Revising the nitrogen cycle in the Peruvian oxygen minimum zone

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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 these waters. Without detectable denitrification, it is unclear how NH_4^+ is remineralized from organic matter and sustains anammox or how secondary NO_2^- maxima arise within the OMZ. Here we show that in the ETSP-OMZ, anammox obtains 67% or more of NO_2^- 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 NH_4^+ requirement for anammox. The remaining NH_4^+ came from remineralization via nitrate reduction and probably from microaerobic respiration. Altogether, deep-sea NO_3^- accounted for only ≈50% of the nitrogen loss in the ETSP, rather than 100% as commonly assumed. Because oceanic OMZs seem to be expanding because of global climate change, it is increasingly imperative to incorporate the correct nitrogen-loss pathways in global biogeochemical models to predict more accurately how the nitrogen cycle in our future ocean may respond.

anammox | dissimilatory nitrate reduction to ammonium | nitrogen loss | functional gene expression | remineralization

Nitrogen often is a limiting nutrient to biological production in the oceans, and nitrogen cycling is linked intimately to biological CO_2 sequestration via various feedback loops (1, 2). In the conventional paradigm of oceanic nitrogen cycling, dinitrogen gas (N_2) becomes bioavailable via N_2 fixation. This fixed nitrogen remains in the oceans in various organic and inorganic forms until it is lost to the atmosphere when facultative anaerobic microorganisms respire nitrate (NO_3^-) in the absence of oxygen and produce N_2. Known as "heterotrophic denitrification," this process for decades has been the only known pathway for oceanic nitrogen loss. This paradigm now is challenged by the recent findings of anammox, the anaerobic ammonium (NH_4^+) oxidation by nitrite (NO_2^-) to yield N_2 (3), as the likely predominant pathway for nitrogen loss in oceanic oxygen minimum zones (OMZs) off Namibia, Peru, and Chile (4–7). Although OMZ waters constitute only about 0.1% of the ocean volume worldwide, 20% to 40% of the total loss of oceanic nitrogen is estimated to occur in these zones (2, 8, 9).

Anammox is a chemolithoautotrophic process that fixes inorganic carbon with the energy harnessed from N_2 production, as opposed to the degradation of organic matter in heterotrophic denitrification. Denitrification is a stepwise reduction process involving a number of intermediates ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), but only when the process proceeds all the way to N_2 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 NH_4^+ from organic matter when anaerobically respiring NO_3^-. Nonetheless, the expected NH_4^+ accumulations have not been observed in the OMZs (11). Although the occurrence of anammox could explain this lack of NH_4^+ accumulation, the exact NH_4^+ sources for anammox become unclear without detectable denitrification (4, 7). Moreover, processes leading to secondary NO_2^- maxima (as opposed to primary NO_2^- 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 NO_2^- production: anaerobic nitrate reduction and aerobic ammonia oxidation. Nitrate reduction to NO_2^- 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, NH_4^+ 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 NH_4^+. Another possible source of NH_4^+ 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 NO_2^- and NH_4^+ 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,

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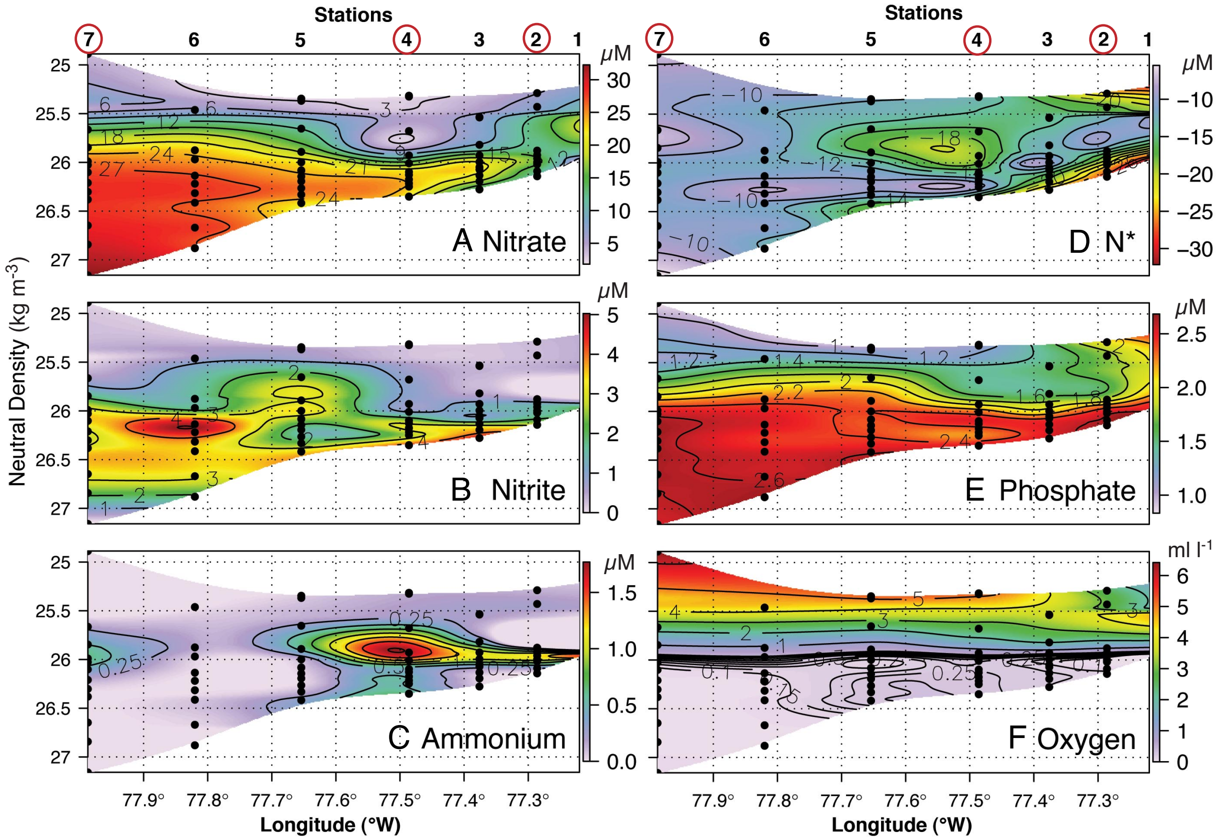

Fig. 1. Hydrochemical properties along an east–west transect at 12°S: distribution of (*A*) nitrate, (*B*) nitrite, (*C*) ammonium, (*D*) N*, (*E*) phosphate, and (*F*) oxygen plotted against neutral density (kg m^{-3}). Black-filled circles denote discrete sampling depths at Stations 1 to 7. Station numbers circled in red indicate sampling stations from which the parallel ^{15}N-rate measurements and gene expression data presented in the current study were obtained.

denoted by strongly negative N* (9, 15), were particularly apparent 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 from 3 sampling stations, representative of the inner-shelf (Station 2), mid-shelf (Station 4), and offshore open ocean (Station 7) areas of the Peruvian OMZ.

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 by16S-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. 2*B*), 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* ($\leq$ 63% sequence identity) (supporting information (SI) Fig. S2). Despite the high expression-to-gene ratio (mRNA:DNA) of typical denitrifier *nirS* when detected (mean values = 139% compared with 49% for Scalindua-*nirS*), denitrifier *nirS* showed much lower gene abundance and expression levels that often were close to detection limits (Figs. 2*B* and S3). The apparent predominance of Scalindua-*nirS* was consistent with the ^{15}N rate measurements (4), which revealed substantial anammox activities but no detectable denitrification. Hence, Scalindua-*nirS* is an effective functional gene biomarker for anammox in environmental samples.

Sources of Nitrite. Nitrate, the preferred electron acceptor after O$_2$, was reduced to NO$_2^-$ at high rates ($\leq 3\,07 \pm 26$ nM d^{-1}) throughout

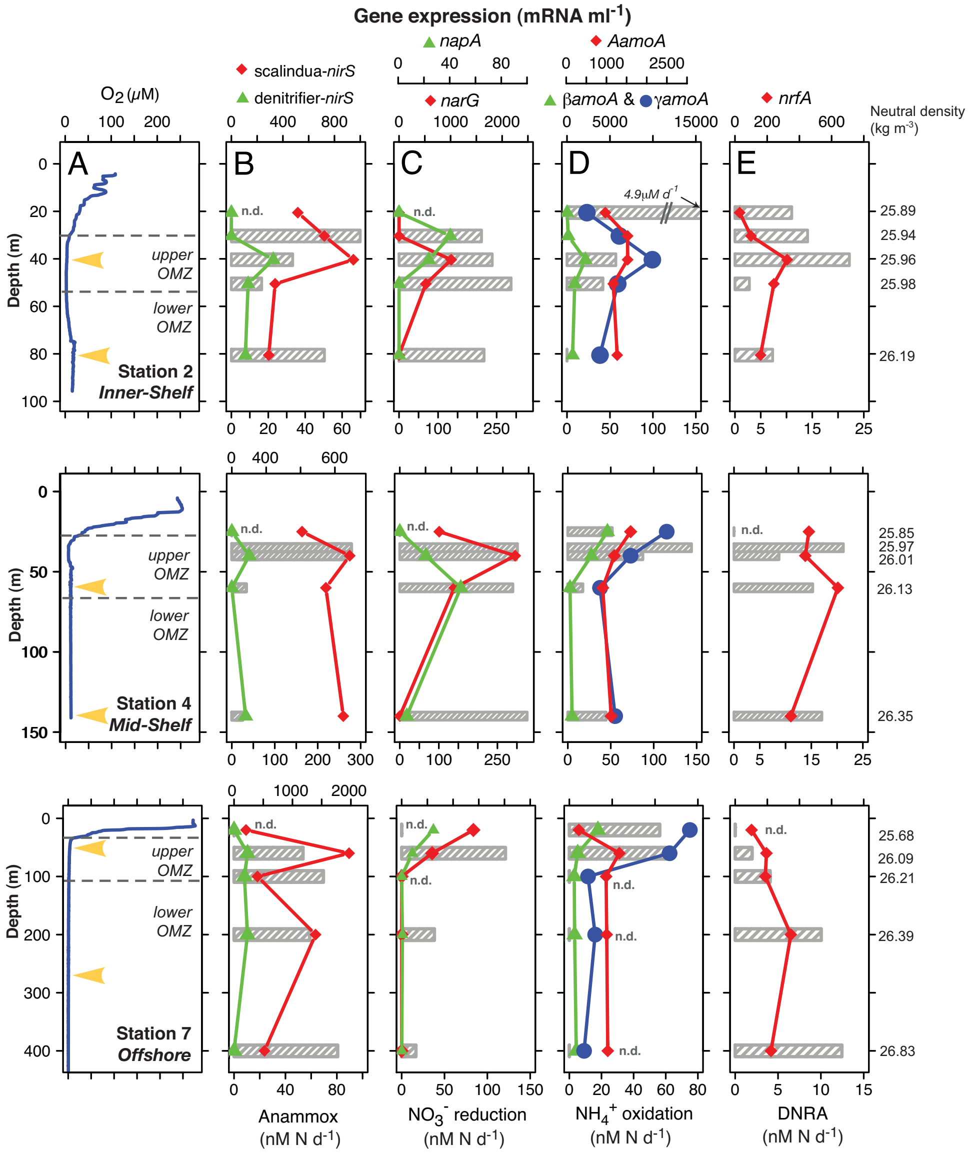

Fig. 2. Vertical distribution of oxygen (*A*) and the various measured ^{15}N rates along with corresponding functional gene expression (*B–E*) at Stations 2 (inner shelf), 4 (mid-shelf), and 7 (offshore). (*B*) Anammox rates (*bars*) with Scalindua (*red*) and denitrifier (*green*) *cd*$_1$-containing nitrite reductase (*nirS*) gene expression. (*C*) net $^{15}NO_3^-$ reduction rates (*bars*) with membrane-bound (*narG*) (*red*) and periplasmic (*napA*) (*green*) nitrate reductase gene expression. (*D*) net $^{15}NH_4^+$ oxidation rates (*bars*) with expression of crenarchaeal, β-, and γ- proteobacterial ammonia monooxygenase (*amoA*) genes (*red*, *green*, and *blue*, respectively). (*E*) net ^{15}N DNRA rates (*bars*) with cytochrome *c* nitrite reductase gene (*nrfA*) expression (*red*). n.d. denotes nondetectable reaction rate. Arrows indicate approximate depths of nitrite maxima. Please note the different scales used for the measured rates in these plots.

the OMZ, thereby providing anammox with NO_2^- (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, *narG*, within the OMZ. The expressed sequences at the anammox rate maximum (Station 4) were verified to be *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. S4*A*). Both the abundance and expression of *narG* exceeded those of Scalindua-*nirS* (Figs. 2 and S3), but the mRNA:DNA ratio of *narG* was far below that of Scalindua-*nirS* (mean = 1% and 49%, respectively). This difference might reflect the facultative 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 (*napA*) also was considerably elevated at anammox depths. The identities of these expressed *napA* genes were confirmed via cDNA sequence analyses. Their closest relatives included estuarine sediment clones and the photosynthetic, nitrate-reducing α-proteobacterium *Rhodobacter capsulatus* (Fig. S4*B*). 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.

Table 1. Estimated depth-integrated NO and NH_4^+ sources and sinks in the Peruvian OMZ, calculated as net fluxes with the unit of mmol N m^{-2} d^{-1}

	Inner Shelf: Station 2			Mid-shelf: Station 4			Offshore: Station 7		
Sources and Sinks	Upper OMZ 25–50 m	Lower OMZ 50–94 m	Overall OMZ	Upper OMZ 25–60 m	Lower OMZ 60–140 m	Overall OMZ	Upper OMZ 25–100 m	Lower OMZ 100–600 m	Overall OMZ
NO_2^- sources									
NH_4^+ oxidation	1.6	0.6	*2.2*	2.5	2.5	*4.9*	3.4	0	*3.4*
NO_3^- reduction	5.1	9.9	*15.0*	7.8	23.1	*30.9*	4.7	7.3	*12.0*
Total	6.7	10.5	*17.2*	10.3	25.6	*35.8*	8.1	7.3	*15.4*
NO_2^- sinks									
[a]NO_2^- oxidation	3.8	4.3	*8.1*	5.7	14.0	*19.7*	2.4	12.4	*14.8*
Anammox	0.6	0.9	*1.5*	2.9	1.2	*4.1*	1.8	14.2	*16.0*
Total	4.4	5.2	*9.6*	8.6	15.2	*23.8*	4.2	26.6	*30.8*
NH_4^+ sources									
[b]NO_3^- reduction	0.4	0.8	*1.1*	0.6	1.7	*2.3*	0.4	0.6	*0.9*
[c]DNRA	0.6	0.4	*0.9*	0.6	1.7	*2.3*	0.2	4.0	*4.2*
[d]Missing sources	1.7	0.8	*2.6*	4.8	0.8	*5.6*	6.6	[e]0.6	*17.2*
Total	2.7	2.0	*4.7*	6.0	4.2	*10.3*	7.2	15.1	*22.3*
NH_4^+ sinks									
NH_4^+ oxidation	1.6	0.6	*2.2*	2.5	2.5	*4.9*	3.4	0	*3.4*
[a]Assimilation	0.5	0.4	*1.0*	0.7	0.6	*1.3*	1.8	1.0	*2.8*
Anammox	0.6	0.9	*1.5*	2.9	1.2	*4.1*	1.8	14.2	*16.0*
Total	2.7	1.9	*4.7*	6.1	4.3	*10.3*	7.0	15.2	*22.2*

[a] From Lipschultz et al. (12).

[b] Amounts of NH_4^+ produced based on the measured $^{15}NO_{3-}$ -reduction rates and stoichiometry of Eq. 1.

[c] Amounts of NH_4^+ produced based on the measured ^{15}N-DNRA rates and stoichiometry of Eq. 2.

[d] Amounts of additional NH_4^+ source(s) required to achieve an assumed NH_4^+ balance.

[e] Likely attributed to DNRA in this case.

Despite the very low to nondetectable oxygen concentrations (conventional detection limit: 1.5–2 μM), high $^{15}NH_4^+$ oxidation rates (17–144 nM N d^{-1}), measured as $^{15}NO_2^-$ production in $^{15}NH_4^+ + ^{14}NO_2^-$ 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. 2*D*). No significant $^{15}NO_2^-$ production was observed when allylthiourea, an inhibitor of aerobic ammonium oxidation, was added in parallel incubations, indicating the occurrence of microaerobic ammonium oxidation. Although $^{15}NH_4^+$ 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. 2*D*). These results were consistent with some previous reports on nitrification in the ETSP (12, 21). Further support for microaerobic ($\leq$ 10 μM O_2) NH_4^+ oxidation was provided by an independent study of *amoA* expression in unmanipulated water samples. The functional gene *amoA* encodes for the subunit A of ammonia monooxygenase, a key enzyme in aerobic ammonia oxidation that requires oxygen for activation. Strong expression of *amoA* was exhibited by both crenarchaeal and bacterial ammonia oxidizers, especially in the upper OMZ (Fig. 2*D*). The expressed crenarchaeal *amoA* formed 2 subclusters with other marine pelagic sequences, whereas the expressed β- and γ- proteobacterial *amoA* were affiliated with *Nitrosospira* spp. and *Nitrosococcus oceani*, respectively (Fig. S5). Similar to the Black Sea suboxic zone (13), crenarchaeal *amoA* was more abundant than its bacterial counterparts with respect to gene abundance, but at lower expression levels (Fig. S3). There were tight associations between anammox and crenarchaeal *amoA* gene abundance based on correlation (Spearman R = 0.57, $P < 0.005$) and principal component analyses (*SI Text*, Table S1, and Fig. S6). The difference in the Peruvian OMZ, however, was that crenarchaeal *amoA* was expressed alongside anammox. Because different groups of organisms may have different characteristic rate-to-gene-expression relationships, our data here could not determine the relative importance of bacterial versus crenarchaeal ammonia oxidizers in nitrification. These data, nonetheless, do indicate that both groups are actively involved and at which depths where individual groups are more likely to be active.

Aerobic ammonium oxidation produced at least 65% (> 100% in all but 2 cases) of the NO_2^- required for anammox, or 6% to 33% of the total NO_2^- production, in the upper OMZ, but it was undetectable in the lower OMZ offshore (Station 7), based on ^{15}N-rate measurements corroborated by gene expression analyses (Fig. 2*D*). Sixty-seven percent to 94% of the total NO_2^- production in the upper OMZ was attributed to nitrate reduction, which was the sole source of NO_2^- in the lower OMZ. Together, ammonia oxidation and nitrate reduction often supplied more than enough NO_2^- for anammox in the Peruvian OMZ. Taking nitrite oxidation (12) into account, depth-integrated estimates of NO_2^- fluxes (Table 1) indicate substantial net production on the shelf but net consumption further offshore. This finding highlights the likelihood that the secondary NO_2^- maxima frequently observed in the offshore ETSP OMZ were largely the results of shelf production and horizontal advection, a possibility that is supported by the NO_2^- maxima trailing off the shelf along the 12°S-transect (Figs. 1 and S1).

Sources of Ammonium. Apart from NO_2^- production, nitrate reduction as a heterotrophic process involves the degradation of organic matter, whereby 16 moles of NH_4^+ are released for every mole of organic matter remineralized:

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 212\ NO_3^- + 16\ H^+ \rightarrow 106\ CO_2 + 16\ NH_4^+ + 212\ NO_2^- + 106\ H_2O + H_3PO_4 \qquad [1]$$

Calculations from our rate measurements and stoichiometry of Eq. 1 reveal that nitrate reduction could meet a substantial proportion of the NH_4^+ 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 $^{15}NO_3^-$-reduction rates and NH_4^+ (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 would be DNRA, in which NH_4^+ originates from both NO_3^- and organic matter:

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 53\ NO_3^- + 122\ H^+ \rightarrow 106\ CO_2 + 69\ NH_4^+ + 53\ H_2O + H_3PO_4 \quad [2]$$

Indeed, significant $^{15}NH_4^+$ production could be detected in $^{15}NO_x^-$ incubations throughout the OMZ, with the highest rates reported for the upper OMZ on the shelf (Fig. 2*E*) coinciding with high anammox rates. The biomarker functional gene for DNRA, cytochrome *c* nitrite reductase gene *nrfA*, also was strongly expressed throughout the OMZ (Fig. 2*E*). These expressed sequences were verified to be *nrfA* (Fig. S7) by cDNA sequence analyses. Their phylogenetic affiliations with known sequences perhaps are not very informative at this point, because most *nrfA* 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 $\approx 10\ \mu M$ ($\approx 0.25\ ml\ l^{-1}$) 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 ($NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (22), and anammox bacteria have been found to be microaerotolerant (active up to $\approx 10\ \mu M\ O_2$) 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 ≤ 1.5–$2\ \mu M$). 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 $0.7\ \mu M$ of O_2, or less than $1.2\ \mu M$ 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 $^{14}NO_2^-$ concentrations in our incubations enabled us to capture most, if not all, of the $^{15}NO_2^-$ 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 $^{15}NO_x^-$ incubations might have been taken up by other NH_4^+-consuming processes and gone undetected. Thus, the net DNRA rates measured are likely 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 *nrfA* expression and DNRA rates with depth within this zone, where nitrate reduction rates (as well as *narG* 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.

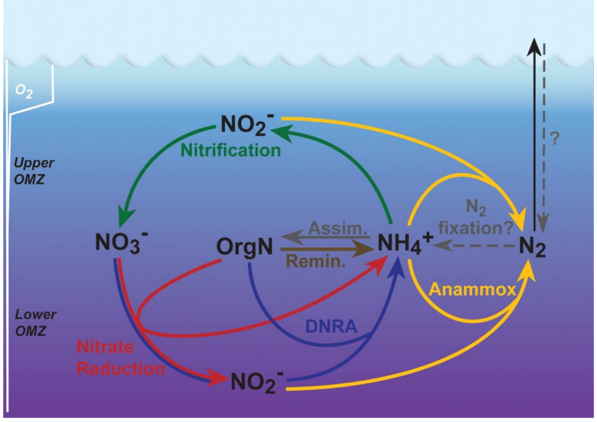

Fig. 3. A revised nitrogen cycle in the Peruvian OMZ. Anammox (*yellow*) has been found to be the predominant pathway for nitrogen loss and was coupled directly to nitrate reduction (*red*) and aerobic ammonia oxidation (the first step of nitrification, *green*) for sources of NO_2^-. The NH_4^+ required 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.

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 ^{15}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^- and NH_4^+. DNRA, a process usually consideed important only in peripheral environments, occurs throughout the OMZ and sometimes could supply most of the anammox need for NH_4^+. Meanwhile, aerobic ammonia oxidation supplies substantial amounts of NO_2^-, particularly in the upper OMZ, and strongly suggests the presence of microaerobic conditions that would enable microaerobic remineralization. Assuming sufficient additional microaerobic remineralization and DNRA (in the case of lower OMZ offshore) to balance NH_4^+, crude estimates of depth-integrated NO_2^- and NH_4^+ fluxes in the Peruvian OMZ (Table 1) suggest that 52% to 64% of nitrogen loss (instead of the 100% commonly assumed) originates from upwelled deep-sea NO_3^- (NO_3^- consumed in NO_3^- reduction and DNRA) and that the rest originates from remineralized nitrogen. Remineralized NH_4^+ 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 NH_4^+ 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 N_2 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 N_2 (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 CO_2 sequestration (29), but whether positive or negative feedbacks may ensue via subsequent remineralization of organic matter and 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 ^{15}N-Isotope Pairing Experiments. Water sampling was conducted in April 2005. Details of site descriptions, sampling, physico-chemical analyses, and ^{15}N stable-isotope pairing experiments measuring anammox and the denitrification rate have been described previously (4). In the same ^{15}N incubations, the rates of nitrate reduction and aerobic ammonia oxidation were determined as net $^{15}NO_2^-$ production in the $^{15}NO_3^-$ and $^{15}NH_4^+ + ^{14}NO_2^-$ incubations, respectively, measured after conversion to N_2 (13) or N_2O (30). Activities of ammonia oxidation were verified further by performing negative controls in selected samples, in which allylthiourea, a specific inhibitor of ammonia oxidation, was added to the $^{15}NH_4^+ + ^{14}NO_2^-$ incubations (86 μM final concentration). No significant $^{15}NO_2^-$ production could be detected in those tested samples. All incubations were conducted at nondetectable O_2 levels (after degassing with He for 15 min) except for $^{15}NH_4^+ + ^{14}NO_2^-$ incubations of the shallowest sampling depth, in which in situ O_2 levels were used (4). To determine DNRA rates, net $^{15}NH_4^+$ production in $^{15}NO_x^-$ incubations was analyzed as N_2 on gas chromatography isotopic ratio mass spectrometry after an alkaline hypobromite conversion (31) of a 5-ml subsample along with added $^{14}NH_4^+$ (final concentration increase of 5 μM). These net rates then were corrected for the percentage of ^{15}N in the original substrate pools but not for any other concurrent production or consumption processes during our incubations. All rates presented were calculated from time-series incubations (0, 6, 12, and 24 h), and only cases in which the measured products increased linearly and 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 mg 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 (Epicentre) was used for cloning. Primers used in various gene detections are listed in Table S2.

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1. Arrigo KR (2005) Marine microorganisms and global nutrient cycles. *Nature* 437:349–355.
2. Gruber N (2004) The dynamics of the marine nitrogen cycle and its influence on atmospheric CO_2 variations in *The Ocean Carbon Cycle and Climate, NATO ASI Series*, eds. M. Follows & T. Oguz. (Kluwer Academic, Dordrecht, pp. 97–148.
3. van de Graaf AA, Mulder A, de Bruijn P, Jetten MSM, Kuenen JG (1995) Anaerobic oxidation of ammonium is a biologically mediated process. *Appl Environ Microbiol* 61:1246–1251.
4. Hamersley MR, et al. (2007) Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. *Limnol Oceanogr* 52:923–933.
5. Galán A, et al. (2008) Anammox bacteria and the anaerobic oxidation of ammonium in the oxygen minimum zone off northern Chile. *Deep Sea Research Part II: Topical Studies in Oceanography*, 10.1016/j.dsr2.2008.09.016.
6. Kuypers MMM, et al. (2005) Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc Natl Acad Sci USA* 102:6478–6483.
7. Thamdrup B, et al. (2006) Anaerobic ammonium oxidation in the oxygen-deficient waters off northern Chile. *Limnol Oceanogr* 51:2145–2156.
8. Codispoti LA, et al. (2001) The oceanic fixed nitrogen and nitrous oxide budgets: Moving targets as we enter the Anthropocene? *Scientia Marina* 65:85–105.
9. Gruber N, Sarmiento JL (1997) Global patterns of marine nitrogen fixation and denitrification. *Global Biogeochemical Cycles* 11:235–266.
10. Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* 61:533–616.
11. Richards FA (1965) Anoxic basins and fjords in *Chemical Oceanography*, eds. J.P. Riley & G. Skirrow. (Academic, New York), pp. 611–645.
12. Lipschultz F, et al. (1990) Bacterial transformations of inorganic nitrogen in the oxygen-deficient waters of the Eastern Tropical South Pacific Ocean. *Deep-Sea Res* 37:1513–1541.
13. Lam P, et al. (2007) Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acad Sci USA* 104:7104–7109.
14. Kartal B, et al. (2007) Anammox bacteria disguised as denitrifiers: Nitrate reduction to dinitrogen gas via nitrite and ammonium. *Environmental Microbiology* 9:635–642.
15. Deutsch C, Gruber N, Key RM, Sarmiento JL, Ganachaud A (2001) Denitrification and N2 fixation in the Pacific Ocean. *Global Biogeochemical Cycles* 15:483–506.
16. Sogin ML, et al. (2006) Microbial diversity in the deep sea and the underexplored ‘‘rare biosphere.’’ *Proc Natl Acad Sci USA* 103:12115–12120.
17. van de Vossenberg J, et al. (2008) Enrichment and characterization of marine anammox bacteria associated with global nitrogen gas production. *Environmental Microbiology* 10:3120–3129.
18. Strous M, et al. (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature* 440:790–794.
19. Jayakumar DA, Francis CA, Naqvi SWA, Ward BB (2004) Diversity of nitrite reductase genes (*nirS*) in the denitrifying water column of the coastal Arabian Sea. *Aquatic Microbial Ecology* 34:69–78.
20. Richardson DJ, Berks BC, Russell DA, Spiro S, Taylor CJ (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cellular and Molecular Life Sciences* 58:165–178.
21. Ward BB, Glover HE, Lipschultz F (1989) Chemoautotrophic activity and nitrification in the oxygen minimum zone off Peru. *Deep-Sea Res* 36:1031–1051.
22. Korner H, Zumft WG (1989) Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture *of Pseudomonas stutzeri*. *Appl Environ Microbiol* 55:1670–1676.
23. Jensen MM, Kuypers MMM, Lavik G, Thamdrup B (2008) Rates and regulation of anammox in the Black Sea. *Limnol Oceanogr* 53:23–36.
24. Redfield A, Ketchum BH, Richards FA (1963) The influence of organisms on the composition of sea-water in *The Sea*, ed. M.N. Hill. (Wiley-Interscience, New York), Vol. 2, pp. 26–77.
25. Van Mooy BAS, Keil RG, Devol AH (2002) Impact of suboxia on sinking particulate organic carbon: Enhanced carbon flux and preferential degradation of amino acids via denitrification. *Geochim Cosmochim Acta* 66:457–465.
26. Deutsch C, Sarmiento JL, Sigman DM, Gruber N, Dunne JP (2007) Spatial coupling of nitrogen inputs and losses in the ocean. *Nature* 445:163–167.
27. Capone DG, Knapp AN (2007) Oceanography: A marine nitrogen cycle fix? *Nature* 445:159–160.
28. Stramma L, Johnson GC, Sprintall J, Mohrholz V (2008) Expanding oxygen-minimum zones in the tropical oceans. *Science* 320:655–658.
29. Duce RA, et al. (2008) Impacts of atmospheric anthropogenic nitrogen on the open ocean. *Science* 320:893–897.
30. McIlvin MR, Altabet MA (2005) Chemical conversion of nitrate and nitrite to nitrous oxide for nitrogen and oxygen isotopic analysis in freshwater and seawater. *Anal Chem* 77:5589–5595.
31. Warembourg FR (1993) Nitrogen fixation in soil and plant systems in *Nitrogen isotopes techniques*, eds. K. Knowles & T. H. Blackburn (Academic, New York), pp. 157–180.