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Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants

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Abstract

Numerous past studies have shown members of the genus *Nitrospira* to be the predominant nitrite-oxidizing bacteria (NOB) in nitrifying wastewater treatment plants (WWTPs). Only recently, the novel NOB "*Candidatus* Nitrotoga arctica" was identified in permafrost soil and a close relative was enriched from activated sludge. Still, little is known about diversity, distribution, and functional importance of *Nitrotoga* in natural and engineered ecosystems. Here we developed *Nitrotoga* 16S rRNA-specific PCR primers and FISH probes, which were applied to screen activated sludge samples from 20 full-scale WWTPs. *Nitrotoga*-like bacteria were detected by PCR in 11 samples and reached abundances detectable by FISH in seven sludges. They coexisted with *Nitrospira* in most of these WWTPs, but constituted the only detectable NOB in two systems. Quantitative FISH revealed that *Nitrotoga* accounted for nearly 2% of the total bacterial community in one of these plants, a number comparable to *Nitrospira* abundances in other WWTPs. Spatial statistics revealed that *Nitrotoga* co-aggregated with ammonia oxidizing bacteria, strongly supporting a functional role in nitrite oxidation. This activity was confirmed by FISH-MAR, which revealed nitrite-dependent autotrophic carbon fixation by *Nitrotoga in situ*. Correlation of presence or absence with WWTP operational parameters indicated low temperatures as a main factor supporting high *Nitrotoga* abundances, although in incubation experiments these NOB remained active over an unexpected range of temperatures, and also at different ambient nitrite concentrations. In conclusion, this study demonstrates that *Nitrotoga* can be functionally important nitrite oxidizers in WWTPs and can even represent the only known NOB in engineered systems.

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Keywords

Nitrotoga; nitrite-oxidizing bacteria; nitrification; activated sludge; wastewater treatment

Introduction

The anthropogenic release of large amounts of nitrogen has detrimental effects on the environment such as eutrophication of inland and coastal water bodies, leading to algal blooms and hypoxia (Diaz and Rosenberg 2008). As high concentrations of ammonia and nitrite are also toxic for many organisms, their discharge into the environment must be regulated to protect ecosystems and drinking water supplies (Camargo and Alonso 2006, Conley *et al*. 2009). Besides agriculture, human sewage is one of the largest sources of inorganic nitrogen, in particular ammonia from urea degradation. Therefore, WWTPs designed for nutrient removal are essential for protecting aquatic ecosystems from nitrogen contamination. Most WWTPs exploit nitrifying and denitrifying microbes to aerobically oxidize ammonia via nitrite to nitrate (nitrification) and to subsequently reduce the produced nitrate to gaseous dinitrogen under anaerobic conditions (denitrification). Complete nitrification depends on the mutualistic interaction of ammonia-oxidizing microbes and nitrite-oxidizing bacteria (NOB) and thus both groups have been intensively studied (for reviews see Gujer 2010, Wagner *et al*. 2002).

Before cultivation-independent molecular methods became available, members of the alphaproteobacterial genus *Nitrobacter* were thought to be mainly responsible for nitrite oxidation in sewage treatment because the isolation of these NOB from activated sludge was straightforward (e.g., Henze *et al*. 1997). This view changed radically when molecular tools revealed that *Nitrobacter* occurs in many WWTPs only in small numbers close to or even below the detection limit of microscopy-based methods such as fluorescence *in situ* hybridization (FISH; Wagner *et al*. 1996). Instead, yet uncultured *Nitrospira* turned out to be the key NOB in most engineered systems (Daims *et al*. 2001b, Juretschko *et al*. 1998, Schramm *et al*. 1998). Since this discovery, research on NOB in WWTPs has focused mainly on *Nitrospira*, which belong to the distinct bacterial phylum *Nitrospirae*, display a considerable phylogenetic diversity, and possess genetic and physiological features that clearly distinguish them from other known NOB (Daims *et al*. 2001a, Foesel *et al*. 2008, Lücker *et al*. 2010, Maixner *et al*. 2006, Maixner *et al*. 2008, Schramm *et al*. 1999).

However, novel nitrite oxidizers are still being discovered (e.g., Schott *et al*. 2010). Recently, Alawi *et al*. (2007) enriched the novel nitrite-oxidizing betaproteobacterium "*Candidatus* Nitrotoga arctica" from permafrost soil. *N. arctica* only grows at low temperatures between 4 to 17 °C. At only 0.3 mM (Alawi *et al*. 2007), its nitrite concentration optimum is approximately one order of magnitude below the nitrite concentrations usually applied to cultivate *Nitrospira* isolates (Lebedeva *et al*. 2008) and even two orders of magnitude below the nitrite concentrations used to grow *Nitrobacter* (Prosser 1989). Interestingly, a closely related *Nitrotoga* strain was also enriched from a full-scale WWTP (Alawi *et al*. 2009), fuelling speculations that these novel NOB might be relevant for nitrite oxidation in engineered systems. This question cannot be answered by

cultivation-based approaches because very few *Nitrotoga* cells could be sufficient as inoculum for a successful enrichment culture, whereas their *in situ* numbers may be low and thus irrelevant for the nitrification process in the system.

In this study we therefore applied the full-cycle rRNA approach (Amann *et al*. 1995) to investigate whether *Nitrotoga* are hitherto overlooked key nitrifiers in full-scale sewage treatment systems. Based on in-depth phylogenetic analyses of the new candidate genus *Nitrotoga*, cultivation-independent molecular tools for the specific detection of *Nitrotoga*-like bacteria were developed. These were then applied to detect, visualize, and quantify *Nitrotoga* in nitrifying full-scale WWTPs and to investigate their spatial distribution patterns relative to ammonia-oxidizing bacteria (AOB) within activated sludge flocs. Furthermore, FISH in combination with microradiography (FISH-MAR) was used to test for the chemolithoautotrophic capacity of *Nitrotoga*-like NOB across a range of nitrite concentrations and temperatures.

Material and Methods

Activated sludge sampling and fixation

Activated sludge samples were obtained from full-scale sequencing batch reactors (SBRs) operated with or without differential internal cycling (DIC) (Holm 2003), from conventional activated sludge basins, fixed bed reactors, and a membrane filtration plant. The selected WWTPs are located in Germany and Switzerland and treat municipal wastewater, which in some cases is mixed with industrial sewage or animal rendering waste (Table 1). In addition, highly enriched *N. arctica* was grown according to Alawi *et al*. (2007) and used for probe and primer evaluation.

For FISH analysis, activated sludge and enrichment culture samples were fixed with paraformaldehyde (PFA) according to Daims *et al*. (2005). Fixed biomass was stored at −20°C. Unfixed samples for DNA extraction were harvested by centrifugation (13,000 × g, 10 min, 4°C) and stored at −20°C.

Probe and primer design and evaluation

16S rRNA-targeted FISH probes and 16S rRNA gene-targeted PCR primers were designed and evaluated using the probe design and probe match functions of ARB (Ludwig *et al*. 2004) and a manually curated SILVA 16S rRNA database (version SSURef_NR99_115) (Quast *et al*. 2013). In short, the 16S rRNA sequence database was updated by importing (i) all high quality, near full-length 16S rRNA sequences (≥1250 nucleotides, pintail quality scores ≥75%) included in the SILVA SSU r117 web release and classified as *Candidatus* Nitrotoga (http://www.arb-silva.de/browser/), and (ii) all sequences with an identity >96% to *N. arctica* strain 6680 (DQ839562) from the NCBI nr database. Sequences with a pintail value <75% were regarded as potential chimeras (Ashelford *et al*. 2005) and excluded from further analyses. For the purpose of this study, the genus *Nitrotoga* was defined on the basis of phylogenetic analyses (see below) and only included sequences that formed a stable monophyletic group with *N. arctica*. The definition of the family *Gallionellaceae* was based on SILVA classification. High quality sequences of family members were extracted from the

SILVA guide tree and comprised the genera *Nitrotoga*, *Gallionella*, and *Sideroxydans*, as well as a large number of sequences of unresolved affiliation within the family.

Optimal PCR conditions for the new *Nitrotoga*-targeting primer pairs were determined by temperature gradient PCR. Optimal hybridization conditions for newly developed FISH probes were determined as described previously (Daims *et al*. 1999). Non-target organisms were not included, as they were not available in pure culture or contained in our clone libraries for Clone-FISH (Schramm *et al*. 2002). The probes were thus used at the highest hybridization stringency (i.e., formamide concentration in the hybridization buffer) that still yielded bright fluorescence signals for the target organisms and in combination with unlabeled competitor probes specific for the non-target organisms with the fewest number of mismatches at the respective probe binding site. Moreover, in all experiments at least two probes labeled with different fluorochromes were used in combination to unambiguously identify the target organisms according to the multiple probe concept (Ludwig *et al*. 1998). Probes used for FISH were 5′-labeled with the dyes 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS), Cy3, or Cy5. Labeled probes, unlabeled competitors, and PCR primers were obtained from Thermo Scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA). All FISH probes used in this study are listed in Tables 2 and S1.

DNA extraction, PCR, and cloning of 16S rRNA genes

Genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA, USA) according to manufacturer's instructions. Preliminary test PCRs were performed using the general bacterial primers 616V and 1492r to ensure that the DNA was of sufficient quantity, quality, and purity for PCR as described elsewhere (Juretschko *et al*. 1998, Kane *et al*. 1993). For the specific amplification of *Nitrotoga*-like 16S rRNA genes, the genus *Nitrotoga*-specific primer combination S-G-Ntoga-0124-a-S-19 (Ntoga124F, 5′- ATC GGA ACG TAC CCG GAA A-3′) and S-G-Ntoga-1462-a-A-18 (Ntoga1462R, 5′- CGA ACC CTA CCG TGG CAA C-3′) were used. Reaction mixtures were prepared according to the manufacturer's recommendations in a total volume of 50 µl with 2 mM $MgCl_2$, 0.5 µM of each primer, and 1.25 U of Taq polymerase (Fermentas, St. Leon-Rot, Germany). Additionally, 5 µg of BSA were added to circumvent PCR inhibition. PCR cycling consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and elongation at 72°C for 1 min 30 s, and was completed by a final elongation step at 72°C for 10 min. The presence and purity of amplicons were confirmed by agarose gel electrophoresis. Cloning and sequencing of amplified 16S rRNA genes were performed as described elsewhere (Juretschko *et al*. 1998). Cloned genes to be sequenced were selected based on different restriction fragment length polymorphism (RFLP) patterns, with 1 - 5 clones per pattern randomly chosen. For RFLP, 5 µl of M13 (Invitrogen, Carlsbad, CA, USA) PCR product were digested with 1 µl restriction enzyme MspI (Fisher Scientific, Vienna, Austria) and 1 µl buffer (universal buffer "Tango", Fisher Scientific, Vienna, Austria) at 37°C for 3 h. Fragment patterns were separated and visualized by gel electrophoresis using a 2.5% (w/v) agarose gel.

Phylogenetic analyses

For phylogenetic tree calculations, a manually curated 16S rRNA database was created as described above (see chapter Probe and primer design and evaluation). All sequences imported into the ARB software (Ludwig *et al*. 2004) were automatically aligned using the tools implemented in ARB, followed by manual refinement of the alignment. Phylogenetic analyses were performed using maximum-likelihood and maximum-parsimony methods as provided by ARB, or Bayesian inference using MrBayes (Ronquist and Huelsenbeck 2003). A 50% conservation filter for the family *Gallionellaceae* was used (resulting in 1481 informative positions) and only near full-length sequences (>1320 nucleotides) were included in tree calculations. Bootstrap values were estimated using the maximum-likelihood and maximum-parsimony algorithms with 100 iterations, or MrBayes run with parameters stoprule=yes and stopval=0.01, causing the program to stop when the average standard deviation of the topological convergence diagnostics has reached a value <0.01.

FISH, microscopy, and digital image analysis

Aliquots of PFA-fixed biomass were spotted onto microscope slides and FISH was performed as described elsewhere (Daims *et al*. 2005). Probe-conferred fluorescence was recorded on a LSM 510 confocal laser scanning microscope (CLSM, Carl Zeiss AG, Oberkochen, Germany) equipped with one argon ion (450 to 514 nm) and two helium neon lasers (543 and 633 nm) for the detection of FLUOS, Cy3, and Cy5, respectively, or a Leica LSM SP8 equipped with a white light laser (Leica Microsystems, Wetzlar, Germany). For determining probe dissociation profiles (Daims *et al*. 1999) of newly designed FISH probes (Table 2), highly enriched *N. arctica* biomass was used for hybridization with the respective probe and 10 images per formamide concentration were recorded for subsequent image analysis. For quantifying relative biovolume fractions (Daims and Wagner 2007, Schmid *et al*. 2000), the activated sludge samples were hybridized to probe Ntoga122 and the EUB338 probe mix (Tables 2 and S1), and 40 image pairs containing each probe signal were taken at random fields of view. For analyzing spatial distribution patterns of *Nitrotoga*-like bacteria within activated sludge flocs (Daims *et al*. 2006), cells were stained by probes Ntoga122, Cluster6a192, and BET42a (Tables 2 and S1) and 40 images of these probe signals were recorded at random positions. All digital image analysis tasks were carried out by using the software *daime* (Daims et al., 2006).

FISH and microautoradiography

Activated sludge was sampled from two DIC-SBR reactors (Bad Zwischenahn and Deuz, Germany; Table 1) and transferred to the laboratory at 4°C within 24h. Prior to the incubations, sludge samples were diluted at a ratio of 1:5 with sterile filtered supernatant from the same reactor and pre-incubated at room temperature overnight to ensure that all endogenous ammonia and nitrite were consumed. Concentrations of these electron donors were monitored by Nessler's reagent (Sigma-Aldrich, Vienna, Austria) and Merckoquant test stripes (Merck, Vienna, Austria), respectively. Subsequently, 5 ml sample aliquots were transferred to 100 ml glass serum bottles. All experiments were performed in duplicates and PFA-fixed biomass from the same reactor was used as dead control. To start the incubation, $NaNO_2$ was added to the desired concentration (see below) and each vessel was

supplemented with 10 μCi [^{14}C]-H_2CO_3. The bottles were sealed air tight with rubber stoppers. Assuming that parallel incubations behaved similarly, nitrite consumption was followed in an additional sample for each incubation condition which contained non-radioactive bicarbonate. Nitrite was replenished when necessary with an aseptic needle without opening the radioactive culture bottles. Incubations were performed using six different nitrite concentrations (0, 0.1, 0.5, 1, 5, and 10 mM NO_2^-, at 14°C) and at five different temperatures (4°C, 10°C, 14°C, 20°C, and 27°C, with 0.5 mM NO_2^-). After 6 h, incubations were stopped by harvesting the biomass (25,500 × g, 5 min, 4°C) and performing PFA fixation as described elsewhere (Daims *et al*. 2005). In order to disintegrate the sludge flocs, one volume of PFA-fixed sample was diluted with four volumes 1x phosphate buffered saline (PBS), transferred to a 2 ml screw-cap reaction tube containing a ¼-inch ceramic sphere, and vortexed horizontally for 10 min at maximum speed. Following two washing steps in 1 × PBS, a small aliquot was spotted onto a cover slip and FISH-MAR was carried out as described by Lee *et al*. (1999) with an exposure time of 7 days.

Results and discussion

Phylogeny and environmental distribution of the candidate genus *Nitrotoga*

N. arctica is the proposed type strain of the new candidate genus *Nitrotoga* (Alawi *et al*. 2007), which is affiliated with the family *Gallionellaceae* (Skerman *et al*. 1980) of the order *Gallionellales* (Weiss *et al*. 2007), and contains the only known nitrite oxidizers in the *Betaproteobacteria*. Besides *Nitrotoga*, the *Gallionellaceae* comprise two genera of iron-oxidizing organisms, *Gallionella* (Garrity *et al*. 2005, Henrici and Johnson 1935) and *Sideroxydans* (Weiss *et al*. 2007), some of which were reported to couple Fe(II)-oxidation to nitrate reduction (Blöthe and Roden 2009).

In this study, the genus *Nitrotoga* was defined based on phylogenetic support and comprised only sequences that consistently formed a monophyletic cluster with *N. arctica* in our analyses. The affiliation of partial 16S rRNA sequences with the genus was confirmed by individually adding them to a representative tree without changing the overall tree topology. According to this conservative approach, the candidate genus *Nitrotoga* contains, besides the WWTP-derived sequences from this study and the enrichments described by Alawi *et al*. (2007, 2009), organisms thriving in diverse habitats (Figure 1). *Nitrotoga*-like sequences were detected by cultivation-independent approaches in different wastewater and drinking water treatment systems (Ji and Chen 2010, Kong *et al*. 2007, Kwon *et al*. 2010, Maestre *et al*. 2009, White *et al*. 2012), soil (Sattin *et al*. 2009), groundwater and cave-derived water (Chen *et al*. 2009, Flynn *et al*. 2013), and in lake, river, and marine water and sediment samples (Brümmer *et al*. 2003, Li *et al*. 2011, Liu *et al*. 2012, Martiny *et al*. 2011, Na *et al*. 2011, Percent *et al*. 2008, Schwarz *et al*. 2007, Tamminen *et al*. 2011). While this wide distribution of *Nitrotoga*-like organisms indicates that these novel NOB might contribute to nitrification in a great variety of habitat types, none of these studies provided evidence for their function in the respective ecosystem. Thus, their abundances and *in situ* functions in these systems, as well as their competitive success compared to other NOB such as *Nitrospira* and *Nitrobacter*, remain to be elucidated.

Probe design and evaluation

16S rRNA-targeted probes for the specific detection of *Nitrotoga*-like bacteria by FISH (Table 2) were designed according to the "multiple probe approach" (Ludwig *et al*. 1998), ensuring the unambiguous identification of *Nitrotoga* cells by phylogenetically nested probes for the candidate genus *Nitrotoga* and most members of the family *Gallionellaceae*. Probes Ntoga122 and Ntoga1424 were designed to target the candidate genus only, whereas probes FGall221b and Ntoga438 also include some sequences of uncertain affiliation within the *Gallionellaceae*, which have a high sequence similarity (95.8 to 97.9%) to *N. arctica* but did not cluster consistently with the genus *Nitrotoga* in our phylogenetic analyses. Probe FGall178 and the FGall221a+b probe mixture target most known members of the family *Gallionellaceae* for which sequence information is available in the respective region of the 16S rRNA (Table 2).

When tested on highly enriched *N. arctica* biomass, all probes yielded bright fluorescence signals, irrespective of the dye used for probe labeling. Only signals of probe Ntoga1424 were relatively dim, which is consistent with a low brightness of probes targeting the homologous region of the 16S rRNA in *E. coli* (Behrens *et al*. 2003). *In silico* evaluation indicated a good specificity of all probes, with the respective perfect-match probe binding sites found in very few non-target organisms only. Still, the new probes should always be used by combining the three genus-specific, or two genus- with one family-specific probe, all labeled in different colors. Organisms detected by all applied probes can be regarded as *Nitrotoga*-like bacteria with a high degree of confidence because the current 16S rRNA databases contain no non-target organism with the binding sites for three of the *Nitrotoga* or *Gallionellaceae*-specific probes. Still, as some closely related bacteria (mainly *Betaproteobacteria*) have few and hard to discriminate mismatches at the respective probe binding site, we recommend using the new probes in combination with the respective unlabeled competitor oligonucleotides (Table 2). In this study, unambiguous identification of *Nitrotoga* in the activated sludge samples was ensured by first hybridizing the sample with multiple *Nitrotoga*-specific probes as described above. As these experiments revealed that all applied probes bound to the same cell clusters and no cells were detected by only a subset of these probes, the new probes were subsequently also used in combination with probes targeting organisms other than *Nitrotoga* or *Gallionellaceae* (see below).

Occurrence of *Nitrotoga*-like NOB in WWTPs

Utilizing the new genus *Nitrotoga*-specific primer pair Ntoga124F/Ntoga1462R, we screened 20 full-scale WWTPs by PCR for the presence of *Nitrotoga*-like bacteria. With SBRs, DIC-SBRs, and conventional activated sludge systems, these plants represented different reactor types (Table 1). PCR amplicons were obtained for 11 samples, indicating that *Nitrotoga*-like NOB were present in as many as 55% of the screened WWTPs (Table 1). All of these 11 plants received municipal sewage, which in some cases was mixed with different amounts of industrial wastewaters. The applied PCR assay did not detect *Nitrotoga*-like NOB in any of the systems treating animal rendering waste.

To confirm the specificity of the primer pair, the PCR products obtained from four WWTP samples, including one plant where *Nitrotoga* was detectable by PCR but not by FISH (see

below), were cloned and in total 61 of the cloned 16S rRNA genes were Sanger-sequenced. Indeed, none of the clone libraries contained any non-target organism. Although the sequences obtained in this study shared high sequence similarities ≥98%, they formed several sub-clusters within the candidate genus *Nitrotoga* in phylogenetic analyses (Figure 1). These sub-clusters might reflect microdiversity of closely related and coexisting *Nitrotoga* strains or the presence of multiple *rrn* operons with small sequence dissimilarities in *Nitrotoga* genomes. Alternatively, the observed sequence differences could be artifacts introduced by PCR or sequencing errors.

FISH confirmed the presence of *Nitrotoga*-like organisms in seven WWTPs, which all were also PCR-positive (Table 1). In these samples, the *Nitrotoga* cells occurred as dense clusters of heterogeneous shape located within the sludge flocs (Figure 2). The cells in these aggregates were irregularly shaped rods or cocci, resembling the morphologies described for *N. arctica* (Alawi *et al*. 2007). Interestingly, all WWTPs that harbored *Nitrotoga* in sufficiently high quantities for detection by FISH (Amann *et al*. 1995) were operated at temperatures between 7 and 16°C (Table 1). This observation is fully consistent with the optimal growth temperature range of enriched *Nitrotoga* cultures (Alawi *et al*. 2009) and temperature hence seems to be a major factor affecting the growth of *Nitrotoga*-like NOB in full-scale WWTPs.

Positive PCR results could not be confirmed by FISH for three of the SBRs and for one fixed-bed reactor (Table 1). Possible reasons for this discrepancy may be a cellular ribosome content of *Nitrotoga* below the detection limit of the applied standard FISH protocol (1400 ribosomes per cell; Hoshino *et al*. 2008) or PCR-amplification of DNA from lysed cells or extracellular DNA. Considering that PCR is at least tenfold more sensitive than FISH (Amann *et al*. 1995), the abundance of *Nitrotoga* most likely was too low for FISH detection in these activated sludges, implying that *Nitrotoga* were not functionally important and probably were allochthonous organisms unable to establish large stable populations in these WWTPs.

Quantification of *Nitrotoga*-like bacteria in activated sludge

In most of the analyzed WWTPs containing *Nitrotoga*-like bacteria, they were of low abundance (<1% of the total bacterial biomass) and coexisted with NOB of the genus *Nitrospira* (Table 1). Intriguingly, however, no known NOB except *Nitrotoga* were detected in the WWTPs Langenzenn and Bad Zwischenahn. The sludge from Langenzenn harbored only few *Nitrotoga* cell clusters (<1% of total bacterial biomass), and high ammonia concentrations were measured in the effluent of this plant at the time of sampling, although AOB related to *Nitrosomonas oligotropha* were present in this sample (data not shown). On the contrary, the activated sludge from Bad Zwischenahn contained comparably large amounts of *Nitrotoga*-like bacteria, which according to quantitative FISH constituted between 1% and 2% of the total bacterial biovolume (measured in four technical replicates). The low concentrations of ammonia and nitrite in the effluent of this WWTP (Table 1) imply complete nitrification. As *Nitrotoga*-like bacteria were the only detected NOB in this plant, this finding suggests that *Nitrotoga* can be solely responsible for nitrite oxidation in full-scale WWTPs.

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Spatial co-localization of *Nitrotoga*-like NOB with AOB

AOB and NOB are partners in a mutualistic symbiosis where AOB oxidize ammonia to nitrite, which then serves as substrate for NOB whose activity prevents the accumulation of nitrite that could otherwise be toxic to AOB (Stein and Arp 1998). The strong interdependence of the two functional groups is often reflected by a close spatial co-aggregation of AOB and NOB in nitrifying activated sludge and biofilm samples, as observed frequently for *Nitrospira* with various AOB (Juretschko *et al*. 1998, Maixner *et al*. 2006, Okabe *et al*. 1999, Schramm *et al*. 1999). The spatial arrangement patterns of microbial populations in complex samples can be analyzed by a combination of FISH, image analysis, and spatial statistics (Daims *et al*. 2006) and already confirmed the co-localization of *Nitrospira* and AOB in WWTPs (Daims *et al*. 2006, Maixner *et al*. 2006).

To test whether *Nitrotoga*-like bacteria also co-localize with AOB, their spatial distribution patterns in two activated sludge samples were analyzed by the aforementioned method. The first WWTP addressed by this approach was Bad Zwischenahn, where no other known NOB except *Nitrotoga* had been detected (Table 1). In this sludge sample, all known AOB were detected by probe Cluster6a192 as shown in test hybridizations in combination with the AOB probe mix (Table S1). Visual observation already indicated that most *Nitrotoga* cell clusters occurred in close vicinity of AOB within the sludge flocs, sometimes even enclosing the AOB cell aggregates (Figure 2). Quantitative analysis confirmed a pronounced co-aggregation of AOB and *Nitrotoga*-like bacteria at distances below 50 μm between the cell clusters (Figure 3, panel A), suggesting that *Nitrotoga* preferably grew in the close vicinity of AOB in this sludge. The degree of clustering was highest at distances between 2 and 40 μm with two local maxima, one at 12 and a more pronounced one at 26 μm. These two peaks might reflect the presence of at least two *Nitrotoga* subpopulations, which would be in line with the apparent microdiversity of *Nitrotoga* found in the clone libraries (including clones from WWTP Bad Zwischenahn, Figure 1). Such slightly different spatial distribution patterns relative to AOB were also observed for NOB belonging to sublineage I and II of the genus *Nitrospira*, which have different nitrite concentration optima and thus occurred at different distances from AOB, which are the source of nitrite (Maixner *et al*. 2006). At distances larger than 50 μm no co-aggregation of *Nitrotoga*-like bacteria and AOB was detected and the pair cross-correlation function was not significantly different from one (indicating a random distribution pattern) or was even below this threshold. However, it should be noted that low pair cross-correlation values at large distances can also result from the absence of nitrifiers outside the activated sludge flocs, a bias that occurs if the analyzed distances are close to or exceed the average floc size (Daims *et al*. 2006).

To confirm the quantified co-aggregation of *Nitrotoga* and AOB, the analysis was repeated with a negative control that was artificially derived from the recorded FISH images. First, the *Nitrotoga* and AOB probe signals (both groups are affiliated with the *Betaproteobacteria*) were digitally subtracted from the BET42a probe signal (targeting all *Betaproteobacteria*), resulting in images containing only other betaproteobacterial cells. These cells did not belong to any known nitrifying population and thus were not expected to have any specific functional link to *Nitrotoga*. Subsequently, the spatial arrangement pattern of *Nitrotoga* relative to the non-nitrifying *Betaproteobacteria* was quantified, resulting in

pair cross-correlation values close to one over the whole range of tested distances (Figure 3, panel B). This result indicates a random distribution of *Nitrotoga* relative to non-nitrifying *Betaproteobacteria* in the sludge flocs. When only the *Nitrotoga* probe signal was subtracted from the BET42a signal, the presence of AOB within the remaining BET42a probe-defined population restored the observed co-aggregation pattern at distances between 6 and 50 μm (Figure 3, panel C). Thus, the co-aggregation between *Nitrotoga* and AOB was specific and most likely caused by a direct physiological interaction between these organisms.

The spatial distribution of *Nitrotoga*-like NOB relative to AOB was quantified also in WWTP Deuz, where in addition to *Nitrotoga* also *Nitrospira* had been detected by FISH (Table 1). While the *in situ* analyses of the sludge from WWTP Bad Zwischenahn had suggested that *Nitrotoga* can functionally replace *Nitrospira*, it remained to be shown if *Nitrotoga*-like bacteria can also compete with *Nitrospira* for niches in the close neighborhood of AOB when both NOB groups co-occur. Indeed, spatial arrangement analyses showed that both *Nitrotoga* and *Nitrospira* co-aggregated with AOB in WWTP Deuz. Intriguingly, however, the quantified co-aggregation patterns where distinctly different. For the more abundant *Nitrospira*, a strong co-aggregation signal at distances ranging from 5 to 35 μm was obtained (Figure 3, panel D). *Nitrotoga*-like bacteria also strongly co-aggregated with AOB, but the distance range was much more narrow with a clear peak around 10 μm distance (Figure 3, panel E). This outcome strongly suggests that both NOB groups are functionally linked to AOB, but apparently show ecological niche partitioning. *Nitrospira*-like bacteria appear to be more flexible regarding the symbiosis with AOB, for example because they could be adapted to a relatively broad range of nitrite concentrations found at different distances from AOB, or due to the presence of several sublineages (Maixner *et al*. 2006). In contrast, *Nitrotoga* seemed to inhabit a narrower niche where they could successfully compete for nitrite or might be involved in other yet uncharacterized biological interactions with AOB. Taken together, our analyses confirmed that in two different WWTPs *Nitrotoga*-like NOB specifically co-aggregated with AOB at short distances and thus strongly supported the hypothesis that *Nitrotoga* grow by oxidizing nitrite in full-scale WWTPs.

In situ chemolithoautotrophic activity of *Nitrotoga*-like NOB

FISH and spatial arrangement analyses of activated sludge samples already strongly indicated that *Nitrotoga*-like organisms grow by nitrite oxidation in full-scale WWTPs, but a physiological proof of this lifestyle was lacking. Thus, activated sludge from two different WWTPs was incubated in the presence or absence of nitrite, and nitrite-dependent inorganic carbon fixation by the autotrophic *Nitrotoga*-like bacteria was monitored at the single-cell level by FISH-MAR. In one of the plants analyzed (WWTP Bad Zwischenahn; Figures 4 and S1) *Nitrotoga* constituted the only known NOB, whereas in the second plant (WWTP Deuz; Figure S2) they coexisted with *Nitrospira*. Indeed, in both samples *Nitrotoga*-like bacteria readily incorporated carbon from [^{14}C]-H_2CO_3 when the activated sludge was incubated at a low nitrite concentration (0.5 mM NO_2^-) and temperature (14°C), conditions reported optimal for growth of *N. arctica* and a closely related WWTP isolate (Alawi *et al*. 2007, Alawi *et al*. 2009). Hence, these FISH-MAR data clearly demonstrate the role of uncultured *Nitrotoga*-like bacteria as novel NOB in the two WWTPs analyzed. Moreover,

they turned out to be active over a broad range of nitrite concentrations and temperatures. They readily incorporated ^{14}C-labeled bicarbonate with as little as 0.1 mM nitrite and still remained active in presence of 10 mM nitrite (Figures S1 and S2), which was far above the tolerance limit of 1.2 mM nitrite reported for *N. arctica* (Alawi *et al*. 2007).Furthermore, they actively fixed carbon at temperatures from 4°C up to 27°C (Figures S1 and S2), an unexpected broad range of incubation temperatures as *Nitrotoga* isolates were reported to proliferate at low temperatures only (Alawi *et al*. 2007, Alawi *et al*. 2009). Similarly, carbon incorporation by the coexisting *Nitrospira*-like NOB in WWTP Deuz could be detected with all nitrite concentrations and at all temperatures tested (Figure S2). Altogether, our FISH-MAR experiments revealed that the uncultured *Nitrotoga*-like NOB were able to fix inorganic carbon across a much greater span of environmental conditions than previously anticipated based on the behavior of cultured representatives. However, it should be noted that the metabolic activity observed in our short-term incubations not necessarily shows growth, but rather that *Nitrotoga* can remain active under conditions that may be less favorable for them. Such flexibility is certainly beneficial in fluctuating natural environments or in WWTPs where the operational conditions change frequently, like in SBRs or in regions with pronounced daily temperature shifts. Since the *Nitrotoga* strains cultured so far were outcompeted by other NOB in long-term enrichments at high nitrite concentrations or temperatures (Alawi *et al*. 2009), and as we found high *Nitrotoga* abundances by FISH only in low-temperature WWTPs (Table 1), we expect differences between short-term activity and long-term ecological success of *Nitrotoga*-like NOB. Furthermore, the influence on their growth rates of additional factors like presence of organic substrates and salts remains to be investigated in future research.

Conclusions

This study demonstrates that the recently discovered *Nitrotoga* are functionally important nitrite oxidizers in full-scale WWTPs, where they often co-exist with *Nitrospira* but occasionally represent the only known NOB populations. Thus, *Nitrotoga* should be included in studies of nitrification in WWTPs in addition to *Nitrospira*, *Nitrobacter*, and *Nitrolancea* (Sorokin *et al*. 2014). With the newly developed *Nitrotoga*-specific PCR primers and FISH probes, we provide molecular tools for the reliable *in situ* detection, visualization, and quantification of *Nitrotoga*-like bacteria. Clearly, to achieve encompassing insights into the microbiology of nitrification we will need further research on *Nitrotoga* reaching from environmental distribution surveys to functional, genomic, and post-genomic analyses. Especially in the context of wastewater treatment, future work should determine key ecophysiological parameters such as the nitrite oxidation kinetics of *Nitrotoga*-like bacteria, and should address their competition and co-existence with other NOB. A highly interesting topic will be the sensitivity or resilience of *Nitrotoga* to disturbances during reactor operation. Such information is urgently needed for all NOB relevant in WWTPs because problems and failures of the nitrification process in engineered systems are still frequently encountered, but the causes are mostly unknown and a reliable strategy to prevent such events has not been developed yet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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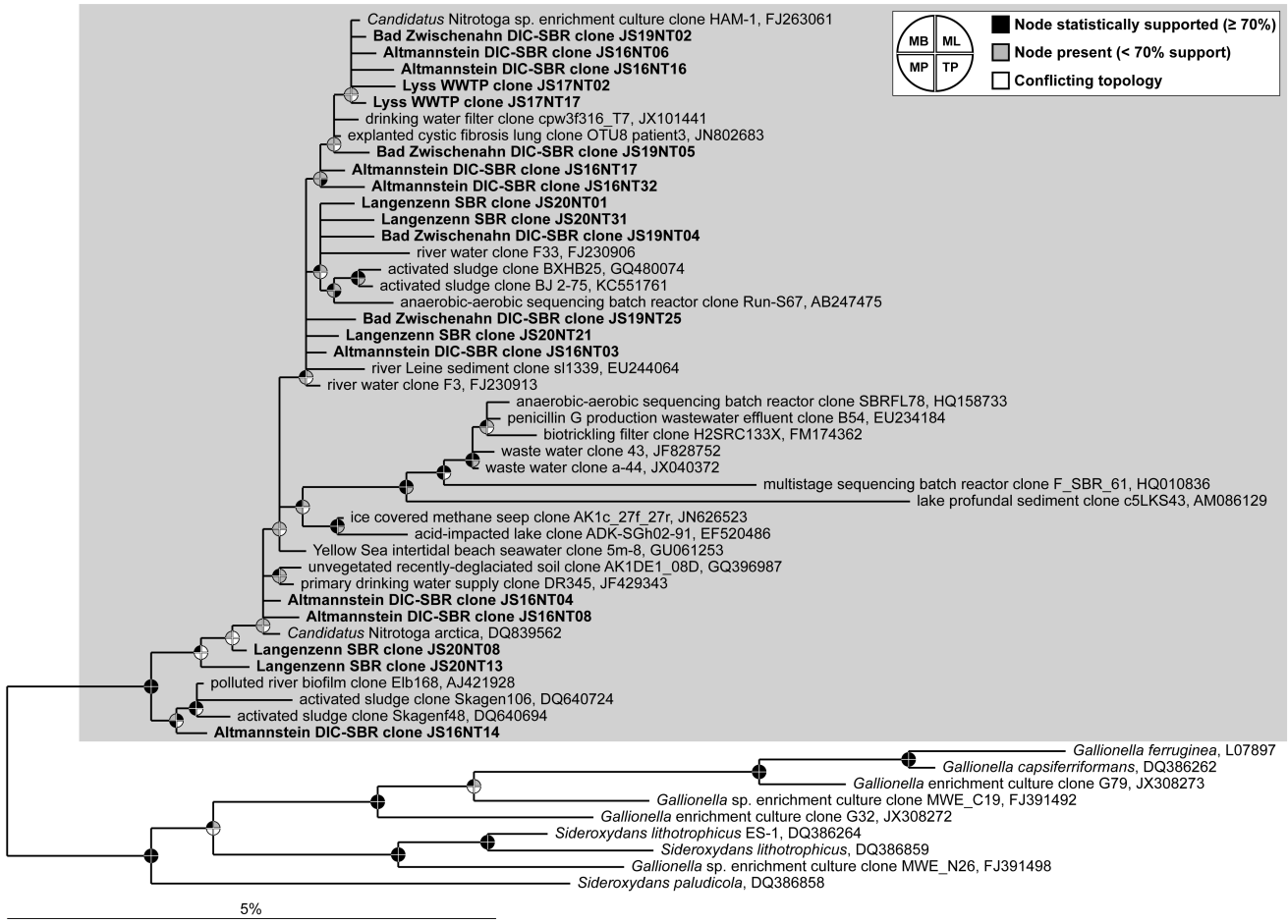

Figure 1. Phylogenetic analysis of the candidate genus *Nitrotoga* (gray box) and selected members of the family *Gallionellaceae*.

Displayed is a Bayesian interference tree (s.d. = 0.009882) including representative nearly full-length 16S rRNA gene sequences related to *N. arctica* strain 6680. Pie charts indicate statistical support of nodes based on bootstrap analysis or Bayesian inference. Bootstrap values are based on 100 iterations. Sequences obtained in this study are printed in bold. The scale bar corresponds to 5% estimated sequence divergence. MB, Bayesian inference; ML, maximum likelihood; MP, maximum parsimony; TP, Treepuzzle.

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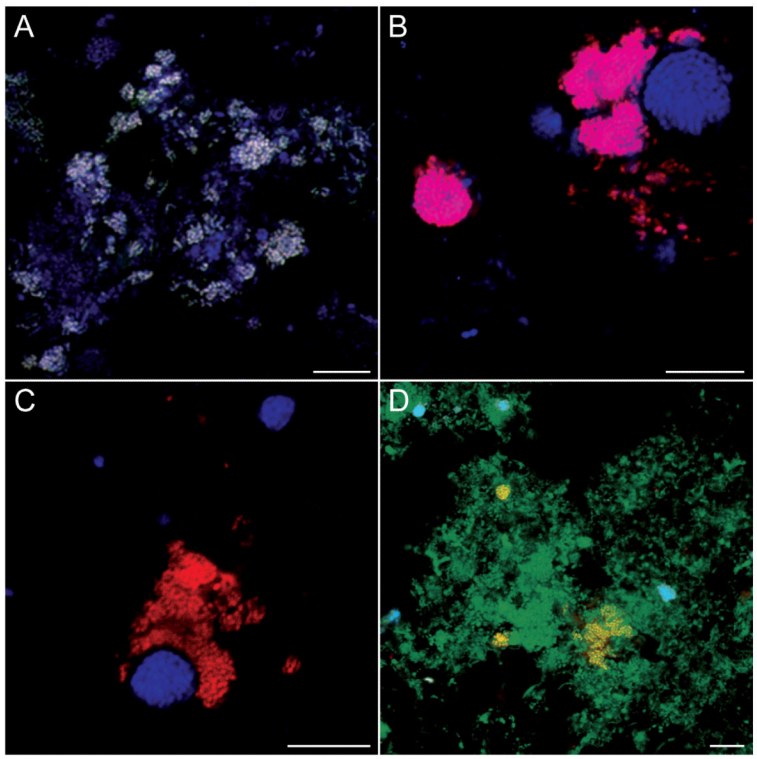

Figure 2. Confocal micrographs of FISH-stained *Nitrotoga*-like bacteria in activated sludge samples from WWTPs Bad Zwischenahn (A-C) and Deuz (D).

(A) *Nitrotoga* cell aggregates hybridized to probes Ntoga122 (green), FGall221b (red), and EUB338mix (blue). *Nitrotoga* appear white due to overlay of all probe signals. (B) *Nitrotoga* detected by probes FGall221b (red) and EUB338mix (blue) at high magnification. *Nitrotoga* appear magenta. (C) Simultaneous detection of *Nitrotoga* and AOB cell clusters by probes FGall221b (red) and Cluster6a192 (blue) at high magnification. Note the close vicinity of *Nitrotoga* and AOB, reflecting their metabolic interaction. (D) Simultaneous detection of *Nitrotoga* and *Nitrospira* by probes Ntoga122 (red), Ntspa662 (blue), and EUB338mix (green). *Nitrotoga* appear yellow, *Nitrospira* cyan. For probe details refer to Tables 2 and S1. The scale bar in all micrographs equals 10 μm.

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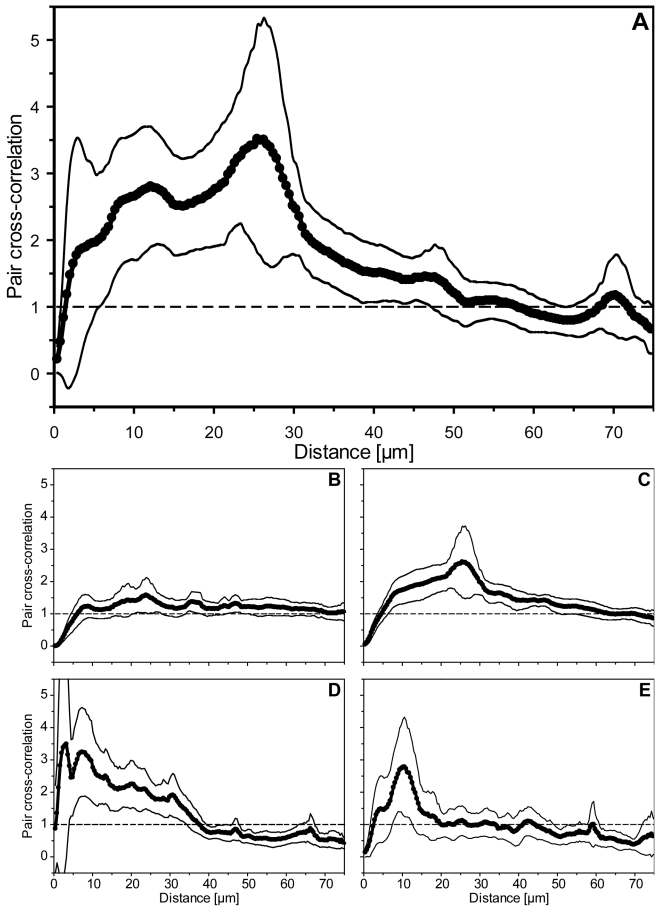

Figure 3. Statistical analyses of the spatial arrangement patterns of NOB and AOB in the activated sludge samples from the Bad Zwischenahn (A-C) and Deuz (D, E) WWTPs.

(A-C) Spatial arrangement of *Nitrotoga*-like bacteria relative to (A) AOB, (B) all other *Betaproteobacteria* excluding AOB, and (C) all other *Betaproteobacteria* including AOB. (D, E) Spatial arrangement of (D) *Nitrospira*-like and (E) *Nitrotoga*-like bacteria relative to AOB. Black circles depict the mean pair cross-correlation function, and the upper and lower lines delimit 95% confidence intervals. Values >1 indicate co-aggregation, values <1 repulsion, and values =1 (dashed horizontal line) random distribution at the corresponding distance (Daims *et al*. 2006).

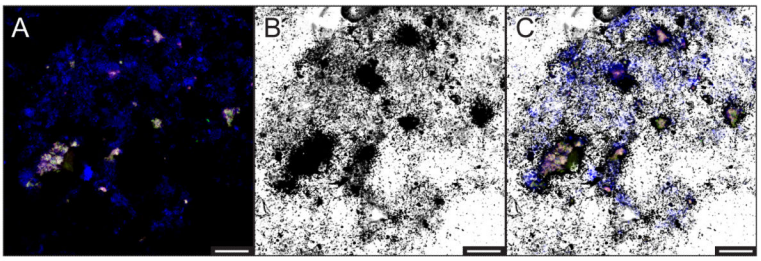

Figure 4. Confocal micrographs of FISH-stained *Nitrotoga*-like bacteria with corresponding MAR signal.

Shown are representative images from the incubation of activated sludge from WWTP Bad Zwischenahn with 0.5 mM NO_2^- at 14°C. (A) *Nitrotoga* cell aggregates hybridized to probes Ntoga122 (red), FGall221b (green), and EUB338mix (blue); (B) corresponding DIC image showing silver grain deposition above radioactively labeled bacteria; (C) overlay of FISH and MAR signal. The scale bar in all images corresponds to 25 μm.

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Table 1

Characteristics of the analyzed wastewater treatment plants.

WWTP	Reactor type[a]	Type of treated sewage	Detection of *Nitrotoga*[b]		*Nitrospira* sublineage[c,d]	Temp.[e] [°C]	Influent[e] [mg/l]	Effluent[e] [mg/l]			Sampling date (2007)
			PCR	FISH			NH_4^+	NH_4^+	NO_2^-	NO_3^-	
Altmannstein	SBR	municipal	+	+	I + II	7	54.7	9.18	0.48	0.72	March 24
Ampfing	SBR	municipal, slaughter, and dairy waste	−	−	II	13	nd	0.1	0.04	3.24	March 26
Bad Zwischenahn	DIC-SBR	municipal and industrial	+	+	−	16	60	0.25	0.15	6.5	May 23
Bruchmühlen	DIC-SBR	municipal	+	+	I	15	36	0.53	0.09	4.53	May 22
Deuz	DIC-SBR	municipal	+	+	I + II	13	nd	0.33	0.09	3.46	May 21
Hettstedt	DIC-SBR	municipal and external activated sludge	−	−	I + II	15	56	12.35	0.24	3	May 24
Huntlosen	DIC-SBR	municipal	−	−	I + II	17	68	0.13	0.03	2.2	May 23
Ingolstadt	SBR	activated sludge drainage	−	−	I + II	27	856	0.3	<0.1	20.4	May 09
Kraftisried	single-stage activated sludge basin	animal rendering	−	−	I + II	7	397.5	35.3	6.2	17.4	January 29
Langenzenn	SBR	municipal	+	+	−	9	21.25	7.96	0.42	3.1	March 14
Lyss (ARA)	fixed bed reactor	municipal	+	−	I	12	20	1	0.1	18	January 29
Lyss (GZM)	membrane filtration plant	animal rendering	−	−	I + II	30	700	<1	<0.5	14	January 29
Oberding	fixed bed reactor	animal rendering	−	−	I + II	26	450	<1	<0.5	4	January 29
Plattling	two-stage activated sludge basin	animal rendering	−	−	I + II	30	750	1	<0.5	3	January 29
Radeburg	DIC-SBR	municipal	+	−	I	14	nd	0	0.05	3.3	May 24
Rosenheim	SBR	municipal	−	−	I + II	36	970	nd	nd	nd	May 30
Seefeld	SBR	municipal	+	−	I	nd	8.32	1.59	nd	1.73	March 28
Spenge	DIC-SBR	municipal	+	+	I	14	24	<0.2	0.05	1.38	May 22
Waldsassen	SBR	municipal and industrial	+	+	I	9	18.5	<0.1	nd	3.45	March 27
Weisstal	DIC-SBR	municipal	+	−	I + II	nd	nd	0	0.02	4.4	May 21

[a] SBR, sequencing batch reactor; DIC-SBR, differential internal cycling SBR.

[b] +, *Nitrotoga* detected; −, *Nitrotoga* not detected.

[c] detection of *Nitrospira* by FISH: I, *Nitrospira* sublineage I; II, *Nitrospira* sublineage II; −, *Nitrospira* not detected.

[d] *Nitrobacter*, *Nitrococcus*, *Nitrolancea*, and *Nitrospina*-like NOB were not detected by FISH in any sample

[e] Temperature was measured on sampling date; nd, not determined.

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Table 2

FISH probes designed in this study.

Probe full name[a]	Short name	Sequence 5′-3′	Binding position[b]	FA%[c]	Target group	Coverage[d]	
						Probe	Comb.
S-G-Ntoga-0122-a-A-19	Ntoga122	TCC GGG TAC GTT CCG ATA T	122 - 140	40	genus *Nitrotoga*	98.5%	98.5%
cS-G-Ntoga-0122-a-A-19 [e]	c1Ntoga122	TCW GGG TAC GTT CCG ATA T	122 - 140		–	–	
cS-G-Ntoga-0122-b-A-19 [e]	c2Ntoga122	TCY GGG TAC GTT CCG ATG T	122 - 140		–	–	
S-F-Gall-0178-a-A-18	FGall178	TCC CCC TYA GGG CAT ATG	178 - 195	30	family *Gallionellaceae*	98.9%	98.9%
cS-F-Gall-0178-a-A-18[e]	cFGall178	TCC CCC TYA GGG CKT ATG	178 - 195		–	–	
S-F-Gall-0221-a-A-18	FGall221a	TAT CGG CCA CTC CGA AAG	221 - 238	30	family *Gallionellaceae*	71.6%	90.2%
cS-F-Gall-0221-a-A-18 [e]	c1FGall221a	TAT CGG CCA CTC CTA AAG	221 - 238		–	–	
S-F-Gall-0221-b-A-18[f]	FGall221b	TAT CGG CCG CTC CGA AAA	221 - 238	30	genus *Nitrotoga*	97.9%	
					family *Gallionellaceae*	18.6%	
cS-F-Gall-0221-b-A-18	cFGall221b	CAT CGG CCG CTC CGA AAG	221 - 238		–	–	
S-*-Gall-0438-a-A-18	FGall438	GTT TTC TTT CCG GCT GAA	438 - 455	25	genus *Nitrotoga*	89.4%	89.4%
cS-*-Gall-0438-a-A-18[e]	c1FGall438	GAT TTC TTT CCG GCT GAA	438 - 455		*Zoogloea* spp.	–	
cS-*-Gall-0438-b-A-18[e]	c2FGall438	GTT TTC TTC CCG GCT GAA	438 - 455		*Thaurea/Dechloromonas* spp.	–	
cS-*-Gall-0438-c-A-18[e]	c3FGall438	GTT TTC TTT CCG TCT GAA	438 - 455		*Azoarcus* spp.	–	
S-G-Ntoga-1424-a-A-18	Ntoga1424	CTA GCT GCT TCT GGT AGA A	1424 - 1442	20	genus Nitrotoga	82.2%	82.2%
cS-G-Ntoga-1424-a-A-18[e]	c1Ntoga1424	CTA ACT GCT TCT GGT AGA A	1424 - 1442		*Sterolibacterium/Dechlorosoma* spp.	–	
cS-G-Ntoga-1424-b-A-18[e]	c2Ntoga1424	CTA GCT GCT TCT GGT ACA A	1424 - 1442		*Acidothiobacillus* spp.	–	

[a] Probe nomenclature according to (Alm *et al.* 1996).

[b] Probe binding position according to *E. coli* 16S rRNA gene numbering.

[c] Percent formamide (v/v) added to the hybridization buffer for optimal hybridization conditions.

[d] Group coverage was calculated as the fraction of organisms with a full sequence match to the respective probe relative to the number of sequences within the respective probe target group as defined by phylogenetic analyses (see main text for details). Column “Probe” lists the coverage of single probes, column “Comb.” that of probe combinations.

[e] Competitor probes were added to the hybridization buffer as unlabeled oligonucleotides and in equimolar amounts as the labeled probes to increase hybridization specificity.

[f] Probe can be used alone for detection of the genus *Nitrotoga* or in combination with probe FGall221a for detection of the family *Gallionellaceae*.