

ORIGINAL ARTICLE

Nitrite oxidation in the upper water column and oxygen minimum zone of the eastern tropical North Pacific Ocean

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Nitrogen (N) is an essential nutrient in the sea and its distribution is controlled by microorganisms. Within the N cycle, nitrite (NO₂⁻) has a central role because its intermediate redox state allows both oxidation and reduction, and so it may be used by several coupled and/or competing microbial processes. In the upper water column and oxygen minimum zone (OMZ) of the eastern tropical North Pacific Ocean (ETNP), we investigated aerobic NO₂⁻ oxidation, and its relationship to ammonia (NH₃) oxidation, using rate measurements, quantification of NO₂⁻-oxidizing bacteria via quantitative PCR (QPCR), and pyrosequencing. ¹⁵NO₂⁻ oxidation rates typically exhibited two subsurface maxima at six stations sampled: one located below the euphotic zone and beneath NH₃ oxidation rate maxima, and another within the OMZ. ¹⁵NO₂⁻ oxidation rates were highest where dissolved oxygen concentrations were <5 μM, where NO₂⁻ accumulated, and when nitrate (NO₃⁻) reductase genes were expressed; they are likely sustained by NO₃⁻ reduction at these depths. QPCR and pyrosequencing data were strongly correlated ($r^2 = 0.79$), and indicated that *Nitrospina* bacteria numbered up to 9.25% of bacterial communities. Different *Nitrospina* groups were distributed across different depth ranges, suggesting significant ecological diversity within *Nitrospina* as a whole. Across the data set, ¹⁵NO₂⁻ oxidation rates were decoupled from ¹⁵NH₄⁺ oxidation rates, but correlated with *Nitrospina* ($r^2 = 0.246$, $P < 0.05$) and NO₂⁻ concentrations ($r^2 = 0.276$, $P < 0.05$). Our findings suggest that *Nitrospina* have a quantitatively important role in NO₂⁻ oxidation and N cycling in the ETNP, and provide new insight into their ecology and interactions with other N-cycling processes in this biogeochemically important region of the ocean.

The ISME Journal (2013) 7, 2192–2205; doi:10.1038/ismej.2013.96; published online 27 June 2013

Subject Category: Geomicrobiology and microbial contributions to geochemical cycles

Keywords: nitrification; nitrite oxidation; *nitrospina*; oxygen minimum zone; pyrosequencing

Introduction

Nitrogen (N) is a required nutrient for all organisms and limits primary production across large areas of the ocean (Mills *et al.*, 2004). Although N is present in many different chemical forms in the sea, dissolved nitrite (NO₂⁻) alone may be used by microorganisms either as a reductant (under aerobic conditions) or oxidant (under anaerobic conditions) owing to its intermediate redox state. In the aerobic ocean, NO₂⁻ is oxidized to nitrate (NO₃⁻) by NO₂⁻-oxidizing bacteria (NOB); this constitutes the second step of nitrification and is expected to be

tightly coupled to the first step, oxidation of ammonia (NH₃) by NH₃-oxidizing bacteria and NH₃-oxidizing Archaea. No single organism is known to carry out both steps of nitrification. Costa *et al.* (2006) argue that this reflects optimization of pathway length to maximize energy production, and that because NO₂⁻ oxidation is energetically less favorable, it must occur more rapidly than NH₃ oxidation and consume NO₂⁻ nearly instantaneously. However the coupling between these groups is fundamentally unstable: Graham *et al.* (2007) demonstrated that perturbation of NH₃ oxidation in bioreactors lead to chaotic behavior among NOB, and evidence for such temporal decoupling in the ocean was observed off southern California (Beman *et al.*, 2010). NH₃ and NO₂⁻ oxidation can also be decoupled spatially; light is thought to directly or indirectly inhibit NH₃-oxidizing Archaea, NH₃-oxidizing bacteria and NOB to varying degrees, with NOB being more sensitive (reviewed by Lomas

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Received 18 December 2012; revised 23 April 2013; accepted 17 May 2013; published online 27 June 2013

and Lipschultz (2006). This may contribute to the accumulation of NO₂⁻ at the base of the euphotic zone—a widespread feature known as the primary NO₂⁻ maximum (PNM). Although prevailing evidence indicates that PNM primarily results from incomplete phytoplankton reduction of NO₃⁻ (Lomas and Lipschultz, 2006, Beman *et al.*, 2012), this remains uncertain because direct quantitative comparisons between NH₃ and NO₂⁻ oxidation, and the microorganisms involved, are rare (Ward *et al.*, 1989a, Lipschultz *et al.*, 1990).

Of particular relevance is the recent discovery that marine Thaumarchaeota (formerly marine group 1 Crenarchaeota) oxidize NH₃ (reviewed by Francis *et al.* (2007); these organisms number up to 10²⁸ cells in the ocean (Karner *et al.*, 2001) and even modest per cell rates of archaeal NH₃ oxidation imply substantial global N turnover. In fact, recent studies indicate that NH₃ oxidation occurs more rapidly and over a broader depth range than previously appreciated (Yool *et al.*, 2007, Beman *et al.*, 2008, Lam *et al.*, 2009, Santoro *et al.*, 2010, Beman *et al.*, 2012). A NO₂⁻ rarely accumulates in the ocean (discussed above), this necessitates a corresponding sink for NO₂⁻. Mincer *et al.* (2007) proposed that correlation between NH₃-oxidizing Archaea and relatives of known NO₂⁻-oxidizing *Nitrospina* bacteria is evidence of metabolic coupling; these groups showed similar distributions in both Monterey Bay and the North Pacific Subtropical Gyre. More recently, Füssel *et al.* (2012) reported that *Nitrospina* numbered up to 5.4% of microbial cells and were detected where NO₂⁻ was oxidized in the oxygen minimum zone (OMZ) along the Namibian coast. This included depths where dissolved oxygen (DO) concentrations were extremely low, indicating that NO₂⁻ oxidation persists even under low DO. In fact, NO₂⁻-based metabolism is central in the world's OMZs (Francis *et al.*, 2007; Wright *et al.*, 2012), where DO concentrations fall below 20 μM because of microbial respiration (Paulmier and Ruiz-Pino, 2009). The accumulation of NO₂⁻ within OMZs is a diagnostic of anaerobic N and sulfur cycling (Ulloa *et al.*, 2012), and among the processes that use NO₂⁻ as an oxidant under these conditions, anammox and denitrification lead to gaseous N loss from the ocean (Lam and Kuypers, 2011). However, the source of NO₂⁻ that fuels these processes is poorly constrained. NH₃ oxidation may contribute *ca.* 50% of NO₂⁻ supply to anammox in the Black Sea (Lam *et al.*, 2007), whereas NO₃⁻ reduction coupled to anammox was most important (at least 67% of NO₂⁻ demand) in the eastern tropical South Pacific (ETSP) OMZ (Lam *et al.*, 2009), and Ward *et al.* (2009) demonstrated that conventional denitrification was dominant in the Arabian Sea.

The eastern tropical North Pacific Ocean (ETNP) is the largest oceanic OMZ (Paulmier and Ruiz-Pino, 2009), and so has an important role in oceanic—and global—N cycling. Annually, ~26 Tg of dissolved N is converted to gaseous forms in the ETNP (DeVries

et al., 2012) via microbial processes that require oxidized N. Our work and that of others has also identified relatively high rates of NH₃ oxidation in the ETNP (Ward and Zafiriou, 1988, Sutka *et al.*, 2004, Beman *et al.*, 2008, 2012); yet NO₂⁻ oxidation has not been previously measured in this region. As NO₂⁻ oxidation can persist under <1 μM DO, NOB may directly compete with other organisms for NO₂⁻ in OMZs (Lipschultz *et al.*, 1990, Füssel *et al.*, 2012); in the ETNP OMZ, the accumulation of NO₂⁻ and its isotopic composition suggest that NO₃⁻ may be continually reduced and reoxidized through dynamic N and NO₂⁻ cycling (Casciotti and McIlvin, 2007). Adding to this complexity, OMZs are expanding as a consequence of climate change (Stramma *et al.*, 2008, Keeling *et al.*, 2010), potentially altering the rates and distribution of N transformations in the water column (Gilly *et al.*, 2013). In the ETNP, N loss rates have varied several-fold over the past few decades in response to variations in the DO (Deutsch *et al.*, 2011). Placing quantitative bounds on microbially driven biogeochemical processes occurring in OMZs—and specifically the ETNP—is therefore essential.

We quantified NO₂⁻ oxidation rates and NOB distributions across an oceanographic transect through northern portions of the ETNP and the southern Gulf of California (GOC). Using an *in situ* floating array and stable isotope tracer, ¹⁵NO₂⁻ oxidation rates were measured at high resolution with depth; NOB were quantified using real-time quantitative PCR (QPCR) and next-generation pyrosequencing of bacterial communities in corresponding DNA samples. This parallels our work on NH₃ oxidation for the same stations and depths (Beman *et al.*, 2012). Dual study of these processes—which presumably are biogeochemically coupled, and together constitute nitrification—are rare in the literature. We compared ¹⁵NO₂⁻ oxidation rate profiles with ¹⁵N-labeled ammonium (¹⁵NH₄⁺) oxidation rates, depth profiles of NOB and key environmental factors such as NO₂⁻ concentrations and DO concentrations.

Materials and methods

Sampling and biogeochemical measurements

Samples were collected in July and August of 2008 aboard the *R/V New Horizon*; seven stations were occupied for several days each. A Seabird Electronics (Bellevue, WA, USA) SBE 9 conductivity-temperature-depth sensor package equipped with additional sensors was used to measure conductivity, temperature, depth, DO concentrations, chlorophyll fluorescence, light transmission and photosynthetically active radiation. Water samples were collected using 10 l polyvinyl chloride bottles deployed on the conductivity-temperature-depth rosette; samples for nutrient measurements and Winkler titrations were typically collected on the

first cast and analyzed within hours of collection to characterize the chemical structure of the water column. We selected depths for rate measurements and collection of DNA samples based primarily on nutrient concentrations: 1–3 depths above the primary NO₂⁻ and NH₄⁺ maxima were sampled, followed by 5–7 depths spaced every 5 m and several additional depths spaced every 10–20 m. ¹⁵NO₂⁻ oxidation rates were measured at each depth on a free-floating array (see below), and DNA samples were collected at the same depths (see below). Water samples for rate measurements and DNA samples were collected on the same cast at all stations.

Details of biogeochemical measurements are provided in Beman *et al.* (2012). Briefly, oxygen concentrations were measured using a SBE oxygen sensor and corrected on the basis of Winkler titrations ($r^2 = 0.997$ for 187 samples for the cruise). Winkler-corrected SBE oxygen data are shown for the casts where rate and DNA samples were collected. NH₄⁺ concentrations were measured using the fluorometric method of Holmes *et al.* (1999), and NO₂⁻ and NO₃⁻ concentrations were measured using standard colorimetric techniques (Strickland and Parsons, 1972); NO₃⁻ was reduced to NO₂⁻ with cadmium for measurement. r^2 for standard curves were: 0.996–0.999 (oxygen measurements), 0.959–0.999 (NH₄⁺ measurements) and 0.993–0.999 (NO₂⁻ and NO₃⁻ measurements).

In situ free-floating array

We deployed a free-floating array to mimic *in situ* conditions for ¹⁵NO₂⁻ oxidation rate measurements (Beman *et al.*, 2012). ¹⁵NO₂⁻ oxidation was measured via addition of ¹⁵N-labeled NO₂⁻ to sample bottles suspended at multiple depths in the water column; water samples were collected into 250 ml polycarbonate bottles in the dark using silicone tubing, samples were overfilled by three volumes to avoid oxygen contamination, chilled ¹⁵N-labeled NaNO₂ was added and bottles were sealed. Bottles were attached to the array immediately before deployment at dawn, and the array was deployed for *ca.* 24 h under *in situ* temperature and light conditions. Our deepest array-based incubations were placed at 160 m depth, and samples collected from depths > 160 m were incubated onboard the ship at 12 °C in the dark within temperature-controlled incubators; this includes 18 samples reported here. Following array recovery, samples were filtered through 0.2 μm syringe filters, collected in 50 ml high-density polyethylene bottles, residual ¹⁵NO₂⁻ was removed (see below) and samples frozen at -20 °C until analysis at the University of Hawaii.

¹⁵NO₂⁻ oxidation rate measurements

¹⁵NO₂⁻ oxidation rates were measured by adding 98 atom percent (atom%) ¹⁵NO₂⁻ to a final concentration of 48–192 nmol l⁻¹ (depending on *in situ* NO₂⁻

concentrations) and measuring the accumulation of ¹⁵N label in the NO₃⁻ pool after incubation for ~24 h; excess ¹⁵NO₂⁻ was removed using methods modified from Granger and Sigman (2009). Upon recovery of samples from the *in situ* array, sulfamic acid (~8–10 μl ml⁻¹) was added, the samples were shaken and allowed to sit for >5 min, and then samples were neutralized by adding NaOH (4 M, ~11 μl ml⁻¹) before storing frozen (-20 °C) until analysis. The efficiency of ¹⁵NO₂⁻ removal using this method was tested in the laboratory and our results indicated a minor amount of ¹⁵NO₃⁻ was present after the sulfamic acid treatment, most likely owing to a small quantity of ¹⁵NO₃⁻ contamination in K¹⁵NO₂ (see Olson, 1981; Ward, 1987; Lipschultz *et al.*, 1990). However, the amount of ¹⁵N label remaining was not significantly higher at concentrations of ¹⁵NO₂⁻ < 200 nmol l⁻¹, and so does not affect our rate calculations.

The δ¹⁵N value of N₂O produced from NO₃⁻ using the denitrifier method (Sigman *et al.*, 2001) was measured using methods described in Popp *et al.* (1995) and Dore *et al.* (1998). Briefly, N₂O produced from NO₃⁻ was transferred from a reaction vial, cryofocused, separated from other gases using a 0.32-mm inner diameter 25 m × 5 μm CP-PoraBOND Q (Agilent Technologies, Santa Clara, CA, USA) capillary column at room temperature and introduced into the ion source of a MAT252 mass spectrometer through a modified GC-C I interface (Thermo Scientific, Bremen, Germany). Isotopic reference materials (United States Geological Survey-32, National Institute of Standards and Technology-3 and University of Hawaii NaNO₃) bracketed every nine samples and δ¹⁵N values measured online were linearly correlated ($r^2 = 0.979$) with accepted reference material δ¹⁵N values. Accuracy and precision were further evaluated by multiple analyses of a sodium NO₃⁻ solution for which the δ¹⁵N value of the solid NaNO₃ (that is, University of Hawaii NaNO₃) had been previously determined using an online carbon-N analyzer coupled with an isotope ratio mass spectrometer (ConFlo II Delta-Plus; Thermo Scientific), and were found to be < ±0.5‰ (±0.00018 atom% ¹⁵N) for samples containing >2.5 nmol of NO₃⁻.

Initial atom% enrichment of the substrate at the beginning of the experiment (*no*NO₂⁻, see Equation (1)) was calculated by isotope mass balance based on NO₂⁻ concentrations, assuming that the ¹⁵N activity of unlabeled NO₂⁻ was 0.3663 atom% ¹⁵N. Rates of ¹⁵NO₂⁻ oxidation (¹⁵R_{ox}) were calculated using Equation (1) from Ward *et al.* (1989b):

$${}^{15}\text{R}_{\text{ox}} = \frac{(n_t - n_{\text{onNO}_3^-}) \times [\text{NO}_3^-]}{(n_{\text{NO}_2^-}) \times t} \quad (1)$$

where n_t is the atom% ¹⁵N in NO₃⁻ measured at time t , $n_{\text{onNO}_3^-}$ is the measured atom% ¹⁵N of unlabeled NO₃⁻, [NO₃⁻] is the concentration of the NO₃⁻ pool

and $n_{\text{NO}_2^-}$ is the exponential average atom% of NO₂⁻ over time t . $n_{\text{NO}_2^-}$ was calculated based on ambient NO₂⁻ concentrations and atom% (assumed to be 0.3663), added ¹⁵N-labeled NO₂⁻, and measured ¹⁵NH₄⁺ oxidation rates (producing unlabeled NO₂⁻) reported by Beman *et al.* (2012).

DNA and RNA extraction

At each depth, 4 l of seawater was collected from the conductivity-temperature-depth rosette and divided into two sets of 2-l samples, which were then filtered through separate 25 mm diameter 0.2 μm Suppor filters (Pall Corporation, Port Washington, NY, USA) using a peristaltic pump. Filters were flash-frozen in liquid N and stored at -80 °C until DNA and RNA extraction, with one set of filters dedicated to each. Details of DNA extraction were reported in Beman *et al.* (2012). Filters for RNA extraction were frozen in RLT buffer with beta-mercaptoethanol added (Qiagen, Valencia, CA, USA), and RNA was extracted using the Qiagen RNeasy kit following Church *et al.* (2005) but with the following modifications: tubes containing filters, glass beads and buffer were first agitated for 80 s on a FastPrep machine (MP Biomedicals, Solon, OH, USA) at setting 5.5; each sample and an equal volume of 70% ethanol solution was then bound to the RNeasy spin column, and purified and eluted following the manufacturer's instructions. We used Turbo DNase (Ambion, Life Technologies Corporation, Carlsbad, CA, USA) to remove carry-over DNA, and samples were treated with 10% DNase buffer and 5% DNase for 20 min at 37 °C.

Complementary DNA was generated from extracted RNA using the Invitrogen SuperScript III Reverse Transcriptase kit (Life Technologies Corporation), following the manufacturer's instructions. In brief, ten microliters of RNA extract, 1 μl each of random hexamers and 1 μl 10 mM dNTPs were incubated at 65 °C for 5 min, and then placed on ice for 1 min; samples were then incubated with 10 μl of complementary DNA synthesis mix (2 μl 10 × reverse transcriptase buffer, 4 μl 25 mM MgCl₂, 2 μl 0.1 M dithiothreitol, 1 μl RNase OUT (40 U - μl⁻¹) and 1 μl SuperScript III Reverse Transcriptase) at 25 °C for 10 min, then 55 °C for 50 min, and the reverse transcriptase reaction was terminated at 85 °C for 5 min. Remaining RNA was removed through incubation with RNase H at 37 °C for 20 min.

Quantitative PCR

QPCR assays were performed on a Stratagene MX3005P (Agilent Technologies), using the following reaction chemistry: 12.5 μl SYBR Premix F (Epicentre Biotechnologies, Madison, WI, USA), 2 mM MgCl₂, 0.4 μM of each primer, 1.25 units AmpliTaq polymerase (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA), 40 ng μl⁻¹ bovine serum albumin and 1 ng DNA in

a final volume of 25 μl. 16S rRNA genes from *Nitrospina* were amplified in triplicate using the primers NitSSU_130F (5'-GGGTGAGTAACACGTGAATAA-3') and NitSSU_282R (5'-TCAGGCCGGCTAAMCA-3'; Mincer *et al.*, 2007). Coefficient of variations of triplicate QPCR reactions averaged 5%. Cycling conditions were modified from Mincer *et al.* (2007): the total number of cycles was reduced from 50 to 30, and the length of the detection step was increased from 1 to 7 s. The partial 16S rRNA sequence from the uncultured marine *Nitrospinaceae* bacterium recovered on BAC EB080L20_F04 was used as a QPCR standard; this sequence was synthesized at Blue Heron Biotechnologies (Bothell, WA, USA) and used in tenfold dilutions from 10⁷ to 10² 16S rRNA genes per μl to generate standard curves ($r^2 = 0.987-0.994$). *narG* and *napA* assays followed Lam *et al.* (2009) and were used to screen select complementary DNA samples (see text); negative controls did not exhibit amplification.

Pyrosequencing

DNA samples collected at all depths at stations 1-6 were sequenced using Titanium chemistry on the Roche 454 (Branford, CT, USA) FLX platform at Research and Testing Laboratories. No samples from station 7 were sequenced. Bacteria-specific primers 27F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GWNTTACNGCGGCKGCTG-3'; Engelbrekton *et al.*, 2010) modified with additional degeneracies (Ns in primer sequence) were used to amplify a portion of the bacterial 16S rRNA gene. Relevant linkers were attached to primers, as were eight-base barcodes (Hamady *et al.*, 2008) used to sort individual samples; for the 60 samples, we recovered 714 245 sequences with a median read length of 475 bp. We used the approach of Huse *et al.* (2007) that is commonly used in the literature for quality control (Brown *et al.*, 2009; Galand *et al.*, 2009). Using the program mothur (<http://www.mothur.org>; Schloss *et al.* 2009), we discarded sequences: > ± 100 bp from the median sequence length, containing any ambiguous bases, containing homopolymers > 8 bp, of < 25 average quality score, and that did not exactly match the forward primer and barcode sequence. The final criterion is where most (21% of all sequences) sequences were removed. (We did not screen sequences based on the reverse primer, as some of the reads are high quality sequences that did not extend to the reverse primer). In total, 282 030 sequences did not meet quality control criteria and were excluded from subsequent analyses.

Bacterial 16S rRNA sequences were aligned to the Greengenes alignment (DeSantis *et al.*, 2006) in mothur. The alignment was optimized to the start position and we discarded sequences starting after the position that 95% of the sequences start; the alignment was then manually curated to remove misaligned sequences ($n = 25$). Community

composition of individual samples was determined by classification of 16S rRNA sequences based on the ARB SILVA data set (Pruesse *et al.*, 2007) in mothur, and data are reported for a consensus confidence threshold of 65%. The 8997 *Nitrospina* 16S rRNA gene sequences identified here were clustered into operational taxonomic units (OTUs) based on 97% sequence identity, and sequences from the 476 OTUs have been deposited in GenBank under accession numbers KC774803-KC775278. We generated representative sequences for each OTU using the get.oturep command in mothur, and BLASTed these sequences against the Genbank nr/nt nucleotide database using blastn and default parameters (database accessed 8 March 2013).

Data analysis

Oceanographic data were visualized in Ocean Data View (<http://www.odv.awi.de/>). To compute the abundance of different *Nitrospina* OTUs, we used QPCR data and the proportion of *Nitrospina* sequences that were affiliated with the different OTUs; that is, the OTU abundance equals the QPCR-based *Nitrospina* abundance at a particular depth, multiplied by the fraction of sequences drawn from a particular *Nitrospina* OTU in proportion to the total number of *Nitrospina* sequences recovered at that depth. This approach is similar to using the relative abundance of terminal-restriction fragment-length polymorphism OTUs and cell counts to examine patterns in SAR11 ecotypes (Carlson *et al.*, 2009), but in this case we focus on *Nitrospina* and use pyrosequencing and QPCR data. All statistical analyses were conducted in MATLAB version 7.6.0 (R2008a; MathWorks, Natick, MA, USA).

Results and discussion

Oceanographic variability

The OMZ of the ETNP extends from the west coast of North America thousands of kilometers across the Pacific Ocean (Figure 1). Low oxygen waters also

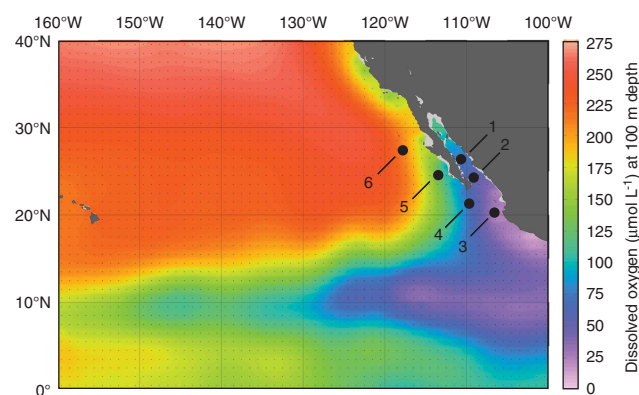


Figure 1 Cruise track in the GOC and ETNP. Station locations are plotted on DO concentrations (μM) at 100 m depth from the World Ocean Atlas in Ocean Data View.

extend into the GOC and we sampled a series of six stations in the GOC and ETNP; station 1 was located in the Carmen Basin of the GOC, station 2 was located near the mouth of the GOC and station 3 was located in the core of the ETNP OMZ (Figure 1; Supplementary Table S1), where oxygen concentrations declined rapidly with depth and dropped to $20 \mu\text{mol kg}^{-1}$ by 111 m (Figure 2o). Three additional stations extended north from the tip of Baja California Sur (station 4) to several hundred kilometers off the coast of northern Baja (Figure 1). (A seventh station sampled for $^{15}\text{NH}_4^+$ oxidation rates (Beman *et al.*, 2012) was not sampled for $^{15}\text{NO}_2^-$ oxidation). General trends in temperature, DO and nutrient concentrations were reported previously (Beman *et al.*, 2012): sea surface temperatures increased from north to south, reaching nearly 30°C at station 3; the OMZ also broadened and shoaled to the south and DO reached lower absolute values (Figures 2 and 3e, j, o). The concentrations of NH_4^+ and NO_2^- were highest at discrete depths in the water column (Figures 2 and 3d, i, n). These depths were greater at stations 3 and 6 and relatively shallow at stations 1 and 4, with stations 2 and 5 being intermediate. The PNM was offset from the NH_4^+ maximum by 5–10 m at stations 3 and 4, and was collocated with the NH_4^+ maximum at stations 1, 2 and 5 (Figures 2 and 3). Prominent secondary NO_2^- maxima were observed at stations 2 and 3 in OMZ waters, and NO_2^- concentrations at these depths were an order of magnitude greater than that in the PNM (up to $4.2 \mu\text{mol l}^{-1}$ at stations 2 and 3; Figures 2i, n and 4a).

NO₂⁻ oxidation rates and NH₃ oxidation rates

Within this oceanographic context, we found that $^{15}\text{NO}_2^-$ oxidation rates varied strongly with depth and from station to station (Figures 2 and 3b, g, l). They typically exhibited peaks at the base of the euphotic zone, decreased slightly with depth, and in some cases, increased again on the edge of the OMZ. Maximum rates at each station ranged from $44.5\text{--}213 \text{ nmol l}^{-1} \text{ d}^{-1}$, and minimum rates ranged from undetectable to $50.4 \text{ nmol l}^{-1} \text{ d}^{-1}$ across stations. These values are in line with previous studies that have measured rates of $0\text{--}600 \text{ nmol l}^{-1} \text{ d}^{-1}$ across different regions of the ocean (Ward, 1987; Lipschultz *et al.*, 1990; Dore and Karl, 1996; Bianchi *et al.*, 1999; Clark *et al.*, 2008; Füssel *et al.*, 2012). Higher rates have typically been observed in OMZs and other productive regions of the ocean—such as the ETSP, in the Benguela upwelling region and in the Southern California Bight (Ward, 1987; Lipschultz *et al.*, 1990; Füssel *et al.*, 2012)—than within oligotrophic regions (Dore and Karl, 1996; Bianchi *et al.*, 1999; Clark *et al.*, 2008). Overall, $^{15}\text{NO}_2^-$ oxidation rates were correlated with NO_2^- concentrations ($r^2 = 0.344$, $P < 0.05$; Figure 4a) in the GOC and ETNP, and relationships between rates and NO_2^- were strongest at stations 2 and 5 (Table 1).

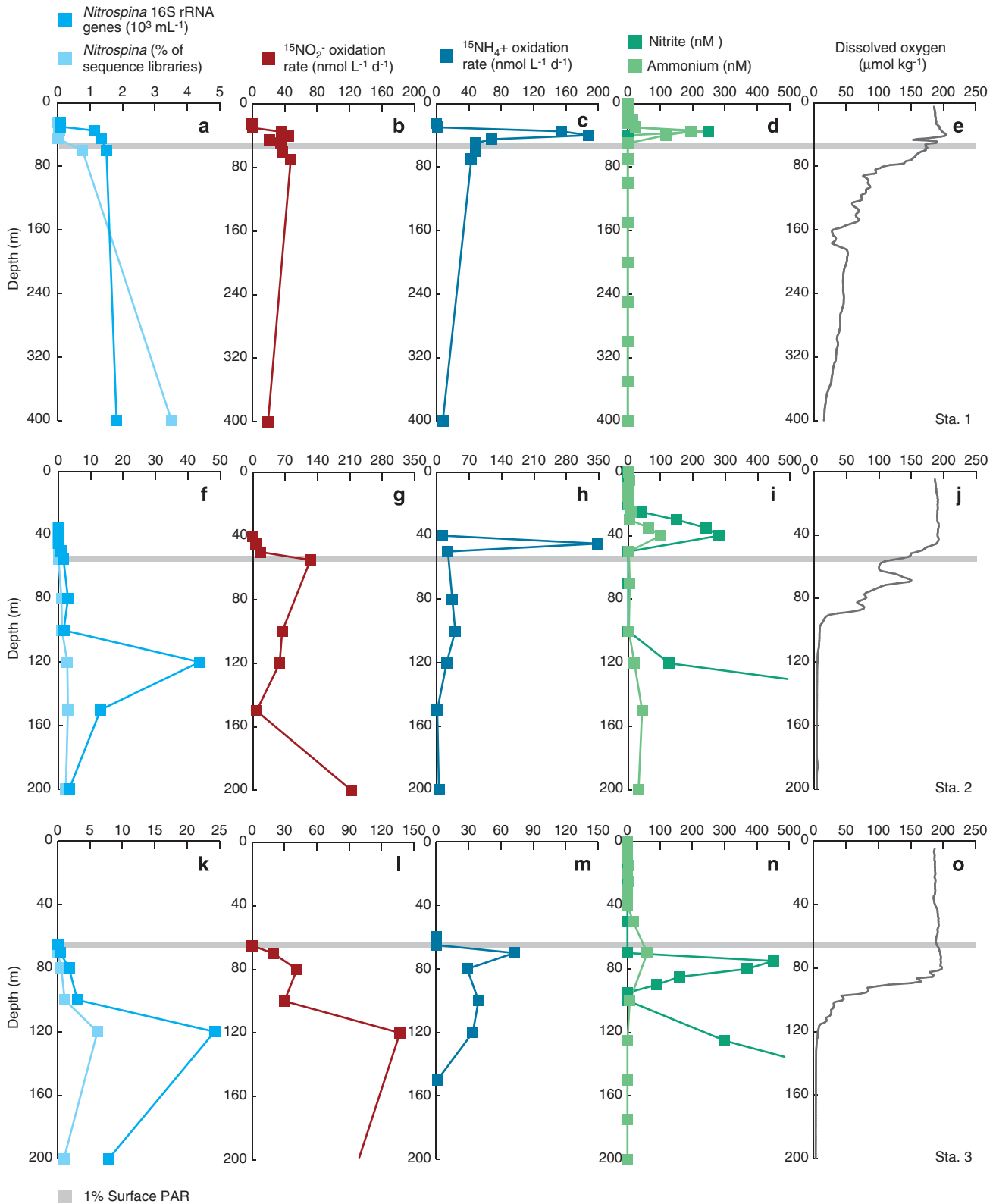


Figure 2 Depth profiles from (a–e) station 1, (f–j) station 2 and (k–o) station 3 in the ETNP and GOC. (a, f, k) QPCR data (10³ 16S rRNA genes per ml) and pyrosequencing data (expressed as % of total 16S rRNA sequence for a given sample) for *Nitrospina*; (b, g, l) ¹⁵NO₂⁻ oxidation rates (in nmol l⁻¹ d⁻¹); (c, h, m) ¹⁵NH₄⁺ oxidation rates; (d, i, n) NH₄⁺ and NO₂⁻ concentrations (nmol l⁻¹); and (e, j, o) DO concentrations (μmol kg⁻¹). s.e. for QPCR fall within the width of the data points, and the gray line denotes the depth of the euphotic zone (that is, 1% of surface photosynthetically active radiation). All data are plotted to 200 m depth, with the exception of station 1 data, which are plotted to 400 m depth. Note that horizontal axes vary between a, f and k, and between b, g and l, but c, h and m are identical to corresponding b, g and l to allow comparison between ¹⁵NO₂⁻ and ¹⁵NH₄⁺ oxidation rates.

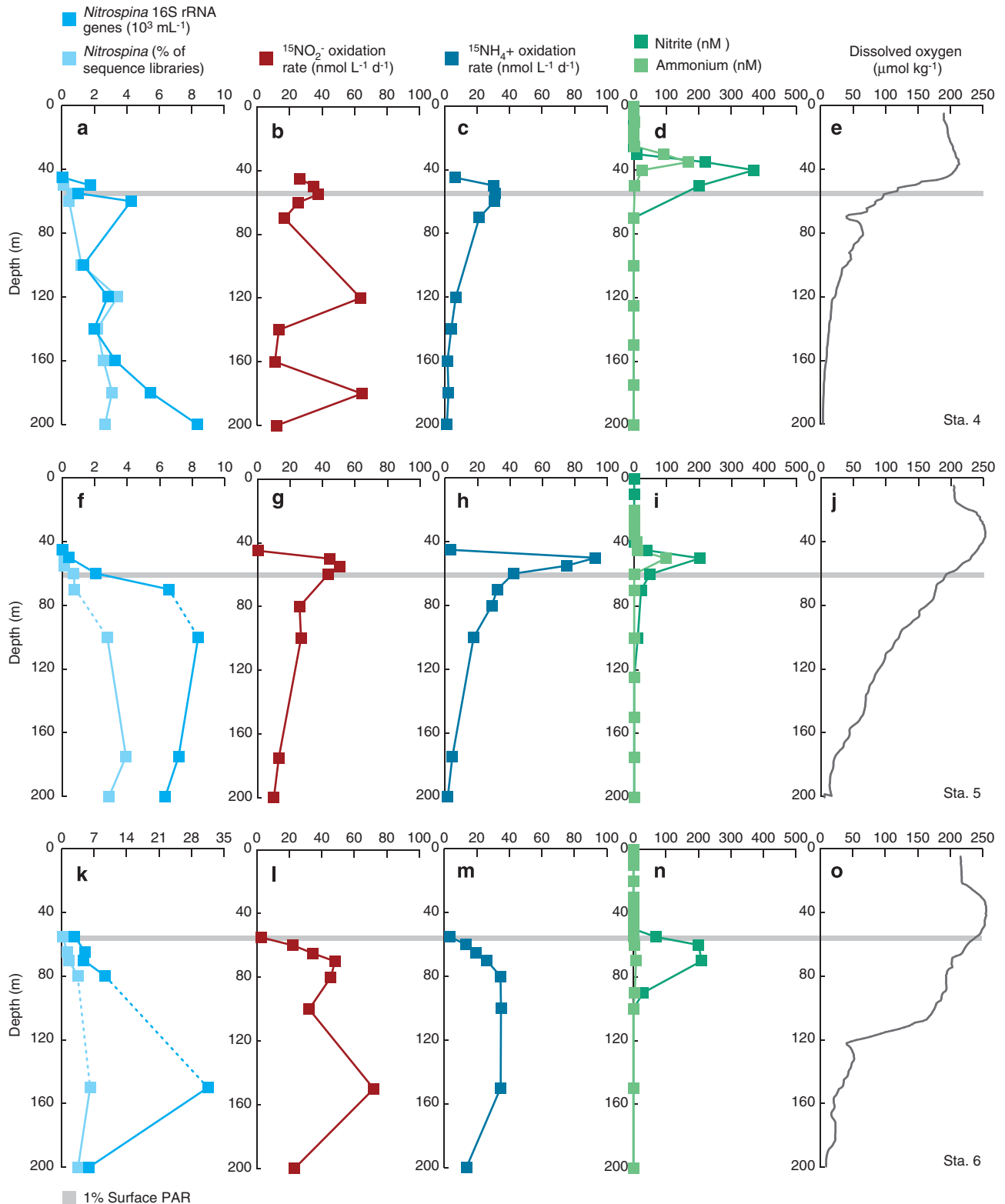


Figure 3 Depth profiles from (a–e) station 4, (f–j) station 5 and (k–o) station 6 in the ETNP and GOC. (a, f, k) QPCR data (10³ 16S rRNA genes per ml) and pyrosequencing data (expressed as % of total 16S rRNA sequence for a given sample) for *Nitrospina*; (b, g, l) ¹⁵NO₂⁻ oxidation rates (in nmol l⁻¹ d⁻¹); (c, h, m) ¹⁵NH₄⁺ oxidation rates; (d, i, n) NH₄⁺ and NO₂⁻ (nmol l⁻¹); and (e, j, o) DO concentrations (μmol kg⁻¹). Standard errors for QPCR fall within the width of the data points, and dashed lines connecting data points denote presence of DNA samples with QPCR inhibition (which are subsequently excluded from statistical analyses). The gray line denotes the depth of the euphotic zone, and all data are plotted to 200 m depth. Note that horizontal axes vary between a, f and k, but b, g, l and c, h, m are plotted on identical axes to allow comparison between ¹⁵NO₂⁻ and ¹⁵NH₄⁺ oxidation rates.

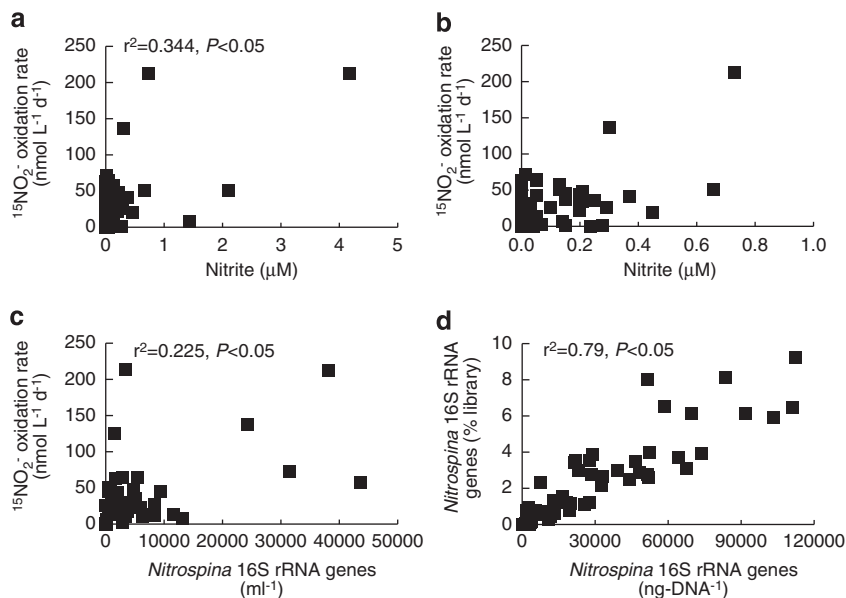


Figure 4 Correlations between ¹⁵NO₂⁻ oxidation rates and (a and b) NO₂⁻ concentrations and (c) *Nitrospina* 16S rRNA gene abundance (ml⁻¹) based on QPCR. In b, data points with >1 μM NO₂⁻ are not shown in order to show points near the axes. (d) Correlation between *Nitrospina* as a percentage of pyrosequencing libraries (vertical axis) and *Nitrospina* 16S rRNA gene abundance (ng-DNA⁻¹) based on QPCR (horizontal axis). QPCR data are normalized per ng-DNA rather than per ml to reflect *Nitrospina* 16S rRNA genes as a proportion of community DNA.

Table 1 *r*² values for the relationships between nitrite oxidation rates, ammonia oxidation rates, NOB abundance and dissolved nitrite

Station	Nitrite oxidation vs nitrite	Nitrite oxidation vs ammonia oxidation	Nitrite oxidation vs <i>Nitrospina</i>
1	0.021	0.401*	0.126
2	0.443*	0.038 (negative)	0.135
3	0.013	0.003	0.881*
4	0.003	0.001	0.004 (negative)
5	0.443*	0.790*	0.085 (negative)
6	0.027	0.731*	0.647*
Overall	0.344*	0.000	0.225*

**P* < 0.05.

Most other studies have either not analyzed this relationship or have too few data points to adequately do so, but Ward (1987) proposed that substrate supply may limit NO₂⁻ oxidation, and our results are consistent with this suggestion. Lipschultz *et al.* (1990) also reported strong correspondence between NO₂⁻ concentrations and NO₂⁻ oxidation rates in the OMZ of the ETSP.

¹⁵NO₂⁻ oxidation and ¹⁵NH₄⁺ oxidation were correlated (*r*² = 0.40–0.79, *P* < 0.05) at stations 1, 5 and 6 consistent with biogeochemical coupling between the two steps of nitrification (Figures 2 and 3). However, ¹⁵NH₄⁺ oxidation occurred up to 11 times more rapidly than ¹⁵NO₂⁻ oxidation in the upper water column at stations 1 and 5, and 56 times more rapidly at station 2. At all of these stations, these rate differences fell within 5 m depth of the PNM where NO₂⁻ accumulates to concentrations of

hundreds nM. In the most extreme case, ¹⁵NH₄⁺ oxidation rates were 342 nmol l⁻¹ d⁻¹ greater than ¹⁵NO₂⁻ oxidation rates at 45 m at station 2 (Figures 2g–i). At station 1, ¹⁵NH₄⁺ oxidation was 119 nmol l⁻¹ d⁻¹ greater than ¹⁵NO₂⁻ oxidation, and the PNM was 249 nM at 35 m (Figures 2b–d); at station 5, the rate mismatch was 48.1 nmol l⁻¹ d⁻¹ at the 203 nM PNM (Figures 3g–i). At these depths and stations, decoupling between NH₃ and NO₂⁻ oxidation could potentially produce the PNM in 2–5 days and possibly in <1 day at station 2. This is consistent with previous estimates from the Atlantic by Clark *et al.* (2008); they calculated that NH₃ oxidation could replace >50% of the NO₂⁻ pool within 24 h at most of the stations investigated. The data presented here also support our previous contention that NH₃ oxidation contributes to generation of the PNM at stations 1 and 5 in the GOC and ETNP (Beman *et al.*, 2012). With our coupled ¹⁵NH₄⁺ and ¹⁵NO₂⁻ oxidation data, we note that differential sensitivity to light does not appear to be a driving factor, because ¹⁵NO₂⁻ oxidation occurred in the euphotic zone and was correlated with ¹⁵NH₄⁺ oxidation in some cases. This is an important distinguishing factor from previous work, which has not measured ¹⁵NH₄⁺ and ¹⁵NO₂⁻ oxidation at high resolution with depth (Ward, 1987; Lipschultz *et al.*, 1990; Dore and Karl, 1996; Bianchi *et al.*, 1999; Clark *et al.*, 2008; Füssel *et al.*, 2012), as we have here (5–10 m intervals in the upper water column). On the basis of our data, the fact that NH₃ oxidation rates exceed NO₂⁻ oxidation rates appears to be the key in generating the PNM within the upper water column.

OMZ NO₂⁻ oxidation

At stations 2, 3 and 4, ¹⁵NO₂⁻ was oxidized most rapidly (63.7–213 nmol l⁻¹ d⁻¹) at depths >120 m, where oxygen concentrations were low (1.8–8.4 μmol kg⁻¹). Here, ¹⁵NO₂⁻ oxidation rates were much greater than ¹⁵NH₄⁺ oxidation rates; ¹⁵NH₄⁺ oxidation was detected in the OMZ, but was typically <5 nmol l⁻¹ d⁻¹ and declined with decreasing DO, such that ¹⁵NH₄⁺ oxidation rates were positively correlated with DO concentrations at stations 1–5 ($r^2 = 0.51\text{--}0.98$; Beman *et al.*, 2012). In contrast, ¹⁵NO₂⁻ oxidation was uncorrelated with DO, and ¹⁵NO₂⁻ oxidation was up to 50 times more rapid than ¹⁵NH₄⁺ oxidation. This represents decoupling between NH₃ and NO₂⁻ oxidation in the OMZ, and is a probable result of the high concentrations of available NO₂⁻. At stations 2 and 3, NO₂⁻ accumulates to concentrations of up to 4.2 μM at 150–200 m; our highest measured rates (213 nmol l⁻¹ d⁻¹) were measured at 200 and 250 m in the OMZ at station 2. NO₂⁻ concentrations were not as high in the OMZ at station 4, but they were greater than that at stations 1, 5 and 6 (for example, 114 nM at 400 m at station 4). These accumulations of NO₂⁻ evidently allow NOB to oxidize NO₂⁻ within the OMZ in spite of low oxygen concentrations, and are consistent with previous work by Lipschultz *et al.* (1990) and a more recent work by Füssel *et al.* (2012) in other OMZs. Lipschultz *et al.* (1990) found rates of up to 600 nmol l⁻¹ d⁻¹ at ~1 μM DO in ETSP, and Füssel *et al.* (2012) reported rates of 372 nmol l⁻¹ d⁻¹ at <1 μM DO off Namibia. NO₂⁻ oxidation appears to be an important form of microbial metabolism in OMZs (for example, Wright *et al.*, 2012, Ulloa *et al.*, 2012), and the high rates we observed within the oceans' largest OMZ lend further support to this hypothesis.

In previous studies, NO₂⁻ oxidation persisted under low DO because these conditions permit NO₃⁻ reduction (Lipschultz *et al.*, 1990, Füssel *et al.*, 2012), which produces NO₂⁻ that may be oxidized back to NO₃⁻, or used by anammox or denitrification as an oxidant. Alongside measurements of NO₂⁻ oxidation, both Lipschultz and colleagues (1990) and Füssel *et al.* (2012) measured NO₃⁻ reduction in the ETSP and Namibian OMZs, finding generally good correspondence between NO₃⁻ reduction and NO₂⁻ oxidation in magnitude and depth distribution. Lam *et al.* (2009) also found that NO₃⁻ reduction rates were high in the Peruvian OMZ, and detected NO₃⁻ reductase gene expression (*narG* and *napA*) where NO₃⁻ reduction was also measured. We screened OMZ RNA samples where dissolved NO₂⁻ exceeded 500 nM for *narG* and *napA* expression; this NO₂⁻ threshold defines anoxic waters based on recent measurements in the ETSP (Thamdrup *et al.*, 2012, Ulloa *et al.*, 2012), and includes samples from 150–300 m at station 2 and 200–300 m at station 3. We found expression of *narG*, *napA* or both, in all of these samples,

indicating that NO₃⁻ reduction is a possible source of NO₂⁻ that may then be oxidized back to NO₃⁻.

Taking a step back, our results indicate that coupling between NH₃ and NO₂⁻ oxidation is uncommon in the GOC and ETNP. Station 6 was the only station where ¹⁵NH₄⁺ and ¹⁵NO₂⁻ oxidation rates were correlated and similar in magnitude. Instead, quantitative differences in ¹⁵NH₄⁺ and ¹⁵NO₂⁻ oxidation rates in the upper water column, and high ¹⁵NO₂⁻ oxidation rates measured within the OMZ, indicate that NH₃ and NO₂⁻ oxidation are not tightly connected, and shift in and out of equilibrium in the ETNP. This does not mean that these processes are decoupled over long time scales: they cannot be, as NO₂⁻ would accumulate where NH₃ oxidation or NO₃⁻ reduction exceeds NO₂⁻ oxidation, or be rapidly consumed where NO₂⁻ oxidation exceeds NO₂⁻ supply.

NOB communities

Of the different NOB that may oxidize NO₂⁻, *Nitrospina* bacteria are hypothesized to have a key role in the oceanic NO₂⁻ oxidation (Mincer *et al.*, 2007) and comprised up to 9.25% 16S rRNA gene sequences recovered in our sequence libraries (250 m at station 2). This indicates that *Nitrospina* constitute a substantial proportion of the bacterial community in the ETNP, and raises the possibility that *Nitrospina* may be abundant elsewhere in the ocean. *Nitrospina* typically increased in relative proportions up to 100–200 m depth (light blue lines in Figures 2 and 3a, f, k), and their maximum proportions ranged from 3.09 to 6.52% across the other stations. Throughout our extensive pyrosequencing data set, we found little evidence that other NOB are present in GOC and ETNP. Out of 420 240 sequences, *Nitrococcus* and *Nitrobacter* were never detected, and a single *Nitrospira* 16S rRNA gene sequence was detected at 100 m at station 4. In the Namibian OMZ, Füssel *et al.* (2012) found that *Nitrospina* (up to 5.4% of microbial cells) and *Nitrococcus* (up to 4.9% of microbial cells) were both abundant, and the sum of these groups (0.3–9%) was similar to the relative abundance of *Nitrospina* that we observed (although their direct counts include Archaea, whereas our data are based on relative proportions within bacterial sequence libraries). We also found strong positive correlation between the proportion of *Nitrospina* in our pyrosequencing data set and *Nitrospina* 16S rRNA genes based on QPCR (normalized per ng-DNA, to reflect gene abundance relative to total extracted microbial DNA; $r^2 = 0.79$, $P < 0.05$; Figure 4d). This correlation with QPCR data indicates that the pyrosequencing data reflect the proportions of different groups within the water column, and that other NOB are therefore substantially less abundant than the *Nitrospina* in GOC and ETNP.

We examined different *Nitrospina* ecotypes present in the GOC and ETNP by clustering all

classified *Nitrospina* sequences into OTUs based on 97% sequence identity. We found a few dominant OTUs, with 66.2% of *Nitrospina* sequences affiliated with just three of them: 24.6% were affiliated with the most abundant OTU, 21.7% with the next most abundant and 19.9% with the third most abundant OTU (Table 2). *Nitrospina* represented 2.14% of all recovered 16S rRNA sequences, so each of these top three *Nitrospina* OTUs constitute 0.4–0.5% of all 16S rRNA gene sequences. The remaining 473 *Nitrospina* OTUs each contained 3.6% or fewer of the *Nitrospina* sequences. With a few abundant OTUs, and many uncommon OTUs, *Nitrospina* OTU rank abundance was power law-scaled ($r^2 = 0.923$; *Nitrospina* sequences in OTU = $1003.1 * \text{OTU rank}^{-1.209}$). This is a general property of many ecological communities (Fuhrman, 2009) that *Nitrospina* also follow in the GOC and ETNP. Meta-analysis by Wright *et al.* (2012) showed that other common OMZ bacteria—such as SUP05/ARCTIC96BD-19 and SAR324—are likewise divided into multiple ecotypes, including some that are dominant and nearly ubiquitous, and many others that are less common. Although *Nitrospina* are frequently detected in OMZs and may have an important role in N cycling within them, Wright *et al.* (2012) did not analyze *Nitrospina* in a similar way; our results indicate that *Nitrospina* communities share similar properties to other typical OMZ bacteria.

GOC and ETNP *Nitrospina* OTUs also exhibited 98–99% similarity to database 16S rRNA sequences recovered from other regions of the ocean (Table 2). This includes surface waters (25–49 m depth) of the Arabian Sea (99% identical to OTU 10; Fuchs *et al.*, 2005), suboxic to anoxic waters at 119–130 m depth in the Namibian upwelling system (OTU 7; Woebken *et al.*, 2007), suboxic waters at 890 m depth in the San Pedro Basin (OTU 1; Brown *et al.*, 2005; Beman *et al.*, 2010) and 100 m in the Saanich Inlet, which is typically anoxic below this depth (OTUs 4 and 9; Walsh *et al.*, 2009). OTUs 5, 8 and 11 were similar to sequences recovered from 200 to 800 m depth in the Gulf of Mexico or at Station Aloha in the North Pacific subtropical gyre (Rich *et al.*, 2011; Swan *et al.*, 2011; Redmond and Valentine, 2012), whereas OTUs 2, 3 and 6 were 99% identical to sequences previously recovered in the GOC (Dick and Tebo, 2010) or ETNP (Ma *et al.*, 2009). Given the high sequence similarity to these database sequences and the diversity of oceanic regions represented by them, all of these *Nitrospina* OTUs appear widely distributed and may be important NO₂⁻ oxidizers elsewhere in the ocean. In the ETNP, our data provide an in-depth view of *Nitrospina* in world's largest OMZ, and indicate that thousands of *Nitrospina* sequences and hundreds of *Nitrospina* OTUs are spread across a range of DO concentrations.

Table 2 Dominant *Nitrospina* operational taxonomic units (OTUs), percentage of sequences, distribution in the water column and similarity to database sequences

OTU	% of <i>Nitrospina</i> sequences	Depth range		<i>Nitrospina</i> GenBank accession number	Nearest GenBank sequence (accession number)	Depth (m)	Shared identity (%)	Reference
		Minimum (m)	Maximum (m)					
1	24.6	60	800	KC775278	San Pedro Basin, California (DQ009478)	890	99	Brown <i>et al.</i> (2005)
2	21.7	55	800	KC775277	Carmen Basin, Gulf of California (FJ981406)	2000	99	Dick and Tebo (2010)
3	19.9	100	800	KC775276	Eastern Tropical North Pacific (AY726827)	100	99	Ma <i>et al.</i> (2009)
4	3.6	50	550	KC775275	Saanich Inlet, Canada (GQ349069)	100	99	Walsh <i>et al.</i> (2009)
5	2.7	70	800	KC775274	Hawaii Ocean Time-series (HQ675664)	770	99	Swan <i>et al.</i> (2011)
6	2.0	375	800	KC775273	Guaymas Basin, Gulf of California (FJ981193)	1503	99	Dick and Tebo (2010)
7	1.5	50	550	KC775272	Namibian upwelling system, clone N67e_42 (EF646085)	119–130	99	Woebken <i>et al.</i> (2007)
8	1.4	55	750	KC775271	Gulf of Mexico, non-plume sample W65–11 (JN018903)	800	99	Redmond and Valentine (2012)
9	1.1	60	600	KC775270	Saanich Inlet, Canada (GQ349960)	100	99	Walsh <i>et al.</i> (2009)
10	1.1	50	550	KC775269	Arabian Sea, station B high-nucleic acid sample (AY907818)	25–49	98	Fuchs <i>et al.</i> (2005)
11	1.0	50	500	KC775268	Hawaii Ocean Time-series (GU474877)	200	98	Rich <i>et al.</i> (2011)

Nitrospina and NO₂⁻ oxidation

For the entire data set, QPCR-determined *Nitrospina* 16S rRNA gene abundances (ml⁻¹) were significantly correlated with ¹⁵NO₂⁻ oxidation rates ($r^2 = 0.225$ $P < 0.05$; Figure 4c). Like other variables we examined, the strength of this relationship varied from station to station, with strongest correspondence at stations 3 and 6 (Table 1). We computed the distribution of different *Nitrospina* OTUs throughout the water column based on these QPCR data and the proportion of *Nitrospina* sequences that were affiliated with the different OTUs (Figure 5; see Materials and Methods and Carlson *et al.*, 2009). Although these PCR-based approaches introduce uncertainties that preclude further quantitative analysis, there are few practical alternatives, as OTU-specific QPCR primers likely have their own biases, as would microarrays with OTU-specific probes; we use these data to demonstrate the vertical distribution of the *Nitrospina* OTUs.

Across this data set, no single OTU was found at all depths where NO₂⁻ was oxidized, indicating that multiple *Nitrospina* OTUs must govern the process across the GOC and ETNP. The three dominant *Nitrospina* OTUs showed slightly different depth distributions, with the third most abundant OTU confined to a smaller depth range than the other two (Table 2), but displaying greater constancy with depth (Figure 5b). All three of these OTUs were uncommon in the upper water column, where high rates of ¹⁵NO₂⁻ oxidation nevertheless occur; a good example of this is the peak in ¹⁵NO₂⁻ oxidation rates found at 55 m at station 2, where none of the dominant OTUs were present. OTUs 7, 10 and 11 were the only *Nitrospina* detected at this depth, as well as in other samples collected at < 100 m depth (Figure 5c). Their presence where other *Nitrospina* are absent suggests that they contribute to NO₂⁻ oxidation in the upper water column.

Together with the fact that only a single 16S rRNA gene from other NOB was detected in our pyrosequencing data set, and that *Nitrospina* abundance based on QPCR was correlated with ¹⁵NO₂⁻ oxidation rates, these findings indicate that (1) *Nitrospina* oxidize NO₂⁻ in the ETNP and GOC and (2) a mixture of *Nitrospina* OTUs contribute at different depths. Untangling the relative contributions of these OTUs may be possible through quantifying OTU-specific NO₂⁻ oxidoreductase (*nxr*) gene expression, but requires (meta)genomic/transcriptomic data for the different *Nitrospina* OTUs. In the absence of such published data, our approach identifies *Nitrospina* that are candidates for additional study in other OMZs and other regions of the sea. More specifically, the more abundant OTUs identified here are widespread in the ETNP OMZ, whereas OTUs 7, 10 and 11 are less abundant overall, but appear important in the well-oxygenated upper water column.

Conclusions

We report the first ¹⁵NO₂⁻ oxidation measurements from the ETNP, and show that the two steps of nitrification were rarely connected. NO₂⁻ oxidation may be coupled to anaerobic processes that produce NO₂⁻ in the OMZ. NO₂⁻ concentrations correlated with ¹⁵NO₂⁻ oxidation rates and the highest rates were observed in the OMZ, where NO₂⁻ concentrations were elevated. High *Nitrospina* abundances (based on pyrosequencing), the lack of other detectable NOB and significant correlations with ¹⁵NO₂⁻ oxidation rates (based on QPCR) all indicate that *Nitrospina* are dominant NOB in the GOC and ETNP. For the first time, we show that *Nitrospina* constitute a large percentage of the bacterial community (up to 9.25%) and are grouped into

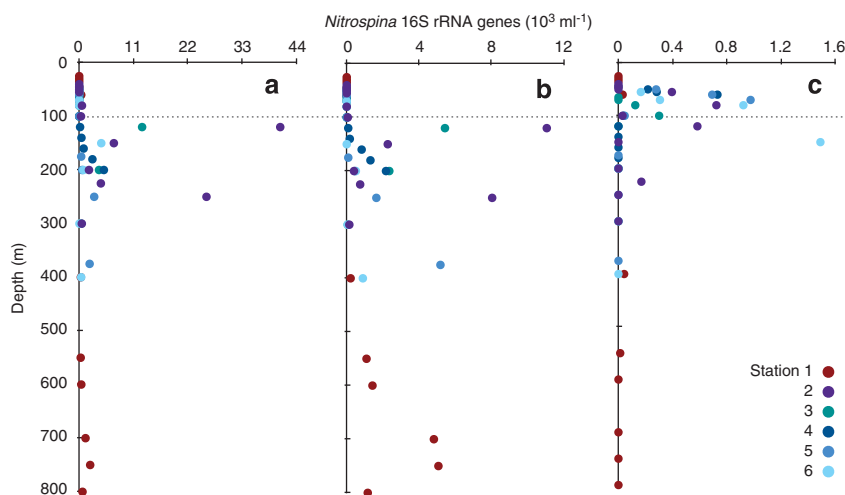


Figure 5 Distribution of *Nitrospina* ecotypes with depth across stations: (a) the most abundant OTU, (b) the third most common OTU and (c) the 7th most abundant OTU. Colors indicate different stations and data are expressed as 10³ 16S rRNA genes per ml.

multiple OTUs that are distributed heterogeneously with depth. Many of these OTUs are highly similar to sequences recovered from other areas of the ocean and may represent biogeochemically important populations of *Nitrospina*. Given this sequence diversity and ecological diversity within the *Nitrospina*, we suggest that the genomic and metabolic diversity of these putative ecotypes should be explored, such that their relative contributions to NO₂⁻ oxidation at different depths and in different oceanic regions may be determined. In the ETNP and other OMZs, NO₂⁻ cycling is central and *Nitrospina* are widespread, abundant and diverse. Our findings support the idea that *Nitrospina* are key contributors to oceanic NO₂⁻ oxidization, and provide new insight into their ecology and rates of N cycling, within the oceans' largest, and expanding OMZ.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

We thank Fred Prahl (chief scientist), Jackie Mueller, Natalie Wallsgrove, Jason Smith, Julie Fliegler and the officers and crew of the R/V *New Horizon* for assistance in the field. We also thank Rachel Foster for supplying critical DNA samples, Susan Alford, Molly Carolan and Natalie Wallsgrove for assistance with laboratory analyses. This work was supported by National Science Foundation Oceanography Award 10-34943 to JMB and BNP, and this is School of Ocean and Earth Science and Technology Contribution number 8809.

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