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Intensive nitrogen loss over the Omani Shelf due to anammox coupled with dissimilatory nitrite reduction to ammonium

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A combination of stable isotopes (^{15}N) and molecular ecological approaches was used to investigate the vertical distribution and mechanisms of biological N_2 production along a transect from the Omani coast to the central–northeastern (NE) Arabian Sea. The Arabian Sea harbors the thickest oxygen minimum zone (OMZ) in the world's oceans, and is considered to be a major site of oceanic nitrogen (N) loss. Short ($<48\,h$) anoxic incubations with ^{15}N-labeled substrates and functional gene expression analyses showed that the anammox process was highly active, whereas denitrification was hardly detectable in the OMZ over the Omani shelf at least at the time of our sampling. Anammox was coupled with dissimilatory nitrite reduction to ammonium (DNRA), resulting in the production of double-^{15}N-labeled N_2 from $^{15}NO_2^-$, a signal often taken as the lone evidence for denitrification in the past. Although the central–NE Arabian Sea has conventionally been regarded as the primary N-loss region, low potential N-loss rates at sporadic depths were detected at best. N-loss activities in this region likely experience high spatiotemporal variabilities as linked to the availability of organic matter. Our finding of greater N-loss associated with the more productive Omani upwelling region is consistent with results from other major OMZs. The close reliance of anammox on DNRA also highlights the need to take into account the effects of coupling N-transformations on oceanic N-loss and subsequent N-balance estimates.

Introduction

Nitrate is the highest energy-conserving oxidant for respiration after oxygen. In oxygen-deficient seawater, nitrate is generally believed to be reduced in a stepwise manner to N_2O or N_2 ($2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2$) for the respiratory oxidation of organic matter. Known as heterotrophic denitrification, this process has been considered an important factor in the global N-loss from the ocean (Naqvi, 1987; Gruber and Sarmiento, 1997; Codispoti et al.,

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2001), affecting indirectly biological productivity and thus sequestration of atmospheric CO_2 (Capone, 2000; Gruber, 2004). The major oxygen minimum zones (OMZs) in the world, found in the Arabian Sea and the eastern tropical North and South Pacific, are responsible for 20–40% of global N-loss from the oceans, whereas 10–20% is thought to occur in the Arabian Sea (Naqvi, 1987; Codispoti et al., 2001; Gruber, 2004; Devol et al., 2006). Recent findings from incubation experiments with ^{15}N-labeled compounds indicated substantial N-loss due to anammox ($NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$) in the OMZs off Peru, Chile and Namibia, whereas denitrification was generally below detection (Kuypers et al., 2005; Thamdrup et al., 2006; Hamersley et al., 2007). These studies strongly question the traditional view that heterotrophic denitrification is the only significant driver of oceanic N-loss.

In the Arabian Sea OMZ, however, N-loss rates have rarely been measured directly. Despite the

detection of signature lipid biomarker (Jaeschke et al., 2007) and gene sequences (Woebken et al., 2008) of anammox bacteria in the Arabian Sea OMZ, anammox activities had only been inferred (Devol et al., 2006) until recently. Nicholls et al. (2007) carried out incubation experiments using ^{15}N-labeled substrates and reported indications of very low anammox rates at three depths in the central–northeastern (NE) Arabian Sea. A few years later, anammox rates were measured in the same region (Ward et al., 2009; Bulow et al., 2010). Interestingly, in the latter study, denitrification was reported to be the dominant and often the sole active N-loss process in most samples investigated. Nonetheless, as pointed out a few decades ago (Richards, 1965), if denitrification is the only active N-loss pathway, as a heterotrophic and presumably the major remineralization process in the OMZs, denitrification should liberate large amounts of ammonium (NH_4^+) from the oxidation of organic matter. On the contrary, such expected NH_4^+ accumulations have not been observed in the OMZs (Cline and Richards, 1972; Codispoti and Christensen, 1985; Naqvi, 1987). Thus, the occurrence of anammox, which removes NH_4^+ while producing N_2, is highly probable.

Anammox has been reported to co-occur with multiple N-cycling processes, including nitrification, nitrate reduction and dissimilatory nitrate/nitrite reduction to ammonium (DNRA), in OMZs off the coasts of Peru and Namibia where denitrification was not detectable (Kartal et al., 2007a; Lam et al., 2009). Coupling of these reactions with anammox may in fact lead to the production of double-^{15}N-labeled N_2 ($^{15}N^{15}N$) and complicate data interpretation of isotope-pairing experiments. For instance, coupling between ammonia oxidation to nitrite and anammox can generate $^{15}N^{15}N$ in $^{15}NH_4^+$ incubations in the presence of little ambient NO_2^- (Lam et al., 2007; Jensen et al., 2008). It is also theoretically possible to have $^{15}N^{15}N$ produced from the interactions between anammox bacteria and organisms capable of DNRA in $^{15}NO_2^-$ incubations, with results mimicking the signature of denitrification (Kartal et al., 2007a). In this case, $^{15}NO_2^-$ can be reduced via DNRA to $^{15}NH_4^+$, which is then combined with $^{15}NO_2^-$ at a 1:1 ratio through anammox to generate $^{15}N^{15}N$ (Supplementary Figure S1). In the Arabian Sea OMZ, the occurrence of DNRA has been suggested on the basis of an excess production of single-^{15}N-labeled N_2 ($^{14}N^{15}N$) that could not be explained by either denitrification or anammox (Nicholls et al., 2007). However, the actual occurrence of DNRA and the presence of DNRA-capable microorganisms in the Arabian Sea remain unexplored.

Unlike the eastern tropical South Pacific where there is perennial upwelling except for El Niño years, biogeochemical processes in the Arabian Sea are heavily influenced by the seasonal reversal of monsoonal winds (Wiggert et al., 2005). Surface production is enhanced by upwelling along the western boundary and to a lesser extent in the central region during the Southwest monsoon (June–August), and by deep convective mixing in the north during the Northeast monsoon (December–February; Marra and Barber, 2005). In the two intermonsoonal periods, conditions in the Arabian Sea approach oligotrophy. Because persistent oxygen-deficient conditions overlap with nitrite accumulations and large N-deficits primarily in the central–NE Arabian Sea, most though not necessarily exclusive N-loss activities have been postulated to occur in this part of the basin (Naqvi, 1991; Bange et al., 2005). In comparison, the mid-water column underlying the productive Omani upwelling zone rarely reaches complete anoxia, and so has not been considered important for N-loss (Naqvi, 1991; Bange et al., 2005). This apparent decoupling of N-loss and surface production in the Arabian Sea contrasts with other OMZs of eastern tropical Pacific and South Atlantic. However, no actual N-loss measurements have been made over the Omani shelf to date.

In this study, we examined the activities and mechanisms of N_2 production in the Arabian Sea OMZ, through a combination of various ^{15}N-stable-isotope-pairing experiments, expression analyses of biomarker functional genes and the enumeration of anammox bacterial cells. We conducted experiments for six depths through the vertical expanse of the OMZ (100–900 m) at seven stations, encompassing the Omani shelf and the central–NE Arabian Sea, to compare the relative importance of N-loss from these two regions.

Materials and methods

Water sampling and analyses of nutrients, particulate organic carbon and particulate nitrogen

Sampling took place in September/October 2007, just at the beginning of the autumn intermonsoon (Table 1). We followed the cruise track of the former US Joint Global Ocean Flux Study (Morrison et al., 1999; Figure 1). Water sampling was conducted with a conductivity–temperature–depth rosette equipped with 10 l bottles on board the *R/V Meteor*. The standard oxygen sensor of the conductivity–temperature–depth (Seabird) and an additionally mounted microsensor were used together to identify the OMZ. Besides, we deployed the highly sensitive STOX (Switchable Trace amount OXygen) sensor (Revsbech et al., 2009; detection limit: 90 nM during our deployments) at stations 956–958 in the central–NE Arabian Sea. Water samples were analyzed for NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} (detection limits 20, 30, 100 and 100 nM, respectively) at 10 to 25-m intervals for 12 stations (Figure 1). NH_4^+ and NO_2^- concentrations were measured immediately after sampling using fluorometric and spectrophotometric techniques, respectively (Grasshoff, 1983; Holmes et al., 1999). Samples for NO_3^- and PO_4^{3-} were stored frozen

Table 1 Summary of ^{15}N-incubation experiments. Numbers in parentheses in the last column refer to the final concentrations of the ^{15}N- and ^{14}N-species added into the incubations

Station	Sampling date	Bottom depth (m)	Sampling depth (m)	Substrate additions (μM)
944	21 September	653	120, 150, 200, 450, 500, 550	$^{15}NH_4^+$ (5)
			[a]	$^{15}NO_2^-$ (5)
			[a]	$^{15}NH_4^+ + ^{14}NO_2^-$ (5+5)
			[a]	$^{15}NO_2^- + ^{14}NH_4^+$ (5+5)
946	22 September	430	100, 125, 150, 200, 300, 400	$^{15}NH_4^+$ (5)
			[a]	$^{15}NO_2^-$ (5)
949	24 September	3985	150, 175, 375, 500, 675, 900	$^{15}NH_4^+$ (5)
			[a]	$^{15}NO_2^-$ (5)
			[a]	$^{15}NH_4^+ + ^{14}NO_2^-$ (5+5)
			[a]	$^{15}NO_2^- + ^{14}NH_4^+$ (5+5)
950	25 September	3916	150, 175, 300, 500, 700, 900	[b]
953	27 September	3549	125, 225, 350, 500, 700, 900	[b]
955	29 September	3254	115, 200, 300, 500, 700, 900	[b]
957	30 September	3242	110, 200, 300, 500, 700, 900	[b]

[a]Same depths as listed for the one above.
[b]Same ^{15}N treatments as listed for St 949.
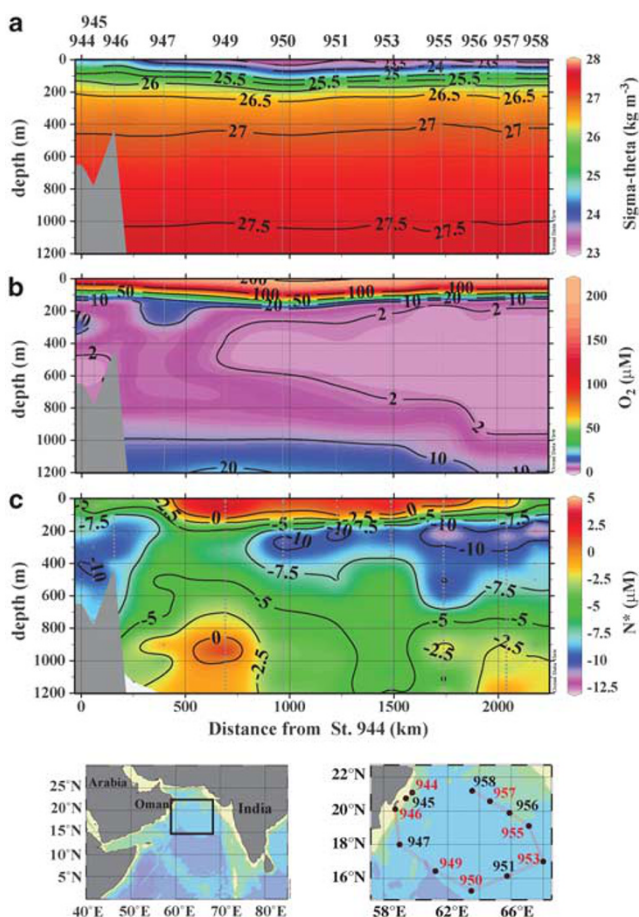
Figure 1 Distributions of (**a**) density, (**b**) oxygen and (**c**) N* along the cruise track from the Omani coast to the central–northeastern Arabian Sea. Gray-filled circles denote sampling depths. Maps show sampling area, sampling locations and station numbers along our cruise track. Station numbers in red indicate sampling stations where ^{15}N-incubation experiments were conducted.

and measured spectrophotometrically (Grasshoff *et al.*, 1999) with an autoanalyzer (TRAACS 800, Bran & Lubbe, Hamburg, Germany) in a shore-based laboratory. Particulate organic carbon and nitrogen were collected by filtering 3–30 l of seawater onto precombusted glass fiber filters. Particulate organic carbon and nitrogen were quantified on a flush combustion CNS Carlo Erba 1500 analyzer (Carlo Erba, Milan, Italy) after carbonate removal with 1 M HCl.

^{15}N-isotope pairing experiments
Time-series ^{15}N-incubation experiments were carried out to determine N-loss rates with various ^{15}N- and ^{14}N-amendments, following previously described protocols (Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2005). Different combinations of ^{15}N-labeled and unlabeled NH_4^+ and/or NO_2^- were added to helium-purged seawater samples, and dispensed into Exetainer vials (Labco, High Wycombe, Buckinghamshire, UK) for incubation (Table 1). Purging with helium removed O_2 to below the detection limit of an oxygen microsensor ($\leqslant 0.5 \mu$M; MPI, Bremen, Germany). Samples were incubated at *in situ* temperature and in the dark for about 0, 6, 12, 24 and 48 h. Isotopic compositions and concentrations of the N_2 and N_2O produced were analyzed on a gas chromatography-isotope ratio mass spectrometer (Fisons VG Optima, Fison, Manchester, UK). Afterwards, net DNRA rates were determined as the net accumulation of $^{15}NH_4^+$ from the same $^{15}NO_2^-$ incubations. The isotopic composition of NH_4^+ was analyzed after chemical conversion into N_2 with alkaline hypobromite (NaOBr; Warembourg, 1993). Further details are provided in Supplementary SI Text.

All rates were derived from linear regressions of ^{15}N-production (N_2, N_2O or NH_4^+) as a function of time, and only productions remaining linear without lag-phase and with slopes significantly greater than zero (one- or two-tailed *t*-tests, $P < 0.05$) were reported (unless otherwise stated; detection limits: 150–200 pM per day, 0.5 nM per day and 0.5 nM per day for N_2, N_2O and NH_4^+, respectively). Anammox and denitrification rates were calculated from the

regression slopes and the mole fractions of ^{15}N in substrate pools (Thamdrup and Dalsgaard, 2002; Thamdrup et al., 2006; Supplementary SI Text). In the case of DNRA–anammox coupling, total anammox rates were calculated in a similar manner as in nitrification–anammox coupling (Jensen et al., 2008; Supplementary SI Text).

Molecular ecological analyses

Anammox bacteria were enumerated via catalyzed reporter deposition–fluorescence in situ hybridization (CARD–FISH; Pernthaler et al., 2002) with 16S rRNA targeted probes BS820/BS820c (Hamersley et al., 2007; Supplementary SI Text). For simultaneous DNA and RNA extractions, seawater samples (10–15 l) were filtered through Sterivex filters (0.22 μm pore size, Millipore GmbH, Schwalbach, Germany) and stored at $-80\,^{\circ}C$ until extraction (Supplementary SI Text). The biomarker functional genes for anammox (Scalindua-type cd_1-nitrite reductase or nirS), denitrification (denitrifier-nirS) and DNRA (cytochrome c nitrite reductase, or nrfA) were quantified along with their expressions (transcription as mRNA) via real-time PCR, as previously described (Lam et al., 2009). Detailed protocols are provided in the Supplementary SI Text and Table S1. Clone libraries were also constructed for the expressed Scalindua-type and denitrifier-nirS, and the complementary DNA inserts were sequenced for phylogenetic analyses (Supplementary SI Text). Active gene transcription in unmanipulated seawater samples may provide independent support for an active process detected via manipulated incubation experiments, though the relationships between rates and gene expressions are not necessarily straightforward. This is because the different measurement types have vastly different detection limits, and that gene expression is also affected by physiological states, stresses and other (post)transcriptional factors. As a lot remains unexplored in the immense oceanic microbiome, we do not claim an exhaustive coverage of the functional gene targets by the selected primers.

Results and discussion

Physicochemical settings

Consistent with previous observations (Morrison et al., 1999), the water column at the time of our sampling became more strongly stratified from the western boundary towards the central basin (Figures 1a and b). Enhanced surface chlorophyll a, lower sea surface temperatures and shoaling of isopycnals westwards indicate residual upwelling near the Omani shelf (Figure 1a, Supplementary Figure S2). Oxygen concentrations declined sharply below ~ 25–50 m, and reached $<10\,\mu M$ at about ~ 85–100 m. This oxygen-deficient OMZ extended to ~ 1000 m depth (Figure 1b). Using the highly

sensitive STOX sensor at stations 956–958, apparent anoxic conditions (<90 nM) were found between ~ 110 and 800 m water depths (Supplementary Figure S3). Strong deficits of combined inorganic nitrogen relative to phosphate (PO_4^{3-}; Redfield et al., 1963), shown here as strongly negative N* (in $\mu M = [NO_3^- + NO_2^- + NH_4^+] - 16 \times [PO_4^{3-}] + 2.9\,\mu mol\,kg^{-1} \times density$ in $kg\,l^{-1}$; Gruber and Sarmiento, 1997), were the most pronounced in the upper part of the central–NE OMZ (Figure 1c), overlapping with the strong secondary nitrite maximum (Lam et al., 2011). Meanwhile, contrary to previous reports (Naqvi, 1991; Bange et al., 2005), N-deficits were also observed over the Omani shelf from 200 m water depth to the seafloor, coincident with apparently anoxic conditions (<1–2 μM O_2 measured by a microsensor; Figures 1 and 2b), indicating past and/or present substantial N-loss from these waters.

High N-loss via anammox over the Omani Shelf

Indeed, significant N-loss activity was detected at the shelf stations (944 and 946). In both incubations with $^{15}NH_4^+$ and $^{15}NO_2^-$, the production of single ^{15}N-labeled N_2 ($^{14}N^{15}N$) occurred right from the start and increased linearly with time over 48 h (for example, Figure 3). At the upper four sampled depths at station 946, and 120 m at station 944, all of the produced ^{15}N-labeled N_2 in the $^{15}NH_4^+$ incubations was recovered as $^{14}N^{15}N$, whereas no production of $^{15}N^{15}N$ was detected (Figures 2d and 3a, Supplementary Figure S4a). Such exclusive production of $^{14}N^{15}N$ without delay clearly indicated active occurrence of anammox. In parallel, there was comparable production of $^{14}N^{15}N$ in the $^{15}NO_2^-$ incubations, further verifying anammox activity (Figures 2d and 3b).

Similar to the findings in the Peruvian and Chilean OMZ (Thamdrup et al., 2006; Hamersley et al., 2007), anammox rates determined from both ^{15}N-incubations were the highest in the upper OMZ over the shelf (~ 2.8 nmol of $N_2\,l^{-1}$ per day at station 944; 21–39 nmol of $N_2\,l^{-1}$ per day at station 946), coincident with local maxima of NH_4^+ and NO_2^- (Figures 2c; Supplementary Table S2). Anammox rates dropped considerably to ~ 1 nmol of $N_2\,l^{-1}$ per day below 200 m at station 946, where NO_2^- returned to low levels. Anammox bacterial abundance, as measured via CARD–FISH, showed a similar vertical distribution to anammox rates (Figure 2e,d) (Spearman $R = 0.766$, $P < 0.05$ and $R = 0.755$, $P < 0.05$ for rates from $^{15}NH_4^+$ and $^{15}NO_2^-$ incubations, respectively). Expression of the anammox functional gene biomarker, Scalindua-nirS, which encodes for the anammox (Scalindua)-specific cytochrome cd_1-containing nitrite reductase (Strous et al., 2006), was detectable at most depths examined, with the highest level found in the upper OMZ (Figure 2e, Supplementary Figure S5). Phylogenetic analyses revealed that the Arabian Sea expressed Scalindua-nirS sequences formed two tight clusters with some South China Sea sediment clones,
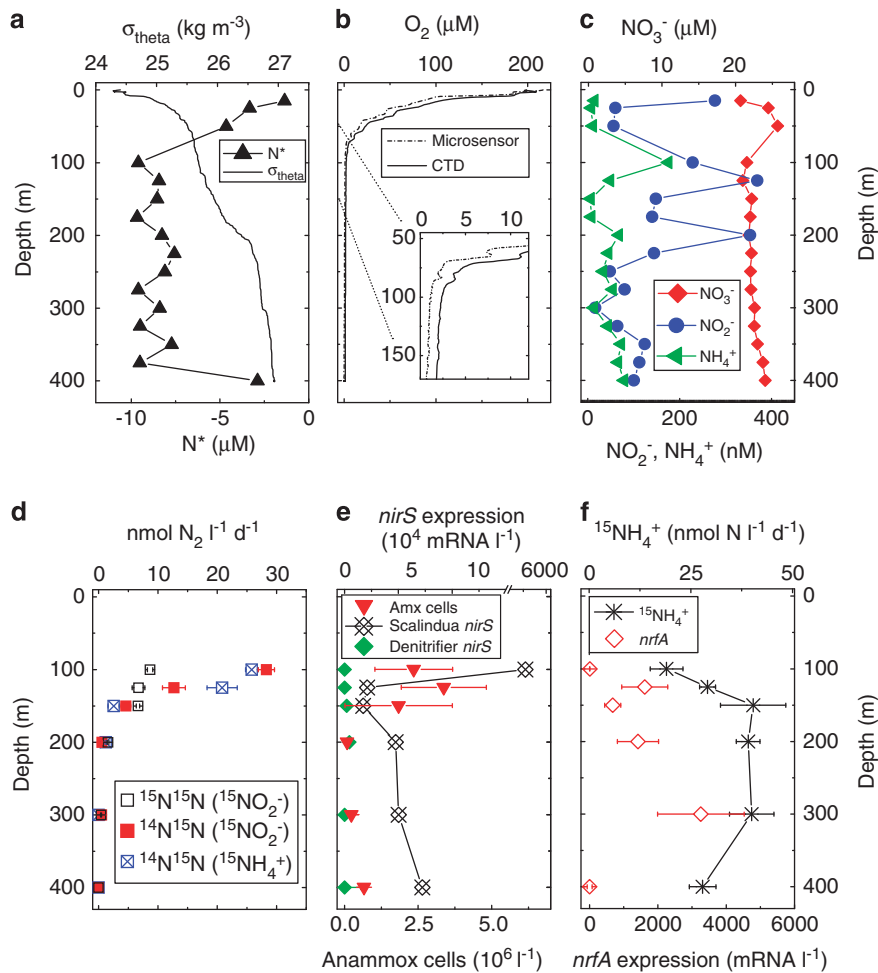
Figure 2 Vertical distribution of (**a**) potential density anomaly (σ_θ) and N*, (**b**) oxygen, (**c**) inorganic nitrogen, (**d**) $^{14}N^{15}N$ and $^{15}N^{15}N$ production rates (mean ± s.e.) in various ^{15}N-incubations, (**e**) anammox bacterial abundance (mean ± s.e.) measured by CARD–FISH and the expression (mean ± s.e.) of anammox (Scalindua-type) and denitrifier cd_1-containing nitrite reductase nirS genes, and (**f**) DNRA rates (mean ± s.e.) as net $^{15}NH_4^+$ production from $^{15}NO_2^-$ and expression of the corresponding biomarker functional gene, nrfA (mean ± s.e.)—all measured at station 946.

whereas they were clearly distinct from the Peruvian OMZ cluster (71–90% similarity) and the cultured Candidatus Scalindua sp. T23 (81–91%; Supplementary Figure S6). This pattern is consistent with the 16S rRNA-based phylogeny (Woebken et al., 2008). In fact, with support from internal transcribed spacer sequence analyses, Arabian Sea anammox bacteria seem to form a novel species Candidatus Scalindua arabica (Woebken et al., 2008) that cannot be targeted by the oligonucleotide Amx820 (Schmid et al., 2000), commonly used to quantify anammox bacteria in environmental studies (for example, Ward et al., 2009).

Interestingly, there was substantial production of $^{15}N^{15}N$ without lag in the $^{15}NO_2^-$ incubations for most depths at station 946, which is normally taken as an evidence for concurrent denitrification activity (Figures 2d and 3b). The $^{15}N^{15}N$ production rates decreased with depth, from 8.7 nmol of $N_2 l^{-1}$ per day at 100 m to 0.41 nmol of $N_2 l^{-1}$ per day at 300 m (Figure 2d). Nevertheless, if denitrification and

anammox co-occurred, anammox bacteria likely depended on denitrification for the released NH_4^+ from organic matter. Consequently, the proportional contribution of anammox to total N_2 production would be constrained by the stoichiometric ratios of organic matter remineralized during denitrification (Dalsgaard et al., 2003). Assuming a C:N:P ratio of 106:16:1 in the organic matter (Redfield et al., 1963), anammox should in theory account for 29% of the total N_2 production (Supplementary SI text), or ≤50% of total N_2 production in the extreme case when only proteins were remineralized (Van Mooy et al., 2002; Dalsgaard et al., 2003). Instead, anammox contributed to 41–77% of total N_2 production in our samples, suggesting that additional or alternative pathways were at play. The expression of the denitrifier-nirS, a biomarker functional gene encoding cd_1-containing nitrite reductase specific for denitrifiers, was not detectable at most depths at station 946 (Figure 2e). Neither detectable was the production of $^{15}N-N_2O$ from $^{15}NO_2^-$, further pointing
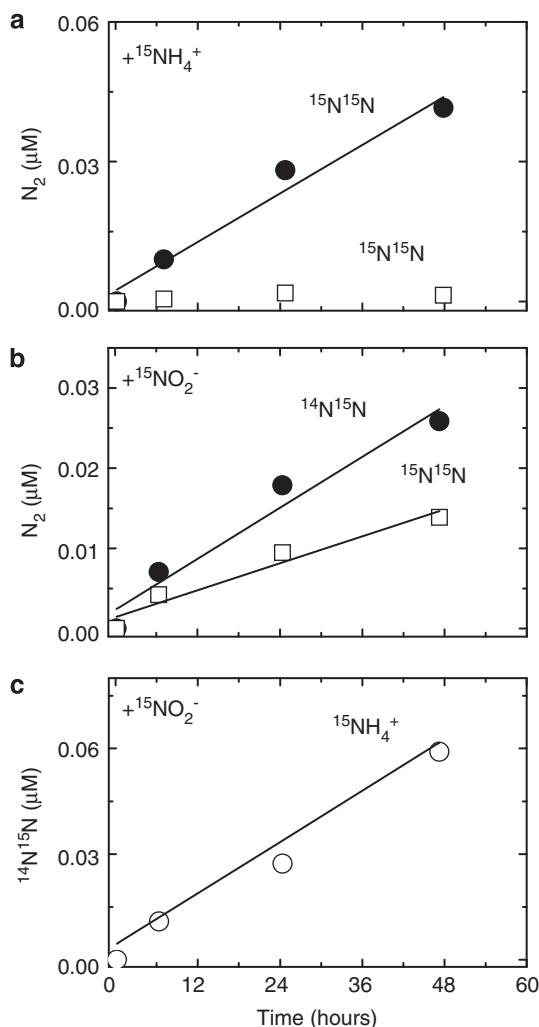
Figure 3 Examples of the linear production of ^{15}N-labeled N_2 during incubations (**a**) with $^{15}NH_4^+$ and (**b**) with $^{15}NO_2^-$. (**c**) DNRA measured as linear production of $^{15}NH_4^+$ in the same $^{15}NO_2^-$-incubations. Production rates of $^{14}N^{15}N$, $^{15}N^{15}N$ and $^{15}NH_4^+$ were calculated from the slopes in linear regression analyses. Data came from 125 m at station 946 over the Omani shelf.

to only low, if any, denitrification activity. Similarly, although $^{15}N^{15}N$ production was detected at 120 m at station 944, it only occurred after a 14-h lag (Supplementary Table S1), whereas denitrifier-*nirS* expression and $^{15}N\text{-}N_2O$ were not detected.

DNRA–anammox coupling
Substantial $^{15}NH_4^+$ production from the $^{15}NO_2^-$ pool indicates high net DNRA rates over the Omani shelf, with $\leqslant 40 \pm 5.5$ and 3.55 ± 0.40 nmol of N l^{-1} per day measured at station 946 (Figures 2d,f and 3c) and station 944 (at 120 m with ~ 5 h lag), respectively, where high anammox rates were also measured (Supplementary Table S2–3). These net DNRA rates were in the upper range of those reported for the Peruvian OMZ (Lam *et al.*, 2009). Further evidence for DNRA came from the significantly correlating

(Spearman $R = 0.538$, $P < 0.01$) expression of *nrfA*, the biomarker functional gene encoding the enzyme cytochrome *c* nitrite reductase that is essential in the DNRA reaction (Figure 2f, Supplementary Figure S5c, f). Together, the relatively high net DNRA rates, the consistently detectable active expression of *nrfA* and Scalindua-*nirS* versus the sporadically detected low denitrifier-*nirS* expression, strongly suggest that the $^{15}N^{15}N$ production resulted from the reduction of $^{15}NO_2^-$ to $^{15}NH_4^+$, followed by a one-to-one combination of the produced $^{15}NH_4^+$ and the added $^{15}NO_2^-$ through anammox (Supplementary Figure S1). Although heterotrophic denitrification is thermodynamically more favorable than DNRA, a recent study using pure cultures of denitrifying and ammonifying (DNRA capable) bacteria revealed that the former conserved less energy and so had lower growth yields than ammonifying bacteria (4.6–6 g biomass per mol of NO_2^- by denitrifiers versus 8.4 g by ammonifiers; Strohm *et al.*, 2007). Hence, DNRA and anammox bacteria (growth yield 1.3–2 g biomass per mol e$^-$-acceptor; Strous *et al.*, 2006) acting together could have an energetic advantage over denitrifiers in the competition for substrates in oceanic OMZs.

Both measured DNRA rates and *nrfA* expression increased with depth in the upper OMZ at station 946, corresponding to a decrease in ambient NH_4^+ levels. The ^{15}N mole fraction of NH_4^+ resulting from DNRA is estimated to increase from 24% at 100 m to 61% at 150 m, which reflects an increase in the proportion of anammox activity fueled by DNRA (Supplementary SI text). In other words, when ambient NH_4^+ is low and DNRA is highly active, the generated NH_4^+ by DNRA becomes more important for anammox. A tight DNRA–anammox coupling can then produce $^{15}N^{15}N$ from $^{15}NO_x^-$, a signal that could easily be mistaken as a signature of denitrification only (Kartal *et al.*, 2007a). Meanwhile, deeper in the water column where NH_4^+ was scarce and no N-loss activity was detected, the calculated NH_4^+ turnover (generation) due to DNRA alone was rather short (2–3 days). This might indicate additional *in situ* NH_4^+ assimilation, or large advective losses due to stronger current regimes over shelf regions that have not been simulated in our incubation experiments, whereas substrate stimulation in DNRA rate measurements were unlikely given the high availability of ambient nitrate.

Nevertheless, at two out of six depths (100 and 200 m) at station 946, DNRA–anammox coupling was insufficient to fully explain the observed % of $^{15}N^{15}N$ in total N_2 production from $^{15}NO_2^-$. The observed 23 and 74% at 100 and 200 m, respectively, were double those of the expected 10 and 34% based on DNRA and anammox rates and the ambient NH_4^+ concentrations (Supplementary SI Text). Part of this $^{15}N^{15}N$ production could probably be explained by intracellular DNRA–anammox coupling by

anammox bacteria themselves, which has not been accounted for in the above calculations. Such a capability has been shown in cultures (Kartal *et al.*, 2007a) though it awaits verification in environmental studies. Alternatively, it might be partly attributable to denitrification, which would be in concordance with the detectable denitrifier-*nirS* expression at 150 and 200 m. Sequence analyses of these expressed denitrifier-*nirS*, as amplified with the primers nirS1F-6R (Braker *et al.*, 1998) and subsequently cloned, revealed relatively low diversity (only six OTUs based on 97% nucleotide sequence cutoff) out of the 87 obtained sequences (Supplementary Figure S6), in contrast with the high diversity observed at the DNA level in a nearby region (Jayakumar *et al.*, 2009). Most sequences were affiliated with *Paracoccus denitrificans* and *Roseobacter denitrificans*, whereas three OTUs were more closely related to other environmental sequences, including some previously retrieved from the Arabian Sea.

Low and sporadic N-loss in the central–NE OMZ
In the central–NE Arabian Sea OMZ (stations 950, 953, 955 and 957), contrary to the expected high N-loss activities (Naqvi, 1991; Bange *et al.*, 2005), N_2 production was mostly undetectable except for perhaps 1–2 sporadic depths at each station (Supplementary Figure S4 c–f). Only at station 949, just outside the prominent secondary nitrite maximum, was anammox detected right below the oxycline (150 m) as significant $^{14}N^{15}N$ productions in all four sets of incubations: $^{15}NH_4^+$, $^{15}NO_2^-$, $^{15}NH_4^+ + {}^{14}NO_2^-$ and $^{15}NO_2^- + {}^{14}NH_4^+$ (Supplementary Figure S4b, Supplementary Table S2). The detectable N_2 production at the other central–NE stations only appeared in the form of $^{14}N^{15}N$, and lacked consistency over the four different ^{15}N-treatments (Supplementary Figure S4 c–f). Although experiments with $^{15}NH_4^+$ alone almost never yielded any ^{15}N-labelled N_2 to unambiguously confirm anammox activity, there was no production of $^{15}N^{15}N$ from $^{15}NO_2^-$ ($\pm {}^{14}NH_4^+$) without initial time lag to verify *in situ* denitrification either. When detected, the rates ($\leqslant 1$ nmol of $^{14}N^{15}N$ l^{-1} per day) were close to the detection limit, and were often found with both substrates (NH_4^+ and NO_2^-) added. Hence, these N-loss rates in the central–NE Arabian Sea should be regarded as potential rather than *in situ* rates. Anammox potentials were further corroborated by the consistently detectable expression of Scalindua-*nirS* (Supplementary Figure S5), and the presence of anammox bacteria identified via CARD–FISH, though their cellular abundance was too low to give reliable microscopic cell counts ($<0.1\%$ total microbial abundance). These results are consistent with another recent study conducted during the same intermonsoon period, when patchy anammox activity was reported at two stations nearby (Ward *et al.*, 2009; Bulow *et al.*, 2010). DNRA–anammox

coupling was not evident in the central–NE basin at the time of our sampling. DNRA rates were assessed at two central–NE stations (957 and 955), and were only detectable at two depths (200 m at station 955 and 900 m at station 957) with very low rates near detection limit.

Despite the greater abundance of denitrifiers over anammox bacteria detected at the gene level, which agreed with the recent study in the central–NE Arabian Sea (Ward *et al.*, 2009), there was only low to undetectable active expression of the dentrifier-*nirS* gene (Supplementary Figure S5). Although the primers (Michotey *et al.*, 2000; Throbäck *et al.*, 2004) used for quantification (quantitative PCR) may not cover all environmental *nirS* sequences and thereby underestimated denitrifiers at the gene level, these primers have been tested to exhibit high specificity (Throbäck *et al.*, 2004) and cover the vast majority of the expressed complementary DNA sequences except for two OTUs (six sequences) retrieved in our study. In contrast, the primer nirS3R (Braker *et al.*, 1998) used to quantify denitrifiers by Ward *et al.* (2009) has sequence mismatches with all complementary DNA sequences obtained in our study, as well as with a fair number of cultured strains (Braker *et al.*, 1998) and published environmental sequences (for example, 67 out of the 113 denitrifier sequences shown in Supplementary Figure S6), including some retrieved from the Arabian Sea. If the stringency of quantitative PCR conditions is relaxed to cover more sequences, this primer may additionally bind to a second region 18 bases downstream and result in double or unspecific quantification. Therefore, the primer nirS3R is unsuitable and not chosen for use in quantitative PCR. Overall, our gene expression analyses showed little indication of actively expressed denitrifier-*nirS* in these waters, albeit new designs for more universal *nirS*-targeting primers suitable for quantitative PCR, or the use of PCR-independent methods, such as metagenomics/metatranscriptomics, are necessary for a more comprehensive appraisal.

Altogether, the lack of readily detectable denitrifier-*nirS* expression at most stations and depths in the central–NE Arabian Sea, was in line with the absence of any significant $^{15}N^{15}N$ production detected from $^{15}NO_2^-$ incubations, as well as the lack of measurable ^{15}N-labeled N_2O production. Interestingly, in the incubations with $^{15}NO_2^-$ ($\pm {}^{14}NH_4^+$) at these stations, an abrupt N_2 production often occurred after 24–50 h time lag (Supplementary Figure S7). This sharp delayed N_2 production included both $^{14}N^{15}N$ and $^{15}N^{15}N$. Although denitrification might be inhibited by $\geqslant 2$–4 µM of O_2 (Devol, 1978) and anammox by $\geqslant 13$ µM (Jensen *et al.*, 2008), <90 nM of O_2 was detected in the core of the central–NE OMZ *in situ* with the STOX sensor, and $\leqslant 0.5 \pm 0.09$ µM O_2 measured in the exetainers with an oxygen microsensor in the current and previous studies (<0.2 µM by Dalsgaard *et al.*, 2003 and Jensen *et al.*, 2008). Hence, oxygen

inhibition of N-loss processes at sub-micromolar levels can most likely be discounted. Instead, although reflecting an apparent artifact after prolonged incubation and not counted as rates here, the lagged $^{15}N^{15}N$ production from $^{15}NO_2^-$ ($\pm$ $^{14}NH_4^+$) likely indicated stimulated denitrification after long incubation hours, and would agree with the observed high gene abundance but inconsistent expression of denitrifier-*nirS*. Similarly, time lags could be observed in the recent study by Ward *et al.* (2009) that was further detailed in Bulow *et al.* (2010). In that study, there was no significant ^{15}N-labeled N_2 production from $^{15}NO_2^-$ nor from $^{46}N_2O$ in the first 24 h of incubation, at least for the representative sampled depth shown and for which their highest denitrification rate (25 ± 9.1 nmol of $N_2 l^{-1}$ per day) was reported. In other words, despite the persistent denitrification potentials present in the central–NE Arabian Sea OMZ, there is limited evidence for their *in situ* activities. However, active denitrification cannot be excluded at other times, as it is likely subjected to strong spatiotemporal variabilities.

Distribution and variability of N-loss in the Arabian Sea

Considering the entire basin of the Arabian Sea, our measured anammox rates ranged from undetectable to 27 ± 1.1 nmol of $N_2 l^{-1}$ per day (from $^{15}NH_4^+$ incubations), or up to 38 ± 1.9 nmol of $N_2 l^{-1}$ per day taking into account the coupling with DNRA (from $^{15}NO_2^-$ incubations; Table 2). When integrated over the depth range of the OMZ, the highest N-loss due to anammox occurred over the Omani shelf at a rate of up to 4.5 ± 0.4 mmol of N m^{-2} per day (Figure 4), comparable with those reported for the Namibian and Peruvian shelves (Kuypers *et al.*, 2005; Hamersley *et al.*, 2007). Together with

previous findings of substantial anammox, but low to undetectable denitrification in other marine oxygen-deficient waters (Kuypers *et al.*, 2005; Thamdrup *et al.*, 2006; Hamersley *et al.*, 2007; Jensen *et al.*, 2008), the current study confirms the importance of anammox in global oceanic N-loss. At just about 500 km offshore, the depth-integrated N-loss was reduced by two orders of magnitude, and down to only potential rates in the central–NE basin (Figure 4). Even if the measured potential rate profiles were integrated for the central–NE OMZ,
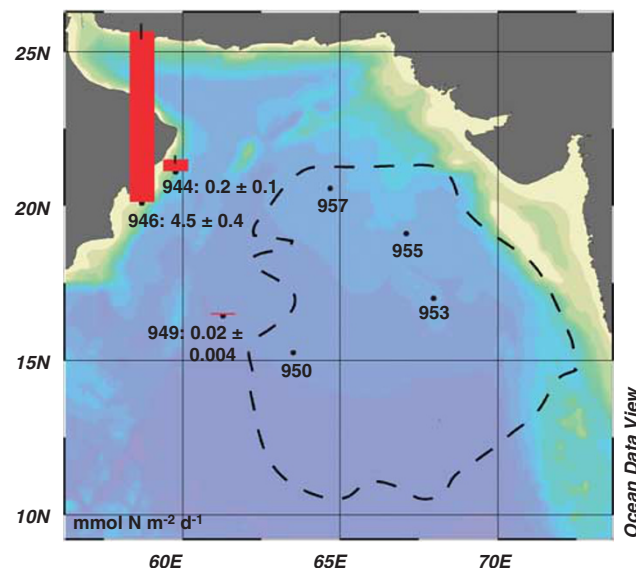

Figure 4 Estimates of depth-integrated anammox rates ($\pm$ s.e.) measured in Arabian Sea OMZ in this study. Rates are calculated from incubations with $^{15}NO_2^-$, and only rates that showed consistency across various isotope combinations have been included. The dashed line marks the approximate geographical extent of the prominent secondary nitrite maximum ($\geqslant 0.5$ μM) as the postulated major denitrification region in the Arabian Sea (Naqvi, 1991).

Table 2 Summary of volumetric and depth-integrated rates of anammox and denitrification in various studies of oceanic OMZs.

	Anammox (nmol of $N_2 l^{-1}$ per day)	Denitrification (nmol of $N_2 l^{-1}$ per day)	Anammox (mmol of N m^{-2} per day)	Denitrification (mmol of N m^{-2} per day)	Reference
Off Chile	2–17[a]	ND	0.6–1.5[b]		Thamdrup *et al.*, 2006
Off Peru[c]	0–164[a]	ND	1.3–34		Hamersley *et al.*, 2007
Off Namibia	10–180[a]	ND	1–5		Kuypers *et al.*, 2005
Arabian Sea				~1.3–1.9[d]	Devol *et al.*, 2006
Arabian Sea	0.2–4.3	0.2–25			Ward *et al.*, 2009
Arabian Sea	0–27[a]		0.02–3.2[a]		This study
Arabian Sea	0–38[e]		0.02–4.5[e]		This study

Abbreviations: ND, not detectable; OMZs, oxygen minimum zones.
[a]Rates from $^{15}NH_4^+$ incubations.
[b]Approximate rates as calculated based on rates from Figure 5 of Thamdrup *et al.* (2006). Anammox rates were integrated over a depth range of 30–175 m at two stations sampled within 3 days.
[c]Potential rates calculated from non-substrate-limited $^{15}NH_4^+ + ^{14}NO_2^-$ incubation.
[d]Approximate rates as calculated based on rates from Figure 4 of Devol *et al.* (2006). The N-loss rates were determined as the conversion of $^{15}NO_3^-$ to $^{14}N^{15}N$ in incubated water samples from the open-ocean Arabian Sea. Therefore, denitrification measurements did not directly include N_2 production by anammox, nor could anammox be excluded. Rates were integrated over a depth range of 150–350 m.
[e]Rates from $^{15}NO_2^-$ incubations.
Depth-integrated rates were calculated as $IR = \sum_{n=1} (R_n h_n)$ where IR denotes the integrated rate, R_n is the measured rate for depth interval n, and h_n denotes the height of the water column for interval n (Dalsgaard *et al.*, 2003). The boundary for each interval n was demarcated by the midpoint between two consecutive measurements. The upper boundary was set at the depth where $O_2 < 2$ μM.

there would be at most 0.3–0.6 mmol of N m^{-2} per day N-loss. This finding of greater N-loss associated with the more productive upwelling waters toward the coast, on one hand coheres with observations from other OMZs that are also linked to upwelling systems (Kuypers et al., 2005; Hamersley et al., 2007). On the other hand, it contradicts with the conventional presumption of high N-loss in the central–NE Arabian Sea deduced from nitrite accumulation or N-deficits (Naqvi, 1991; Bange et al., 2005), and the moderate denitrification rates measured previously (Ward et al., 2009; Bulow et al., 2010).

The fact that high N-loss rates were obtained over the more productive Omani shelf waters suggested a close link between N-loss and the availability of organic matter. Indeed, our measured N-loss rates were significantly and positively correlated with surface particulate organic carbon (Spearman correlation $R = 0.50$, $P < 0.0001$) and particulate nitrogen (Spearman $R = 0.53$, $P < 0.0005$; Supplementary Figure S8). Both anammox and denitrification have to rely on organic matter, either indirectly for the source of remineralized NH_4^+ by the former, or directly for sources of carbon and electrons by the latter or both (Kartal et al., 2007b). Thus, organic matter would be a logical controlling factor for N-loss via either pathway. This is consistent with a study from the eastern tropical South Pacific where surface primary production and not oxygen appeared to be the primary factor regulating N-loss (Lipschultz et al., 1990). Surface production in the Arabian Sea is heavily influenced by the seasonal monsoons, and nutrients are delivered to the open ocean via mesoscale eddies more than open-ocean Ekman pumping (Marra and Barber, 2005; Wiggert et al., 2005). Consequently, high spatiotemporal variabilities are especially apparent in the central–NE region, as reflected in the patchiness in surface chlorophyll a during our sampling period (Supplementary Figure S2; Lam et al., 2011). Over just a 2-month period around our sampling, a high degree of week-to-week patchiness could be seen especially in the central–NE Arabian Sea; whereas the effects of upwelling persisted westward until early October 2007 (Supplementary Figure S2b). The recent study by Ward et al. (2009) sampled in close proximity to stations 950 and 955 at 1 week after and before this study, respectively. They reported moderate N-loss rates through mainly denitrification. Although their observations might not have truly differed much from ours if the same criteria were used for rate calculations (for example, exclusion of data series with significant initial time lags), the disparities between the two studies may also be attributed to the high spatiotemporal variabilities in the Arabian Sea. The detected N-loss activity might reflect N-loss in response to episodic pulses of organic matter sinking from surface water. Therefore, the spatiotemporal variabilities of N-loss and N-cycling in the Arabian Sea certainly require further assessment.

Conclusions and perspectives

Our current study reports for the first time a direct link between DNRA and anammox in the ocean through a production of $^{15}N^{15}N$ from $^{15}NO_2^-$—signal easily mistaken as a signature for denitrification only. This DNRA–anammox coupling mediates the highest N-loss rates reported at this time in the Arabian Sea, and was found in a hitherto overlooked region over the productive Omani shelf. By contrast, only low potential rates at best were detected in the presumed active N-loss zone in the central–NE OMZ. Despite being the world's thickest oceanic OMZ, the calculated depth-integrated N-loss from the Arabian Sea OMZ was only a fraction of that in the Peruvian OMZ (Table 2). This suggests that the common estimate of the Arabian Sea accounting for $\sim 20\%$ of global oceanic N-loss is perhaps too high, and that the strongly negative N*, as a time-integrated signal, might reflect an accumulation of low or episodic N-loss within a poorly ventilated water layer in the central–NE basin (Lam et al., 2011). Although we might have only captured a snapshot in space and time, if N-loss is indeed intimately linked to organic matter availability, considering the Omani upwelling being the most productive waters in the basin on an annual basis, the detected N-loss therein might represent a major contribution to the yearly N-budget that has so far been ignored. The observed DNRA–anammox coupling may also occur at the eastern and northern boundaries of the Arabian Sea, where the OMZ impinges on the Indian and Pakistani shelves underlying some relatively productive waters at least on a seasonal basis. These shelf regions may together be responsible for significant total N-loss that needs to be taken into N-budget calculations. Further studies with higher temporal and spatial resolutions are necessary to elucidate the true spatial extent of N-loss, and how exactly N-loss and the overall N-cycling respond to seasonality and short-term variabilities in the Arabian Sea, and whether there is a dominance or succession of N-loss mechanisms. Besides, our results highlight the need to examine possibly concurrent N-cycling processes together with N-loss in oxygen-deficient environments, as these concurrent processes may complicate reliable estimates of total N-loss.

Acknowledgements

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