Low Temperature and Neutral pH Define “*Candidatus Nitrotoga* sp.” as a Competitive Nitrite Oxidizer in Coculture with *Nitrospira defluvii*

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ABSTRACT Nitrification is an essential process for N removal in activated sludge to avoid toxicity of ammonium and nitrite. Besides *Nitrospira*, “*Candidatus Nitrotoga*” has been identified as a key nitrite-oxidizing bacterium (NOB) performing the second step of nitrification, nitrite oxidation to nitrate, in wastewater treatment plants (WWTPs). However, the driving forces for the dominance of *Nitrotoga* in certain plants have often remained unclear and could not be explained solely by temperature effects. In this study, we characterized the physiology of the ammonium-dependent *Nitrotoga* sp. BS with regard to temperature and pH variations and evaluated its competitiveness against *Nitrospira defluvii*. Both NOB originated from the same WWTP and shared a comparable pH optimum of 7.3. Based on these results, coculturing experiments with these NOB were performed in batch reactors operated at either 17°C or 22°C to compare their abundances under optimal (pH 7.4) or sub-optimal (pH 6.4) conditions using 1 mM nitrite. As revealed by quantitative PCR (qPCR), fluorescence *in situ* hybridization (FISH), and 16S amplicon sequencing, *Nitrotoga* sp. BS was clearly favored by its optimal growth parameters and dominated over *Ns. defluvii* at pH 7.4 and 17°C, whereas a pH of 6.4 was more selective for *Ns. defluvii*. Our synthetic communities revealed that niche differentiation of NOB is influenced by a complex interaction of environmental parameters and has to be evaluated for single species.

IMPORTANCE “*Ca. Nitrotoga*” is a NOB of high environmental relevance, but physiological data exist for only a few representatives. Initially, it was detected in specialized niches of low temperature and low nitrite concentrations, but later on, its ubiquitous distribution revealed its critical role for N removal in engineered systems like WWTPs. In this study, we analyzed the competition between *Nitrotoga* and *Nitrospira* in bioreactors and identified conditions where the *K* strategist *Ns. defluvii* was almost replaced by *Nitrotoga* sp. BS. We show that the pH value is an important factor that regulates the composition of the nitrite-oxidizing enrichment with a dominance of *Nitrotoga* sp. BS versus *Ns. defluvii* at a neutral pH of 7.4 in combination with a temperature of 17°C. The physiological diversity of novel *Nitrotoga* cultures improves our knowledge about niche differentiation of NOB with regard to functional nitrification under suboptimal conditions.

KEYWORDS *Nitrospira*, *Nitrotoga*, activated sludge, bioreactors, niche separation, nitrification, nitrite oxidation, nitrite-oxidizing bacteria, physiology, qPCR

Nitrification is an important detoxifying process in the environment and in technical applications related to water purification. Harmful ammonia is oxidized via toxic nitrite to less critical nitrate. The second step from nitrite to nitrate is performed by nitrite-oxidizing bacteria (NOB), and members of the genus *Nitrospira* were recognized as the most important key players about 20 years ago (1, 2). However, this finding has

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been challenged by the discovery of the cold-adapted "*Candidatus Nitrotoga* spp." (here *Nitrotoga*) in wastewater treatment plants (WWTPs) (3, 4). They mostly coexisted with *Nitrospira* but were also found to be the only NOB in some of these facilities for unknown reasons (4–7). Additionally, *Nitrotoga* was detected in biofilters treating groundwater (8) as well as in freshwater and marine recirculation aquaculture systems (RASs) (9, 10). Thus, these NOB are of importance in water processing, especially in systems with low temperature. Natural habitats of *Nitrotoga* include, among other cold environments, permafrost and periglacial soils (11, 12), cave systems (13), subglacial lakes (14, 15), and coastal sand (16). Due to this environmental distribution, it was believed that temperature was the main determining factor for niche differentiation between *Nitrotoga* and *Nitrospira* (3, 16). Accordingly, cultivated members of the genus *Nitrotoga* show optimal growth at between 10°C and 22°C (9, 11, 16). However, the paradigm of strict cold adaptation of the genus *Nitrotoga* has been questioned just recently by the isolation of the mesophilic *Nitrotoga fabula* from a WWTP (17). Previously, it was shown by Kruse and colleagues (18) that the *Nitrotoga*-typical fatty acid 16:1 *cis*9 was labeled by [¹³C]bicarbonate using 3 mM and 30 mM nitrite, indicating metabolically active cells in activated sludge at 28°C.

In addition to low temperature, a slightly acidic pH was reported later on as a selective criterion for enrichment and successful separation of *Nitrotoga* from other NOB (9). In contrast, cultivated *Nitrospira* species have their optimum in the neutral to slightly alkaline range (19–21), and a low pH value avoids flourishing of most NOB cultures (our unpublished result). Therefore, the goal of this study was to prove whether the low-pH strategy can be transferred to *Nitrotoga* sp. BS from the WWTP in Hamburg-Dradenau. It was cocultivated with *Nitrospira defluvii*, a member of sublineage I and known as the most important NOB in activated sludge (2, 22, 23).

Most competition experiments for NOB have so far been conducted between *Nitrospira* and *Nitrobacter* and focused on the availability of nitrite (24–28). Recently, a study by Kinnunen et al. (29) revealed the dominance of *Nitrotoga* over *Nitrospira* at nitrite levels close to their respective K_m values. We took a different approach by coculturing *Nitrotoga* sp. BS and *Ns. defluvii* at different pH values and temperatures and analyzed their relative abundances by quantitative PCR (qPCR), fluorescence *in situ* hybridization (FISH), and amplicon sequencing after the consumption of 1 mM nitrite. Whereas temperature-dependent distribution patterns of NOB, including *Nitrotoga*, were initially investigated (3), experiments focusing on distinct pH values are still missing. For quantification by qPCR, *Nitrotoga*-specific 16S rRNA primers were designed.

Physiological experiments with *Nitrotoga* sp. BS were complemented by kinetic investigations because the affinity for nitrite as an energy source determines the success of NOB in distinct habitats (24). *Nitrospira* are *K* strategists with low K_m values, whereas *Nitrotoga* cultures analyzed so far have exhibited a lower affinity for nitrite but comparable maximum activity (V_{max}) (16, 30, 31). We defined conditions to assess niche separation of *Nitrotoga* versus *Nitrospira* and selected parameters (17°C and 22°C in combination with pH 6.4 and pH 7.4) that are of relevance for the treatment of wastewater.

RESULTS AND DISCUSSION

Cultivation. *Nitrotoga* sp. BS originated from a nitrite inhibition test with *Nitrotoga* sp. HAM-1, which was initially enriched from activated sludge of the WWTP in Hamburg-Dradenau (3). This culture tolerated high levels of nitrite, and *Nitrotoga* sp. BS was subcultured from an enrichment grown with 8 mM substrate. As revealed by 16S rRNA amplicon sequencing, the relative abundance of the enrichment *Nitrotoga* sp. BS derived from the nitrite inhibition test amounted to approximately 87%, and that of its preculture *Nitrotoga* sp. HAM-1 amounted to about 44% (see Fig. S1 in the supplemental material). Both nitrite oxidizers coexisted with heterotrophic bacteria, namely, *Nocardioides* sp. and *Parvibaculum* sp. *Nocardioides* was already detected in a moderate thermophilic *Nitrospira* culture and might be an abundant accompa-

FIG 1 (A) Temperature optima of *Nitrotoga* sp. BS (●) and *Ns. defluvii* (□), incubated at pH 7.4. Maximum nitrite turnover rates at the optimal temperature were 201 μ M nitrite per day for *Nitrotoga* sp. BS and 117 μ M nitrite per day for *Ns. defluvii*. (B) pH optima of *Nitrotoga* sp. BS (●) incubated at 17°C and *Ns. defluvii* (□) incubated at 28°C. Maximum nitrite turnover rates at pH 7.3 were 268 μ M nitrite per day for *Nitrotoga* sp. BS and 440 μ M nitrite per day for *Ns. defluvii*. Error bars for *Nitrotoga* sp. BS in panels A and B are from three biological replicates, which were inoculated with independent precultures grown at 17°C and pH 7.4. The symbols are partly too small to see. Data for *Ns. defluvii* in panels A and B were reported previously (9).

nying bacterium of NOB due to its capability for nitrate reduction (20). *Nitrotoga* sp. BS shared 16S rRNA gene similarities of 98.5% with *Nitrotoga* sp. HAM-1 (GenBank accession number [FJ263061.1](#)) and of 99.1% with *Nitrotoga arctica* (GenBank accession number [DQ839562.1](#)) (Table S1).

Ammonium dependence. Whereas growth of *Nitrotoga* sp. BS was stable in early enrichments, nitrite oxidation became more and more fastidious over time. Its reproducible growth could be recovered by amendment with at least 0.1 mM ammonium (Fig. S2), and this approach also worked for the preculture *Nitrotoga* sp. HAM-1. In accordance, growth of *Nitrotoga* sp. AM1 was also enhanced by ammonium (16), but further *Nitrotoga* representatives (*Nt. arctica* and *Nitrotoga* sp. HW29) did not benefit from its addition (not shown). So far, *Nitrolancea hollandica* has been the only NOB that necessarily requires ammonium supplements (32, 33), and genome analyses revealed the absence of a known assimilatory nitrite reductase as a gene for nitrite reduction to ammonia (32). The presence of such an enzyme in *Nitrotoga* sp. BS still awaits investigation, but reported *Nitrotoga* genomes contain genes for assimilatory nitrite reduction to ammonia (17, 34).

Physiological diversity of *Nitrotoga*. Members of the genus *Nitrotoga* have so far been considered psychrotolerant NOB, which was also the case for *Nitrotoga* sp. BS. The culture grew best at 17°C to 22°C and showed longer lag phases at lower temperatures. About 50% of the activity was still reached at 4°C compared to the optimum temperature of 17°C (Fig. 1A). These results correspond to the preferred temperatures of *Nitrotoga* sp. AM1 (16) and *Nt. arctica* (11), whereas *Nitrotoga* sp. HW29 favored slightly elevated temperatures of about 22°C (9). In contrast, the overall temperature profile of *Nt. fabula* was similar to those of some *Nitrospira* species (17), especially *Ns. defluvii*, which exhibited an optimum temperature of 28°C to 32°C and a reduction of nitrite consumption to about 10% at 10°C (Fig. 1A).

TABLE 1 Kinetic parameters for nitrite oxidation^a

Culture (optimal temp [°C]) ^c	Mean $V_{\max}$ ($\mu\text{mol NO}_2^-/\text{mg protein} \cdot \text{h}$) $\pm$ SD	Mean K_m ($\mu\text{M NO}_2^-$) $\pm$ SD
<i>Nt. arctica</i> ^b (17)	26 $\pm$ 3	58 $\pm$ 28
<i>Nitrotoga</i> sp. HAM-1 (17)	38 $\pm$ 4	43 $\pm$ 9
<i>Nitrotoga</i> sp. HW29 (22)	52 $\pm$ 4	60 $\pm$ 11
<i>Ns. defluvii</i> ^b (28)	48 $\pm$ 2	9 $\pm$ 3

^aStandard deviations for *Nitrotoga* sp. HAM-1 and *Nitrotoga* sp. HW29 were calculated from two biological replicates.

^bData from reference 30.

^cMeasurements were performed at optimal temperatures (in parentheses).

As described previously, *Nitrotoga* sp. HW29 revealed a pH optimum in the neutral to slightly acidic range (9), which could not be confirmed for *Nitrotoga* sp. BS. Both investigated cultures, *Nitrotoga* sp. BS as well as *Ns. defluvii*, revealed pH optima of 7.3 (Fig. 1B), which is comparable to the favored pH range of *Nt. fabula* (17). Therefore, a tolerance against low pH values is not a common feature of *Nitrotoga* but might represent an adaption to the natural habitat. *Nitrotoga* sp. HW29 originated from an aquaculture plant with a pH of 6.8 in the process water, close to its pH optimum (9), while *Nitrotoga* sp. BS and *Ns. defluvii* were sampled from activated sludge with a pH of 7.6 to 7.8 (22). Information about NOB in low-pH environments is scarce (35), and the only acidophilic NOB culture that has been isolated so far originates from acidic soil and belongs to the genus *Nitrobacter* (36). While *Ns. defluvii* was well adapted to a slightly acidic pH of 6.5 with a nitrite consumption rate of about 75% in comparison to that at pH 7.3 (Fig. 1B), nitrite oxidation of *Nitrotoga* sp. BS ceased between pH 6.7 and 6.3 (Fig. 1B). A closer look at this pH range confirmed that *Nitrotoga* sp. BS still grows at pH 6.5 but with a prolonged lag phase and less activity of about 45% compared to that at the optimum pH of 7.3 (Fig. S3).

As previously described for *Nitrospira* (22), different *Nitrotoga* cultures vary in their responses to nitrogen compounds, such as ammonium, nitrite, and nitrate. Similar to *Nitrotoga* sp. AM1, the ammonium tolerance of *Nitrotoga* sp. BS was quite high, because growth continued in the presence of 30 mM ammonium chloride at neutral pH (Table S2). In contrast to NH_4^+ , free ammonia (NH_3) is inhibitory for most NOB (25, 31) but not for relatives of *Nitrospira calida* (37). In accordance with results for *Nt. arctica* (11), *Nitrotoga* sp. BS was sensitive to low levels of nitrite, and both cultures were inhibited at concentrations above 4 mM. In contrast, *Nt. fabula* resisted high nitrite concentrations of 30 mM (17), comparable to values reported for *Ns. defluvii* (38). The accumulation of the end product of nitrification is also detrimental, and *Nitrotoga* sp. BS as well as *Nt. fabula* tolerated similar concentrations of 20 and 25 mM nitrate, respectively.

As a control, *Nitrotoga* sp. BS was cultivated in the presence of different NaCl concentrations and tolerated up to 1% salt (results not shown). This corresponds to about 171 mM NaCl and by far exceeded the amounts of ammonium, nitrite, and nitrate applied in the above-mentioned inhibition tests.

Nitrite oxidation kinetics of *Nitrotoga*. Previous investigations revealed that *Nitrospira* is a *K* strategist (30, 39) that prefers low nitrite concentrations and can be characterized by a high substrate affinity and low maximum growth rate. In comparison, *Nitrotoga*-like NOB appear to have a moderate affinity for nitrite. The cultures of *Nitrotoga* sp. HW29 and *Nitrotoga* sp. HAM-1 investigated here had K_m values of 60 and 43 μM nitrite (Table 1 and Fig. S4 and S5), with higher K_m values than those of most *Nitrospira* species (Fig. S6) but higher affinities than those of *Nt. fabula* and *Nitrobacter* species, except for *Nitrobacter vulgaris* (16, 17, 30, 31). However, the maximum specific activities ($V_{\max}$) of *Nitrotoga* are in the same range as those of lineage I and II *Nitrospira* species and rather indicate a lifestyle as a *K* strategist (Fig. S7) (30).

Cocultivation of *Nitrospira* and *Nitrotoga*. During competition experiments in batch reactors with 1 mM nitrite as the substrate, *Nitrotoga* sp. BS was clearly supported by its favored pH and temperature conditions. As shown by qPCR (Fig. S8 and S9), it

FIG 2 NOB abundance during cocultivation, depicted as copy numbers of the 16S rRNA gene per milliliter, before and after each run at pH 6.4 and 7.4. Three independent processes were conducted for *Nitrotoga* sp. BS and *Ns. defluvii* at 17°C (runs 1 to 3) (A) and at 22°C (runs 4 to 6) (B). White columns, *Nitrotoga*; black columns, *Nitrospira*. Error bars show standard deviations of data from three technical replicates. The symbols are partly too small to see.

outcompeted *Ns. defluvii* at pH 7.4 and 17°C (Fig. 2A and Table S3A), although both NOB shared the same optimum pH of 7.3 (Fig. 1B). The suboptimal temperature of 17°C was probably disadvantageous for *Ns. defluvii* (Fig. 1A). At pH 6.4, however, *Ns. defluvii* was more successful, as the NOB distribution shifted toward *Nitrospira* in two of three processes. When the experiments were repeated at 22°C, *Nitrotoga* sp. BS was clearly suppressed, with only about 1% of the NOB content at pH 6.4. One of three experiments failed, probably because a temperature of 22°C and pH 6.4 were suboptimal conditions for both NOB. Nevertheless, at pH 7.4, *Nitrotoga* sp. BS dominated the nitrifying enrichment in two of three experiments at 22°C (Fig. 2B and Table S3B). At pH 6.4, there is a general trend that *Nitrospira* is dominant over *Nitrotoga*. In contrast, *Nitrotoga* is dominant at pH 7.4 regardless of the temperature. So it seems that pH plays a more important role than temperature in shaping NOB communities under the selected conditions. A further indicator that pH 7.4 is more favorable for *Nitrotoga* sp. BS was the increase of its 16S rRNA gene copy number that outmatched the increase of the *Ns. defluvii* copy number in almost every cocultivation at 17°C or 22°C (Table S4).

Control reactions in Erlenmeyer flasks at 25°C with 1 mM nitrite as the substrate revealed a striking dominance of *Ns. defluvii* independent of the applied pH (Fig. S10 and S11), emphasizing the adverse effect of this temperature on *Nitrotoga* sp. BS. Further control experiments at 17°C with different substrate concentrations of 0.3 and 3 mM nitrite also supported pH 7.4 as being favorable for *Nitrotoga* sp. BS (Fig. S12).

FIG 3 FISH for cocultivation experiments with *Nitrotoga* sp. BS and *Ns. defluvii* at pH 6.4 (left) and pH 7.4 (right) at 17°C (run 1). For *Nitrotoga*, magenta is Cy3; for *Ns. defluvii*, yellow is Cy5; and for all cells, blue is DAPI.

However, in-depth evaluation of the influence of the substrate on the distribution of *Nitrotoga* sp. BS and *Ns. defluvii* will be the subject of further studies.

FISH qualitatively supported the NOB distribution found by qPCR (Fig. S13 and S14) and visualized the high abundance of *Nitrotoga* sp. BS at pH 7.4 and 17°C. *Ns. defluvii* was easily recognized by its characteristic microcolonies, whereas *Nitrotoga* formed more loose aggregates or occurred as single cells (Fig. 3). The results were also confirmed by amplicon sequencing of the 16S rRNA gene, with a higher abundance of *Nitrotoga* sp. BS at pH 7.4 (Fig. S15 and S16). Non-NOB sequences belonged to *Parvibaculum*, *Nocardioides*, and *Hyphomicrobium*.

Since the experiments were performed at substrate saturation using 0.3 to 3 mM, the affinity for nitrite was irrelevant for the competition between *Ns. defluvii* and *Nitrotoga* sp. BS. Although a pH of 7.4 was more selective for *Nitrotoga* sp. BS (Table S4), long-term studies with 0.3 mM nitrite at this pH revealed that *Nitrospira* outcompeted *Nitrotoga* and *Nitrobacter* in enrichments from activated sludge (3, 28), and *Ns. defluvii* showed high ^{13}C incorporation at 17°C and 28°C in this substrate range (18).

The selective force of different substrate affinities was actually tested by Kinnunen and colleagues, and they demonstrated that *Nitrotoga* dominated a nitrifying community at a nitrite concentration of 70 μM but was replaced by lineage II *Nitrospira* at a reduced concentration of 7 μM (29). Kinetic data are available for a few NOB, and investigated species of *Nitrospira* and *Nitrotoga* possess comparable V_{max} values (Fig. S7). Assuming comparable maximum specific activities of *Ns. defluvii* and *Nitrotoga* sp. BS under optimal conditions, reduction of the temperature to 17°C was critical for *Ns. defluvii* since its nitrite consumption decreased to about 50% compared to that at the optimum temperature (Fig. 1A).

In general, complete nitrite oxidation was achieved faster in bioreactors operating at pH 7.4, and about 43 to 78% activity was observed at pH 6.4 (Table S3). Nitrite was stoichiometrically oxidized to nitrate consistently without oxygen limitation (Fig. S17 and S18), and ammonium was not consumed (not shown).

***Nitrotoga* competes successfully with *Nitrospira*.** Considering its wide distribution in natural and technical habitats, *Nitrotoga* is a key NOB besides *Nitrospira* (4–6, 9). In accordance with their respective K_m values, *Nitrospira* is better adapted to lower nitrite concentrations that dominate in most natural habitats, whereas *Nitrotoga* is more likely to proliferate in niches with locally elevated nitrite concentrations (40). However, distinct physiological preferences within *Nitrospira* and *Nitrotoga* are well documented (18, 22, 34). Our experiments confirmed that temperature is the main driver for the successful niche occupation of *Nitrotoga*-like NOB. Although *Ns. defluvii* favors temperatures of between 28°C and 32°C, as for other *Nitrospira* species (9, 22), these NOB also occur in cold environments such as permafrost soils (41), and a sublineage II *Nitrospira* strain could be grown at 10°C together with *Nitrotoga* (22). Nevertheless, the recent description of mesophilic *Nt. fabula* dissipated a clear niche separation between *Nitrotoga* and *Nitrospira* with regard to temperature (17).

While it was reported previously that a slightly acidic pH is also advantageous for *Nitrotoga* in direct competition with *Nitrospira* (9), it became obvious that this appears to be an exception, since most *Nitrotoga* cultures prefer neutral to slightly alkaline conditions (16, 17; this study). Additionally, no acidophilic *Nitrospira* culture could be obtained (our unpublished result), even though samples from acidic biofilms were used for enrichment (42). Therefore, it was surprising that *Nitrospira* dominated most of the experiments performed at low pH, indicating that niche differentiation depends on the interaction of several parameters, and competition success might differ for every species. As a consequence, further species of NOB await physiological characterization before empirical data can be used for the generation of nitrification models.

MATERIALS AND METHODS

Cultures. A pure culture of *Ns. defluvii* was isolated from activated sludge of the wastewater treatment plant (WWTP) in Hamburg-Dradenau (22, 23). *Nitrotoga* sp. BS was subcultured from *Nitrotoga* sp. HAM-1, which was enriched from the same WWTP at low temperature (3).

Cultivation and physiological experiments. Cultivation of *Nitrotoga* sp. BS, its preculture *Nitrotoga* sp. HAM-1, and *Ns. defluvii* was performed at 17°C in 300-ml Erlenmeyer flasks with mineral salts medium (43) adjusted to pH 7.4 and supplemented with trace elements by Widdel and Bak (44). The cultures were kept in the dark, not agitated, and fed stepwise to a total amount of 5 mM nitrite before the start of physiological experiments. Medium for *Nitrotoga* sp. BS was additionally supplemented with 0.1 mM ammonium. Cultures were regularly checked for the degree of contamination by heterotrophs and fungi on plates with modified complex medium (45) containing 0.5 g NaCl, 1 g KH_2PO_4 , 1 g yeast extract, 5 g meat extract, 5 g casein hydrolysate, and 1.5% (wt/vol) agar per liter. Plates were incubated for 3 weeks at 28°C.

Experiments to obtain temperature and pH optima of *Nitrotoga* sp. BS were set up in biological triplicates in 100-ml Erlenmeyer flasks with 50 ml mineral salts medium containing 1 mM nitrite and supplemented with 0.1 mM ammonium. Oxidation of nitrite was monitored until the whole substrate was consumed, and the optima were evaluated from the slope with the highest consumption rates. The influence of ammonium on *Nitrotoga* sp. BS was tested in triplicates in 100-ml Erlenmeyer flasks with 50 ml mineral salts medium and 1 mM nitrite as the substrate. Ammonium was supplemented at concentrations from 0 to 10 mM. All batches were inoculated with 1% (vol/vol) preculture if not stated otherwise. Tests for pH optima and the influence of ammonium were performed at 17°C. The temperature optimum was obtained at a pH of 7.4. Samples were taken regularly to measure nitrite and nitrate concentrations.

Inhibition tests for ammonium, nitrite, nitrate, and NaCl for *Nitrotoga* sp. BS were conducted at 17°C in biological duplicates in test tubes containing 10 ml medium and 1 mM nitrite as the substrate, except for nitrite inhibition, when up to 20 mM NaNO_2 was applied. Inhibition by ammonium was tested from 0 to 40 mM NH_4Cl , and inhibition by nitrate was tested from 0 to 20 mM NaNO_3 . Salt inhibition was evaluated from 0.05 to 3% NaCl (corresponding to 8.6 to 514 mM NaCl). The Griess-Ilosvay spot test (46) was performed daily to determine the time until complete nitrite consumption.

K_m and $V_{\max}$ values for nitrite oxidation of *Nitrotoga* sp. HW29 and *Nitrotoga* sp. HAM-1 were determined based on measurements by a microrespiration system as described previously (30). Kinetic parameters for nitrite were calculated from oxygen consumption at various substrate concentrations in biological duplicates. Experiments were performed at the optimal temperature for each culture at pH 7.4 (17°C for *Nitrotoga* sp. HAM-1 and 22°C for *Nitrotoga* sp. HW29).

Competition experiments. Cocultivations were set up in two 500-ml MiniBioBundle reactors (Applicon Biotechnology BV, The Netherlands) with 350 ml of the above-mentioned mineral salts medium containing 1 mM nitrite and 0.1 mM ammonium. The process was controlled via *my-Control* software, and parameters were monitored by BioXpert Lite v1.12 software (both Applicon Biotechnology BV, The Netherlands). One reactor was adjusted to pH 6.4, the other was adjusted to pH 7.4, and the temperature was kept constant at 17°C or 22°C. Each temperature was tested in three independent experiments. Aeration was not applied, and stirring was kept low at 10 rpm, since freshly transferred cultures of NOB are sensitive to shearing stress. The reactors were inoculated with 1% (vol/vol) preculture of each NOB (*Ns. defluvii* plus *Nitrotoga* sp. BS). Complete oxidation of nitrite to nitrate indicated the end of each process.

Several control reactions were performed in 300-ml Erlenmeyer flasks with 100 ml of medium, and the mixtures were incubated without agitation. One experiment mixture was incubated at 25°C with 1 mM nitrite as the substrate, and a further batch incubation was done at 17°C. These flasks, containing 0.3 or 3 mM nitrite as the substrate, were inoculated with the same preculture.

Amplicon sequencing. The diversity of the nitrifying cultures of each reactor process, of *Nitrotoga* sp. BS, and of *Nitrotoga* sp. HAM-1 was evaluated by amplicon sequencing. Genomic DNA was extracted with the Ultra-Clean microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions, and 10 ng was used to generate 16S rRNA amplicons. Primers 515F and 806R (47) were used for preparative PCR, followed by Illumina MiSeq (bioreactor processes; *Nitrotoga* sp. BS) with a sequencing depth of 20,000 reads. Primers 341F and 785R (48) were used for preparative PCR, followed by 454 pyrosequencing (*Nitrotoga* sp. HAM-1) with a sequencing depth of 3,000 reads. Both reactions were performed at a sequencing service facility (MR DNA, Shallowater, TX, USA). Denoised sequences were grouped into operational taxonomic units (OTU), and chimeras were removed, accord-

TABLE 2 Oligonucleotide primers and probes used in this study

Primer or probe	Sequence (5'–3')	Target	Reference
Primers			
27F	AGA GTT TGA TCM TGG CTC AG	16S rRNA gene, <i>Bacteria</i>	55
515F	GTG CCA GCM GCC GCG GTA A	16S rRNA gene, V4 region, <i>Bacteria</i>	47
806R	GGA CTA CHV GGG TWT CTA AT	16S rRNA gene, V4 region, <i>Bacteria</i>	47
341F	CCT ACG GGN GGC WGC AG	16S rRNA gene, V3–4 region, <i>Bacteria</i>	48
785R	GAC TAC HVG GGT ATC TAA TCC	16S rRNA gene, V3–4 region, <i>Bacteria</i>	48
Nsp1158R	CCC GTT MTC CTG GGC AGT	16S rRNA gene, genus <i>Nitrospira</i>	24
Ntg124F	ATC GGA ACG TAC CCG GAA A	16S rRNA gene, genus <i>Nitrotoga</i>	4
Ntg840R	CTA AGG AAG TCT CCT CCC	16S rRNA gene, genus <i>Nitrotoga</i>	11
Ntg8-2F	TAA GCC CGG GGA TTT CAC AT	qPCR, 16S rRNA gene, genus <i>Nitrotoga</i>	This study
Ntg8-2R	ATA CCC TGT GTG GAT GAC GG	qPCR, 16S rRNA gene, genus <i>Nitrotoga</i>	This study
Nsp15F	ATT GCT ACC TCG TCA GGC TT	qPCR, 16S rRNA gene, genus <i>Nitrospira</i>	This study
Nsp15R	CGC ATT AAG TAT CCC GCC TG	qPCR, 16S rRNA gene, genus <i>Nitrospira</i>	This study
SP6	ATT TAG GTG ACA CTA TAG	SP6 promotor, pGEM-T-Easy vector	
T7	TAA TAC GAC TCA CTA TAG GG	T7 promotor, pGEM-T-Easy vector	
Probes			
Ntg122	TCC GGG TAC GTT CCG ATA T	Genus <i>Nitrotoga</i> , Cy3 labeled	4
Ntg122-Ca	TCW GGG TAC GTT CCG ATA T	Competitor	4
Ntg122-Cb	TCY GGG TAC GTT CCG ATA T	Competitor	4
Nsp662	GGA ATT CCG CGC TCC TCT	Genus <i>Nitrospira</i> , Cy5 labeled	2
Nsp662-C	GGA ATT CCG CTC TCC TCT	Competitor	2
Nsp712	CGC CTT CGC CAC CGG CCT TCC	Phylum <i>Nitrospirae</i> , Cy5 labeled	2
Nsp712-C	CGC CTT CGC CAC CGG TGT TCC	Competitor	2

ing to in-house analysis pipelines (MR DNA, Shallowater, TX, USA). Taxonomic classification of OTUs was performed by using BLASTn against the Greengenes, RDP II, and NCBI databases. Primers are listed in Table 2.

Chemical analyses. Samples were taken daily to quantify nitrite and nitrate by high-performance liquid chromatography (HPLC) via ion pair chromatography on a LiChrospher RP-18 column (125 by 4 mm; Merck KGaA, Germany) with UV detection in an automated system (Hitachi LaChrom elite; VWR International GmbH, Darmstadt, Germany). Data acquisition and processing were performed with the integrated software EZChrom Elite 3.3.2. Ammonium was measured by an *ortho*-phthalaldehyde assay (49, 50).

Standard plasmids and primer design for quantitative PCR. Standard plasmids for quantitative PCR (qPCR) contained regions of the 16S rRNA genes of *Ns. defluvii* and *Nt. arctica*. Specific DNA regions were amplified by PCR with semispecific primer pair 27F/Nsp1158R (Table 2) for *Nitrospira* and specific primer pair Ntg124F/Ntg840R (Table 2) for *Nitrotoga*. PCR was performed in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories GmbH, Munich, Germany) with a 4-min initial denaturation step at 95°C and subsequently 31 cycles of 35 s of denaturation at 95°C, 45 s of annealing at 58°C for *Nitrospira* or 65°C for *Nitrotoga*, and 90 s of elongation at 72°C, followed by a final elongation step at 72°C for 8 min. PCR products were cleaned up with the GenepHlow gel/PCR kit (catalog number DFH300; Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions. The fragments were cloned into the pGEM-T Easy vector (Promega Corp., USA) according to the manufacturer's recommendations. Positive clones were sequenced (Eurofins Genomics GmbH, Germany). Plasmids were isolated via a high-speed plasmid minikit (catalog number PD300; Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions and diluted in diethyl pyrocarbonate (DEPC)-H₂O to 10⁹ plasmids per μ l as the stock solution for further use as standard plasmids. Primers (Table 2) for qPCR were derived from the same plasmids using NCBI primer-BLAST (51) and synthesized at Eurofins Genomics GmbH (Germany). Primer pairs were checked for specificity on plasmids, pure cultures, and the competitor NOB (see Fig. S8 and S9 in the supplemental material).

Quantitative PCR. Cells were harvested from 50 ml after each bioreactor run by centrifugation. DNA was isolated from the pellet with the Ultra-Clean microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Reaction mixtures for qPCR were prepared with Maxima SYBR green qPCR master mix (Thermo Fisher Scientific, Waltham, MA, USA). Samples were run in triplicates on a Bio-Rad CFX96 C1000 touch real-time PCR detection system (Bio-Rad Laboratories GmbH, Germany). The reactions comprised a 30-s initial denaturation step at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing for 20 s at 59°C for both NOB, and 30 s of elongation at 72°C. The SYBR signal was monitored each cycle after elongation. After complete qPCR, a melt curve was generated by increasing the temperature stepwise from 65°C to 95°C at 0.5°C intervals for 10 s and signal measurement at each step. Copy numbers were calculated automatically by the accompanying software Bio-Rad CFX Manager 3.1, based on a regression curve obtained from standard plasmids ranging from 10⁷ copies down to 10² copies per μ l. Reactions were quality checked according to MIQE guidelines (52). Copy numbers of *Nitrotoga* were divided by a factor of 2, since this NOB contains two copies of the 16S rRNA gene (34).

FISH. Cells were harvested from 50 ml after each bioreactor run by centrifugation, and the pellet was fixed with 4% paraformaldehyde as described previously (53). Fluorescence *in situ* hybridization (FISH)

was modified after the protocol by Manz et al. (54). Hybridization was performed at 40% formamide with the Cy3-labeled probe Ntg122 and the Cy5-labeled probes Nsp662 and Nsp712, targeting the genera *Nitrotoga* and *Nitrospira*, respectively, as well as competitors for each probe (Table 2). Following hybridization, samples were stained with 4',6'-diamidino-2-phenylindole (DAPI) and embedded in CitiFluor AF1 (CitiFluor Ltd., London, United Kingdom). Slides were examined with an Axio ImagerM2 epifluorescence microscope (Carl Zeiss Microscopy GmbH, Germany).

Accession number(s). The 16S rRNA gene sequence of *Nitrotoga* sp. BS was deposited at GenBank (National Center for Biotechnology Information) under accession number [MF555727](https://doi.org/10.1093/ajph.2017.11.2229).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02569-18>.

SUPPLEMENTAL FILE 1, PDF file, 2.9 MB.

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