The discovery of the Anammox process and beyond.

J.Gijs Kuenen[1)], B.Kartal[2)], M.S.M.Jetten[1,2)]

(1) Delft University Technology, Biotechnology, Julianalaan 67, NL-2628 BC, Delft, NL

(2) Radboud University Nijmegen, Microbiology, Toernooiveld 1, NL-6525ED Nijmegen, NL

The anammox reaction ($NH_4^+ + NO_2^- \rightarrow N_2$) was discovered and validated to be mediated by microbes in the early nineties. In 1999, the responsible bacteria were positively identified (Strous et al. 1999). These slowly growing microorganisms belong to the order Brocadiales and are affiliated to the Planctomycetes (Jetten et al, 2010). Cultivation of these bacteria at sufficient biomass density and rate (t_d=10-14 days), as well as the lack of pure cultures, have challenged the study of these extra-ordinary organisms. Membrane reactors have now made possible the cultivation of up to 95% enriched cell suspensions of anammox bacteria (Van der Star et al 2008).

Anammox bacteria are characterized by a compartmentalized cell architecture featuring a central cell compartment, the "anammoxosome" that is present in all anammox bacteria investigated so far (van Niftrik et al 2008 a,b) and assumed to be the locus of the anammox catabolism. Thus far unique "ladderane" lipid molecules have been identified as part of their membrane systems surrounding the different cellular compartments. Recently, a division ring could visualized by electron tomography (van Niftrik et al 2009) and further EM-evidence has shown that the anammoxosome is a separate, highly folded, organelle responsible for energy metabolism, not only packed with hydrazine oxidoreductase, but also with high concentrations of cytochrome-c along a 150 nm ring in the inside of the compartment. The anammoxosome is vertically inherited from mother to daughter cells. The cells undergo binary fission via a division ring, possibly encoded by a new type of gene, kustd1438, which was unrelated to *ftsZ* as found in other bacteria. Immunogold localization specifically localized kustd1438 to the cell division ring.

Nitrogen formation in the anammox pathway involves the intermediary formation of hydrazine, a very reactive and toxic compound. The genome of the anammox bacterium *Kuenenia stuttgartiensis* was assembled from a complex microbial community grown in a sequencing batch reactor (74% enriched in this bacterium) using a metagenomics approach. The assembled genome, combined with a variety of experimental data, allowed the *in silico* reconstruction of the metabolic pathway of the anammox reaction and identification of genes most likely involved in the process (Strous et al. 2006). Nitric oxide (NO) -rather than hydroxylamine as proposed earlier- is the most likely candidate- intermediate combining with ammonium to give hydrazine. The genome analysis showed the presence of a complete set of genes involved in the acetyl-CoA-pathway that is most likely responsible for autotrophic carbon assimilation, as previously predicted by stable carbon isotopic fractionation of ladderane lipids of anammox bacteria, and later confirmed by enzyme assays (Schouten et al 2004; Strous et al 2006).

Recent research (Kartal et al 2007a, 2008) has shown that anammox bacteria can also oxidize, but not directly assimilate, organic acids such as formate, acetate, propionate and methylamines. In this process the organic compound appears to be completely

oxidized with the concomitant reduction of nitrate or nitrite to ammonium. Hence the anammox bacteria are capable of producing a mixture of nitrite and ammonium, which can be used for its energy metabolism. This has consequences for the interpretation of field measurements using ^{15}N-labeled nitrate or nitrite, because doubly labeled dinitrogen gas is produced when anammox bacteria employ this pathway. It is common practice to ascribe the production of $^{15,15}N$-N_2 to regular, 'canonical' denitrification. Therefore, when organic compounds are available in the samples, it remains a challenge to experimentally discriminate between this canonical denitrification on one hand and the dissimilatory nitrate/nitrite reduction by either heterotrophic or by anammox bacteria on the other hand (Kartal et al 2007b, Jensen et al 2007; Lam et al 2009).

The habitat of anammox bacteria requires the simultaneous presence of ammonium and nitrite, which can be found mostly at or near the aerobic/anaerobic interface of sediments and water bodies. Anammox activity in nature was first demonstrated with ^{15}N-labeled ammonium by Thamdrup and Dalsgaard (2002). The essential evidence that the mixed ^{14,15}N-label was due to anammox bacteria was presented by Kuypers at al (2003) during an expedition to the Black Sea. This study also showed that there is a strong positive correlation between presence of ladderane lipids and anammox activity. Recent evidence, as discussed by Thamdrup and by Kuypers in this symposium, indicates that anammox activity is responsible for at least 50% of the N-turnover in oxygen limited marine environment. The habitat of anammox bacteria has recently been further extended as anammox activity, ladderane lipids and 16S rRNA gene libraries indicated the presence in hot springs (Jaeschke et al 2009), hydrothermal vent (Byrne et al 2009), and many fresh water wetland ecosystems (Ehler et al 2009).

The Anammox reaction is now also applied for large scale ammonium removal from municipal and industrial waste water (Van der Star et al. 2007). In such systems a combination of aerobic nitrification and anaerobic anammox conversion is required. The simplest version is a two-reactor process as described by Van Dongen (2001). Under oxygen limitation aerobic nitrification- and anammox reactions may simultaneously occur as demonstrated in lab and now also applied in industrial reactors for nitrogen removal (Third et al 2001, Van der Star et al. 2007). In such "model" systems aerobic nitrifiers and anammox bacteria coexist in different configurations, depending on the specific regime, (semi)continuous or discontinuous, of the operation. Study of these systems may greatly help in understanding the complex behavior of anammox processes in Nature such as the Peruvian OMZ (Lam et al 2009). Recent anammox reviews are by Kuenen (2008) and by Jetten et al (2009)

N. Byrne, M. Strous, V. Crépeau et al., *ISME J,* **3** (1), 117 (2009)
D.V. Erler, B.D. Eyer, L. Davison, *Environ Sci Technol*, **42** 9144 (2008)
A. Jaeschke , H.J. Op den Camp, H. Harhangi, et al., *FEMS Microbiol Ecol,* **67**, 343 (2009)
M.M. Jensen, B.Thamdrup, T. Dalsgaard, *Appl Environ Microbiol* **73** 3151 (2007)
M.S.M. Jetten, L. van Niftrik, M. Strous et al., *Crit Rev Bioch & Mol Biol*, **1**, 1–20 (2009)
M.S.M. Jetten, H.J.M. Op den Camp, J.G. Kuenen, M. Strous, *Bergey's Manual of Systematic Bacteriology* **in press** (2010)

B. Kartal, J. Rattray, L. A. van Niftrik et al., *Syst Appl Microbiol* **30**, 39 (2007a).
B. Kartal, M. M. M. Kuypers, G. Lavik et al., *Environ Microbiol* **9**, 635 (2007b).
B. Kartal, L. van Niftrik, J Rattray et al., *FEMS Microbiol Ecol* **63**, 46 (2008)
M.M.M.Kuypers, A.O..Sliekers, G.Lavik et al., *Nature* **422**, 608 (2003)
J.G.Kuenen, *Nature Rev Microbiol.* **6**, 320 (2008)
P. Lam, G. Lavik, M .M. Jensen et al, *P.N.A.S.* **106**, 4752 (2009)
S.Schouten, M.Strous, M.MM.M.Kuypers et al., *Appl Environ Microbiol* **70**, 3785 (2004)
M. Strous, J. A. Fuerst, E. H. Kramer et al., *Nature* **400**, 446 (1999).
M. Strous, E. Pelletier, S. Mangenot et al., *Nature* **440**,790 (2006)
B.Thamdrup, T.Dalsgaard, *Appl Environ Microbiol* **68**, 1312 (2002)
K.A. Third, A.O. Sliekers, J. G. Kuenen et al, *Sys Appl Microbiol* **24**, 588 (2001).
W.R.L. Van der Star, W. R. Abma, D. Blommers et al., *Water Res* **41**, 4149 (2007)
W.R.L. Van der Star, A.L. Miclea, U.van Dongen et al., *Biotech Bioeng* **101**, 286 (2008).
U.Van Dongen, M.S.M.Jetten, M.CC.M.Van Loosdrecht., *Water Sci.Technol* **44**,153 (2001)
L. Van Niftrik, W.J.C. Geerts, B.M. Humbel et al., *Mol Microbiol* **73**, 1009 (2009)

Distribution and controls of anaerobic ammonium oxidation in natural environments

Bo Thamdrup, Institute of Biology, University of Southern Denmark

Pathways of anaerobic ammonium oxidation

Ammonium is the thermodynamically stable state of nitrogen under anoxic, reducing conditions in aquatic environments, but oxidation to N_2 is exergonic in the presence of electron acceptors such as iron or manganese oxides, or nitrate at typical environmental concentrations. Oxidation with sulfate is also slightly exergonic at pH ≥ 7. Oxidation to nitrite, with some accumulation of this product, is thermodynamically feasible with nitrate and manganese oxide, but not with iron oxides or sulfate.

The metal oxides have been hypothesized to support anaerobic ammonium oxidation in sediments, aquifers, and anoxic waters (Luther et al. 1997, Hulth et al. 1999, Clement et al. 2005), and such processes would be relevant to nitrogen cycling during Earth's early, oxygen deficient history. Yet, no clear direct evidence of such processes – either spontaneous or biologically catalyzed – has yet been reported from natural environments, and experiments using ^{15}N as a tracer generally constrain the processes to very low potential importance in estuarine and marine environments (e.g., Thamdrup and Dalsgaard 2000, 2002, Engström et al. 2005, 2009). One recent study suggests that anaerobic ammonium oxidation to nitrite is linked to iron oxide reduction in wetland soil (Shrestha et al. 2009), although such a reaction seems to be endergonic at typical environmental conditions.

By contrast, ammonium oxidation coupled to nitrate reduction is documented in a wide range of marine sediments, anoxic waters and other environments, by both chemical distributions and tracer experiments. All available evidence indicates that anammox bacteria convey this process, with nitrite as the direct oxidant (Dalsgaard and Thamdrup 2002, Kuypers et al. 2003), and observations of nitrate- or nitrite-linked ammonium oxidation are now generally referred to as "anammox". A definite link between process and organism is, however, difficult to establish in many cases, particularly in sediments, and the potential involvement of other organisms or metabolic pathways cannot be fully excluded.

Environmental distribution of anammox activity

To date, anammox activity has mainly been detected in marine environments, including sediments and oxygen minimum zones, where the process seems to be essentially ubiquitous, as well as sea ice and hydrothermal vents. In contrast to this, there are as yet extremely few reports of anammox activity in freshwater environments, with the exception of locations that receive wastewater. The process has been detected in a stratified lake and an aquifer, but neither in lake sediments nor soils. The general exclusion of a microbial guild such as the anammox bacteria from terrestrial environments would be highly unusual. Also, several candidate genera of anammox bacteria have been described from (fresh) wastewater treatment systems, where the metabolism was first described, while only the candidate genus "Scalindua" dominates in the marine realm. Future searches for the anammox process in freshwater systems might benefit from

focusing at environments that are geochemically more similar to the marine environments where the process is important. Key factors seem to include the redox state, moderate organic loading, and some temporal stability.

Rates and controls of the anammox process

Through major contributions to N_2 production in many environments, anaerobic ammonium oxidation is an important component of the global nitrogen cycle. However, its contribution varies from 0 to 100% between environments, and the controls of this variation are only superficially understood. In marine sediments, which have been most extensively studied, water depth is a good predictor for much of the variation (Figure 1). Thus, anammox typically contributes little to N_2 production in shallow coastal sediments, but the contribution increases with water depth, and beyond ~50 m contributions are generally ~30% or higher. During the complete degradation of organic matter of Redfieldian composition through denitrification, with oxidation of the released ammonium through anammox, anammox accounts for about 29% of the N_2 production (Dalsgaard et al. 2003). Since both anammox and denitrification in marine systems are largely fuelled by reductants originating from organic matter (ammonium vs. organic C or Fe(II) or reduced sulfur compounds geretaed during anaerobic mineralization), large deviations from 29% indicate an imbalance in the relative efficiency of the two pathways in scavenging their respective electron donors. In shallow sediments with high organic loading it thus appears that ammonium from anaerobic mineralization escapes anammox, which suggests that anammox

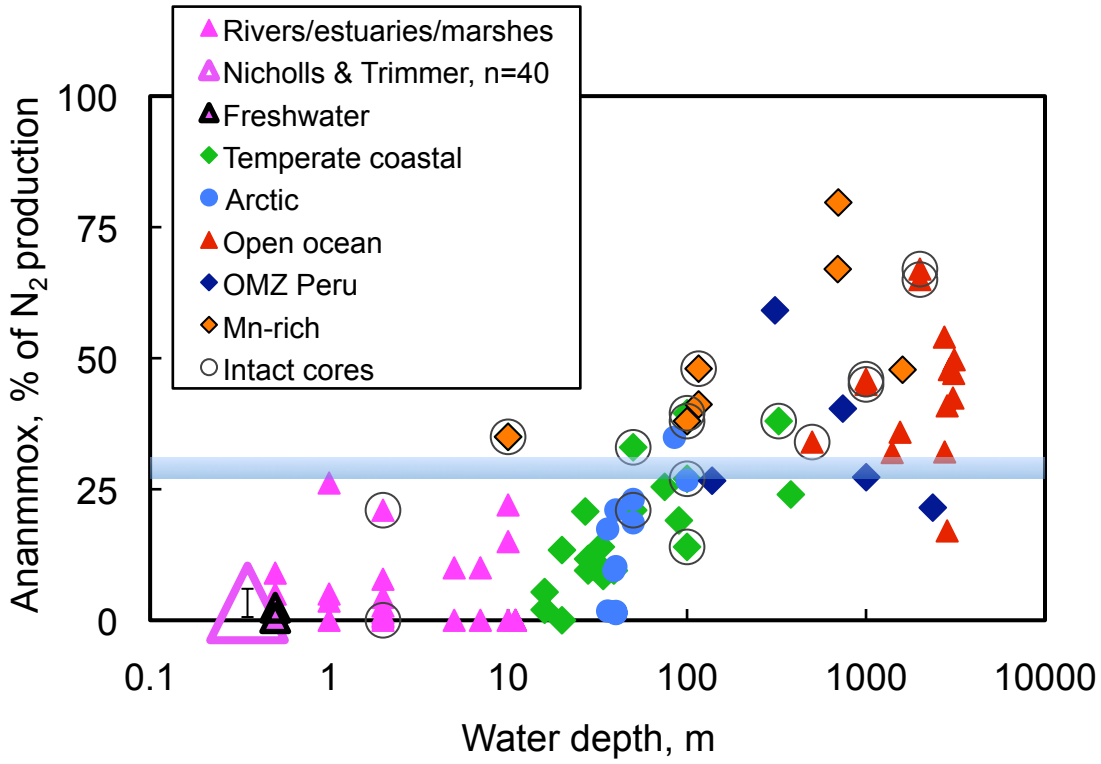

Figure 1 Compilation of the relative contribution of anammox to N_2 production in marine sediments and a few freshwater sites, based on experimental determinations. Most data are from incubations of homogenized sediment. Circles indicate data from intact cores. The blue line indicates a contribution of 29% as predicted from stoichiometric considerations (see text). After Thamdrup and Dalsgaard 2008 with newer data added. A list of data sources is available from the author.

is limited by the availability of nitrate or nitrite. Conversely, in deeper sediments, contributions from anammox up to 80% may be explained by a partial inhibition of denitrification through competition with dissimilatory manganese reduction. Based on the availabe data, it seems realistic that anammox contributes about 30% of the N_2 production on average in marine sediments.

In anoxic water columns, an even larger range in the relative importance of anammox is observed in an essentially bimodal distribution (Figure 2). At or near sulfidic interfaces, denitrification dominates completely, while anammox has been found to account for al N_2 production in anoxic, non-sulfidic systems as found in oxygen minimum zones and the suboxic zone of the Black Sea. Sulfide inhibition of anammox and temporal instability, may contribute to its absence from sulfidic systems (Jensen et al. 2009), but the complete exclusion of denitrification from oxygen minimum zones is paradoxical. Anammox depends on the release of ammonium from the mineralization of organic matter, but which type of respiration conveys this mineralization if not denitrification? Possible explanations include methodological artefacts, spatial heterogeneity, and other pathways of nitrate reduction (Kuypers et al. 2005, Thamdrup et al. 2006, Lam et al. 2009). Most recently, denitrification was found to dominate at locations in the OMZ of the Arabian Sea, suggesting that spatial heterogeneity plays a role (Ward et al. 2009). While further investigations are strongly needed to resolve this issue, the available data do indicate a substantial role for anammox in N_2 production in anoxic waters.

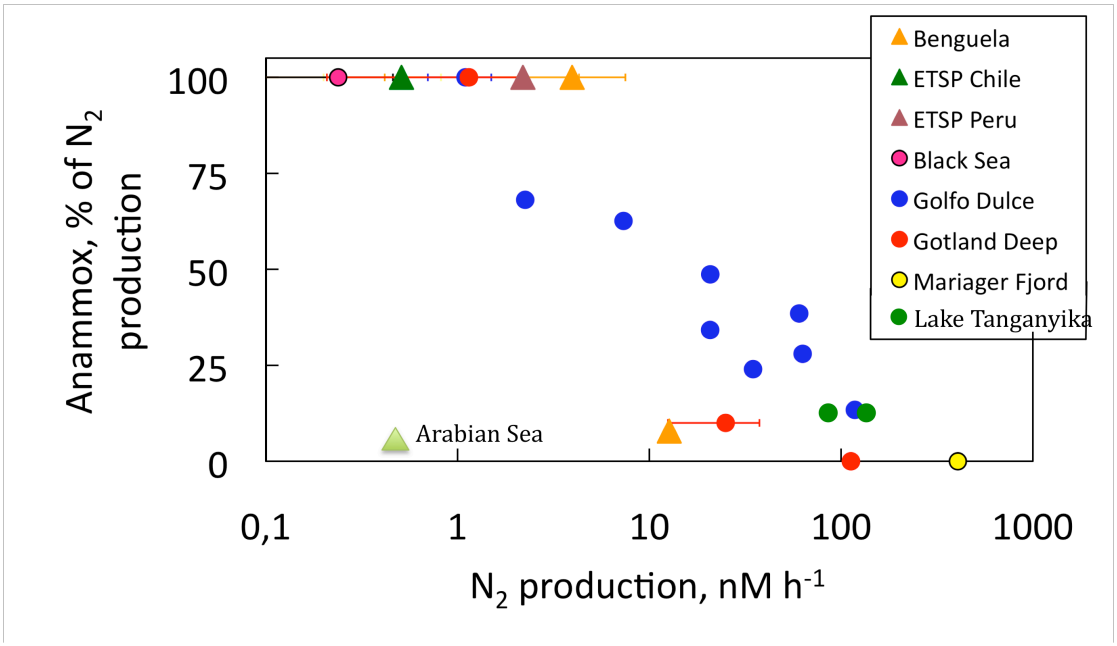

Figure 2 Compilation of the relative contribution of anammox to N_2 production in anoxic waters, based on experimental determinations. Triangles represent oxygen minimum zones while circles represent anoxic basins. A list of data sources is available from the author.

References

Clement J. C., Shrestha J., Ehrenfeld J.G., and Jaffe P.R. (2005) Ammonium oxidation coupled to dissimilatory reduction of iron under anaerobic conditions in wetland soils. *Soil Biol. Biochem.***37**, 2323-2328.

Dalsgaard T., Canfield D.E., Petersen J., Thamdrup B., and Acuña-Gonzalez J. (2003) N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature* **422**, 606-608.

Dalsgaard T. and Thamdrup B. (2002) Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. *Appl. Environ. Microbiol.* **68**, 3802-3808.

Engström P., Daldsgaard T., Hulth S., and Aller R.C. (2005) Anaerobic ammonium oxidation by nitrite (anammox): implications for N_2 production in coastal marine sediments. *Geochim. Cosmochim. Acta* **69**, 2057-2065.

Engstrom P., Penton C.R., and Devol A.H. (2009) Anaerobic ammonium oxidation in deep-sea sediments off the Washington margin. *Limnol. Oceanogr.* **54**, 1643-1652.

Hulth S., Aller R.C., and Gilbert F. (1999) Coupled anoxic nitrification/manganese reduction in marine sediments. *Geochim. Cosmochim. Acta* **63**, 49-66.

Jensen M.M., Petersen J., Dalsgaard T., and Thamdrup B. (2009) Pathways, rates, and regulation of N_2 production in the chemocline of an anoxic basin, Mariager Fjord, Denmark. *Mar. Chem.* **113**, 102-113.

Kuypers M.M.M., Lavik G., Woebken D., Schmid M., Fuchs B.M., Amann R., Jørgensen B.B., and Jetten M.S.M. (2005) Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc. Nat. Acad. Sci. U.S.A.* **102**, 6478-6483.

Lam P., Lavik G., Jensen M.M., van de Vossenberg J., Schmid M., Woebken D., Dimitri G., Amann R., Jetten M.S.M., and Kuypers M.M.M. (2009) Revising the nitrogen cycle in the Peruvian oxygen minimum zone. *Proc. Nat. Acad. Sci. U.S.A.* **106**, 4752-4757.

Luther G.W., III, Sundby B., Lewis B.L., and Brendel P.J. (1997) Interactions of manganese with the nitrogen cycle: alternative pathways to dinitrogen. *Geochim. Cosmochim. Acta* **61**, 4043-4052.

Shrestha J., Rich J.J., Ehrenfeld J.G., and Jaffe P.R. (2009) Oxidation of Ammonium to Nitrite Under Iron-Reducing Conditions in Wetland Soils Laboratory, Field Demonstrations, and Push-Pull Rate Determination. *Soil Sci.* **174**, 156-164.

Thamdrup B. and Dalsgaard T. (2000) The fate of ammonium in anoxic manganese oxide-rich marine sediment. *Geochim. Cosmochim. Acta* **64**, 4157-4164.

Thamdrup B. and Dalsgaard T. (2002) Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Appl Environ. Microbiol.* **68**, 1312-1318.

Thamdrup B., Dalsgaard T., Jensen M.M., Ulloa O., Farias L., and Escribano R. (2006) Anaerobic ammonium oxidation in the oxygen-deficient waters off northern Chile. *Limnol. Oceanogr.* **51**, 2145-2156.

Thamdrup B. and T. D. (2008) Nitrogen cycling in sediments. In *Microbial Ecology of the Oceans, 2nd edition* (ed. D. L. Kirchman), pp. 527-568. John Wiley & Sons.

Ward B.B., Devol A.H., Rich J.J., Chang B.X., Bulow S.E., Naik H., Pratihary A., and Jayakumar A. (2009) Denitrification as the dominant nitrogen loss process in the Arabian Sea. *Nature* **461**, 78-81.

Interactions of anammox with other microbial N-cycle processes

(Phyllis Lam[1], Gaute Lavik[1], Marlene Jensen[1], Mike Jetten[2], **Marcel Kuypers[1]**)

1Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany.
2Microbiology, IWWR, Radboud University Nijmegen, Nijmegen, the Netherlands.

In marine suboxic environments, various N cycling processes may be occurring simultaneously. Because the product of one nitrogen transformation is often the substrate of another, co-occurring processes are often difficult to detect using conventional chemical rate measurements. However, due to the microbially-mediated nature of the N-cycle, one may identify concurrent active processes via the expression of functional gene biomarkers. Based on whole-genome analysis of a marine anammox species *Scalindua* sp. T23, we identified potential functional gene biomarkers for the anammox process. One of the selected genes is a putative cd_1-containing nitrite reductase gene (*nirS*) unique to anammox bacteria. Quantitative gene abundance and expression of this anammox-*nirS* were proved to be in good agreement with parallel rate measurements via ^{15}N-isotope-pairing experiments. In several marine suboxic settings, we qualitatively and quantitatively compared the genetic potentials and active expressions of this anammox-*nirS* with the biomarker functional genes of various other N transformations. The identified active processes were further verified by ^{15}N incubation experiments. For example, in the central Black Sea, we found that anammox bacteria coexist with γ-proteobacterial ammonia-oxidizers within a narrow suboxic layer, with the latter providing 50% of the nitrite needed to fuel the anammox process. Meanwhile, it was crenarchaea that were the active nitrifiers in the immediately overlying oxic water instead, accounting for 75% of nitrite variations therein.

Using similarly multidisciplinary approaches, we found that in the Peruvian OMZ, the majority of nitrite for anammox came from nitrate reduction, whereas up to one-third came from aerobic ammonia oxidation. Anammox bacteria acquired ammonium from the degradation of organic matter associated with nitrate reduction, but also from dissimilatory nitrate reduction to ammonium (DNRA). Intriguingly, there was little evidence of N-loss in the central-northern Arabian Sea, previously considered the main region of N-loss. Instead, substantial N-loss occurred in the Omani upwelling area, via a close coupling between anammox and DNRA. Strong spatio-temporal variabilites are apparent in the Arabian Sea N-loss, which might be associated with the highly variable organic matter production in these waters.