Presence and distribution of heterotrophic denitrifying bacteria in the Eastern Tropical North Pacific oxygen minimum zone in relation to environmental parameters

Sara Geurtsen

University of Washington

School of Oceanography, Box 355351

Seattle, WA 98195-5351

sarag18@u.washington.edu

Non-technical Summary

The marine nitrogen cycle plays an important role in primary productivity by supplying primary producers with the essential nutrient nitrate. The expansion of oxygen minimum zones in the oceans is causing increasing concerns about its implications for nutrient cycles such as the nitrogen cycle. Oxygen minimum zones are sites of a process called heterotrophic denitrification, which results in the removal of large amounts of biologically reactive nitrogen from the oceans. Heterotrophic denitrifying bacteria perform this process by converting nitrate into dinitrogen gas. Dinitrogen gas is not very soluble in seawater and escapes to the atmosphere. I investigated the presence of heterotrophic denitrifying bacteria in the Eastern Tropical North Pacific oxygen minimum zone to assess their contribution to nitrogen removal in this area. I found heterotrophic denitrifying bacteria at two different stations and evidence of active heterotrophic denitrification at multiple stations. This suggests that heterotrophic denitrifying bacteria play an active role in nitrogen removal in the Eastern Tropical North Pacific oxygen minimum zone.

Acknowledgments

I would like to thank Michael Carlson for spending many hours explaining molecular techniques to me and answering millions of questions. I would like to thank the instructors of Ocean 443/444 for taking us on this research cruise, which was an amazing experience. I would also like to thank the students of Ocean 443/444, especially

Annie Thomson, Matt Knight, and Will Kammin. Special thanks also to the captain and crew of the Thomas G. Thompson.

Abstract

The removal of fixed nitrogen from the oceans via denitrification pathways has important implications for oceanic primary productivity and climate dynamics. Heterotrophic denitrification is a process limited to oxygen minimum zones (OMZs) and hemipelagic sediments during which heterotrophic denitrifying bacteria convert nitrate (NO_3^-) to dinitrogen gas (N_2). These bacteria may use nitrate instead of oxygen as an electron acceptor when oxygen becomes depleted. Nitrate is an important nutrient that fuels primary productivity. Dinitrogen gas however escapes from the ocean due to its low solubility in seawater, causing a loss of biologically reactive nitrogen from the system. In this study the presence of heterotrophic denitrifying bacteria in the Eastern Tropical North Pacific (ETNP) OMZ was investigated to assess their contribution to nitrogen removal in this area. This was done by means of polymerase chain reaction (PCR) amplifications of the *nirS* gene, which encodes the enzyme nitrite reductase, and is present in all species of heterotrophic denitrifying bacteria. In addition, microbial abundance was determined via DAPI staining followed by epifluorescent microscopy. Heterotrophic denitrifying bacteria were detected at Station 6 (160 m, 200 m, and 300 m) and Station 24 (50 m, 150 m, and 300 m). The presence of heterotrophic denitrifying bacteria corresponded to low oxygen conditions. Evidence of heterotrophic denitrification was found at multiple stations. This suggests that heterotrophic denitrifying bacteria play

an active role in nitrogen removal in the Eastern Tropical North Pacific oxygen minimum zone.

Introduction

The Eastern Tropical North Pacific (ETNP) contains earth's largest oxygen minimum zone (OMZ) with an area of 12.4×10^6 km^2 (Paulmier and Ruiz-Pino 2009). An OMZ is defined by dissolved oxygen (DO) concentrations of less than 20 µmol L^{-1} (Paulmier and Ruiz-Pino 2009). Whereas some microorganisms thrive in OMZs, most macro-organisms cannot survive in such low oxygen environments. In recent decades OMZs have been expanding in what is believed to be a response to climate change (Paulmier and Ruiz-Pino 2009). This expansion is in part due to increased water column stratification that has occurred in response to warming of the oceans (Stramma et al. 2008). Increased water column stratification prevents deep water from being replenished with oxygen at the surface due to decreased circulation and may therefore lead to the formation of OMZs. The expansion of OMZs is causing increased concerns about its implications for marine macro-organisms, as well as the major nutrient cycles of the oceans (carbon and nitrogen), which are involved in important climate feedback loops and the food web of the oceans.

The question how the expansion of OMZs might affect the nitrogen cycle has become a topic of great interest. The nitrogen cycle plays an important role in primary productivity by supplying primary producers with the essential nutrient nitrate (NO_3^-). Nitrogen loss from the oceans is predicted to increase as a result of expanding OMZs

because oxygen deficiency encourages two processes known as heterotrophic denitrification and anammox. There are concerns that nitrogen loss could become unmanageable in the future and lead to decreases in primary productivity. Heterotrophic denitrification is limited to OMZs and hemipelagic sediments because it only occurs when oxygen concentrations reach values between 20 and 1 µmol L^{-1} (Jayakumar et al. 2009). Heterotrophic denitrification is performed by heterotrophic denitrifying bacteria, which may use nitrate (NO_3^-) instead of oxygen (O_2) as an electron acceptor when oxygen concentrations are depleted (Jayakumar et al. 2009). These bacteria possess a gene that encodes the enzyme "nitrite reductase," which allows them to perform this function (Jayakumar et al. 2009). During the process of heterotrophic denitrification heterotrophic denitrifying bacteria convert nitrate (NO_3^-) to dinitrogen gas (N_2) through a series of steps represented in the following reaction (Arrigo 2005):

$C_{106}H_{175}O_{42}N_{16}P + 104\ NO_3^- = 4\ CO_2 + 102\ HCO_3^- + 60\ N_2 + 36\ H_2O + HPO_4^{2-}$

(Sarmiento and Gruber 1997).

Dinitrogen gas (N_2) is no longer available for biological productivity because only a few select phytoplankton, mostly cyanobacteria, can utilize this form of nitrogen and convert it back into organic nitrogen compounds through a process called N_2-fixation (Lalli and Parsons 1993). The majority of dinitrogen gas (N_2) escapes to the atmosphere because this gas is not very soluble in seawater. Heterotrophic denitrifying bacteria are not the only community of microorganisms responsible for this nitrogen loss from the oceans. In recent decades another group of bacteria that perform a process known as "anammox"

has been identified (Arrigo 2005). Anammox is an autotrophic process during which anammox bacteria oxidize ammonium (NH_4) to dinitrogen gas (N_2), using nitrite (NO_2) as an oxidant (Arrigo 2005). Similar to heterotrophic denitrification, anammox occurs only in OMZs and hemipelagic sediments (Jayakumar et al. 2009). N_2-fixation rates are not high enough to balance the nitrogen losses caused by heterotrophic denitrification and anammox (Arrigo 2005). Together heterotrophic denitrifying bacteria and anammox bacteria convert between 30 and 50% of global fixed nitrogen to biologically unavailable nitrogen in oceanic OMZs (Bulow et al. 2010, Jayakamur et al. 2009). Nitrogen is therefore considered the limiting nutrient of primary production in the oceans (Arrigo 2005).

Nitrogen deficits may have detrimental effects on primary productivity rates. Phytoplankton require the uptake of nutrients in a specific stoichiometric ratio in order to successfully carry out primary production (Arrigo 2005). If nitrogen becomes dramatically reduced, primary productivity will likely decline. Since phytoplankton make up the base of the food chain, higher trophic levels will be affected. Decreased nitrogen concentrations will also reduce the ocean's ability to absorb CO_2. Phytoplankton take up CO_2 during primary productivity and produce organic carbon (Lalli and Parsons 1993). Some of this organic matter sinks to the ocean floor and becomes incorporated into the sediments. This is an efficient way of preventing large accumulations of CO_2 in the atmosphere, which contribute to global warming. When nitrogen concentrations are low, phytoplankton take up less CO_2 in order to adhere to the required nutrient ratio. This then results in higher atmospheric CO_2 concentrations and increased global warming. Global warming has harmful effects on terrestrial and marine ecosystems alike by causing sea

level rise, ice melt and ocean acidification, increasing the frequency of storms, and eliminating habitat for cold-weather organisms.

The ETNP's OMZ is especially interesting in relation to heterotrophic denitrification because it contains earth's largest denitrification zone with an area of 7.8 x 10^6 km^2 (Paulmier and Ruiz-Pino 2009). A denitrification zone is defined by nitrate (NO_3) deficits greater than 10 µmol L^{-1} (Paulmier and Ruiz-Pino 2009). There are some uncertainties about how much each process, heterotrophic denitrification or anammox, contributes to the volume of the measured denitrification zone in the ETNP (Paulmier and Ruiz-Pino 2009). The indices used to capture the size of the denitrification zone were not capable of distinguishing between different N_2-generating processes (Paulmier and Ruiz-Pino 2009). Previous studies aiming to analyze the magnitude of heterotrophic denitrification vs. anammox have been conducted in the world's other two major OMZs, which lie in the Eastern Tropical South Pacific (ETSP) and the Arabian Sea but no such study has been conducted in the ETNP OMZ (Jayakumar et al. 2009). Heterotrophic denitrification has been found to dominate in the Arabian Sea OMZ, whereas anammox has been found to dominate in the ETSP OMZ (Ward and Jayakumar 2010). Heterotrophic denitrification is believed to dominate over anammox in the Arabian Sea OMZ because the Arabian Sea receives a greater carbon supply than the ETSP (Ward et al. 2008). Heterotrophic denitrifying bacteria require a steady supply of organic matter in order to perform heterotrophic denitrification (Ward et al. 2008). Bag incubation experiments showed that the ETSP and ETNP were limited in carbon relative to the Arabian Sea (Ward et al. 2008).

The purpose of this study was to investigate the presence of heterotrophic

denitrifying bacteria in the ETNP OMZ and to compare these findings to heterotrophic denitrification rates, environmental parameters (oxygen, nitrate and nitrite concentrations) and microbial abundance data in order to gain insight into the significance of heterotrophic denitrification in the ETNP OMZ. It is important to study heterotrophic denitrifying bacteria specifically because of their ability to exist in non-oxygen deficient conditions, as well as oxygen deficient conditions. Denitrifying bacteria switch to aerobic growth at normal oxygen concentrations and may therefore be present in areas where denitrification is not necessary (Jayakumar et al. 2009). This allows them to have a more widespread distribution than anammox bacteria, which can exist only in anaerobic environments. Heterotrophic denitrifying bacteria have the potential to more quickly colonize areas that have recently been converted to OMZs than anammox bacteria. Knowledge of heterotrophic denitrifying bacteria may be more pertinent in the current stage of climate change, which has caused the expansion of OMZs but not progressed as far as making OMZs completely anoxic.

I hypothesized that I would be able to detect heterotrophic denitrifying bacteria throughout the vertical extent of the ETNP OMZ and that I would find evidence of active heterotrophic denitrification in the areas where denitrifying bacteria had been discovered. First, I predicted that heterotrophic denitrifying bacteria would be found throughout the entire vertical extent of the ETNP OMZ, including areas above and below the OMZ, because heterotrophic denitrifying bacteria denitrify over a wide range of oxygen conditions, ranging from 20 μmol L^{-1} to 1 μmol L^{-1}, and may exist in areas where oxygen is not limited (Jayakamur et al. 2009). Second, I expected that heterotrophic denitrifying bacteria would be absent from areas where DO concentrations fell below 1 μmol L^{-1}

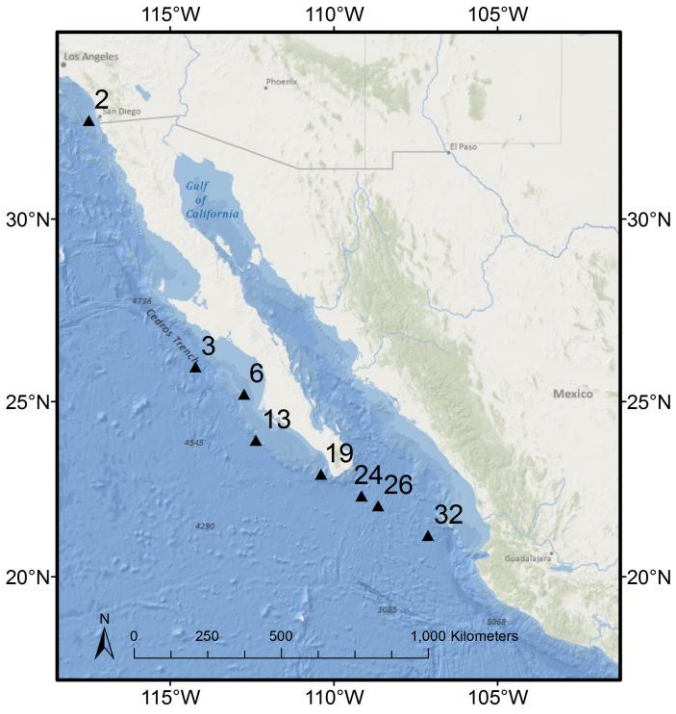
because these conditions are intolerable even for denitrifying bacteria (Jayakumar et al. 2009). Third, I anticipated that areas where heterotrophic denitrifying bacteria had been detected would show increased concentrations of nitrite (NO_2), which is an intermediary in the conversion of nitrate (NO_3^-) to dinitrogen gas (N_2). Fourth, I predicted that microbial abundance would be highest in the rim of the OMZ and would decrease with depth towards the core of the OMZ because the rim of the OMZ would contain sufficient DO to accommodate a variety of microbes other than denitrifying bacteria, whereas the inner region of the OMZ would not contain sufficient DO to accommodate microbes other than heterotrophic denitrifiers and anammox bacteria, leading to lower overall cell counts. Microbial abundance would also decrease from north (San Diego) to south (Manzanillo) because the waters in front of San Diego lie outside of the OMZ and are therefore more suitable habitat for a larger variety of microbes.

Methods

Study Area and Sample Collection

The Ocean 443/444 cruise took place between March 16, 2012 and March 27, 2012 on the *R/V Thomas G. Thompson* of the University of Washington. The ship departed from San Diego, California and cruised along the Baja Peninsula towards Manzanillo, Mexico. A vertical survey of the water column was conducted at seven different stations (Figure 1). Station 2 (San Diego) was chosen as a control station, located outside of the OMZ. Stations 6, 13, and 19 were chosen to represent the coastal ocean and stations 24, 26, and 32 were chosen to represent the open ocean. Water

samples were collected in the Niskin bottles of a CTD Sea-Bird Electronics SBE 911plus rosette. At every station six depths were chosen based on the oxygen profile provided by the computer linked to the downward-traveling CTD. Every station included a surface sample taken from above the OMZ, one sample from inside the oxycline, three samples from within the OMZ, and one sample from below the OMZ. Once the CTD was aboard the ship, 1 L of water was extracted into brown bottles from each Niskin bottle that had taken a sample and filtered through filtering towers onto 0.22 mm, 47 mm diameter Millipore filters. The filtering towers were connected to a vacuum pump. The filters were subsequently packaged into 1.7 ml microfuge tubes and stored at -80°C until further analysis.



Station 2: -117.48 E, 32.61 N
Station 3: -114.22 E, 25.97 N
Station 6: -112.74 E, 25.20 N
Station 13: -112.38 E, 23.91 N
Station 19: -110.39 E, 22.95 N
Station 24: -109.16 E, 22.33 N
Station 26: -108.65 E, 22.05 N
Station 32: -107.12 E, 21.20 N

Fig. 1. Map of the Eastern Tropical North Pacific study side locations.

Microbial abundance

1 ml of each 1 L sample was used for bacterial abundance analysis, which was carried out by means of DAPI staining and epifluorescence microscopy on board the ship according to a protocol by Yamaguchi et al. 2006. First the 1 ml water sample was filtered onto a 0.2 μm polycarbonate membrane filter by vacuum filtering. The filter was then incubated in staining buffer [0.1 mol/l phosphate buffer (pH 8.5), 5% (w/v) NaCl, 0.5 mmol/l EDTA (ethylenediaminetetraacetic acid)] containing DAPI (final concentration, 1 μg/ml) and CFDA (final concentration, 150 μg/ml) in a filtration funnel for 5 min at room temperature (25 °C). Next the DAPI solution was removed by vacuum filtering. The filter was covered with a coverslip, placed under the epifluorescent microscope, and visualized in the UV channel.

Nucleic acid extraction and PCR

Molecular analysis was conducted on only four of the seven stations (6, 13, 24, and 32). First, DNA extractions were performed on the samples according to a phenol-chloroform extraction protocol by Thurber et al. 2009. DNA extractions were followed by polymerase chain reaction (PCR). PCR amplification was performed according to Braker et al. 1998. *NirS1F* was used as the forward primer and *nirS6R* was used as the reverse primer. Amplifications occurred in a total volume of 25 ml composed of 2.5 ml 10X PCR buffer, 2.5 ml forward primer, 2.5 ml reverse primer, 1.25 ml dNTPs, 1.25 ml $MgCl_2$, 0.2 Taq polymerase, 5 ml DNA, and 9.8 ml deionized water. PCR began with a 5-min denaturation step at 95°C, followed by a 30 s denaturation step at 95°C, a primer-annealing step of 40 s, and an elongation step of 40 s at 72°C. 30 cycles were performed

and then concluded with a 7-min incubation at 72°C. Annealing temperatures were decreased by 0.5°C for every new cycle during the first 10 cycles with temperatures beginning at 45°C and ending at 40°C. The following 20 cycles were conducted at a temperature of 43°C. Once PCR was completed, the samples were analyzed via gel electrophoresis on 1.5% agarose gels. A fragment size of 890 bp was expected to indicate the amplification of nitrite reductase on the gel.

Table 1. Primer sequences and positions used to amplify fragments from *nirS* nitrite reductase.

Primer[a]	**Position**[b]	**Primer sequence (5'-3')**
nirS1F	763-780	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T
nirS6R	1638-1653	CGTTGAACTT(A/G)CCGGT

Results

Microbial abundance

Microbial abundance decreased from surface to bottom at all stations sampled (Fig. 2). The highest surface microbial abundance value of $9.3x10^5$ cells ml^{-1} was found at Station 6. Surface abundance otherwise ranged between $8.6x10^5$ cells ml^{-1} and $8.1x10^4$ cells ml^{-1}. Surface abundance showed a slight decreasing trend from northern towards southern stations with Stations 2, 3, and 6 showing higher surface abundances than the more southern stations. Bottom microbial abundance values typically ranged between $1.3x10^4$ cells ml^{-1} and $6.4x10^4$ cells ml^{-1}. Station 6 was the only station where microbial abundance increased to a value of $7.2x10^5$ ml^{-1} at depth. The more southern stations

showed less variation in microbial abundance from the surface to the bottom of the water column.

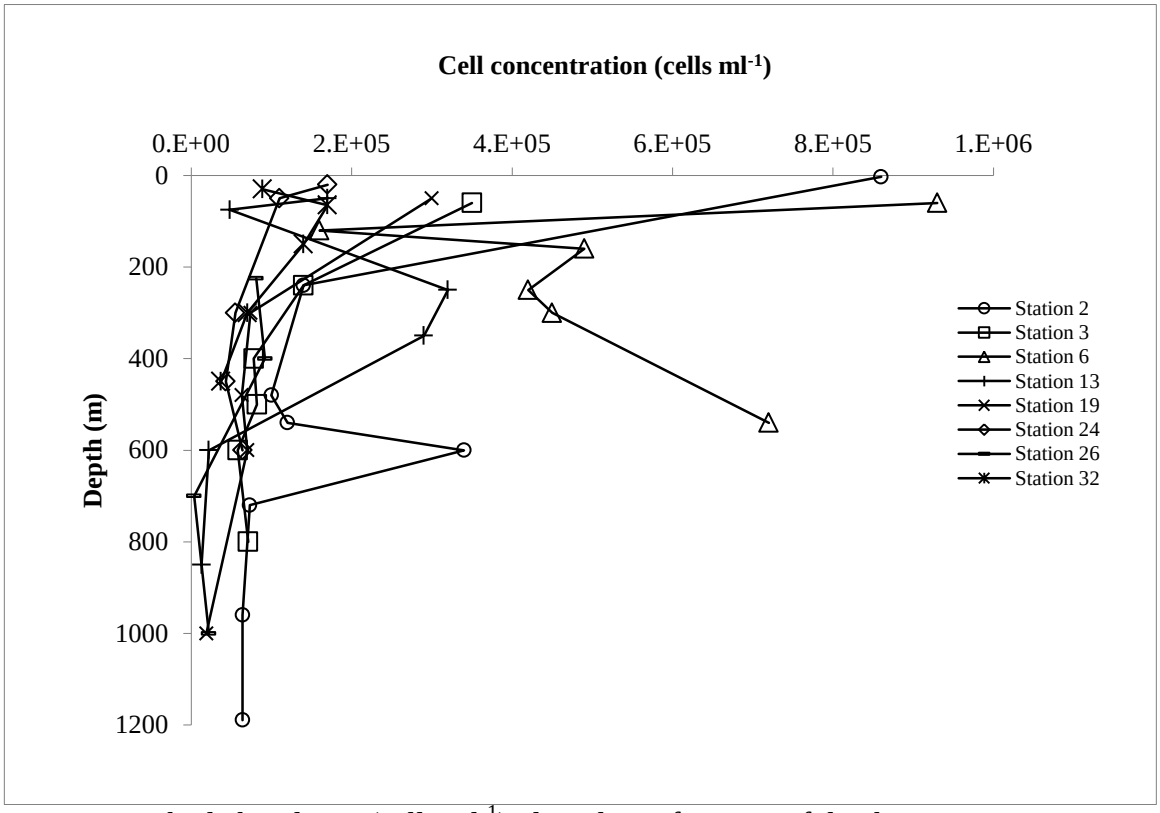


Fig. 2. Microbial abundance (cells ml^{-1}) plotted as a function of depth.

Molecular analysis

Heterotrophic denitrifying bacteria were detected at 160 m, 200 m, and 300 m at Station 6 (Fig. 3a). No heterotrophic denitrifying bacteria were detected at 60 m and 540 m at Station 6. Heterotrophic denitrifying bacteria were detected at 50 m, 150 m, and 300 m at Station 24 (Fig. 3c). No heterotrophic denitrifying bacteria were detected at 450 m and 600 m at Station 24. Heterotrophic denitrifying bacteria were not detected at any of the depths sampled at Stations 13 and 32.

Environmental parameters

Oxygen concentrations decreased from the surface towards the bottom at all four stations. Surface oxygen values (~3 m) ranged between 235.2 µmol kg^{-1} (Station 6) and 210.5 µmol kg^{-1} (Station 32) and deep water oxygen values (~600 m) ranged between 2.9 µmol kg^{-1} (Station 13) and 1.0 µmol kg^{-1} (Stations 24 and 32). Oxygen values from the surface to the bottom showed a slight decrease from northern stations towards southern stations.

Nitrate concentrations increased with depth at all four stations and were similar at all four stations from surface to bottom. Surface nitrate values (~3 m) were low with values between 0.0 µmol L^{-1} (Stations 6 and 24) and 1 µmol L^{-1} (Station 32). Deep water nitrate concentrations ranged between 25.0 µmol L^{-1} (Station 6, 600 m) and 45.4 µmol L^{-1} (Station 13, 1000 m).

Nitrite concentrations were very low from surface to bottom at all stations except for Station 32. Many of the sampling depths did not contain any nitrite. There was no clear pattern regarding surface vs. bottom nitrite concentrations. Overall nitrite concentrations ranged between 0.06 µmol L^{-1} (Station 6) and 6.60 µmol L^{-1} (Station 32).

Station 6 (Fig. 3a)

Oxygen concentrations ranged between 235.2 µmol kg^{-1} at the surface (~3 m) and decreased to 2.1 µmol kg^{-1} at depth (~538 m). Nitrate concentrations ranged between 0 µmol L^{-1} at the surface (~3 m) and 25.0 µmol L^{-1} at depth (~538 m). Nitrite was detected at 60 m and 538 m with values of 0.17 µmol L^{-1} and 0.06 µmol L^{-1} respectively.

Station 13 (Fig.3b)

Oxygen concentrations ranged between 230.5 µmol kg^{-1} (~4 m) and 2.9 µmol kg^{-1} (~600 m). Oxygen increased slightly at the bottom (~1000 m) to 12.0 µmol kg^{-1}. Nitrate concentrations ranged between 0.2 µmol L^{-1} (~4 m) and 45.4 µmol kg^{-1} (~1000 m). Nitrite was detected at 50 m and 100 m with values of 0.01 µmol L^{-1} and 0.08 µmol L^{-1} respectively.

Station 24 (Fig. 3c)

Oxygen concentrations ranged between 211. 0 µmol kg^{-1} (~3 m) and 1.2 µmol kg^{-1} (600 m). Oxygen increased slightly at the bottom (~1000 m) to 12.9 µmol kg^{-1}. Nitrate concentrations ranged between 0.0 µmol L^{-1} at the surface (~3 m) and 41.9 µmol L^{-1} at depth (~1000 m). Nitrite was detected at every depth sampled with values averaging around 0.04 µmol L^{-1}. A peak in nitrite concentration was detected at 50 m with a value of 0.16 µmol L^{-1}.

Station 32 (Fig. 3d)

Oxygen concentrations ranged between 210.5 µmol kg^{-1} (~3 m) and 0.8 mmol kg^{-1} (65-100 m). Oxygen increased towards the bottom (~2000 m) to 77.1 µmol kg^{-1}. Nitrate concentrations ranged between 1.0 µmol L^{-1} at the surface (~3 m) and 44.7 µmol L^{-1} at depth (~1000 m). Nitrite was detected at all depths sampled between 30 m and 200 m with values ranging between 0.3 µmol L^{-1} and 6.6 µmol L^{-1}.

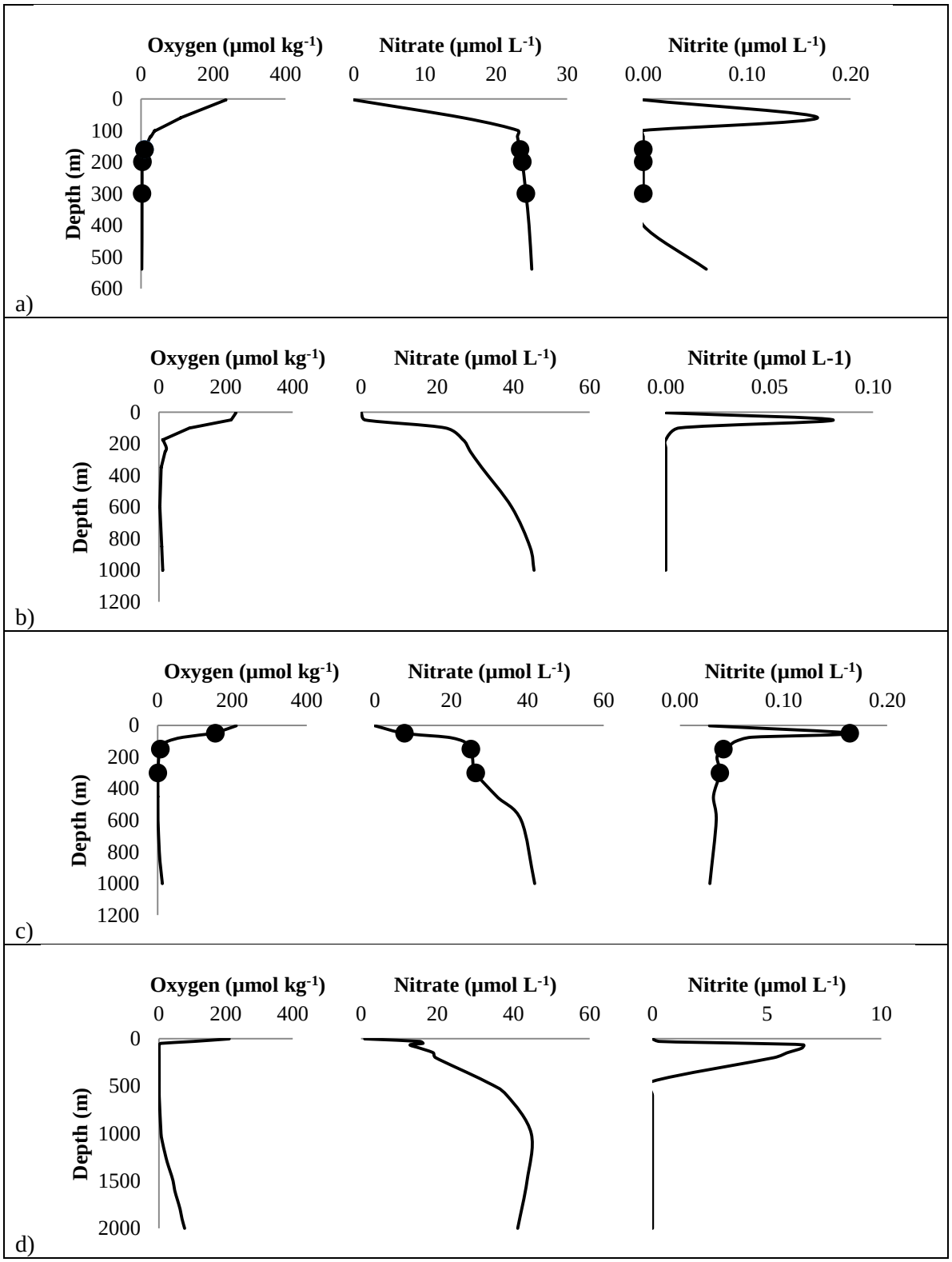


Fig. 3. Environmental parameters at a) Station 6 b) Station 13 c) Station 24 and d) Station 32. Circular markers indicate where heterotrophic denitrifying bacteria were detected (Station 6: 160 m, 200 m, 300 m and Station 24: 50 m, 150 m, 300 m).

Discussion

Microbial abundance patterns followed my predictions, apart from several exceptions. Abundance typically decreased from the surface to the bottom of the water column. This was expected because oxygenated surface waters contain higher phytoplankton densities than deoxygenated deep waters (Lalli and Parsons 1993). Microbes depend on the particulate detritus, excretion, and exudation generated by phytoplankton and zooplankton as food sources (Lalli and Parsons 1993). Their abundance is therefore directly related to the abundance of phytoplankton and zooplankton. Chlorophyll data from the cruise indicated much higher chlorophyll concentrations in surface waters than at depth. Microbial surface abundances decreased slightly from San Diego towards more southern stations due to a slight decrease in surface oxygen concentrations from north to south and consequently decreasing chlorophyll concentrations. Several patterns were unexpected. Cell abundance at Station 6 increased at 540 m for example. The CTD scraped the bottom at this depth, which suggests that sediment infiltrated the sample. Sediment typically contains large numbers of microbes (Lalli and Parsons 1993), which indicates that this led to the high cell counts for this depth. At Station 13 abundance increased between 250 m and 350 m. This increase can be explained by a corresponding increase in oxygen at these depths.

Heterotrophic denitrifying bacteria were detected at the expected depth ranges at Station 6 and Station 24, with the exception of the 50 m sample from Station 24. Heterotrophic denitrifying bacteria were not detected at Station 13 and Station 32. Oxygen concentrations were low where denitrifying bacteria were discovered (<20 μmol L^{-1}), excluding the 50 m sample from Station 24 where the oxygen concentration was

153.6 μmol L^{-1} and therefore much above the expected threshold for heterotrophic denitrification. Nitrite concentrations were low at all stations, excluding Station 32, and did not surpass denitrification stage 1 (0-35% of NO_3^- reduced; Table 2).

No nitrite was detected at the Station 6 depths where heterotrophic denitrifying bacteria were found, which suggests that either no heterotrophic denitrification was occurring or that denitrification had reached Stage 4 (90 - 100% NO_3^- reduced; Table 2). Another possible explanation is that anammox was occurring at this Station. Anammox bacteria were discovered at 160 m and 300 m at Station 6. Since anammox bacteria use nitrite as an oxidant (Arrigo 2005) it is possible that they used up all of the nitrite generated by heterotrophic denitrifying bacteria in this area. The presence of heterotrophic denitrifying bacteria in areas where no nitrite was detected may also be explained by the fact that heterotrophic denitrifying bacteria often use oxygen as an electron acceptor when oxygen conditions are not completely depleted (Wilson and Bouwer 1997). Oxygen concentrations at Station 6 were between 2.9 and 9.9 μmol kg^{-1} in the areas where heterotrophic denitrifying bacteria were detected. The oxygen threshold above which denitrification is inhibited varies over a wide range of concentrations depending on the preference of the species of bacteria (Jayakumar et al. 2009). Different species begin denitrifying at different oxygen concentrations (Jayakumar et al. 2009).

Table 2. Adapted from Jayakumar et al. 2009. Stages of denitrification defined by % NO_3^- reduced.

Stage of denitrification	**Nitrite concentration μmol L^{-1}**	**% of NO_3^- reduced**
0	0	0
1	0-1	0-35
2	1-2	35-45
3	3-5	45-90
4	>5	90-100
5	0	100% (not SO_4^- reducing)
6	SO_4^- reducing	

The fact that no heterotrophic denitrifying bacteria were detected at 60 m at Station 6 was expected because oxygen concentrations were approximately 110.5 μmol kg^{-1} at this depth and therefore too high for heterotrophic denitrification to occur (Jayakumar et al. 2009). No heterotrophic denitrifying bacteria were detected at 540 m at Station 6, which was also not surprising as oxygen conditions at this depth were very low with a value of 2.1 μmol kg^{-1}.

Nitrite was very low at Station 13 and only detected at two depths (50 m and 100 m) with values of 0.01 μmol L^{-1} and 0.08 μmol L^{-1}. Since heterotrophic denitrifying bacteria were not found at Station 13 and there was no evidence of heterotrophic denitrification detected at this station as determined by fellow student Greg Ikeda (Ikeda 2012), this suggests that heterotrophic denitrifying bacteria do not inhabit this area. One additional possibility is that since only three depths (50 m, 150 m, 300 m) were processed from this station, a large sample area that might have contained heterotrophic denitrifying bacteria was eliminated.

The 50 m depth of Station 24 showed the most elevated nitrite concentration besides Station 32 with a value of 0.2 μmol L^{-1}. Since heterotrophic denitrifying bacteria were found at this depth, this suggests that heterotrophic denitrification was occurring, even though oxygen concentrations were sufficient to allow aerobic respiration. Previous research indicates that heterotrophic denitrification may occur in oxygenated areas due to the existence of anoxic microenvironments in these areas (Wilson and Bouwer 1997). These anoxic microenvironments are found in the oxygen-depleted interiors of particles in the euphotic zone (Dore 1995). The interiors of particles may become anoxic when bacteria colonize them and use up the oxygen while performing microbial processes such

as decomposition. It is possible that heterotrophic denitrifying bacteria in this area were attached to particles. Nitrite concentrations at Station 24 were slightly elevated at 150 m and 300 m (the two other areas where heterotrophic denitrifying bacteria were detected), which suggests that heterotrophic denitrification was occurring in very limited terms (Stage 1: 0-35% of NO_3^- reduced; Table 2). The fact that no heterotrophic denitrifying bacteria were detected at 450 m and 600 m at Station 24 was not surprising because oxygen conditions at these depths were very low with values between 1.0 and 1.2 μmol kg^{-1}.

Station 32 showed very high nitrite concentrations that were defined by denitrification stage 4 (90-100% NO_3^- reduced; Table 2). Regarding these high nitrite concentrations, the absence of heterotrophic denitrifying bacteria was surprising. I expected to find heterotrophic denitrifying bacteria in the 150 m sample due to the high nitrite concentration and low oxygen at this depth. The absence of heterotrophic denitrifying bacteria and large presence of nitrite in the subsurface waters of this station suggest that the nitrite excess was a result of phytoplankton excretion or an imbalance between ammonia oxidation and nitrite oxidation instead of the result of heterotrophic denitrification (Lomas and Lipschultz 2006). Another possibility is that since PCR was performed on only two depths for this station (150 m and 600 m), a large sample area that could have contained heterotrophic denitrifying bacteria was eliminated, reducing the chance of finding denitrifiers at this station when they were actually present.

Results from this study suggest that heterotrophic denitrification plays an important role in nitrogen loss in the ETNP OMZ. A study conducted by fellow student Matt Bessee suggests that heterotrophic denitrification, and not anammox, is the

dominant N_2-generating process in the ETNP (Bessee 2012). These findings are similar to those of the study conducted by Jayakumar et al. 2009 in the Arabian Sea OMZ, where heterotrophic denitrification was found to dominate over anammox. In addition, this data suggests that communities of heterotrophic denitrifying bacteria have the potential to increase in abundance with the expansion of OMZs, whereas overall microbial diversity will decrease. Decreases in microbial abundance with decreasing oxygen and decreasing chlorophyll suggest that diversity also decreases with decreasing oxygen and decreasing chlorophyll because fewer species can tolerate these conditions. The Jayakumar et al. 2009 study found that diversity decreased in the core (low oxygen and chlorophyll) of the OMZ, where overall microbial abundance was low. The area was dominated by several species of heterotrophic denitrifying bacteria. This suggests that as OMZs expand, microbial abundance will potentially decrease in these areas of the ocean and that these areas could become dominated by a few different heterotrophic denitrifying species such as observed in the Jayakumar et al. 2009 study.

The next interesting step would be to study which different species of bacteria inhabit the ETNP OMZ, as well as a species to habitat relationship analysis. This would assist in confirming that heterotrophic denitrification is the dominant nitrogen-removal process in the ETNP. In addition it would be interesting to determine if other patterns found in the Arabian Sea study persist in the ETNP. The 2009 Jayakumar et al. study for example observed that diversity of denitrifying bacteria was high in the rim of the Arabian Sea OMZ, whereas denitrifying bacteria abundance relative to overall microbial abundance was low. Surveys of the core of the OMZ showed that denitrifying bacteria abundance relative to overall microbial abundance had increased but diversity had

decreased. The conclusion was that several species of denitrifying bacteria had outcompeted the other microbes in the core, leading to low overall diversity (Jayakumar et al. 2009). Further investigation of elements, which might influence the occurrence of heterotrophic denitrification vs. anammox would also be helpful. As mentioned previously, heterotrophic denitrifying bacteria require organic matter in order to perform denitrification (Ward et al. 2008). It would be interesting to identify other essential components of heterotrophic denitrification. This would assist in better understanding this process and facilitate predictions of where it might intensify as the climate continues to change.

This study was limited by a variety of factors, the main one being time constraint, which prevented me from performing DNA extractions and PCR analysis of the remaining stations. In addition, it would have been necessary to clean up or entirely redo some of the samples that I performed DNA extractions on. Some of these samples ended up with very small amounts of DNA and large amounts of contamination, which prevented me from using them in the PCR analysis. I only ran three samples for Station 13 and two samples for Station 32 due to the large contamination or lack of adequate DNA in the other samples from these stations. It is possible that I did not detect heterotrophic denitrifying bacteria at these stations due to the low number of samples that I processed. Six samples were run for each Station 6 and Station 24, increasing the probability of finding denitrifiers at these stations. More nitrite measurements would have been helpful in order to determine a more accurate picture of denitrification rates at the stations sampled.

Conclusions

(I) Presence of heterotrophic denitrifying bacteria and evidence of heterotrophic denitrification suggest that heterotrophic denitrifying bacteria play an active role in nitrogen removal in the ETNP OMZ.

(II) Heterotrophic denitrifying bacteria were detected primarily in oxygen-depleted environments. This suggests that this is their primary habitat and that they could therefore increase in abundance in the case of the expansion of oxygen minimum zones.

(III) Microbial abundance decreases with decreasing oxygen and decreasing chlorophyll concentrations. This suggests that overall diversity might also decrease with depth. In the case of the expansion of oxygen minimum zones microbial communities might become less diverse and dominated by a few different denitrifying species such as observed in the core of the OMZ in the Arabian Sea study by Jayakumar et al. (2009).

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