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Comparison of Oxidation Kinetics of Nitrite-Oxidizing Bacteria: Nitrite Availability as a Key Factor in Niche Differentiation

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Nitrification has an immense impact on nitrogen cycling in natural ecosystems and in wastewater treatment plants. Mathematical models function as tools to capture the complexity of these biological systems, but kinetic parameters especially of nitrite-oxidizing bacteria (NOB) are lacking because of a limited number of pure cultures until recently. In this study, we compared the nitrite oxidation kinetics of six pure cultures and one enrichment culture representing three genera of NOB (*Nitrobacter*, *Nitrospira*, *Nitrotoga*). With half-saturation constants (K_m) between 9 and 27 μM nitrite, *Nitrospira* bacteria are adapted to live under significant substrate limitation. *Nitrobacter* showed a wide range of lower substrate affinities, with K_m values between 49 and 544 μM nitrite. However, the advantage of *Nitrobacter* emerged under excess nitrite supply, sustaining high maximum specific activities (V_{max}) of 64 to 164 μmol nitrite/mg protein/h, contrary to the lower activities of *Nitrospira* of 18 to 48 μmol nitrite/mg protein/h. The V_{max} (26 μmol nitrite/mg protein/h) and K_m (58 μM nitrite) of "*Candidatus* Nitrotoga arctica" measured at a low temperature of 17°C suggest that *Nitrotoga* can advantageously compete with other NOB, especially in cold habitats. The kinetic parameters determined represent improved basis values for nitrifying models and will support predictions of community structure and nitrification rates in natural and engineered ecosystems.

Aerobic nitrite oxidation is the second microbially mediated part of nitrification, a key process in the global nitrogen cycle, catalyzed by autotrophic, slow-growing nitrite-oxidizing bacteria (NOB). Under nitrifying conditions, the growth of NOB is directly linked to the nitrite production rate and the kinetics of nitrite oxidation (1). Nitrification occurs in almost every aquatic and terrestrial ecosystem, in natural as well as in artificial environments like wastewater treatment plants (WWTPs). The intermediate nitrite hardly accumulates, but local or temporary peaks might appear especially when conditions suddenly change or adverse conditions like alkaline pH values impair NOB activity (2). In WWTPs, disturbances can cause nitrite peaks after destabilization of the NOB guild (3). In soils, nitrite concentrations can vary from ~0.01 to ~100 μg of nitrogen g of soil^{-1}, with the highest values measured in fertilized samples (4). Therefore, the concentration of nitrite as the substrate of NOB varies and is regarded as one major factor providing niche differentiation (5). In the ecological context, field studies are important to characterize nitrifying communities with specific activities and affinities for nitrite, but for a better understanding of wastewater treatment processes, mathematical models are required (6). Such models include ecophysiological kinetic data, which are known primarily for ammonia-oxidizing bacteria (AOB) (7). They provide the substrate nitrite for the second major step of nitrification. However, the development of two-step nitrification models also requires kinetic parameters of NOB (8, 9).

Because of their earlier discovery and the ease with which pure cultures can be obtained, studies of the nitrite oxidation kinetics of NOB were previously performed mainly with *Nitrobacter* bacteria, initially regarded as a model NOB in WWTPs (10). The first cultures of *Nitrospira*, later identified as key NOB in activated sludge (11, 12), were obtained from 1986 on (13, 14). In the beginning, estimations of the oxidation kinetics of *Nitrospira* populations were done by microsensor measurements of biofilm samples (15, 16). Differences in growth kinetics between NOB can be described in terms of *K* and *r* strategists (16, 17). *Nitrobacter* bacteria (*r* strategists) have relatively high maximum nitrite oxidation activity (or growth rate) and low substrate affinities, while *Nitrospira* bacteria (*K* strategists) are characterized by low maximum activity and high substrate affinities. These contrary strategies were confirmed by biotechnological approaches in reactor systems (18–20) but not with pure cultures until now.

Besides WWTPs, members of the genus *Nitrospira* represent the most dominant nitrite oxidizers in many natural and engineered ecosystems in terms of diversity (12, 21–23). An open question is the relevance of the new candidate genus *Nitrotoga* (24) for the treatment of wastewaters. These NOB were detected primarily in soils (24) but also occur in cave biofilm (25), freshwater (26), and activated sludge (27–29).

Here we present a broad comparison of directly measured oxidation kinetics of nonmarine NOB by microsensor measurements. In addition to *Nitrobacter*, the study includes pure cultures of lineage I and II *Nitrospira* from freshwater and activated sludge and the first analysis of a highly enriched *Nitrotoga* culture from permafrost soil. Hence, we investigated representatives of three different phyla, namely, *Alpha-* and *Betaproteobacteria* and *Nitrospirae* (14, 24, 30). Not included are the marine NOB genera *Nitrococcus* and *Nitrospina* (31) within the phyla *Gammaproteobac-*

Received 22 August 2014 **Accepted** 10 November 2014

Accepted manuscript posted online 14 November 2014

Citation Nowka B, Daims H, Spieck E. 2015. Comparison of oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as a key factor in niche differentiation. Appl Environ Microbiol 81:745–753. doi:10.1128/AEM.02734-14.

Editor: G. Voordouw

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doi:10.1128/AEM.02734-14

teria and "*Nitrospinae*" (75), respectively, and the novel strain *Nitrolancea hollandica* within the phylum *Chloroflexi* (32).

The lab-scale kinetic parameters provided show the nitrite-dependent niche differentiation of NOB and might be translated to full-scale mathematical models (like an activated-sludge model [33]) to improve the efficiency of biological nitrification in WWTPs and reactor systems.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Seven different NOB species of three genera were investigated in this study. *Nitrobacter hamburgensis* X14 and *Nitrobacter winogradskyi* strain "Engel" were both isolated from soil (34, 35), and *Nitrobacter vulgaris* Ab_1 was from sewage (36). The isolates of "*Candidatus* Nitrospira defluvii" A17 (lineage I) and *Nitrospira* strain BS10 (lineage II) were derived from activated sludge (37; B. Nowka, S. Off, H. Daims, and E. Spieck, unpublished data), and *Nitrospira moscoviensis* M-1 (lineage II) originated from a heating water system (14). "*Candidatus* Nitrotoga arctica" 6680 was highly enriched from permafrost soil (24). Although no pure culture was investigated here, *Nitrotoga* was the only type of cells visible by light microscopy when grown in mineral salts medium with nitrite.

All *Nitrobacter* and *Nitrospira* bacteria were grown as batch cultures in 500 ml mineral medium with the following composition: 1 liter of demineralized water, different concentrations of $NaNO_2$ as the sole energy source, 0.007 g of $CaCO_3$, 0.5 g of NaCl, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.15 g of KH_2PO_4, and the following trace elements: 33.8 μg of $MnSO_4 \cdot H_2O$, 49.4 μg of H_3BO_3, 43.1 μg of $ZnSO_4 \cdot 7H_2O$, 37.1 μg of $(NH_4)_6Mo_7O_{24}$, 973.0 μg of $FeSO_4 \cdot 7H_2O$, and 25.0 μg of $CuSO_4 \cdot 5H_2O$. For the "*Ca*. Nitrotoga arctica" enrichment, a modified trace element composition was used: 100.0 μg of $MnCl_2 \cdot 4H_2O$, 30.0 μg of H_3BO_3, 144.0 μg of $ZnSO_4 \cdot 7H_2O$, 36.0 μg of $Na_2MoO_4 \cdot 2H_2O$, 2.1 mg of $FeSO_4 \cdot 7H_2O$, 2.0 μg of $CuCl_2 \cdot 2H_2O$, 190.0 μg of $CoCl_2 \cdot 6H_2O$, and 24.0 μg of $NiCl_2 \cdot 6H_2O$. Before demineralized water was added, these supplements were dissolved in 12.5 ml of HCl (25%) (38). The pH was adjusted to 8.4 to 8.6 and changed to 7.4 to 7.6 2 days after autoclaving. The cultures were started with inocula of 1% (vol/vol) and incubated in the dark at temperatures of 17°C ("*Ca*. Nitrotoga arctica"), 28°C (all *Nitrobacter* strains, "*Ca*. Nitrospira defluvii," and strain BS10), and 37°C (*N. moscoviensis*). After the first detection of nitrite consumption, the *Nitrospira* and *Nitrobacter* cultures were stirred moderately (100 to 300 rpm). The "*Ca*. Nitrotoga arctica" enrichment was incubated without agitation.

Chemical analyses. Nitrite and nitrate concentrations were determined by high-performance liquid chromatography (HPLC) via ion pair chromatography with a LiChrospher RP-18 column (5 μm, 4 by 125 mm; Merck KGaA, Darmstadt, Germany) (39) and UV detection in an automated system (LaChrom Elite HPLC system; VWR International GmbH, Darmstadt, Germany). Cell protein concentrations were measured by the bicinchoninic acid method (40) after cell lysis in 0.15 M NaOH and incubation at 90°C for 30 min.

Disruption of "*Ca*. Nitrospira defluvii" aggregates and microcolonies. Since "*Ca*. Nitrospira defluvii" forms extensive cell aggregates and microcolonies under the growth conditions used, we developed a method for disruption to enable single-cell counting. In the first step, 1 ml of cell culture was shaken with a Mikro-Dismembrator S (Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 40 min at a frequency of 1,500 oscillations/min in a 3-ml polytetrafluoroethylene shaking flask with a chromium steel grinding ball (diameter, 5 mm). Afterwards, the cell culture was diluted in 10 ml of distilled H_2O (dH_2O) and sonicated for 10 min at a frequency of 24 kHz and an amplitude of 0.5 (UP200S Ultrasonic Processor; Hielscher Ultrasonics GmbH, Teltow, Germany). This procedure removed the bulk of the extracellular polymeric substances and resulted in single cells suitable for cell counting.

Cell counting. Aliquots of cell cultures were diluted in 10 to 15 ml of dH_2O. Cells were collected by filtration with a Milli Pure Filter System (Sartorius GmbH, Göttingen, Germany) with membrane filters with a 0.2-μm pore size (Merck Millipore, Billerica, MA). For cell counting, the filters were dried and pieces of the filters were subsequently stained with 0.01 mg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) for 3 min immersed in dH_2O and 70% ethanol for a few seconds. Cells were counted on dried filters by fluorescence microscopy (Axio Imager.M2; Carl Zeiss, Göttingen, Germany) and by use of a counting ocular (area of a square: 0.0004 mm^2 at ×1,000 magnification). Cell counts were calculated by the following equation: $cells_{overall}$ = [filter area (380 mm^2)/square area (0.0004 mm^2)] · (cell count per square). The cell count per square was generated from the average cell number of 30 squares.

Activity measurements. Activity measurements were performed with early-stationary-phase cells in nitrite-limited mineral medium by the following procedure. Cultures (500 ml) of "*Ca*. Nitrospira defluvii" and the three *Nitrobacter* strains were started with a nitrite concentration of 9 mM, and *N. moscoviensis* was started with a nitrite concentration of 5.7 mM. To avoid inhibition of cell growth, cultures of *Nitrospira* strain BS10 and "*Ca*. Nitrotoga arctica" were started with low nitrite concentrations of 0.3 and 0.7 mM, respectively, and replenished with these amounts when nitrite was consumed. Nitrite consumption and nitrate production were monitored frequently by HPLC. Between 12 and 48 h after complete nitrite consumption (early stationary phase), 50-ml aliquots of the cultures were transferred to 100-ml flasks, stirred, and incubated in thermostat-regulated rooms at corresponding temperatures until the start of the measurements ("*Ca*. Nitrotoga arctica" at 17°C; all *Nitrobacter* strains, "*Ca*. Nitrospira defluvii," and strain BS10 at 28°C; *N. moscoviensis* at 37°C). Nitrite-dependent oxygen consumption was measured in a microrespiration system (Unisense AS, Denmark). This system consisted of a one-channel oxygen sensor amplifier (OXY-Meter), a Clark-type oxygen microsensor (OX-MR; polarized for at least 48 h before use), a stirring system with glass-coated magnets, 2-ml glass chambers with glass stoppers, a rack for eight chambers, and the data acquisition software MicOx 3.0. The response time (90%) of the oxygen microsensor was <15 s, and the oxygen uptake of the microsensor was below 1 nM day^{-1} (41). All measurements were carried out in a recirculated water bath in thermostat-regulated rooms with stirring at 200 rpm. Subsamples of the early-stationary-phase cells were transferred to the 2-ml glass chambers, sealed with glass stoppers, and immersed in the water bath. The microrespiration sensor was inserted through a capillary hole inside the glass stopper. The measurements started with an initial equilibration for 15 to 30 min until the signal from the sensor was stable; nitrite from stock solutions was then added through a second capillary hole with a syringe.

Calculation of oxidation kinetics and other properties. Nitrite oxidation kinetics were estimated from multiple oxygen consumption rates at various defined nitrite concentrations. Measurements of every nitrite-oxidizing strain were performed with at least three independently grown cultures. The amounts of consumed nitrite were calculated from oxygen consumption according to the ratio of nitrite oxidation to oxygen consumption of 1:0.5. By using the data analysis software SigmaPlot 12.0 (Systat Software GmbH, Erkrath, Germany), oxygen uptake rates were plotted according to the total nitrite concentration and kinetic characteristics were obtained by fitting a Michaelis-Menten kinetic with the following equation to the data: $V = (V_{max} \cdot [S])/(K_m + [S])$. Here, V is activity, V_{max} is the maximum specific activity (μmol/mg protein/h), K_m is the half-saturation constant for nitrite oxidation (μM), and $[S]$ is the nitrite concentration (μM). Although the experiments were not performed with purified enzymes, we prefer to use the terms K_m and V_{max}, since we determined kinetics in short-term activity assays within a few hours, where growth can be neglected.

Nitrite consumption and cell growth were determined at the nitrite concentrations shown at the corresponding temperatures (described above) in 150- to 500-ml Erlenmeyer flasks containing mineral medium inoculated with 1% (vol/vol) early-stationary-phase cells. Subsamples were withdrawn for determination of nitrite and nitrate concentrations (1 ml), cell counts (1 to 10 ml), and protein concentrations (50 ml) as described above. Values of generation time (g in hours) and cell activity (k in

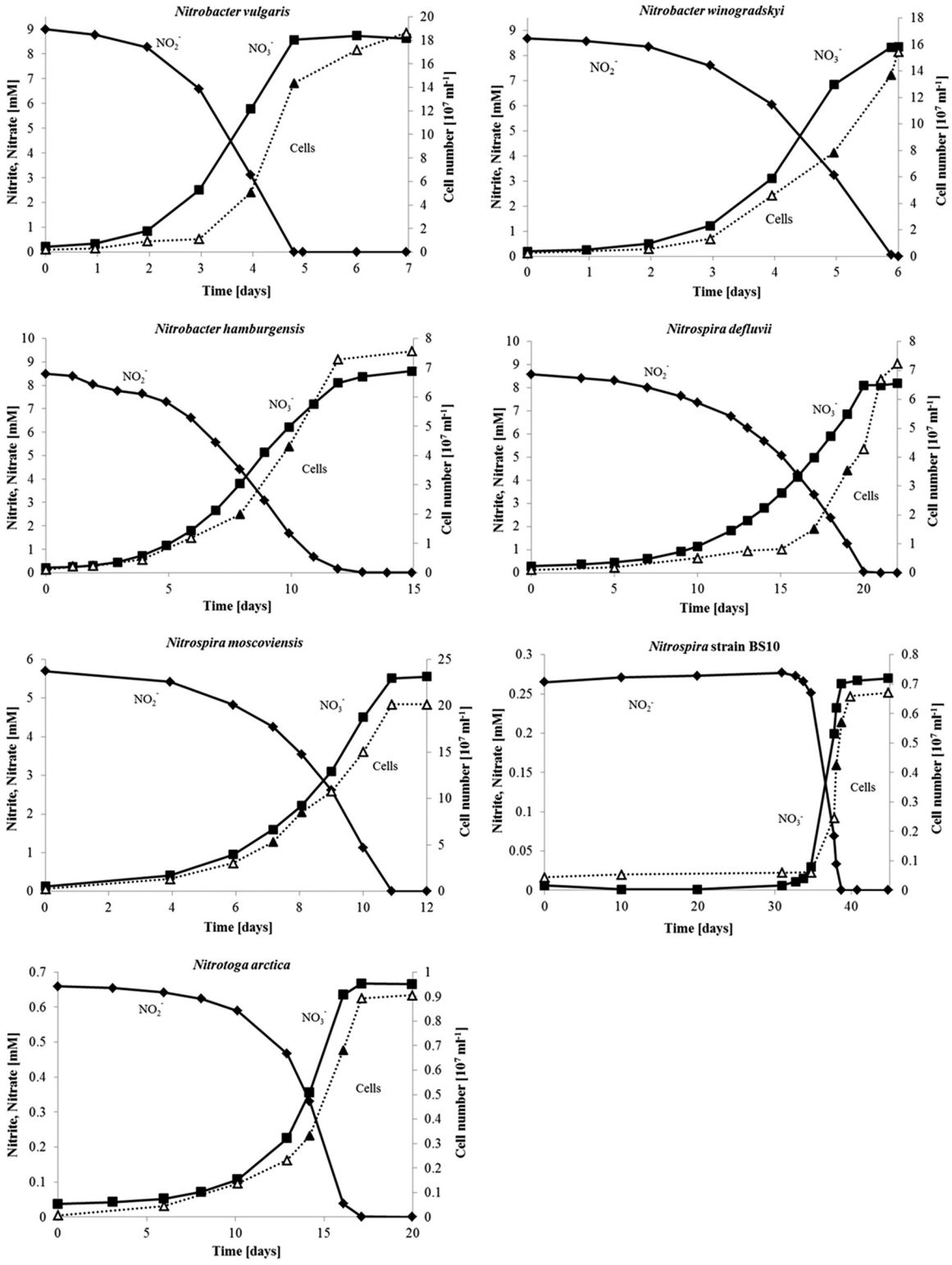

FIG 1 Growth of NOB in nitrite-limited mineral medium as batch cultures. *Nitrobacter vulgaris*, *Nitrobacter hamburgensis*, *Nitrobacter winogradskyi*, "*Ca*. Nitrospira defluvii," *Nitrospira moscoviensis*, and *Nitrospira* strain BS10 were pure cultures, and "*Ca*. Nitrotoga arctica" was an enrichment culture. The initial provided nitrite was set to noninhibiting concentrations. After complete depletion of nitrite, the cultures entered the stationary phase. The time points at which generation times were calculated are marked by filled triangles.

TABLE 1 Growth parameters of selected NOB cultures

Organism (temp [°C])	Generation time (g)[j]	Max cell sp act (k)[k]	Growth yield	
			Protein (Y_p)[l]	Cells (Y_c)[m]
Nitrobacter vulgaris[a] (28)	13 (12)[d]	13.1	0.099	10.32
Nitrobacter hamburgensis[a] (28)	43 (40, 63–84)[e]	2.9 (3.7–7.4, 1.0–3.3)[c,f]	0.108	9.95
Nitrobacter winogradskyi[a] (28)	26 (8–14, 41–87)[g]	2.5 (1.9–3.7, 12)[h]	0.083	10.26
"*Ca.* Nitrospira defluvii"[a] (28)	37	2.8	0.122	9.93
Nitrospira moscoviensis[a] (37)	32 (12)[i]	0.6	0.213 (0.120)[i]	10.55
Nitrospira strain BS10[a] (28)	37	2.1	0.142	10.4
"*Ca.* Nitrotoga arctica"[b] (17)	44	2	0.102	10.14

[a] Pure culture.
[b] Enrichment culture.
[c] Mixed culture.
[d] Value in parentheses is from reference 36.
[e] Values in parentheses are from references 35 and 45, respectively.
[f] Values in parentheses are from references 45 and 46, respectively.
[g] Values in parentheses are from references 45 and 72, respectively.
[h] Values in parentheses are from references 45 and 73, respectively.
[i] Value in parentheses is from reference 14.
[j] In hours.
[k] In femtomoles of NO_2^- per cell per hour.
[l] In mg of protein per millimole of nitrite.
[m] Log number of cells per millimole of nitrite.

fmol NO_2^- cell^{-1} h^{-1}) were calculated from the exponential growth phase as described previously (42). Protein growth yield (Y_p in mg protein mmol^{-1} nitrite) and cell growth yield (Y_c in log cell counts mmol^{-1} nitrite) were calculated on the basis of cells or protein produced per millimole of nitrite oxidized.

RESULTS

Growth of NOB in batch cultures. Prior to the respirometry measurements, the cells were grown in batch cultures in nitrite-limited mineral medium. Figure 1 summarizes the exemplary cell growth, nitrite consumption, and equivalent nitrate formation of such NOB batch cultures. Since the growth of *Nitrospira* strain BS10 and "*Ca.* Nitrotoga arctica" was inhibited by high nitrite concentrations, the substrate concentrations were reduced to 0.3 and 0.7 mM nitrite, respectively. In contrast, the *Nitrobacter* cultures and "*Ca.* Nitrospira defluvii" grew well with 9 mM nitrite and *N. moscoviensis* grew well with 5.7 mM nitrite. For *Nitrobacter*, minimum generation times (g) of exponentially growing cells (Table 1) between 13 h (*N. vulgaris*) and 43 h (*N. hamburgensis*) were obtained. The generation times of the *Nitrospira* cultures ranged from 32 h (*N. moscoviensis*) to 37 h ("*Ca.* Nitrospira defluvii," strain BS10), and the slowest growth was that of "*Ca.* Nitrotoga arctica" (g = 44 h). Cell activities (k) were calculated during the exponential growth phase by simultaneous determination of cell counts and nitrite oxidation rates (Table 1). The results are, by tendency, consistent with the V_{max} values measured via oxygen consumption (see below). *N. vulgaris* showed the highest cell activity (13.1 fmol of NO_2^- cell^{-1} h^{-1}), and *N. moscoviensis* showed the lowest (0.6 fmol of NO_2^- cell^{-1} h^{-1}). Nitrite-dependent growth yields of produced protein (Y_p) and cell concentrations (Y_c) are listed in Table 1. In general, *Nitrospira* cultures (0.122 to 0.213 mg of protein mmol^{-1} nitrite) revealed higher yields of nitrite conversion for protein synthesis than *Nitrobacter* (0.083 to 0.108 mg of protein mmol^{-1} nitrite) and *Nitrotoga* (0.102 mg of protein mmol^{-1} nitrite) cultures, but all of the species produced comparable cell amounts per unit of nitrite (Table 1).

Oxidation kinetics. Key parameters of nitrite oxidation kinetics, maximum specific activities (V_{max}), and half-saturation constants (K_m), were measured by microsensor-based oxygen consumption of the *Nitrotoga* enrichment (Fig. 2) and the six NOB isolates (Fig. 3; Table 2). Nitrite-depleted, early-stationary-phase cells of all of the cultures consumed very low concentrations of oxygen and started O_2 uptake rapidly, within a few minutes after nitrite addition (Fig. 2A). The oxygen consumption rates were dependent on the nitrite concentrations provided (Fig. 2B) and followed Michaelis-Menten kinetics (Fig. 2C). The highest maximum oxidation activities obtained were those of members of the genus *Nitrobacter*. *N. vulgaris* from activated sludge showed by far the highest activity (164 ± 9 μmol of NO_2^- mg of protein^{-1} h^{-1}), followed by soil-derived *N. winogradskyi* and *N. hamburgensis* (78 ± 5 and 64 ± 1 μmol of NO_2^- mg of protein^{-1} h^{-1}, respectively). All of the *Nitrospira* strains studied reached lower maximum activities, though lineage I "*Ca.* Nitrospira defluvii" (48 ± 2 μmol of NO_2^- mg of protein^{-1} h^{-1}) enriched (37) and later isolated from activated sludge (Nowka et al., unpublished data) reached nitrite consumption rates similar to those of *Nitrobacter* strains from soil. Both of the lineage II *Nitrospira* isolates investigated, *N. moscoviensis* from heating water and strain BS10 from activated sludge, showed less than half of this maximum activity. "*Ca.* Nitrotoga arctica" (26 ± 3 μmol of NO_2^- mg of protein^{-1} h^{-1}) oxidized nitrite with a slightly higher maximum activity than both lineage II *Nitrospira* isolates.

A comparison of the K_m values also revealed a clear differentiation of nitrite affinities between the NOB investigated. "*Ca.* Nitrospira defluvii" and *N. moscoviensis* had by far the greatest affinity for nitrite of the seven NOB investigated (both 9 ± 3 μM NO_2^-), whereas strain BS10 featured a K_m value three times as high (27 ± 11 μM NO_2^-). Of the three *Nitrobacter* strains investigated, *N. vulgaris* showed the best adaption to low nitrite concentrations (49 ± 11 μM NO_2^-), though its affinity was still 1.8 to 5.4 times lower than that of the *Nitrospira* species. The lowest

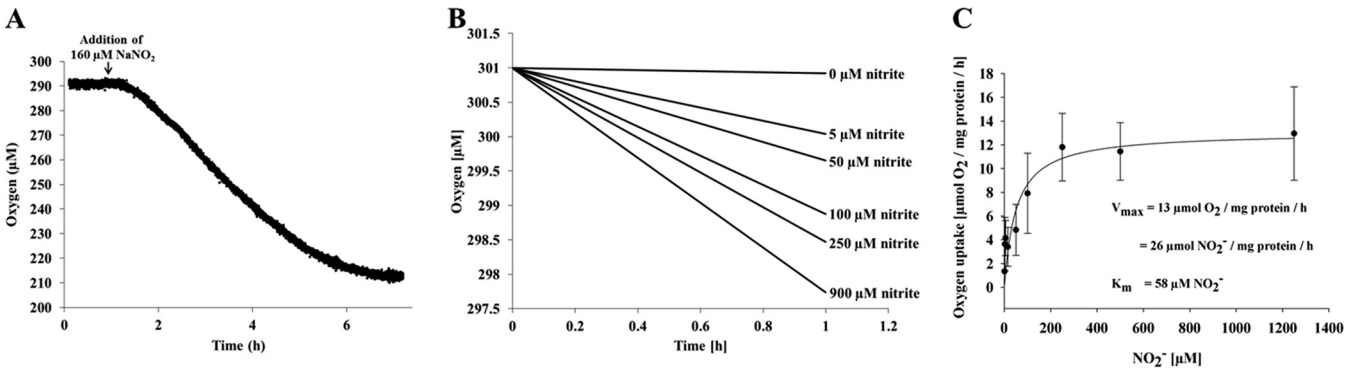

FIG 2 Nitrite oxidation kinetics of "*Ca*. Nitrotoga arctica." (A) Nitrite-dependent oxygen uptake of early-stationary-phase cells at 17°C. After equilibration of the cell aliquot and a stable oxygen concentration signal for ~1 h, the experiment was started by the addition of 160 µM $NaNO_2$. (B) Nitrite-dependent oxygen uptake of early-stationary-phase cells at 17°C at the nitrite concentrations shown. (C) Michaelis-Menten plot of oxygen uptake according to the nitrite concentration. The experiments were performed with early-stationary-phase cells at 17°C. Average values and standard deviations were calculated from 3-fold measurements. The kinetic parameters were calculated by fitting a Michaelis-Menten equation to the data.

affinities measured were those of *N. hamburgensis* and *N. winogradskyi* (544 ± 55 and 309 ± 92 µM NO_2^-, respectively). The nitrite affinity of "*Ca*. Nitrotoga arctica" (58 ± 28 µM NO_2^-) was comparable to that of *N. vulgaris*, in the midrange of the NOB studied.

We compared the K_m values we determined with those of other studies (Fig. 4), which demonstrated that the affinities of NOB for nitrite differ over 2 orders of magnitude. The highest affinities found were those of *N. moscoviensis* retrieved from heating water and "*Ca*. Nitrospira defluvii" obtained from activated sludge, whereas the soil-derived NOB had relatively high K_m values, with "*Ca*. Nitrotoga arctica" being most competitive representative. By tendency, the affinities of NOB originating from activated sludge and freshwater were higher than those of NOB from soil.

DISCUSSION

Knowledge of the community and activity of nitrifying bacteria is essential to improve operation strategies and design biotechnological processes in WWTPs (6). Ammonia oxidation, as the initial step of nitrification, is catalyzed by AOB and ammonia-oxidizing archaea, which revealed significantly different affinities for ammonia (43). Nitrification kinetics measured by respirometry often lack information about nitrite oxidation (9), and the range of nitrite affinities of NOB besides *Nitrobacter* is still a black box. Our goal was to generate kinetic data for most of the known NOB species of the genera *Nitrobacter*, *Nitrospira*, and *Nitrotoga* to close this gap. Although scientific interest in activated-sludge modeling may be declining (44), the behavior of single organisms in activated-sludge systems might enhance the understanding of such processes.

Reported key growth parameters of pure NOB cultures like the maximum specific activity (V_{max}) and the half-saturation constant for nitrite (K_m) are known mainly for *Nitrobacter* (1, 45, 46). The current data on *Nitrospira* were determined with enrichments but not with pure cultures (16, 20). Since the oxidation kinetics of cells are affected by different growth conditions, values can fluctuate greatly (45, 46) and shift through different growth phases with changing substrate concentrations (47). Therefore, the aim of this study was to determine the nitrite oxidation kinetics of NOB under standardized laboratory conditions, adjusting only strain-specific temperatures and substrate preferences. We are aware that these batch culture experiments rely on artificial growth conditions with the disadvantage of changing growth conditions (e.g., substrate concentration, cell density) in the system over time (48). Since the natural conditions of the NOB studied most are probably more complex and diverse, the results obtained should be seen as one vital component for the determination of NOB kinetics.

Nitrite oxidation kinetics. Previous studies demonstrated that the availability of nitrite is an important factor for the competition of NOB, suggesting different nitrite oxidation kinetics for members of the genera *Nitrobacter* and *Nitrospira* (16, 18–20). Moreover, coexisting *Nitrospira* populations revealed distinct preferences for nitrite (49). These findings indicate that substrate availability may account for the distribution of the different NOB in natural and artificial ecosystems. The comparison of V_{max} and K_m values for nonmarine NOB strains, in addition to results from the literature, strongly supports this assumption (Table 2 and Fig. 4), revealing specific adaptations for *Nitrospira*, *Nitrobacter*, *Nitrotoga*, and their sublineages, and a distinct clustering of populations obtained from different ecosystems.

Nitrospira. Members of the genus *Nitrospira* showed by far the highest affinities of the NOB investigated here. With a K_m value of 9 ± 3 µM nitrite, "*Ca*. Nitrospira defluvii" and *N. moscoviensis* had the lowest half-saturation constant determined for *Nitrospira* so far. Interestingly, these species belong to phylogenetically separate *Nitrospira* lineages I and II, which are widely distributed in natural habitats and were reported to be the main nitrite oxidizers in man-made ecosystems like WWTPs (12, 37, 50). This dominance can be explained by their advantage of oxidizing nitrite with high affinity and an energetically favorable periplasmic nitrite oxidoreductase (NXR) (51). It is interesting that their K_m values often resemble *in situ* nitrite concentrations. In the WWTP, where "*Ca*. Nitrospira defluvii" and strain BS10 originated, nitrite concentrations of 7 to 8 µM in activated-sludge samples (52) and 21 to 571 µM in sewage influents (Hamburg Wasser, personal communication, 2014) were measured. *Nitrospira* bacteria have commonly been detected in oligotrophic ecosystems such as subsur-

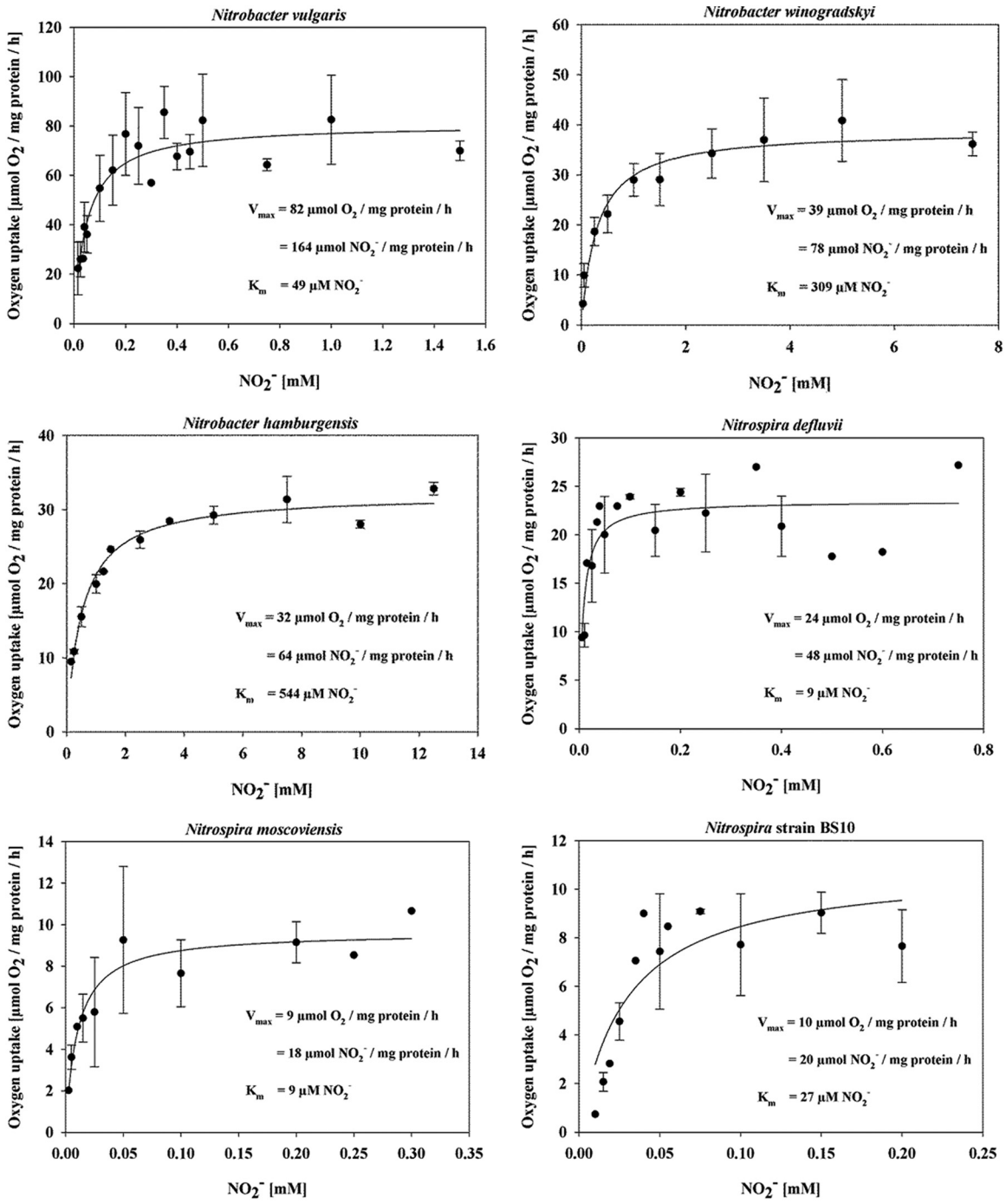

FIG 3 Nitrite oxidation kinetics of NOB. Michaelis-Menten plots of oxygen uptake at various nitrite concentrations are shown. Experiments were performed with early-stationary-phase cultures at defined incubation temperatures of 28°C (*Nitrobacter vulgaris*, *Nitrobacter hamburgensis*, *Nitrobacter winogradskyi*, "*Ca.* Nitrospira defluvii," *Nitrospira* strain BS10) and 37°C (*Nitrospira moscoviensis*). Average values and standard deviations were calculated from 3-fold measurements. The kinetic parameters were calculated by fitting a Michaelis-Menten equation to the data. See experimental details and calculations in Materials and Methods.

face fluids (53) and freshwaters with nitrite at nanomolar concentrations and below the detection limit, respectively (54, 55). These findings and the oxidation kinetics measured in this study support the suggested classification of *Nitrospira* bacteria as *K* strategists (16). Intriguingly, *Nitrospira* bacteria are not restricted to environments with low nitrite concentrations but can also be found at millimolar concentrations reported in subsurface fluids (56) or sequencing batch reactors (12). If such elevated nitrite levels are only transient, "*Ca.* Nitrospira defluvii" competes with the low-affinity *r* strategists of the genus *Nitrobacter*. However, continuously high nitrite concentrations were shown to select for *Nitrobacter* (19, 57).

TABLE 2 Kinetic constants of selected NOB cultures

Organism (temp [°C])	Mean max sp act[f] ± SD	Mean saturation constant for activity[g] ± SD
Nitrobacter vulgaris[a] (28)	164 ± 9	49 ± 11
Nitrobacter hamburgensis[a] (28)	64 ± 1	544 ± 55 (540–1,370, 706–1,240)[c,d]
Nitrobacter winogradskyi[a] (28)	78 ± 5	309 ± 92 (36–260, 170–1,380)[e]
"*Ca.* Nitrospira defluvii"[a] (28)	48 ± 2	9 ± 3
Nitrospira moscoviensis[a] (37)	18 ± 1	9 ± 3
Nitrospira strain BS10[a] (28)	20 ± 2	27 ± 11
"*Ca.* Nitrotoga arctica"[b] (17)	26 ± 3	58 ± 28

[a] Pure culture.
[b] Enrichment culture.
[c] Mixed culture.
[d] Values in parentheses are from references 45 and 46, respectively.
[e] Values in parentheses are from references 45 and 74, respectively.
[f] V_{max} in micromoles of NO_2^- per milligram of protein per hour.
[g] K_m in micromolar NO_2^-.

Comparing the three *Nitrospira* species studied, lineage II *N. moscoviensis* and strain BS10 revealed considerably lower maximum activities than lineage I "*Ca.* Nitrospira defluvii," which supports the hypothesis of Maixner et al. (49) that lineage I *Nitrospira* bacteria outcompete lineage II *Nitrospira* bacteria at high nitrite concentrations. Interestingly, the V_{max} of "*Ca.* Nitrospira defluvii" was much higher than that of *N. moscoviensis*, although the experimental temperature was 9°C lower. This finding suggests a high efficiency or a higher expression of the NXR inside "*Ca.* Nitrospira defluvii."

In contrast to the maximum activities, the nitrite affinities determined allow no differentiation between the *Nitrospira* strains from different phylogenetic lineages tested. The indicated advantage of "*Ca.* Nitrospira defluvii" over strain BS10 at low as well as high nitrite concentrations raises the question of whether there are other selective factors promoting the growth of strain BS10 in wastewater treatment, such as pH, affinity for oxygen, availability of organics, and temperature.

Nitrobacter. While *Nitrobacter* species showed the highest maximum activities (V_{max}) of NOB, their affinities for the substrate nitrite are generally low. One reason besides the kinetics of the NXR might be low affinities of the transporters that are needed to shuttle nitrite across the cytoplasmic membrane. There are *narK* homologs identified in the genomes of *N. hamburgensis* and *N. winogradskyi* that could function as nitrite/nitrate transporters (58, 59), but their kinetics await experimental clarification. However, the K_m values for nitrite indicate a great diversity of *Nitrobacter* ecotypes, which is consistent with a large genetic heterogeneity within *Nitrobacter* communities (60, 61). In this study, *N. vulgaris* derived from activated sludge revealed the highest nitrite affinity and maximum activity of the species of this genus investigated. The low nitrite affinities of soil-derived isolates of both *N. winogradskyi* and *N. hamburgensis* indicate that environmental factors other than nitrite concentration may account for *Nitrobacter* distribution. It was recently demonstrated that *Nitrospira* dominated in nutrient-limited environments such as surface soil (62), whereas *Nitrobacter* preferred a high supply of organic matter like that present in the rhizosphere or after fertilization (63, 64). Indeed, the potential of growing mixotrophically and chemoorganotrophically (65, 66) and even anaerobically via nitrate reduction (67) gives *Nitrobacter* a versatile metabolism, which may compensate for its low affinity for nitrite.

Nitrotoga. The nitrite oxidation kinetics of "*Ca.* Nitrotoga arctica" bacteria from permafrost soil revealed that they are rather *K* strategists like *Nitrospira*. Both NOB are characterized by a wide periplasmic space, which contains the NXR in *Nitrospira* (51, 68) and in *Nitrotoga* (E. Spieck, unpublished data). The maximum specific activity was slightly higher than that of *N. moscoviensis* and strain BS10 but lower than the activity of other NOB, especially the soil-derived *Nitrobacter* strains. The K_m value of *Nitrotoga* connects the affinity ranges of *Nitrospira* and *Nitrobacter* (Fig. 4) and indicates better adaption of "*Ca.* Nitrotoga arctica" than of the soil-inhabiting *Nitrobacter* strains to low nitrite concentrations. However, it should be considered that the measurements of *Nitrotoga* kinetics were performed with an enrichment culture and the presence of heterotrophic bacteria (despite a very low number) could have supported the activity and growth of "*Ca.* Nitrotoga arctica." Therefore, final conclusions have to await the isolation of these NOB and the availability of pure cultures to confirm the results. The preferred incubation temperature of 17°C, in contrast to the higher preferred incubation temperatures of the other cultures (28 to 37°C), shows why *Nitrotoga* is apparently widely distributed in cold-affected environments (24, 26), in particular, in pristine soils (69) or freshwater systems (25). Nevertheless, *Nitrotoga* is not restricted to nutrient-limiting habitats, since it is also abundant in sewage (27, 29). *Nitrotoga* overgrew *Nitrospira* and *Nitrobacter* during long-term cultivation at 5 and 10°C, respec-

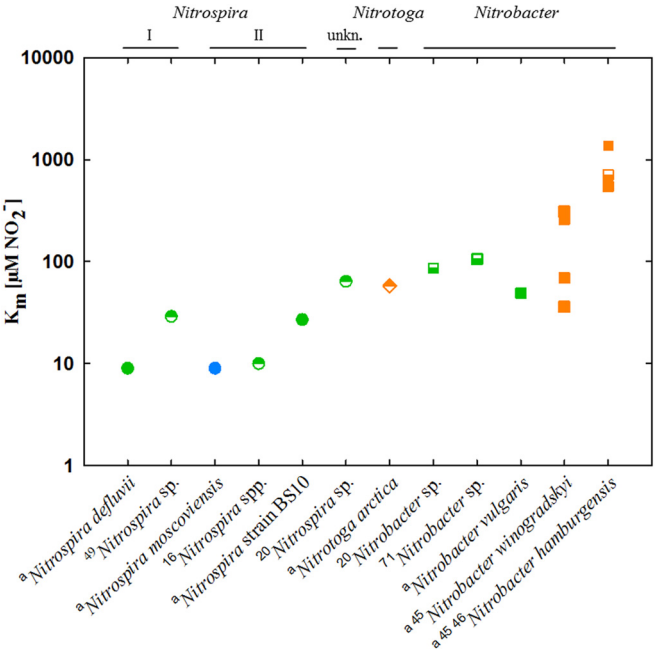

FIG 4 Nitrite affinities of NOB. K_m values of members of the genera *Nitrospira* (circles), *Nitrotoga* (diamond), and *Nitrobacter* (squares) are shown (pure cultures, filled symbols; enrichment cultures, half-filled symbols). Cultures were derived from activated sludge (green), freshwater (blue), and soil (orange). Phylogenetic affiliations, including *Nitrospira* lineages I and II, are displayed at the top (unkn., unknown). The values shown were obtained from this study (indicated by a superscript letter a) and references 16, 20, 45, 46, 49, and 71 (indicated by superscript numbers).

tively (27, 70), but extended competition experiments with *Nitrotoga* and the other genera of NOB are missing so far and will be a subject for the future.

ACKNOWLEDGMENTS

This research was funded by the Vienna Science and Technology Fund, WWTF (project LS09-40), and the German Research Foundation, DFG (project SP 667/10-1).

We thank Hamburg Wasser for support.

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