5$  cells  $\times \text{mL}^{-1}$  to  $4.3 \times 10^4$  at 450m and then increased to  $6.4 \times 10^4$  cells  $\times \text{mL}^{-1}$  at 600m.

### *Station 32*

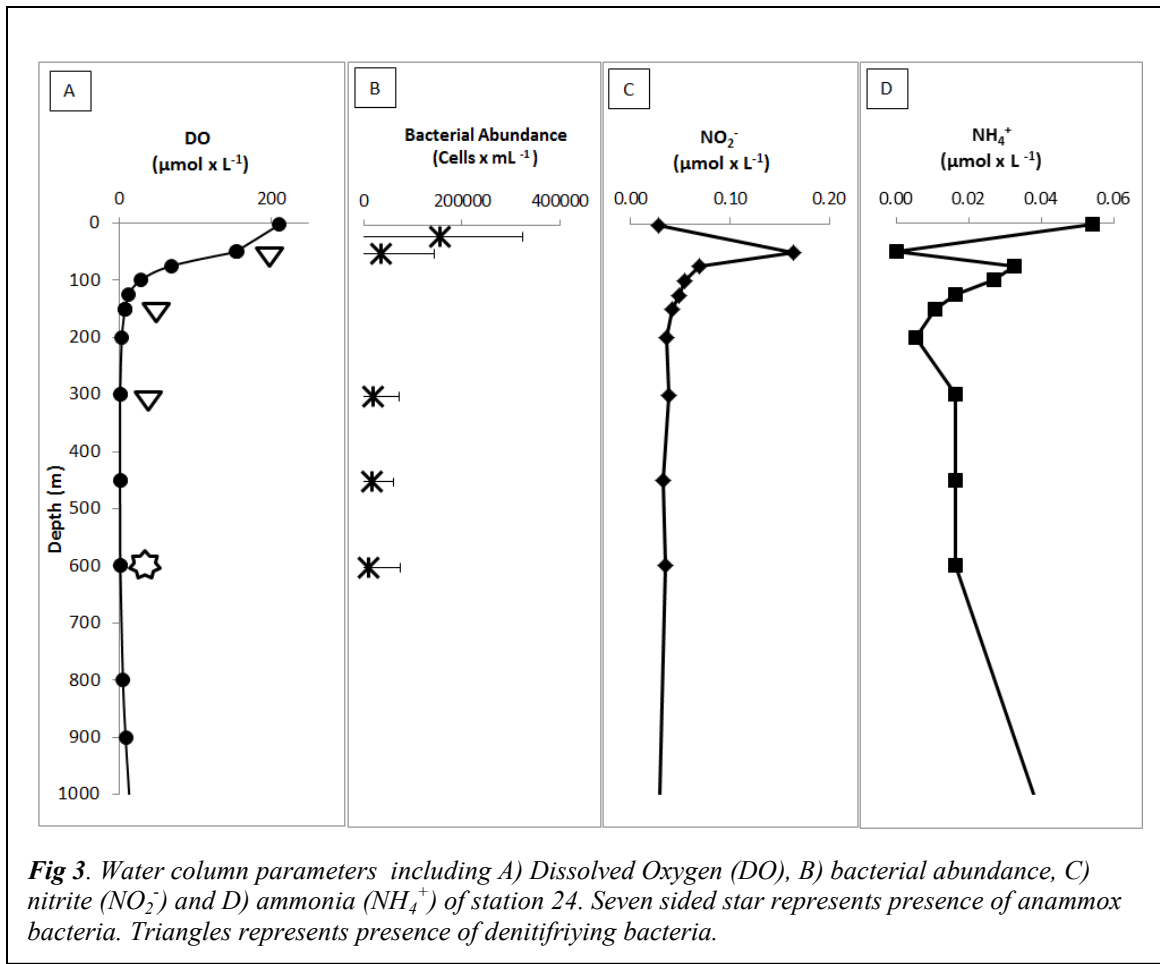
Anammox and denitrifying bacteria were undetectable at Station 32 when examined on a 0.2 $\mu\text{m}$  filter at 150m and 600m. The oxygen dropped to less than 1  $\mu\text{mol L}^{-1}$  by 50m and stayed below 2  $\mu\text{mol L}^{-1}$  until 800m. A peak in  $\text{NO}_2^-$  of 6.6  $\mu\text{mol L}^{-1}$  was located at 65m. The  $\text{NO}_2^-$  levels declines to an undetectable amount by 450m.  $\text{NH}_4^+$  was undetectable throughout the water column.

**Table 1.** Anammox presence with surrounding oxygen, nitrite ( $\text{NO}_2^-$ ) and ammonia ( $\text{NH}_4^+$ ) concentrations.

Station	Depth (m)	DO ( $\mu\text{mol L}^{-1}$ )	$\text{NO}_2^-$ ( $\mu\text{mol L}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )
6	160 (0.2 $\mu\text{m}$ )	9.37	0	0
6	300 (0.2 $\mu\text{m}$ )	2.75	0	0
24	600 (30 $\mu\text{m}$ )	1.14	0.04	0.02



**Fig 2.** Water column parameters including A) Dissolved Oxygen (DO), B) bacterial abundance, C) nitrite ( $\text{NO}_2^-$ ) and D) ammonia ( $\text{NH}_4^+$ ) of station 6. Seven sided stars represents presence of anammox bacteria. Triangles represents presence of heterotrophic denitrifying bacteria.



## DISCUSSION

Stations 6 and 24 demonstrate unique aspects of anammox ecology.

### Station 6

In the Soledad Basin the OMZ shoals up onto the shelf of the Baja Peninsula. The oxygen profile is unique because there is no lower oxycline so the OMZ runs into the sea floor. Anammox bacteria were found in the oxycline at 160m and in the core of the OMZ at 300m demonstrating a range in depths and a range in DO from  $9.37 \mu\text{mol L}^{-1}$  at 160m to  $2.75 \mu\text{mol L}^{-1}$  at 300m. Both  $\text{NO}_2^-$  and  $\text{NH}_4^+$  were undetectable from 100m to 400m. This segment of undetected nutrient concentrations spans the depths of anammox presence. This suggests that anammox bacteria has used up all available  $\text{NO}_2^-$  and  $\text{NH}_4^+$ .

Filter sizes provided insight to the denitrification going on at station 6. At 160m both anammox bacteria and denitrifying bacteria were found. Anammox bacteria were found on a  $0.2 \mu\text{m}$  filter and heterotrophic denitrifying bacteria were found on a  $30 \mu\text{m}$  filter. Additionally both anammox bacteria and heterotrophic denitrifying bacteria were found on a  $0.2 \mu\text{m}$  filter. This finding supports the hypothesis that autotrophic anammox bacteria would be found on a  $0.2 \mu\text{m}$  filter, which are associated with free floating bacteria, and denitrifying bacteria would be found on a  $30 \mu\text{m}$  filter, which assumes bacteria present are associated with particle attachment. Station 6 demonstrates the ability of anammox bacteria and heterotrophic denitrifying to fill separate niches at the same depth but also to be present at the same depth.

Heterotrophic denitrifying bacteria were found at three depths at station 6. First at 160m on a 30 $\mu$ m filter and below 160m were found on 0.2 $\mu$ m filters. Depth of presence the filter size on which they were found may be explained by depth. Particles may become smaller and less abundant with depth, so it is logical that denitrifying bacteria may have been present 30 $\mu$ m filter and on 0.2 $\mu$ m at depth.

Station 6 had abnormally high cell abundance at 540m compared to other stations at that depth. This abundance was unexpected and may be explained by either the CTD hitting some sediment at the bottom. Sediment may explain higher cell abundance due to higher levels of organic material. Setting aside the abundance at 540m, there was decrease in bacterial abundance with depth into the OMZ. Anammox bacteria presence also exists as depth increased. Anammox bacteria demonstrate the ability to fill a unique niche of low oxygen that the general bacterial populations did not fill.

#### *Station 13*

Understanding the absence of anammox bacteria is equally important as understanding presence. There are countless reasons why anammox bacterial may have been undetectable at station 13 including environmental parameters as well as error in methods. Oxygen levels may have influenced anammox presence. The shape and definition of the OMZ at station 13 greatly differs from other station. The OMZ never dropped below 2.74 $\mu$ mol L<sup>-1</sup> and lacked a core holding a minimum oxygen concentration for one depth, making the OMZ narrow and not as distinguished. The lack of shape and intensity of the OMZ core of station 13 may have been made colonization by anammox bacteria difficult. Additionally NO<sub>2</sub><sup>-</sup> was undetectable below 100m, making anammox activity difficult at 250m and 350m. Lack of shape and intensity of the OMZ core, and low NO<sub>2</sub><sup>-</sup> acted as environmental restraints making

station 13 a bad candidate for anammox bacterial presence.

#### *Station 24*

Anammox bacteria were present at a depth of 600m, in the lower oxycline of the OMZ. Anammox bacteria were never present at the same depth as denitrifying bacteria at this station. Anammox bacteria were found in the bottom half of the core right before the lower oxycline and denitrifying bacteria were found from the oxycline all the way down through the core of the OMZ. This suggests that there may be specific niches between anammox and denitrifying bacteria by depth in the water column. Anammox bacteria were found on the 30 $\mu$ m filters at 600m suggesting particle association. Denitrifying bacteria were also found on 30 $\mu$ m filters suggesting particle association. It is important to note that at station 24 the water fraction on the 0.2 $\mu$ m filter was not examined for bacteria presence. All though the fraction of water on the 0.2 $\mu$ m filters was not examined, the 30 $\mu$ m filters interesting results. It was unexpected that anammox bacteria were found on a 30  $\mu$ m filters at this location. The DO at this location was the lowest of any condition of anammox bacteria presence in this study. Particle association of anammox was expected at depths where DO was relatively high (above 10 $\mu$ m) so anammox could use an anoxic micro environment within the particle (Woebken et al. 2007). This demonstrates the ability of anammox bacteria in the ETNP to fill a second kind of niche, a micro niche with in particles. It is possible that anammox bacteria were filling these micro niches for other reasons besides low DO. NO<sub>2</sub><sup>-</sup> concentrations were low at this depth. Anammox bacteria may have been present in a particle microniche to seek out these essential nutrients for anammox.

### *Station 32*

Following the trend of southern stations in the ETNP, the OMZ at station 32 was bigger with lower DO. Station 32 had a well defined OMZ core. The DO in the station 32 OMZ had qualities suitable for anammox presence including a minimum oxygen of  $0.9 \mu\text{mol L}^{-1}$  and  $\text{NO}_2^-$  concentration of  $19.04 \mu\text{mol L}^{-1}$ . However  $\text{NH}_4^+$  concentrations were undetectable throughout the OMZ.  $\text{NH}_4^+$  may have been the limiting nutrient for anammox and the reason for their absence at station 32 at 150m and 300m.

### *Implications of environmental conditions of anammox bacterial presence*

DO concentration in water of anammox bacteria presence (under  $9.37 \mu\text{mol L}^{-1}$ ) was consistent with the past study in the ETNP where anammox presence was found in water with DO concentrations below  $10 \mu\text{mol L}^{-1}$  (Rush et al. 2011). Station 6 demonstrates anammox bacteria presence without the necessary nutrients present and station 24 demonstrates presence with very low concentrations of nutrients present. In two out of the three instances of anammox bacterial presence  $\text{NO}_2^-$  was undetectable. In the third instance the concentration of  $\text{NO}_2^-$  was  $0.04 \mu\text{mol L}^{-1}$ . These concentrations were only a fraction of what Rush et al. typically found necessary ( $0.2 \mu\text{mol L}^{-1}$ ) for presence of anammox bacteria (Rush et al. 2011). In one case Rush et al. found ladderane presence with no detectable  $\text{NO}_2^-$  present (Rush et al. 2011).

How is it possible for population of anammox bacteria survive in a water column that has no necessary nutrients? There is some kind of nutrient cycling going in the water over some time scale. One possibility is that anammox bacteria recently used up the nutrients these necessary nutrients are frequently cycled back into the water. Another possibility is anammox used up all the

nutrients, but have been in the water column for a longer period, no longer anammoxing. This possibility may be facilitated by the anammox bacteria generation time, which has been estimated between 10-30 days (Nifrik et al. 2004).

Station 6 demonstrated anammox bacteria occupying similar depths as heterotrophic denitrifying bacteria. Anammox bacteria (found on  $0.2 \mu\text{m}$  filter) and heterotrophic denitrifying bacteria (found on  $30 \mu\text{m}$  filter) occupied 160m. At 300m both bacteria were found on  $0.2 \mu\text{m}$  filter. Station 24 demonstrated separation of anammox bacteria spatially the water column. Station 24 also demonstrated particle association of anammox bacteria. Particles offer two advantages to anammox bacteria. There are decreased oxygen levels within the particle due to respiration of heterotrophic bacteria and also remineralization of  $\text{NH}_4^+$  (Woebken et al. 2007). Anammox have been found attached to particles in OMZs off of Namibia and Peru but this is the first known instance of particle association of anammox bacteria found in the ETNP (Woebken et al. 2007).

All though anammox bacteria are confined to OMZs the environmental parameters they were governed by in this study were surprising. Presence in a nutrient deprived water column may give insight into the ability of anammox bacteria to colonize the growing areas of the ocean OMZs. Suggested by their presence in stations 6 and 24, anammox bacteria have some kind of mechanism allowing them to be stable in low nutrient environments. Anammox bacteria association with particles to seek nutrients and anaerobic microniches may act as an additional mechanism in which anammox bacteria colonize a new OMZ or spreading OMZ. Understanding how anammox bacteria colonize new and spreading OMZ water columns has important implications to the future oceans. Particle occupation of

anammox bacteria may be a mechanism anammox bacteria use to move into growing OMZs and understanding colonization processes is an important study for the future.

## CONCLUSIONS

- As bacterial abundances decreased with depth into the OMZ, there was a detection of anammox bacterial presence. This demonstrates the ability of anammox bacteria fill a special niche within the world's ocean.
- Anammox bacteria were found on 0.2µm and 30µm filters, demonstrating the ability of anammox bacteria to live within anoxic microniches of particles and also as free floating bacteria.
- Anammox bacteria and heterotrophic denitrifying bacteria were found at the same depth on the same filters and also at the same depth on differing filter sizes.
- Anammox bacteria were present in water with DO concentrations under 10 µmol L<sup>-1</sup> and low concentrations of NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>.
- Both NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> acted as limiting nutrients for anammox bacteria in different locations of the ETNP.

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