# 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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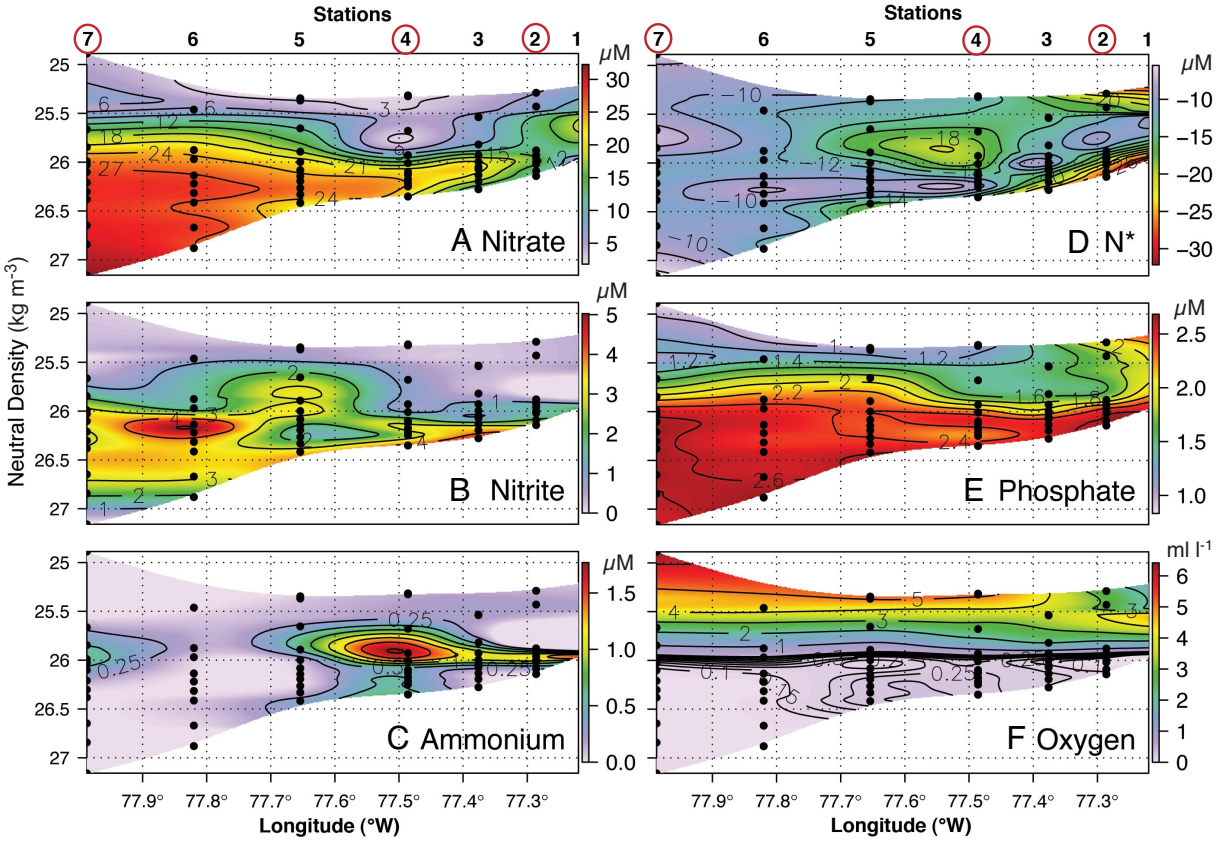


**Fig. 1.** Hydrochemical properties along an east–west transect at 12°S: distribution of (*A*) nitrate, (*B*) nitrite, (*C*) ammonium, (*D*) N*, (*E*) phosphate, and (*F*) oxygen plotted against neutral density (kg m$^{-3}$). Black-filled circles denote discrete sampling depths at Stations 1 to 7. Station 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 $\mu$M, 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

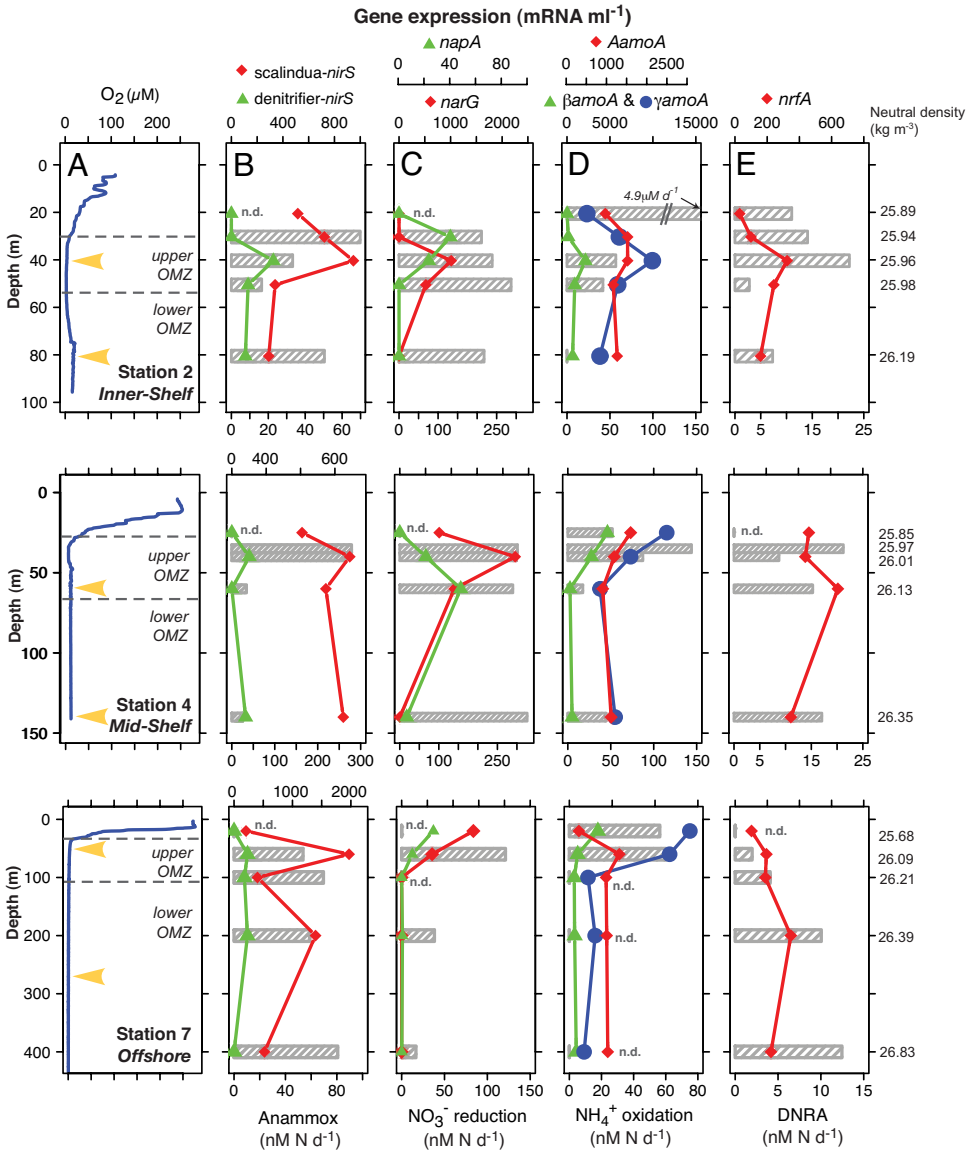


**Fig. 2.** Vertical distribution of oxygen (*A*) and the various measured $^{15}N$ rates along with corresponding functional gene expression (*B–E*) at Stations 2 (inner shelf), 4 (mid-shelf), and 7 (offshore). (*B*) Anammox rates (*bars*) with Scalindua (*red*) and denitrifier (*green*) $cd_1$-containing 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 $\mu$M), 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 $\mu$M $O_2$) $NH_4^+$ 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 $\beta$- and $\gamma$- 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:

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

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 ≈10 $\mu$M (≈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 ≈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.

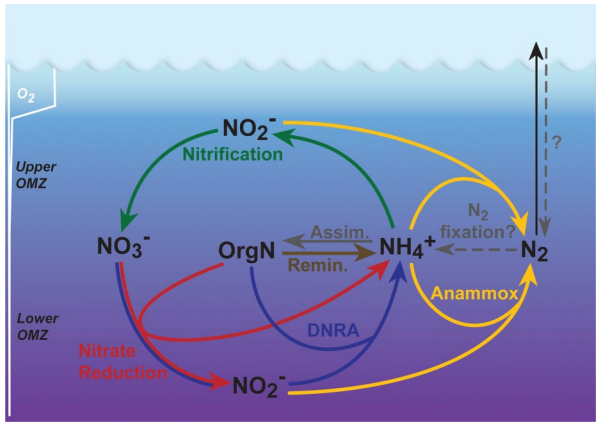


**Fig. 3.** A revised nitrogen cycle in the Peruvian OMZ. Anammox (*yellow*) has been found to be the predominant pathway for nitrogen loss and was coupled directly to nitrate reduction (*red*) and aerobic ammonia oxidation (the first step of nitrification, *green*) for sources of $NO_2^-$. The $NH_4^+$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

alized 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 $\mu$M 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 $\mu$M). 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 $\mu$m (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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