Microbial Nitrogen Cycling Processes in Oxygen Minimum Zones

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Abstract

Oxygen minimum zones (OMZs) harbor unique microbial communities that rely on alternative electron acceptors for respiration. Conditions therein enable an almost complete nitrogen (N) cycle and substantial N-loss. N-loss in OMZs is attributable to anammox and heterotrophic denitrification, whereas nitrate reduction to nitrite along with dissimilatory nitrate reduction to ammonium are major remineralization pathways. Despite virtually anoxic conditions, nitrification also occurs in OMZs, converting remineralized ammonium to N-oxides. The concurrence of all these processes provides a direct channel from organic N to the ultimate N-loss, whereas most individual processes are likely controlled by organic matter. Many microorganisms inhabiting the OMZs are capable of multiple functions in the N- and other elemental cycles. Their versatile metabolic potentials versus actual activities present a challenge to ecophysiological and biogeochemical measurements. These challenges need to be tackled before we can realistically predict how N-cycling in OMZs, and thus oceanic N-balance, will respond to future global perturbations.

INTRODUCTION

Oxygen (O_2) is the preferred electron acceptor for the respiration of organic matter. Our modern oceans are so largely well oxygenated that most marine organisms lead an oxic mode of life. In <4% of global ocean volume, however, oxygen drops to below 90 μmol kg^{-1}, a lethal level to many macroorganisms; in <0.05% of global ocean volume, 4.5 μmol kg^{-1} of oxygen remain in seawater, such that oxic respiration can hardly be sustained (Karstensen et al. 2008, Keeling et al. 2010). Although most life avoids such low-O_2 conditions, those microorganisms that can exploit alternative electron acceptors for respiration, or those capable of harnessing energy from various redox reactions to fix their own organic carbon, flourish in these suboxic waters, also known as oxygen minimum zones (OMZs), and form unique microbial communities distinct from those living in oxic waters.

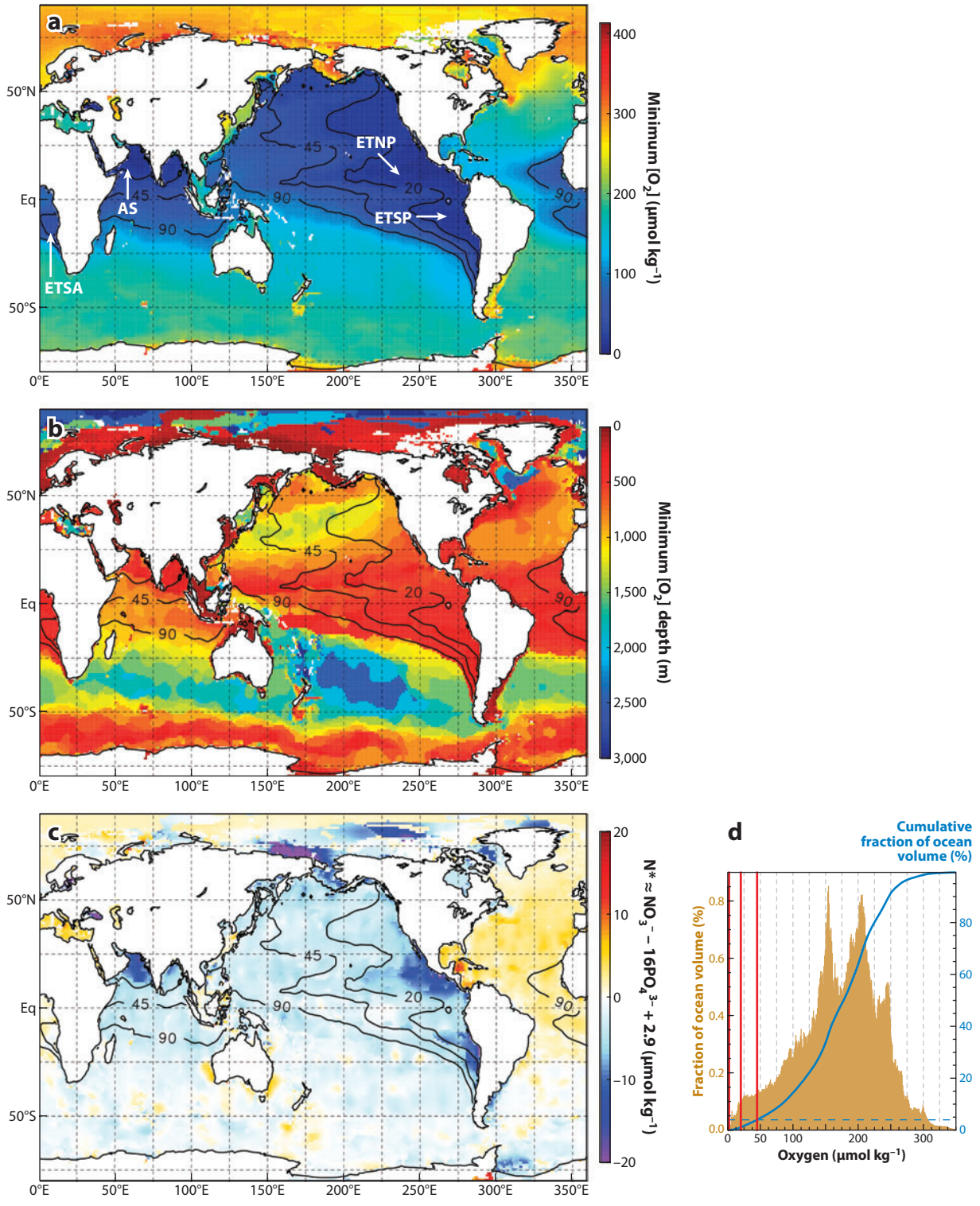
The occurrence of oceanic OMZs, usually at water depths of 100–1,000 m underlying highly productive surface waters, is likely due to a combination of sluggish ventilation and oxygen consumption via degradation of sinking organic matter (Wyrtki 1962). The upper oxygen thresholds chosen to define OMZs have been rather spurious, ranging from <2 μM to ~90 μM, depending on the context of the study (Codispoti et al. 2005, Karstensen et al. 2008, Paulmier & Ruiz-Pino 2009). The lack of clear O_2 definition is also due partly to the lack of reliable methods to accurately measure O_2 levels below 5 μM, that is, until the recent development of a novel oxygen sensor that can achieve accuracies at nanomolar levels (Revsbech et al. 2009). Subsequent measurements made in the OMZ off Peru revealed that O_2 levels could reach as low as <2 nM, or practically anoxic, at least in parts of the OMZ. In the current review, we loosely adapt the criterion $O_2 \leq$ 20 μM to include the maximum O_2 level at which the use of an alternative electron acceptor has been reported in a water column (Smethie 1987). As a result, the so-defined OMZs constitute ~1% of global ocean volume (**Figure 1**).

The Electron Tower and Enhanced Redox Cycling

According to the electrochemical series (**Figure 2**), nitrate (NO_3^-) is the next preferred electron acceptor for respiration after oxygen (considering the reduction to dinitrogen, N_2) and can yield similar amounts of free energy ($\Delta G°$) as that from oxic respiration of organic matter (Froelich et al. 1979). The expected energy yield of the reaction $NO_3 \rightarrow N_2$ is closely followed by those of manganese (IV) and iodate (IO_3^-) reduction, whereas those of iron (III) and sulfate (SO_4^{2-}) respiration are significantly lower (**Table 1**). Nitrate and iodate occur at relatively high concentrations (~30 μM and 0.2–0.5 μM, respectively) in typical seawater, compared with only (sub)nanomolar levels of both manganese and iron. Therefore, the former two are likely much more important in suboxic respiration in seawater; whereas, despite its high abundance (~28 mM) in seawater,

Figure 1

(*a*) Minimum O_2 concentrations (μmol kg^{-1}) in the vertical water column at each 1° × 1° grid in the world's oceans. (*b*) Corresponding depths at which O_2 minima are found. (*c*) A map of the corresponding N* at the O_2 minima indicates severely negative N*, particularly at the eastern tropical North and South Pacific (ETNP and ETSP, respectively) and the Arabian Sea (AS). Isolines (*a–c*) mark the minimum O_2 values of 20, 45, and 90 μmol kg^{-1} that are frequently used to define oxygen minimum zones (OMZs) or hypoxic zones. The eastern tropical South Atlantic (ETSA; panel *a*) is an expanding OMZ where active nitrogen loss has been detected. (*d*) Fractions of global ocean volume at various O_2 concentrations and the corresponding cumulative percentage of total ocean volume (*blue line*). Red lines highlight the O_2 levels of 2, 20, and 45 μmol kg^{-1}. Data from World Ocean Atlas 2005 (Garcia et al. 2006). Figure adapted for publication, courtesy of Yves Plancherel.



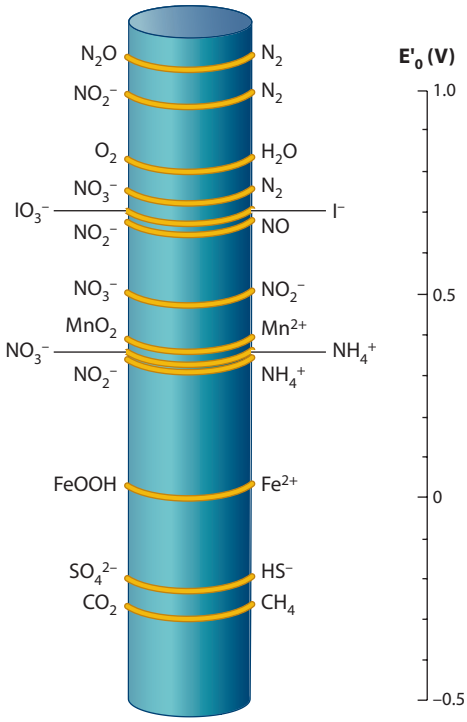


Figure 2

The electron tower illustrating the electrode potentials (E'_0) of various redox couples at environmentally relevant concentrations in the oxygen minimum zones. The values are calculated for pH 7 and otherwise standard conditions. Redrawn after Canfield et al. (2005).

the low electrode potential and $\Delta G°$ of SO_4^{2-} reduction relegate it to a much later point in the sequence, after most other electron acceptors have been exhausted.

Because of such suboxic respiration, a plethora of reduced chemical species is produced, which is normally rare in the oxygenated world's oceans. Some so-called chemolithoautotrophic microorganisms are able to reoxidize reduced chemicals with trace amounts of oxygen available in the OMZ, or with other alternative electron acceptors to gain energy to support carbon fixation. Examples in the OMZs include autotrophic ammonia oxidation (Ward & Zafiriou 1988, Ward et al. 1989) and autotrophic sulfide oxidation with NO_3^- (Lavik et al. 2009) (**Table 1**). In other words, recently reduced chemical species are cycled back to their higher valence states in close proximity to their origins. Hence, OMZs represent key regions of enhanced cycling of biologically active elements in the oceans.

Nitrogen Cycling and Modern OMZs

The oceanic nitrogen cycle is perhaps the one elemental cycle that is most affected by oceanic OMZs. NO_3^- is the first preferred alternative electron acceptor, and its reduction to gaseous products N_2O or N_2 via canonical denitrification, or to N_2 via anammox (anaerobic ammonium oxidation; Mulder et al. 1995), leads to a loss of nitrogen from the oceans to the atmosphere. Active nitrification ($NH_4^+ \rightarrow NO_2 \rightarrow NO_3$) in or near OMZs fosters return of nitrogen remineralized from organic matter back to the highest oxidation state, thus further promoting nitrogen loss

Table 1 Standard Gibbs free energy ($\Delta G°$) calculated for the major respiratory pathways using acetate as the electron donor, as well as chemolithoautotrophic pathways of interest in oxygen minium zones[a]

Reaction		$\Delta G°$ (kJ per reaction)
Organotrophy		
N_2O reduction[b]	$2\,N_2O + {}^1/_2\,C_2H_3O_2^- \rightarrow 2\,N_2 + HCO_3^- + {}^1/_2\,H^+$	−600
NO reduction[b]	$4\,NO + {}^1/_2\,C_2H_3O_2^- \rightarrow 2\,N_2O + HCO_3^- + {}^1/_2\,H^+$	−530
Oxic respiration	$\mathbf{O_2 + {}^1/_2\,C_2H_3O_2^- \rightarrow HCO_3^- + {}^1/_2\,H^+}$	**−402**
Denitrification	${}^4/_5\,NO_3^- + {}^1/_2\,C_2H_3O_2^- + {}^3/_{10}\,H^+ \rightarrow {}^2/_5\,N_2 + HCO_3^- + {}^2/_5\,H_2O$	−398
Mn reduction	$2MnO_2 + {}^1/_2\,C_2H_3O_2^- + {}^7/_2\,H^+ \rightarrow 2\,Mn^{2+} + HCO_3^- + 2\,H_2O$	−385
Nitrite reduction (to NO)[b]	$4\,NO_2^- + {}^1/_2\,C_2H_3O_2^- + {}^7/_2\,H^+ \rightarrow 4\,NO + HCO_3^- + 2\,H_2O$	−371
Nitrite reduction (to N_2)[b]	$NO_2^- + {}^1/_2\,C_2H_3O_2^- \rightarrow {}^1/_2\,N_2 + HCO_3^- + {}^1/_2\,H^+$	−355
Iodate reduction	${}^2/_3\,IO_3^- + {}^1/_2\,C_2H_3O_2^- \rightarrow {}^2/_3\,I^- + HCO_3^- + {}^1/_2\,H^+$	−341
DNRA (with NO_3^-)	${}^1/_2\,NO_3^- + {}^1/_2\,C_2H_3O_2^- + {}^1/_2\,H^+ + {}^1/_2\,H_2O \rightarrow {}^1/_2\,NH_4^+ + HCO_3^-$	−257
Nitrate reduction[b]	$2\,NO_3^- + {}^1/_2\,C_2H_3O_2^- \rightarrow 2\,NO_2^- + HCO_3^- + {}^1/_2\,H^+$	−244
Fe reduction	$4\,FeOOH + {}^1/_2\,C_2H_3O_2^- + {}^{15}/_2\,H^+ \rightarrow 4\,Fe^{2+} + HCO_3^- + 6\,H_2O$	−241
DNRA (with NO_2^-)	${}^2/_3\,NO_2^- + {}^1/_2\,C_2H_3O_2^- + {}^5/_6\,H_2O \rightarrow {}^2/_3\,NH_4^+ + HCO_3^-$	−222
Sulfate reduction	${}^1/_2\,SO_4^{2-} + {}^1/_2\,C_2H_3O_2^- + {}^1/_2\,H^+ \rightarrow {}^1/_2\,H_2S + HCO_3^-$	−43.8
Methanogenesis	${}^1/_2\,C_2H_3O_2^- + {}^1/_2\,H_2O \rightarrow {}^1/_2\,CH_4 + {}^1/_2\,HCO_3^-$	−19.9
Chemolithotrophy		
Autotrophic denitrification (with $HS^- \rightarrow S°$)	$2NO_3^- + 5HS^- + 7H^+ \rightarrow N_2 + 5S° + 6H_2O$	−1,260
Anammox	$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O$	−358
Ammonia oxidation	$NH_3 + {}^3/_2\,O_2 \rightarrow HNO_2 + H_2O$	−278
Nitrite oxidation	$NO_2^- + {}^1/_2\,O_2 \rightarrow NO_3^-$	−82

[a]Pathways are arranged in descending order of the calculated $\Delta G°$. Oxic respiration of organic matter has been highlighted in bold. All values are calculated for 25°C and at pH 7, with unit activity for all reactants and products. $\Delta G°$s for respiratory pathways are standardized to a 4 e^- transfer equivalent to the oxidation of 1 mol of organic carbon.
[b]Individual steps in the canonical denitrification.

(**Figure 3**). Globally, it has been estimated that OMZ waters are responsible for approximately 30–50% of nitrogen loss from the world's oceans, or 16–27% from land and oceans combined (Codispoti et al. 2001; Gruber 2004, 2008). These large nitrogen losses from the OMZs have left notable imprints in seawater nutrient ratios of nitrogen to phosphorus, with especially negative deviations from the otherwise relatively constant value of 16 (Redfield et al. 1963). These so-called nitrogen deficits are commonly expressed as N^* ($N^* = [\text{Total inorganic nitrogen}] - 16[PO_4^{3-}] + 2.9\ \mu mol\ kg^{-1}$) (Gruber & Sarmiento 1997, Deutsch et al. 2001). Three major OMZs, in particular, can be identified with strongly negative N^*, implying nitrogen loss (Gruber & Sarmiento 1997).

Two of the major OMZs are found associated with the productive eastern boundary upwelling systems in the tropical North and South Pacific (ETNP and ETSP), and the third is located in the northeastern Arabian Sea (Gruber & Sarmiento 1997). Additionally, in the eastern tropical South Atlantic (ETSA), suboxic/anoxic conditions occur in a narrow zone along the continental margin off Namibia, associated with the Benguela upwelling system (Calvert & Price 1971), and active nitrogen loss has also been reported (Kuypers et al. 2005).

Despite the numerous studies on microbial processes, especially nitrogen cycling in the OMZs in the later half of the twentieth century (e.g., Brandhorst 1959, Wooster et al. 1965, Fiadeiro & Strickland 1968, Cline & Richards 1972, Codispoti & Christensen 1985, Naqvi 1987,

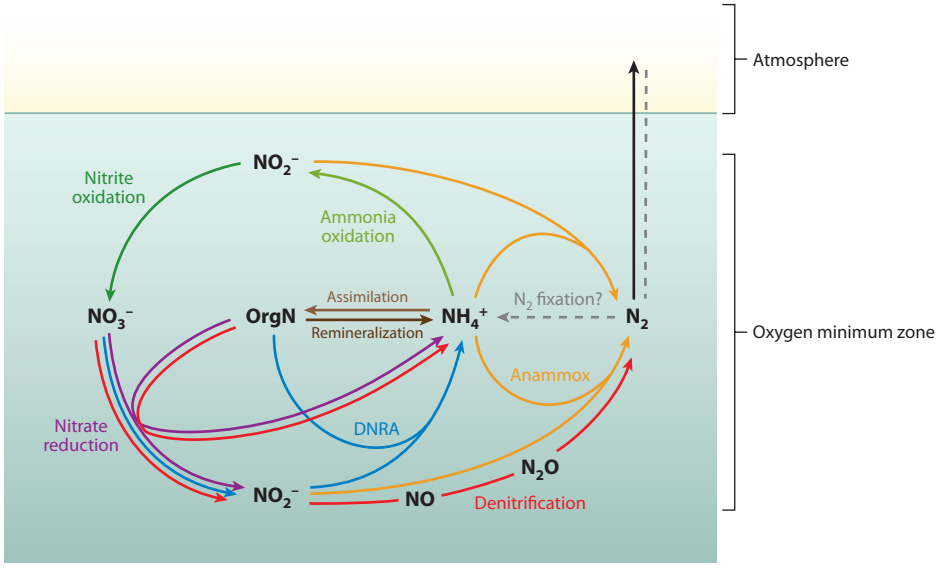


Figure 3

The cycling of nitrogen in oceanic oxygen minimum zones (OMZs). N_2 can be produced by both anammox (*orange*) and denitrification (*red*). In the latter, each step can also be considered individually, with possible release of intermediates, and each could be responsible for further remineralization, though shown only in the first step here. Both nitrate reduction (*purple*) and dissimilatory nitrate reduction to ammonium (DNRA; *blue*) are also remineralization processes, producing additional NO_2^- and NH_4^+, respectively. Nitrification is divided into two steps: ammonia oxidation (*light green*) and nitrite oxidation (*dark green*). Potential N_2 fixation is also shown (*gray dashed*) but has not been tested in the OMZ. Adapted from Lam et al. (2009).

Ward & Zafiriou 1988, Lipschultz et al. 1990), the past decade has witnessed a resurgence of research interest in this field. One of the reasons is the discovery of anammox as an alternative nitrogen loss pathway to heterotrophic denitrification in both marine sediments and water columns (Thamdrup & Dalsgaard 2002, Dalsgaard et al. 2003, Kuypers et al. 2003). In addition, a number of new key microbial players in the marine nitrogen cycle have been reported, including archaeal nitrifiers that appear to be abundant in mesopelagic oceans (Francis et al. 2005, Könneke et al. 2005). The significance of these processes in the OMZs thus requires further evaluation.

Moreover, recent analyses of contemporary and historical data sets have observed deoxygenation of global oceans at a rate of 0.09–0.34 μmol kg^{-1} y^{-1} in the past five decades, resulting in global expansion of the OMZs, especially in the Atlantic and Pacific (Stramma et al. 2008). Oceanic OMZs currently impinge on over 1 million km^2 of permanently hypoxic shelves and continental margins worldwide (Helly & Levin 2004), whereas coastal shelf hypoxia, or even anoxia, has been increasing in frequency and intensity (Diaz & Rosenberg 2008, Naqvi et al. 2000). Hence, any potential impacts of ocean warming or increasing anthropogenic pressures on biogeochemical cycling in either the OMZ waters or the continental margins would likely be magnified by the effects on the other.

In the current review, we examine some recent findings in relation to existing knowledge of microbial processes in oceanic OMZs centered on the nitrogen cycle. Some closely related processes of other elemental cycles are also discussed. Our focus lies on water-column processes rather than those in the sediments. This review will then close with some emerging puzzles on the subject.

DENITRIFICATION

The term denitrification itself literally means the loss of nitrogen from a system regardless of the exact pathways. However, in a strict sense, denitrification refers to the sequential reduction of nitrate to the gaseous products N_2O and/or N_2 via nitrite (NO_2^-) and nitric oxide (NO): $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. This is also known as heterotrophic denitrification, as organic matter is respired in the process. For decades, heterotrophic denitrification has been the only known nitrogen loss pathway in nature until the recent discovery of anammox. Hence, most past and present estimates of oceanic nitrogen budget were derived from the stoichiometry (Deutsch et al. 2001, Gruber & Sarmiento 1997) or stable isotope effects (Ganeshram et al. 2000, Brandes & Devol 2002, Deutsch et al. 2004) associated with denitrification only.

A wide variety of microorganisms, including over 40 genera of bacteria, halophilic archaea, fungi, and foraminifera (Mancinelli & Hochstein 1986, Shoun et al. 1992, Zumft 1997, Cabello et al. 2004, Piña-Ochoa et al. 2010), have the capability to denitrify but rarely are they strict anaerobes (Zumft 1997). In fact, most of them can switch between oxic and NO_3^--dependent modes of respiration. The transcription and subsequent building of denitrifying enzymes do not start until their mode switch, which is mainly regulated by oxygen and NO_2^-/NO_3^- availability (Ferguson 1994). Because denitrification is divided into four steps, each step requires mediation by a different enzyme. Each enzyme (both transcription and activities) responds differently to oxygen, NO_3^-, NO_2^-, NO, and N_2O, and their activities are not necessarily coordinated with considerable interspecies variations (Ferguson 1994). For example, except for the reaction of membrane-bound nitrate reductase (NAR) that occurs in the cytoplasm, enzymatic reactions of the next three steps of denitrification all take place in the periplasm, which means that, following its production, NO_2^- must be transported across the membrane back to the periplasm for subsequent reduction (Zumft 1997). Some denitrifying bacteria possess a periplasmic form of nitrate reductase (NAP), whereas denitrifying archaea seem to have all the involved enzymes membrane-bound but with reactive sites on the periplasmic side (Cabello et al. 2004). Because of the different efficiencies and regulations of various enzymes, intermediates like NO_2^- and N_2O often accumulate in the surroundings. Such accumulations of intermediates seem to increase with decreases in pH and availabile organic carbon (Blaszczyk 1993, Schalk-Otte et al. 2000, Güven 2009).

Denitrification in the OMZs

It was precisely the NO_2^- accumulations within oceanic OMZs, the so-called secondary nitrite maxima, that prompted the early denitrification studies (Gilson 1937, Brandhorst 1959, Wooster et al. 1965, Fiadeiro & Strickland 1968). It has been found that NO_3^--dependent respiration is dominant over oxic respiration only when oxygen drops to below 2–4 μM (Devol 1978), though denitrifying activities at up to 20 μM of O_2 have been reported (Smethie 1987). Most denitrification rate measurements for the OMZs predate the nineties, including those for the ETSP (Codispoti & Packard 1980, Codispoti & Christensen 1985, Lipschultz et al. 1990), ETNP (Cline & Richards 1972, Codispoti & Richards 1976), and the Arabian Sea (Naqvi 1987, Devol et al. 2006). These studies used a variety of methods, such as direct N_2 quantification, N_2:Ar ratios, ^{15}N tracer experiments, mass balance or stoichiometric approaches, and electron transport activity assays (Groffman et al. 2006). However, none of these methods has been able to distinguish N_2 production resulting from denitrification or anammox.

In 2002, a modified ^{15}N isotope pairing technique was successfully applied in marine sediments for this purpose (Thamdrup & Dalsgaard 2002). Anammox indeed made a significant contribution to N_2 production in many of the marine and estuarine sediments examined, but denitrification is often the dominant N_2 production pathway, except for some deep-station sediments

(e.g., Trimmer et al. 2003; Rysgaard et al. 2004; Engström et al. 2005, 2009; Rich et al. 2008). Nevertheless, the OMZ waters showed a different picture. Here, a dominance of anammox was reported, first in the ETSA OMZ off the Namibian coast (Kuypers et al. 2005), and later also in the ETSP (Thamdrup et al. 2006, Hamersley et al. 2007, Galán et al. 2009, Ward et al. 2009). There was little or no evidence for active occurrence of denitrification in these studies, except for some sporadic depths in the ETSP (B. Thamdrup, unpublished data). Interestingly, in a lot of these incubations, signs of denitrification were actually observed after a considerable time lag (>24 h) (Kuypers et al. 2005; Thamdrup et al. 2006; M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data) but were more likely a result of incubation artifacts rather than in situ activities. In congruence, although denitrifying bacteria are readily detected in the ETSP OMZ based on their biomarker functional gene *nirS* (Castro-Gonzalez et al. 2005, Lam et al. 2009, Ward et al. 2009), its active gene expression or transcription was barely detectable or not at all (Lam et al. 2009). The *nirS* gene encodes for cytochrome cd_1-containing nitrite reductase that mediates the key reaction $NO_2^- \rightarrow NO$. Hence, both the rate measurements and the gene expression data strongly suggest that denitrifying potentials are abundant in the OMZs, but actual denitrifying activities are not always induced in situ. As facultative anaerobes, denitrifying bacteria may have alternative modes of living, at least in the ETSA and ETSP OMZs. They may also be dormant for certain periods of time, as dormancy is likely a widespread phenomenon and a major factor in structuring microbial diversity in the environment (Jones & Lennon 2010).

The only report for consistently active denitrification in an OMZ determined by isotope pairing experiments came from a recent study in the Arabian Sea (Ward et al. 2009) and was further detailed in Bulow et al. (2010). Three stations were sampled in the central northeastern basin within the postulated denitrification zone delineated by the prominent secondary nitrite maximum (Naqvi 1991). Isotope pairing experiments were conducted at three to four depths of the upper ~300 m of the OMZ, and active denitrification was reported for most sampled depths, with an average rate of 8.5 ± 2.8 nM N_2 d^{-1} (up to 25 ± 9 nM N_2 d^{-1}) (Ward et al. 2009, Bulow et al. 2010). In comparison, another study conducted in the same region at approximately the same time reported only low N_2 production rates at sporadic depths, corroborated by gene expression analyses (M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data). This latter study saw no convincing $^{15}N^{15}N$ production from $^{15}NO_2^-$ to verify denitrification, nor consistent $^{15}N^{14}N$ production from various $^{15}NH_4^+$ experiments to confirm anammox, and therefore the N_2 production pathway was considered ambiguous. However, looking closely at the original time-series data from both studies, the results may not be all that different if the same criteria were used for rate determination (e.g., exclusion of data series with initial time lag, or of data points in the exponential production phase, would yield low N_2 production rates in both studies).

ANAMMOX

Anammox is the coined term for the anaerobic ammonium oxidation with nitrite that produces N_2 ($NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$) (van de Graaf et al. 1995). This reaction yields $\Delta G°$ of -357 kJ mol^{-1} and can support chemolithoautotrophic growth (van de Graaf et al. 1996). The possible existence of such a lithoautotroph (Broda 1977) and the occurrence of this reaction in marine suboxic water columns (Richards 1965, Cline & Richards 1972) were postulated decades ago based on thermodynamics calculations and absence of significant NH_4^+ accumulations, but direct evidence for this microbial process was not available until 1995 from a wastewater treatment study (Mulder et al. 1995, van de Graaf et al. 1995). The exact pathway of the anammox reaction, however, is not completely understood to date. A combination of whole-genome analyses and experiments with

cultured species indicates that it likely involves a cytochrome cd_1-nitrite reductase (NirS) that reduces NO_2^- to NO, which then reacts with NH_4^+ to produce hydrazine (N_2H_2) that is eventually oxidized to N_2 (Strous et al. 2006). Alternatively, hydroxylamine (NH_2OH) may also be involved. When added in the absence of NO_x^- in an enrichment culture, NH_2OH was disproportionated into NH_4^+ and N_2, though how exactly this fits in the overall anammox reaction requires further verification with direct measurement of NH_2OH in the course of the anammox process (van der Star et al. 2008). In either pathway, the N_2 production is likely mediated by octahaem cytochrome *c* hydroxylamine/hydrazine oxidoreductases, as several copies of genes encoding this enzyme have been identified in an anammox genome (Strous et al. 2006). Interestingly, for every 1 mol of N_2 produced, 0.3 mol of NO_3^- is also produced from NO_2^- alongside. This reaction is believed to be important for replenishing electrons for the acetyl-CoA carbon fixation pathway (van de Graaf et al. 1997, Strous et al. 2006).

In addition to a lithoautotrophic lifestyle, anammox bacteria can be rather versatile in their metabolisms. Some have been shown to utilize small organic acids as electron donors to reduce NO_3^-/NO_2^-, as well as Mn(IV) and Fe(III) as alternative electron acceptors (van de Graaf et al. 1997, Güven et al. 2005, Strous et al. 2006, Kartal et al. 2007b). At the same time, they can also perform dissimilatory NO_3^- reduction to NH_4^+ (dissimilatory nitrate reduction to ammonium, or DNRA), which is subsequently combined with NO_2^- to produce N_2, thus mimicking denitrification (Kartal et al. 2007a).

All anammox bacteria identified thus far belong to a monophyletic group in the phylum Planctomycetes, which is further divided into two subgroups. The first subgroup consists primarily of cultured species enriched from wastewater treatment plants and comprises the genera "*Candidatus* Brocadia," "*Ca.* Kuenenia," "*Ca.* Anammoxoglobus," and "*Ca.* Jettenia" (Strous et al. 1999, Schmid et al. 2000, Kartal et al. 2007b, Quan et al. 2008). Most environmental sequences, especially those from marine settings, fall into the second subgroup "*Ca.* Scalindua" (Schmid et al. 2007). It includes the first reported marine anammox bacterium "*Ca.* S. sorokini" from the Black Sea (Kuypers et al. 2003), sequences from the Namibian and Peruvian OMZs (Kuypers et al. 2005, Hamersley et al. 2007, Woebken et al. 2008), enrichment cultured species "*Ca*. S. brodae" (Schmid et al. 2003), and another from marine sediments (van de Vossenberg et al. 2008). They shared ≥97% sequence identity and so are considered to be one species. Recently, another species, "*Ca*. S. arabica," was found in the Arabian Sea and ETSP OMZs, Lake Tanganyika, and the South China Sea (95–97% sequence identity with "*Ca.* S. sorokini") (Schubert et al. 2006, Woebken et al. 2008, Galán et al. 2009). Finally, "*Ca.* Scalindua" also includes a more distant single species "*Ca*. S. wagneri" enriched from wastewater (93.6% sequence identity as "*Ca*. S. sorokini") (Schmid et al. 2003).

Despite their potential importance in biogeochemical cycling, anammox bacteria have never constituted more than 4% of the total microbial community in the marine suboxic water columns examined (Kuypers et al. 2005; Hamersley et al. 2007; M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data). They are slow growing, with a doubling time of ∼11 days, even under optimal growth conditions in bioreactors (Strous et al. 1998). The anammox reaction takes place within anammoxosomes, the intracytoplasmic compartments that are bounded mostly by dense ladderane lipids unique to anammox bacteria. Ladderanes are less permeable than regular biomembranes, so they can protect other cellular components by containing the highly toxic intermediates like hydrazine (Lindsay et al. 2001, Sinninghe Damste et al. 2002, van Niftrik et al. 2004). Because anammoxosomes constitute >50% individual cell volumes, they give characteristic ringed shapes to anammox cells when stained and viewed under epifluorescence microscopes (Strous et al. 1999).

Anammox in the OMZs

The occurrence of anammox in seawater was first reported for the stratified water columns of the Black Sea (Kuypers et al. 2003) and Gulfo Dulce (Dalsgaard et al. 2003), at depths where neither oxygen nor sulfide were detectable. Within this suboxic zone, NO_3^- concentrations dropped from micromolar levels in the oxic zone to barely detectable at these depths, whereas NH_4^+ started to increase toward the underlying sulfidic layer. NO_2^- accumulated at this intersection of NO_3^- decrease and NH_4^+ increase, and it was in this zone where active anammox was detected. Both studies employed stable-isotope pairing techniques, whereas the Black Sea study was further supported by the identification of anammox bacteria via their signature 16S rRNA and ladderane biomarkers. Subsequently, the occurrence of anammox was found in the narrow ETSA OMZ over the Namibian shelf (Kuypers et al. 2005), then also in the more extensive ETSP OMZs (Thamdrup et al. 2006, Hamersley et al. 2007, Galán et al. 2009), and more recently in the Arabian Sea OMZ (Ward et al. 2009; M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data).

In all cases, anammox seemed to occur at zones of NO_3^- decrease and moderate NO_2^- accumulation, whereas NH_4^+ concentrations could range from micromolar levels (e.g., Namibian and Peruvian shelf OMZs) (Kuypers et al. 2005, Hamersley et al. 2007) to sometimes below detection (e.g., Chilean OMZ and open ocean OMZs off Peru and the Arabian Sea) (Thamdrup et al. 2006; Hamersley et al. 2007; M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data). These discoveries of anammox in the OMZs reconcile the enigma of the apparent lack of observed NH_4^+ accumulation despite active remineralization of organic matter in these waters, which heterotrophic denitrification as a remineralization process itself cannot explain (Richards 1965). Anammox activities are usually found to be the highest at the base of oxyclines and in the upper portions of the OMZs ($\leq$139 nM N_2 d^{-1} in the Peruvian OMZ). There are exceptions, however, over the shelf regimes (e.g., in Namibian and Peruvian OMZs), in which anammox activities could be elevated just above the seafloor (up to 172 nM N_2 d^{-1} in the Namibian OMZ)—possibly under the influence of resuspension and benthic microbial processes (Hamersley et al. 2007, Kuypers et al. 2005). When measured, anammox bacterial abundance generally shows positive correlation with the measured anammox rates (Kuypers et al. 2005; Hamersley et al. 2007; M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data), further supporting the activity distribution of this process in the OMZs. With respect to horizontal rate distribution, results from the three OMZs investigated thus far reveal generally higher volumetric rates over the shelf than in the open ocean, for example, in descending order: Namibian shelf, Peruvian shelf, Arabian Sea–Omani shelf, Peruvian open ocean OMZ, Chilean OMZ (open ocean), and Arabian Sea central northeastern OMZ (open ocean). However, when integrated over the thickness of the OMZ, anammox in the open ocean OMZ may make a larger contribution (though not always) to the total nitrogen loss (**Table 2**).

The finding of higher volumetric nitrogen loss rates over shelf OMZs is consistent with the conventional association between high surface productivities of upwelling regions and higher nitrogen loss in the underlying OMZ. Nevertheless, it is a surprising finding for the Arabian Sea. Based on the nitrite distribution in the basin, the previous consensus was that most nitrogen loss occurs in the central northeastern part of the basin rather than the productive western basin (Naqvi 1991). In contrast, the latest actual rate measurements off the Omani coast reported rates several-fold higher than in the open ocean (central northeastern basin), and this finding was corroborated by gene expression analyses (M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data).

Table 2 Total nitrogen loss rates (volumetric rates and depth-integrated rates) over shelf and open ocean oxygen minimum zones in three ocean basins

Oxygen minimum zones	Shelf		Open Ocean		Reference(s)
	Volumetric nitrogen loss (nM N_2 d^{-1})	Depth-integrated nitrogen loss (mmol N m^{-2} d^{-1})	Volumetric nitrogen loss (nM N_2 d^{-1})	Depth-integrated nitrogen loss (mmol N m^{-2} d^{-1})	
Southeast Atlantic off Namibia	10–180	1–5	—	—	Kuypers et al. 2005
Eastern tropical South Pacific off Peru	0–164	1.3–11.5	0–40	1.5–34	Hamersley et al. 2007
Eastern tropical South Pacific off Chile	—	—	2–17	0.6–1.5	Thamdrup et al. 2006
Arabian Sea	—	—	0.2–25	—	Ward et al. 2009[b]
Arabian Sea	0–39	0.2–4.5	0–1.8[a]	0–0.6[a]	M.M. Jensen, P. Lam, N.P. Revsbech, B. Nagel, B. Gaye, et al., unpubl. data

[a]Potential rates only.
[b]Rates are not depth-integrated for this study due to the limited sampled depths in relation to the thick oxygen minimum zone.

Regulation of Anammox

Although anammox is an anaerobic process and was found to be highly sensitive to low levels (~1 μM) of oxygen in enrichment cultures from wastewater (Strous et al. 1997, van de Graaf et al. 1996), oxygen did not seem to fully inhibit anammox in seawater until ~13.5 μM, according to O_2 manipulation experiments (Jensen et al. 2008). At the same time, anammox is sensitive to low levels of sulfide, which would explain the lack of anammox detected in sulfidic waters such as the bottom layers of the Black Sea and Gulfo Dulce (Jensen et al. 2008). Anammox is also inhibited by methanol and acetylene, the latter of which is a common inhibitor to both aerobic ammonia oxidation and nitrous oxide reduction (the last step of denitrification) (Jensen et al. 2007).

Despite the conventional association between NO_2^- accumulation and nitrogen loss, no apparent relationship can be discerned between NO_2^- concentration and anammox rates measured across three OMZs (**Figure 4*a***). This is consistent with the results from the only NO_2^- uptake kinetics experiment that was performed in marine sediments (Dalsgaard & Thamdrup 2002), in which the half-saturation coefficient (K_m) was determined to be below 3 μM and possibly as low as 0.1 μM. Further increase in NO_2^- did not stimulate anammox in that sediment study, and most OMZ water samples examined thus far indeed carried more than 0.1 μM NO_2^-. In comparison, a moderate but significant correlation (Spearman $R = 0.60$, $p < 10^{-13}$) can be observed between NH_4^+ concentrations and anammox rates (**Figure 4*b***). Because the anammox reaction utilizes NH_4^+ and NO_2^- in a stoichiometric ratio of approximately 1:1, and the ambient concentrations of NH_4^+ in the OMZs are generally lower than that of NO_2^-, anammox in the OMZs may be more frequently limited by NH_4^+ rather than NO_2^-. In congruence, in the water column of the Gulfo Dulce, an addition of 10 μM of NH_4^+ stimulated anammox rates by two- to fourfold in samples where NH_4^+ was originally close to the detection limit (Dalsgaard et al. 2003).

NH_4^+ is a recycled form of nitrogen and usually originates from the remineralization of organic matter. In the OMZs, where oxygen ceases to be the predominant electron acceptor for

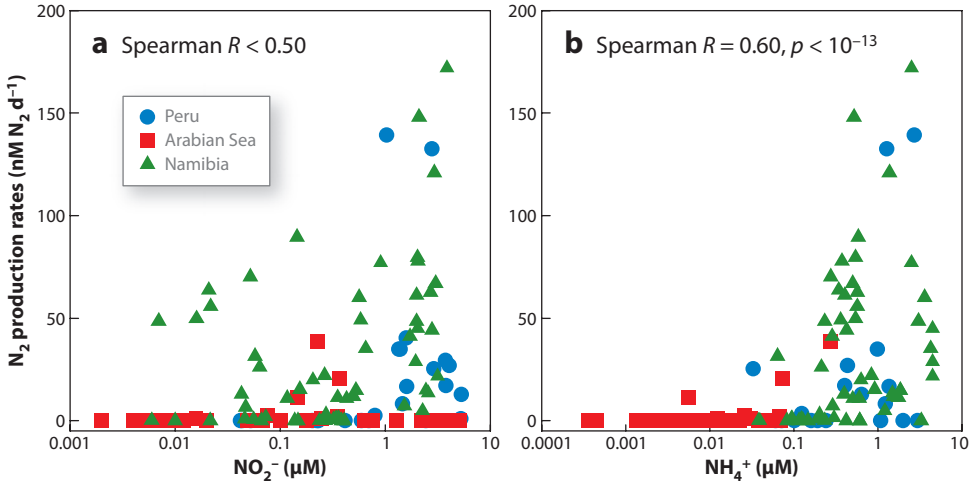


Figure 4

N_2 production rates due to anammox in relation to nitrite (*a*) and ammonium (*b*) across three oxygen minimum zones (OMZs) in different ocean basins. Also shown in (*b*) is the significant Spearman correlation between anammox rates and ammonium concentrations for all data, but no significant correlation with nitrite concentrations was found for the three OMZs combined.

respiration, it was thought that denitrification would be responsible for the majority of organic matter remineralization and thus NH_4^+ production. Hence, in theory, when denitrification and anammox co-occur, as they did in the Gulfo Dulce, for every 1 mol of Redfieldian organic matter (Redfield et al. 1963) remineralized, 16 mol of NH_4^+ will be released and combined with 16 mol of NO_2^- from partial denitrification, thus producing 16 mol of N_2, along with the 39.2 mol from denitrification. In other words, anammox would be responsible for 29% of the N_2 production (Dalsgaard et al. 2003). Nevertheless, problems arose when anammox rates sometimes exceeded denitrification rates in the Gulfo Dulce, or when denitrification was mostly not detected in the Namibian and ETSP OMZs. Therefore, remineralization and thus generation of NH_4^+ would have to rely on some other means. In the Peruvian OMZ, anammox was found to be closely coupled with nitrate reduction to nitrite, as well as dissimilatory NO_3^-/NO_2^- reduction to NH_4^+ (DNRA), for its NH_4^+ requirements. Meanwhile, ammonium oxidation also co-occurred with anammox and provided the latter with additional NO_2^- sources (Lam et al. 2009).

NITRATE REDUCTION

The dissimilatory reduction of nitrate to nitrite ($NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + 2H_2O$) is the first and essential step in denitrification and DNRA, but it is also a stand-alone process. More organisms are capable of nitrate reduction to nitrite without the ability to denitrify or ammonify (Gonzalez et al. 2006, Zumft 1997). Under certain conditions, denitrifiers are also known to accumulate NO_2^- without reducing it further (Betlach & Tiedje 1981). Two forms of dissimilatory nitrate reductases, as discussed above, have been found so far: One is membrane-bound (NAR) and the other occurs in the periplasm (NAP). Because of its location, NAR is directly involved in respiration via the generation of transmembrane proton motive force for ATP synthesis (Jormakka et al. 2003). The functions of NAP vary from species to species and include redox balancing, adaptation to aerobic–anaerobic transitions, nitrate scavenging, ammonification,

and denitrification. It is incorporated into an electron-transport chain with a proton-translocating enzyme to create a proton gradient, though the energy yielded is less conserved than that from NAR (Richardson 2000). Some organisms possess both forms of nitrate reductases (e.g., *Escherichia coli*, *Paracoccus denitrificans*, *Rhodobacter eutropha*) and some only one (see review by Zumft 1997). Although the redox couple NO_3^-/NO_2^- ($E_0' = 0.42$ V) is placed lower than the downstream denitrification steps in the electron tower (**Figure 1**) and the energy yield ($\Delta G°$) is also lower (**Table 1**), NAR and NAP are the only enzymes in the denitrification sequence that are directly associated with the generation of transmembrane proton motive force.

The occurrence of nitrate reduction in the OMZs was already noted in early reports on nitrite accumulations (Brandhorst 1959). Because the degree of nitrite accumulation could not totally match the missing nitrate, denitrification (as the only known nitrogen loss pathway at the time) was thought to take place in the OMZs (Thomas 1966). Subsequently, research focus has usually been placed on denitrification. Nevertheless, the prevalence of nitrate reduction can be seen from early observations and modeled results, in which 40–80% of nitrate reduced accumulates as NO_2^- in the OMZs (Anderson et al. 1982, Fiadeiro & Strickland 1968).

The first actual rate measurements for nitrate reduction using ^{15}N-tracers were conducted in the ETSP OMZ (Lipschultz et al. 1990). Nitrate reduction was detected throughout the OMZ at rates of 25–400 nM d^{-1}, with higher and more variable rates in the upper parts of the OMZ. Almost two decades later, another study in the same region detected similar rates but extended their measurements into shelf regions (Lam et al. 2009). Consistently higher rates were found over the shelf (182–305 nM d^{-1}) than at deep stations (16–117 nM d^{-1}). This finding was additionally supported by up to 25-fold-higher expression of the biomarker genes encoding NAR and NAP over the shelf. A similar rate distribution was also observed in the Arabian Sea OMZ, with rates an order of magnitude higher over the Omani shelf than in the central northeastern basin (P. Lam, M. Jensen, A. Kock, Y. Plancherel, G. Lavik, H. Bange, and M. Kuypers, unpubl. data). Although nitrate respiration may not become dominant until $\leq$2–4 μM of O_2 (Devol 1978), nitrate reduction remained active at ~20 μM of O_2 despite a 75% rate reduction from ambient O_2 level (Lipschultz et al. 1990).

Compared to anammox rates that were measured in the same incubations, nitrate reduction almost always occurred at higher rates and could account for 67–100% of total NO_2^- production. This NO_2^- could then be channeled to the anammox reaction (Lam et al. 2009). Because nitrate reduction is a heterotrophic process, for every 1 mol of Refieldian organic matter respired with nitrate reduction, 16 mol of NH_4^+ will be released. Hence, nitrate reduction can also act as an NH_4^+ supplier for anammox, fulfilling 16–100% of the NH_4^+ requirement over the ETSP OMZ shelf regime, and $\leq$34% in the open ocean (Lam et al. 2009). Consistent with findings in the ETSP, nitrate reduction in the Arabian Sea was found to be responsible for the majority of NO_2^- production, and its rates were significantly correlated with NO_2^- distribution in the central northeastern part of the basin (P. Lam, M. Jensen, A. Kock, K. Lettmann, Y. Plancherel, G. Lavik, H. Bange, and M. Kuypers, unpubl. data).

DISSIMILATORY NITRATE/NITRITE REDUCTION TO AMMONIUM

Apart from the reduction to N_2 via denitrification or anammox, nitrate/nitrite can also be reduced to ammonium. The DNRA process, also known as nitrate/nitrite ammonification ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$), usually starts with NAP-mediated nitrate reduction, though the reaction may also begin with NO_2^- directly. Some microorganisms use this reaction as an electron sink, thereby replacing intermediates during fermentation and allowing ATP generation via substrate-level phosphorylation with organic substrates (Cole & Brown 1980). Perhaps more commonly, DNRA

is involved in the electron transport phosphorylation during respiration (Simon 2002). The respiratory nitrite ammonification, in particular, is mediated by the enzyme cytochrome *c* nitrite reductase (NrfA), which sits at the periplasmic membrane surface (Simon et al. 2000). Apart from NO_2^-, NrfA can also reduce NO and hydroxylamine to NH_4^+ (Burlat et al. 2005).

A diverse group of microorganisms is known to carry the ammonifying trait, including representatives of γ-, δ-, and ε-Proteobacteria, such as *E. coli* and some that are capable of other alternative respiratory pathways like dissimilatory iron, sulfate, and sulfur reduction (Simon 2002). In addition, nitrate/nitrite ammonifying capability has been found in chemolithotrophs like the Fe(II), hydrogen, and sulfide oxidizers, and also in anammox bacteria (Kartal et al. 2007a, Simon 2002). As a result, DNRA may be linked to other branches of the nitrogen cycle as well as other elemental cycles.

In theory, the redox couple NO_2^-/NH_4^+ has an electrode potential (E'_0) of 0.34 V (**Figure 2**) and should yield $\Delta G°$ of −222 kJ per 0.5 mol acetate respired (**Table 1**), which is lower than that calculated for denitrification. However, a recent study indicates that because energy yield is not efficiently conserved in the series of enzymatic reactions of denitrification, nitrate ammonifiers can in fact achieve higher growth yields than denitrifiers (Strohm et al. 2007). This phenomenon would be even more pronounced under NO_3^--limiting conditions, as more NO_3^- is required per mole of organic substrate respired via denitrification, which yields a lower $\Delta G°$ per mole of NO_3^-. As a result, nitrate/nitrite ammonifiers should have certain competitive advantages over denitrifiers in natural environments, especially in nitrate-limiting and organic-rich settings.

Indeed, the significance of DNRA has been recognized for some time in marine sediments (Sørensen 1987). In the OMZ water column, it was first reported only a few years ago in the Namibian OMZ close to the seafloor (Kartal et al. 2007a), and later also at depths free from sediment influence throughout the shelf and open ocean Peruvian OMZ (2–22 nM d^{-1}) (Lam et al. 2009). Assuming heterotrophic respiratory DNRA, which remineralizes Refieldian organic matter, DNRA was estimated to account for up to 100% of the anammox NH_4^+ requirement over the shelf region, though only ≤34% in the open ocean (Lam et al. 2009). The latest study in the Arabian Sea also detected very active DNRA (≤42 nM d^{-1}), especially over the Omani shelf (M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data). Interestingly, perhaps due to the relatively low ambient NH_4^+, co-occurrence of anammox and DNRA resulted in the production of double-^{15}N-labeled N_2 ($^{15}N^{15}N$), which increased in proportion to total N_2 production, with an increase in measured DNRA rates and a decrease in NH_4^+. Because $^{15}N^{15}N$ production is usually a signature of active denitrification (**Table 3**), effects from such DNRA-anammox coupling may call for reevaluations of some previous denitrification measurements via the isotope pairing technique.

Table 3 Suite of six stable isotope-pairing experiments originally designed to distinguish anammox and denitrification that can be used to target various concurrent nitrogen cycling processes at the same time, when the corresponding products are analyzed

No.	Isotope combination	Targeted process	Experiments	Targeted product(s)
1	$^{15}NH_4^+$	Anammox	1–6	N_2 ($^{15}N^{14}N$)
2	$^{15}NH_4^+ + {}^{14}NO_2^-$	Denitrification	3–6	N_2 ($^{15}N^{15}N$, $^{15}N^{14}N$)
3	$^{15}NO_2^-$	Nitrate reduction	5, 6	$^{15}NO_2^-$
4	$^{15}NO_2^- + {}^{14}NH_4^+$	Dissimilatory nitrate reduction to ammonium	5, 6	$^{15}NH_4^+$
5	$^{15}NO_3^-$ (+ $^{14}NO_2^-$)	Ammonia oxidation	1, 2	$^{15}NO_2^-$
6	$^{15}NO_3^- + {}^{14}NH_4^+$	Nitrite oxidation	3, 4	$^{15}NO_3^-$

NITRIFICATION

Nitrification is the major oxidative branch of the nitrogen cycle, connecting organic nitrogen to the highest oxidation state of nitrogen (NO_3^-). It consists of two steps: oxidation of NH_4^+/NH_3 to NO_2^-, and NO_2^- oxidation to NO_3^-. Each step is performed by separate groups of microorganisms, mostly known to be chemilithoautotrophs. Both processes generally require oxygen as their terminal electron acceptor, but there are some exceptions.

Ammonia Oxidation

In ammonia-oxidizing bacteria (AOB), this step of nitrification can be further divided into two more steps with hydroxylamine (NH_2OH) as an intermediate. Firstly, NH_3 is oxidized to NH_2OH via the membrane-bound ammonia monooxygenase (AMO) enzyme: $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$. Subsequently, a periplasmic haem-containing enzyme, the hydroxylamine oxidoreductase (HAO), oxidizes NH_2OH to NO_2^-: $NH_2OH + H_2O \rightarrow HNO_2 + 4e^- + 4H^+$. The evolved electrons are channeled to cytochromes c_{552} and c_{m552}, where they are partitioned to the AMO to meet the e^- needs of the former reaction, and to the terminal oxidase through the ubiquinone pool (Whittaker et al. 2000). It is at the terminal oxidase where a proton motive force is generated ($2H^+ + 0.5O_2 + 2e^- \rightarrow H_2O$) and, consequently, ATP generation follows (Wood 1986). The overall reaction ($NH_3 + 1.5O_2 + \rightarrow HNO_2 + H_2O$) thus yields $\Delta G°$ of -278 kJ (mol^{-1} NH_3) at standard conditions.

Both AMO and HAO can oxidize a number of other substrates to various extents. For instance, AMO can oxidize C–H bonds to alcohols, C=C bonds to epoxides, and sulfides to sulfoxides (Hyman & Wood 1984, Juliette et al. 1993, Hooper et al. 1997). HAO, on the other hand, can oxidize hydrazine to dinitrogen, produce NO from NH_2OH and possibly, N_2O from NO_2^- (Hooper & Terry 1979, Poth & Focht 1985). AOB has several ways to adapt to low-O_2 conditions and even to continue growth via NH_3 oxidation with gaseous nitrogen oxide or tetraoxide (NO_2 or N_2O_4) as electron acceptors. In this case, NO_2^- and NO are formed with NH_2OH as an intermediate, and part of the NO_2^- produced is then reduced to N_2 (and N_2O) (Schmidt & Bock 1997). Hence, nitrification and denitrification co-occur within the same cells. However, NO_2 and N_2O_4 are rather scarce in the environment, thus the ecological significance of this anaerobic pathway in nature remains unclear and little investigated. Some AOB are also capable of NO_2^- reduction to N_2O and N_2 (nitrifier denitrification) with hydrogen, hydroxylamine, or organic compounds (Ritchie & Nicholas 1972, Stüven et al. 1992, Bock et al. 1995). Denitrifying genes *nirK* and *norB* encoding the copper-containing nitrite reductase and nitric oxide reductase, respectively, have also been identified in various AOB (Casciotti & Ward 2001, 2005; Garbeva et al. 2006; Cantera & Stein 2007).

For over a century, organisms capable of oxidizing ammonia autotrophically have been found exclusively in three groups of Proteobacteria: the β-proteobacterial *Nitrosomonas* and *Nitrosospira*, and the γ-proteobacterial *Nitrosococcus*. To date, most knowledge of ammonia oxidation has thus come from AOB. However, a chemolithoautotrophic ammonia-oxidizing archaeon, *Nitrosopumilus maritimus*, was isolated from a marine aquarium a few years ago (Könneke et al. 2005). This archaeon falls into a group of mesophilic *Crenarchaeota* that have been found to be ubiquitous in the oceans (Karner et al. 2001). Meanwhile, a gene (*amoA*) encoding an enzyme homologous to the bacterial AMO has been detected in a marine crenarchaeal metagenome (Venter et al. 2004), then also in the isolate *N. maritimus* (Könneke et al. 2005), as well as in various environments including ETNP (Francis et al. 2005). Therefore, ammonia-oxidizing archaea (AOA) may also contribute to NO_2^- production in the OMZs, along with AOB previously documented (Ward et al. 1989,

Molina et al. 2007). Lastly, some heterotrophic bacteria, including aerobic denitrifiers *Paracoccus denitrificans* and *Alcaligenes faecalis*, are known to be capable of NH_3 oxidation, yet without energy conservation (Robertson et al. 1989, Richardson et al. 1998). Most nitrifying activities in the environment have been attributed thus far to the lithoautotrophic forms. Potential contributions from heterotrophic nitrifiers have hardly been investigated, owing in part to technical challenges to differentiation between the lithotrophic and heterotrophic variants.

The significance of ammonia oxidation in or at the boundaries of the OMZs was recognized before the 1960s (Brandhorst 1959), and the ability of ammonia oxidizers to nitrify under low-O_2 conditions (<4.5 μM) has been tested with marine isolates (Carlucci & Strickland 1968). However, direct measurement of nitrification rates in the OMZs using ^{15}N-tracers was not available until decades later: first in the ETNP (Ward & Zafiriou 1988) and then in the ETSP (Lipschultz et al. 1990, Ward et al. 1989). In both studies, ammonia oxidation rates were highest near the upper boundaries of the OMZs. Rates sometimes remained detectable within the OMZ core, as supported in the ETSP by the presence of both β- and γ-proteobcterial AOB detected via AMO-targeted immunofluorescence (Ward et al. 1989).

A recent revisit to the ETSP OMZ observed similar rate distributions (Lam et al. 2009). In addition, ammonia oxidation was found to account for 6–33% of total NO_2^- production in the upper OMZ, and exceeded the NO_2^- requirement by anammox that was occurring simultaneously at these depths. Ammonia oxidation was, however, undetectable in the lower OMZ in the open ocean. Organisms potentially responsible for this reaction were also examined but via analyses of the biomarker functional gene *amoA*. Active *amoA* expressions were observed for both β- and γ-proteobacterial AOB, as well as for their archaeal counterparts. γ-proteobacterial *amoA* usually showed the highest expression (transcription), but archaeal *amoA* was the most abundant at the gene level. Together, these data seem to suggest that all three groups of ammonia oxidizers contributed to ammonia oxidation in the ETSP OMZ and confirm the postulated importance of AOA in the OMZs (Francis et al. 2005). Likewise, all three kinds of *amoA* were actively expressed in the Arabian Sea OMZ, yet archaeal *amoA* expression in particular showed a significant correlation with the detected ammonia oxidation rates (P. Lam, M. Jensen, A. Kock, K. Lettmann, Y. Plancherel, G. Lavik, H. Bange, and M. Kuypers, unpubl. data).

Interestingly, although ammonia oxidation presumably requires oxygen, experiments with artificially increased O_2 levels (~20 μM) did not stimulate ammonia oxidation in the ETSP (Lipschultz et al. 1990). In comparison, anoxic incubations (under N_2) caused only <50% rate reduction compared to ambient O_2 (2.5 μM), and high ammonia oxidation rates were mostly associated with low O_2 (down to ~1–2 μM) (Lipschultz et al. 1990). Apparently, ammonia oxidizers are well adapted to low-O_2 conditions, while probably taking advantage of the higher NH_4^+ availability within the OMZs. They seem to undergo, at least in part, nitrifier denitrification that releases N_2O and so contributes to nitrogen loss.

N_2O is a potent greenhouse gas as well as a stratospheric ozone-depleting agent. In the oceans, approximately 25–50% of oceanic N_2O production is estimated to originate from oceanic OMZs (Suntharalingam et al. 2000). N_2O can be produced as an intermediate and also as a terminal product by denitrifying bacteria, depending on the species. Meanwhile, nitrifiers can also produce N_2O via hydroxylamine or nitrite, the so-called nitrifier denitrification. Because of an often observed correlation between N_2O and apparent oxygen utilization (AOU), and the fact that N_2O maxima are usually found in the upper OMZ, N_2O has mainly been attributed to nitrifiers (Nevison et al. 2003), though recent stable isotopic and isotopomer analyses suggest some contribution from denitrifiers in certain cases (Naqvi et al. 1998, Yamagishi et al. 2007, Farias et al. 2009). Further ^{15}N-incubation experiments in the Arabian Sea indeed indicated ^{15}N-N_2O production via both pathways (Nicholls et al. 2007).

Nitrite Oxidation

Nitrite is oxidized to nitrate with water via nitrite oxidoreductase (NXR) ($NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$), and the electrons produced are passed on to a terminal oxidase that consumes O_2 ($2H^+ + 2e^- + 0.5O_2 \rightarrow H_2O$) (Bock & Wagner 2006, Kumar et al. 1983). The whole reaction ($NO_2^- + 0.5O_2 \rightarrow NO_3^-$) produces modest amounts of energy ($\Delta G° = -82$ kJ mol^{-1}), which may explain the slow growth rates usually observed in nitrite oxidizers. Nitrite oxidizers have conventionally been considered obligate chemolithoautotrophs, but at least some species can grow chemo-organotrophically or mixotrophically using simple organic compounds like acetate and/or pyruvate (Bock 1976, Freitag et al. 1987, Daims et al. 2001). Nitrite oxidizers can also respire nitrate in the absence of oxygen, thus producing gaseous nitrogen oxides or NH_4^+ (Freitag et al. 1987, Bock et al. 1988). As the name suggests, nitrite oxidoreductase can mediate reactions in either direction (Sundermeyer-Klinger et al. 1984), and an additional nitrite reductase has been found in at least *Nitrobacter* (Ahlers et al. 1990). Both enzymes may participate in denitrifying reactions. All nitrite oxidizers found so far fall into five genera: *Nitrobacter*, *Nitrococcus*, *Nitrospina*, *Nitrospira* (Spieck & Bock 2005), and the recently discovered *Nitrotoga* (Alawi et al. 2007). Except for *Nitrospira*, which falls into the phylum Nitrospirae, nitrite oxidizers belong to different subclasses of Proteobacteria, among which *Nitrococcus*, *Nitrospina*, and perhaps sometimes *Nitrobacter*, are more common in marine environments.

Despite the potential importance of nitrite oxidation in suboxic waters, rate measurements for this process have been reported only once in the OMZ. In the ETSP OMZ (Ward et al. 1989, Lipschultz et al. 1990), nitrite oxidation rates exceeded ammonia oxidation rates in general and occasionally also nitrate reduction rates (Lipschultz et al. 1990). The local rate maxima of nitrite oxidation penetrated deeper into the OMZ than those of ammonia oxidation and appeared rather insensitive to suboxic/anoxic conditions. A rate reduction of only 28% was observed in anoxia, whereas an artificial increase to ~20 μM of O_2 resulted in a slight decrease in rates instead. As with ammonia oxidation, higher nitrite oxidation rates are generally linked to low O_2 conditions ($\leq$2.5 μM) (Lipschultz et al. 1990).

Altogether, it is apparent that the low-O_2 conditions found in the OMZs permit co-occurrence of both oxidative and reductive processes. Nitrite oxidation rates are not limited by ammonia oxidation rates, as they normally are under more oxic conditions due to an additional NO_2^- source from nitrate reduction. Nitrite oxidation recycles a fair proportion of the reduced NO_3^- (i.e., NO_2^- from NO_3^- reduction) back to the highest N-oxidation state (i.e., NO_3^-) and thus, to a certain extent, dampens the rate of nitrogen loss from the OMZs.

AUTOTROPHIC DENITRIFICATION

When surface production becomes intensely high, increased carbon export into (and so respiration within) the OMZ may lead to the depletion of O_2 followed by other major alternative electron acceptors. Sulfate reduction then ensues, resulting in a build-up of hydrogen sulfide. Sulfide is toxic to most metazoans and inhibits various microbial metabolic pathways, including ammonia oxidation and anammox. These nitrogen cycling processes become inhibited under euxinic conditions, whereas other sulfide-tolerant reactions such as DNRA and denitrification prevail. A number of sulfate reducers and sulfide/sulfur oxidizers are capable of DNRA (Simon 2002), and sulfate-reducing *Desulfovibrio* spp. have been isolated from the Peruvian OMZ (Finster & Kjeldsen 2010). Some of the sulfide/sulfur oxidizers can utilize NO_3^-/NO_2^- as electron acceptors and release gaseous N_2 and/or N_2O, while the evolved energy is harnessed to fix inorganic carbon. This unique nitrogen loss pathway is referred to as chemolithoautotrophic denitrification.

Chemolithautotrophic denitrification can also be linked to hydrogenotrophy, methanotrophy, and iron oxidation, but these will not be considered further in this review.

Like heterotrophic denitrification, chemolithoautotrophic denitrification is a stepwise reaction with NO_2^-, NO, and N_2O as intermediates, which may or may not be reduced further, depending on the organisms or environmental conditions involved (Robertson & Kuenen 2006). Chemolithoautotrophic denitrifiers utilize the same types of denitrifying enzymes as their heterotrophic counterparts. Different sulfur-containing compounds, including sulfide, elemental sulfur, and thiosulfate, may be oxidized even by the same species, whereas the end product can range from zero-valent sulfur to sulfate, depending on physiological conditions (Robertson & Kuenen 2006, Ghosh & Dam 2009). A typical and simplified reaction can be written as $2NO_3^- + 5HS^- + 7H^+ \rightarrow N_2 + 5S^\circ + 6H_2O$, which is highly favorable energetically ($\Delta G^\circ = -1{,}260$ kJ reaction^{-1}) for chemosynthesis. Although more energy can be gained ($\Delta G^\circ = -3{,}841$ kJ reaction^{-1}) by oxidizing HS^- to sulfate (SO_4^{2-}), four times as much NO_3^- is required ($8NO_3^- + 5HS^- + 3H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 4H_2O$), so that the former reaction becomes more favorable energetically under NO_3^--limiting conditions. Autotrophic denitrifying sulfur/sulfide oxidizers found so far mainly fall into different subclasses of Proteobacteria. These include the well-studied *Thiobacillus denitrificans* (β-subclass); *Magnetospirillum* (α-subclass); the nitrate-storing, filamentous *Beggiatoa* (γ-subclass) often found on surfaces of sulfidic sediments; the haloalkaphilic *Thioalkalivibrio denitrificans*; and a variety of ε-Proteobacteria such as *Thiomicrospira*, *Sulfurimonas*, and *Arcobacter* (McHatton et al. 1996, Gevertz et al. 2000, Sorokin et al. 2001, Inagaki et al. 2003, Ghosh & Dam 2009).

Using culture-independent approaches, representatives of the ε- and/or γ-proteobacterial–like variants have recently been detected in euxinic water layers of the Baltic Sea (Brettar et al. 2006), Saanich Inlet (Zaikova et al. 2010), and Namibian OMZ (Lavik et al. 2009). In the latter study, parallel ^{15}N-stable isotope pairing experiments further verified the activities of denitrification that coincided with the prevalence of both ε- and γ-proteobacterial chemolithoautotrophic denitrifiers during one of the sulfidic episodes over the Namibian shelf. Although sulfidic events may not be a regular feature in oceanic OMZs, episodic events have been noted in at least the Peruvian-ETSP OMZ (Dugdale et al. 1977) and the Arabian Sea off the Indian coast (Naqvi et al. 2000). Even during nonsulfidic conditions, similar ε- and/or γ-proteobacterial autotrophic denitrifiers have been found in the Chilean-ETSP OMZs (Stevens & Ulloa 2008) and Arabian Sea OMZs (Fuchs et al. 2005), thus implying persistent potentials of chemolithoautotrophic denitrification in oceanic OMZs. In fact, more sulfidic episodes in subsurface waters could easily have gone undetected due to a buffering effect by the actions of such autotrophic denitrifiers well below sea surface (Lavik et al. 2009). Hence, chemolithoautotrophic denitrification may be playing a more important role in oceanic nitrogen balance than previously assumed.

INTERACTIONS WITH OTHER ELEMENTAL CYCLES

Apart from sulfur cycling, both oxidative and reductive nitrogen transformations in the OMZs could be linked directly or indirectly to other elemental cycles, the most prominent ones probably being manganese (Mn), iron (Fe), and iodine (I). For both Mn and Fe, the highest oxidation states, Mn(IV) and Fe(III), occur as oxyhydroxides that are suspended particulates in seawater. Mn(IV) and Fe(III) concentrations decrease within the OMZs, whereas their dissolved and reduced forms (Mn^{2+} and Fe^{2+}) increase in abundance. Such pheonomena have been observed in at least the ETNP and Arabian Sea OMZs (Landing & Bruland 1987, Lewis & Luther 2000, Moffett et al. 2007). Similarly, iodine usually occurs at its highest oxidation state, iodate (IO_3^-), in the largely oxygenated oceans, but it is mostly replaced by the reduced form iodide (I^-) in the

OMZs, coinciding with the observed secondary nitrite maxima (Farrenkopf et al. 1997b, Rue et al. 1997, Farrenkopf & Luther 2002). Therefore, the vertical distributions of these three elements strongly suggest the occurrence of Mn, Fe, and IO_3^- reduction within the OMZs and are most likely microbially mediated. The electrode potential of the redox couple IO_3^-/I^- is placed even higher than NO_3^-/NO_2^- and is closely followed by Mn(IV)/Mn^{2+} (**Figure 2**). At the same time, the potential energy yields ($\Delta G°$) of IO_3^- and Mn reduction in respiration are comparable to complete denitrification or are even higher than nitrate reduction (**Table 1**). Hence, IO_3^- and Mn(IV) are logically favorable alternative electron acceptors to NO_3^- and could be responsible for a fair amount of organic matter remineralization and NH_4^+ production within the OMZs. In addition, Mn(IV) and at least I_2 have been suggested as alternative electron acceptors for nitrification in anoxic conditions but remain to be tested in OMZ waters (Cline & Richards 1972, Luther et al. 1997).

Interestingly, most Fe(III)-reducing microorganisms are also Mn(IV)-reducers, including the widely known *Geobacter* spp. and *Shewanella* spp. (Lovley 2006). Many of these species can reduce NO_3^- to NO_2^- and can perform DNRA (Richardson 2000, Simon 2002, Lovley 2006) or even complete denitrification (Brettar et al. 2002). Several *Shewanella* spp. have been isolated from the Arabian Sea OMZ, out of which one (*S. oneidensis*, formerly *S. putrefaciens*) has been found capable of IO_3^- reduction (Farrenkopf et al. 1997a). Besides *S. oneidensis*, some marine nitrate-reducing *Pseudomonas*, *Bacillus*, *Achromobacter*, and *Vibrio* species have also been observed to reduce IO_3^-, likely via their nitrate reductase enzymes (Tsunogai & Sase 1969), though a periplasmic iodate reductase has recently been identified in a denitrifying, IO_3^--reducing *Pseudomonas* species (Amachi et al. 2007).

In other words, many of the above-mentioned microorganisms are very versatile and can be involved in multiple elemental cycles, such as Fe/Mn-reducers being IO_3^- -reducers and/or NO_3^--reducers or even denitrifiers. Whether the same happens in the OMZs, and under which conditions, and whether multiple reduction processes may co-occur or shift in dominance within the same organisms remains to be investigated.

CURRENT KNOWLEDGE AND REMAINING PUZZLES

In summary, suboxic conditions in the OMZs permit the co-occurrence of multiple oxidative and reductive nitrogen transformations, resulting in an almost complete nitrogen cycle. Nitrogen loss is governed by the concerted actions of all these processes rather than just the process that leads to direct N_2 production, and there exists a close connection between nitrogen loss and organic matter via the remineralization product NH_4^+. At the same time, these nitrogen cycling processes are closely linked to other elemental cycles such as those of manganese, iron, iodine, and sulfur. Each microorganism may have more than one ecological and biogeochemical function, but different processes and different microorganisms that mediate these processes might predominate under different conditions. Many questions of when, where, and how remain regarding these microbial biogeochemical cycling processes in the OMZs, and more questions have emerged. They need to be addressed through the development of novel techniques, better sampling resolution in time and space, and more importantly, collaborative efforts across disciplines that involve microbiologists, geochemists, and physical oceanographers. Below are some of the emerging issues.

1. There is a consensus from several studies that anammox is the predominant nitrogen loss pathway in the ETSP OMZ and that denitrification occurs sporadically at most. However, in the Arabian Sea OMZ, the question of which is the dominant nitrogen loss pathway persists, whereas the same question remains untested in the ETNP. Hence, nitrogen loss in

especially these latter two major OMZs is undoubtedly an urgent subject for investigation. The fact that both denitrifiers and anammox bacteria are present in the OMZs implies that the potential for both processes exists. Nevertheless, which process is active and predominates in situ likely varies with changing environmental conditions. Meanwhile, the effects of such environmental changes on co-occurring nitrogen transformations should not be neglected in view of the interdependence of nitrogen cycling processes. Further studies should include more comprehensive examination of all of these processes together, while integrating geochemical and molecular ecological measurements with water circulation analyses within the OMZs.

2. The co-occurrence of multiple nitrogen cycling processes means that as soon as the product of one process is released, it is quickly taken up as a reactive substrate for another process. To disentangle this complex nitrogen cycle, stable isotope pairing experiments have proved useful to directly trace the fate of nitrogen through various transformations simultaneously (**Table 3**). Nevertheless, the results might be misleading at times. For instance, the production of $^{15}N^{15}N$ from $^{15}NO_2^-$ is usually a signature of denitrification, but a combination of anammox and DNRA could give the same results (Kartal et al. 2007a; M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data). Examination of ^{15}N-products in various nitrogen pools in the same incubation is thus necessary. At the same time, rate calculations are heavily reliant on the known ^{15}N fraction in the substrate pool, which is continuously being altered in the course of experiment due to the multiple consuming and producing processes that occur simultaneously. Rate corrections are difficult unless all nitrogen transformations are determined. Although some cases saw indistinguishable N_2 production rates calculated from various stable isotope combinations and those modeled from nutrient profiles, thus lending confidence to the determined rates (Jensen et al. 2008), inconsistencies also arise that can render nitrogen loss pathways ambiguous (M. Jensen, P. Lam, N. Revsbech, B. Nagel, B. Gaye, M. Jetten, and M. Kuypers, unpubl. data). Thus, it remains a challenge to determine the in situ gross rates of all co-occurring nitrogen transformations.
3. The coupling of anammox with heterotrophic processes, nitrate reduction, and DNRA, as well as ammonia oxidation, implies that substantial portions of nitrogen loss originate from the organic nitrogen pool (as remineralized nitrogen). In order to meet this remineralized nitrogen demand, it is nonetheless impossible to have much higher remineralization of Redfieldian organic matter occurring than currently estimated, owing to the constraints imposed by the closely related carbon cycle (Gruber 2004). The best explanation for the unaccounted source of remineralized nitrogen could be a preferential degradation of organic nitrogen over carbon in the OMZs (Van Mooy et al. 2002), or remineralization of nitrogen-enriched organic matter resulting from spatially coupled N_2 fixation over the OMZ (Deutsch et al. 2007). The occurrence of N_2 fixation in or near the OMZs, however, remains to be tested in situ.
4. Apart from anammox and heterotrophic denitrification, more incidences of sulfidic events on shelf OMZs have been reported in recent years, suggesting more frequent occurrences of chemolithoautotrophic denitrification (Naqvi et al. 2000, Diaz & Rosenberg 2008, Lavik et al. 2009). In light of the global expansion of OMZs in response to ocean warming, and the increasing natural or anthropogenically induced coastal eutrophication, the importance of chemolithoautotrophic denitrification in oceanic nitrogen loss is likely to increase, along with the emission of N_2O, an ozone-depleting and greenhouse gas, from these waters (Naqvi et al. 2000). Moreover, many shelf regions are important areas for fisheries, which are deleteriously affected by euxinic conditions. Hence, it is biogeochemically, ecologically, and

economically important to more closely examine the frequency and extent of these events in shelf OMZs.

5. Most of the nitrogen transformation processes reviewed here are heterotrophic processes and are thus dependent on the availability of organic matter. Although nitrification and anammox processes are chemolithoautotrophic, their requirement for NH_4^+, a remineralization product, renders an indirect dependency on organic matter. Even in chemolithoautotrophic denitrification, euxinia results from intense organic loading. Organic matter is thus likely the paramount regulating factor for oceanic nitrogen loss. Nevertheless, currently available data on organic matter in the OMZs are usually lumped into particulate versus dissolved pools, whereas only selected compounds, such as lipids, are analyzed in more detail. It is unlikely that the composition of organic matter is the same throughout the OMZs and at all times, or that different organic compounds have the same influence on various microbial processes. More detailed organic geochemical analyses in these waters are thus called for.
6. The reliance on organic matter by various nitrogen cycling processes might also explain the higher nitrogen transformation rates and overall nitrogen loss often detected in shelf OMZ waters. Moreover, shelf OMZs are often in direct contact with sediments, which are conducive to more active nitrogen loss. Nonetheless, current oceanic nitrogen budget estimates, as well as modeling studies using global nutrient data sets, often have to exclude the vicinity of continental shelf regions (e.g., Gruber & Sarmiento 1997, Deutsch et al. 2001), due in part to the greater errors that result from a paucity of data, and in part from the subsequent interpolation required for these regions in global nutrient data sets. Therefore, there is a strong need for high-quality biogeochemical and physical measurements, along with modeling efforts to accurately determine diffusive and advective nitrogen fluxes between shelf and open ocean regimes, that can be incorporated into global circulation–based nitrogen balance estimates.
7. Another remaining challenge comes from the organismal side. Many microorganisms are capable of multiple functions in the nitrogen cycle. For example, nitrifiers can conduct denitrification; some heterotrophic nitrifiers are aerobic denitrifiers; some denitrifiers can be nitrogen-fixers (Zumft 1997); and anammox bacteria are capable of DNRA, nitrate reduction, and nitrite oxidation. In addition, microorganisms involved in the nitrogen cycle also have a role in other elemental cycles. These metabolic traits have been found in cultures but have not necessarily been verified in the environment, though with potentially important biogeochemical consequences. It is, however, a challenge to determine which of the metabolic traits is being used by an organism. If sequences of nitrogen cycling biomarker functional genes (**Table 4**) of certain microorganisms are known, gene expression analyses are useful in identifying the active building of these key enzymes, thus serving as parallel evidence for the active occurrence of the targeted process (e.g., Lam et al. 2009). However, these analyses are mainly based on reverse-transcription polymerase chain reactions (RT-PCR), which are limited by the primer designs that may not cover all members of the targeted functional group due to the many unknowns in the immense biosphere. Neither can regular (RT)PCR-based methods make a direct link between cell activities and identities to distinguish alternative metabolic pathways from those of the microorganisms that are "normally" considered responsible. Recent advances in molecular biology such as metagenomics and metatranscriptomics (DeLong 2009), and various combined single-cell and stable isotope analytical techniques (Wagner 2009), may help shed light on the actual active roles played by various microorganisms under different conditions, at different time and space.

Table 4 Biomarker functional genes for the various nitrogen transformations likely relevant in oxygen minimum zones

Process	Gene	Encoding enzyme
Nitrate reduction[a]	*narG*	Membrane-bound dissimilatory nitrate reductase
	napA	Periplasmic dissimilatory nitrate reductase
Nitrite reduction[a]	*nirS*	Cytochrome cd_1 nitrite reductase
	nirK	Copper-containing nitrite reductase
Nitric oxide reduction[a]	*norB*	Nitric oxide reductase
Nitrous oxide reduction[a]	*nosZ*	Nitrous oxide reductase
Anammox	*nirS*	Cytochrome cd_1 nitrite reductase
	hzo	Hydrazine oxidoreductase
Dissimilatory nitrate reduction to ammonium	*nrfA*	Cytochrome *c* nitrite reductase
Ammonia oxidation	*amo*	Ammonia monooxygenase
	hao	Hydroxylamine oxidoreductase
Nitrite oxidation	*nxr*	Nitrite oxidoreductase

[a]Processes and functional genes necessary in canonical denitrification.

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Errata

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