Revising the nitrogen cycle in the Peruvian oxygen minimum zone

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Edited by David M. Karl, University of Hawaii, Honolulu, HI, and approved January 21, 2009 (received for review December 8, 2008)

The oxygen minimum zone (OMZ) of the Eastern Tropical South Pacific (ETSP) is 1 of the 3 major regions in the world where oceanic nitrogen is lost in the pelagic realm. The recent identification of anammox, instead of denitrification, as the likely prevalent pathway for nitrogen loss in this OMZ raises strong questions about our understanding of nitrogen cycling and organic matter remineralization. In recent years, there has been a considerable increase in the number of reports documenting the occurrence of anammox in marine ecosystems, particularly in oxygen-depleted zones. Here we show that in the ETSP-OMZ, anammox obtains 67% or more of NO3 from nitrate reduction, and 33% or less from aerobic ammonia oxidation, based on stable-isotope pairing experiments corroborated by functional gene expression analyses. Dissimilatory nitrate reduction to ammonium was detected in an open-ocean setting. It occurred throughout the OMZ and could satisfy a substantial part of the NH4 requirement for anammox. The remaining NH4 came from remineralization via nitrate reduction and probably from microaerobic respiration. Altogether, deep-sea NO3 maxima arise within the OMZ. Anammox is a chemolithoautotrophic process that fixes inorganic carbon with the energy harnessed from N2 production: anaerobic nitrate reduction and aerobic ammonia oxidation. Nitrate reduction to N2 has been measured previously as a proxy for denitrification in the Eastern Tropical South Pacific (ETSP) (12), but its significance as a standalone process has not been evaluated thus far. Direct coupling between anammox and aerobic ammonia oxidation was reported for the Black Sea suboxic zone even though oxygen concentrations were below detection limits (13). Given the similar suboxic conditions and nitrogen availability, nitrification–anammox coupling also would be highly probable in oceanic OMZs. Meanwhile, in the absence of detectable denitrification in the ETSP, NH4 for anammox still would have to be remineralized from organic matter via other microbial processes. If nitrate reduction indeed occurs as a heterotrophic process, it also would release NH4. Another possible source of NH4 is dissimilatory nitrate reduction to ammonium (DNRA). Until its recent detection in the Namibian inner-shelf bottom waters (14), most studies on DNRA were restricted to fully anoxic, sulfide-rich environments; its potential occurrence in the open ocean remains unexplored.

Here we aimed to assess the microbial processes responsible for the generation of NO2 and NH4 for anammox in the ETSP OMZ off Peru and the microorganisms involved. Along a 12°S-transect from the inner shelf to offshore open ocean, anammox was detected throughout the OMZ with especially high rates in the upper part of the OMZ on mid-shelf (4). Strong deficits of fixed nitrogen, denoted by strongly negative N* (9, 15), were particularly apparent in the OMZ. Nonetheless, the expected NH4 accumulations have not been observed in the OMZs (11). Although the occurrence of anammox could explain this lack of NH4 accumulation, the exact NH4 sources for anammox become unclear without detectable denitrification (4, 7). Moreover, processes leading to secondary NO3 maxima (as opposed to primary NO3 maxima that occur at shallower depths and probably result from phytoplankton growths) and their interactions with anammox in the OMZs are also poorly understood.

Two microbial processes may lead to NO3 production: anaerobic nitrate reduction and aerobic ammonia oxidation. Nitrate reduction to N2 has been measured previously as a proxy for denitrification in the Eastern Tropical South Pacific (ETSP) (12), but its significance as a standalone process has not been evaluated thus far. Direct coupling between anammox and aerobic ammonia oxidation was reported for the Black Sea suboxic zone even though oxygen concentrations were below detection limits (13). Given the similar suboxic conditions and nitrogen availability, nitrification–anammox coupling also would be highly probable in oceanic OMZs. Meanwhile, in the absence of detectable denitrification in the ETSP, NH4 for anammox still would have to be remineralized from organic matter via other microbial processes. If nitrate reduction indeed occurs as a heterotrophic process, it also would release NH4. Another possible source of NH4 is dissimilatory nitrate reduction to ammonium (DNRA). Until its recent detection in the Namibian inner-shelf bottom waters (14), most studies on DNRA were restricted to fully anoxic, sulfide-rich environments; its potential occurrence in the open ocean remains unexplored.

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Anammox is a chemolithoautotrophic process that fixes inorganic carbon with the energy harnessed from N2 production, as opposed to the degradation of organic matter in heterotrophic denitrification. Denitrification is a stepwise reduction process involving a number of intermediates (NO3 → NO2 → NO → N2O → N2), but only when the process proceeds all the way to N2 does it meet the strict definition of denitrification (10). Apart from being a nitrogen sink, heterotrophic denitrification is regarded as the major remineralization pathway in the OMZs, such that heterotrophic bacteria release NH4 from organic matter when anaerobically respiring NO3. Nonetheless, the expected NH4 accumulations have not been observed in the OMZs (11). Although the occurrence of anammox could explain this lack of NH4 accumulation, the exact NH4 sources for anammox become unclear without detectable denitrification (4, 7). Moreover, processes leading to secondary NO3 maxima (as opposed to primary NO3 maxima that occur at shallower depths and probably result from phytoplankton growths) and their interactions with anammox in the OMZs are also poorly understood.

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on the shelf along the seafloor or in mid-water at or just above the oxycline on mid-shelf. These deficits coincided with lower NO$_3$ but higher NH$_4$ concentrations, the apparent presence of very low levels of oxygen ($\leq 10$ µM, or 0.25 ml l$^{-1}$) (Fig. 1 and Fig. S1), and the highest measured anammox rates (4). Using $^{15}$N stable-isotope pairing techniques, we measured nitrogen transformations potentially co-occurring with anammox in the same incubations and present those measurements here as net rates. These processes were verified further by quantifying the active expression of biomarker functional genes (i.e., when cell machineries are actively signaled to build the encoding key enzymes in the particular processes). Although we selected or designed primers that are as universal as possible for each biomarker functional gene examined, we do not claim to have a truly exhaustive coverage for these genes because of the immensity of the oceanic microbiome (16). Instead, because nucleic acids were collected from unmanipulated water samples, positive gene expression may serve as independent evidence for processes that are active in situ and give insight into the diversity of organisms involved in these processes. We identified a functional gene biomarker for anammox and examined its expression pattern relative to rate measurements. The potential sources of NO$_2$ and NH$_4$ for anammox then were evaluated using similar multidisciplinary approaches. Presented in the following sections are results relative to rate measurements. The potential sources of NO$_2$ gene biomarker for anammox and examined its expression pattern specifically the putative cytochrome cd$_1$-containing nitrite reductase gene ($nirS$) that is unique to Candidatus Scalindua but is distinct from denitrifier $nirS$. The encoding enzyme, similar to that of the anammox bacterium Candidatus Kuenenia stuttgartiensis, is believed to be responsible for the initial nitrite reduction to nitric oxide in anammox (18). Indeed, Scalindua $nirS$ genes were detected in the Peruvian OMZ in an abundance significantly correlated with that determined by 16S-rRNA gene-targeted quantitative PCR (4) (Pearson correlation $r = 0.84$, $P < 0.0001$). Furthermore, Scalindua-$nirS$ was strongly expressed, as determined by quantitative RT-PCR, especially in the upper part of the OMZ where anammox rates were high (Fig. 2B), and was positively correlated with anammox bacterial abundance (Spearman $R = 0.66$, $P < 0.05$) (4). These expressed Scalindua-$nirS$ were fairly diverse, but all clustered with the $nirS$ present in the Candidatus Scalindua genome assembly (73%–93% nucleotide sequence identity) and in 2 sequences obtained from the Arabian Sea (19); however, they were clearly different from typical denitrifier $nirS$ ($\approx 63\%$ sequence identity) (4).

**Results and Discussion**

**Functional Gene Expression Analyses for Anammox.** Based on the whole-genome data of an enriched marine anammox bacterium, Candidatus Scalindua sp. T23 (17), primers were designed to target specifically the putative cytochrome cd$_1$-containing nitrite reductase gene ($nirS$) that is unique to Candidatus Scalindua but is distinct from denitrifier $nirS$. The encoding enzyme, similar to that of the anammox bacterium Candidatus Kuenenia stuttgartiensis, is believed to be responsible for the initial nitrite reduction to nitric oxide in anammox (18). Indeed, Scalindua $nirS$ genes were detected in the Peruvian OMZ in an abundance significantly correlated with that determined by 16S-rRNA gene-targeted quantitative PCR (4) (Pearson correlation $r = 0.84$, $P < 0.0001$). Furthermore, Scalindua-$nirS$ was strongly expressed, as determined by quantitative RT-PCR, especially in the upper part of the OMZ where anammox rates were high (Fig. 2B), and was positively correlated with anammox bacterial abundance (Spearman $R = 0.66$, $P < 0.05$) (4). These expressed Scalindua-$nirS$ were fairly diverse, but all clustered with the $nirS$ present in the Candidatus Scalindua genome assembly (73%–93% nucleotide sequence identity) and in 2 sequences obtained from the Arabian Sea (19); however, they were clearly different from typical denitrifier $nirS$ ($\approx 63\%$ sequence identity) (4).

**Sources of Nitrite.** Nitrate, the preferred electron acceptor after O$_2$, was reduced to NO$_3$ at high rates ($\approx 3.07 \pm 26$ nM d$^{-1}$) throughout the shelf along the seafloor or in mid-water at or just above the oxycline on mid-shelf. These deficits coincided with lower NO$_3$ but higher NH$_4$ concentrations, the apparent presence of very low levels of oxygen ($\leq 10$ µM, or 0.25 ml l$^{-1}$) (Fig. 1 and Fig. S1), and the highest measured anammox rates (4). Using $^{15}$N stable-isotope pairing techniques, we measured nitrogen transformations potentially co-occurring with anammox in the same incubations and present those measurements here as net rates. These processes were verified further by quantifying the active expression of biomarker functional genes (i.e., when cell machineries are actively signaled to build the encoding key enzymes in the particular processes). Although we selected or designed primers that are as universal as possible for each biomarker functional gene examined, we do not claim to have a truly exhaustive coverage for these genes because of the immensity of the oceanic microbiome (16). Instead, because nucleic acids were collected from unmanipulated water samples, positive gene expression may serve as independent evidence for processes that are active in situ and give insight into the diversity of organisms involved in these processes. We identified a functional gene biomarker for anammox and examined its expression pattern relative to rate measurements. The potential sources of NO$_2$ and NH$_4$ for anammox then were evaluated using similar multidisciplinary approaches. Presented in the following sections are results relative to rate measurements. The potential sources of NO$_2$ gene biomarker for anammox and examined its expression pattern specifically the putative cytochrome cd$_1$-containing nitrite reductase gene ($nirS$) that is unique to Candidatus Scalindua but is distinct from denitrifier $nirS$. The encoding enzyme, similar to that of the anammox bacterium Candidatus Kuenenia stuttgartiensis, is believed to be responsible for the initial nitrite reduction to nitric oxide in anammox (18). Indeed, Scalindua $nirS$ genes were detected in the Peruvian OMZ in an abundance significantly correlated with that determined by 16S-rRNA gene-targeted quantitative PCR (4) (Pearson correlation $r = 0.84$, $P < 0.0001$). Furthermore, Scalindua-$nirS$ was strongly expressed, as determined by quantitative RT-PCR, especially in the upper part of the OMZ where anammox rates were high (Fig. 2B), and was positively correlated with anammox bacterial abundance (Spearman $R = 0.66$, $P < 0.05$) (4). These expressed Scalindua-$nirS$ were fairly diverse, but all clustered with the $nirS$ present in the Candidatus Scalindua genome assembly (73%–93% nucleotide sequence identity) and in 2 sequences obtained from the Arabian Sea (19); however, they were clearly different from typical denitrifier $nirS$ ($\approx 63\%$ sequence identity) (4).
the OMZ, thereby providing anammox with NO$_3^-$ (Fig. 2). The measured rates of nitrate reduction were congruent with previously reported values (12) and usually were greater than those of anammox, sometimes by more than an order of magnitude, except in the lower oxic zone offshore (Station 7). Further evidence for nitrate reduction was given by the high abundance and strong expression of the membrane-bound nitrate reductase gene, $\text{narG}$, within the OMZ. The expressed sequences at the anammox rate maximum (Station 4) were verified to be $\text{narG}$ by cDNA cloning and comparative sequence analyses. They were affiliated with environmental clones obtained from soils or estuarine sediments, or some with known denitrifiers and nitrate reducers (Fig. S4A). Both the abundance and expression of $\text{narG}$ exceeded those of Scalindua-$\text{nirS}$ (Figs. 2 and S3), but the mRNA:DNA ratio of $\text{narG}$ was far below that of Scalindua-$\text{nirS}$ (mean = 1% and 49%, respectively). This difference might reflect the facilitative nature of nitrate-reducing (narG) potential despite its relative ubiquity among microbes, if the stability of the 2 types of mRNA were similar. Nevertheless, the transcriptional regulatory network and behavior for these 2 genes in various microbes are not sufficiently understood at this point to verify this interpretation further. Although periplasmic nitrate reductase (NAP), unlike the membrane-bound counterpart (NAR), is not necessarily used in respiratory nitrate reduction (20), the expression of the encoding gene ($\text{napA}$) also was considerably elevated at anammox depths. The identities of these expressed $\text{napA}$ genes were confirmed via cDNA sequence analyses. Their closest relatives included estuarine sediment clones and the photosynthetic, nitrate-reducing $\text{Rhodobacter capsulatus}$ (Fig. S4B). Nitrate reduction is the first essential step in denitrification, but more organisms are capable of nitrate reduction than of complete denitrification (10). Hence, the finding of nitrate reduction but no denitrification in the Peruvian OMZ is not surprising.
Despite the very low to nondetectable oxygen concentrations (conventional detection limit: 1.5–2 μM), high 15NH₄⁺ oxidation rates (17–144 nM N d⁻¹), measured as 15NO₂⁻ production in 15NH₄⁺/15NO₂⁻ incubations, were observed within the upper OMZ along with high anammox rates (16–279 nM N d⁻¹) (4), sometimes even exceeding those in shallower oxic depths (e.g., Stations 4 and 7) (Fig. 2D). No significant 15NO₂⁻ production was observed when allythiourea, an inhibitor of aerobic ammonium oxidation, was added in parallel incubations, indicating the occurrence of microaerobic ammonium oxidation. Although 15NH₄⁺ oxidation was still detectable in the lower OMZ on shelf stations (Stations 2 and 4), it was undetectable in the lower OMZ offshore (Station 7) (Fig. 2D). These results were consistent with some previous reports on ammonia oxidation with particulate organic carbon and nitrogen (Spearman R = 0.001). These findings highlight the likelihood that the secondary NO₂⁻ maxima frequently observed in the offshore ETSP OMZ were largely the result of shelf production and horizontal advection, a possibility that is supported by the NO₂⁻ maxima trailing off the shelf along the 12°S-transect (Figs. 1 and S1).

Sources of Ammonium. Apart from NO₂⁻ production, nitrate reduction as a heterotrophic process involves the degradation of organic matter, whereby 16 moles of NH₄⁺ are released for every mole of organic matter remineralized:

\[
\text{(CH₂O)}_{106} (\text{NH₄H})_{34} \text{H₃PO₄} + 212 \text{ NO}_3^- + 16 \text{ H}^+ \rightarrow 106 \text{ CO}_2 + 16 \text{ NH}_4^+ + 212 \text{ NO}_2^- + 106 \text{ H}_2\text{O} + \text{H}_3\text{PO}_4^- \]

Calculations from our rate measurements and stoichiometry of Eq. 1 reveal that nitrate reduction could meet a substantial proportion of the NH₄⁺ requirement by anammox on shelf stations (16%–100%) and > 100% in the upper and lower OMZs, respectively) and up to 34% offshore (Station 7). The significant role of nitrate reducers in remineralization also is shown in the correlation of narG expression with particulate organic carbon and nitrogen (Spearman R = 0.87 and 0.87, respectively; P < 0.05), as well as between 15NO₃⁻ reduction rates and NH₄⁺ (Spearman R = 0.75, P = 0.001). These
associations were supported further by principal component analyses (SI Text).

Nevertheless, a large NH$_4^+$ source still was unaccounted for in the upper OMZs at all stations where the highest anammox rates were measured, as well as in the lower OMZ offshore (Station 7). Another potential NH$_4^+$ source could be DNRA, in which NH$_4^+$ originates from both NO$_3^-$ and organic matter:

\[
(\text{CH}_2\text{O})_{106}(\text{NH}_3)_{16}\text{H}_3\text{PO}_4 + 53 \text{ NO}_3^- + 122 \text{ H}^+ \rightarrow 106 \text{ CO}_2 + 69 \text{ NH}_4^+ + 53 \text{ H}_2\text{O} + \text{H}_3\text{PO}_4
\]

Indeed, significant $^{15}$NH$_4^+$ production could be detected in $^{15}$NO$_3^-$ incubations throughout the OMZ, with the highest rates reported for the upper OMZ on the shelf (Fig. 2E) coinciding with high anammox rates. The biomarker functional gene for DNRA, cytochrome c nitrite reductase gene mrfA, also was strongly expressed throughout the OMZ (Fig. 2E). These expressed sequences were verified to be mrfA (Fig. S7) by cDNA sequence analyses. Their phylogenetic affiliations with known sequences perhaps are not very informative at this point, because most mrfA sequences currently available in public databases come from genome sequences of culture collections in which the majority of cultures are pathogens. Most research on DNRA to date has focused on strictly anoxic environments, but DNRA never has been identified as a significant NO$_3^-$ sink in an open-ocean setting and linked to nitrogen loss. In the Peruvian OMZ, our measured DNRA rates were sufficient to fuel 7% to 134% and 7% to 34% of the NH$_4^+$ needed by anammox at the shelf and offshore stations, respectively.

Although nitrate reduction and DNRA combined appeared to produce more than enough NH$_4^+$ for anammox in the lower OMZ on the shelf, if all potential NH$_4^+$ sources and sinks were considered, some sources of NH$_4^+$ still needed to be identified at all stations (Table 1). The occurrence of ammonia oxidation and nitrite oxidation (12, 21), particularly in the upper OMZ, strongly suggested microaerobic conditions. In fact, oxygen concentrations up to $\sim 10$ μM ($\sim 0.25$ ml l$^{-1}$) were detected in the lower OMZ on mid-shelf (Stations 3–5) (Figs. 1 and S1). Nitrate reduction may be less sensitive to oxygen than the subsequent steps in the denitrification sequence (NO$_2^- \rightarrow$ NO$\rightarrow$ N$_2$O$\rightarrow$ N$_2$) (22), and anammox bacteria have been found to be microaerotolerant (active up to $\sim 10$ μM O$_2$) in the marine environment (23). Therefore, the detection of nitrate reduction and anammox was in line with the suggested microaerobic conditions in the upper OMZ just below the oxycline, as well as in the lower OMZ on the shelf. Lipschultz et al. (12) also pointed out the possible presence of oxygen in the ETSP OMZ and detected nitrate reduction therein. However, the exact extent of oxygen penetration in the OMZ would require further verification with more sensitive oxygen measurements (detection limit $\approx 1.5$–2 μM). Because oxygen is the most preferred electron acceptor, microaerobic remineralization of organic matter could proceed and release more NH$_4^+$ than nitrate reduction and DNRA at these depths. Its occurrence also would be consistent with the elevated levels of NH$_4^+$ in the upper boundaries of the OMZs, as well as in the lower OMZ on mid-shelf where O$_2$ seemed to be slightly elevated (Fig. 1). Even at the anammox rate maxima, the required remineralization would need less than 0.7 μM of O$_2$, or less than 1.2 μM taking into account the O$_2$ requirement by ammonia oxidation, a level that remains below the limits of conventional methods of O$_2$ detection. Such microaerobic remineralization could release enough NH$_4^+$ to fulfill the remaining needs for NH$_4^+$ on the shelf and in the upper OMZ offshore.

In the lower OMZ offshore (Station 7), the low to nondetectable nitrification rates and amoA expression indicated that microaerobic remineralization is not significant. Although the presence of relatively high $^{14}$NO$_3^-$ concentrations in our incubations enabled us to capture most, if not all, of the $^{15}$NO$_3^-$ produced for nitrate reduction and ammonium oxidation rate measurements, the same did not always apply for the $^{15}$NH$_4^+$ production measurements for DNRA. The ambient NH$_4^+$ concentrations were especially close to or below detection level in the lower OMZ offshore, so that some of the $^{15}$NH$_4^+$ produced in the $^{15}$NO$_3^-$ incubations might have been taken up by other NH$_4^+$-consuming processes and gone undetected. Thus, the net DNRA rates measured are likely to be lower than the actual gross rates. Consequently, DNRA, a process that does not consume oxygen, might be an even more important source of NH$_4^+$ in the offshore lower OMZ. This possibility also would be consistent with the increase in mrfA expression and DNRA rates with depth within this zone, where nitrate reduction rates (as well as nrfA and napA expression) were reduced, but anammox rates remained comparable to those in the overlying upper OMZ. On the other hand, the possibilities that anammox bacteria might themselves perform DNRA in the presence of small organic compounds (14) or that NH$_4^+$ might be released in fermentative reactions cannot be fully excluded at this point.

Conclusions and Perspectives

A considerably different and complex picture of nitrogen cycling has emerged in the Peruvian OMZ (Fig. 3). Our results based on both $^{14}$N-incubation experiments and molecular analyses indicate that anammox is the predominant pathway for nitrogen loss (4) and is coupled directly to multiple aerobic and anaerobic nitrogen transformations. Nitrate reduction provides anammox with NO$_2^-$, which, as shown in Fig. 3, is the predominant nitrogen source for anammox. Microaerobic conditions, at least in the upper part of the OMZ, were suggested by the occurrence of nitrification, which diminishes in importance from shelf to open ocean and in the lower OMZ. In contrast, NH$_4^+$ production caused by nitrate reduction and DNRA became increasingly important in the lower OMZ and offshore. Assim (gray) denotes assimilation. Remin (brown) denotes remineralization. Nitrogen fixation (gray dashes) might be coupled spatially to nitrogen loss near the OMZ but has not been assessed in this study.

Fig. 3. A revised nitrogen cycle in the Peruvian OMZ. Anammox (yellow) has been found to be the predominant pathway for nitrogen loss and was coupled directly to nitrate reduction (red) and aerobic ammonia oxidation (the first step of nitrification, green) for sources of NO$_3^-$. The NH$_4^+$ required by anammox originated from DNRA (blue) and remineralization of organic matter via nitrate reduction and probably from microaerobic respiration. Microaerobic conditions, at least in the upper part of the OMZ, were suggested by the occurrence of nitrification, which diminishes in importance from shelf to open ocean and in the lower OMZ. In contrast, NH$_4^+$ production caused by nitrate reduction and DNRA became increasingly important in the lower OMZ and offshore. Assim (gray) denotes assimilation. Remin (brown) denotes remineralization. Nitrogen fixation (gray dashes) might be coupled spatially to nitrogen loss near the OMZ but has not been assessed in this study.
alized nitrogen. Remineralized NH₄⁺ thus may play a much more important role in oceanic nitrogen loss than previously thought. It would require the remineralization of about 3.5 to 7 times the amount of Redfieldian organic matter (C:N:P = 106:16:1) (24) than the estimates based on denitrification stoichiometry. However, because of the constraints imposed by other closely associated elemental cycles (e.g., carbon and phosphorus) (2), such an increase in the remineralization of Redfieldian organic matter may not be realistic. Alternatively, remineralized NH₄⁺ might come from preferential degradation of organic nitrogen over carbon in suboxic settings (25), or the remineralization of nitrogen-enriched organic matter might result from the spatially coupled N₂ fixation over the OMZ (26, 27). In either case, calculations of nitrogen loss based on nitrate deficit alone would be underestimates, possibly explaining the discrepancies between the estimates of nitrogen loss based on nitrate deficits and excess N₂ (see ref. 8). However, the degree of such underestimations would need evaluated further via larger-scale experiments and modeling studies. The OMZs are expanding in global oceans (28), and more ocean volumes are becoming subjected to nitrogen loss. At the same time, atmospheric anthropogenic nitrogen input is increasing rapidly (29). In theory, this additional input would increase marine primary production and thus marine CO₂ sequestration (29), but whether positive or negative feedbacks may ensue via subsequent remineralization of organic nitrogen and marine nitrogen loss becomes an urgent research question. At this time of rapid global change, it is increasingly imperative to incorporate the correct nitrogen-loss mechanisms in global biogeochemical models, in order to more accurately assess the current oceanic nitrogen balance accurately and to more precisely predict how the closely linked nitrogen and carbon cycles in the future Ocean will respond.

Materials and Methods

Water Sampling and ¹⁵N-Isotope Pairing Experiments. Water sampling was conducted in April 2005. Details of site descriptions, sampling, physico-chemical analyses, and ¹⁵N stable-isotope pairing experiments measuring ammonium and the denitrification rate have been described previously (4). In the ¹⁴N incubations, the rates of nitrate reduction and aerobic ammonia oxidation were determined as net ¹⁴NO₂⁻ production in the ¹⁴NO₃⁻ and ¹⁵NH₄⁺ and ¹⁴NH₄⁺ incubations, respectively, measured after conversion to N₂ (13) or N₂O (30). Activities of ammonia oxidation were verified further by performing negative controls in selected samples, in which all-thiouracil, a specific inhibitor of ammonia oxidation, was added to the ¹⁵NH₄⁺ and ¹⁴NO₃⁻ incubations (86 μM final concentration). No significant ¹⁴NO₂⁻ production could be detected in those tested samples. All incubations were conducted at nondetectable O₂ levels (after degassing with He for 15 min) except for ¹⁴NH₄⁺ and ¹⁴NO₃⁻ incubations of the shallowest sampling depth, in which in situ O₂ levels were used (4). To determine DNA rates, net ¹⁴NO₂⁻ production in ¹⁴NO₃⁻ incubations was analyzed as N₂ on gas chromatography: isotopic ratio mass spectrometry after an alkaline hypobromite conversion (31) of a 5-ml subsample along with added ¹⁵NH₄⁺ (final concentration increase of 5 μM). These net rates then were corrected for the percentage of ¹⁴N in the original subsample pools but not for any other concurrent production or consumption processes during our incubations. All rates presented were calculated from time-series incubations (6, 6, 12, and 24 h), and only cases in which the measured ¹⁵N enrichment signal remained significantly with time, without lag-phase, were considered for rate calculations.

Molecular Ecological Analyses. Nucleic acids samples were collected from unmanipulated seawater samples by filtering 200–400 ml of seawater onto polycarbonate membrane filters with a pore size of 0.2 μm (Millipore) and were frozen immediately at −80 °C until extraction in the laboratory. Nucleic acids were extracted using Total DNA/RNA kit (Qiagen) with additional 15-min cell lysis (10 ng ml⁻¹ lysozyme in 10 mM Tris-EDTA, pH 8; 4 units of SUPERasein, Ambion), and bead beating (3 × 30 s, FastPrep Instrument, QBioGene) before extraction. Qualitative and quantitative PCR, reverse transcription, and phylogenetic analyses followed protocols in Lam et al. (13), except that the CopyControl PCR Cloning Kit (Epitope) was used for cloning. Primers used in various gene detections are listed in Table S2.

ACKNOWLEDGMENTS. We sincerely thank the Government of Peru for permitting research in their waters, Admiral Hugo Árvalo Escaro, President of Instituto del Mar del Perú, and his administration, as well as Ambassador Roland Klessow (German Embassy to Peru) for their support that enabled this expedition to take place. We are grateful for the technical and analytical assistance of Gabriele Klockgether, Robert Hammersley, Shobhit Agrawal, Christoph Walcher, Daniela Francke, Stefanie Pietsch (Max Planck Institute for Marine Microbiology), Marc Stross, Boran Kartal, Wim Geerts (Radbound University Nijmegen), Michelle Grad (Instituto del Mar del Perú), Siegfried Krüger (Baltic Sea Research Institute Warnemünde), and the captain and crew of RV José Olaya. We thank Yves Plancheur (Princeton University) for assistance in plotting and helpful discussions. Funding came from the Max Planck Gesellschaft, from the Deutsche Forschungsgemeinschaft, Grant Ku1550/3-1 to P.L.) and from the BioGeoSphere Program of the Netherlands Organisation for Scientific Research.