Collaborative Research: Dinitrogen fixation rates and diazotrophic communities in contrasting oxygen regimes of the Eastern Pacific Ocean

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Rationale

In much of the world ocean, the bioavailability of dissolved nitrogen (N) limits primary production in surface waters. While dinitrogen (N_2) is abundant in marine waters, it is biologically unavailable to all but certain groups of prokaryotic marine organisms that fix N_2 (diazotrophs). Diazotrophs can stimulate biological production via the introduction of new N into otherwise N-depleted oceanic systems. However, in current models N_2 fixation is restricted to the euphotic zone of tropical and subtropical seas where autotrophic cyanobacterial diazotrophs are known to occur (Gruber 2008). While we know that diazotrophs inhabit other aphotic habitats including microbial mats in shallow benthic environments (Bebout et al. 1987, Paerl et al. 1996, Herbert 1999), sea grass communities (Capone 1983, McGlathery 2008), coral reef systems (O'Neil & Capone 2008), and hydrothermal vent communities (Metha et al. 2005, Mehta & Baross 2006), these habitats are geographically limited and so their contribution to marine N_2 fixation is thought to be small (Carpenter & Capone 2008). Recent work suggests that planktonic diazotrophs are geographically more widely distributed than previously thought and pelagic diazotrophs have now been identified in higher latitude temperate oceanic regions (Moisander et al. 2010), temperate coastal systems (Moisander et al. 2010, Mulholland et al. 2012), and coastal Arctic Seas (Blais et al. 2012, Sipler et al. submitted). In addition, recent studies suggest that there is active N_2 fixation in relatively warm (14-23^{o}C) aphotic oxygenated pelagic waters (Rahav et al. 2013) and in aphotic waters within oxygen deficient zones (ODZs) (Fernandez et al. 2011, Hamersley et al. 2011, Jayakumar et al. 2012). Because the volume of aphotic water in the ocean is large, if N_2 fixation is widely distributed at sub-euphotic depths, this could result in a dramatic upward revision of global N inputs via this process. However, at present there are few measurements of rates and we know little about how vertical chemical and physical gradients affect N_2 fixation and the diazotrophic communities mediating these N inputs.

The global balance between oceanic N inputs via N_2 fixation and N losses via denitrification (including annamox) impacts the oceanic inventory of bioavailable nitrogen and therefore, primary productivity in the oceans (Gruber & Galloway 2008). On geologic timescales, these processes are thought to be balanced (Falkowski 1997, Deutsch et al. 2007). However, rate measurements suggest that N losses from the ocean via denitrification exceed N inputs via N_2 fixation at present (Codispoti et al. 2001, Codispoti 2007, Gruber & Galloway 2008). Consequently, the balance between nitrogen (N) inputs to and N losses from the world's ocean and the distributions of microorganisms mediating these processes have been the subject of spirited debate over the last century. While denitrification is limited to oxygen depleted zones, there are vast volumes of suboxic waters associated with three major ODZs in the Arabian Sea, the ETNP, and the ETSP and the volume of suboxic water is projected to increase under future climate scenarios (Stramma et al. 2008) affecting marine productivity (Behrenfeld et al. 2006, Polovina et al. 2008, Stramma et al. 2010, 2011). N_2 fixation could offset N losses in these regions if it is quantitatively significant. The goal of the proposed research is to resolve rates of N_2 fixation and diazotrophic diversity with respect to <u>vertical</u> gradients of oxygen, light, and dissolved nitrogen (N), within and adjacent to the ETNP and ETSP ODZs where there are contrasting productivity regimes as well as profound losses of oceanic N due to denitrification.

Project Goals

We examined dinitrogen (N_2) fixation rates and *nifH* gene diversity in the context of light, nutrient, and oxygen gradients (and necessarily temperature gradients) along vertical profiles that penetrate into to the eastern tropical North Pacific (ETNP) and eastern tropical South Pacific (ETSP) oxygen deficient zones (ODZs). These oceanic realms have contrasting surface productivity which may control rates of microbial growth and processes at depth. We compared rates of N_2 fixation and diazotrophic community composition in vertical profiles within the ODZs to those in water masses adjacent to the ODZs. Rates were measured using stable isotope tracer techniques that account for slow gas dissolution and that we have already applied successfully elsewhere; we refine those methods as part of this project. We compared rate measurements of N_2 fixation with the abundance and expression of *nifH* genes and *nirS* genes as proxies for active N_2 fixation and denitrification, respectively, in the region to better understand the juxtaposition of these two processes in association with ODZs.

The overarching questions that we were addressing during our cruises were:

1. *What is the contribution of diazotrophy to the total productivity in the euphotic zone of the ETP?*
2. *Is N_2 fixation occurring in and above the ETP ODZs and if so, how do the rates compare to N_2 fixation rates in euphotic and aphotic N_2 fixation in adjacent oxic waters?*
3. *How does the community composition of N_2 fixing microbes vary with respect to the vertical gradients of light, oxygen, and dissolved inorganic N concentrations with depth?*
4. *What is the contribution of heterotrophic N_2 fixation to depth integrated N_2 fixation in the ETP both in and adjacent to the ODZs?*
5. *Is the rate of N_2 fixation (N inputs) within and above the ETP ODZs enough to partially offset denitrification (N losses) from these regions?*

During our cruise from 12/31/14 to 1/23/15 to the Eastern Tropical South Pacific (ETSP) aboard the *R/V Atlantis* we were able to implement all of our objectives despite our inability to obtain clearances to sample Peruvian coastal waters until mid-way through the cruise during January 2015.

Primary production and N_2 fixation

During the ETSP cruise we occupied 19 stations; usually one per day (Figure 1). At each station we conducted CTD casts to obtain hydrographic measurements and to collect water using Niskin bottles mounted on the CTD rosette. Water was collected from 3 to 5 euphotic depths at a pre-dawn cast to measure net primary productivity and N_2 fixation in surface waters. Detailed vertical profiles of nitrite concentrations were obtained by pooling results from multiple CTD casts. Water was collected from sub-oxic depths using a pump profiling system comprised of a CTD and tubing through which to pump water to the surface. Incubation bottles were filled and capped under an anoxic headspace. Primary productivity was measured in surface water incubations using ^{13}C-labeled bicarbonate. Experiments were initiated by adding ~10% of the calculated ambient bicarbonate concentration as $NaH^{13}CO_3^-$ (~200 μM additions) to triplicate light and duplicate dark bottles. Light bottles and bottles filled from sub-oxic depths (4L amber glass bottles) were capped and then enriched with $^{15}N_2$ (1 mL/L). Bottles were gently inverted for about 15 minutes to maximize gas dissolution, and then the gas bubble was removed to prevent further enrichment over the course of incubations. To terminate incubations, bottles

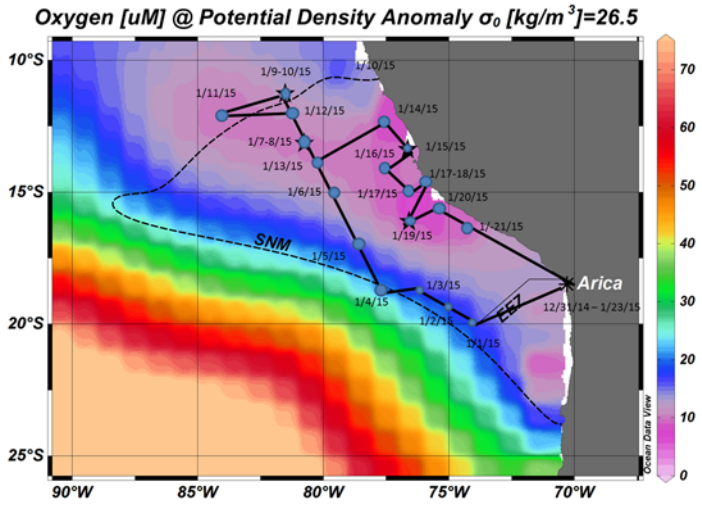

Figure 1. Station locations overlain on O_2 concentrations plotted on an isopycnal (from the World Oceans Atlas).

were uncapped and then an 8 ml sample was immediately removed using a gastight syringe and injected into a He-purged exetainer for analysis of ^{15}N enrichment of the dissolved N_2 pool. The remaining sample was filtered through a pre-combusted (450°C for 2 hours) GF75 filter (nominal pore size of 0.3 µm). Filters were placed into cryotubes and then frozen and transported to Mulholland's laboratory at Old Dominion University (ODU) for analysis. Exetainers were shipped by surface to Princeton University.

Anammox and denitrification

N loss measurements (anammox and denitrification) were made at 8 stations during the ETSP cruise and at 15 stations during the ETNP cruise. Seawater samples were collected from two or more depths (up to a maximum of five depths covering the span of low oxygen depths). Experiments were set up as described below to measure N loss and nitrous oxide production. For these experiments seawater was collected from the 10L Niskin into 320mL ground glass bottles which were overflowed three times to maintain anoxia. In an N_2-flushed glove bag, tracer solutions ($^{15}NH_4^+ + {}^{14}NO_2^-$ and $^{15}NO_2^- + {}^{14}NH_4^+$) were added to the bottles to reach final concentrations of 3 µM ^{15}N. 12mL exetainers (Labco) were filled with 8mL of tracer-spiked seawater, removed from the glove bag, and flushed with helium at 3 psi for 5 minutes. Incubations lasted 24 hours, during which triplicate vials were sacrificed every 8 hours by injecting 0.1mL 50% (w/v) $ZnCl_2$ solution. These samples were transported to Princeton where they were placed on autosampler using ultra-high purity (UHP) He as the carrier gas and injected into a Europa 20/20 mass spectrometer to measure N_2 concentration and isotope ratio (m/z = 28, 29, 30) measurements. Rates of denitrification and anammox were calculated according to Ward et al. (2009).

N_2O production

N_2O production (^{15}N incubation) experiments were targeted at depths where oxygen concentrations changed dramatically (oxycline) and remained below detection (ODZ). Seawater was sampled from 10L Niskin bottles into the bottom of acid washed, 50mL glass serum bottles (Wheaton, USA) and then allowed to overflow two to three times the volume before sealing it with a three prong butyl septum and aluminum ring. 5mL headspace was created by replacing the volume with UHP He. Three suites of ^{15}N tracer solutions ($^{15}NH_4^+$ plus $^{14}NO_2^-$, $^{15}NO_2^-$ plus $^{14}NH_4^+$, $^{15}NO_3^-$ plus $^{14}NH_4^+$ and $^{14}NO_2^+$) were applied to enrich $^{15}NH_4^+$, $^{15}NO_2^-$ and $^{15}NO_3^-$ to 0.5 and 1 µM (final concentration), respectively. Tracer solutions were made from nitrogen free deionized water, and were degassed before adding 0.1mL into each sample. Incubations lasted 24 hours at *in situ* temperatures. Duplicate samples were preserved every 6 hours by adding 0.1mL saturated $HgCl_2$. N_2O samples for initial concentrations and isotopic enrichment were preserved immediately after collection from Niskin bottles. Samples were transported to Princeton and

placed in an autosampler in line with a cryo-focusing unit with UHP He as the carrier gas. They were injected into a Thermo-Finnigan Delta V for concentration and isotope ratio (m/z = 44, 45, 46) measurements. N_2O production was calculated from the progressive increase in $^{45}N_2O$ and $^{46}N_2O$ in time course experiments. After N_2O measurements, samples spiked with $^{15}NH_4^+$ and $^{15}NO_3^-$ were also measured for $^{15}NO_2^-$ to determine rates of ammonium oxidation and nitrate reduction. Briefly, NO_2^- was quantitatively converted to N_2O using sodium azide in acetic acidic solution. The resulting N_2O was measured on a Thermo-Finnigan Delta V mass spectrometer.

Nutrient and biomass measurements

Dissolved nitrite and ammonium were analyzed colorometrically and fluorometrically, respectively, onboard ship (Grasshof et al. 1983, Holmes et al 1999). Chlorophyll *a* concentrations were analyzed fluorometrically onboard (Welschmeyer 1994). Additional water samples were filtered through 0.2 μm membrane Millipore filters, and filtrate was placed in sterile vials for nitrate, phosphate, and urea analysis using an Astoria Pacific Autoanalyzer at ODU (stored at -20 ^oC). Filtered (0.2 μm) samples were also collected and stored at -80 ^oC for cyanate analysis at Old Dominion University using high performance liquid chromatography (Widner et al. 2013). Samples were also collected to measure particulate carbon (PC) and nitrogen (PN) concentrations and the natural abundance of particulate ^{15}N and ^{13}C in the environment. These samples were filtered onto pre-combusted (450^oC for 2 hours) GF75 filters (nominal pore size of 0.3 μm), placed in cryovials, frozen, and transported to ODU for analysis. At ODU, isotope enrichment and PN/PC were measured on a Europa 20/20 isotope ratio mass spectrometer equipped with an automated nitrogen and carbon analyzer.

Molecular sample collection

Samples for molecular analysis were collected from more than 150 depths covering the major process study stations, specifically, the depths where samples were collected for N_2 fixation and nitrogen loss. Upon completion of analysis or rate measurements, molecular samples will be selected for analysis.

Data Management

We are preparing data for submission to our BCO-DMO webpage, http://www.bco-dmo.org/project/472492, the preferred archival database of the National Science Foundation.

Project Results

The aims of the sampling program were to get a 3-dimensional picture of where active N_2 fixation occurs with respect to gradients of dissolved oxygen, light, nutrients and productivity. As such, our cruise plan was designed to capture both horizontal and vertical gradients in these properties. At each station we conducted a pre-dawn vertical cast to capture the basic hydrographic properties and collect samples to guide subsequent casts.

The most significant findings so far include:

1) Measurements of active N_2 fixation in surface and subsurface waters and N loss at suboxic depths.
2) Identification and quantification of unique assemblages of diazotrophic microbes.
3) Identification of cyanate as a contributor to N losses in oxygen deficient zones.

In particular, we found that rates of N_2 and C fixation were highest in surface waters at the coastal stations, within the oxic zone (Figures 2, 3, and 4). Rates of N_2 fixation were measureable in deeper anoxic waters but rates were low.

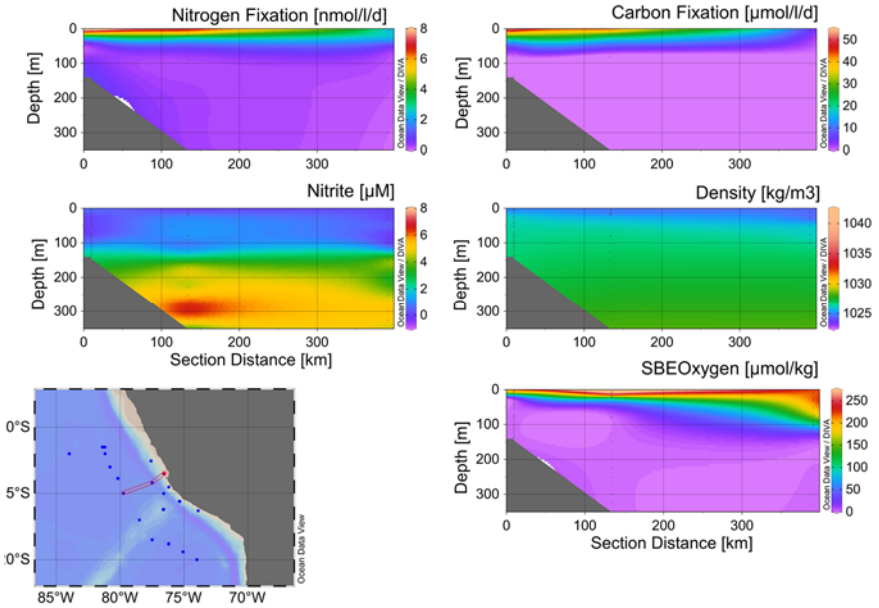

Figure 2. Sections showing rates of N_2 and C fixation, nitrite and dissolved O_2 concentrations and density from an onshore-offshore transect between 3 - 5º South latitude near the coast of Peru.

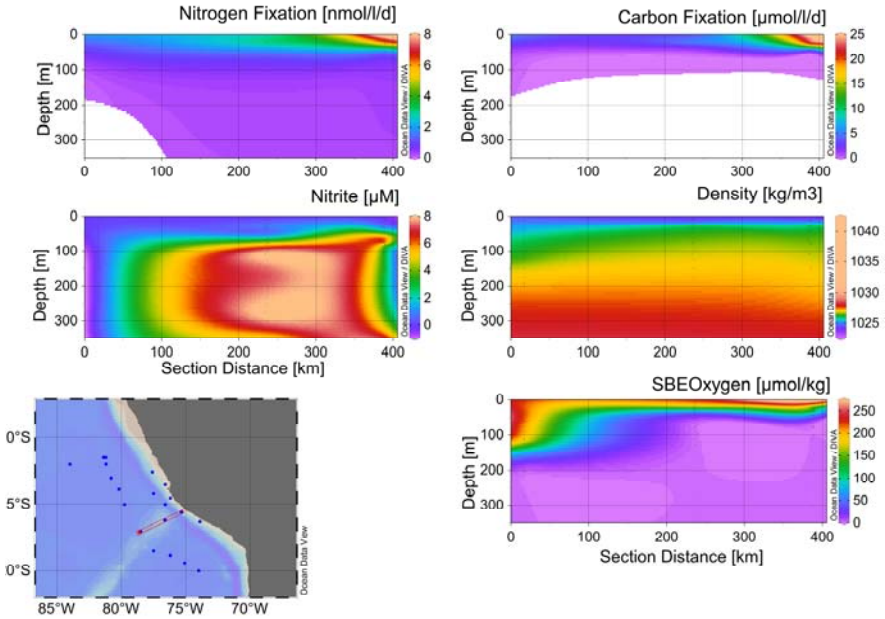

Figure 3. Sections showing rates of N_2 and C fixation, nitrite and dissolved O_2 concentrations and density from an onshore-offshore transect between 5 - 7º South latitude near the coast of Peru.

Some samples still need to be analysed.

Products to date

Two manuscripts have been prepared, one submitted to *Limnology and Oceanography* and the other will be submitted shortly to the *ISME Journal*.

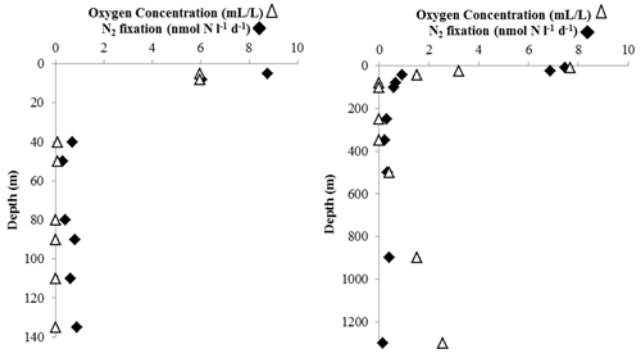

Figure 4. Depth profiles of N_2 fixation and O_2 concentrations from the two nearshore stations shown in Figures 2 and 3. The panel on the left is the more northern station and the panel on the right is the southern station.

A talk was presented at the SCOR WG144 meeting in Goa, India in December 2016 and a poster was presented at the AGU fall meeting in San Francisco, CA. Another talk and poster were presented at the ASLO Ocean Sciences meeting in New Orleans, LA (February 21-26, 2016). A poster was also presented at the Aquatic Sciences meeting in Granada, Spain (February 22-27, 2015). An invited oral presentation was given at the Gordon Research Conference (July 27-31, 2015). One manuscript has been submitted that includes results from this cruise and many more are in preparation

Training

Brittany Widner was a graduate student supported by the project and she has gained further laboratory and field experience as she participated in all aspects of cruise planning and sample collection and processing. Two other graduate students from ODU participated in the cruise, Shannon Cofield and Stephen Stone, as well as Mulholland's laboratory manager, Peter Bernhardt. A graduate student from Princeton University, Qixing Ji, also participated in the cruise and made measurements of denitrification. In addition, a graduate student, two post-doctoral researchers, and a research technician from Chile and two researchers from Peru participated in the cruise.

In addition to researchers from Old Dominion University and Princeton, there were 4 participants from the University of Concepción in Chile (Mr. Gadiel Eugenio Alarcón Coronado, Dr. Maria Consuelo Gazitua Zavala, Dr. Alejandro Andrés Murillo Córdova, and Ms. Montserrat Gabriela Aldunate Chinchón), and 2 participants from the Instituto del Mar del Perú (IMARPE) (Mr. Sergio Bances and Mr. Alberto Saturnino Lorenzo Puitiza).

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