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Biogeosciences Discussions

Interactive comment on "Production of oceanic nitrous oxide by ammonia-oxidizing archaea" *by* C. R. Loescher et al.

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Biogeosciences Discuss., 9, C738–C743, 2012 www.biogeosciences-discuss.net/9/C738/2012/ Biogeosciences Discussions Interactive comment on "Production of oceanic nitrous oxide by ammonia-oxidizing archaea" by C. R. Loescher et al. Anonymous Referee #2 Received and published: 22 April 2012

We thank the anonymous reviewer #2 for inspiring thoughts and comments and moreover for considering our dataset exciting. We modified the manuscript and addressed the general and detailed comments as follows:

Part 1. General comments. The discovery of ammonia oxidation within the domain

Archaea has challenged century-old paradigms about the biological control of nitrification and nitrous oxide production. Several recent studies have demonstrated that particularly in the marine water column putative ammonia oxidizing archaea (AOA) are significantly more abundant than ammonia-oxidizing bacteria (AOB). As such, AOA may have a significant impact on the nitrogen cycle of the coastal and open oceans. Indeed, very recently it was shown that AOA release N2O, an important greenhouse gas, that has long been known to be linked to nitrification. To this end, the study by Loescher et al. provides further insights into the distribution and potential sources of nitrous oxide in the oceans. The authors report nitrous oxide concentrations along with archaeal and bacterial ammonia monooxygenase gene phylogeny and abundances in the water column of the Eastern Tropical North Atlantic (ETNA) and Eastern Tropical South Pacific (ETSP). Furthermore, the authors provide long awaited data on the production of nitrous oxide by N. maritimus, the only marine ammonia-oxidizing archaeon available in pure culture. The authors show that N. maritimus produces N2O in pure culture, thus, corroborating previous measurements by Santoro et al. in AOA enrichment cultures. By comparison of laboratory and field data, the authors conclude that N2O in the tropical oceanic areas may derive predominantly from ammonia-oxidizing archaea. I really enjoyed reading the manuscript by Loescher et al. The manuscript provides exciting data, is reasonably well edited, and the figures are clear and provide easy access to the data. However, as I have outlined in my detailed comments, most of the data remain correlative and particularly the presentation of the molecular data and culture experiments require significant improvement in order to support the conclusions drawn by the authors. Thus, the high potential for novelty remains at least somewhat unfulfilled.

Part 2 Detailed comments. Q1: Page 2099 line 12ff: Based on the data shown in Fig. 3, I somewhat disagree with the statement "High copy numbers of archaeal amoA genes and high N2O concentrations co-occurred in the ETNA". Based on Fig. 3 elevated archaeal amoA gene copy numbers were associated with moderately high N2O concentrations. However, only rarely N2O maxima directly correlated with maximum

amoA gene copy numbers. Please rephrase.

R1: We agree that absolute maxima co-occur only at certain sampling depths and stations. However, we argue that there is an overall pattern, particularly when N2O and amoA gene copies decrease towards surface and deeper waters, which is roughly comparable, whereas the main peak areas overlap to a significant degree. Moreover, the amoA gene abundance does not necessarily translate into gene expression or metabolic activity and thus does only reflect the potential presence of AOA at certain stations and depths in the water body. We rephrased the respective sentence:

'A comparable pattern of the distribution of archaeal amoA genes and N2O was observed in the water column of the ETNA (Fig. 3) suggesting a potential coherence between AOA abundance and N2O accumulation (Pearson correlation coefficient r = 0.63; statistical significance is indicated) in the layers with low O2 (Fig. 4)...'

Q2: Page 2099 line 14ff: In the Methods section the authors describe PCR assays for nirS, nirK, nosZ, and hzo. Were neither of them detected, or were nirK and nosZ not tested in the ETNA samples? Please clarify!

R2: We agree with the reviewer that this was not very clearly stated. All genes mentioned (nirS, nirK, nosZ, and hzo) were tested by PCR in samples of the ETSP, nirS, nirK and hzo were also tested by PCR in samples from the ETNA, but were found to be absent, here. nosZ was not tested in ETNA samples, as it didn't make sense to us to test for nosZ when nirS was not detectable. We now rephrased the respective sentence:

'...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.'

Q3: Page 2099 line 15f: According to Schmidt et al. (1998) in anammox bacteria the hzo gene codes for the hydrazine oxidoreductase and hao for hydroxylamine oxidore-

ductase. Here, I think you would like to refer to hydrazine oxidoreductase, correct?

R3: We apologize for our mistake and thank the reviewer for pointing to it, we now changed the sentence to 'hzo coding for the hydrazine oxidoreductase'

Q4: Page 2099 line 23ff: Please correct reference: Loescher et al. 2011 or 2012?

R4: We corrected the reference to Loescher (2011), PhD thesis, Sensitivity to the biological oceanic nitrogen cycle to changes in dissolved oxygen, 120pp. Kiel

Q5: Page 2099 line 26f: Beta- and gammaproteobacterial amoA gene sequences were detected in separate qPCR assays. This is a rare and valuable dataset! Very few data are available on the distribution of gammaproteobacterial amoA genes. Thus, please report separately the numbers for beta- and gammaproteobacteria in ETNA and ETSP samples!

R5: We agree with the reviewer and added the beta- and gammaproteobacterial amoA gene distribution to a supplemental figure (Fig. S1, see below) and changed the sentence as follows:

'Gene abundances of β- and y-proteobacterial amoA gene were much lower (up to 950 and 1078 copies mL–1 in the ETNA and ETSP, respectively; Fig. S1).'

Fig. S1: Distribution of β- and γ- Proteobacterial amoA gene abundance along vertical profiles of N2O from the eastern tropical North Atlantic Ocean (upper 3 panels) and from the eastern tropical South Pacific Ocean (lower 3 panels). The locations of sampling stations are indicated with asterisks in the map, Fig.1. Selected vertical profiles (I-VI) 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.

Q6: Page 2100 line 6ff: The authors present amoA gene and transcript copy numbers for both ETNA and ETSP samples, respectively. The authors nicely discuss the potential pitfalls for interpretation of transcript copy numbers from environmental samples! Nonetheless, albeit no transcript numbers are reported for N. maritimus, one

should be able to assume that higher transcriptional activity is associated with higher metabolic activity. Can such a correlation also be found between amoA transcript numbers and N2O production? Or may N2O production be associated with lower gene copy to transcript ratios?

R6: This is a highly interesting point. We agree that one should assume that higher transcriptional activity should lead and be associated to higher metabolic activity. The argumentation with regard to transcript copy numbers from environmental samples refers to the difference in DNA and RNA copy numbers. However, in case of N2O production we can just speculate on a similar correlation. As N2O is a by-product, usually occurring at conditions which are not completely favorable for the organisms and which has (at least to our knowledge) no biological function for the organisms, one can probably not draw the same conclusion as for NH4+ oxidation to NO2-. Moreover, it could also be the case that higher N2O production is associated to lower metabolic activity. However, in our pure culture experiments, we were not able to monitor changes in amoA transcript numbers, as the incubation volumes were too small to get a representative amount of RNA, thus we unfortunately cannot draw any conclusion for the environmental observations from amoA transcript numbers in N. maritimus pure culture experiments. Nevertheless, we consider this idea as very interesting and we will try to follow up this point with larger volume incubations and further field experiments, in the future.

Q7: Page 2100, line 16ff: The phylogenetic analysis of the clone library data remains very superficial. The authors state that DNA samples from 15 stations with 12 depths each were included in the clone library analysis. Thus, from about 180 individual samples a total of _300 clones were sequenced, i.e. on average < 2 sequences per sample. This is definitely not a representative clone library for any sample and the conclusions withstand no statistical scrutiny whatsoever. I believe the point the authors are trying to make, that cluster B sequences are preferentially associated with low oxygen concentrations, is demonstrated more clearly in the following way: The authors could pool all

clone sequences obtained from stations and depth within the OMZ of the ETNA, and ETNP, respectively, and those from outside the OMZ (e.g. based on oxygen concentrations below and above 20 μM) and in a table summarize the numbers and percentage of cluster A and cluster B sequences in each group. This pooled analysis should reveal with much higher statistical confidence whether cluster A or B may be associated with high or low oxygen concentrations, respectively.

R7: We apologize for the confusion, as we did of course not sequence the full vertical profile with 12 depths from every station but rather compared OMZ samples vs. non-OMZ samples which showed amoA gene presence in PCR amplification tests. We tested all 12 depths for amoA presence by PCR, here we found amoA present in most of the samples, but we sequenced 15-30 single clones from up to 3 depths of the 15 different stations. We changed the sentences as follows and added the information on pooled sequences from OMZ waters (<60μM O2, as ETNA waters usually do not have O2 concentrations below 40-50μM) and surrounding waters in a table (Table S1):

'The diversity of AOA present in the ETNA was determined based on the analysis of $\sim$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 amoA presence by PCR.' 'The sequences split into two main clusters, with sequences from the O2 minimum clustering mainly in cluster B (Fig. 5, Tab. S1). Only 11,5 % of sequences derived from samples from the O2 minimum fall into cluster A. Sequences derived from depths between the surface and the upper oxycline were present in both clusters (Fig. 5). 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, Tab. S1), as already observed for the sequences from the O2 minimum in the Atlantic Ocean. ' Tab S1: Abundance of clusters A and B in clone libraries generated from samples from the ETNA and ETSP

Q8: Page 2100, line 16ff: It remains unclear how the DNA samples were processed.

Were the DNA samples pooled and then a single clone library was constructed? Or were 180 cloning reactions performed and between 1-3 clones selected from each library for sequencing? Please clarify.

R8: We apologize for the confusion. We set up 20 separate cloning reactions from 20 single samples (not pooled) and sequenced 15-30 single clones per reaction/ sample. We did not pool the DNA as we wanted to see potential differences between coastal and open ocean stations and different depths, however, exclusively differences between OMZ waters and surrounding waters turned out to be present. We changed manuscript accordingly.

Q9: Page 2101, line 1: The authors point out the importance of amoA cluster B-associated archaea for N2O production. Is there any basis for this distinction? Do the authors have actual evidence for any difference in N2O production in members of cluster A and B? (Compare also comments page 2102 line 24ff below).

R9: Unfortunately, N. maritimus is the only planktonic representative of AOA in available pure cultures. As N. maritimus is affiliated to cluster A, we can only speculate on cluster B AOA. The observation by Hallam et al. (2006) and Molina et al. (2010) however indicate that cluster B organisms are predominantly present in niches with low O2, which in turn favors N2O production. So, we argue that if those organisms are somehow adapted to low O2 conditions, they might have a larger impact on N2O production with further decreasing ocean deoxygenation. It is tempting to speculate that they produce higher amounts of N2O than N. maritimus (cluster A AOA).

Q10: Page 2101, line 17: The authors show that the chemical compound N-guanyldiaminoheptane (GS7) significantly reduces the production of N2O in samples from ETNA. The authors used 1 mM of this archaeal cell cycle inhibitor GS7 in these water samples. This is a major finding and could strongly facilitate the future analysis of archaeal nitrification. However, in situ doubling times of microorganisms between 5-14 days are not unusual. Does the inhibition of the archaeal cell cycle indeed inhibit am-

monia oxidation activity, and thus N2O production, significantly in 24h incubations? Or alternatively, could the high concentration of this compound also affect other organisms (e.g. eukaryotes) that excrete ammonia, and thereby diminishing the supply of ammonium to nitrification? I am sure the authors are also aware that AOB are sensitive to a wide range of chemical compounds. To the best of my knowledge, there are no data on the impact that GS7 may have on the activity of AOB. Did the authors test any AOB for sensitivity to GS7? Without actual confirmation that 1 mM of this compound does not inhibit N2O production by AOB this result remains at least somewhat inconclusive. The authors should discuss these data more comprehensively.

R10: This is an interesting aspect, we are actually not aware of potential effects on eukaryotes in our setup. However, an effect on the ammonia excretion (positively or negatively) cannot completely be ruled out. The three incubation, we presented here show a certain decrease in N2O production, however, we used water from the core of the OMZ in the Atlantic, where probably sinking organic particles are the major supply of ammonium to the water body compared to zooplankton excretion. The sensitivity of AOB was checked using N. oceani and N. marina pure cultures. Different concentrations up to 1.5 mM GC7 were applied on the cultures which did not affect ammonia oxidation or growth behavior. In contrast, N. maritimus showed a decrease in ammonia oxidation and growth when applying GC7 concentrations higher than 0.2 mM and shut down nitrification when applying GC7 in a concentration of $\sim$ 0.8 mM. This information has now been included in the methods section.

Q11: Page 2102, line 4ff: The authors report significantly higher N2O yields in N. maritimus than in their tested AOB strains. Judging from Fig. 7, the N2O production rate was likely not constant throughout the experiments? How much variability was there, actually, and under which conditions were the highest rates observed? Goreau et al. report at least one order of magnitude higher N2O yields in the tested marine Nitrosomonas strains than the authors present in Table 1. The statement in line 12f. should be revised accordingly.

R11: Highest rates were generally observed in incubations with AOA and AOB cultures at lowest O2 concentration when nitrification started. After some time, the organisms appeared to have adjusted to the respective condition and produced comparably lower amounts of N2O. Goreau et al. used a slightly different setup (e.g. a different source of NH4+) as we did, thus the dataset is not 100% comparable to ours, however, the N2O production per cell presented by Goreau et al. in table 1of his paper does from our point of view average to what we detected in N. marina under the respective O2 conditions. In our manuscript, we compared our production rates to those measured by Goreau, we did not refer to N2O yields as shown in table 2 in Goreau et al.. In this table (Goreau et al, tab.2) it is however visible, that his NO2- concentrations are up to ∼4 times higher, than those measured in our experiment.

Q12: Page 2102, line 24ff: This is a great comparison! However, the authors neglected a further very interesting and important comparison: How do the N2O yields in N. maritimus compare to the in situ N2O production rates shown in Fig. 6? Assuming AOA in situ at the ETNA and N. maritimus have the same N2O yield of 0.026% (Table 1), the GS7-sensitive in situ nitrification rates associated with 1.5 and 3 nM N2O production per day would amount to 5,700 to 11,500 nmol ammonia oxidation per liter per day at 20.001W, and 20.999W, respectively (=> 140 to 288 fmol per AOA cell per day). Are such high nitrification rates realistically possible in these areas?

R12: Clark et al. (2008) reported NH4+ to NO2- oxidation rates in the ETNA ranging from 0-150 nmol L-1 d-1, which is obviously much lower than those calculated from our experimental dataset. N2O production in the seawater incubation setup might thus have been overestimated as oxygen consumption in the serum bottles might have led to increased N2O production.

Q13: On the other hand, assuming AOA carry a single amoA gene copy, approximately 4x10e7 cells per liter (= 4 x 10e4 archaeal amoA per ml average in Fig. 1, panel I and II) producing between 1.5 and 3 nmol N2O per liter per day (Fig. 6) would have a 1.5 to 3-fold higher daily rate of N2O production than N. maritimus. Thus, either the

gene copy numbers must be underestimated by at least one order of magnitude, the N2O production in the samples overestimated by an order of magnitude, or one could speculate that the N2O yields of AOA in situ at the OMZ may be significantly higher than in N. maritimus. Thus, if OMZs are indeed dominated by cluster B-affiliated AOA; these organisms may have significantly higher N2O yields than cluster A-affiliated AOA (including N. maritimus). A future expansion of OMZ and potentially cluster B-affiliated AOA could thus have important consequences for the oceanic N2O budget.

R13: Thanks for this inspiring comment, we can just speculate on difference of productivity between cluster A and B, but this would make a strong point for the importance of cluster B. However, we cannot completely rule out that N2O production in the seawater incubation setup might have been overestimated as oxygen consumption in the serum bottles might have led to increased N2O production (see above).

Q14: Page 2102, line 4ff: In Fig. 7 the authors report oxygen concentrations over the course of the experiment. According the methods section, the time course experiments were conducted in 125ml serum vials and oxygen concentrations were determined by Winkler titrations. To the best of my knowledge this can't be possible. Winkler titrations would require significantly bigger water samples. Please add more detailed explanation on how the experiment was carried out.

R14: We conducted Winkler titrations using a volume of 50 mL, which is a volume usually applied on environmental samples or pure culture incubations according to the original method described in Grasshoff, 1976 and Grasshoff, 1999.

We now added the information on the Winkler titration to the methods section:

'Hydrographic parameters and nutrients Samples for salinity, O2 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 mL sampling volumes. . .'

‘Culture experiments N2O concentrations were measured by gas chromatography using the headspace method as described above, oxygen concentrations were determined using Winkler titration in 50mL Winkler bottles.’

Q15: Page 2105, line 19ff: Several conclusions of this manuscript are based on the absence of nirS, nirK, nosZ, and hzo genes in ETNA samples. Please report the detection limits of your quantitative PCR assays for the specific genes.

R15: Those genes were PCR detected in the ETNA but quantified exclusively in samples of the ETSP. However, the PCR detection system should give a comparable detection limit as the respective qPCR would give (using the same Primers and PCR conditions) so that a detection limit of the respective genes should be in the range of 1 copy L-1 for nirS up to 4 copies L-1 in case of the other genes (deducted from the standard calibration curve in the qPCR assays). This information has now been added to the methods section.

Q16: Page 2106, line 9ff. Please add more detailed description of DNA processing, sample pooling, and number of clone libraries constructed (see comment above)

R16: see above R8

Q17 Page 2107, line 3: Were the experiments shown in Fig. 7 really carried out in 125ml serum bottles? What were the actual culture and headspace volumes? And what were the incubation temperatures for the different strains?

Q17: Culture experiments were carried out as described in 125mL serum bottles containing 75 mL culture and 50 mL headspace. The incubation temperature was 28°C, which has also been used in previous studies with AOA and AOB. This information has been now added to the methods section:

‘Pure cultures of Nitrosopumilus maritimus SCM1, Nitrosococcus oceani NC10 and Nitrosomonas marina NM22 were grown in triplicates in 125 mL serum bottles (containing 75mL culture and 50mL headspace) at 28°C according to Goreau et al. and

Könneke et al. (Könneke et al., 2005;Goreau et al., 1980).’

Q18: Page 2107, line 4: Check publication years.

R18: We corrected the publication years (see above).

Q19: Page 2107, line 6f: Were the cell abundances reported in Fig. 7 generated by the DAPI method, the flow cytometry method, or both? Please clarify.

R19: The presented cell counts were derived from flow cytometry (using a FACScalibur, Becton, Dickinson), after staining the cells with Sybr Green I (Invitrogen). However, before applying flow cytometry, we checked microscopically (using the DAPI method) whether cell counts of the flow cytometer are correct. As this is obviously unclear in the methods section, we changed the respective sentences as follows:

‘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).’

Q20: Page 2115, Fig. 1: DNA and RNA profiles are indistinguishable from each other. Please correct. Panels 1-VI are supposed to reflect dots in the maps. There are at least 30 dots. Please clearly mark stations I-VI in the maps. Remove other dots or add further explanation in caption. From which cruises originate the data?

R20: We agree and adjusted the map and the DNA and RNA profiles (see response to referee 1); stations corresponding to the profiles are indicated with asterisks and profile numbers corresponding to the vertical profiles, below. RNA/DNA profiles are distinguishable (dashed and solid lines). The data originated from the ATA03 cruise (ETNA) and the M77/3 cruise (ETSP).

Q21: Page 2122, Fig. 7: It should be stated whether a representative growth curve or averages of the three replicates are shown.

R21: The figure shows averages of three replicates which grew in this case quite similar. The error ranges are indicated by error bars, which are sometimes very small. This has already been stated in the figure legend, and was now also added to the methods section:

'Cell abundances from the triplicate samples were monitored by flow cytometry (FACScalibur, Becton, Dickinson) after staining with Sybr Green I (Invitrogen, Carlsbad). '

Q23: END OF REVIEW and evening.

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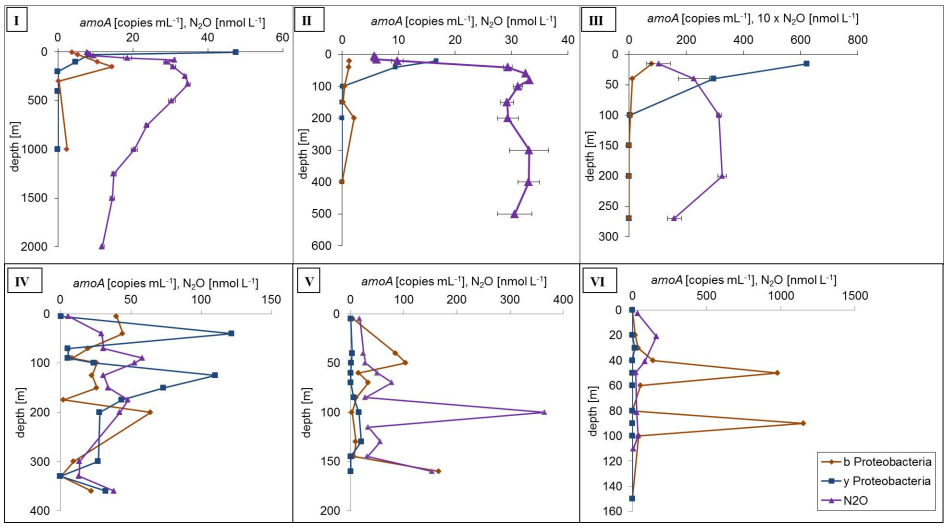

Fig. 1. Fig. S1:Fig. S1: Distribution of β- and γ- Proteobacterial amoA gene abundance along vertical profiles

Area	O_2	No. of sequenced clones	% Cluster A sequences	% Cluster B sequences
ETNA OMZ	< 60µmol/L	96	11.5	88.5
ETNA surrounding waters	> 60µmol/L	91	48.4	50.6
ETSP OMZ	< 30µmol/L	70	8.6	91.4
ETSP surrounding waters	> 30µmol/L	66	56.1	43.9

Fig. 2. Tab S1: Abundance of clusters A and B in clone libraries generated from samples from the ETNA and ETSP