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 Pomerening-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 nitrification/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 chemooorganotrophic 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.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 (Oorschel 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).
Nitrosomonas cluster 7 – primers and probes

| Cluster | Species/Primer | Mismatch (MM) | Primer | Taqprobe |
|---------|----------------|---------------|--------|----------|
| Cluster 8 | Nitrosomonas nitroa | 0 | A198-2 | amoA-2R-4 |
|         | Nitrosomonas nitrona | 1 | A198-3 | amoA-2R-4 |
|         | Nitrosomonas ar. N141 | 2 | A198-4 | amoA-2R-4 |
|         | Nitrosomonas ar. N858 | 3 | A189-4 | amUni1F |
|         | Nitrosomonas ar. N833 | 0 | A189-3 | amUni1F |
|         | Nitrosomonas communis | 1 | A189-2 | amUni1F |
|         | Nitrosomonas ar. N8104 | 2 | A189-1 | amUni1F |
|         | Nitrosomonas heliophila | 3 | A188-5 | amUni1F |
|         | Nitrosomonas heliophila | 0 | A188-4 | amUni1F |
|         | Nitrosomonas stercola | 1 | A188-3 | amUni1F |
|         | Nitrosomonas ar. A7 | 2 | A188-2 | amUni1F |
|         | Nitrosomonas eutropha | 3 | A188-1 | amUni1F |
|         | Nitrosomonas ar. C31 | 0 | A187-5 | amUni1F |
|         | Nitrosomonas sp. EN31 | 1 | A187-4 | amUni1F |
|         | Nitrosomonas europaea | 2 | A187-3 | amUni1F |
|         | Nitrosomonas ar. Nm103 | 3 | A187-2 | amUni1F |
|         | Nitrosomonas ar. LT41 | 0 | A187-1 | amUni1F |
|         | Nitrosomonas sp. A2 | 1 | A186-5 | amUni1F |
|         | Nitrosomonas sp. LT2 | 2 | A186-4 | amUni1F |
|         | Nitrosomonas sp. Nn148 | 3 | A186-3 | amUni1F |
|         | Nitrosomonas sp. Nn205 | 0 | A186-2 | amUni1F |
|         | Nitrosomonas sp. RN73 | 1 | A186-1 | amUni1F |
|         | Nitrosomonas sp. Tn614 | 2 | A185-5 | amUni1F |
|         | Nitrosomonas sp. Tn614-4 | 3 | A185-4 | amUni1F |
|         | Nitrosomonas sp. C-113a | 0 | A185-3 | amUni1F |
|         | Nitrosomonas sp. Nm105 | 1 | A185-2 | amUni1F |
|         | Nitrosomonas ar. Nm156 | 2 | A185-1 | amUni1F |
|         | Nitrosomonas ar. LT574 | 3 | A184-5 | amUni1F |
|         | Nitrosomonas ar. C-45 | 0 | A184-4 | amUni1F |
|         | Nitrosomonas ar. E-224 | 1 | A184-3 | amUni1F |
|         | Nitrosomonas ar. AL212 | 2 | A184-2 | amUni1F |
|         | Nitrosomonas ar. AL212 | 3 | A184-1 | amUni1F |
| Cluster 9 | Nitrosomonas oligotropha | 0 | A183-5 | amUni1F |
|         | Nitrosomonas sp. NL7 | 1 | A183-4 | amUni1F |
|         | Nitrosomonas sp. Nm97 | 2 | A183-3 | amUni1F |
|         | Nitrosomonas sp. En466 | 3 | A183-2 | amUni1F |
|         | Nitrosomonas sp. C132 | 0 | A183-1 | amUni1F |
|         | Nitrosomonas sp. Nm98 | 1 | A182-5 | amUni1F |
|         | Nitrosomonas sp. Nn464 | 2 | A182-4 | amUni1F |
|         | Nitrosomonas sp. bs78A3 | 3 | A182-3 | amUni1F |
|         | Nitrosomonas unam | 0 | A182-2 | amUni1F |
|         | Nitrosomonas sp. AL212 | 1 | A182-1 | amUni1F |

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an AOB 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 amoNoTaqD729 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 (amoRI27542f, amoRI27677r and amoARIbhq651r) for quantification of only N. nitroa 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...
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

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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 R² values from qPCR analysis for ner primer set, net primer set and Rott primer set.

Table S5. AmoA abundance analyzed with qPCR for differ-

ent DNA mixtures of 4 different AOB. The ner_net pp re-

presents 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 treat-

ment plants (Unspiked) and the spiked environmental sam-

ples 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 consid-

ered for the sequence alignment: *Nitrosococcus mobilis* (AF037108.1), *Nitrosomonas cryotolerans* (AF314753.1), *Nitrosomonas nitrosa* (AF272404), *Nitrosospira brienesis* (U76553.1), *Nitrosolobus multiformis* (U31649.1), *Nitrosospi-

ra* sp. NpAV (JXQM0100023.1), *Nitrosomonas sp. JL21* (AF327919.1), *Nitrosomonas oligotropha* (AF272406.1), *Nitrosomonas oligotropha* Nm45 (AJ298709.1), *Nitro-

somonas 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* ATCC 19718 (AL954747.1), *Nitrosomonas europaea* (L08050.1), *Nitrosomonas eutropha* C91 (CP000450.1), *Nitrosomonas eutropha* strain Nm14 (FMTW0100055.1), *Nitrosomonas eutropha* strain Nm38 (FNNM1000040.1), *Nitrosomonas eutropha* strain Nm56 (FNYF0100053.1), *Nitrosomonas eutropha* (U72670.1), *Nitrosomonas eutropha* (AY177932.1), *Nitrosomonas eutropha* (US1630.1).

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 (*Nitro-

somonas 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 (Pseu-

domonas nitroreducens), Lane 7 = NC, Lane 8 =100 bp lad-

der.

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 (*Nitro-

somonas 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 (Pseu-

domonas nitroreducens), Lane 7 = NC, Lane 8 =100 bp lad-

der.

Fig. S4. Abundance of amoA genes for 4 different DNA mix-

tures 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*.