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Production of oceanic nitrous oxide by ammonia-oxidizing archaea

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Abstract. The recent finding that microbial ammonia oxidation in the ocean is performed by archaea to a greater extent than by bacteria has drastically changed the view on oceanic nitrification. The numerical dominance of archaeal ammonia-oxidizers (AOA) over their bacterial counterparts (AOB) in large parts of the ocean leads to the hypothesis that AOA rather than AOB could be the key organisms for the oceanic production of the strong greenhouse gas nitrous oxide (N_2O) that occurs as a by-product of nitrification. Very recently, enrichment cultures of marine ammonia-oxidizing archaea have been reported to produce N_2O.

Here, we demonstrate that archaeal ammonia monooxygenase genes (*amoA)* were detectable throughout the water column of the eastern tropical North Atlantic (ETNA) and eastern tropical South Pacific (ETSP) Oceans. Particularly in the ETNA, comparable patterns of abundance and expression of archaeal *amoA* genes and N_2O co-occurred in the oxygen minimum, whereas the abundances of bacterial *amoA* genes were negligible. Moreover, selective inhibition of archaea in seawater incubations from the ETNA decreased the N_2O production significantly. In studies with the only cultivated marine archaeal ammonia-oxidizer *Nitrosopumilus maritimus* SCM1, we provide the first direct evidence for N_2O production in a pure culture of AOA, excluding the involvement of other microorganisms as possibly present in enrichments. *N. maritimus* showed high N_2O production rates under low oxygen concentrations comparable to concentrations existing in the oxycline of the ETNA, whereas the N_2O production from two AOB cultures was comparably low under similar conditions. Based on our findings, we hypothesize that the production of N_2O in tropical ocean areas results mainly from archaeal nitrification and will be affected by the predicted decrease in dissolved oxygen in the ocean.

1 Introduction

Atmospheric nitrous oxide (N_2O) is a strong greenhouse gas (Forster et al., 2007) and a major precursor of stratospheric ozone depleting radicals (Ravishankara et al., 2009). The ocean is a major source of N_2O contributing approximately 30 % of the N_2O in the atmosphere (Denman et al., 2007). Oceanic N_2O is exclusively produced during microbial processes such as nitrification (under oxic to suboxic conditions) and denitrification (under suboxic conditions; Bange et al., 2010; Codispoti, 2010). The formation of N_2O as a by-product of nitrification (oxidation of ammonia, NH_3, via hydroxylamine, NH_2OH to nitrite, NO_2^-) was reported for ammonia-oxidizing bacteria (AOB) (Frame and Casciotti, 2010; Goreau et al., 1980). In the case of nitrifier-denitrification NO_2^- can further be reduced to nitric oxide (NO) and N_2O (Poth and Focht, 1985; Shaw et al., 2006). The accumulation of oceanic N_2O is favored in waters with low oxygen (O_2) concentrations, which is attributed to an enhanced N_2O yield during nitrification (Goreau et al., 1980; Stein and Yung, 2003). The frequently observed linear correlation between ΔN_2O (i.e. N_2O excess) and the apparent oxygen utilization (AOU) is usually taken as indirect evidence for N_2O production via nitrification (Yoshida et al., 1989).

The traditional view that oceanic NH_3 oxidation is exclusively performed by AOB has been challenged by (1) the presence of archaeal *amoA* genes in metagenomes of various environments (Lam et al., 2009; Schleper et al., 2005; Treusch et al., 2005; Venter et al., 2004), (2) the successful isolation of the ammonia-oxidizing archaeon *N. maritimus* (Könneke et al., 2005) and (3) the fact that archaea capable of ammonia oxidation have been detected in various oceanic regions throughout the water column and in sediments (Church et al., 2009; Francis et al., 2005; Lam et al., 2009; Santoro et al., 2010; Wuchter et al., 2006). Moreover, *N. maritimus* appears to be adapted to perform ammonia oxidation even under the oligotrophic conditions (Martens-Habbena et al., 2009) that dominate in large parts of the open ocean. These observations point towards an important role of ammonia-oxidizing archaea (AOA, now constituting the novel archaeal lineage of *Thaumarchaeota*; Brochier-Armanet et al., 2008; Spang et al., 2010) for the oceanic nitrogen (N) cycle, which has been overlooked until recently (Francis et al., 2007; Schleper, 2010). Archaeal N_2O production has been proposed to contribute significantly to the upper ocean N_2O production in the central California Current and has recently been demonstrated to occur in two AOA enrichment cultures (Santoro et al., 2011). However, the ability of AOA to independently produce N_2O as a by-product of nitrification has not been directly demonstrated in pure cultures or in the ocean.

The eastern tropical North Atlantic (ETNA) and the eastern tropical South Pacific (ETSP) Oceans represent two contrasting oceanic O_2 regimes: while O_2 concentrations in the ETNA are commonly above $40\,\mu mol\,l^{-1}$, the ETSP regime is characterized by a pronounced depletion of O_2 in intermediate waters between ~ 75 and 600 m, resulting in an oxygen minimum zone (OMZ) with O_2 concentrations close to or even below the detection limit ($\sim 2\,\mu mol\,l^{-1}$) of conventional analytical methods.

The *amoA* gene coding for the alpha subunit of the ammonia monooxygenase is present in archaea as well as in β- and γ-proteobacterial ammonia-oxidizers and is commonly used as a functional biomarker for this group (Hallam et al., 2006b; Schleper et al., 2005; Treusch et al., 2005; Venter et al., 2004). Thus, in order to identify whether archaeal or bacterial *amoA* was associated with the maximum in N_2O concentration in the ocean, we determined the archaeal and bacterial *amoA* gene abundances and expression in relation to N_2O concentrations along vertical profiles during three cruises (in February 2007, February 2008, and June 2010) to the ETNA and one cruise (in January 2009) to the ETSP. Further, we demonstrated N_2O production in a pure culture of *N. maritimus* SCM1, which was found to be strongly O_2 sensitive and is thus suggested to be of highest impact at times of ocean deoxygenation (Stramma et al., 2010). N_2O production from pure cultures of the two marine nitrifying bacteria *Nitrosococcus oceani* NC10 and *Nitrosomonas marina* NM22 was low compared to the rates achieved by the archaeal isolate in our experiments.

2 Methods summary

2.1 Hydrographic parameters and nutrients

Samples for salinity, O_2 concentrations and nutrients were taken from a 24-Niskin-bottle rosette equipped with a CTD-sensor. Oxygen concentrations were determined following the Winkler method using 50 or 100 ml sampling volumes, and salinity and nutrient concentrations were determined as described in Grasshoff et al. (1999).

2.2 Determination of dissolved N_2O concentrations

Triplicate samples for N_2O analysis were taken from CTD casts during the cruises P348 (February 2007), ATA03 (February 2008), P399 (June 2010) to the ETNA and M77/3 (January 2009) to the ETSP. N_2O concentrations were measured with a GC headspace equilibration method as described in Walter et al. (2006); ΔN_2O and AOU were calculated as described therein.

2.3 Molecular genetic methods

2.3.1 Sampling

Seawater samples were taken from a minimum of 12 depths from the CTD casts. For the extraction of DNA and RNA a volume of about 2 l seawater was rapidly filtered (~ 30 min filtration time for samples from the ETNA, for samples from the ETSP exact filtration volumes and times were determined and recorded continuously) through 0.2 µm polyethersulfone membrane filters (Millipore, Billerica, MA, USA). The filters were immediately frozen and stored at $-80\,^{\circ}C$ until further analysis.

2.3.2 Nucleic acid purification

DNA and RNA was extracted using the Qiagen DNA/RNA AllPrep Kit (Qiagen, Hilden, Germany) according to the manufacturers protocol with a small modification. A lysozyme treatment ($50\,\mu g\,ml^{-1}$ for 10 min at room temperature) followed by a proteinase K treatment was performed prior to starting the extraction. Extracts of DNA and RNA were quantified fluorometrically using a NanoDrop 2000 (Thermo Scientific Fischer). A treatment with Dnase I (Invitrogen, Carlsbad, CA) was performed with the extracted RNA to remove any residual DNA; purity of RNA was checked by 16S rDNA PCR amplification before reverse transcription.

Table 1. Primers and PCR conditions: for real-time qPCR the initial denaturing step was 10 min at 95 °C, annealing temperatures were the same as in the end point PCRs, no final extension step took place, 40 cycles were performed followed by melting curve analysis. A fragment of 175 bp was amplified in qPCRs of archaeal *amoA*.

Target organism	Target gene	Oligonucleotide	Sequence ($5' \rightarrow 3'$)	PCR conditions	Reference
β-proteobact. ammonia-oxidizers	*amoA*	amoA1F′ amoA2R amoA-1F (qPCR) amoAR_new (qPCR)	GGGGTTTCTACTGGTGG CCTCKGSAAAGCCTTCTTC GGGGTTCTACTGGTGGT CCCCTCGGCAAAGCCTTCTTC	94 °C for 5 min, 30 × (94 °C for 20 s, 55 °C for 1 min, 72 ° C for 1 min), 72 ° C for 10 min	Rotthauwe et al. (1997)
γ-proteobact. ammonia-oxidizer	*amoA*	amoA3F amoA4R A189 (qPCR) A682 (qPCR)	GGTGAGTGGGYTAACMG GCTAGCCACTTTCTGG GGCGACTGGGACTTCTGG GAACGCCGAGAAGAACGC	94 °C for 5 min, 30 × (94 °C for 20 s, 48 °C for 1 min, 72 °C for 1 min), 72 °C for 10 min	Purkhold et al. (2000)
Archaeal ammonia-oxidizers	*amoA*	Arch-AmoAF Arch-AmoAR AamoA_for (qPCR) AamoA_rev (qPCR)	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT GGGCGACAAAGAAGAATAAACACTCGC ACCTGCGGTTCTATCGGCGT	95 °C for 5 min, 30 × (94 °C for 45 s, 50 °C for 1 min, 72 °C for 1 min), 72 °C for 10 min	Francis et al. (2005) this study

2.3.3 PCR and quantitative PCR

The extracted RNA was reverse transcribed to cDNA by using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturers' protocol.

Bacterial and archaeal *amoA* as marker genes for nitrification were PCR-amplified from DNA and cDNA. PCR and quantitative PCR conditions and primers are listed in Table 1. *nirS, nirK* and *nosZ* as marker genes for denitrification and the key functional marker gene for anammox, *hzo*, were PCR amplified according to established protocols (Lam et al., 2007; Schmid et al., 2008). The presence of key genes for anammox and denitrification was tested by PCR in the ETNA but quantified exclusively in samples of the ETSP. Assuming that the PCR detection system has a detection limit comparable to the respective qPCR (using the same Primers and PCR conditions), it should be in the range of 1 copy l^{-1} for *nirS* up to 4 copies l^{-1} in case of the other genes (deduced from the standard calibration curve in the qPCR assays). All PCRs were performed using 0.1 µl FlexiTaq (Promega Corporation, USA).

Absolute quantification of bacterial and archaeal *amoA* was performed with standard dilution series; quantification was performed in duplicates. Standards for quantitative PCRs were obtained from *Nitrosococcus oceani* NC10, *Nitrosomonas marina* NM22 and NM51 for bacterial *amoA* and from an environmental clone for archaeal *amoA* (GenBank accession number JF796147). The specifity of the newly developed qPCR primers detecting archaeal *amoA* was checked according to the Miqe guidelines (Bustin et al., 2009) by cross amplification tests and re-cloning and sequencing of the products. Reactions were performed in a final volume of 25 µl using 0.5 µl of each primer, 6.5 µl nuclease free water and 12.5 µl SYBR qPCR Supermix W/ROX (Invitrogen, Carlsbad, CA). Reactions were performed using an ABI 7300 Real Time PCR system (Applied Biosystems, Carlsbad, CA) according to Lam et al. (2007).

2.3.4 Construction of clone libraries and phylogenetic analysis

Cloning of PCR amplicons was performed using the Topo TA Cloning® Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Sequencing was carried out by the Institute of Clinical Molecular Biology, Kiel. Sequences for archaeal *amoA* were analyzed using the ClustalW multiple alignment tool on a 495 bp fragment (sequences were submitted to GenBank under accession numbers JF796145–JF796179); sequence differences were set on a minimum of 5 %, and phylogenetic trees were made using distance-based neighbour-joining analysis (Saitou and Nei, 1987).

2.4 Seawater incubations

Seawater incubations were performed at three different stations in the ETNA (cruise P399). The 25 ml serum bottles were filled with seawater from 200–250 m depth from the CTD casts, closed with an air-tight butyl rubber stopper and aluminum crimp-capped. Triplicate samples were taken to determine the initial N_2O concentration; six bottles were incubated, one triplicate as a control and one triplicate was treated with 1 mM of the hypusination inhibitor N1-guanyl-1,7-diaminoheptane (GC_7) (Jansson et al., 2000). Prior to the experiment, the sensitivity of AOA and AOB was checked using *Nitrosopumilus maritimus* SCM1, *Nitrosococcus oceani* NC10 and *Nitrosomonas marina* NM22 pure cultures. Different concentrations up to 1.5 mM GC7 were applied to the cultures, which did not affect ammonia oxidation

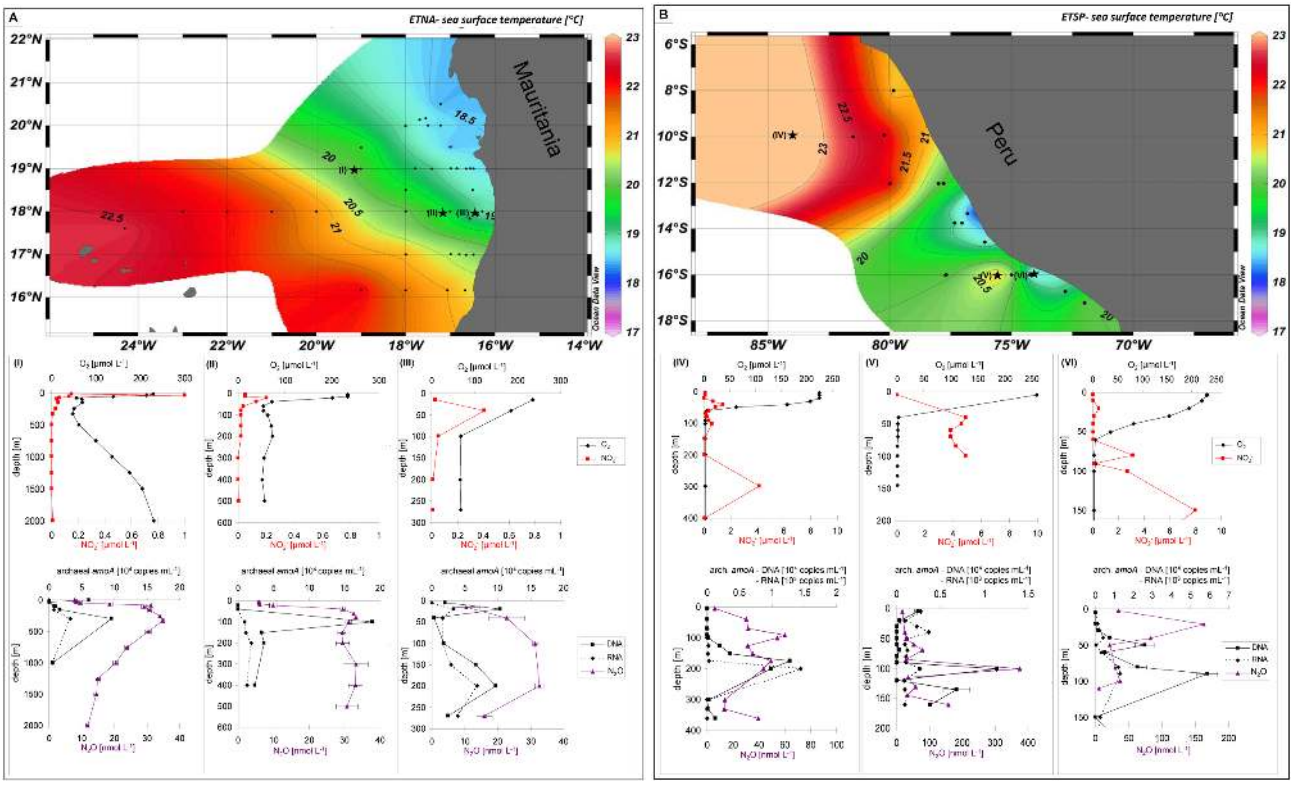

Fig. 1. Maps of sea surface temperatures **(A)** from the eastern tropical North Atlantic Ocean and **(B)** from the eastern tropical South Pacific Ocean. The locations of sampling stations are indicated with asterisks in the maps. Selected vertical profiles (I–VI) of O_2, NO_2^-, N_2O (measured in triplicates) and archaeal *amoA* (measured in duplicates by qPCR) are shown; (I) and (IV) are located offshore, (II) and (V) are located on the continental slope, and (III) and (VI) are onshore/coastal stations.

or growth behavior in AOB. In contrast, *N. maritimus* showed a decrease in ammonia oxidation and growth when applying GC_7 concentrations higher than 0.2 mM and shut down nitrification when applying GC_7 in a concentration of $\sim$ 0.8 mM.

Incubations were kept for the duration of the experiment (24 h) in the dark at 8 °C. The experiment was stopped by $HgCl_2$ addition, followed by the determination of the final N_2O concentrations.

2.5 Culture experiments

Pure cultures of *N. maritimus*, *N. oceani* and *N. marina* were grown in triplicates in 125 ml serum bottles (containing 75 ml culture and 50 ml headspace) at 28 °C according to Könneke et al. (2005) and Goreau et al. (1980). Serum bottles were closed with an air-tight butyl rubber stopper and aluminum crimp-capped. Cultures were kept for the duration of the experiment in the dark. Cell abundances from the triplicate samples were monitored by flow cytometry (FACScalibur, Becton, Dickinson) after staining with Sybr Green I (Invitrogen, Carlsbad). The accuracy of the flow cytometry was previously checked microscopically after staining the cells with the fluorescent DNA-binding dye 40, 6 0-diamidino-2-phenylindole (DAPI). Cultures were checked for contaminants microscopically and by 16S rDNA analysis. While tests for the bacterial 16S rDNA gene were negative, the analysis of 84 clones of archaeal 16S rDNA showed that all analyzed sequences matched the identity of the *N.maritimus* culture. The 16S rDNA gene was PCR amplified with universal primers, followed by Topo TA cloning and sequencing. Sequence analysis did not show any contaminants.

N_2O concentrations were measured by gas chromatography using the headspace method as described above, oxygen concentrations were determined using Winkler titration in 50mL Winkler bottles. NH_4^+ and NO_2^- concentrations were determined at several time points over the complete incubation time frame (Grasshoff et al., 1999). In order to exclude chemical N_2O production from the medium, cultures toxified with mercury chloride were measured in parallel; no N_2O production could be detected.

Isotopomeric studies were performed with cultures of 0.5 l volume, grown in serum bottles supplemented with $^{15}NH_4^+$ (10 % of total NH_4^+). Measurements were performed as described in Fehling and Friedrichs (2010) and Nakayama et al. (2007).

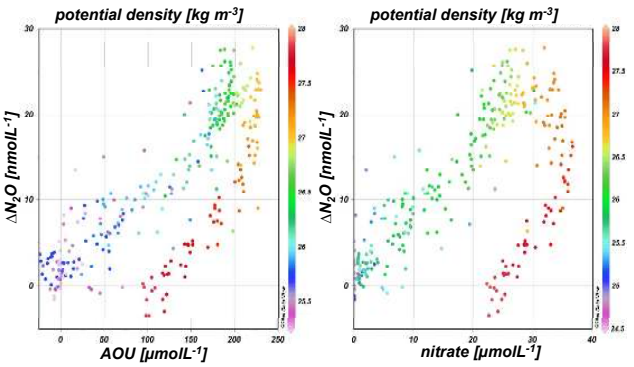

Fig. 2. ΔN_2O versus the apparent oxygen utilization (AOU) and nitrate in the ETNA (data from cruises ATA03, P348 and P399), the potential density is colour-coded.

3 Results and discussion

3.1 Vertical distribution of AOA and AOB along N_2O depth profiles

Vertical profiles of N_2O showed a distribution with concentrations between 10 and 35 nmol l^{-1} in the ETNA, whereas the N_2O concentrations in the ETSP were in the range from 10 to 374 nmol l^{-1} (Fig. 1). In the majority of the sampled stations in the ETNA and the ETSP, the accumulation of dissolved N_2O was associated with minimum O_2 concentrations, as expected (Codispoti, 2010). Maximum N_2O concentrations in the ETNA were generally lower compared to the ETSP, probably as a result of extremely depleted O_2 concentrations in the ETSP below 75 m resulting in enhanced N_2O accumulation (Suntharalingam et al., 2000; Codispoti, 2010).

The well-established linear correlation between ΔN_2O and AOU as well as NO_3^- (Nevison et al., 2003) was found for the ETNA (Fig. 2), indicating that nitrification was the likely pathway for N_2O production. A comparable pattern of the distribution of archaeal *amoA* genes and N_2O was observed in the water column of the ETNA (Fig. 3), strongly suggesting a correlation between AOA abundance and [N_2O] accumulation (Pearson correlation coefficient $r = 0.63$, statistical significance is indicated) in the layers with low O_2 (Fig. 4). The key genes for denitrification and anammox (*nirS* and *nirK* coding for nitrite reductases and *hzo* coding for the hydrazine oxidoreductase; Lam et al., 2007; Schmid et al., 2008) were not present in detectable amounts. A co-occurrence of N_2O and archaeal *amoA* genes was detected at certain depths, e.g. at profile V at 100 m water depth (Fig. 1) in the ETSP, but was not a general feature, possibly resulting from additional N_2O production via other processes such as denitrification, nitrifier-denitrification or anammox (Kartal et al., 2007) at present suboxic conditions. The presence of key genes of anammox and denitrification assayed and predominantly detected at coastal stations of the ETSP but also present in large parts of the area off Peru further points to

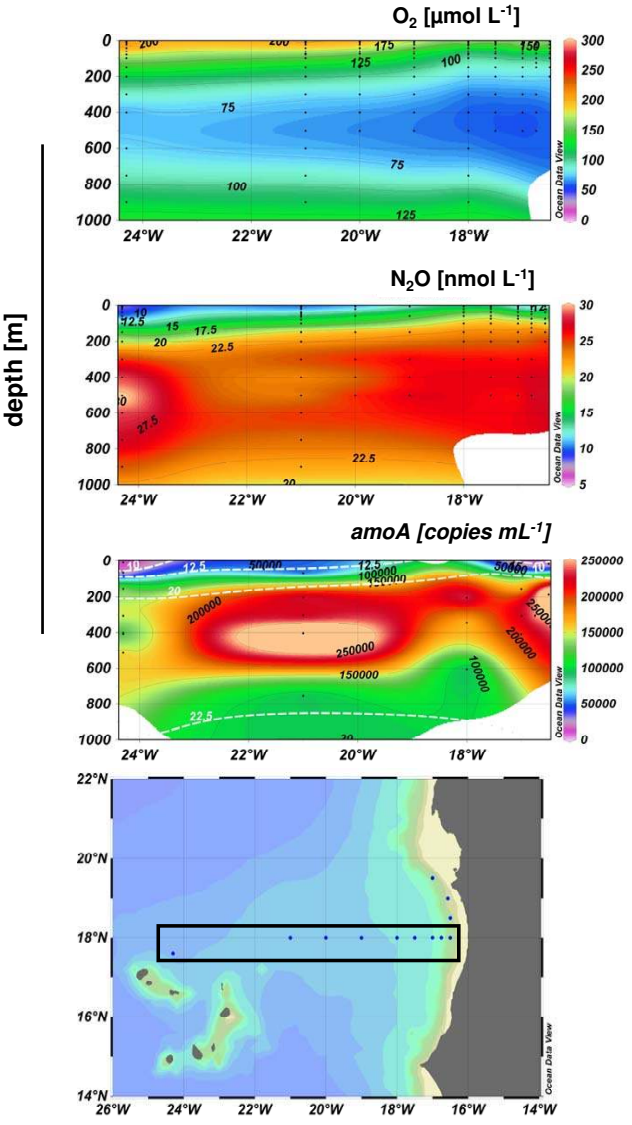

Fig. 3. Distribution of O_2 [µmol l^{-1}], N_2O [nmol l^{-1}] and archaeal *amoA* [copies ml^{-1}] along 18° N in the ETNA, detected during the cruise P399. Archaeal *amoA* abundances are overlaid by the detected N_2O concentration (dashed white line).

an active contribution of mixed processes to N_2O production in the ETSP (the complete dataset of the ETSP can be seen in Löscher (2011). N_2O production by mixed processes may explain the lack of correlation between ΔN_2O and AOU as well as NO_3^- in the ETSP (Ryabenko et al., 2012). Gene abundances of archaeal *amoA* in the ETNA and ETSP were detectable throughout the water column and reached values of up to 1.9×10^5 and 6×10^4 copies ml^{-1}, respectively (Fig. 1). Gene abundances of β- and γ-proteobacterial *amoA* were much lower (up to 950 and 1178 copies ml^{-1} in the ETNA and ETSP, respectively; Fig. S1 in the Supplement). This is in line with previous studies reporting 1–4 orders of magnitude higher abundances of AOA than AOB

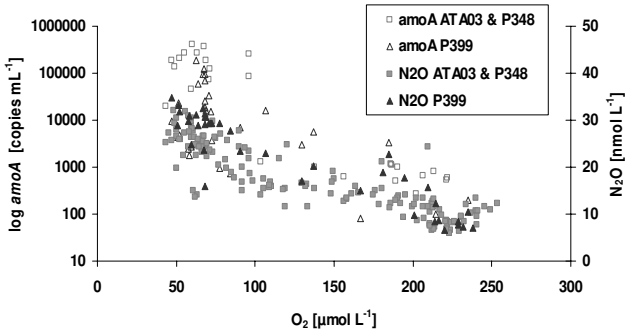

Fig. 4. Archaeal *amoA* and N_2O versus O_2 in the ETNA (data from the cruises ATA03, P348 and P399/2). A similar trend has been detected during the three cruises.

in various oceanic regions (Wuchter et al., 2006; Santoro et al., 2010; Lam et al., 2009; Francis et al., 2005; Church et al., 2009). Thus, we hypothesize that a significant part of the N_2O production occurs via archaeal nitrification in the ETNA and might also be present in parts of the water column of the ETSP. A difference of one order of magnitude between archaeal *amoA* copies in RNA and in DNA has been observed in vertical profiles of the ETSP, with copy numbers up to 7×10^4 copies ml^{-1} in the DNA and up to 1.5×10^3 copies ml^{-1} in the RNA. A similar tendency is detectable in the ETNA; however, the difference is less pronounced compared to the ETSP. This discrepancy, already reported by Lam et al. (2009), is hypothesized to be due to a diurnal cycle of ammonia-oxidation and therefore changing *amoA* expression. Moreover, a sampling bias due to comparably long filtration times (up to 30 min) might have led to RNA degradation, as previous studies reported transcript half-lives of down to 0.5 min in *Prochlorococcus* (Steglich et al., 2010).

3.2 Phylogenetic diversity of archaeal *amoA*

The diversity of AOA present in the ETNA was determined based on the analysis of ~ 300 *amoA* sequences from 15 stations of 3 cruises (P348, ATA03, and P399). Sequences were derived from up to 3 depths between the ocean surface and 1000 m, which showed archaeal *amoA* presence by PCR. The sequences split into two main clusters, with sequences from the O_2 minimum clustering mainly in cluster B (Fig. 5, Table S1 in the Supplement). Only 11.5 % of sequences derived from samples from the O_2 minimum fall into cluster A. Sequences derived from depths between the surface and the upper oxycline were present in both clusters to equal amounts (Fig. 5, Table S1 in the Supplement). In the ETSP, sequences from within as well as from depths above the OMZ separated into both clusters, with the majority of absolute sequence numbers from the OMZ affiliating with cluster B (Fig. 5, Table S1 in the Supplement), as already observed for the sequences from the O_2 minimum in the Atlantic Ocean.

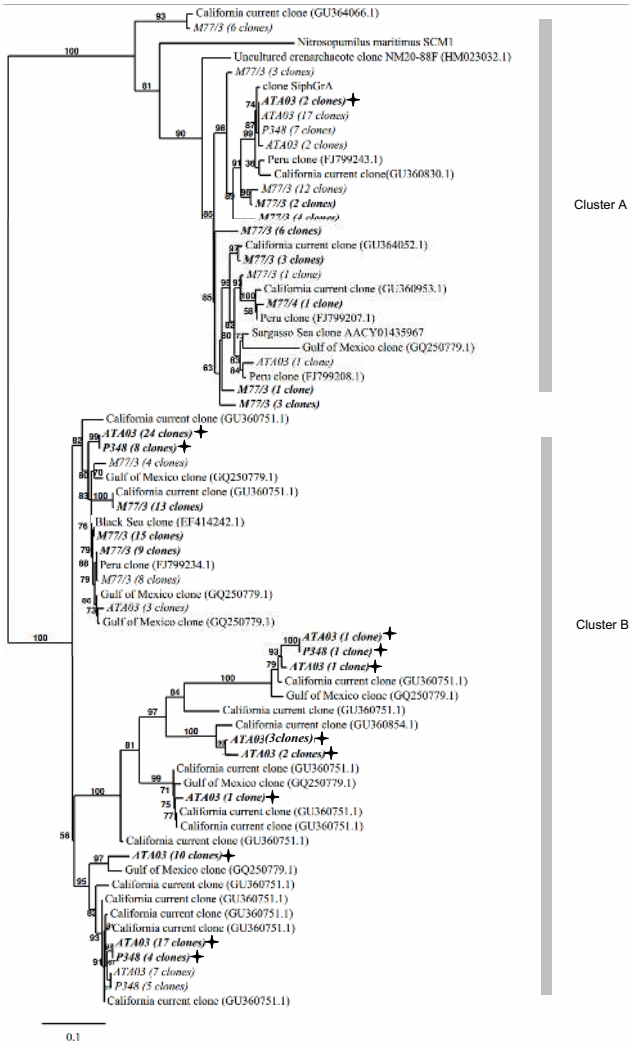

Fig. 5. Distance-based neighbour-joining phylogenetic tree of archaeal *amoA* sequences from the ETNA (cruises ATA03 and P348) and ETSP (cruise M77/3). Sequences derived from the oxygen minimum zone (OMZ) of the ETNA are in italics, bold and marked with solid stars; sequences from above the OMZ are in italics. Sequences from the OMZ of the ETSP are in italics and bold; sequences from above the OMZ are in italics.

3.3 Potential importance of cluster B affiliated Thaumarchaeota for N_2O production

The distribution of archaeal *amoA* genotypes along vertical profiles in the ETNA with the majority of cluster B sequences present in clone libraries from the OMZ suggest a production of N_2O by *Thaumarchaeota* affiliated with cluster B, previously reported to be a deep marine cluster (Hallam et al., 2006a) associated mainly with O_2 and NH_4^+ poor waters (Molina et al., 2010). A niche separation based on O_2 concentrations of cluster B affiliated AOA in the ETNA seems to be very likely, which is consistent with our data from the ETSP. Regarding the on-going decrease in dissolved O_2

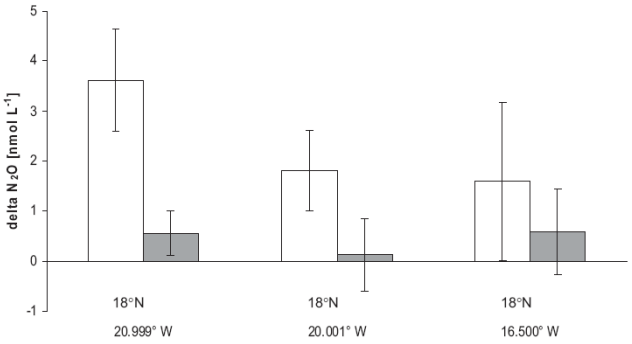

Fig. 6. N_2O production determined from seawater incubations at three different stations (1–3) from the ETNA (P399). ΔN_2O was calculated as the difference of N_2O concentrations over the incubation time (i.e. 24 h). Open columns represent samples with no inhibitor, filled columns represent samples with the archaeal inhibitor GC_7. Error bars indicate the standard deviation of three technical replicates.

concentrations in tropical ocean areas (Stramma et al., 2010), we hypothesize that cluster B affiliated AOA might dominate the production of N_2O and the balance between reduced and oxidized nitrogen species in the ocean, as those organisms are likely more adapted to low O_2 concentrations.

Both observed clusters were present in a similar distribution along vertical profiles during all cruises to the ETNA (Figs. 4 and 5, Table S1 in the Supplement). The community of AOA in this area appears therefore to be stable over the time investigated.

3.4 N_2O production in the ETNA

At three different stations in the ETNA, 24h seawater incubations using seawater from the N_2O maximum (at the depth of the OMZ) were performed. In two out of three experiments, N_2O production was significantly lower in samples treated with N^1-guanyl-1,7-diaminoheptane (GC_7) (Fig. 6), a hypusination inhibitor shown to selectively inhibit the cell cycle of archaea (Jansson et al., 2000), but which appears not to affect AOB (for detailed experimental data see Sect. 2). In the third experiment performed at a coastal station, a similar trend was observed; however it was not statistically significant. These findings further support our hypothesis that N_2O production in large parts of the ETNA occurs within the OMZ and is mainly carried out by archaea.

3.5 N_2O production in *N. maritimus*

Direct evidence for the production of N_2O by archaea was obtained from experiments with pure cultures of *N. maritimus*. The purity of the cultures was confirmed by extensive 16S rDNA analysis (see Sect. 2). *N. maritimus* cultures grew at comparable rates under the varying O_2 conditions and showed similar nitrification rates. Production of

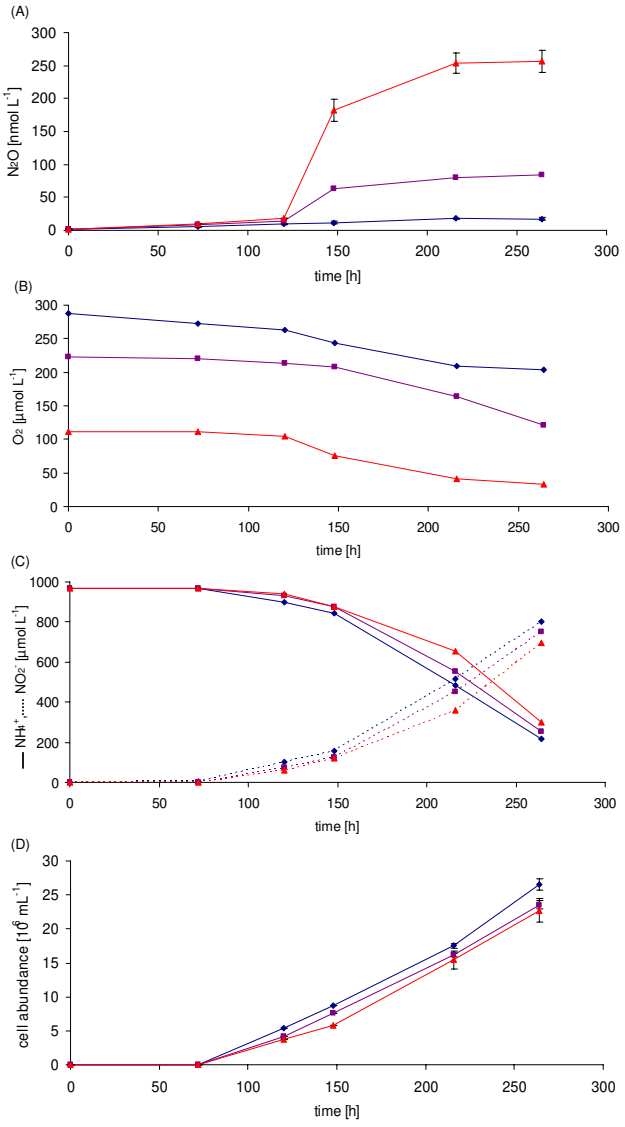

Fig. 7. N_2O **(A)**, O_2 **(B)**, NH_4^+ and NO_2^- **(C)** as well as cell abundances **(D)** determined from incubation experiments with pure cultures of *N. maritimus*. Experiments are colour-coded according to their initial O_2 concentrations: red (112 µmol l^{-1}); violet (223 µmol l^{-1}); and blue (287 µmol l^{-1}). N_2O and cell abundances were measured in triplicates and the associated error ranges are indicated (please note that in the most cases the error bars are too small to be visible in the figure).

N_2O in *N. maritimus* cultures was inversely correlated to O_2 concentrations (Fig. 7) which were chosen according to the O_2 concentrations present along the oxycline in the ETNA (112, 223 and 287 µmol l^{-1}, Fig. 1). N_2O production rates from two AOB cultures (*Nitrosomonas marina* NM22 and *Nitrosococcus oceani* NC10) were considerably lower compared to the N_2O produced by *N. maritimus* (Fig. 7, Table 2). The N_2O yields (N_2O/NH_4^+), which appear to result from ammonia oxidation, ranged from 0.002 %–0.03 %

Table 2. N_2O production in culture experiments: mean O_2 and N_2O concentrations (in triplicate samples) of pure cultures of *N. maritimus*, *N. marina* and *N. oceani* after 264 h incubation, the initial NH_4^+ concentration ($\sim 1\,mmol\,l^{-1}$) was completely converted to NO_2^- in the end of the experiment in AOB cultures, NO_2^- was below the detection limit at the initial time point of the incubation.

Culture	N_2O [nmol l^{-1}] after 264 h incubation	O_2 [µmol l^{-1}]	Ratio N_2O/ NH_4^+ [%]	Max. N_2O production [nmol l^{-1} day^{-1} 10^{-6} $cells^{-1}$]
Nitrosopumilus maritimus SCM1	254.75 ± 16.86	33.5	0.026	24.27
	82.63 ± 1.89	121.1	0.009	5.6
	15.57 ± 2.38	203.2	0.002	0.44
Nitrosomonas marina NM22	41.71 ± 0.039	44.7	0.006	4.17
	14.4 ± 0.4	142.9	0.003	1.44
Nitrosococcus oceani NC10	36.78 ± 1.33	49.8	0.005	3.68
	11.91 ± 0.33	163.7	0.001	1.21

in the culture of *N. maritimus* to 0.001 %–0.006 % in the AOB cultures. The N_2O production rates per cell derived from our AOB experiments are in agreement with those reported by Goreau et al. (1980), even though a different experimental setup was used. Culture experiments, such as those presented here, were performed with AOB cell densities ($\sim 10^5$ cells ml^{-1}), which are much higher than usually found in the ocean (10^2–10^3 cells ml^{-1}) (Wuchter et al., 2006; Lam et al., 2009). Thus, the N_2O production rates from the AOB cultures are probably overestimated and not representative as N_2O production per cell by AOB also depends on the present cell densities (Frame and Casciotti, 2010), with high cell densities leading to enhanced N_2O production. In contrast, the AOA cell densities in our culture experiment ($\sim 10^5$–10^7 cells ml^{-1}) were comparable to those present in the oceanic environment ($\sim 10^5$ cells ml^{-1}) and thus seem to be reasonably representative of the rates expected in natural populations of AOA.

Using the observed archaeal N_2O production rate for low O_2 conditions derived from our experimental results (140 nmol l^{-1} d^{-1}; normalized to 10^6 cells ml^{-1} yielding ~ 24 nmol l^{-1} d^{-1}, see Table 2), an upper estimate for the potential archaeal N_2O production would be around 14 nmol m^{-2} s^{-1} (however, NH_4^+ concentrations in our culture experiments were significantly higher than in the environment), assuming a thickness of about 50 m for the low O_2 layer as typically found in the ETNA. Compared to estimates of N_2O emissions from the ETNA to the atmosphere of up to 2 nmol m^{-2} s^{-1} (Wittke et al., 2010), potential oceanic archaeal N_2O production might be indeed significant.

3.6 Potential pathways for archaeal N_2O production

AOB can produce N_2O from NH_2OH during nitrification or from NO_2^- during nitrifier-denitrification (Kool et al., 2010; Shaw et al., 2006). In AOA however, the pathway of ammonia oxidation is yet not understood. So far, no equivalent to the hydroxylamine-oxidoreductase, which catalyses the oxidation of NH_2OH to NO_2^- during nitrification in AOB, has been identified (Könneke et al., 2005; Martens-Habbena et al., 2009; Walker et al., 2010), indicating that AOA likely use a different pathway than AOB do when producing N_2O. The detection of the nitrite reductase gene *nirK* in the sequenced genomes of cultured *Thaumarchaeota* (Walker et al., 2010) led to the theory that AOA might produce N_2O by nitrifier-denitrification, which might particularly impact at low O_2concentrations. To identify the origin of N_2O formation, isotopomeric studies were performed with *N. maritimus* pure cultures. Using the lowest O_2 concentration of the three chosen (112 µM), a ^{15}N site preference (SP_{N_2O}) in N_2O of 34 ± 12 ‰ was detected, consistent with results from AOA enrichments (Santoro et al., 2011), which is in agreement with the SP_{N_2O} of ~ 33 ‰ typically found in AOB cultures performing ammonia oxidation (Sutka et al., 2006) (for comparison: nitrifier-denitrification of AOB results in a SP_{N_2O} of about 0 ‰). Thus, our dataset points towards a production of N_2O via the oxidation of NH_4^+ to NO_2^-, potentially via an unknown intermediate as we were not able to detect NH_2OH in *N. maritimus* cultures using the method described in Schweiger et al. (2007). However, taking $\delta^{18}O$ data into account, Santoro et al. (2011) suggested a reduction of NO_2^- to N_2O. As we have not performed O_2 isotopomeric studies, we cannot exclude N_2O production via nitrifier-denitrification, particularly when O_2 becomes limiting as previously described for the Arabian Sea (Nicholls et al., 2007) where O_2 concentrations drop far more than in our experiments (lowest O_2 concentration ~ 112 µM).

4 Summary

Taken together, the high abundance of archaeal *amoA* relative to AOB, the frequently obtained comparable patterns of N_2O accumulation and archaeal *amoA*, the inhibition of N_2O production in seawater experiments in the presence of the archaeal inhibitor GC_7 as well as the N_2O production by *N. maritimus* add to the mounting evidence that, in large parts of the ocean, N_2O is produced by archaeal rather than by

bacterial nitrification. Further, the archaeal N_2O production appears to be highly sensitive to the dissolved O_2 concentration, with highest N_2O production rates at low O_2 concentrations such as those present in the OMZ of the ETNA. The predicted expansion of OMZs in the future in many parts of the ocean (Stramma et al., 2008) may lead to an enhanced N_2O production in the ocean (Naqvi et al., 2010) and therefore may have severe consequences for the budget of N_2O in the atmosphere as well.

Supplementary material related to this article is available online at: http://www.biogeosciences.net/9/2419/2012/bg-9-2419-2012-supplement.pdf.

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