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Brief Report

Lost in translation: the quest for *Nitrosomonas* cluster 7-specific *amoA* primers and TaqMan probes

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Summary

The choice of primer and TaqMan probes to quantify ammonia-oxidizing bacteria (AOB) in environmental samples is of crucial importance. The re-evaluation of primer pairs based on current genomic sequences used for quantification of the *amoA* gene revealed (1) significant misrepresentations of the AOB population in environmental samples, (2) and a lack of perfect match primer pairs for *Nitrosomonas europaea* and *Nitrosomonas eutropha*. We designed two new *amoA* cluster 7-specific primer pairs and TaqMan probes to quantify *N. europaea* (nerF/nerR/nerTaq) and *N. eutropha* (netF/netR/netTaq). Specificity and quantification biases of the newly designed primer sets were compared with the most popular primer pair (amoA1f/amoA2r) using DNA from various AOB cultures as individual templates as well as DNA mixtures and environmental samples. Based on the qPCR results, we found that the newly designed primer pairs and the most popular one performed similarly for individual templates but differed for the DNA mixtures and environmental samples. Using the popular primer pair introduced a high underestimation of AOB in environmental samples, especially for *N. eutropha*. Thus, there is a strong need for more specific primers and probes to understand the occurrence and competition between *N. europaea* and *N. eutropha* in different environments.

Introduction

The chemolithotrophic aerobic ammonia-oxidizing bacteria (AOB) carry out the first step of nitrification, the biological oxidation of ammonium to nitrite. AOB are an integral component of the nitrogen cycle in natural environments as well as wastewater treatment systems, and therefore, significant attention has been given to determine the quantitative dynamics of AOB. It is known that the 16S rRNA gene sequence-based approach (using the AOB targeting CTO primer pair) may result in co-amplification of sequences belonging to other microbial groups (Rotthauwe *et al.*, 1997; Sekido *et al.*, 2008; Baptista *et al.*, 2014). Therefore, AOB are routinely quantified with real-time PCR (qPCR) targeting ammonia monooxygenase alpha subunit (*amoA*) gene (Aakra *et al.*, 2001), in both natural and engineered systems (Sharma *et al.*, 2007; Orschler *et al.*, 2019).

Two decades back, Rotthauwe *et al.* (1997) designed the popular primer set amoA1f/amoA2r (hereafter called 'Rott pp') with two degenerate positions to generate a 491-bp-long PCR product for broader coverage of the AOB. This primer pair is, in most cases, the first choice (a commonly used primer pair), although covering the entire bandwidth of all AOB with a universal *amoA* primer pair is challenging (Rotthauwe *et al.*, 1997). Since the development of the Rott pp, there has been rapid development in molecular biology, leading to continuous expansion of our understanding of complex microbiomes. Also, more genomic sequences have become available for the diverse AOB. Consequently, the lack of 100% sensitivity and specificity (i.e. a perfect match (PM) primer set) of the Rott pp for all β-subclass AOB has been pointed out recurrently (Purkhold *et al.*, 2000; Dionisi *et al.*, 2002; Harms *et al.*, 2003; Hornek *et al.*, 2006; Junier *et al.*, 2009; Dechesne *et al.*, 2016). Also, the Rott pp has a number of mismatches with the *amoA* gene of some AOB, which affect their quantification in unknown microbiomes (Okano *et al.*, 2004; Meinhardt *et al.*, 2015). The Rott pp was designed with the idea of a 'universal primer to maximize coverage of AOB diversity', instead of fine-scale quantification of diverse AOB subgroups, which is more useful for understanding and controlling an engineered ecosystem. In an attempt to

circumvent this problem, Harms *et al.* (2003) designed a primer pair targeting the *amoA* gene and a TaqMan probe for specific quantification of *Nitrosomonas oligotropha* type, which belongs to cluster 6A (Koops and Pommerening-Röser, 2001). Layton *et al.* (2005) developed new primer sets for *N. nitrosa*, to identify AOB population dynamics in a wastewater treatment plant (WWTP). However, specific primers for *N. europaea* and *N. eutropha* are missing, even though *N. europaea* and *N. eutropha* have been reported as the most abundant AOB in several environments like freshwater (Stehr *et al.*, 1995) or WWTPs (Siripong and Rittmann, 2007). *Nitrosomonas* cluster 7 members (*N. europaea* and *N. eutropha*) are predominantly found in WWTP due to their environmental niches of nitrogen-rich environments (Wagner *et al.*, 1995; Purkhold *et al.*, 2000; Bollmann *et al.*, 2002). This includes conventional biological nitrogen removal systems, as well as partial nitritation/anammox (PN/A) systems (Vlaeminck *et al.*, 2010).

Understanding the role of AOB in various environments adequately and studying their response to dynamic changes in the environment requires a robust method with highly specific primer pairs. The lack of perfect match bacterial *amoA* primers can lead to over- or underestimation of the AOB population (Dionisi *et al.*, 2002; Dechesne *et al.*, 2016), which is critical for understanding inter- and intra-microbial group competition. With degenerated primers, it is possible to achieve perfect match primer pairs for multiple representatives of a microbial group. However, during the synthesis of degenerate primers, it is unlikely that equimolar proportions of all the individual primers will be produced. As a result, non-equimolar proportions of the primers in the mixture may also result in disproportional representation of the general AOB in the sample (Green *et al.*, 2015). Additionally, a high specificity is required, to understand if and why ammonia-oxidizing archaea (AOA) outcompete AOB in various environments like soil (Leininger *et al.*, 2006), freshwater (Bollmann *et al.*, 2014) or wastewater (Gwak *et al.*, 2019), and how we put these results in scientific context.

The use of multiple primer sets targeting the *amoA* gene has already been recommended (Bru *et al.*, 2008; Meinhardt *et al.*, 2015; Dechesne *et al.*, 2016). Considering multiple primer sets when exploring AOB in unknown communities is a labour and time-intensive suggestion, but indispensable. For example, Stein *et al.* (2007) revealed several properties of the *N. eutropha* genome, which are distinct from the closely related *N. europaea*

that support niche specialization, such as different chemoorganotrophic growth of *N. eutropha* and *N. europaea* under anoxic conditions with nitrite as terminal electron acceptor (Schmidt, 2009). It is also reported that *N. eutropha* can tolerate nitrite accumulation better than *N. europaea* (Zart and Bock, 1998). This highlights how *N. eutropha* and *N. europaea* may respond differently to a variety of WWTP operational conditions. Therefore, it is important to perform differential quantification of *N. europaea* and *N. eutropha*, which is not possible with universal primer sets like the Rott pp.

Here, we compared previously published *amoA* primers (Supplementary information, Table S1) with the currently available *amoA* gene nucleotide sequences from the Functional Gene Repository (http://fungene.cme.msu.edu), to determine the coverage and specificity for group-specific AOB. Based on this information, we designed two primer sets, including forward and reverse primers with TaqMan probes for *N. europaea* and *N. eutropha* (Supplementary information, Table S2, Fig. S1). These primer sets were evaluated by both PCR and qPCR and compared with the Rott pp for pure culture DNA and environmental samples. Shotgun sequencing gave an overall insight into the *amoA* gene sequences that were found in the environmental samples.

Results and discussion

We performed systematic in silico PCR analysis to determine the coverage of previously published primers and TaqMan probes targeting the *amoA* gene (Fig. 1 and Supplementary Information for details). To consider previously published primer pairs and TaqMan probes to be a perfect match (PM) with the *amoA* gene diversity, both, the primers and TaqMan probes (if available), were required to share 100% nucleotide identity over a minimum of 15 bp of the primer length. We found that the foremost primer pair (i.e. A189-A682) is not a PM for any cluster of beta-proteobacteria AOB, except for *Nitrosospira* sp. NpAV. This primer pair contains degeneracy in both primers, which can result in 4 variants of the forward and 16 variants of the reverse primer. Our analysis revealed that the coverage of the most commonly used primer pair, the Rott pp (Rotthauwe *et al.*, 1997), is different for different clusters of AOB (Orschler *et al.*, 2019). In case of cluster 7 (*N. europaea* lineage), it is not a PM primer pair (Fig. 1). The forward primer (amoA1F) has one base pair (bp) mismatch (MM) to *N.*

Fig. 1. *In silico* PCR of all known *amoA* primer pairs/TaqMan probes represented in a neighbour-joining phylogenetic tree inferred from AOB *amoA* gene sequences; the heatmap represents the amplification for the respective primer pair/TaqMan probe; dark green shows perfect match (PM) primer pairs, olive green represents one mismatch (MM) pp, orange represents two MM pp, red represents three MM pp, and no colour denotes more than 3 MM pp. Clustering was based on Koops (1992).

europaea, and the reverse primer (amoA-2r), which is a degenerate primer, has one bp MM to *N. eutropha.* Also, for cluster 6A (*N. oligotropha* lineage) it is not a PM primer set. However, for some members of cluster 3 (*Nitrosospira* lineage) it is a PM primer pair. Afterwards, a modified amoA-2r was designed having degenerated bases which resulted in 18 sequence variants, to better capture AOB diversity (Hornek *et al.*, 2006). A decade after the Rott pp was designed, the TaqMan probes (i) amoA-NM3 having degeneracy for cluster 7; (ii) amoA-NM4 for cluster 6A; and (iii) amoA-Ns having degeneracy for cluster 3 were designed for improved specific quantification of the respective AOB (Regan *et al.*, 2007). We found that amoA-NM3 and amoA-Ns are PM probes for cluster 7 and cluster 3 due to the presence of degeneracy, but amoA-NM4 is a MM probe for cluster 6a (Fig. 1). It was realized very early that it is difficult to design a single primer pair and TaqMan probe targeting the *amoA* gene to detect all AOB. Therefore, Harms *et al.* (2003) designed a primer pair (i.e. amoNo550D2f containing two degenerate bases and amoNo754r) and TaqMan probe (i.e. amoNoTaq729) for quantification of *N. oligotropha*-like bacteria. Primer pair amoNo550D2f and amoNo754r combined with TaqMan Probe amoNo-TaqD729 have high coverage for cluster 6A. However, only one possible forward primer was a PM primer, although three sequence variants are possible due to degenerated bases. Similarly, Layton *et al.* (2005) designed a primer pair and TaqMan probe (amoRl27542f, amoRl2767r and amoARlbhq651r) for quantification of only *N. nitrosa* having MM for cluster 6A, 7 and 3 (Supplementary information, Table S1). The recently designed primer pair amoA-1Fmod/GenAOBr without degeneracy (Meinhardt *et al.*, 2015) is a PM primer pair for members of cluster 3 but not for cluster 6A (Fig. 1). This study even reported less efficient amplification of *N. europaea.* We designed primer pairs and TaqMan probes without introducing degeneracy for specific quantification of *N. europaea* (nerF/nerR combined with nerTaq as TaqMan probe) and *N. eutropha* (netF/netR with netTaq probe; Fig. 1), respectively. In addition, PCR followed by gel electrophoresis showed no amplification for a set of non-target microorganisms and efficient amplification for target microorganisms (Supplementary information, Figs S2 and S3).

The effect of internal primer–template mismatch is highly variable and can lead to dramatic effects. It may lower PCR efficiency depending on the position of the mismatch and detection sensitivity if the annealing temperature is elevated to compensate the primer MM (Sekido *et al.*, 2008; Wu *et al.*, 2017). These mismatches introduce bias in determining community composition.

We performed comparative qPCR analysis using *N. europaea* and *N. eutropha* as pure cultures as well as in different mixtures (detailed information in the supplementary), for Rott pp and new developed ner and net primer pairs/TaqMan probe (Fig. 2, Fig. S4). The abundance measured in the PCR reaction mix containing 100% *N. europaea* DNA template was highly similar between the Rott pp and the ner primer/probe set. Less similarity for reactions containing 100% *N. eutropha* was observed between the Rott pp and the net primer/probe set. A significant difference between the abundance measured using the Rott pp and both newly designed primer/probe sets was observed for mixed DNA templates (i.e. not 100% target DNA in the qPCR reaction; Fig. 2, Fig. S4). Based on percentual discrepancy, the Rott pp showed an underestimation of 50.78% for mixture 1 and 48.66 % for mixture 2. Mixture 3 with four different AOB pure cultures resulted in an underestimation of 50.78%, and 48.64% for mixture 4. These results clearly show that presence of more than one target AOB in the PCR reaction impacts the performance of the Rott pp. The abundance measured using the Rott pp differed by almost half compared to the abundance measured using the newly developed primer/probe sets. Our results are in consensus with the previously expressed opinion about an underestimation of *amoA*-targeted quantification using the Rott pp (Layton *et al.*, 2005; Meinhardt *et al.*, 2015; Dechesne *et al.*, 2016).

Metagenomics was performed to determine the composition of the AOB community in unknown environmental samples. *AmoA* gene-targeted assembly was performed for the environmental samples (details in the Supplementary Information). The composition of AOB differed for each sample (Fig. 3), containing AOBs from clusters 6a/7/*N. cryotolerans* lineage. To compare the performance of the Rott pp with ner and net primer/probe sets, the abundance of AOB in these environmental samples was quantified using respective primer/probe sets (Fig. 4). *N. eutropha* was not found in environmental samples using net primer/probe set. These results fit very well with the metagenomic composition, too. In case of the ner primer/probe set, 4.02 ± 0.18 log copies ng^{-1} DNA in sample 2 and 3.2 ± 0.05 log copies ng^{-1} DNA in sample 3 were detected, whereas 4.51 ± 0.03 log copies ng^{-1} DNA in sample 2 and 3.43 ± 0.11 log copies ng^{-1} DNA in sample 3 were detected based on the Rott pp. No detection of *N. europaea* in sample 1 and sample 4 was also in consensus with metagenomic composition. The abundance estimate based on the Rott pp was only 0.5 log copies ng^{-1} DNA for sample 2 and 0.3 log copies ng^{-1} DNA for sample 3 more than estimates based on ner primer/probe set, though the samples contained more of other AOB as shown in Fig. 3. These results suggest that use of Rott pp can lead to misinterpretation in terms of the total abundance of the AOB population because in general
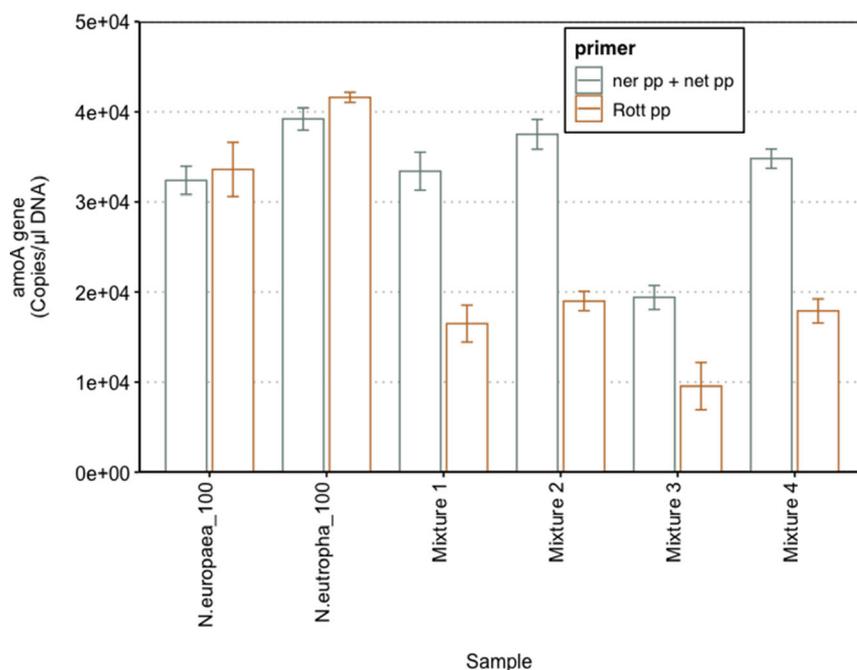
Fig. 2. Bacterial *amoA* counts from different DNA mixtures of 4 different AOB. The ner pp + net pp represents the sum of the abundance detected using ner and net pp. N.europaea_100 corresponds to 100% *N. europaea*, N.eutropha_100 corresponds to 100 % *N. eutropha*, Mixture 1 corresponds to *N. europaea: N.eutropha* – 3: 1, Mixture 2 corresponds to *N. europaea: N.eutropha* – 1: 3, Mixture 3 corresponds to *N. europaea: N.eutropha: others* – 1: 1: 2, Mixture 4 corresponds to *N. europaea: N.eutropha* – 1: 1. See supplementary for details on sampling, DNA extraction, for qPCR conditions and efficiency, and plotted qPCR data in Table S5.
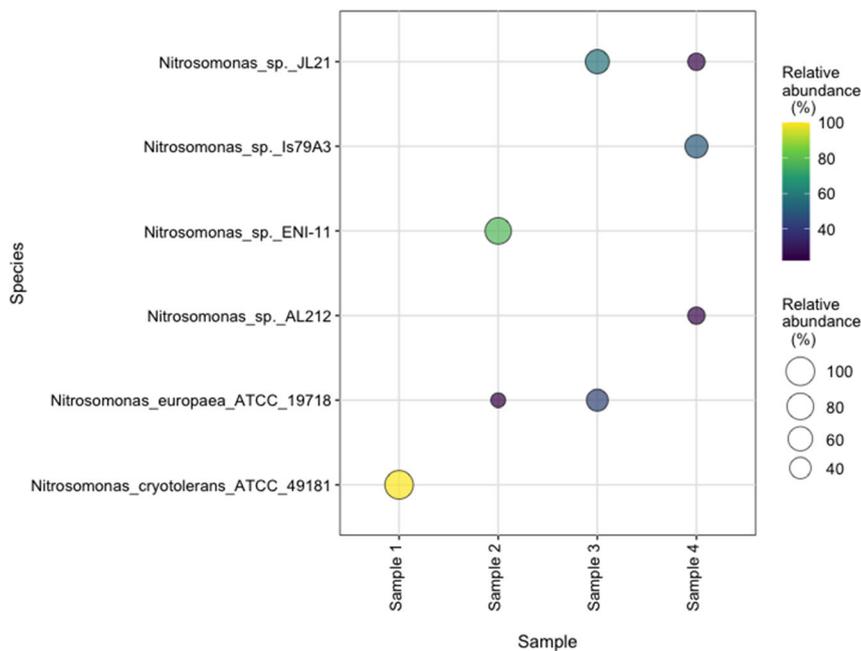
Fig. 3. Community composition of AOB from wastewater treatment plant samples based on shotgun sequencing. Colour bar and circle size represent the relative abundance in %. See Supplementary Information for details on DNA extraction and shotgun sequencing.

(especially for WWTP operators and environmental engineers) it is assumed that the use of universal primer pairs like Rott pp allows for the detection of all the AOB present in the WWTP.

Moreover, a significant (*P*-value < 0.01, one-way analysis of variance (ANOVA) analysis) difference was observed in AOB concentration when all samples were spiked with a defined concentration of 5 log copies per
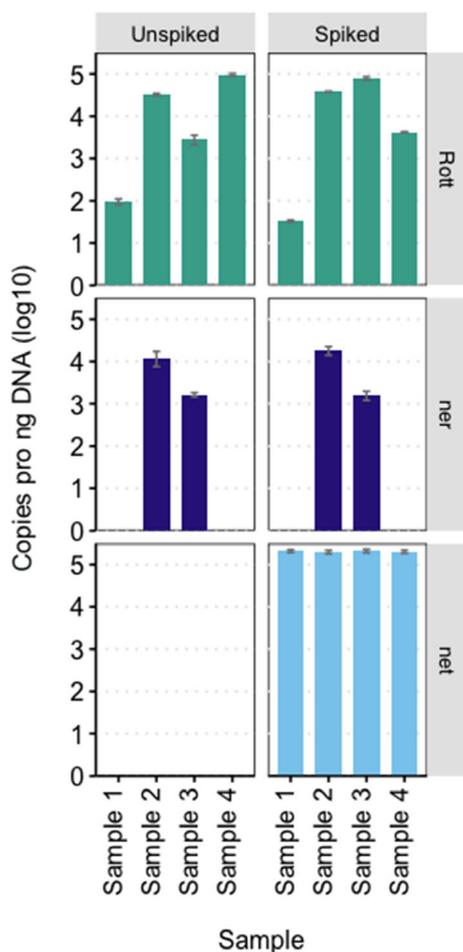
Fig. 4. *AmoA* abundance analysed with qPCR for the ner (dark blue) and net (light blue) primer sets and the Rott primer pair (amoA1f/amoA2r) (green); the three graphs on the left side (with 'Unspiked') are the original environmental samples from the wastewater treatment plants, and the three graphs on the right side (with 'Spiked') show the results after spiking the samples with a defined concentration of 5 log copies ng^{-1} DNA of *N. eutropha*. See supplementary for details on sampling, DNA extraction, for qPCR conditions and efficiency, and plotted qPCR data in Table S6.

ng DNA of *N. eutropha* to evaluate coverage of the Rott pp in comparison with the ner and net primer/probe sets. No significant change in the measured concentrations of AOB was observed using the Rott pp in comparison with the unspiked samples (Fig. 4). Using the net primer/probe set, it was possible to measure the abundance of the spiked *N. eutropha* (Fig. 4).

Because of the high specificity of the primers and probe sets to detect specific AOB, the principle of nesting (adding abundance of each specific AOB) can be applied for quantification of total AOB as suggested previously (Layton *et al.*, 2005; Lim *et al.*, 2008; Sekido *et al.*, 2008). This helps to overcome the major problem of under- or over-estimating AOB abundances in environmental samples. For example, nitrite accumulation in engineered environments

may disproportionately affect *N. europaea* and *N. eutropha* populations, as each AOB differs in their ability to tolerate increased nitrite conditions (Zart and Bock, 1998; Tan *et al.*, 2008; Cua and Stein, 2011). The primers designed in this study, in contrast to the Rott pp, would be able to detect these species-specific population trends. Thus, abundance estimate using the Rott pp, which is not suitable for *N. eutropha,* may not recognize species-specific impact of nitrite accumulation on AOB. This approach can further be used to analyse competitive dynamics amongst different AOB present in samples. The primer pairs and TaqMan probes developed in this study will additionally help to (i) have a closer look at environments where competitions between *N. europaea* and *N. eutropha* are expected; and (2) overcome the underestimation of AOB, together with use of primers and probes developed for *N. oligotropha* cluster (Harms *et al.*, 2003; Meinhardt *et al.*, 2015) and *Nitrospira* cluster.

Conflict of interest

The authors declare no financial and non-financial conflict of interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Published primers/TaqMan probes to target ammonium-oxidizing bacteria for *amoA* gene with the respective target group and the sequence.

Table S2. Primer and TaqMan Probes for *N. europaea* and *N. eutropha* designed in this study.

Table S3. PCR Protocol.

Table S4. Efficiencies and R^2 values from qPCR analysis for ner primer set, net primer set and Rott primer set.

Table S5. *AmoA* abundance analyzed with qPCR for different DNA mixtures of 4 different AOB. The ner_net pp represents the sum of the abundance detected using ner and net pp. N.europaea_100 corresponds to 100% *N. europaea*, N.eutropha_100 corresponds to 100 % *N. eutropha*, Mixture 1 corresponds to *N. europaea: N.eutropha* – 3 : 1, Mixture 2 corresponds to *N. europaea: N.eutropha* – 1 : 3, Mixture 3 corresponds to *N. europaea: N.eutropha: others* – 1 : 1: 2, Mixture 4 corresponds to *N. europaea: N.eutropha* – 1 : 1. Data are shown as mean and standard deviation (SD) of triplicate qPCR runs.

Table S6. *AmoA* abundance analyzed with qPCR for the original environmental samples from the wastewater treatment plants (Unspiked) and the spiked environmental samples from the wastewater treatment plants (Spiked). Original environmental samples from the wastewater treatment plants were spiked with a defined concentration of 5 log copies/ng DNA of *N. eutropha*. Data are shown as mean and the corresponding standard deviation of triplicate qPCR runs.

Fig. S1. Sequence alignment of the amoA gene sequences of various AOB. List of microbial members that were considered for the sequence alignment: *Nitrosococcus mobilis* (AF037108.1), *Nitrosomonas cryotolerans* (AF314753.1), *Nitrosomonas nitrosa* (AF272404), *Nitrosospira briensis* (U76553.1), *Nitrosolobus multiformis* (U31649.1), *Nitrosospira* sp. NpAV (JXQM0100023.1), *Nitrosomonas* sp. JL21 (AF327919.1), *Nitrosomonas oligotropha* (AF272406.1), *Nitrosomonas oligotropha* Nm45 (AJ298709.1), *Nitrosomonas ureae* (AF272403.1), *Nitrosomonas* sp. AL212 (AF327918.1), *Nitrosomonas* sp. AL212 (CP002552.1), *Nitrosomonas* sp. Nm10 (AF272411.1), *Nitrosomonas* sp. JL21 (AF327919.1), *Nitrosomonas* sp. AL212 (AF327918.1), *Nitrosomonas europaea* ATCC 19718 (AL954747.1), *Nitrosomonas europaea* (AF058692.1), *Nitrosomonas europaea* Nm35 (FOID01000069.1), *Nitrosomonas europaea* strain ATCC 25978 (FUWK01000078.1), *Nitrosomonas europaea* strain Nm50 (FNNX01000068.1), *Nitrosomonas europaea* ATCC 19718 (AL954747.1), *Nitrosomonas europaea* (L08050.1), *Nitrosomonas eutropha* C91 (CP000450.1), *Nitrosomonas eutropha* strain Nm14 (FMTW01000055.1), *Nitrosomonas eutropha* strain Nm38 (FNNM01000040.1), *Nitrosomonas eutropha* strain Nm56 (FNYF01000053.1), *Nitrosomonas eutropha* (U72670.1), *Nitrosomonas eutropha* (AY177932.1), *Nitrosomonas eutropha* (U51630.1).

Fig. S2. PCR product of net primer pair visualized on a pre-cast 2% agarose E-Gel with Annealing Temperature of 65 °C. Lane M=100 bp ladder, Lane 1= DSM 101675 (*Nitrosomonas eutropha*), Lane 2= DSM 28437 (*Nitrosomonas europaea*), Lane 3 = DSM 28438 (*Nitrosomonas nitrosa*), Lane 4= DSM 28436 (*Nitrosomonas communis*), Lane 5= DSM 428 (*Cupriavidus necator*), Lane 6= DSM 1650 (*Pseudomonas nitroreducens*), Lane 7= NC, Lane 8=100 bp ladder.

Fig. S3. PCR product of ner primer pair visualized on a pre-cast 2% agarose E-Gel with Annealing Temperature of 65 °C. Lane M=100 bp ladder, Lane 1= DSM 101675 (Nitrosomonas eutropha), Lane 2= DSM 28437 (Nitrosomonas europaea), Lane 3 = DSM 28438 (Nitrosomonas nitrosa), Lane 4= DSM 28436 (Nitrosomonas communis), Lane 5= DSM 428 (Cupriavidus necator), Lane 6= DSM 1650 (Pseudomonas nitroreducens), Lane 7= NC, Lane 8=100 bp ladder.

Fig. S4. Abundance of *amoA* genes for 4 different DNA mixtures of different AOB: Mixture 1: *N. europaea: N.eutropha* – 3 : 1; Mixture 2: *N. europaea: N.eutropha* – 1 : 3; Mixture 3: *N. europaea: N.eutropha: others* – 1 : 1: 2; Mixture 4: *N. europaea: N.eutropha* – 1 : 1. And, 100% *N. europaea*, 100 % N. eutropha.