Oxygen at Nanomolar Levels Reversibly Suppresses Process Rates and Gene Expression in Anammox and Denitrification in the Oxygen Minimum Zone off Northern Chile

Tage Dalsgaard, Frank J. Stewart, Bo Thamdrup, Loreto De Brabandere, Niels Peter Revsbech, Osvaldo Ulloa, Don E. Canfield, Edward F. DeLong

ABSTRACT A major percentage (20 to 40%) of global marine fixed-nitrogen loss occurs in oxygen minimum zones (OMZs). Concentrations of O2 and the sensitivity of the anaerobic N2-producing processes of anammox and denitrification determine where this loss occurs. We studied experimentally how O2 at nanomolar levels affects anammox and denitrification rates and the transcription of nitrogen cycle genes in the anoxic OMZ off Chile. Rates of anammox and denitrification were reversibly suppressed, most likely at the enzyme level. Fifty percent inhibition of N2 and N2O production by denitrification was achieved at 205 and 297 nM O2, respectively, whereas anammox was 50% inhibited at 886 nM O2. Coupled metatranscriptomic analysis revealed that transcripts encoding nitrous oxide reductase (nosZ), nitrite reductase (nirS), and nitric oxide reductase (norB) decreased in relative abundance above 200 nM O2. This O2 concentration did not suppress the transcription of other dissimilatory nitrogen cycle genes, including nitrate reductase (narG), hydrazine oxidoreductase (hzo), and nitrate reductase (nirK). However, taxonomic characterization of transcripts suggested inhibition of narG transcription in gammaproteobacteria, whereas the transcription of anammox narG, whose gene product is likely used to oxidatively replenish electrons for carbon fixation, was not inhibited. The taxonomic composition of transcripts differed among denitrification enzymes, suggesting that distinct groups of microorganisms mediate different steps of denitrification. Sulfide addition (1 μM) did not affect anammox or O2 inhibition kinetics but strongly stimulated N2O production by denitrification. These results identify new O2 thresholds for delimiting marine nitrogen loss and highlight the utility of integrating biogeochemical and metatranscriptomic analyses.

IMPORTANCE The removal of fixed nitrogen via anammox and denitrification associated with low O2 concentrations in oceanic oxygen minimum zones (OMZs) is a major sink in oceanic N budgets, yet the sensitivity and dynamics of these processes with respect to O2 are poorly known. The present study elucidated how nanomolar O2 concentrations affected nitrogen removal rates and expression of key nitrogen cycle genes in water from the eastern South Pacific OMZ, applying state-of-the-art 15N techniques and metatranscriptomics. Rates of both denitrification and anammox responded rapidly and reversibly to changes in O2, but denitrification was more O2 sensitive than anammox. The transcription of key nitrogen cycle genes did not respond as clearly to O2, although expression of some of these genes decreased. Quantifying O2 sensitivity of these processes is essential for predicting through which pathways and in which environments, from wastewater treatment to the open oceans, nitrogen removal may occur.

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Address correspondence to Tage Dalgaard, tda@dmu.dk.

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Oxygen (O2) plays a key role in regulating the major biogeochemical cycles in the marine environment (1). The present-day ocean is generally well oxygenated, but in some environments, the demand for O2 exceeds the rate of supply, causing low-O2 environments to develop. This is the case in oxygen minimum zones (OMZs) (2–6) and sediments (7), which may be anoxic or at least have O2 concentrations below the detection limits of the methods used for analysis of O2. In these environments, a suite of anaerobic processes may occur that utilize nitrate (NO3⁻), nitrite (NO2⁻), sulfate (SO4²⁻), and metal oxides as terminal electron acceptors (8). In OMZs, NO3⁻ and NO2⁻ concentrations are generally high and nitrogen oxyanions are believed to be the main terminal electron acceptors (9). It has also recently been shown that a cryptic sulfur cycle operates in the eastern South Pacific.
OMZ, in which sulfate reduction oxidizes organic matter and produces sulfide. However, sulfide does not accumulate, presumably because it is immediately oxidized by \( \text{NO}_3^- \) and \( \text{NO}_2^- \)-reducing chemoeautotrophic microbes (10). The OMZs play an important role in the marine nitrogen cycle, and the ocean’s three major OMZs (eastern South Pacific, eastern North Pacific, and Arabian Sea) are estimated to harbor 20 to 40% of the oceanic reactive nitrogen loss (11, 12). The conversion of reactive nitrogen to \( \text{N}_2 \) occurs through microbial denitrification and anammox, but the relative importance of these sinks is debated (13–15), and the final conversion to \( \text{N}_2 \) appears to proceed in close interaction with other nitrogen, carbon, and sulfur transformations, which together drive dissimilatory reduction of \( \text{NO}_3^- \) to \( \text{NO}_2^- \) and of \( \text{NO}_2^- \) to ammonium (\( \text{NH}_4^+ \)), as well as aerobic oxidation of \( \text{NH}_4^+ \) and \( \text{NO}_2^- \) (e.g., see references 15 and 16).

The distribution of \( \text{O}_2 \) in OMZs and its effect on both aerobic and anaerobic nitrogen transformations is of central importance for understanding the role of OMZs in the marine nitrogen cycle and predicting changes in response to environmental forcing. Recent results obtained using highly sensitive switchable trace oxygen (STOX) sensors (17, 18) indicate that the core of the OMZs is functionally anoxic, with \( \text{O}_2 \) levels below the detection limit of a few nanomolar (2), but with episodic intrusions of more oxygenated waters (6, 19). At the boundaries of the core and after mixing events, \( \text{O}_2 \) is present at low concentrations, potentially allowing interactions between aerobic and anaerobic processes (e.g., see reference 20). \( \text{N}_2 \) production is particularly intense in these transition zones (14, 16), which emphasizes the need for quantification of the \( \text{O}_2 \) sensitivity and dynamic response of the individual processes. Existing estimates of the \( \text{O}_2 \) tolerance of denitrification and anammox differ greatly. Experimental studies with oxygen addition have found anammox in OMZs and the Black Sea to require 2 to 16 \( \mu \text{M} \) \( \text{O}_2 \) for 50% inhibition (21–23), while the abrupt increase in anammox activity at the upper oxic-anoxic interface of the OMZ off Peru and Chile suggests a much greater sensitivity to oxygen exposure in situ (14). A single experimental study of the oxygen sensitivity of denitrification in an OMZ found the process to be completely inhibited at 3 \( \mu \text{M} \) \( \text{O}_2 \), while anammox still proceeded at low rates (23). This could indicate, as suggested earlier (24), that denitrification in OMZs may be more sensitive than anammox to \( \text{O}_2 \), but whether this is more general remains to be experimentally verified, and denitrification is often assumed to be active at micromolar \( \text{O}_2 \) levels (e.g., see references 25 and 26). The assumed \( \text{O}_2 \) sensitivity of these processes plays a vital role when the volume of water in the oceans that can be assumed to participate in \( \text{N}_2 \) production is estimated, and this volume may vary greatly depending on which threshold is chosen; assuming inhibition at nanomolar \( \text{O}_2 \) concentrations would result in a significantly smaller zone of \( \text{N}_2 \) production than if inhibition is assumed to occur up to 20 \( \mu \text{M} \) \( \text{O}_2 \) (26).

Rates of nitrogen transformation in OMZs are determined experimentally in batch incubations with \( ^{15}\text{N} \)-labeled compounds (15, 27). These measurements reflect the metabolism of the microbial community as a whole and can reveal the bulk kinetics of the processes with respect to environmental parameters such as \( \text{O}_2 \). Such kinetics represent the composite of the responses of different types of organisms with distinct physiologies. In another approach, patterns of gene transcription can be analyzed to estimate the activities of different functional pathways and taxonomic members of the microbial community, potentially providing insight into microbial \( \text{O}_2 \) sensitivity of individual clades. Gene expression over vertical \( \text{O}_2 \) gradients in OMZs has been assessed via quantitative PCR (qPCR) using reverse-transcribed RNA and gene-specific primers (e.g., see reference 15). This approach typically targets only a subset of metabolic processes and is subject to biases due to primer-template specificity, particularly in environments with high numbers of uncharacterized taxa. Alternatively, high-throughput sequencing and analysis of community cDNA (metatranscriptomics) can identify coexpression patterns of thousands of genes from diverse community members without requiring a priori knowledge of sequence identity (28). However, metatranscriptomics typically does not yield absolute measurements of transcript abundance and, depending on sequencing depth, may not detect subtle transcriptional shifts in low-frequency taxa (29). Determining whether metatranscriptome patterns can be proxies for biogeochemical activity requires experiments that couple community RNA sequencing with metabolic rate measurements. Few such studies have been conducted for natural microbial communities, and no studies have examined potential linkages between community transcription and metabolic rates at the nanomolar \( \text{O}_2 \) concentrations predicted for the Eastern Pacific OMZs. This is due in part to the challenge of sampling anoxic water columns without concurrent changes in community expression and \( \text{O}_2 \) contamination (28, 30).

This challenge can be met by studying natural communities in microcosm (bioreactor) experiments that combine time series rate measurements, RNA collection, and high-sensitivity control of dissolved-\( \text{O}_2 \) levels, although \( \text{O}_2 \) contamination during experimentation is almost inevitable (31). In the present study, we performed \( \text{O}_2 \) manipulation experiments in bioreactors. Rates of anammox and denitrification were quantified over variable \( \text{O}_2 \) treatments, and the expression of key \( \text{N} \) cycle genes (Fig. 1) at the endpoint of each experiment was analyzed using metatranscriptomics. Each bioreactor was equipped with a STOX sensor, allowing us to directly couple molecular and biogeochemical rate measurements with the precise monitoring of \( \text{O}_2 \) at the nanomolar concentration range reflective of the in situ OMZ environment. Although enclosure in bioreactors has been shown to alter the metatranscriptional profile of some OMZ community members (i.e., bottle effects) (32), comparisons between bioreactors with contrasting oxygen treatments can help identify gene expression patterns suggestive of differential oxygen sensitivity.

**RESULTS**

**Concentrations of \( \text{O}_2 \) and \( \text{H}_2\text{S} \).** Oxygen concentrations were below the detection limit in the OMZ source water, but water sampling using the pump profiling system (PPS) introduced minor amounts of \( \text{O}_2 \) contamination into the bioreactors. The \( \text{O}_2 \) concentration in the water leaving the hose of the PPS was as low as 20 nM (10), but \( \text{O}_2 \) levels were always higher when measured inside the reactors placed in the water bath in the lab (designated “initial” in Table 1). \( \text{O}_2 \) contamination was reduced from the first to the third experiment, with initial concentrations exceeding 100 nM in four reactors in experiment 1, in two reactors in experiment 2, and in none of the reactors in experiment 3. All reactors were sparged with helium within the first few hours after arriving in the lab, which brought the \( \text{O}_2 \) concentrations to between undetectable and ca. 20 nM. The average and maximum \( \text{O}_2 \) concentrations during the first and second halves of the experiments are listed in Table 1. The discrete additions of \( \text{O}_2 \) during the experi-
ments resulted in fluctuating O₂ concentrations, with the worst case shown in Fig. 2B (Table 1). Leakage into the reactors increased O₂ concentrations during the first half of the incubation to an average of 20 to 90 nM in experiment 1 and 4 to 37 nM in experiments 2 and 3 (Table 1). The source of this leakage was probably release of O₂ from the O rings and the edge of the PVC (polyvinyl chloride) plunger, which were the only nonglass parts in contact with the water, similar to the release of O₂ from rubber

![Diagram of reactions and genes]

**FIG 1** Overview of the genes and processes discussed in the present study based on the work of van de Vossenberg et al. (36) and Zumft (70). Genes: *amoC*, ammonia monoxygenase (ammonia oxidation); *hao*, hydroxylamine oxidoreductase (ammonia oxidation); *nxrB*, nitrite oxidoreductase (nitrite oxidation); *narG*, nitrate reductase (nitrate reduction); *nirK*, nitrite reductase (nitrite reduction); *nirS*, nitrite reductase (nitrite reduction, putatively more common in anammox, though some have nirK); *nrfA*, nitrite reductase (putatively more common in DNRA); *norB*, nitric oxide reductase (nitric oxide reduction); *nosZ*, nitrous oxide reductase (nitrous oxide reduction); *HZS*, hydrazine synthase (anammox; HZS indicates the gene cluster containing *hzsA*, *hzsB*, and *hzsC*); *hzo*, hydrazine oxidoreductase (anammox). Note that the *hao* gene has not been found in the nitrifying archaea (71).

<table>
<thead>
<tr>
<th>Gene expression analyzed</th>
<th>First half</th>
<th>Second half</th>
<th>O₂ concn (nM)¹</th>
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<tr>
<td></td>
<td>Avg</td>
<td>SE</td>
<td>Max</td>
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<tr>
<td>First recorded concentration when the reactor was fitted with a STOX sensor.</td>
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<tr>
<td>Highest recorded O₂ concentrations after the initial helium gassing.</td>
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**TABLE 1** Overview of experiments

<table>
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<th>Experiment</th>
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<th>Reactor</th>
<th>H₂S (µM)b</th>
<th>O₂ concn (nM)c</th>
<th>Gene expression analyzed</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>First half</td>
<td>Second half</td>
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<td></td>
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<td></td>
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<tr>
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<td>L</td>
<td>2</td>
<td>34</td>
<td>14.8</td>
<td>2.7</td>
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a L, continuously low; H, continuously high; LH, low during the first half and high during the last half; HL, high during the first half and low during the last half.
b Average H₂S (sum of H₂S, HS⁻, and S₂⁻) concentrations during the incubations (experiment 3 only).
c SE, standard error of the average.
d First recorded concentration when the reactor was fitted with a STOX sensor.
e Highest recorded O₂ concentrations after the initial helium gassing.
was an immediate decrease in the production rates of N\textsubscript{2} by both processes (e.g., Fig. 2B). Conversely, process rates increased when O\textsubscript{2} concentrations were lowered to levels similar to those in the control incubations (Fig. 2C). This change was evident immediately after the O\textsubscript{2} level reached the low nM range, but the processes could have accelerated even earlier, because the 1-h sparging performed to remove O\textsubscript{2} would also have removed any \textsuperscript{15}N-labeled N\textsubscript{2} formed during this period. Denitrification produced N\textsubscript{2}O at rates similar to the N\textsubscript{2} production rates, although with a higher variation. Nitrous oxide was both produced and consumed, which in some cases resulted in a net decrease in N\textsubscript{2}O concentration, e.g., as seen in the experiment depicted in Fig. 2C. The effects of O\textsubscript{2} on production of N\textsubscript{2} and N\textsubscript{2}O by denitrification were similar, with N\textsubscript{2}O production accelerating through the incubation in the low-O\textsubscript{2} controls, being inhibited by amendment of O\textsubscript{2} (e.g., Fig. 2B), and increasing very rapidly after removal of O\textsubscript{2} (e.g., Fig. 2C). The variation in initial rates of anammox and denitrification in the controls was relatively small between the three experiments, with anammox varying from 0.52 to 0.74 nM N\textsubscript{2} h\textsuperscript{-1} and N\textsubscript{2} and N\textsubscript{2}O production by denitrification varying from 0.16 to 0.34 and 0.96 to 1.38 nM N\textsubscript{2} h\textsuperscript{-1}, respectively (Table 2).

**Inhibition kinetics.** The rates of anammox and denitrification from the reactors in which the O\textsubscript{2} concentration was raised halfway through the incubation, and from the control reactors, were normalized (equation 4) and combined to estimate the inhibition of these processes by O\textsubscript{2} (Fig. 3). Assuming an exponential attenuation of rates (equation 5), the O\textsubscript{2} concentration resulting in a 50% inhibition of the process (C\textsubscript{50}) was calculated for each of the four processes: N\textsubscript{2} production by anammox and N\textsubscript{2}, N\textsubscript{2}O, and N\textsubscript{2} + N\textsubscript{2}O production by denitrification (Table 3). Denitrification was much more sensitive than anammox to O\textsubscript{2}, being 50% inhibited at 200 to 300 nM O\textsubscript{2}, whereas anammox first reached 50% inhibition at approximately 900 nM. The presence of sulfide in experiment 3 apparently did not affect the O\textsubscript{2} inhibition kinetics, and data from all three experiments are included in the analysis (Fig. 3).

**Effects of sulfide.** Addition of 1 \textmu M sulfide had no apparent effect on anammox rates, which decreased 30% in the absence and 22% in the presence of sulfide from the first to the second half of the experiment (Fig. 4; decreases in anammox rates between the first and second halves of the experiment and between the absence and presence of sulfide were not statistically significant). Denitrification, on the other hand, was strongly stimulated by sulfide. In the absence of sulfide, N\textsubscript{2} and N\textsubscript{2}O production increased \textasciitilde2-fold from the first to the second half of the experiment, whereas in the presence of sulfide, the production of these gases increased 4.5- and 6.1-fold, respectively.

**Gene expression.** Associations between oxygen treatments and metatranscriptional profiles suggest differential oxygen sensitivity among key pathways of dissimilatory nitrogen metabolism (Fig. 5 and 6). Because we were unable to sequence replicate reactors for all treatments (Table 1), we cannot statistically confirm the variation between reactor treatments. Nonetheless, several trends in N cycle gene transcript abundance are apparent from Fig. 5 and 6. Compared to treatments with low O\textsubscript{2} throughout or in the second half of the experiment (L and HL in Fig. 5), O\textsubscript{2} addition in experiments 1 and 2 was associated with reductions in the relative abundance of transcripts encoding nirS-type nitrite reductase, nitric oxide reductase (norB), and nitrous oxide reductase (nosZ) (Fig. 5). The suppressive effect of O\textsubscript{2} exposure on nirS,
norB, and nosZ transcription was relatively consistent in both low (~200 nM) and high (~1,700 nM) O2 amendments relative to the controls (Fig. 5, experiment 1). In contrast to these transcript patterns and to rate measurements showing inhibition of anammox and denitrification following O2 addition, O2 amendment caused only minor or inconsistent shifts in the abundances of transcripts for dissimilatory nitrate reductase (narG) and the hydrazine oxidoreductase (hzo) associated with anammox. A dependence on O2 was also not evident for the alternative copper-containing nitrite reductase (nirK) or for the sparingly expressed nrfA, associated with dissimilatory nitrite reduction to ammonium (DNRA). Of the enzymes associated with aerobic nitrification, nitrite oxidoreductase (nxrB) showed no clear trend in gene expression, while the expression of ammonium monooxygenase (amoC) genes from ammonium oxidizers was higher in the O2-amended microcosms. (Details on gene transcription in the source water microbial community and on the response of other genes to low-O2 treatment can be found in the work of Stewart et al. [32])

BLAST analysis of protein-coding transcripts identified a taxonomically diverse assemblage of microorganisms mediating OMZ nitrogen cycling with more than 20 higher-level taxa represented (Fig. 6). Denitrification genes (narG, nirK, nirS, norB, and nosZ) were affiliated with a broad range of taxa, and taxonomic composition was highly variable among these genes. For example, nirS was mainly expressed by Gammaproteobacteria while the expression of nosZ was more evenly distributed between Bacteroidetes/Chlorobi, Gammaproteobacteria, and unidentified organisms. Groups making minor contributions also differed markedly between the two genes. This pattern is consistent with denitrification being the result of individual processes catalyzed by different groups of microorganisms. However, a proportion of these transcripts encode enzymes not involved in classical denitrification. For example, a large part (6 to 52%) of norB sequences were most...
closely related to the quinol-oxidizing variant (qNor) of “Candidatus Methylomirabilis oxyfera” (CBE69496.1 and CBE69502.1), a member of the NC10 candidate division enriched from sediment. In this organism, the Nor enzyme may act as a dismutase to convert nitric oxide into dinitrogen and O2, with the latter then being used to oxidize methane under anaerobic conditions (33, 34).

Several genes in Fig. 5 and 6 were most closely related to those of known anammox bacteria. The vast majority of hzo sequences were either more similar to hzo of the marine planctomycete “Candidatus Scalindua profunda” or to genes annotated as “uncultured ammonia-oxidizing bacteria” (e.g., accession no. AEP17466 in the NCBI database), labeled as “planctomycetes” and “unknown” in Fig. 6, respectively. In addition, the majority (53 to 88%) of narG sequences were most closely related to narG of either “Ca. Scalindua” or the freshwater anammox bacterium “Candidatus Kuene- nia stuttgartiensis.” It is hypothesized that in anammox bacteria, narG acts in reverse as a nitrite oxidoreductase, oxidizing NO2− to NO3− to fuel carbon fixation (35). “Ca. Scalindua”-like nirS transcripts were also detected, accounting for 2 to 42% of the total nirS signal (“planctomycetes” in Fig. 6). However, the relative abundance of these transcripts was an order of magnitude lower than that of “Ca. Scalindua” hzo and narG, which showed similar abundances. Unlike the nirS transcription pattern as a whole, “Ca. Scalindua”-like nirS transcripts did not show consistent patterns of change in response to O2 amendment; however, the low abundances of these transcripts may have prevented the detection of clear patterns. “Ca. Scalindua”-like nitric oxide reductase (norB) sequences were also present, but at low abundances comparable to those of “Ca. Scalindua” nirS. The “Ca. Scalindua” norB sequences (Fig. 6) were most closely related to a gene encoding a quinol-oxidizing NO reductase (qNor) (gene scal02135). In this bacterium, norB may act not in energy metabolism but instead to relieve nitric oxide stress (36), as has been observed for the qNor gene in pathogenic bacteria, though this hypothesis has not yet been confirmed for anammox bacteria. Together, these data highlight variable expression levels among key anammox genes but an overall minor transcriptional response of these genes to O2 fluctuation over the concentration range and time periods examined here. This pattern is in contrast to the clear inhibition of anammox rates following O2 addition (Fig. 2).

**TABLE 3** Parameters for the fit of an exponential-type inhibition kinetics to the measured inhibition by O2 of denitrification and anammox

<table>
<thead>
<tr>
<th>Process</th>
<th>C₃₀ (nM)</th>
<th>R²</th>
<th>k</th>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N₂</td>
<td>205 (±34)</td>
<td>0.8818</td>
<td>0.00337</td>
</tr>
<tr>
<td>N₂O</td>
<td>297 (±139)</td>
<td>0.5557</td>
<td>0.00233</td>
</tr>
<tr>
<td>Total</td>
<td>255 (±88)</td>
<td>0.6968</td>
<td>0.00271</td>
</tr>
<tr>
<td>Anammox</td>
<td>886 (±418)</td>
<td>0.4542</td>
<td>0.00078</td>
</tr>
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</table>

a Data were fitted to the following exponential function: inhibition (%) = 1 − e⁻ᵏ·C₃₀ applying the Levenberg-Marquardt algorithm (Origin 9.0; OriginLab, MA), where A is the variable coefficient given in Table 2 and C is the oxygen concentration.
b O2 concentration at which 50% inhibition was obtained. Standard errors are in parentheses.

The effects of O2 manipulations in coupled measurements of anammox and denitrification rates and community gene transcription were observed for the first time under controlled O2 condi-
FIG 6  Taxonomic representation and relative abundance of reads matching key nitrogen genes at different oxygen levels in bioreactor experiment 1 (right) and 2 (left). Transcript abundance is calculated as read count per gene per kilobase of gene length and then standardized as a percentage of total protein-coding reads per data set. Taxonomic identifications are based on annotations of NCBI reference sequences identified as top matches (above a bit score of 50) in BLASTX searches. Note that the y axis scales differ. Abbreviations for oxygen treatments are as in Fig. 5, and genes are as in Fig. 1.
tions reflective of in situ conditions in the OMZ off Chile. Inhibition of anammox and denitrification activity was observed over the O₂ concentration ranges applied in this study (5 to 2,000 nM), suggesting that the experimental O₂ conditions were ecologically relevant (Fig. 3). In contrast, patterns in transcript abundance at the endpoint of each experiment were not unambiguously linked to trends in the rate data. Nonetheless, some genes showed consistent patterns in response to O₂ amendment. Together, these data highlight the potential effects of O₂ fluctuation on dissimilatory nitrogen transformations in OMZ waters, as well as the extent to which community transcription patterns can complement and inform process rate measurements in experimental systems.

Rates of anammox and denitrification and effects of O₂. We quantified bulk anammox and denitrification activity by measuring gas (N₂ and N₂O) production and analyzed the expression of the genes coding for known enzymes catalyzing individual steps of these processes. In these experiments, ¹⁵N₂ accumulated linearly immediately after the experiments were started, indicating that anammox and denitrification were active in situ. This was evidenced further by transcription of indicator genes for both anammox and denitrification (hzo, nar, nir, nor, and noz) in source water at the time of reactor filling, often at higher relative levels than those observed at the experiment endpoints (see the supplemental methods and Fig. S1 in the supplemental material). We suggest that the rates measured during the first part of the experiments, with linear increases in ¹⁵N₂ concentrations, provide a measure of the in situ activity, under the assumption that the O₂ levels here did not inhibit the processes significantly (see below). Anammox and denitrification activity started as soon as the helium sparging stopped and O₂ had been removed (Fig. 2C), with linear increases in ¹⁵N₂ concentrations over time. Addition of O₂ to a concentration of about 2 µM almost completely and rapidly inhibited these processes (Fig. 2B). These trends suggest an inhibition at the enzyme level, allowing immediate resumption of activity as soon as O₂ levels fall below inhibiting concentrations. If enzyme synthesis had been required for the resumption of anammox and denitrification activity, the transition to active N gas production would have been less abrupt and the rates would have increased over time. Indeed, the denitrifier Paracoccus denitrificans in pure culture at optimal temperature required 10 to 24 h to establish a fully active denitrification enzyme system after a shift from aerobic (90% air saturation) to anaerobic conditions (37, 38). Likewise, if inhibition had occurred at the enzyme production level (transcription or protein synthesis), the reduction in process rates presumably would have been much less abrupt, as residual proteins would have continued to function until internal enzyme levels became depleted. Thus, the response of both metabolic activity and gene expression to changes in O₂ concentration show that the organisms involved in nitrogen transformations are well adapted to an environment where O₂ concentrations fluctuate from anoxia to a few micromolar units on a time scale of hours to days, exactly as is the case around the oxic-anoxic interface where our samples were retrieved (e.g., see reference 39).

The rates of fixed-nitrogen removal (sum of anammox and denitrification) found here (in the first half of the control incubations) (Table 2) compare generally well with the rates measured previously at two stations approximately 10 km east and southeast of station 3 (40) and with the rates measured with the standard Exetainer method during the cruise (39). Also, the rates measured by Dalsgaard et al. (14), at a station (G04) coinciding with our station 5, were in the same range as the rates found in the present study. Furthermore, the rates of anammox were within the range found off Peru by Kalvelage et al. (16) (see the work of Dalsgaard et al. [14] for a more thorough comparison of measured rates in the eastern South Pacific). While the fixed-nitrogen removal rates from the reactor experiments were in the same range as rates from previous cruises in the area, the high contribution of denitrification for N₂ production was found in only one previous survey (14). As suggested by Dalsgaard et al. (14), this may be controlled by the availability of electron donors for denitrification. However, O₂ contamination may also play a role. Exetainer incubations, which were used in most of the published OMZ denitrification/anammox studies, are most likely contaminated with a few hundred nM O₂ (31). If the higher sensitivity of denitrification than anammox to O₂ (see below) is a general phenomenon, denitrification may have been inhibited more than anammox in previous studies, potentially leading to an underestimate of the role of denitrification. The O₂ concentrations in the control reactors in the present study were probably lower than in most Exetainer incubations, which may have contributed to the observed higher contribution of denitrification to removal of fixed N.

The rates observed during the second half of the incubations were different from those observed during the first half, probably due to some sort of a bottle effect. In contrast to most of the published studies on anammox and denitrification in OMZs, we were able to identify and quantify this nonlinearity due to frequent sampling for N gas production. Furthermore, we were able to quantify O₂ contamination. While the rates obtained during the second part of the experiments do not represent in situ rates, the same suite of processes continued from the first half of the experiment. Thus, O₂ inhibition kinetics were investigated by comparing the rates from O₂-amended reactors to the rates that were expected in the absence of O₂ manipulation (see equation 3 below). Another consequence of enclosing the water in a bottle was that in some of the experiments, N₂O accumulated to concentrations higher than are normally found in situ. We have no explanation for this, but we assume that the same enzymes are active in the bioreactors and in situ and, as argued above, that the experimentally determined O₂ inhibition kinetics may be applied to in situ conditions.

The O₂-dependent inhibition of N₂ production by anammox and of N₂ and N₂O by denitrification was concentration dependent, with anammox showing a more variable response to O₂ amendments, while denitrification was more sensitive to O₂ (Fig. 3). Consequently, the C₅₀ for anammox (886 nM O₂) is less well supported than that of denitrification (255 nM O₂ for total denitrification) (Table 3). While anammox was less sensitive to O₂ amendments than denitrification in the present study, the data also indicate that anammox was much more sensitive to O₂ than previously found. Indeed, 50% inhibition of anammox was achieved at 11 to 16 µM in the OMZ off Namibia and from 2 to 11 µM off Peru (22), which is in line with results from the Black Sea, where anammox was reduced by 7 to 8% at about 1 µM O₂ and was fully inhibited by 13 µM O₂ (21). Kalvelage et al. (22) observed a tendency for anammox to become more sensitive to O₂ at stations seaward of the continental shelf, where one station showed no anammox above 2.8 µM O₂. A very similar result was obtained by Babbin et al. (23) from the eastern tropical North Pacific, where anammox still occurred at low rates at 3 µM O₂ and
was completely inhibited at 8 \( \mu M \) \( O_2 \). The two latter observations are very much in line with our results from the two off-shelf stations off Iquique, Chile, where ammonox was recorded at up to 1.7 \( \mu M \) \( O_2 \) in two of the experiments (Fig. 3). The fact that the \( O_2 \) threshold observed in the present study is substantially lower than that found in most previous studies may partly be explained by variation in microbial community composition among studies. It is also possible that activity observed at higher \( O_2 \) bulk levels in previous studies in fact occurred in \( O_2 \)-depleted microzones within aggregates (22, 39). Furthermore, these studies all used nonstirred Extainer incubations. Organisms and particulates may settle out of suspension during such incubations, creating a local low-\( O_2 \) environment at the bottom of the vials, where anaerobic processes may be active at what appear to be relatively high \( O_2 \) concentrations. This would not happen in the stirred bioreactors in the present study, and furthermore, the stirring would increase \( O_2 \) transport into aggregates, leading to a smaller difference between \( O_2 \) concentrations inside and outside aggregates. Consequently, stirred incubations may produce \( O_2 \) sensitivity results that more accurately reflect the responses of the individual organisms.

The \( O_2 \) sensitivities of \( N_2O \) and \( N_2 \) production by denitrification were similar, requiring 200 and 300 nM \( O_2 \) for 50\% inhibition, respectively (Table 3). As most studies of nitrogen removal in OMZs have failed to detect canonical denitrification, experimental evidence of the effect of \( O_2 \) on this process is scarce. However, in one recent \( ^{15}N \)-labeling study, denitrification was measurable and \( O_2 \) amendment experiments showed that at 3 \( \mu M \) \( O_2 \), denitrification was completely inhibited (23). This level is certainly higher than the threshold found in the present study, but since concentrations between 3 \( \mu M \) and anoxia were not tested by Babbin et al. (23), these studies do not contradict each other. In the present study, effects of \( O_2 \) on process rates generally agree well with the metatranscriptomic data. In the experimental reactors with \( O_2 \)-levels from ~200 to 1,800 nM, \( nosZ \) transcript abundances were consistently lower than in the controls, which had \( O_2 \) levels in the tens of nanomolar units. Similarly, inhibition of the transcription of \( nirS, nrfA, \) and \( norB \) genes already occurred at the lowest \( O_2 \) level (201 nM), agreeing with the suggested halt of \( N_2 \) production in the eastern South Pacific OMZ when \( O_2 \) is detectable in situ (14) and with studies of cultured denitrifiers showing \( nirS \) and \( norB \) transcription sensitivity at an \( O_2 \) concentration of \(<~0.5 \mu M \) (41–44).

In contrast, the transcription of \( nirK \)-like nitrite reductase, which was predominantly affiliated with archaea and hence likely with ammonium oxidizers, appeared not to be largely affected by \( O_2 \) exposure, consistent with the results obtained with a \( nirK \)-utilizing denitrifier (43). The fact that the \( N_2O \)-producing and -consuming parts of denitrification otherwise react similarly to \( O_2 \) exposure suggest that low-range fluctuations in \( O_2 \) concentrations will not cause substantial \( N_2O \) accumulation from denitrification. The production of \( N_2O \) by ammonium oxidizers may respond differently, however.

We did not quantify the \( O_2 \) sensitivity of \( NO_3^- \) reduction activity in this study. However, the transcription data generated here show no clear suppression of bulk nitrate reductase gene (\( narG \)) transcription within the range of \( O_2 \) concentrations applied \((-2 \mu M \) \( O_2 \)) (Fig. 5). At a first glance, this suggests that \( NO_3^- \) reduction was less sensitive to \( O_2 \) than the other steps in the denitrification pathway. However, the majority of the \( narG \) transcripts were affiliated with \( narG \) of ammonox bacteria (“\( Ca. \) Scalindua” and “\( Ca. \) Kuenenia”), which likely use this enzyme oxidatively to drive reverse electron transport (35, 45). Closer inspection of the \( narG \) transcript pool suggests that \( NO_3^- \) reduction gene expression may in fact be inhibited in some taxonomic groups, notably the \( Gammaproteobacteria \), by \( O_2 \) concentrations as low as 200 nM (Fig. 6). This result is in contrast to results for some organisms that exhibit relatively stable nitrate reductase expression. In the human pathogen \( Mycobacterium \) \( tuberculosis \), for example, a \( NarGH \) nitrate reductase is constitutively expressed, with enzyme levels independent of oxygen concentrations (46). Nitrate reduction to nitrite in this bacterium is instead regulated via a nitrate transporter (\( NarK \)), whose transcription and activity are inhibited not directly by the presence of molecular oxygen but by the redox state of the cell, allowing a rapid switch to nitrate utilization when transitioning from oxidizing to reducing conditions (47). In contrast, studies of complex multispecies communities in OMZs have described variable and relatively weak effects of \( O_2 \) on nitrate reduction rates. For example, no effect of \( O_2 \) at concentrations of up to 25 \( \mu M \) was found at one station in the Peruvian OMZ, but reductions in nitrate reduction rates of up to 50\% when \( O_2 \) reached 4 \( \mu M \) at other stations off Peru and in the Namibian OMZ were reported (22). This variability in the sensitivity of nitrate reduction to \( O_2 \) may be due partly to differences among microbial communities at the sampling sites but also to variations in other chemical parameters, potentially including nitrate or nitric oxide, which have been shown to regulate nitrate reductase transcription or activity (47, 48).

The relative abundances of denitrification and ammonox gene transcripts highlight the potential for decoupling between transcriptional patterns (at the end of the experiment) and the observed biochemical response to \( O_2 \). Notably, endpoint ammonox-associated gene transcripts (e.g., \( hzo \) and “\( Ca. \) Scalindua”-like \( nirS \)) did not vary appreciably or consistently in relative abundance between the low-\( O_2 \) control and \( O_2 \) addition treatments (Fig. 5), whereas \( O_2 \) addition clearly inhibited ammonox activity (Fig. 3). It is possible that variations in ammonox gene transcription occurred between control and amendment reactors earlier in the experiments and that due to the short half-life of mRNA (minutes or hours), such differences were not captured by endpoint sampling. However, assuming that transcript abundances were equivalent among treatments at the start of experiments, it is hard to envision variations in transcription that explain both the observed enzyme inhibition and the endpoint transcript patterns. The potential alternative would be that \( O_2 \) at low levels does not inhibit transcription to the same extent as it inhibits protein function, i.e., that regulation occurs at the posttranscriptional level for ammonox enzymes, within the range of conditions tested here. Other genes involved in dissimilatory nitrogen metabolism, notably the denitrification genes \( nirS, norB, \) and \( nosZ \) (as well as genes involved in aerobic \( NH_4^+ \) oxidation [\( amoc \); see the supplemental material], showed a tighter coupling to \( O_2 \) levels. These results reinforce prior studies showing that distinct steps of multistep metabolic pathways, such as denitrification, can differ in \( O_2 \) sensitivity (43). Consequently, discrepancies in estimates of the sensitivity of bulk denitrification and ammonox to \( O_2 \) (discussed above) are likely due to a combination of taxonomic variation as well as differences in sensitivity among the various enzymes of each pathway. This is particularly likely for denitrification, in which the overall pathway is mediated by diverse assemblages of
bacteria (49–51). Taxonomic analysis of gene transcripts suggested that there is high taxonomic diversity among the denitrifiers and that the taxonomic composition of the individual denitrification gene assemblages was highly variable, indicating that the denitrification process is indeed the result of a series of individual reactions catalyzed by different groups of organisms. Together, these data indicate that the extent to which transcript abundance patterns in metatranscriptional data sets can be used as proxies for process rate measurements is variable, likely due to complex factors, including the relative dominance of different community members, differences in the level of metabolic regulation (transcriptional, translational, and enzymatic), and the range of environmental conditions being observed.

Effects of sulfide. Genomic analysis has indicated a significant role of sulfur cycling in the OMZs (28, 52, 53) contributing to the discovery of a cryptic sulfur cycle (10). This cycle involves the reduction of sulfate to sulfide, which is immediately oxidized and accumulates only under special circumstances in the absence of NO$_3^-$ and NO$_2^-$ in OMZs without sediment contact (54). Under normal OMZ conditions with high concentrations of NO$_3^-$ and NO$_2^-$, reduction or denitrification is responsible for removal of H$_2$S. In a situation with H$_2$S accumulation, mainly inhibition is probably due to the low H$_2$S concentrations in the experiment (Fig. 4), supposedly as a result of H$_2$S being a quantitatively important electron donor. The effect of O$_2$ on denitrification in the presence of H$_2$S was very similar to the effects seen without added H$_2$S (Fig. 2). This might suggest that the same organisms were active in both experiments or, alternatively, that different groups were active but had similar sensitivities to O$_2$. The latter is supported by the delayed response of denitrification to sulfide amendment, which indicates an induction period for the sulfide-oxidizing community. Sulfide amendment may also have stimulated NO$_3^-$ reduction to NO$_2^-$ as previously observed (10), but the precision of our NO$_2^-$ measurements was not sufficient to quantify this process. It has previously been suggested that H$_2$S may interfere with the nitrogen cycle by inhibiting ammonia (27, 55), and complete inhibition of the process at concentrations of 1.5 to 2.5 μM was observed in Black Sea waters (21). In contrast, we found no significant inhibitory effect of ca. 1 μM H$_2$S on ammonia (Fig. 4). Also, the reduction of N$_2$O in the denitrification pathway may be inhibited by H$_2$S (57), but such inhibition was relatively minor in our experiments. During the first half of the experiment, N$_2$O accounted for 74% and 92% of total gas production in the absence and presence of H$_2$S, respectively. These values were 77% and 94% during the second half. This low degree of inhibition is probably due to the low H$_2$S concentrations in the experiment (ca. 1 μM). In the anoxic water column of the Baltic Sea, the ratio of N$_2$O to total gas production was proportional to the H$_2$S concentration, and first at a concentration of 7 μM H$_2$S, N$_2$O accounted for half of the total gas production from denitrification (58). In pelagic OMZs, H$_2$S only rarely accumulates to the levels applied in our experiments (55). However, in OMZs with sediment contact and in a global warming scenario with expanding OMZs (4), sulfide accumulation may occur more frequently (6, 55, 56), potentially leading to N$_2$O production.

**MATERIALS AND METHODS**

**Study area.** Sampling took place in the eastern South Pacific OMZ off Iquique in northern Chile during the Microbial Oceanography of Oxygen Minimum Zones (MOOMZ-III) cruise on the R/V Agor Vidal Gormaz from 8 to 18 January 2010. At this site, O$_2$-deficient equatorial subsurface water is transported southward by the Peru-Chile undercurrent, and the O$_2$-rich surface water is transported north by the Humboldt current (40). Water was sampled at two locations 30 km (station 3; 20°06'S, 70°25'W) and 69 km (station 5; 20°06'S, 70°48'W) off shore (10). At station 3, water was collected at depths of 75 m (specific density $\sigma_{θ}$, 26.23 kg m$^{-3}$) and 82 m ($\sigma_{θ}$ 26.22 kg m$^{-3}$) for experiments 1 and 3, respectively, and at station 5, water was collected at 86 m ($\sigma_{θ}$ 26.21 kg m$^{-3}$) for experiment 2. The oxic-anoxic (oxic $=$ O$_2$ below the detection limit of the STOX sensor; see below) interface was at 70- and 75-m depths at station 3 during sampling for experiments 1 and 3, respectively, and at 80 m at station 5 during sampling for experiment 2.

**Reactors.** The reactors used for incubations were described previously (32). Briefly, reactors were 2-liter glass cylinders with a PVC piston with two O rings fitting tightly inside the glass cylinder. The end of the piston was covered with a glass plate to prevent leakage of O$_2$ into the incubating water. The glass plate was penetrated with three pieces of PEEK (Upchurch Scientific) tubing to allow gassing, amendment, and sampling. A highly sensitive switchable trace oxygen (STOX) sensor (17, 18) was placed in each of the eight parallel reactors. Water was sampled using a pump profiling system (PPS) (10), which allowed pumping of water from the O$_2$-depleted zone directly into the reactors. The pump system was equipped with a Seabird 25 CTD and both a standard SBE 43 O$_2$ sensor and a STOX sensor, and O$_2$ concentrations were monitored continuously during sampling. Filling of the reactors was described in detail earlier (32). Briefly, the reactor was flushed with nitrogen and then filled with water from below with a counterflow of nitrogen. The water was allowed to overflow for three volume changes before the reactor was sealed. The reactors were transferred to the onboard lab, placed in water at the in situ temperature (ca. 13°C), and protected from light. Circulation inside the reactor was created by a glass-coated, 2-cm-long magnetic stir bar rotating at ca. 60 rpm. Standard Teflon-coated stir bars contain large amounts of O$_2$, which would be released to the incubating water and raise the O$_2$ concentration in the experiments.

**Incubation experiments.** Three incubation experiments (28 to 36 h each) were conducted to examine the effects of O$_2$ concentration on denitrification and ammonox rates and community gene expression. Each experiment involved 7 to 8 reactors representing a range of O$_2$ concentrations from ~10 to 50 nM to 2 μM (Table 1). Each reactor was equipped with a STOX sensor and purged with a gentle flow of helium through a 1/16-in. PEEK tubing for ca. 1.5 h, which removed the O$_2$ that had entered during handling, typically 30 to 60 nM with some variation (Table 1). During purging, some CO$_2$ was removed, causing the pH to increase by ~0.3. Subsequently, all headspace and bubbles were removed, and water samples were taken to measure initial concentrations of NO$_3^-$, NO$_2^-$, and NH$_4^+$ as previously observed (10), to be the terminal electron donor for NO$_3^-$ reduction to NO$_2^-$ in the experiments. However, in the H$_2$S-amended reactors, denitrification to N$_2$O was strongly stimulated in the second half of the experiment (Fig. 4), supposedly as a result of H$_2$S being a quantitatively important electron donor. The effect of O$_2$ on denitrification in the absence of H$_2$S release from the sediment, H$_2$S was shown to be the major electron donor and NO$_3^-$ or NO$_2^-$ to be the terminal electron acceptor through all the individual steps of the denitrification process (55, 56). It is not clear to what degree H$_2$S was the direct electron donor for NO$_3^-$ reduction or denitrification in these experiments. However, in the H$_2$S-amended reactors, denitrification to N$_2$O was strongly stimulated in the second half of the experiment (Fig. 4), supposedly as a result of H$_2$S being a quantitatively important electron donor. The effect of O$_2$ on denitrification in the presence of H$_2$S was very similar to the effects seen without added H$_2$S (Fig. 2). This might suggest that the same organisms were active in both experiments or, alternatively, that different groups were active but had similar sensitivities to O$_2$. The latter is supported by the delayed response of denitrification to sulfide amendment, which indicates an induction period for the sulfide-oxidizing community. Sulfide amendment may also have stimulated NO$_3^-$ reduction to NO$_2^-$ as previously observed (10), but the precision of our NO$_3^-$ measurements was not sufficient to quantify this process. It has previously been suggested that H$_2$S may interfere with the nitrogen cycle by inhibiting ammonia (27, 55), and complete inhibition of the process at concentrations of 1.5 to 2.5 μM was observed in Black Sea waters (21). In contrast, we found no significant inhibitory effect of ca. 1 μM H$_2$S on ammonia (Fig. 4). Also, the reduction of N$_2$O in the denitrification pathway may be inhibited by H$_2$S (57), but such inhibition was relatively minor in our experiments. During the first half of the experiment, N$_2$O accounted for 74% and 92% of total gas production in the absence and presence of H$_2$S, respectively. These values were 77% and 94% during the second half. This low degree of inhibition is probably due to the low H$_2$S concentrations in the experiment (ca. 1 μM). In the anoxic water column of the Baltic Sea, the ratio of N$_2$O to total gas production was proportional to the H$_2$S concentration, and first at a concentration of 7 μM H$_2$S, N$_2$O accounted for half of the total gas production from denitrification (58). In pelagic OMZs, H$_2$S only rarely accumulates to the levels applied in our experiments (55). However, in OMZs with sediment contact and in a global warming scenario with expanding OMZs (4), sulfide accumulation may occur more frequently (6, 55, 56), potentially leading to N$_2$O production.
the start of the experiment lowered to the low nM range halfway through (high to low, designated "HL" in Table 1; for an example, see Fig. 2C). Finally, in some reactors, O2 was kept high throughout the experiment (designated "H" in Table 1). Oxygen was added by injecting air-saturated water obtained from the station when the reactors were filled (1 ml injected gave a rise of ca. 170 nM O2). Oxygen was removed by purging with helium as described above. In the experiments where O2 concentrations were raised, air-saturated water was injected at a sufficient frequency to counteract O2 consumption and maintain the target O2 concentration range. In the experiments starting with high O2 concentrations, air-saturated water was injected right after the initial gassing with helium. The increase in O2 concentration after injection of air-saturated seawater was instantaneous, whereas removal of O2 by helium sparging took about 1 h. In experiment 3, a solution of NaNO3 (5 mM) was added to some of the reactors to a concentration of 1.1 μM. There was no systematic change in total sulfide concentration in experiment 3 after sulfide was added, and the actual concentrations are given as the average throughout the experiment (Table 1). The times from sampling until start of the experiment (i.e., tracer amendment) were 10, 9, and 5 h in experiments 1, 2, and 3, respectively.

Samples for N2 and N2O gas isotopic composition and concentration and for NO3−, NO2−, and NH4+ concentrations were taken eight times at approximately regular intervals during the 28- to 36-h-long incubations. Samples for nitrogen gas were stored in 12-ml glass vials with a butyl rubber septum (Exetainer; Labco) preserved with 100 μl 50% (wt/vol) ZnCl2. Samples for NO3− (experiments 1 and 2) and NH4+ were analyzed immediately whereas samples for NO2− in the presence of sulfide (experiment 3) and all NO3− samples were frozen for later analysis. Endpoint samples for gene expression analysis were obtained by filtering the remaining water in each reactor.

Chemical analysis. Concentrations of NO3− plus NO2− were measured as NO after reduction in hot vanadium chloride (59) on a Thermo Environmental Instruments 42c NO3 analyzer. Nitrite in the absence of sulfide was measured using a standard colorimetric technique (60), and NO3− in the presence of sulfide was analyzed as NO after reduction in cold vanadium chloride (59). Ammonium was quantified fluorometrically by the orthophthalaldehyde method (61) on a Turner Designs Trilogy fluorometer, and sulfide concentration was quantified colorimetrically according to the method of Cline (62). Nitrogen isotopes in N2 and N2O were analyzed on a Thermo Delta V Plus isotope ratio mass spectrometer as described previously (14).

Calculations. Rates of anammox and denitrification in the 15NO2− amended experiments were calculated as described earlier (63). Briefly, knowing the mole fraction of 15N in the NO3− pool (F15N) and the production rates of 29N2 (P29) and 30N2 (P30), N2 production by anammox and denitrification were calculated as follows:

\[ \text{denitrification} = P_{30} \times F_{15N}^{-2} \]  
\[ \text{anammox} = F_{15N}^{-1} \times [P_{29} + 2 \times (1 - F_{15N}^{-1}) \times P_{30}] \]

F15N was estimated from the concentrations of NO3− before and after amendment of 15NO2−, and P29 and P30 were calculated as the slopes of the linear regression of 29N2 and 30N2 concentrations as a function of time. Production of N2O by denitrification was estimated using equation 1 replacing P30 with the production rate of 15N2O, which was calculated as the slope of the linear regression of 15N2O versus time. Manipulation of O2 concentrations took place immediately after the fourth sampling in all experiments, and rates of anammox and denitrification were calculated in each incubation for the period from the first to the fourth sampling (referred to as the first half) (Table 1) and from the fourth to the eighth sampling (referred to as the second half) (Table 1). Dissimilatory nitrite reduction to ammonium (DNRA) was not detectable in parallel incubations from the two stations (39), and hence we ruled out the possibility that denitrification could be substantially overestimated due to a coupling of DNRA and anammox. This is consistent with the very low expression of the nrfA gene (see Results), coding for a key enzyme in the DNRA pathway. Furthermore, the N2O production recorded during incubations and the expression of the genes coding for denitrification enzymes (see results) indicate that denitrification was indeed active.

The effects of O2 concentration on rates of anammox and denitrification were evaluated by comparing rates from the first half to rates from the second half of the incubation in the manipulated reactors. However, process rates also changed from the first to the second half in the control reactors with continuously low O2 concentrations. It was assumed that rates in the manipulated reactors would have undergone the same relative change from the first half to the second half of the incubation if they had not been manipulated. Therefore, the effect of the change in O2 concentration was estimated by comparing the measured rate during the second half of the incubation with the rate expected in the absence of manipulation. There was one set of control reactors for each of the three experiments, and manipulated reactors of one experiment were compared to control reactors from that same experiment. The expected rate in the absence of manipulation (R2expected) was calculated as

\[ R_{2\text{expected}} = R_1 \times (R_2/R_1) \]

where R1 is the rate measured during the first half of the incubation and R1 and R2 are the rates measured in the control during the first and second halves of the incubation. The inhibition of the rate due to a change in O2 concentration halfway through the incubation was calculated as

\[ \text{Inhibition(\%)} = 1 - e^{-kX_c} \]

using the Levenberg-Marquardt algorithm (Origin 9.0; OriginLab, MA), where C is the O2 concentration and k is the variable coefficient (modified from the work of Jensen et al. [64], where rates, rather than relative inhibition, were fitted as a function of inhibitor concentration). From this, the O2 concentration resulting in a 50% inhibition of the rates, C50, was calculated.

Analysis of gene expression. Although enclosure in bioreactors has been shown to alter the metatranscriptional profile of some OMZ community members (i.e., bottle effects) (32), comparisons between bioreactors with contrasting oxygen treatments can help identify gene expression patterns suggestive of differential oxygen sensitivity. High sequencing costs and problems with sample loss during processing prevented replicate sequencing for all treatments, excluding the four low-oxygen controls (Table 1). Therefore, samples for community gene expression analysis were collected from a subset of bioreactors (Table 1). Sample processing and analysis were as described previously (32). Briefly, the endpoint microbial community was collected by filtering the water remaining in each bioreactor (~1.2 liters) at the endpoint of the experiment. Water was filtered through a glass fiber prefilter (47 mm, 1.6 m; Sterivex) using a peristaltic pump. Sterivex cartridges were filled with RNAlater (Ambion), capped, flash-frozen in liquid nitrogen, and stored at −80°C. Less than 15 min elapsed between sample collection (experiment end) and flash-freezing.

Community RNA was extracted using a modification of the mirVana microRNA (miRNA) isolation kit (Ambion) as described by Stewart et al. (32). Briefly, filters were thawed on ice, and the RNAlater surrounding each filter was expelled via syringe and discarded. Cells were lysed directly on the filter by adding lysis/binding and miRNA homogenate additive (Ambion) and vortexing. Lysate was expelled, and nucleic acids were isolated by treatment with acid-phenol–chloroform according to the manufacturer’s protocol. Extracted total RNA was treated with DNase (Turbo DNA free) to eliminate genomic DNA and purified using the RNeasy MinElute cleanup kit (Qiagen).
Prokaryotic and eukaryotic rRNA was removed from RNA extracts via a subtractive hybridization protocol (65) using sample-specific probes developed by Stewart et al. (32). rRNA-depleted RNA was linearly amplified, converted to double-stranded cDNA, and purified as described previously (28, 32, 66). Purified cDNA was sequenced on a Roche FLX genome sequencer using Titanium series chemistry (one full plate per sample, excluding the control [low-O2] bioreactor replicates, which were sequenced using a half-plate run each). Metatranscriptome data sets describing gene expression patterns in the control bioreactors (n = 4) (Table 1) and from the in situ microbial community (filtered from source water at the time of bioreactor filling: n = 2) (Fig. 6) were published previously (32) and are available in the NCBI Sequence Read Archive under accession number SRA049608.1. The data for 6 bioreactors amended with O2 in experiments 1 and 2 (Table 1) are published here. Sequencing reads counts for these data sets are in Table S1.

Metatranscriptomes were analyzed as described previously (28, 32). Duplicate reads (100% similarity, identical lengths), potentially arising from pyrosequencing errors, were identified using CD-HIT (67) and removed from each data set. rRNA transcripts were identified by BLASTN (bit score threshold = 50) against a database of prokaryotic and eukaryotic rRNA sequences compiled from the ARB-SILVA databases and excluded from further analysis. Protein-coding mRNA transcripts were identified by BLASTX against the NCBI nonredundant (nr) protein database (as of April 2012), modified to include the published genome of the marine anammox bacterium “Candidatus Scalindua profunda” (36) (data from Joint Genome Institute, U.S. Department of Energy). For reads matching multiple reference genes with equal bit scores, each reference was counted as a top match, with its count scaled in proportion to the number of genes sharing the top score.

The relative abundances of key N cycle genes (Fig. 5 and 6) were determined via keyword searches of BLASTX results (bit score > 50), as described by Canfield et al. (10) and Ganesh et al. (68). NCBI nr genes representing top BLASTX matches were recovered from GenBank, and each database entry was examined manually to confirm gene identity. Entries with ambiguous annotations were further verified by BLASTX. Gene abundances were normalized based on best approximate gene length (bp), estimated from full-length open reading frames from sequenced genomes: amoC (750 bp), nxrB (1,500 bp), hzo (1,650 bp), narG (3,600 bp), nirK (1,140 bp), nirS (1,620 bp), rrfA (1,440 bp), norB (1,410 bp), and nosZ (1,950 bp). Sequence counts per kilobase of target gene were normalized to data set size (Fig. 6) and to counts of sequences matching the universal, single-copy gene encoding RNA polymerase subunit B (rpoB, 4,020 bp) (Fig. 5) as described by Ganesh et al. (68), such that a value of 1 in Fig. 5 indicates abundance equivalent to that of rpoB (assuming the gene lengths listed above). The taxonomic identities of transcripts were inferred from the matching reference gene annotations, with relative taxon abundances tabulated using MEGAN 4 (69) and shown by color coding in Fig. 6.

Data accession number. All sequence data generated in this paper can be accessed at NCBI under BioProject ID PRJNA263804.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl?doc=10.1128/mBio.01966-14/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Table S1, PDF file, 0.04 MB.

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We declare no conflicts of interest.

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REFERENCES
18. Revsbech NP, Thamdrup B, Dalsgaard T, Canfield DE. 2011. Construction of STOX oxygen sensors and their application for determination of


**Supplemental Material**

*Gene transcription – other dissimilatory nitrogen metabolism genes*

Transcripts associated with aerobic nitrogen transformations showed differing patterns in relation to oxygen. In both experiment 1 and 2, transcripts for ammonium monooxygenase (\(amoC\)), catalyzing aerobic ammonia oxidation, were most abundant in treatments with the highest average \(O_2\) concentration over the last half of the incubation (Figure 4). This was the case even in experiment 1 where there was a relatively small difference in \(O_2\) concentration between the continuously low-treatment (34 nM \(O_2\)) and the lowest \(O_2\) amendment (201 nM \(O_2\)). In contrast, \(nxrB\) transcripts, encoding the nitrite oxidoreductase of aerobic nitrite oxidation, were relatively consistent between low-oxygen control and \(O_2\) addition reactors, and even declined in relative abundance in some treatments, although these trends should be confirmed with replicate treatments.

Compared to marker genes for denitrification (main text), transcripts of aerobic nitrification were affiliated with a relatively narrow range of taxa. Notably, \(amoC\) transcripts were dominated by sequences most closely related to homologs from Thaumarchaea and Crenarchaea. Bacterial \(amoC\) transcripts were either not detected or constituted a very minor (<1%) fraction of the total \(amoC\) pool, consistent with prior studies of *in situ* transcriptional activity in the oxycline above the Chilean OMZ (1). The overwhelming majority of nitrite oxidoreductase (\(nxrB\)) sequences were most closely related to \(nxrB\) of *Ca. Nitrospira defluvii* (Figure 5), a nitrite oxidizing isolate from activated sludge (2). Following our analysis, the genome of the marine nitrite oxidizer *Nitrospina gracilis* was published. *Nitrospina* is closely related evolutionarily to *Ca. Nitrospira* (3), and
the *nXR*B sequences in our data therefore could be re-classified as *Nitrospina*-like upon further analysis. *Nitrospina* and *Ca. Nitrospira* genomes both contain adaptations to low oxygen conditions (3, 4). Indeed, *Nitrospina* has been detected in the Namibian OMZ where nitrite oxidation was measurable at O₂ concentrations below 1 µM (5). This result is consistent with our data showing relatively constant *nXR*B levels over a range of low oxygen concentrations and a minor peak in relative transcript abundance at low (~200 nM) and intermediate (~500 nM) O₂ levels (Figure 4). These trends suggest the potential for nitrite oxidation over a range of O₂ conditions typical of the ETSP OMZ and oxycline.

References


Figure S1: Taxonomic representation and relative abundance of reads matching key nitrogen genes at different oxygen levels in bioreactor experiment 1 (top) and 2 (bottom).
Transcript abundance is calculated as read count per gene per kilobase of gene length, and then standardized as a percentage of total protein-coding reads per dataset. Taxonomic identifications are based on annotations of NCBI reference sequences identified as top matches (above bit score 50) in BLASTX searches. Note: y-axis scales differ. Abbreviations for oxygen treatments and genes as in Figure 4 (main text). IS denotes “in situ”, where in situ corresponds to samples collected from the water column source water prior to the start of each experiment. IS samples were published earlier (Stewart, F. J., T. Dalsgaard, C. R. Young, B. Thamdrup, N. P. Revsbech, O. Ulloa, D. E. Canfield and E. F. Delong, PLoS ONE 7(5): e37118, 2012 doi:10.1371/journal.pone.0037118 (2012) and pyrosequencing data have been deposited in the NCBI Sequence Read Archive under SRA049608.1.
Table S1. Sequencing read statistics

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<th>non-rRNA reads$^2$</th>
<th>nr reads$^3$</th>
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</table>

$^1$ reads matching (bit score > 50) SSU or LSU rRNA sequences via BLASTN

$^2$ non-rRNA reads; duplicate reads (reads sharing 100% nucleotide identity and length)

$^3$ excluded

$^4$ reads matching (bit score > 50) protein-coding genes in the NCBI-nr database (as of April 2012), modified to contain the Ca. Scalindua profunda genome (Taxon Object IDs 2017108002 and 2022004002 at JGI)

$^4$ sequenced on a half-plate (454 GS FLX); all other samples sequenced on a full plate run