RESEARCH ARTICLE

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,[a,b] Frank J. Stewart,[c] Bo Thamdrup,[d] Loreto De Brabandere,[d] Niels Peter Revsbech,[e] Osvaldo Ulloa,[f] Don E. Canfield,[d] Edward F. DeLong[g,h]

Department of Bioscience, Aarhus University, Silkeborg, Denmark[a]; Arctic Research Centre, Aarhus University, Aarhus, Denmark[b]; School of Biology, Georgia Institute of Technology, Atlanta, Georgia, USA[c]; Department of Biology and Nordic Center for Earth Evolution (NordCEE), University of Southern Denmark, Odense, Denmark[d]; Department of Bioscience, Aarhus University, Aarhus, Denmark[e]; Departamento de Oceanografía & Instituto Mileno de Oceanografía, Universidad de Concepción, Concepción, Chile[f]; Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA[g]; Department of Oceanography, University of Hawai'i, Mānoa, Hawai'i, USA[h]

ABSTRACT **A major percentage (20 to 40%) of global marine fixed-nitrogen loss occurs in oxygen minimum zones (OMZs). Concentrations of O_2 and the sensitivity of the anaerobic N_2-producing processes of anammox and denitrification determine where this loss occurs. We studied experimentally how O_2 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 N_2 and N_2O production by denitrification was achieved at 205 and 297 nM O_2, respectively, whereas anammox was 50% inhibited at 886 nM O_2. 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 O_2. This O_2 concentration did not suppress the transcription of other dissimilatory nitrogen cycle genes, including nitrate reductase (*narG*), hydrazine oxidoreductase (*hzo*), and nitrite 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 O_2 inhibition kinetics but strongly stimulated N_2O production by denitrification. These results identify new O_2 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 O_2 concentrations in oceanic oxygen minimum zones (OMZ) is a major sink in oceanic N budgets, yet the sensitivity and dynamics of these processes with respect to O_2 are poorly known. The present study elucidated how nanomolar O_2 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 ^{15}N techniques and metatranscriptomics. Rates of both denitrification and anammox responded rapidly and reversibly to changes in O_2, but denitrification was more O_2 sensitive than anammox. The transcription of key nitrogen cycle genes did not respond as clearly to O_2, although expression of some of these genes decreased. Quantifying O_2 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 Dalsgaard, tda@dmu.dk.

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Oxygen (O_2) 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 O_2 exceeds the rate of supply, causing low-O_2 environments to develop. This is the case in oxygen minimum zones (OMZ) (2–6) and sediments (7), which may be anoxic or at least have O_2 concentrations below the detection limits of the methods used for analysis of O_2. In these environments, a suite of anaerobic processes may occur that utilize nitrate (NO_3^-), nitrite (NO_2^-), sulfate (SO_4^{2-}), and metal oxides as terminal electron acceptors (8). In OMZs, NO_3^- and NO_2^- 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 NO_3^-- and NO_2^--reducing chemoautotrophic 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 N_2 occurs through microbial denitrification and anammox, but the relative importance of these sinks is debated (13–15), and the final conversion to N_2 appears to proceed in close interaction with other nitrogen, carbon, and sulfur transformations, which together drive dissimilatory reduction of NO_3^- to NO_2^- and of NO_2^- to ammonium (NH_4^+), as well as aerobic oxidation of NH_4^+ and NO_2^- (e.g., see references 15 and 16).

The distribution of 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 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, O_2 is present at low concentrations, potentially allowing interactions between aerobic and anaerobic processes (e.g., see reference 20). N_2 production is particularly intense in these transition zones (14, 16), which emphasizes the need for quantification of the O_2 sensitivity and dynamic response of the individual processes. Existing estimates of the 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 μM 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 μM 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 O_2, but whether this is more general remains to be experimentally verified, and denitrification is often assumed to be active at micromolar O_2 levels (e.g., see references 25 and 26). The assumed 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 N_2 production is estimated, and this volume may vary greatly depending on which threshold is chosen; assuming inhibition at nanomolar O_2 concentrations would result in a significantly smaller zone of N_2 production than if inhibition is assumed to occur up to 20 μM O_2 (26).

Rates of nitrogen transformation in OMZs are determined experimentally in batch incubations with ^{15}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 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 O_2 sensitivity of individual clades. Gene expression over vertical 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 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 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-O_2 levels, although O_2 contamination during experimentation is almost inevitable (31). In the present study, we performed O_2 manipulation experiments in bioreactors. Rates of anammox and denitrification were quantified over variable oxygen treatments, and the expression of key 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 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 O_2 and H_2S. 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 O_2 contamination into the bioreactors. The O_2 concentration in the water leaving the hose of the PPS was as low as 20 nM (10), but O_2 levels were always higher when measured inside the reactors placed in the water bath in the lab (designated "initial" in Table 1). 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 O_2 concentrations to between undetectable and ca. 20 nM. The average and maximum O_2 concentrations during the first and second halves of the experiments are listed in Table 1. The discrete additions of O_2 during the experi-

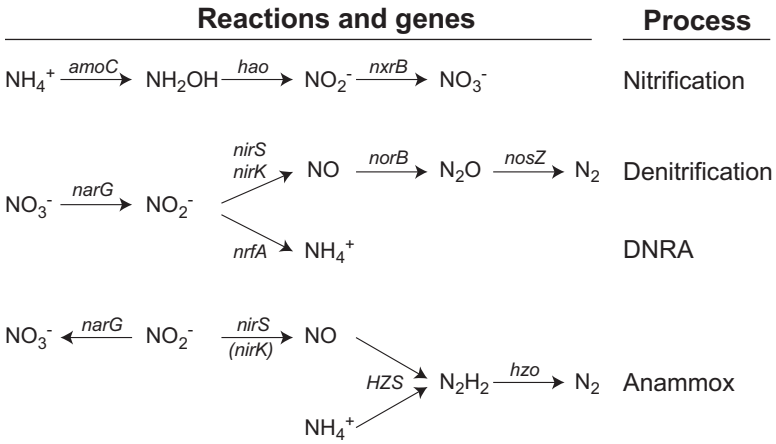

FIG 1 Overview of the genes and processes discussed in the present study based on the work of van de Vossenberg et al. (36) and Zumft (70). Genes: *amoC*, ammonia monooxygenase (ammonia oxidation); *hao*, hydroxylamine oxidoreductase (ammonia oxidation); *nxrB*, nitrite oxidoreductase (nitrite oxidation); *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).

ments resulted in fluctuating O_2 concentrations, with the worst case shown in Fig. 2B (Table 1). Leakage into the reactors increased O_2 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 O_2 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 O_2 from rubber

TABLE 1 Overview of experiments

Experiment	O_2 treatment[a]	Reactor	H_2S (μM)[b]	O_2 concn (nM)[c]							Gene expression analyzed
					First half			Second half			
				Initial[d]	Avg	SE	Max[e]	Avg	SE	Max[e]	
1A	HL	1		145	2,737.8	795.6	3,671.8	13.1	5.4	21.5	
1B	LH	2		94	39.9	6.8	56.6	200.3	47.1	275.3	
1B	LH	3		125	91.9	9.0	106.5	201.4	54.3	288.9	x
1C	LH	4		377	88.6	7.6	105.6	558.3	146.1	819.4	x
1C	LH	5		45	28.3	2.2	32.5	486.7	71.6	607.8	
1 D	LH	6		78	51.0	2.3	56.8	1,766.2	39.7	1,835.4	x
1E	L	7		54	21.2	11.3	56.9	34.2	2.9	39.4	x
1E	L	8		222	41.8	6.9	57.0	72.5	76.7	205.4	x
2A	HL	1		25	1,830.1	148.0	2,105.5	7.1	2.8	16.4	x
2A	HL	6		62	1,781.9	182.3	2,183.7	2.6	4.1	39.4	
2B	H	4		161	1,680.4	283.0	2,176.4	1,630.1	179.0	1,935.2	
2B	H	8		28	1,806.7	198.7	2,138.3	1,723.5	236.9	2,106.3	x
2C	LH	3		1,670	32.3	5.7	43.9	1,537.2	416.9	2,233.4	x
2C	LH	7		19	16.8	3.4	26.9	1,756.2	149.8	2,056.2	
2D	L	2		8	19.4	5.4	29.6	10.5	6.1	57.1	x
2D	L	5		41	11.8	3.1	17.2	7.1	1.9	11.9	x
3A	LH	5	1.1	57	15.0	11.9	99.8	393.7	128.4	706.3	
3A	LH	6	1.0	44	7.7	5.8	21.7	391.2	50.9	479.2	
3B	LH	3	1.2	38	37.3	9.5	49.8	1,789.9	389.2	2,655.2	
3C	L	7	1.3	45	13.4	13.2	43.1	7.8	7.8	34.9	
3C	L	8	1.4	36	4.1	4.1	20.5	3.5	1.8	8.5	
3D	L	1	0.0	68	17.8	3.3	26.6	9.0	3.5	19.8	
3D	L	2	0.0	34	14.8	2.7	19.1	7.0	2.5	12.2	

[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 H_2S (sum of H_2S, HS^-, and S^{2-}) 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 O_2 concentrations after the initial helium gassing.

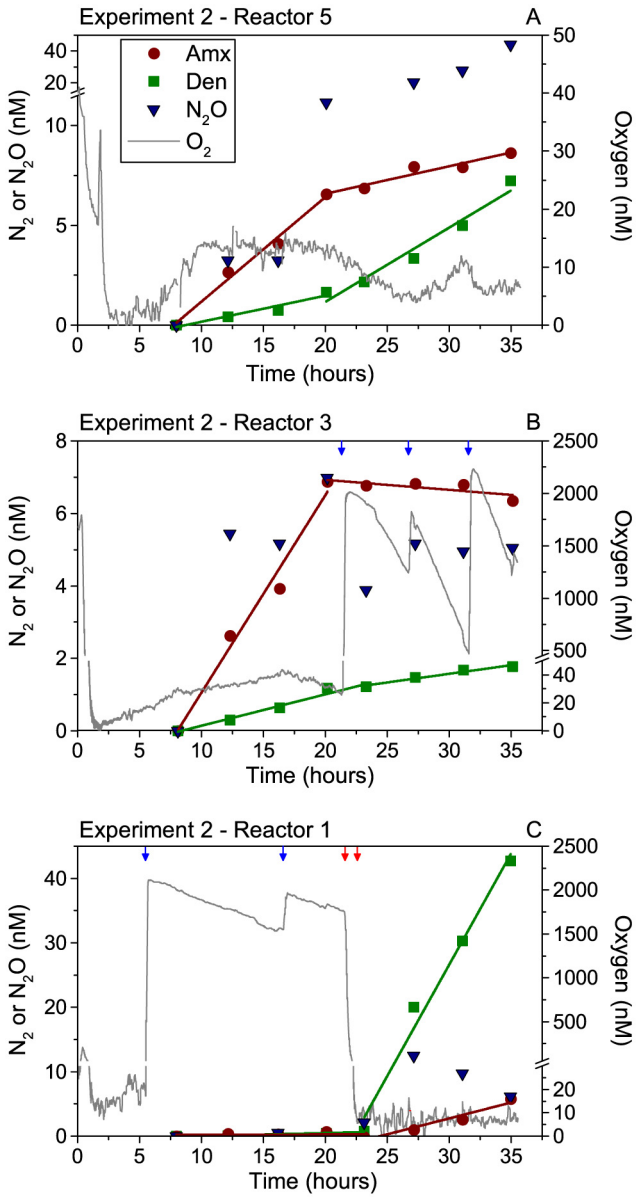

FIG 2 N_2 produced by anammox and denitrification, N_2O produced by denitrification, and O_2 concentrations in reactor experiments at station 5. $^{15}NO_2^-$ was added 20 to 30 min before the first sampling for N_2 and N_2O. Air-saturated water was injected at 21.6, 26.8, and 31.7 h (B) and at 5.7 and 16.7 h (C) (blue arrows). He sparging took place between 21.7 and 22.6 h (C; red arrows). Regression lines indicate the linear changes in N_2 concentrations over the first and second halves of the incubation in the no-oxygen amendment incubation (A) and before and after the increase (B) and decrease (C) in O_2 concentration.

septa demonstrated in glass vials (31). This leakage was not constant, and it was thus not possible to estimate the O_2 respiration in the water accurately.

Production of N_2 and N_2O. In all control experiments with low O_2 throughout, the rate of denitrification increased from the first to the second half of the incubation, whereas the anammox rate decreased (e.g., slope of regression lines in Fig. 2A). The changes were gradual but could be approximated by two linear regressions. In all cases when O_2 concentrations were raised, there was an immediate decrease in the production rates of N_2 by both processes (e.g., Fig. 2B). Conversely, process rates increased when O_2 concentrations were lowered to levels similar to those in the control incubations (Fig. 2C). This change was evident immediately after the O_2 level reached the low nM range, but the processes could have accelerated even earlier, because the 1-h sparging performed to remove O_2 would also have removed any ^{15}N-labeled N_2 formed during this period. Denitrification produced N_2O at rates similar to the N_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_2O concentration, e.g., as seen in the experiment depicted in Fig. 2C. The effects of O_2 on production of N_2 and N_2O by denitrification were similar, with N_2O production accelerating through the incubation in the low-O_2 controls, being inhibited by amendment of O_2 (e.g., Fig. 2B), and increasing very rapidly after removal of O_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_2 h^{-1} and N_2 and $N_2 + N_2O$ production by denitrification varying from 0.16 to 0.34 and 0.96 to 1.38 nM N_2 h^{-1}, respectively (Table 2).

Inhibition kinetics. The rates of anammox and denitrification from the reactors in which the O_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_2 (Fig. 3). Assuming an exponential attenuation of rates (equation 5), the O_2 concentration resulting in a 50% inhibition of the process (C_{50}) was calculated for each of the four processes: N_2 production by anammox and N_2, N_2O, and $N_2 + N_2O$ production by denitrification (Table 3). Denitrification was much more sensitive than anammox to O_2, being 50% inhibited at 200 to 300 nM O_2, whereas anammox first reached 50% inhibition at approximately 900 nM. The presence of sulfide in experiment 3 apparently did not affect the O_2 inhibition kinetics, and data from all three experiments are included in the analysis (Fig. 3).

Effects of sulfide. Addition of 1 μM 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 presence and absence of sulfide were not statistically significant). Denitrification, on the other hand, was strongly stimulated by sulfide. In the absence of sulfide, N_2 and N_2O production increased ≤2-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_2 throughout or in the second half of the experiment (L and HL in Fig. 5), O_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_2 exposure on *nirS*,

TABLE 2 Rates of N_2 production[a]

Station	Depth (m)	Date	Expt	Rate (nM N_2 h^{-1})[b]						Relative anammox (%)[c]		Oxygen concn (nM)	
				D (N_2)		D (total)		A (N_2)					
				Avg	SE	Avg	SE	Avg	SE	N_2	Total	Avg	SE
3	75	12 Jan 2010	1	0.34	0.17	1.38	0.83	0.74	0.24	68	35	31.48	10.3
5	86	14 Jan 2010	2	0.16	0.03	1.16	0.20	0.59	0.07	78	34	15.63	3.72
3	82	16 Jan 2010	3	0.25	0.16	0.96	0.36	0.52	0.12	67	35	16.33	1.50

[a] Data are for the first half of the control incubations, where O_2 concentrations were kept as low as possible. All values are means of data from two reactors.
[b] D (N_2), N_2 production by denitrification; D (total), $N_2 + N_2O$ production by denitrification; A (N_2), anammox.
[c] Relative importance of anammox for N_2 production (N_2) and for $N_2 + N_2O$ production (total).

norB, and *nosZ* transcription was relatively consistent in both low (~200 nM) and high (~1,700 nM) O_2 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 O_2 addition, O_2 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 O_2 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 O_2-amended microcosms. (Details on gene transcription in the source water microbial community and on the response of other genes to low-O_2 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

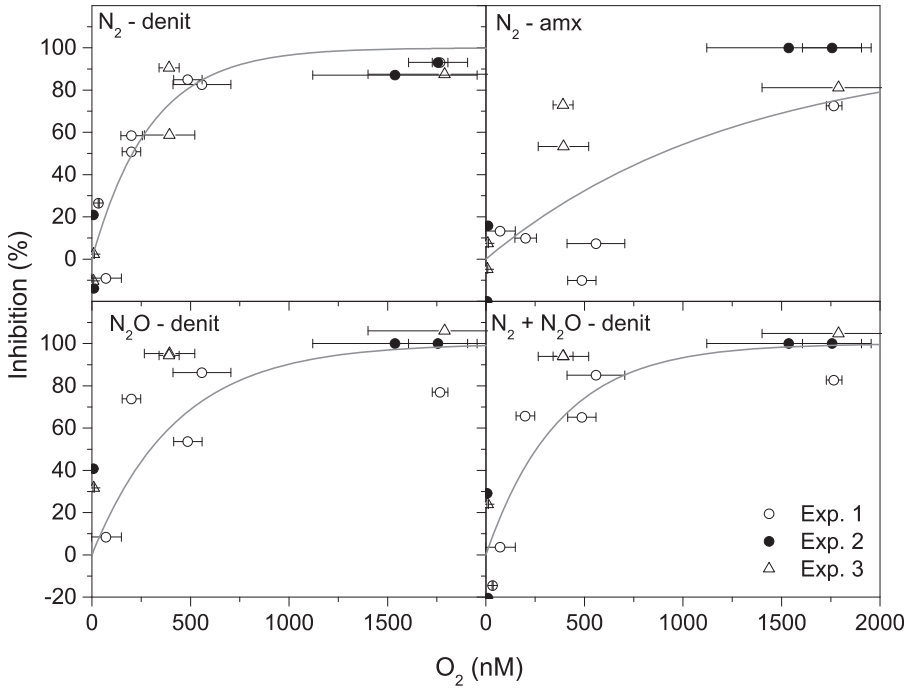

FIG 3 Inhibition of denitrification (N_2, N_2O, and $N_2 + N_2O$ production) and anammox (N_2 production) by O_2 in experiments where O_2 concentration was increased after ca. 20 h. Inhibition was estimated by comparing the rates from before and after O_2 amendment in each individual bioreactor. Error bars show the standard deviations of the average O_2 concentrations for the period after O_2 amendment until the end of the experiment. An exponential-type inhibition kinetics was fitted to the data, as indicated by the lines. See Table 3 for the regression details and results.

TABLE 3 Parameters for the fit of an exponential-type inhibition kinetics to the measured inhibition by O_2 of denitrification and anammox[a]

Process	C_{50} (nM)[b]	R^2	k
Denitrification			
N_2	205 (±34)	0.8818	0.00337
N_2O	297 (±139)	0.5557	0.00233
Total	255 (±88)	0.6968	0.00271
Anammox	886 (±418)	0.4542	0.00078

[a] Data were fitted to the following exponential function: inhibition (%) $= 1 - e^{-k \times C}$, 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] O_2 concentration at which 50% inhibition was obtained. Standard errors are in parentheses.

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 O_2, 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 most 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* Kuenenia stuttgartiensis." It is hypothesized that in anammox bacteria, *narG* acts in reverse as a nitrite oxidoreductase, oxidizing NO_2^- to NO_3^- 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 O_2 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 O_2 fluctuation over the concentration range and time periods examined here. This pattern is in contrast to the clear inhibition of anammox rates following O_2 addition (Fig. 2).

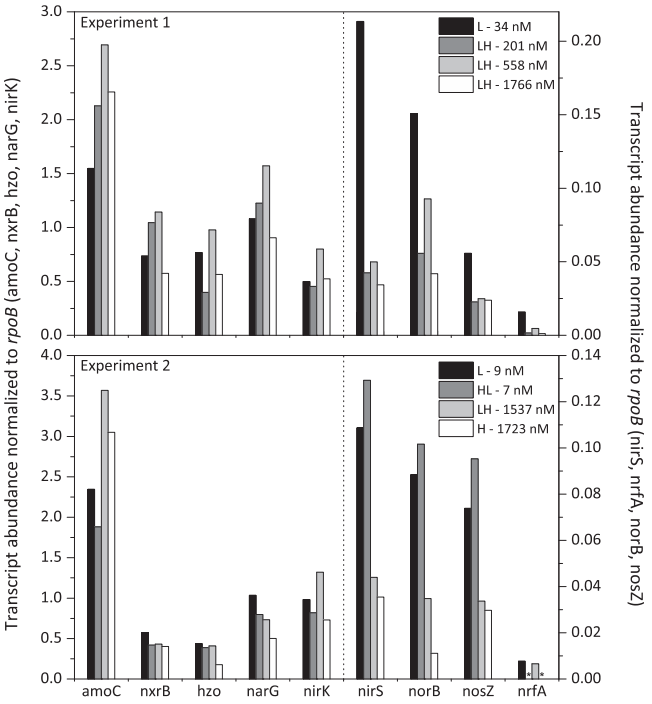

FIG 5 Relative abundance of reads matching key dissimilatory nitrogen metabolism genes in bioreactor experiments. Transcript abundance is calculated as read count per gene per kilobase of gene length and then normalized as a proportion of the abundance of transcripts matching *rpoB* (see the text). O_2 treatments were continuously low (L), continuously high (H), low during the first half and high during the last half of the incubation (LH), and high during the first half and low during the last half (LH); the average oxygen concentration for the last half of the incubation is indicated. Data for continuously low O_2 concentration is the average for two reactors. Bars to the left of the dotted line scale with the left *y* axis, and bars to the right scale with the right *y* axis. Genes are as in Fig. 1. *, below detection limit.

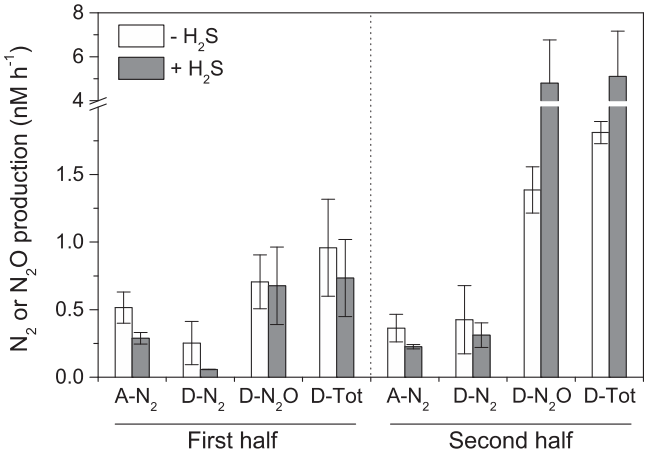

FIG 4 Rates of anammox and denitrification during the first and second half of experiment 3 (reactors 1, 2, 7, and 8) without O_2 amendment, with and without the amendment of ca. 1 μM H_2S (actual concentrations are shown in Table 1). A-N_2 is N_2 production by anammox. D-N_2, D-N_2O, and D-Tot represent N_2 and N_2O production by denitrification and total denitrification, respectively. Each column represents the average from two experiments with four or five time points for the rate estimates.

DISCUSSION

The effects of O_2 manipulations in coupled measurements of anammox and denitrification rates and community gene transcription were observed for the first time under controlled O_2 condi-

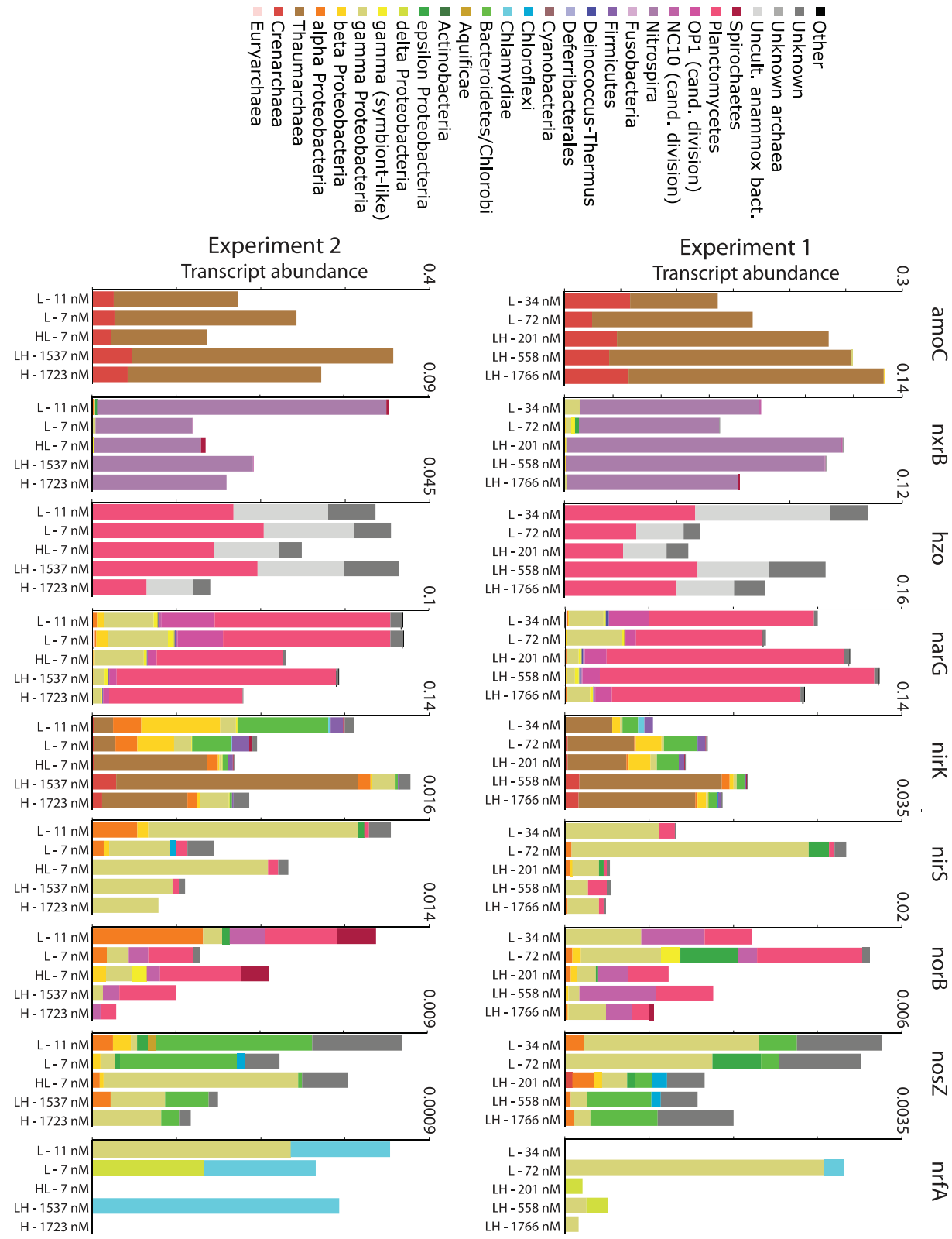

FIG 6 Taxonomic representation and relative abundance of reads matching key nitrogen genes at different oxygen levels in bioreactor experiment 1 (right) and 2 (left). Transcript abundance is calculated as read count per gene per kilobase of gene length and then standardized as a percentage of total protein-coding 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 O_2 concentration ranges applied in this study (5 to 2,000 nM), suggesting that the experimental O_2 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 O_2 amendment. Together, these data highlight the potential effects of O_2 fluctuation on dissimilatory nitrogen transformations in OMZ waters, as well as the extent to which community transcriptome patterns can complement and inform process rate measurements in experimental systems.

Rates of anammox and denitrification and effects of O_2. We quantified bulk anammox and denitrification activity by measuring gas (N_2 and N_2O) production and analyzed the expression of the genes coding for known enzymes catalyzing individual steps of these processes. In these experiments, $^{15}N_2$ 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 $^{15}N_2$ concentrations, provide a measure of the *in situ* activity, under the assumption that the O_2 levels here did not inhibit the processes significantly (see below). Anammox and denitrification activity started as soon as the helium sparging stopped and O_2 had been removed (Fig. 2C), with linear increases in $^{15}N_2$ concentrations over time. Addition of O_2 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 O_2 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 O_2 concentration show that the organisms involved in nitrogen transformations are well adapted to an environment where O_2 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 N_2 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, O_2 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 O_2 (31). If the higher sensitivity of denitrification than anammox to O_2 (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 O_2 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 O_2 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, O_2 inhibition kinetics were investigated by comparing the rates from O_2-amended reactors to the rates that were expected in the absence of O_2 manipulation (see equation 3 below). Another consequence of enclosing the water in a bottle was that in some of the experiments, N_2O 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 O_2 inhibition kinetics may be applied to *in situ* conditions.

The O_2-dependent inhibition of N_2 production by anammox and of N_2 and N_2O by denitrification was concentration dependent, with anammox showing a more variable response to O_2 amendments, while denitrification was more sensitive to O_2 (Fig. 3). Consequently, the C_{50} for anammox (886 nM O_2) is less well supported than that of denitrification (255 nM O_2 for total denitrification) (Table 3). While anammox was less sensitive to O_2 amendments than denitrification in the present study, the data also indicate that anammox was much more sensitive to O_2 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 O_2 and was fully inhibited by 13 μM O_2 (21). Kalvelage et al. (22) observed a tendency for anammox to become more sensitive to O_2 at stations seaward of the continental shelf, where one station showed no anammox above 2.8 μM O_2. 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 O_2 and

was completely inhibited at 8 μM O_2. The two latter observations are very much in line with our results from the two off-shelf stations off Iquique, Chile, where anammox was recorded at up to 1.7 μM 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 Exetainer 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 μM 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 μM 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* denitrification genes already occurred at the lowest O_2 amendment (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 μM (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 transcriptome data generated here show no clear suppression of bulk nitrate reductase gene (*narG*) transcription within the range of O_2 concentrations applied ($<$2 μM) (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 anammox 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 μM was found at one station in the Peruvian OMZ, but reductions in nitrate reduction rates of up to 50% when O_2 reached 4 μM 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 anammox 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 anammox-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 anammox activity (Fig. 3). It is possible that variations in anammox 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 anammox 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 anammox 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 assemblies 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^-, NO_3^- reduction or denitrification is responsible for removal of H_2S. In a situation with H_2S accumulation, mainly resulting from H_2S release from the sediment, H_2S was show 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_2S was the direct electron donor for NO_3^- reduction or denitrification in these experiments. However, in the H_2S-amended reactors, denitrification to N_2O was strongly stimulated in the second half of the experiment (Fig. 4), supposedly as a result of H_2S being a quantitatively important electron donor. The effect of O_2 on denitrification in the presence of H_2S was very similar to the effects seen without added H_2S (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_2S may interfere with the nitrogen cycle by inhibiting anammox (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_2S on anammox (Fig. 4). Also, the reduction of N_2O in the denitrification pathway may be inhibited by H_2S (57), but such inhibition was relatively minor in our experiments. During the first half of the experiment, N_2O accounted for 74% and 92% of total gas production in the absence and presence of H_2S, respectively. These values were 77% and 94% during the second half. This low degree of inhibition is probably due to the low H_2S concentrations in the experiment (ca. 1 μM). In the anoxic water column of the Baltic Sea, the ratio of N_2O to total gas production was proportional to the H_2S concentration, and first at a concentration of 7 μM H_2S, N_2O accounted for half of the total gas production from denitrification (58). In pelagic OMZs, H_2S 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_2O 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 [σ_θ], 26.23 kg m^{-3}) and 82 m (σ_θ, 26.22 kg m^{-3}) for experiments 1 and 3, respectively, and at station 5, water was collected at 86 m (σ_θ, 26.21 kg m^{-3}) for experiment 2. The oxic-anoxic (anoxic = 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 anammox 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 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^+. All reactors were then amended with $^{15}NO_2^-$ to a target concentration of 10 μM. *In situ* NO_2^- concentrations were in the range of 2.2 to 6.7 μM, and $^{15}NO_2^-$ constituted 59 to 86% of the total NO_2^- pool, as determined from concentrations before and after amendment.

All three experiments involved the manipulation of reactor O_2 concentrations. Each experiment included two control reactors that were filled, gassed, incubated, and sampled in parallel to the manipulated reactors. In these controls, the O_2 concentrations were kept as low as possible (designated "L" in Table 1; for an example, see Fig. 2A) in order to represent *in situ* conditions. In some reactors, O_2 was kept low through the first half of the experiment and then raised to a target concentration of 200 nM, 500 nM, or 2 μM (low to high, designated "LH" in Table 1; for an example, see Fig. 2B), while others followed the opposite scheme, with high O_2 from

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, O_2 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 O_2). Oxygen was removed by purging with helium as described above. In the experiments where O_2 concentrations were raised, air-saturated water was injected at a sufficient frequency to counteract O_2 consumption and maintain the target O_2 concentration range. In the experiments starting with high O_2 concentrations, air-saturated water was injected right after the initial gassing with helium. The increase in O_2 concentration after injection of air-saturated seawater was instantaneous, whereas removal of O_2 by helium sparging took about 1 h. In experiment 3, a solution of NaS_2 (5 mM) was added to some of the reactors to a concentration of ca. 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 N_2 and N_2O gas isotopic composition and concentration and for NO_2^-, NO_3^-, and NH_4^+ 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) $ZnCl_2$. Samples for NO_2^- (experiments 1 and 2) and NH_4^+ were analyzed immediately, whereas samples for NO_2^- in the presence of sulfide (experiment 3) and all NO_3^- 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 NO_3^- plus NO_2^- were measured as NO after reduction in hot vanadium chloride (59) on a Thermo Environmental Instruments 42c NO_x analyzer. Nitrite in the absence of sulfide was measured using a standard colorimetric technique (60), and NO_2^- in the presence of sulfide was analyzed as NO after reduction in cold vanadium chloride (59). Ammonium was quantified fluorometrically by the orthophthaldialdehyde method (61) on a Turner Designs Trilogy fluorometer, and sulfide concentration was quantified colorimetrically according to the method of Cline (62). Nitrogen isotopes in N_2 and N_2O were analyzed on a Thermo Delta V Plus isotope ratio mass spectrometer as described previously (14).

Calculations. Rates of anammox and denitrification in the $^{15}NO_2^-$ amended experiments were calculated as described earlier (63). Briefly, knowing the mole fraction of ^{15}N in the NO_2^- pool (F_N) and the production rates of $^{29}N_2$ (P_{29}) and $^{30}N_2$ (P_{30}), N_2 production by anammox and denitrification were calculated as follows:

$$\text{denitrification} = P_{30} \times {F_N}^{-2} \quad (1)$$

$$\text{anammox} = {F_N}^{-1} \times [P_{29} + 2 \times (1 - {F_N}^{-1}) \times P_{30}] \quad (2)$$

F_N was estimated from the concentrations of NO_2^- before and after amendment of $^{15}NO_2^-$, and P_{29} and P_{30} were calculated as the slopes of the linear regression of $^{29}N_2$ and $^{30}N_2$ concentrations as a function of time. Production of N_2O by denitrification was estimated using equation 1 replacing P_{30} with the production rate of $^{46}N_2O$, which was calculated as the slope of the linear regression of $^{46}N_2O$ versus time. Manipulation of O_2 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 N_2O 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 O_2 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 O_2 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 O_2 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 ($R2_{expected}$) was calculated as

$$R2_{expected} = R1 \times (Rc2/Rc1) \quad (3)$$

where $R1$ is the rate measured during the first half of the incubation and $Rc1$ and $Rc2$ 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 O_2 concentration halfway through the incubation was calculated as

$$\text{Inhibition} = -(R2 - R2_{expected})/R2_{expected} \times 100 \quad (4)$$

where $R2$ is the rate measured during the second half of the incubation. The calculated inhibition was plotted individually for each process (N_2, N_2O, and $N_2 + N_2O$ production by denitrification and N_2 production by anammox) as a function of the average O_2 concentration during the second half of the incubation, and data were fitted to the exponential function

$$\text{inhibition}(\%) = 1 - e^{-k \times C} \quad (5)$$

using the Levenberg-Marquardt algorithm (Origin 9.0; OriginLab, MA), where C is the O_2 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 O_2 concentration resulting in a 50% inhibition of the rates, C_{50}, 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 μm GF/A; Whatman) and then a primary collection filter (0.22 μm; 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-O_2] 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 O_2 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), *nrfA* (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.org/lookup/suppl/doi: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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