A refined set of rRNA-targeted oligonucleotide probes for *in situ* detection and quantification of ammonia-oxidizing bacteria

Michael Lukumbuzya [a,*], Jannie Munk Kristensen [b,*], Katharina Kitzinger [a,c], Andreas Pommerening-Röser [d], Per Halkjær Nielsen [b], Michael Wagner [a,b,e,f], Holger Daims [a,f], Petra Pjevac [a,e]

[a] University of Vienna, Centre for Microbiology and Environmental Systems Science, Division of Microbial Ecology, Vienna, Austria.

[b] Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark.

[c] Max Planck Institute for Marine Microbiology, Bremen, Germany.

[d] University of Hamburg, Institute of Plant Science and Microbiology, Microbiology and Biotechnology, Hamburg, Germany.

[e] Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna, Vienna, Austria.

[f] University of Vienna, The Comammox Research Platform, Vienna, Austria.

* equal contributions

Corresponding author: Holger Daims, e-mail: holger.daims@univie.ac.at

Abstract

Ammonia-oxidizing bacteria (AOB) of the betaproteobacterial genera *Nitrosomonas* and *Nitrosospira* are key nitrifying microorganisms in many natural and engineered ecosystems. Since many AOB remain uncultured, fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes has been one of the most widely used approaches to study the community composition, abundance, and other features of AOB directly in environmental samples. However, the established and widely used AOB-specific 16S rRNA-targeted FISH probes were designed up to two decades ago, based on much smaller rRNA gene sequence datasets than available today. Several of these probes cover their target AOB lineages incompletely and suffer from a weak target specificity, which causes cross-hybridization of probes that should detect different AOB lineages. Here, a set of new highly specific 16S rRNA-targeted oligonucleotide probes was developed and experimentally evaluated that complements the existing probes and enables the specific detection and differentiation of the known, major phylogenetic clusters of betaproteobacterial AOB. The new probes were successfully applied to visualize and quantify AOB in activated sludge and biofilm samples from seven pilot- and full-scale wastewater treatment systems. Based on its improved target group coverage and specificity, the refined probe set will facilitate future *in situ* analyses of AOB.

Keywords

Fluorescence *in situ* hybridization; ammonia-oxidizing bacteria; nitrification; wastewater treatment plants; oligonucleotide probes

1. Introduction

Nitrification, a key process in the biogeochemical nitrogen cycle, is the microbially mediated oxidation of ammonia to nitrite and subsequently to nitrate. For many decades nitrification was perceived as a process always performed by two functional groups of aerobic, chemolithoautotrophic microorganisms in cooperation: the ammonia oxidizing bacteria (AOB) and archaea (AOA), which oxidize ammonia to nitrite, and the nitrite oxidizing bacteria (NOB) that oxidize nitrite to nitrate (Bock and Wagner, 2001; Daims et al., 2016; Könneke et al., 2005). Only recently, complete ammonia oxidizers (comammox organisms) have been discovered, which carry out the entire nitrification process alone (Daims et al., 2015; van Kessel et al., 2015).

Currently, all known canonical AOB, oxidizing ammonia to nitrite, belong to one of two phylogenetic lineages within the Proteobacteria. Gammaproteobacterial AOB include the genus *Nitrosococcus*, which is halophilic and occurs in marine habitats and salt lakes (Campbell et al., 2011), and the genus *Nitrosoglobus*, which contains acidotolerant AOB from acidic soils (Hayatsu et al., 2017). In contrast, the AOB that usually dominate in terrestrial and freshwater ecosystems belong to the family *Nitrosomonadaceae* within the (now obsolete, Parks et al., 2018) taxonomic class of Betaproteobacteria (Prosser et al., 2014). Here, we refer to these organisms as β-AOB. All cultivated and described members of the family *Nitrosomonadaceae* are chemolithoautotrophic AOB from the genera *Nitrosomonas* and *Nitrosospira* (Prosser et al., 2014). Representatives of β-AOB are found in almost all oxic environments but are particularly successful in nutrient rich habitats such as fertilized soils or eutrophic freshwater sediments, and also the majority of wastewater treatment plants (WWTPs) (Bollmann et al., 2014; Di et al., 2010; Fan et al., 2011; Verhamme et al., 2011). The low abundance of AOA in activated sludge from most municipal WWTPs (Gao et al., 2013; Mußmann et al., 2011; Wells et al., 2009) has recently been attributed to their sensitivity to

66 copper limitation caused by chemical complexation of copper by organic compounds (Gwak
67 et al., 2019). This effect likely contributes to the commonly observed predominance of β-AOB
68 in these engineered environments.

69 Despite their ubiquity, β-AOB have proven to be exceptionally fastidious and recalcitrant to
70 cultivation. Hence, the number of β-AOB isolates with standing in nomenclature (Parte, 2014)
71 remains low (n=14) despite their broad environmental distribution and high ecological
72 significance. To overcome this problem, researchers use cultivation-independent molecular
73 techniques for studying various aspects of AOB diversity and ecophysiology. One of the most
74 commonly applied molecular methods is rRNA-targeted fluorescence *in situ* hybridization
75 (FISH). This approach uses rRNA-targeted oligonucleotide probes, which are covalently linked
76 to fluorescent dyes and hybridize to the ribosomal RNA of specific microbial populations
77 (Amann et al., 1995; DeLong et al., 1989). The resulting fluorescence signal allows the *in situ*
78 detection and visualization of target organisms in environmental samples. FISH has numerous
79 applications in microbial ecology, which include the *in situ* abundance quantification of
80 populations (Daims and Wagner, 2007; Wagner et al., 1994), and quantitative analyses of the
81 spatial distribution of microorganisms in biofilms and other structurally complex samples
82 (Almstrand et al., 2013; Daims et al., 2006; Dolinšek et al., 2013; Schillinger et al., 2012;
83 Welch et al., 2016). Combinations of FISH with chemical imaging techniques like
84 microautoradiography (Lee et al., 1999), Raman microspectroscopy (Fernando et al., 2019;
85 Huang et al., 2007), and nanometer scale secondary ion mass spectroscopy (NanoSIMS) (Berry
86 et al., 2013; Musat et al., 2012) even permit cultivation-independent physiological studies of
87 discrete microbial populations. FISH can also be used together with bioorthogonal
88 noncanonical amino acid tagging (BONCAT), which is another powerful approach to detect
89 metabolically active microorganisms *in situ* (Hatzenpichler et al., 2014).

FISH has been applied since 1995 (Wagner et al., 1995) in numerous studies to investigate β-AOB in aquatic systems, especially WWTPs, and has proven to be of immense value in this context. For example, *Nitrosomonas* (formerly "*Nitrosococcus*") *mobilis* was identified as the dominant AOB in an industrial WWTP (Juretschko et al., 1998) and later isolated from activated sludge by FISH-assisted screening and propagation of sorted microcolonies (Fujitani et al., 2015). FISH and image analysis were used to quantify the spatial localization patterns of β-AOB in nitrifying biofilms (Almstrand et al., 2013; Gruber-Dorninger et al., 2015, p.; Maixner et al., 2006). By combining FISH detection with microsensor measurements of substrate concentration gradients, both the distribution and activities of β-AOB in biofilms were studied (Okabe et al., 1999; Schramm et al., 1998). Application of this approach to calculate volumetric reaction rates even revealed the *in situ* whole-cell kinetics of uncultured β-AOB (*Nitrosospira* spp.) (Schramm et al., 1999). In another study, FISH and quantitative PCR (qPCR) were used to detect β-AOB in granular activated sludge. These data were the basis for two ecophysiological models, which address the observed (and unexpected) higher *in situ* abundances of NOB over AOB in the granules (Winkler et al., 2012). FISH was also used to quantify the abundance dynamics of diverse β-AOB and anaerobic ammonium oxidizers (anammox) in ammonium- or urea-fed enrichments, revealing different substrate preferences of the populations (Sliekers et al., 2004), and to analyze the spatial organization of β-AOB and anammox organisms in nitrogen-removing biofilms (Pynaert et al., 2003). Since the validation of methods is crucial, a recent comparison of FISH and qPCR as tools to quantify the abundance of β-AOB deserves attention. It revealed that FISH and *amoA*-targeted qPCR yielded consistent results and were superior to qPCR of 16S rRNA genes (Baptista et al., 2014).

These and many other studies used a well-established set of rRNA-targeted oligonucleotide probes to detect all β-AOB or their sublineages by FISH (Adamczyk et al., 2003; Juretschko et al., 2002, 1998; Mobarry et al., 1996; Wagner et al., 1995). However, the most commonly used

115 probes were designed based on a much more limited set of 16S rRNA sequences from β-AOB
116 than is available now, and they do not cover the entire diversity of β-AOB represented in
117 current databases. Moreover, inconsistent *in situ* hybridization patterns were observed, which
118 suggested that some of these probes hybridize to β-AOB outside the expected target groups
119 (Gruber-Dorninger et al., 2015). An incomplete probe coverage and weak specificity can
120 introduce a significant bias in studies that rely on these probes to detect and quantify β-AOB
121 and to distinguish different β-AOB lineages. Hence, an updated set of β-AOB-specific FISH
122 probes is urgently needed.

123 Here we introduce new β-AOB-specific rRNA-targeted oligonucleotide probes, which
124 complement and refine the existing probe set and enable the identification, visualization, and
125 quantification of all currently known β-AOB lineages. Following probe design and evaluation,
126 the specificity and applicability of the new probes were tested with activated sludge and biofilm
127 samples from municipal and industrial WWTPs.

128 **2. Materials and methods**

129 2.1 In silico *design of 16S rRNA-targeted probes and phylogenetic analyses*

130 The new rRNA-targeted oligonucleotide probes (Table 1) were designed using the "probe
131 design" and "probe match" functions of the ARB software package (version 6.0.6) (Ludwig et
132 al., 2004) and the Silva Ref_NR99 (release 132) SSU rRNA sequence database (Quast et al.,
133 2013). The database was amended with 46 additional full-length 16S rRNA gene sequences of
134 β-AOB from full-scale WWTPs in Denmark (Dueholm et al., 2019), which were retrieved from
135 the MiDAS database (McIlroy et al., 2015; Nierychlo et al., 2019). The "probe match" tool of
136 ARB was also used to evaluate the target group coverage and specificity of the previously
137 published FISH probes for β-AOB. A maximum likelihood phylogenetic tree was calculated,
138 based on an alignment (SINA aligner, Pruesse et al., 2012) of 505 reference sequences of β-

AOB from the aforementioned databases, using W-IQ-Tree (Trifinopoulos et al., 2016) with 1,000 bootstrap iterations. TIM3e+G4 was determined by ModelFinder to be the best fitting base substitution model for the calculation (Kalyaanamoorthy et al., 2017). The resulting tree was visualized with iTOL (Letunic and Bork, 2019).

2.2 *Cultivation and fixation of β-AOB pure cultures*

Pure cultures of the β-AOB *Nitrosospira briensis* Nsp1, *Nitrosospira multiformis* NI13, *Nitrosomonas europaea* Nm50, *Nitrosomonas eutropha* Nm57, *Nitrosomonas oligotropha* Nm75, and *Nitrosomonas sp.* Nm51 (an unnamed species from the *Nitrosomonas marina* lineage) were grown as described earlier (Koops et al., 1991). *Nitrosomonas communis* Nm2 was grown in a modified AOB medium according to Zhou *et al.* (2019). All cultures were harvested (~40 ml) during the logarithmic growth phase and centrifuged (4,000×g, room temperature, 20 min) to collect the biomass. The supernatant was removed, and the cell pellets were resuspended in a 3% (w/v) formaldehyde solution for fixation (1 h at room temperature) as detailed elsewhere (Daims et al., 2005). The fixed cultures were washed twice in a 1× PBS (phosphate-buffered saline) solution, centrifuged 12,000×g at room temperature for 8 min, and then resuspended in a 1:1 mixture of 1× PBS and 96% (v/v) ethanol, and stored at -20°C until further processing (Daims et al., 2005).

2.3 *Sampling and fixation of activated sludge*

Activated sludge samples taken at several WWTPs in Austria, Germany, and Denmark were analyzed (Table 2). The samples were centrifuged (20,817×g, 4 °C, 15 min), the supernatant was removed, and the sludge was resuspended in a 2% (v/v) formaldehyde solution for fixation

(3 h, 4 °C). The sludge samples were subsequently washed twice in 1× PBS, resuspended in a 1:1 mixture of 1× PBS and 96% (v/v) ethanol, and stored at -20°C until further processing.

2.4 *Recombinant 16S rRNA expression for Clone-FISH*

We could not obtain cells of the isolates *Nitrosomonas cryotolerans* (targeted by the new probe Nm_CR_998) and *Nitrosomonas sp.* Nm143 (targeted by probe Nm_143_1010) (Table 1). Furthermore, no isolate is available from a *Nitrosomonas communis*-related cluster detected in some WWTPs in Denmark (Table 2), for which we designed the new probe Nm_NI_1026 (Table 1). In order to evaluate the new probes that are specific for these β-AOB and their close relatives, the respective 16S rRNA was heterologously expressed in *E. coli* for Clone-FISH (Schramm et al., 2002). Briefly, synthetic Strings DNA fragments (ThermoFisher Scientific) of full-length 16S rRNA genes were cloned into *E. coli* NovaBlue competent cells using the Novagen pETBlue-1 Perfectly Blunt Cloning Kit (Merck KGaA). The *E. coli* cells were grown to an OD of 0.3-0.4, 1 mM of IPTG was added to the cultures, and the cells were incubated (1 h, 200 r.p.m., 37 °C). Subsequently, chloramphenicol (170 mg l^{-1}) was added and the cells were incubated at 4 °C for 4 hours. Finally, the cells were fixed in formaldehyde as described above for the β-AOB isolates.

2.5 *Fluorescence* in situ *hybridization, microscopy, and digital image analysis*

FISH of all β-AOB pure cultures, *E. coli* cells containing recombinant 16S rRNA (Clone-FISH), and activated sludge samples was performed according to the standard protocol for FISH with rRNA-targeted oligonucleotide probes (Table 1) (Daims et al., 2005; Manz et al., 1992). Briefly, probe solutions had a concentration of 5 pmol μl^{-1} and were applied at a ratio of 1:10 (v/v) in hybridizaton buffer. If applicable, unlabeled competitor oligonucleotides

(Table 1) were used in equimolar concentrations as the probes. Hybridizations were performed at 46 °C for 2 hours. After hybridization, samples were washed in washing buffer for 10 min at 48 °C and shortly dipped into ice-cold MilliQ water. All hybridized samples were also stained with DAPI (4′,6-diamidino-2-phenylindole). For this purpose, 10 µl of 10 mg/ml DAPI was spotted onto hybridized samples, incubated for 5 min at room temperature, and subsequently washed away by dipping samples in 96% (v/v) ethanol. Samples were analysed immediately or stored at -20°C.

Fluorescence micrographs of probe-labelled cells were acquired using an inverted Leica TCS SP8X confocal laser scanning microscope (CLSM). The CLSM was equipped with a UV 405 diode and a supercontinuum white light laser, two photomultiplier (PMT) detectors, three hybrid (HyD) detectors, and the Leica Application Suite AF 3.2.1.9702 or Leica Application Suite X 3.5.6.21594. The settings for excitation and emission wavelengths were adjusted to match the respective fluorochromes (Table 1) as described elsewhere for multicolor FISH (Lukumbuzya et al., 2019). The digital image analysis and visualization software *daime* (version 2.2) (Daims et al., 2006) was used to project 3D confocal *z*-stacks.

To evaluate probe dissociation profiles, β-AOB pure cultures or *E. coli* cells (for Clone-FISH) were hybridized to the respective probes with increasing concentrations of formamide [0 to 70% (v/v)] in the hybridization buffer and corresponding salt concentrations in the wash buffer (Manz et al., 1992). If applicable, competitor oligonucleotides (Table 1) were included. Images for inferring probe dissociation profiles were recorded using the same CLSM settings for all parameters (laser power, confocal pinhole size, and “smart gain”). The probe dissociation profiles were determined, based on the mean fluorescence intensities of the probe-labelled cells, by using the respective tool of the *daime* software. The data were plotted in R, and approximated probe dissociation curves were obtained by non-linear regression with a sigmoidal model.

For the quantification of relative biovolume fractions, activated sludge samples were hybridized to a β-AOB specific probe mix and to the EUB338 I-III probe mix (Table 1), both labeled with different fluorochromes (Daims and Wagner, 2007). The two β-AOB-specific probe mixtures used consisted of previously published or newly designed probes, respectively (Table 1). In the "old" β-AOB probe mix, all probes were labelled with Cy3, and their fluorescence signals were recorded together in the same image. In the "new" β-AOB probe mix, the probes were labelled individually with different dyes, and the recorded images of these fluorescence signals were merged *in silico* for quantifying the relative biovolume fractions of β-AOB. Ten to 40 pairs of images containing the specific probe or EUB338 I-III probe signals, respectively, were acquired at random positions in a sample (Daims and Wagner, 2007). The CLSM settings were adjusted so that cells in the specific probe images had the same size as their counterparts in the EUB338 I-III images (Daims et al., 2005). The respective tool of the *daime* software was used to measure the biovolume fractions of β-AOB based on these image pairs.

3. Results and Discussion

3.1 *Evaluation of existing FISH probes targeting β-AOB*

Most previous studies using FISH to detect β-AOB *in situ* relied on a set of 16S rRNA-targeted oligonucleotide probes, which were designed as long as 16 to 25 years ago. This set consists of the probe Nsm156 (for various *Nitrosomonas* spp.), NEU (for halophilic and halotolerant *Nitrosomonas* spp.), Cl6a192 (for the *Nitrosomonas oligotropha* lineage), NmV (for *N. mobilis*), Ncom1025 (for *Nitrosomonas communis*), Nsv443 (for the *Nitrosospira* lineage), and the two broad-range probes Nso190 and Nso1225 (for most β-AOB) (Adamczyk et al., 2003; Juretschko, 2000; Juretschko et al., 1998; Mobarry et al., 1996; Wagner et al., 1995). In the

present study, we matched the sequences of these probes against a recent 16S rRNA gene sequence database that contained 505 non-redundant, full-length sequences from cultured and uncultured β-AOB. This *in silico* analysis revealed considerable gaps in the target group coverage for some of the probes, whereas others still showed a surprisingly good coverage (Table 3). In particular, the broad-range probe Nso1225 still covers the vast majority of β-AOB, whereas probe Nso190 (originally also designed to target all β-AOB) has a highly incomplete coverage according to current databases. Probe Nsm156, which should target the genus *Nitrosomonas* (Mobarry et al., 1996), still covers a large fraction of this genus. Probe Cl6a192 for the *N. oligotropha* lineage (cluster 6a) covers only ~50% of its target group (Table 3). Furthermore, unexpected hybridization patterns had previously been observed for the probes Cl6a192, NEU, and Ncom1025: although these probes target different lineages of β-AOB, a large proportion of their signals overlapped in FISH experiments with activated sludge (Gruber-Dorninger et al., 2015). These observations are consistent with a lack of specificity of probes NEU and Ncom1025, which becomes apparent when non-target β-AOB-sequences with 1-2 weak nucleotide mismatches to the probes are taken into account (Table 3). Such weak mismatches are often difficult to discriminate in FISH without competitor oligonucleotides. In this case, probe NEU potentially covers ~30% of cluster 6a (the target group of probe Cl6a192) and would probably also bind to members of the *N. mobilis* lineage within cluster 7. Notably, this unspecific hybridization of probe NEU would not be prevented by the published competitor (Table 1). Probe Ncom1025 would also potentially detect the majority of the *N. mobilis* lineage and could thus bind to the same organisms as probe NEU (Table 3). In our study, unspecific probe hybridization was confirmed in tests using biofilm and activated sludge samples from two bioreactors, SBBR1 and KNB (Fig. 1A, C; Table 2). Moreover, in one additional sludge from an industrial WWTP (CP Kelco, Table 2), the probe mix consisting of previously published AOB probes (Table 1) detected numerous microbial

cells that were arranged as tetrads within loose aggregates (Fig. 2A). The morphology of these organisms was very dissimilar from the usual size and shape of the β-AOB cells and cell clusters, which were also present in this sludge (Fig. 2A). Moreover, a test hybridization revealed that the tetrad-shaped cells were also labelled by probe Gam42a (data not shown), suggesting that these organisms were Gammaproteobacteria and unspecifically labelled by the previously published AOB probes. In summary, the *in silico* evaluation and test hybridizations demonstrated that several of the previously published FISH probes for β-AOB suffer from an insufficient target group coverage and specificity. As a consequence, further use of the affected probes for the *in situ* identification and quantification of the respective β-AOB groups should be performed with caution by taking into account their actual specificities (Table 3).

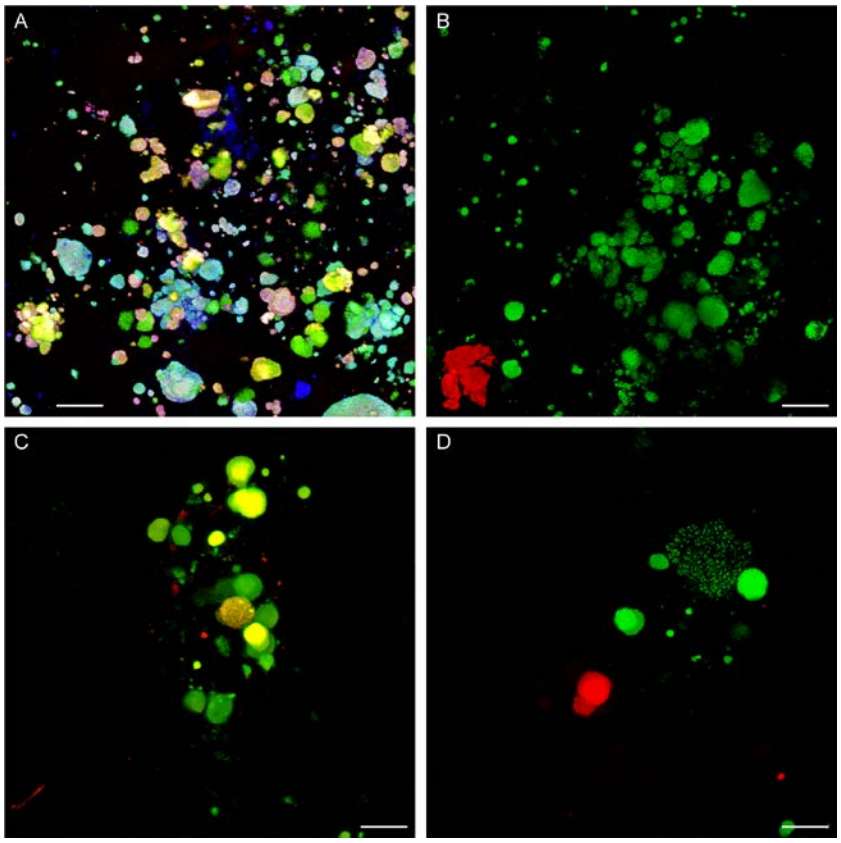


Figure 1. Comparison of hybridization patterns of selected previously published and newly designed FISH probes targeting β-AOB. (**A**) FISH of biofilm from reactor SBBR1 with probes

NEU (red), Cl6a192 (green), and Ncom1025 (blue). Mixed colors (cyan, magenta, yellow, white) indicate binding of multiple probes to the same microcolonies of β-AOB. (**B**) Sample of the same biofilm as in panel A after FISH with probes Nm_EU_136 (red) and Nm_OL_703 (green). No cross-hybridization was observed. Probe Nm_CO_1457 targeting the same lineage as probe Ncom1025 was also applied, but no signals were recorded, indicating that probe Ncom1025 (panel A) detected non-target β-AOB in this sample. (**C**) FISH of sludge from WWTP KNB with probes NEU (red) and Cl6a192 (green). Yellow indicates binding of both probes to the same microcolonies of β-AOB. (**D**) Sample of the same sludge as in panel C after FISH with probes Nm_EU_136 (red) and Nm_OL_703 (green). No cross-hybridization was observed. (**A-D**) All panels show projections of 3D confocal *z*-stacks. Bar = 20 µm.

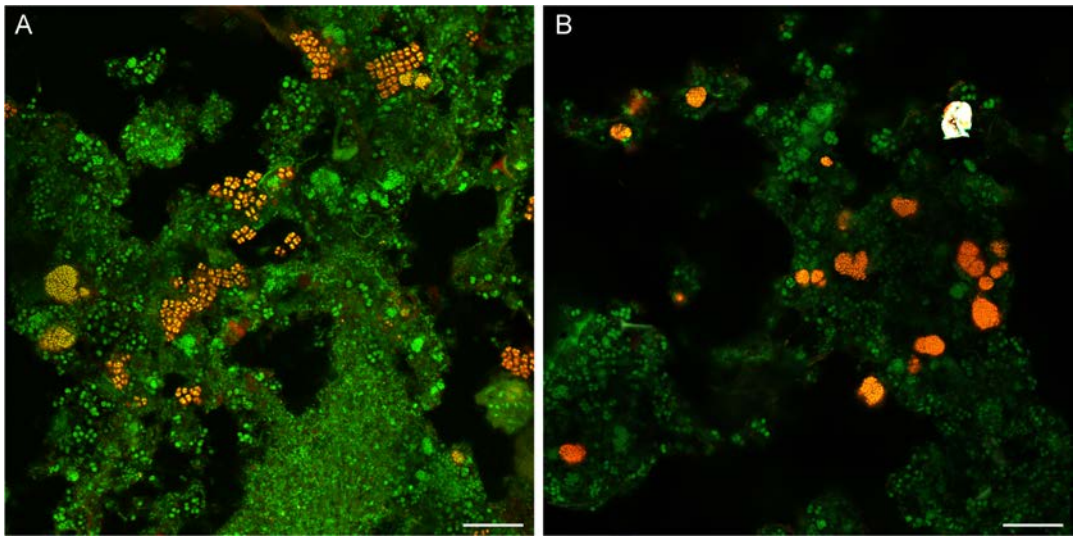


Figure 2. Detection of β-AOB in sludge from the WWTP CP Kelco using previously published and newly designed AOB probe mixtures. (**A**) Application of the old AOB probe mix (red) and probes EUB338 I-III (green). Orange indicates binding of the AOB- and EUB338-probes to the same cells. Note the detection of numerous tetrad-shaped cells by the old AOB mix and the EUB338 probes. (**B**) Application of the new AOB probe mix (red) and probes EUB338 I-III

(green). The white object in the upper right corner is an artifact. (**A, B**) Bar = 20 µm. Details of the probe mixtures are provided in Table 1.

3.2 *Design of a refined, β-AOB cluster-specific 16S rRNA-targeted oligonucleotide probe set*

Based on 16S rRNA and *amoA* gene phylogenies, a subdivision of the β-AOB into distinct lineages ("clusters") was suggested previously (Koops et al., 2003; Purkhold et al., 2003, 2000; Stephen et al., 1996). In that phylogenetic framework, members of the genus *Nitrosospira* belong to the clusters 0 to 4, whereas the remaining clusters 5 to 8 are formed by different lineages of the genus *Nitrosomonas* (Purkhold et al., 2000). Two additional, separate lineages were formed by *Nitrosomonas cryotolerans* and the estuary isolate *Nitrosomonas* sp. Nm143 (Purkhold et al., 2003, 2000). Notably, the obtained tree topologies did not support a clear separation of the *Nitrosospira* and *Nitrosomonas* genera; instead, the analyses indicated that the currently defined genus *Nitrosomonas* is not monophyletic within the β-AOB (Purkhold et al., 2003, 2000). Since the individual *Nitrosomonas* clusters nevertheless represent stable lineages in bootstrap analyses (Purkhold et al., 2003), and a thorough phylogenetic and taxonomic reevaluation of the β-AOB is still pending, we retain the established nomenclature in this study.

Using the β-AOB clusters as a framework we designed six new, 16S rRNA-targeted oligonucleotide probes that are specific for *Nitrosomonas* cluster 6 (including both 6a with *N. oligotropha* and *N. ureae*, and 6b with *N. marina* and *N. aestuarii*), cluster 7 (with *N. europaea*, *N. eutropha*, and *N. mobilis*), cluster 8 (with *N. communis* and *N. nitrosa*), the *N. cryotolerans* cluster, the *N.* sp. Nm143 cluster, and a new environmental cluster "DK-WWTP" related to cluster 8 (Fig. 3). In addition, the old probe Nsv443, which offered good coverage of the target clade but unsatisfactory target specificity, was replaced with the new probe Nsp441 for the

315 genus *Nitrosospira* (comprising clusters 0-4) (Fig. 3). For most of the new probes, we designed
316 competitor oligonucleotides that help discriminate non-target organisms which possess only
317 one or few weak base mismatches to the probe sequence in their 16S rRNA. These competitors
318 were used as unlabeled oligonucleotides and in equimolar concentrations as the labeled probes,
319 in FISH experiments. Details of the probes, their target groups, and the competitors are listed
320 in Table 1. According to an *in silico* analysis, the new probes in combination with the
321 designated competitors (Table 1) display a very good coverage and specificity for their target
322 β-AOB clusters, including cultured isolates as well as environmental sequences from
323 uncultured β-AOB (Table 3). Notably, the newly designed probes (with competitors) also
324 display a very high specificity with respect to non-target matches outside of the β-AOB (*in*
325 *silico* evaluation based on the SILVA SSU NR release 138 and the ARB "probe match" tool
326 with 0-2 weighted mismatches as search criterion). Merely for probe Nm_143_1010, less than
327 ten non-target betaproteobacterial sequences were found that are not covered by the competitor
328 for this probe (Table 1), and probe Nsp441 (Table 1) might hybridize with less than 60 non-
329 target betaproteobacteria (mainly from the genera *Hydrogenophaga* and *Gallionella*).
330 However, the number for Nsp441 is low compared to the previously published probe Nsv443
331 that would potentially detect more than 500 non-target organisms. Considering that any
332 environmental sample likely contains non-target organisms, which are not present in the current
333 sequence databases, we recommend to use the newly designed β-AOB cluster-specific probes
334 in combination with the broad-range probe Nso1225 (Table 3) labelled with a different
335 fluorochrome. Cells detected by both probes should represent the targeted β-AOB lineage. In
336 order to identify the optimal hybridization stringency for each probe, the dissociation profiles
337 of the probes were determined by performing FISH at increasing hybridization and washing
338 stringencies (Manz et al., 1992). Where possible, pure culture cells of the target β-AOB were
339 used in these experiments. As isolates of *N. cryotolerans*, *N.* sp. Nm143, and the uncultured

cluster DK-WWTP were not available, their 16S rRNA genes were heterologously expressed in *E. coli* (Schramm et al., 2002) to determine the dissociation profiles of the respective probes. For all probes, we obtained sigmoid dissociation profiles that were suitable to identify the highest stringency, which still yields bright fluorescence signals with the target organisms (Fig. S1 and S2, Table 1).

All probes were also hybridized to non-target β-AOB cells (or the respective recombinant *E. coli* cells) to test for unspecific hybridization. In these experiments, no fluorescence signal was observed for any probe at the optimal stringency and in the presence of the competitor oligonucleotides (data not shown).

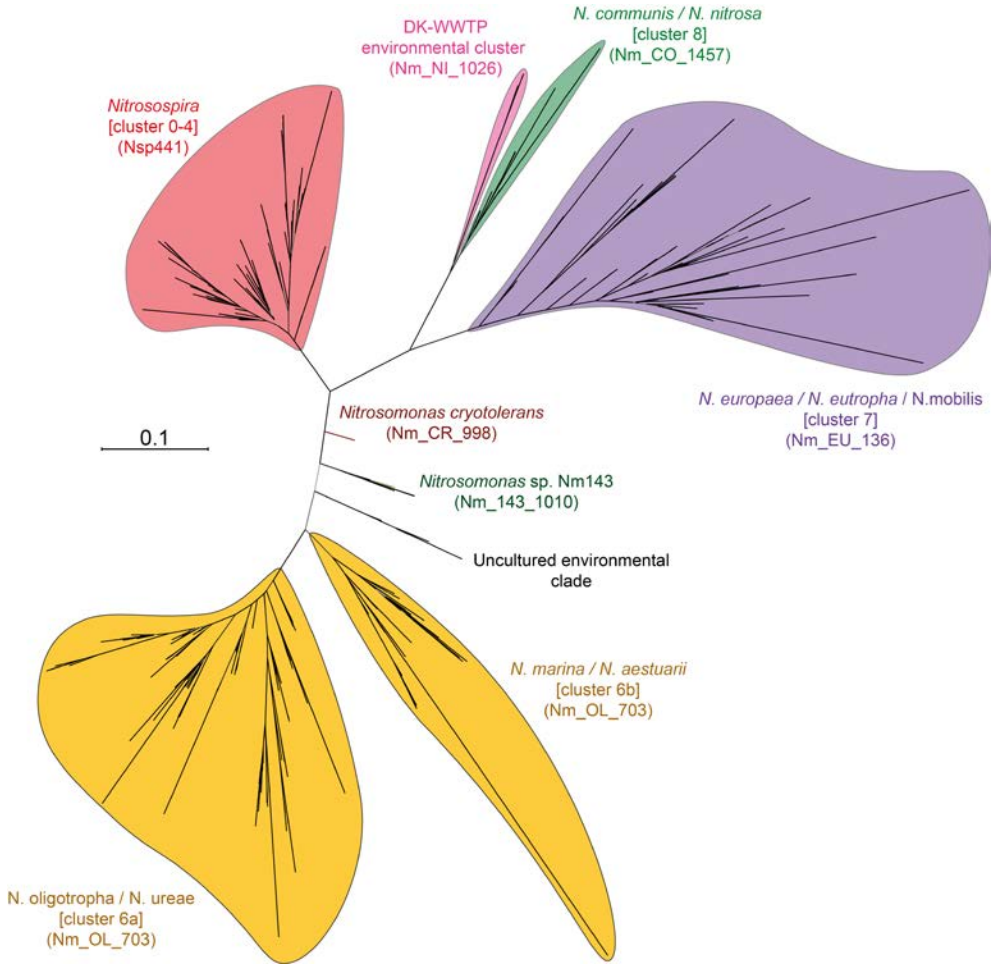


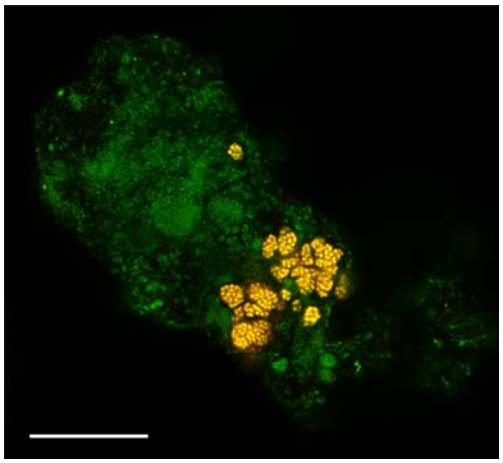
Figure 3. Unrooted maximum likelihood tree showing the major β-AOB lineages. Cluster designations, according to Purkhold *et al*. (2000), are indicated in brackets. The names of newly designed 16S rRNA-targeted oligonucleotide probes are indicated in parentheses. The scale bar depicts 0.1 estimated substitutions per nucleotide.

3.3 *Detection and quantification of β-AOB in activated sludge*

The applicability of the new probe set was tested in FISH experiments with nitrifying activated sludge samples from different WWTPs in Austria, Germany, and Denmark (Table 2). In particular, the newly designed probes targeting *Nitrosomonas* cluster 6 (Nm_OL_703) and cluster 7 (Nm_EU_136) were applied to the same sludge samples that we had already used to confirm the cross-hybridization of the previously published probes Cl6a192, NEU, and Ncom1025 (Fig. 1). With the two new probes, no overlapping fluorescence signals were observed, and the hybridization patterns appeared to be completely consistent (Fig. 1B, D). This result is in agreement with the *in silico* analysis, which predicted that probes Nm_OL_703 and Nm_EU_136 do not target the same sequences in the database (Table 3). Furthermore, when the "new AOB probe mix" (Table 1) was used to detect β-AOB in the sludge from CP Kelco, the conspicuous tetrad-shaped cells, detected by the "old AOB probe mix" were not labelled anymore. Instead, the "new probe mix" detected exclusively cells and cell clusters that displayed the typical morphology of β-AOB, which has been observed in numerous studies of nitrifiers in WWTPs and isolated β-AOB (Daims et al., 2001; e.g., Juretschko et al., 1998; Koops and Pommerening-Röser, 2001) (Fig. 2B). The new probe Nm_NI_1026, which targets the novel uncultured lineage DK-WWTP (Fig. 3), was applied to activated sludge from WWTP Esbjerg East where it showed specific signals with a morphology similar to that typically portrayed by β-AOB (Fig. 4). We could not test the new probes Nm_CR_998, Nm_143_1010,

375 Nm_CO_1457, and Nsp441 with environmental samples, because samples that contained their
376 target β-AOB and were suitable for FISH were not available in the course of this study.
377 However, these probes were successfully evaluated using pure β-AOB cultures or Clone-FISH.
378 Under the assumption that those probes, which could only be tested by Clone-FISH, will also
379 bind to the native ribosomes of their target organisms, all probes should be suitable for FISH
380 analyses of environmental AOB communities.

381



382

383 **Figure 4.** Detection of the novel *Nitrosomonas* lineage DK-WWTP in activated sludge from
384 the WWTP Esbjerg East. Applied probes were EUB338 I-III (green) and NM_NI_1026 (red).
385 Yellow indicates binding of NM_NI_1026 and the EUB338-probes to the same cells. Bar =
386 20 µm.

387

388 A quantitative comparison of the "old" and "new" AOB probe mixes (Table 1) was carried out
389 with activated sludge samples from seven WWTPs (Table 4). For six of these sludge samples,
390 highly similar biovolume fractions of β-AOB were measured by quantitative FISH with either
391 probe set (Table 4). Only for the CP Kelco sludge we obtained a much lower biovolume
392 fraction of β-AOB with the newly designed probe set than with the previously published probes

(Table 4). This difference is most likely explained by the better specificity of the newly designed probes, which did not stain the abundant tetrad-shaped cells in this sludge (Fig. 2). For all samples and experiments, high "congruency" values >90% were obtained (Table 4). This value indicates that the fluorescence signals in the β-AOB probe mix images occupied almost exactly the same area as their counterparts in the general bacterial probe (EUB338 I-III) images. Thus, the β-AOB probes did not detect large amounts of non-bacterial cells and did not bind excessively to non-microbial particles in the samples. Our results confirm that the new β-AOB probe mix (Table 1) is suitable to detect and quantify β-AOB in WWTPs, and, importantly, the data for CP Kelco show that the new mix also offers a better specificity for β-AOB than the previously published probes.

4. Conclusion

The newly designed 16S rRNA-targeted oligonucleotide probes offer a similar or better coverage of different β-AOB lineages than the established probes (Table 3), and they do not show the unspecific hybridization patterns that were observed for some of the previously designed probes when applied to complex microbial communities (Fig. 1 and 2). However, they are not designed to completely replace the established FISH probes for β-AOB. For example, probes Nso1225 and Nsm156 still offer an excellent coverage of their target groups (Table 3), which is why no new broad-range probe for β-AOB was designed in this study. Instead, the new probes can be applied in combination with selected previously published probes to achieve the currently best possible total coverage of β-AOB and to identify specific β-AOB lineages with a high confidence by FISH. Hence, the new probe set will facilitate future studies of β-AOB community composition and population dynamics. It will also enable specific analyses of the spatial localization of members of the different β-AOB lineages in flocs

417 and biofilms. Such spatial analyses can reveal potential niche differentiation and symbiotic
418 interactions with other microorganisms, but were previously hampered by unspecific
419 hybridization patterns of some of the old probes. Although we used only activated sludge and
420 biofilm samples from WWTPs to test the probes in this study, the new probe set can likely also
421 be applied to other types of samples that are suitable for rRNA-targeted FISH. In summary, the
422 new probes will improve virtually all spatial and functional analyses of β-AOB that use FISH
423 to gain insights which can only be obtained by *in situ* approaches.

424

425 **Declaration of competing interest**

426 The authors declare that they have no known competing financial interests or personal
427 relationships that could have appeared to influence the work reported in this paper.

428

429 **Acknowledgements**

430 This research was supported by the Austrian Science Fund (FWF) via project P27319-B21 to
431 H.D. and W1257 to H.D. and M.W., the Comammox Research Platform of the University of
432 Vienna, the Max Planck Society, and the Danish Research Foundation (grant 6111-00617A to
433 P.H.N.).

434

435 **References**

436 Adamczyk, J., Hesselsoe, M., Iversen, N., Horn, M., Lehner, A., Nielsen, P.H., Schloter, M.,
437 Roslev, P., Wagner, M., 2003. The isotope array, a new tool that employs substrate-

mediated labeling of rRNA for determination of microbial community structure and function. Appl. Environ. Microbiol. 69, 6875–6887.

Almstrand, R., Daims, H., Persson, F., Sorensson, F., Hermansson, M., 2013. New methods for analysis of spatial distribution and coaggregation of microbial populations in complex biofilms. Appl. Environ. Microbiol. 79, 5978–5987. https://doi.org/10.1128/aem.01727-13

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

Amann, R.I., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

Baptista, J.D.C., Lunn, M., Davenport, R.J., Swan, D.L., Read, L.F., Brown, M.R., Morais, C., Curtis, T.P., 2014. Agreement between *amoA* gene-specific quantitative PCR and fluorescence *in situ* hybridization in the measurement of ammonia-oxidizing bacteria in activated sludge. Appl. Environ. Microbiol. 80, 5901–5910. https://doi.org/10.1128/AEM.01383-14

Berry, D., Stecher, B., Schintlmeister, A., Reichert, J., Brugiroux, S., Wild, B., Wanek, W., Richter, A., Rauch, I., Decker, T., Loy, A., Wagner, M., 2013. Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing. Proc. Natl. Acad. Sci. U.S.A. 110, 4720–4725. https://doi.org/10.1073/pnas.1219247110

Bock, E., Wagner, M., 2001. Oxidation of inorganic nitrogen compounds as an energy source, in: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E. (Eds.), The Prokaryotes: A Handbook on the Biology of Bacteria. Springer Science+Business Media, New York, pp. 457–495.

463 Bollmann, A., Bullerjahn, G.S., McKay, R.M., 2014. Abundance and diversity of ammonia-
464 oxidizing archaea and bacteria in sediments of trophic end members of the Laurentian
465 Great Lakes, Erie and Superior. PLOS ONE 9, e97068.
466 https://doi.org/10.1371/journal.pone.0097068
467 Campbell, M.A., Chain, P.S.G., Dang, H., Sheikh, A.F.E., Norton, J.M., Ward, N.L., Ward,
468 B.B., Klotz, M.G., 2011. *Nitrosococcus watsonii* sp. nov., a new species of marine
469 obligate ammonia-oxidizing bacteria that is not omnipresent in the world's oceans: calls
470 to validate the names '*Nitrosococcus halophilus*' and '*Nitrosomonas mobilis*.' FEMS
471 Microbiol. Ecol. 76, 39–48. https://doi.org/10.1111/j.1574-6941.2010.01027.x
472 Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M., 1999. The domain-specific
473 probe EUB338 is insufficient for the detection of all *Bacteria*: Development and
474 evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444.
475 Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N.,
476 Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., von Bergen, M., Rattei,
477 T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by
478 *Nitrospira* bacteria. Nature 528, 504–509. https://doi.org/10.1038/nature16461
479 Daims, H., Lücker, S., Wagner, M., 2016. A new perspective on microbes formerly known as
480 nitrite-oxidizing bacteria. Trends Microbiol. 24, 699–712.
481 https://doi.org/10.1016/j.tim.2016.05.004
482 Daims, H., Lücker, S., Wagner, M., 2006. *daime*, a novel image analysis program for microbial
483 ecology and biofilm research. Env. Microbiol. 8, 200–213.
484 Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.H., Wagner, M., 2001. In situ
485 characterization of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater
486 treatment plants. Appl. Environ. Microbiol. 67, 5273–5284.

487 Daims, H., Stoecker, K., Wagner, M., 2005. Fluorescence *in situ* hybridisation for the detection
488 of prokaryotes, in: Osborn, A.M., Smith, C.J. (Eds.), Molecular Microbial Ecology.
489 Bios-Garland, Abingdon, U.K., pp. 213–239.
490 Daims, H., Wagner, M., 2007. Quantification of uncultured microorganisms by fluorescence
491 microscopy and digital image analysis. Appl. Microbiol. Biotechnol. 75, 237–248.
492 https://doi.org/10.1007/s00253-007-0886-z
493 DeLong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA based
494 probes for the identification of single cells. Science 243, 1360–1363.
495 Di, H.J., Cameron, K.C., Shen, J.-P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.-Z.,
496 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen
497 conditions. FEMS Microbiol. Ecol. 72, 386–394. https://doi.org/10.1111/j.1574-
498 6941.2010.00861.x
499 Dolinšek, J., Lagkouvardos, I., Wanek, W., Wagner, M., Daims, H., 2013. Interactions of
500 nitrifying bacteria and heterotrophs: Identification of a *Micavibrio*-like, putative
501 predator of *Nitrospira*. Appl. Env. Microbiol. 79, 2027–2037.
502 https://doi.org/10.1128/AEM.03408-12
503 Dueholm, M.S., Andersen, K.S., Petriglieri, F., McIlroy, S.J., Nierychlo, M., Petersen, J.F.,
504 Kristensen, J.M., Yashiro, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2019.
505 Comprehensive ecosystem-specific 16S rRNA gene databases with automated
506 taxonomy assignment (AutoTax) provide species-level resolution in microbial ecology.
507 bioRxiv 672873. https://doi.org/10.1101/672873
508 Fan, F., Yang, Q., Li, Z., Wei, D., Cui, X., Liang, Y., 2011. Impacts of organic and inorganic
509 fertilizers on nitrification in a cold climate soil are linked to the bacterial ammonia
510 oxidizer community. Microb. Ecol. 62, 982–990. https://doi.org/10.1007/s00248-011-
511 9897-5

512 Fernando, E.Y., McIlroy, S.J., Nierychlo, M., Herbst, F.-A., Petriglieri, F., Schmid, M.C.,
513 Wagner, M., Nielsen, J.L., Nielsen, P.H., 2019. Resolving the individual contribution
514 of key microbial populations to enhanced biological phosphorus removal with Raman–
515 FISH. ISME J. 13, 1933–1946. https://doi.org/10.1038/s41396-019-0399-7
516 Fujitani, H., Kumagai, A., Ushiki, N., Momiuchi, K., Tsuneda, S., 2015. Selective isolation of
517 ammonia-oxidizing bacteria from autotrophic nitrifying granules by applying cell-
518 sorting and sub-culturing of microcolonies. Front. Microbiol. 6.
519 https://doi.org/10.3389/fmicb.2015.01159
520 Gao, J.-F., Luo, X., Wu, G.-X., Li, T., Peng, Y.-Z., 2013. Quantitative analyses of the
521 composition and abundance of ammonia-oxidizing archaea and ammonia-oxidizing
522 bacteria in eight full-scale biological wastewater treatment plants. Bioresour. Technol.
523 138, 285–296. https://doi.org/10.1016/j.biortech.2013.03.176
524 Gruber-Dorninger, C., Pester, M., Kitzinger, K., Savio, D.F., Loy, A., Rattei, T., Wagner, M.,
525 Daims, H., 2015. Functionally relevant diversity of closely related *Nitrospira* in
526 activated sludge. ISME J. 9, 643–655.
527 Gwak, J.-H., Jung, M.-Y., Hong, H., Kim, J.-G., Quan, Z.-X., Reinfelder, J.R., Spasov, E.,
528 Neufeld, J.D., Wagner, M., Rhee, S.-K., 2019. Archaeal nitrification is constrained by
529 copper complexation with organic matter in municipal wastewater treatment plants.
530 ISME J. 1–12. https://doi.org/10.1038/s41396-019-0538-1
531 Hatzenpichler, R., Scheller, S., Tavormina, P.L., Babin, B.M., Tirrell, D.A., Orphan, V.J.,
532 2014. *In situ* visualization of newly synthesized proteins in environmental microbes
533 using amino acid tagging and click chemistry. Env. Microbiol. 16, 2568–2590.
534 https://doi.org/10.1111/1462-2920.12436
535 Hayatsu, M., Tago, K., Uchiyama, I., Toyoda, A., Wang, Y., Shimomura, Y., Okubo, T.,
536 Kurisu, F., Hirono, Y., Nonaka, K., Akiyama, H., Itoh, T., Takami, H., 2017. An acid-

tolerant ammonia-oxidizing gamma-proteobacterium from soil. ISME J. 11, 1130–1141. https://doi.org/10.1038/ismej.2016.191

Huang, W.E., Stoecker, K., Griffiths, R., Newbold, L., Daims, H., Whiteley, A.S., Wagner, M., 2007. Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence *in situ* hybridization for the single cell analysis of identity and function. Env. Microbiol. 9, 1878–1889.

Juretschko, S., 2000. Mikrobielle Populationsstruktur und -dynamik in einer nitrifizierenden/denitrifizierenden Belebtschlammanlage (Ph.D. thesis). Technische Universität München, München.

Juretschko, S., Loy, A., Lehner, A., Wagner, M., 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst. Appl. Microbiol. 25, 84–99.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.-P., Wagner, M., 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64, 3042–3051.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589. https://doi.org/10.1038/nmeth.4285

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543–546.

Koops, H.-P., Böttcher, B., Möller, U.C., Pommering-Röser, A., Stehr, G., 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov.,

562 *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina*
563 sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas*
564 *oligotropha* sp. nov., and *Nitrosomonas halophila* sp. nov. J. Gen. Microbiol. 137,
565 1689–1699.
566 Koops, H.P., Pommerening-Röser, A., 2001. Distribution and ecophysiology of the nitrifying
567 bacteria emphasizing cultured species. FEMS Microbiol. Ecol. 37, 1–9.
568 Koops, H.P., Purkhold, U., Pommerening-Röser, A., Timmermann, G., Wagner, M., 2003. The
569 lithoautotrophic ammonia-oxidizing bacteria, in: Dworkin, M., Falkow, S., Rosenberg,
570 E., Schleifer, K.H., Stackebrandt, E. (Eds.), The Prokaryotes: A Handbook on the
571 Biology of Bacteria. Springer Science+Business Media, New York, pp. 778–811.
572 Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.-H.,
573 Wagner, M., 1999. Combination of fluorescent *in situ* hybridization and
574 microautoradiography - a new tool for structure-function analyses in microbial ecology.
575 Appl. Environ. Microbiol. 65, 1289–1297.
576 Letunic, I., Bork, P., 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new
577 developments. Nucleic Acids Res. 47, W256–W259.
578 https://doi.org/10.1093/nar/gkz239
579 Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhu, K., Buchner, A., Lai, T.,
580 Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O.,
581 Grumann, S., Hermann, S., Jost, R., König, A., Liss T, Lüßmann, R., May, M., Nonhoff,
582 B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M.,
583 Ludwig, T., Bode, A., Schleifer, K.H., 2004. ARB: a software environment for
584 sequence data. Nucleic Acids Res. 32, 1363–1371.

585 Lukumbuzya, M., Schmid, M., Pjevac, P., Daims, H., 2019. A multicolor fluorescence in situ
586 hybridization approach using an extended set of fluorophores to visualize
587 microorganisms. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.01383
588 Maixner, F., Noguera, D.R., Anneser, B., Stoecker, K., Wegl, G., Wagner, M., Daims, H.,
589 2006. Nitrite concentration influences the population structure of *Nitrospira*-like
590 bacteria. Env. Microbiol. 8, 1487–1495.
591 Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.-H., 1992. Phylogenetic
592 oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and
593 solutions. Syst. Appl. Microbiol. 15, 593–600.
594 McIlroy, S.J., Saunders, A.M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A.A., Karst,
595 S.M., Nielsen, J.L., Nielsen, P.H., 2015. MiDAS: the field guide to the microbes of
596 activated sludge. Database, bav062. https://doi.org/10.1093/database/bav062
597 Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., Stahl, D.A., 1996. Phylogenetic
598 probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl.
599 Environ. Microbiol. 62, 2156–2162.
600 Musat, N., Foster, R., Vagner, T., Adam, B., Kuypers, M.M.M., 2012. Detecting metabolic
601 activities in single cells, with emphasis on nanoSIMS. FEMS Microbiol. Rev. 36, 486–
602 511. https://doi.org/10.1111/j.1574-6976.2011.00303.x
603 Mußmann, M., Brito, I., Pitcher, A., Sinninghe Damste, J.S., Hatzenpichler, R., Richter, A.,
604 Nielsen, J.L., Nielsen, P.H., Muller, A., Daims, H., Wagner, M., Head, I.M., 2011.
605 Thaumarchaeotes abundant in refinery nitrifying sludges express *amoA* but are not
606 obligate autotrophic ammonia oxidizers. Proc. Natl. Acad. Sci. U.S.A. 108, 16771–
607 16776. https://doi.org/10.1073/pnas.1106427108
608 Nierychlo, M., Andersen, K.S., Xu, Y., Green, N., Albertsen, M., Dueholm, M.S., Nielsen,
609 P.H., 2019. Species-level microbiome composition of activated sludge - introducing the

MiDAS 3 ecosystem-specific reference database and taxonomy. bioRxiv 842393. https://doi.org/10.1101/842393

Okabe, S., Satoh, H., Watanabe, Y., 1999. *In situ* analysis of nitrifying biofilms as determined by *in situ* hybridization and the use of microelectrodes. Appl. Environ. Microbiol. 65, 3182–3191.

Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.-A., Hugenholtz, P., 2018. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat. Biotechnol. 36, 996–1004. https://doi.org/10.1038/nbt.4229

Parte, A.C., 2014. LPSN—list of prokaryotic names with standing in nomenclature. Nucleic Acids Res. 42, D613–D616. https://doi.org/10.1093/nar/gkt1111

Prosser, J.I., Head, I.M., Stein, L.Y., 2014. The family *Nitrosomonadaceae*, in: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes: Alphaproteobacteria and Betaproteobacteria. Springer, Berlin, Heidelberg, pp. 901–918. https://doi.org/10.1007/978-3-642-30197-1_372

Pruesse, E., Peplies, J., Glöckner, F.O., 2012. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28, 1823–1829. https://doi.org/10.1093/bioinformatics/bts252

Purkhold, U., Pommering-Röser, A., Juretschko, S., Schmid, M.C., Koops, H.-P., Wagner, M., 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. 66, 5368–5382.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Röser, A., Koops, H.P., 2003. 16S rRNA and *amoA*-based phylogeny of 12 novel betaproteobacterial ammonia oxidizing

isolates: extension of the data set and proposal of a new lineage within the Nitrosomonads. Int. J. Syst. Evol. Microbiol. 53, 1485–1494.

Pynaert, K., Smets, B.F., Wyffels, S., Beheydt, D., Siciliano, S.D., Verstraete, W., 2003. Characterization of an autotrophic nitrogen-removing biofilm from a highly loaded lab-scale rotating biological contactor. Appl. Environ. Microbiol. 69, 3626–3635.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219

Schillinger, C., Petrich, A., Lux, R., Riep, B., Kikhney, J., Friedmann, A., Wolinsky, L.E., Göbel, U.B., Daims, H., Moter, A., 2012. Co-localized or randomly distributed? Pair cross correlation of *in vivo* grown subgingival biofilm bacteria quantified by digital image analysis. PLoS ONE 7, e37583. https://doi.org/10.1371/journal.pone.0037583

Schramm, A., de Beer, D., van den Heuvel, J.C., Ottengraf, S., Amann, R., 1999. Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira* spp. along a macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Appl. Environ. Microbiol. 65, 3690–3696.

Schramm, A., de Beer, D., Wagner, M., Amann, R., 1998. Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. 64, 3480–3485.

Schramm, A., Fuchs, B.M., Nielsen, J.L., Tonolla, M., Stahl, D.A., 2002. Fluorescence *in situ* hybridization of 16S rRNA gene clones (Clone-FISH) for probe validation and screening of clone libraries. Env. Microbiol. 4, 713–720.

Sliekers, A.O., Haaijer, S., Schmid, M., Harhangi, H., Verwegen, K., Kuenen, J.G., Jetten, M.S.M., 2004. Nitrification and anammox with urea as the energy source. Syst. Appl. Microbiol. 27, 271–278. https://doi.org/10.1078/0723-2020-00259

Stephen, J.R., McCaig, A.E., Smith, Z., Prosser, J.I., Embley, T.M., 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. 62, 4147–4154.

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., Minh, B.Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 44, W232–W235. https://doi.org/10.1093/nar/gkw256

van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single microorganism. Nature 528, 555–559.

Verhamme, D.T., Prosser, J.I., Nicol, G.W., 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. ISME J. 5, 1067–1071. https://doi.org/10.1038/ismej.2010.191

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., Schleifer, K.-H., 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. 60, 792–800.

Wagner, M., Rath, G., Amann, R., Koops, H.-P., Schleifer, K.-H., 1995. In situ identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. 18, 251–264.

Welch, J.L.M., Rossetti, B.J., Rieken, C.W., Dewhirst, F.E., Borisy, G.G., 2016. Biogeography of a human oral microbiome at the micron scale. Proc. Natl. Acad. Sci. U.S.A. 113, E791–E800. https://doi.org/10.1073/pnas.1522149113

681 Wells, G.F., Park, H.D., Yeung, C.H., Eggleston, B., Francis, C.A., Criddle, C.S., 2009.
682 Ammonia-oxidizing communities in a highly aerated full-scale activated sludge
683 bioreactor: betaproteobacterial dynamics and low relative abundance of *Crenarchaea*.
684 Env. Microbiol. 11, 2310–2328. https://doi.org/10.1111/j.1462-2920.2009.01958.x
685 Winkler, M.K.H., Bassin, J.P., Kleerebezem, R., Sorokin, D.Y., van Loosdrecht, M.C.M.,
686 2012. Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic
687 granular sludge. Appl. Microbiol. Biotechnol. 94, 1657–1666.
688 Zhou, L.-J., Han, P., Yu, Y., Wang, B., Men, Y., Wagner, M., Wu, Q.L., 2019. Cometabolic
689 biotransformation and microbial-mediated abiotic transformation of sulfonamides by
690 three ammonia oxidizers. Water Res. 159, 444–453.
691 https://doi.org/10.1016/j.watres.2019.05.031
692

693 **Tables**

694 **Table 1.** rRNA-targeted oligonucleotide probes used in this study. Probe Gam42a binds to the 23S rRNA, whereas all other listed probes target

695 binding sites on the 16S rRNA.

Probe	**Intended target organisms**	**Probe sequence (5'-3')**	**Competitors and their sequences (5'-3')[a]**	**FA[b] (%)**	**Fluorophore[c]**	**Reference**
L-C-gProt-1027-a-A-17 (Gam42a)	Gammaproteobacteria	GCCTTCCCACATCGTTT	GCCTTCCCACTTCGTTT	35	Cy3	Manz et al., 1992
S-D-Bact-0338-a-A-18 (EUB338-I)	Most bacteria	GCTGCCTCCCGTAGGAGT		35	FITC, Atto565	Amann et al., 1990
S-*-BactP-0338-a-A-18 (EUB338-II)	Planctomycetales and various other bacteria	GCAGCCACCCGTAGGTGT		35	FITC, Atto565	Daims et al., 1999
S-*-BactV-0338-a-A-18 (EUB338-III)	Verrucomicrobia and various other bacteria	GCTGCCACCCGTAGGTGT		35	FITC, Atto565	Daims et al., 1999
S-F-bAOB-1224-a-A-20 (Nso1225)	Most β-AOB	CGCCATTGTATTACGTGTGA		35	Cy3	Mobarry et al., 1996
S-*-Nsm-0651-a-A-18 (NEU)	*Nitrosomonas europaea* (in cluster 7)	CCCCTCTGCTGCACTCTA	TTCCATCCCCCTCTGCCG	35-40	FITC	Wagner et al., 1995
S-*-Nsm6a-0192-a-A-20 (Cl6a192)	*N. oligotropha* lineage (cluster 6a)	CTTTCGATCCCCTACTTTCC	CTTTCGATCCCCTGCTTTCC	35	Cy5	Adamczyk et al., 2003
Ncom1025	*N. communis* lineage (cluster 8)	CTCGATTCCCTTTCGGGCA		35	Atto490LS, Cy3	Juretschko, 2000

"Old AOB probe mix" (Nso1225, Cl6a192, Ncom1025, NEU)	Most β-AOB			35	Cy3, Atto488	As for the single probes
Nm_OL_703	*N. oligotropha, N. ureae, N. aestuarii, N. marina* lineage (cluster 6a+6b)	GCCATCGATGTTCTTCCATATCTC	GCCATCGGTGTTCCTCCATATCTC GCCATCGGTGTTCCTCCACATCTC	35	Atto532	This study
Nm_EU_136	*N. europaea, N. eutropha, N. mobilis* lineage (cluster 7)	CGATGCGTTATTCCCCACTTAAGG	CGATGCGTTATCCCCCACTTAAGG	35	Atto633	This study
Nm_CR_998	*N. cryotolerans* lineage	CTCAGCGAGCTTAGATACAT	CTCAGCGAGCTTCGGTACAT CTCAGCGGGCTTCGATACAT	20	DY-681	This study
Nm_143_1010	*Nitrosomonas sp.* Nm143 lineage	GGGCACGCCTACCTCTCAGT	GGGCACGCCCACCTCTCAGC	35	FITC	This study
Nsp441	*Nitrosospira* lineage (clusters 0-4)	GTGACCGTTTCGTTCCGGCT	GTAACCGTTTCTTTCCGGCT	20	Atto565	This study
Nm_CO_1457	*N. communis* lineage (cluster 8)	AACTCTCACCGTGGCAAACGCCC	GAATCTCACCGTGGCAAACGCCC	35	Atto490LS	This study
Nm_NI_1026	DK-WWTP lineage	GTCTTAATTCCCTTTCGGGC		35	Atto594	This study
"New AOB probe mix" (Nm_OL_703, Nm_EU_136, Nm_CO_1457)	Most β-AOB known to be relevant in WWTPs			35	As for the single probes	This study

[a] Competitors were added as unlabeled oligonucleotides and in equimolar concentrations as the labeled probes.

[b] FA, formamide. The formamide concentration in the hybridization buffer in per cent (v/v) is indicated.

[c] Probes were 5'-labeled with these fluorophores. Details of all listed dyes are provided elsewhere (Lukumbuzya et al., 2019).

699 **Table 2.** Overview of the WWTPs, which were the source of activated sludge samples analyzed in this study.

WWTP (abbreviations)	**Location[a]**	**Sampling date (month/year)**	**Type of WWTP/reactor**
KNB	Klosterneuburg, AT	10/2014, 10/2015	Municipal. Sequencing batch reactor that treats reject water from sludge dewatering after anaerobic digestion.
SBBR1	Ingolstadt, DE	3/1998	Municipal. Previous pilot-size sequencing biofilm batch reactor that treated reject water from sludge dewatering after anaerobic digestion.
ING	Ingolstadt, DE	3/2011	Municipal. Denitrifying-nitrifying tank.
CP Kelco	Lille Skensved, DK	12/2014	Industrial. 300,000 PE full-scale wastewater treatment plant with biological nutrient removal, recirculation and anaerobic digestion.
Esbjerg E	Esberg, DK	8/2016	Municipal. 150,000 PE full-scale wastewater treatment plant with biological nutrient removal, recirculation, primary settling and anaerobic digestion.
Kalundborg	Kalundborg, DK	8/2010	Municipal. 50,000 PE full-scale wastewater treatment plant with enhanced biological phosphorus removal, intermittent aeration.
Lundtofte	Lyngby, DK	8/2010	Municipal. 150,000 PE full-scale wastewater treatment plant with enhanced biological phosphorus removal, intermittent aeration, primary settling, and anaerobic digestion.
Odense NW	Odense, DK	11/2014	Municipal. 51,000 PE full-scale wastewater treatment plant with biological nutrient removal and intermittent aeration, primary settling and anaerobic digestion.

700 [a] Abbreviations: AT, Austria; DE, Germany; DK, Denmark.

701

Table 3. *In silico* analysis of the target group coverage and specificity of previously published and newly designed 16S rRNA-targeted probes for β-AOB. Numbers in the table header (in parentheses) are the numbers of analyzed full-length 16S rRNA gene sequences from the respective lineage in the SILVA Ref_NR99 (release 132) and MiDAS databases. Numbers in all other rows indicate the fractions (in per cent) of the lineages that are targeted by the probes without any nucleotide mismatch. Numbers in parentheses indicate the fractions (in per cent) targeted by the probes with up to 1.5 weighted mismatches according to the ARB "probe match" tool. Table cells of the intended probe target groups are marked grey. Those non-target organisms of a probe, which are discriminated by the competitor listed in Table 1, are not included in the listed fractions.

Probe	All β-AOB (505)	*Nitrosospira* (cluster 0-4) (153)	*N. oligotropha, N. ureae* (cluster 6a) (157)	*N. marina, N. aestuarii* (cluster 6b) (58)	*N. europaea, N. eutropha, N. halophila* (cluster 7) (86)	*N. mobilis* (cluster 7) (11)	*N. communis, N. nitrosa* (cluster 8) (21)	DK-WWTP lineage (3)	*N. cryotolerans* lineage (1)	*N.* sp. Nm143 lineage (1)
Nso1225	94.9	100	93.0	41.4	95.4	45.5	95.2	100	100	100
Nso190	42.6	96.1	0	12.1	58.1	9.1	0	0	100	100
Nsm156	64.2	3.3	90.9	93.1	94.2	90.9	100	0	100	100
Nsv443	15.6	51.6	0	0	0	0	0	0	0	0
Nsp441	15.8	52.3	0	0	0	0	0	0	0	0
Cl6a192	16.2	0	49.7	6.9	0	0	0	0	0	0
Nm_OL_703	36.4	0	80.3	100	0	0	0	0	0	0
NEU	13.1	0 (28.1)	0 (31.21)	0	75.6 (93.0)	0 (63.7)	0	0	0 (100)	0 (100)
NmV	1.4	0	0	1.7	0	54.6	0	0	0	0
Nm_EU_136	14.7	0	0	1.7	75.6	81.8	0	0	0	0
Ncom1025	3.4	0	0	0	0	0 (72.7)	81.0	0	0	0
Nm_CO_1457	3.4	0	0	0	0	0	81.0	0	0	0
Nm_NI_1026	0.6	0	0	0	0	0	0	100	0	0
Nm_CR_998	0.2	0	0	0	0	0	0	0	100	0
Nm_143_1010	0.2	0	0	0	0	0	0	0	0	100

709 **Table 4.** Quantification of the biovolume fractions of β-AOB in seven WWTPs using the old and the new AOB probe mixtures.

	Previously published AOB probe mix		**Newly designed AOB probe mix**	
WWTP	**Biovolume fraction (%)**	**Congruency (%)**	**Biovolume fraction (%)**	**Congruency (%)**
CP Kelco	2.5	97	0.8	98
Esbjerg E	1.1	97	1.3	94
Kalundborg	1.5	99	1.2	99
Lundtofte	0.5	99	0.8	97
Odense NW	1.2	98	1.1	95
KNB	1.3	96	1.6	98
ING	0.9	93	1.4	97

710