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Nitrification activity and community structure of nitrite-oxidizing bacteria in the bioreactors operated with addition of pharmaceuticals

Barbara Kraigher*, Ines Mandic-Mulec

University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Chair of Microbiology, Večna pot 111, 1000 Ljubljana, Slovenia

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ABSTRACT

Pharmaceuticals represent a group of the new emerging contaminants, which might influence microbial communities in the activated sludge. Nitrification activity and *Nitrospira* community structure in the small-scale reactors supplied with different concentrations (0, 50, 200, 500 $\mu g\,L^{-1}$) of the selected pharmaceuticals (ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid) were evaluated. Ammonia removal was not influenced by selected pharmaceuticals. However, in the two reactors operated with 50 $\mu g\,L^{-1}$ of pharmaceuticals (R50 and R50P), the effluent concentration of N-(NO_2^- + NO_3^-) was significantly higher than in the other reactors. *Nitrospira* community structure was assessed by terminal restriction fragment length polymorphism (T-RFLP) and by cloning and sequencing of the partial genes for 16S rRNA. *Nitrospira* spp. were detected in all reactors. The two dominant T-RFs represented the sublineages I and II of the genus *Nitrospira*. Main shifts were observed in the reactors R50 and R50P, where the T-RF representing sublineage II was much higher as compared to the other reactors. Consistent with this, the *Nitrospira* sublineage II was detected only in the clone libraries from the reactors R50 and R50P. Our results suggest that the relative abundance of *Nitrospira* sublineage II could be related to the effluent N-(NO_2^- + NO_3^-) concentration.

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1. Introduction

The occurrence and behaviour of pharmaceuticals in wastewater treatment (WWT) and in the environment have attracted increasing interest since 1990s [1–4]. Many pharmaceuticals, in particular non-steroidal anti-inflammatory drugs (e.g. ibuprofen, diclofenac, naproxen, ketoprofen) and lipid regulators (e.g. clofibric acid) are consumed in tons per year in human and veterinary medicine. Consequently, substantial amounts of pharmaceuticals can reach WWT plants and if they are not properly eliminated, they can be released into surface waters where they can accumulate, reaching detectable and biologically active levels. Numerous pharmaceuticals have been detected in surface waters, groundwater, wastewater and even in drinking water, in concentrations ranging from $ng\,L^{-1}$ to several $\mu g\,L^{-1}$. For example, Ashton et al. [5] have detected 27 $\mu g\,L^{-1}$ and Farré et al. [6] 85 $\mu g\,L^{-1}$ of ibuprofen in the effluent samples, while in the influents from a municipal sewage treatment plant located in the southeast of Spain (Alméria), Gómez et al. [7] have detected even 34–168 $\mu g\,L^{-1}$ of ibuprofen. Mostly, studies focus on the presence and the elimination of pharmaceuticals in the WWT plants. However, since WWT processes rely on the composition and activity of their microbial communities in activated sludge, it is also important to know how different chemicals entering the wastewater (including pharmaceuticals) affect activated sludge microbial communities.

Nitrification, the microbial oxidation of ammonia via nitrite to nitrate, is the initial step in the nitrogen removal from wastewater and is one of the key processes of biological WWT. Nitrification failure can occur easily, since nitrifying bacteria are slow-growing, autotrophic bacteria and appear to be inhibited by several environmental and operating factors such as low temperature, low dissolved oxygen and various chemical inhibitors [8], which can result in water resources pollution and eutrophication. Tran et al. [9] have suggested that nitrification can enhance the biotransformation of pharmaceutical residues. The two steps of nitrification (ammonia oxidation and nitrite oxidation) are carried out by two distinct functional groups of bacteria: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), which are phylogenetically heterogenous [10].

Nitrobacter-like bacteria, which could be relatively easily isolated from many environmental samples, were long considered to be the key nitrite oxidizers in biological WWT. Only recently, the application of cultivation-independent molecular methods revealed that mostly uncultured *Nitrospira*-like microorganisms and not *Nitrobacter* spp., are the dominant nitrite oxidizers in most WWT plants [11–14]. The genus *Nitrospira* is one of the less inten-

* Corresponding author. Tel.: +386 1 320 3410; fax: +386 1 257 3390.
E-mail address: barbara.kraigher@bf.uni-lj.si (B. Kraigher).

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sively studied groups of NOB, which are mostly uncultured and are, according to molecular data, among the most diverse and widespread nitrifiers in natural ecosystems and biological WWT [12]. The knowledge about physiology of *Nitrospira* spp. is still limited since the first enrichment of a sublineage I *Nitrospira* strain (which is mainly present in the WWT plants) from a nitrifying sludge was reported only recently [15]. Sequencing the genome of the 'Candidatus *Nitrospira defluvii*' revealed a gene similar to genes encoding chlorite dismutase [16]. Hence, *Nitrospira* may be involved in (per)chlorate and chlorite degradation, and might provide a link between N cycle and important bioremediation processes in WWT plants [16].

The aim of this study was to explore diversity of nitrite-oxidizing bacteria in the reactors operated with different concentrations of widely used pharmaceuticals (ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid) and to evaluate the potential influence of selected pharmaceuticals on nitrification activity and on the structure of nitrite-oxidizing bacterial communities in the bioreactors. Since previous study [17] indicated differences in bacterial community structure of activated sludge in the presence of selected pharmaceuticals, specifically in nitrite-oxidizing *Nitrospira*-like bacteria, this group was further assessed by terminal restriction fragment length polymorphism (T-RFLP) and by 16S rRNA gene clone libraries of the *Nitrospira*-like communities in the bioreactors.

2. Materials and methods

2.1. Bioreactor description and sampling of activated sludge

Studies were performed in small-scale bioreactors already described in Kosjek et al. [18] and Kraigher et al. [17]. Artificial wastewater was prepared by dissolving a nutrient-mineral composition in tap water (simulating the composition of real municipal wastewater). The chemicals used were: yeast extract (130 mg L^{-1}), casein peptone (130 mg L^{-1}), meat extract (130 mg L^{-1}), CH_3COONH_4 (317 mg L^{-1}), NH_4Cl (40 mg L^{-1}), K_2HPO_4 (24 mg L^{-1}), KH_2PO_4 (8 mg L^{-1}), $CaCO_3$ (100 mg L^{-1}), $MgCO_3$ (100 mg L^{-1}), NaCl (40 mg L^{-1}), and $FeSO_4 \cdot 7H_2O$ (5 mg L^{-1}). In order to follow biomass adaptation under exposure to different concentrations of pharmaceuticals, reactors were operated under continuous input of different concentrations (0, 50, 50, 200, 500 μg L^{-1} in reactors R0, R50, R50P, R200, R500, respectively) of each of the selected pharmaceuticals: ibuprofen, naproxen, ketoprofen, diclofenac, clofibric acid (all provided by Sigma–Aldrich, St. Louis, MO, USA). The R0 served as a control and was supplied with artificial wastewater without pharmaceuticals. Activated sludge from a Slovenian municipal WWTP was used for the reactors R0 and R50 start-up. The reactors R50P, R200 and R500 were set up using activated sludge from reactor R50 as inoculum. Reactors were operated continuously for at least 18 months without changing conditions to allow adaptation of the activated sludge community. The removal efficiency for chemical oxygen demand (COD) was over 90% in all reactors. The removal efficiency of the pharmaceuticals was also followed and was high for ibuprofen, naproxen and ketoprofen (over 86%) and lower for diclofenac and clofibric acid (less than 60%) [18]. Nitrification performance was assessed by measuring N-NH_4^+ and N-(NO_2^- + NO_3^-) concentrations in the influent and in the effluent using continuous flow analyzer (FlowSys Alliance Instruments, Salzburg, Austria).

Sampling of activated sludge in the reactors was performed three times, at approximately 45-day intervals. For each sampling, two (or three for the first sampling) 10-mL samples were collected from each reactor, transferred into sterile plastic tubes and stored at −80 °C until DNA extraction. Sampling of influents and effluents was performed eight times between March and August. For each sampling, 10-mL samples were collected and stored at −20 °C until N-NH_4^+ and N-(NO_2^- + NO_3^-) analyses.

2.2. Analysis of Nitrospira-like community structure by T-RFLP

DNA was extracted from 1-mL subsamples using the UltraClean soil DNA isolation kit (MoBio Solano Beach, CA, USA) according to the manufacturer's instructions. Isolated chromosomal DNA (concentration of 50–100 ng μL^{-1}) was checked on an 1% agarose gel, compared to the Gene Ruler DNA Ladder Mix (Fermentas, Litva) to estimate the size and concentration, and used as a template for PCR with the 16S rRNA gene *Nitrospira* sp. specific primer Nspira-705r [19] in conjunction with the conserved bacterial primer 27f labelled with 6-FAM (6-carboxyfluorescein) at the 5′ end. The 25-μL reaction mixtures were set up and PCR was performed as described in Kraigher et al. [20] except that annealing temperature was 59 °C. Restriction, ethanol precipitation and T-RFLP profiling was performed as described in Kraigher et al. [17]. Profiles were generated using Genescan analysis software (ABI). T-RFs with peak heights of less than 50 fluorescence units and T-RFs that were less than 50 bp long were excluded from the analyses. The size of each T-RF was determined according to Genescan 500 ROX size standard (Applied Biosystems Inc.) with an acceptable error of ±2 bp. Peak heights differing by ±2 bp were summed (based on visual inspection of profiles and on profiles of selected clones containing *Nitrospira* sublineage I or II 16S rRNA gene partial sequences). The data were then normalized by calculating relative peak height as a percentage of the total signal intensity of the corresponding T-RFLP profile, which minimized artifacts associated with different DNA concentrations loaded in capillary electrophoresis. Only the peaks with relative height >1% were considered for analyses.

2.3. Preparation of Nitrospira-specific 16S rRNA gene clone libraries and sequence analysis

Clone libraries were constructed from the reactors R0, R50, R50P and R500. PCR was performed with the thermocycling conditions used for T-RFLP, only that unlabelled forward primer was used. Purification of PCR fragments and cloning was performed as described in Kraigher et al. [17]. White colonies were screened for inserts of the expected size (about 700 bp) using the vector primers SP6 and T7 (Promega, Madison, WI, USA). PCR fragments obtained were subjected to restriction fragment length polymorphism (RFLP) analysis with *Hae*III. Since diversity of the fragments appeared very low (only a few different patterns were detectable in selected fragments for all clone libraries), only 12 clones were selected from each clone library for sequencing by Macrogen Inc. (Seoul, Korea). Plasmids were isolated from colonies and single extension 16S rRNA gene sequencing was performed by applying primer SP6. The sequences obtained were manually proofread and corrected, if necessary, with Chromas Version 2.3. Vector sequences were removed and potential chimeric sequences were detected by the Chimera Check program version 2.7 of the Ribosomal Database Project (RDP) [21] and by the Bellerophon program [22]. The sequences were then compared with available database sequences using the Basic Local Alignment Search Tool (BLAST). GenBank sequences most similar to clone sequences were downloaded and included in phylogenetic tree reconstruction using neighbour-joining method with 500 bootstrap replicates and the Kimura-2-parameter evolutionary model within the MEGA version 4 [23]. Distinct OTUs at different similarity levels were found and analyzed using the Mothur program [24]. In addition, *in-silico* T-RFLP of the sequences from clone libraries was performed using the T-DisctinctiEnz program (http://www.biorcgld.org/tools/restriction/t_DistinctiEnz.pl)

and the lengths of *in-silico* T-RFs were included in the phylogenetic tree.

2.4. Accession numbers

Sequences from the clone libraries were deposited in the GenBank database under accession numbers HQ198808 to HQ198853.

3. Results

3.1. Nitrification performance in the reactors

Nitrification performance was determined from concentrations of N-NH_4^+ in the influent and in the effluent of the five reactors. Concentrations were measured on eight sampling dates over five months and showed a high nitrification activity in all reactors with average removal efficiencies of at least 90% (Fig. 1a). Only on two sampling dates in reactor R500 and on one sampling date in R0, the removal efficiencies were lower than 90% (however, the samples for community analyses were not taken on these sampling dates).

In addition, N-(NO_2^- + NO_3^-) concentrations in the influent and in the effluent were measured. In Fig. 1b, concentrations of N-(NO_2^- + NO_3^-) produced in the reactors (i.e. difference between N-(NO_2^- + NO_3^-) concentrations in the effluent and influent) are shown. They were significantly higher in the reactors R50 and R50P as compared to the reactors R200 and R500 and also higher than in the control reactor R0 ($p < 0.001$). In all reactors, mean effluent concentrations of N-(NO_2^- + NO_3^-) were lower than mean influent concentrations of N-NH_4^+ (9–61 mg L^{-1} and 72–75 mg L^{-1}, respectively).

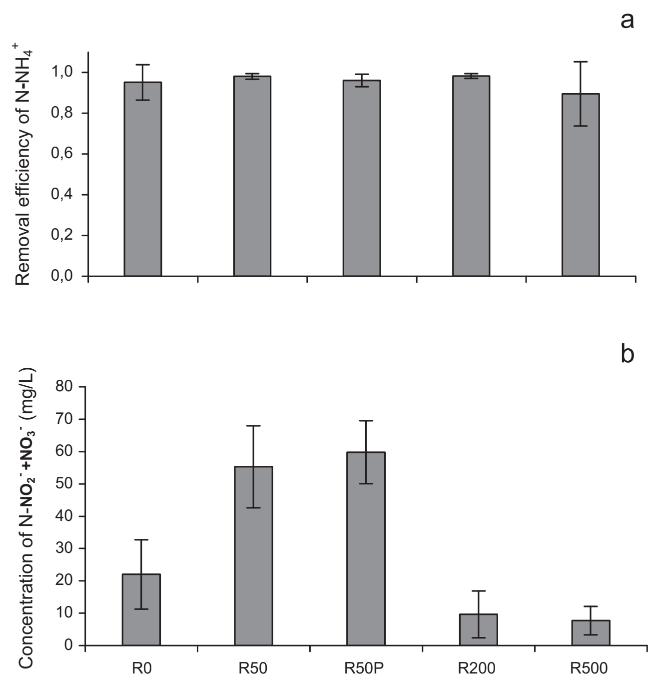

Fig. 1. Removal efficiencies of N-NH_4^+ (a) and differences between effluent and influent concentrations of N-(NO_2^- + NO_3^-) (b) in reactors R0, R50, R50P, R200 and R500 operated with different concentrations of pharmaceuticals: 0, 50, 50, 200, 500 $\mu g\,L^{-1}$, respectively. Bars indicate standard deviations of eight measurements between March and August ($n = 8$).

3.2. Analysis of Nitrospira-like community structure by T-RFLP

The structure of nitrite-oxidizing bacterial communities in the five reactors was evaluated by T-RFLP of *Nitrospira*-specific partial 27f – Nspira-705r 16S rRNA genes that were successfully amplified from all reactors on all sampling dates. Specific reverse primer Nspira-705r [19] was checked by RDP Probe Match (http://rdp.cme.msu.edu/probematch/search.jsp) and it revealed much more matches (993) to the *Nitrospira*-like bacteria in the database as compared to the primers NSR1113f and NSR1264r [13] (only 84 and 133 matches, respectively). Two different concentrations of PCR amplifications (varying for 7.5-fold) were digested by *Hae*III and subjected to T-RFLP analysis. As expected, higher concentration revealed some more distinct T-RFs (data not shown) as compared to the lower concentration profiles. However, some T-RFs that were detected in higher concentration profiles (and sometimes also in lower concentration profiles), were observed also in the T-RFLP profiles of the single clones, indicating that they were most probably artifacts of PCR or digestion. Therefore, only lower concentration T-RFs that were present in at least one reactor in both replicates of the sampling time (with relative height of >1%) were considered for analyses. T-RFLP profiles were compared by calculating the mean relative abundances of individual T-RFs in different reactors on the three sampling dates and are shown as his-

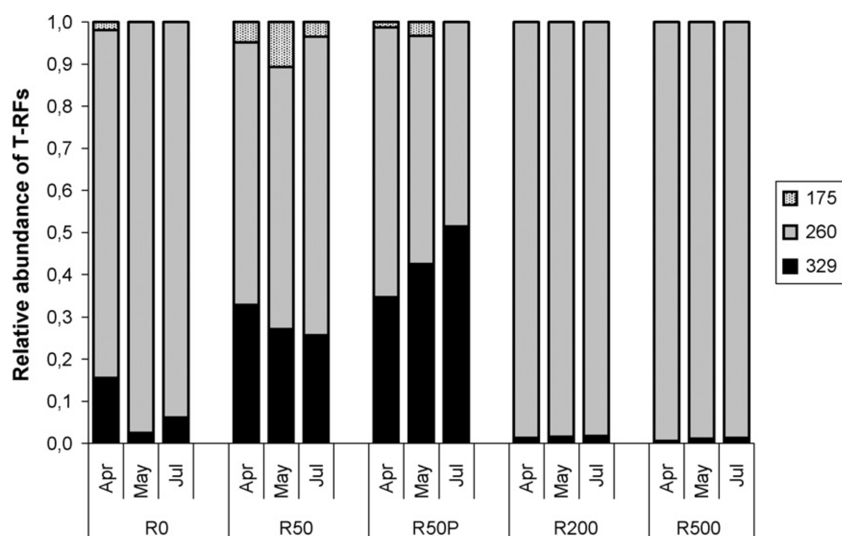

Fig. 2. Histograms of T-RF relative abundances in *Hae*III T-RFLP profiles of *Nitrospira*-like partial 16S rRNA genes in the five reactors (R0, R50, R50P, R200, R500) on three sampling times (April, May, July). Numbers in the key indicate the lengths of the T-RFs.

tograms in Fig. 2. Three distinct T-RFs were found in the reactors and only one T-RF (approximately 260 bp) was found highly abundant (47–99%) in all reactors on all sampling dates. The main difference between the reactors was observed in the T-RF of approximately 329 bp, which was much more abundant in the reactors R50 and R50P (25–50%) as compared to the other reactors (2.5–15% in R0 and 0–1.7% in R200 and R500). To identify the dominant T-RFs, some clones from the previously constructed clone library, which contained *Nitrospira*-like sequences [17], were also subjected to T-RFLP analyses. It was found that T-RFs of 260 bp and 329 bp represent the two *Nitrospira* sublineages I and II, respectively. The T-RF of 175 bp was also represented by one clone sequence in the previous library (0F5, EU499598).

To directly compare the relative abundances of the two *Nitrospira* sublineages in the reactors, the ratios between the fluorescent signals of T-RFs of 329 bp and 260 bp were calculated from each profile as peak height ratio and are shown as mean values of two (or three) replicate samples and three sampling times in Fig. 3. Ratios were higher in the reactors R50 and R50P as compared to the other reactors, which was evident from both peak height and peak area (data not shown) analyses.

Additionally, to evaluate the AOB that perform the first step of nitrification, the gene for ammonia monooxigenase *amo*A was successfully amplified from all reactors and T-RFLP with the restriction enzyme *Taq*I was performed. Since profiles were very variable within and between reactors, data was not shown and further analyzed. However, in all bioreactors (regardless of pharmaceutical concentration) we have found one dominant T-RF of 219 bp, which represents *Nitrosomonas europaea/eutropha* lineage [25] and was very often found as the dominant AOB in WWT [26,27].

3.3. Nitrospira-specific 16S rRNA gene sequence analysis

To obtain a more detailed view of the phylogenetic diversity of *Nitrospira*-like bacteria in the activated sludge of the reactors, partial *Nitrospira*-like 16S rRNA specific gene clone libraries were constructed from reactors R0, R50, R50P and R500. For initial screening of clone libraries, RFLP by *Hae*III restriction enzyme on the cloned sequences was performed and revealed only one digestion pattern in the clone libraries R0 and R500 and two in R50 and R50P (data not shown). Therefore, only 12 clones per library were selected for sequencing. 46 sequences of good quality were obtained and were initially analyzed using BLAST searches at NCBI. They were all affiliated with *Nitrospira* genus (at least 97% similar to the sequences in the database), and were mostly very similar to the sequences found in the WWT plants, some of them were found also in soil. The most similar sequences in the database and some sequences from different *Nitrospira* sublineages were downloaded and included in the phylogenetic tree reconstruction (Fig. 4). All sequences in the four libraries were at least 90% similar to each other. Distribution of the library sequences over different OTUs at

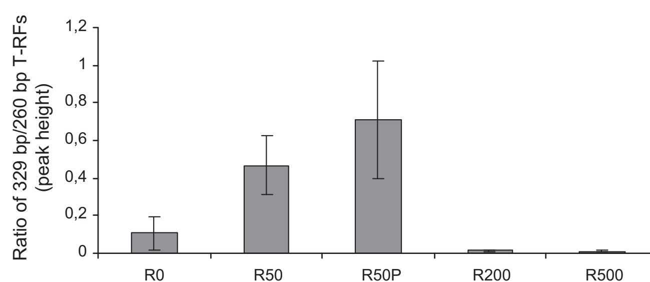

Fig. 3. Ratios of peak heights of two T-RFs with sizes 329 bp and 260 bp in the five reactors (R0, R50, R50P, R200, R500). T-RFs of 260 bp and 329 bp correspond to the *Nitrospira* sublineages I and II, respectively. Average ratios with standard deviations ($n=6$) for the five reactors are shown.

99% similarity level and over the two *Nitrospira* sublineages is summarized in Table 1. Majority of the sequences (37 out of 46) were assigned to the *Nitrospira* sublineage I and were all at least 98% similar to each other. At a genetic distance of 0.01, they fell into two groups (designated in the tree as OTU1 and OTU2). Nine sequences were assigned to the *Nitrospira* sublineage II (with at least 94% similarity to each other), and they were all retrieved from the libraries of the reactors R50 and R50P. At a genetic distance of 0.05, they fell into two groups (designated in the tree as OTU3 and OTU4). In the libraries from reactors R0 and R500, no sequences were assigned to the *Nitrospira* sublineage II.

In-silico T-RFLP (*Hae*III) of the aligned sequences included in the tree reconstruction revealed two dominant terminal fragments (264 bp and 331 bp) representing sublineages I and II of the *Nitrospira* genus. The two sequences in the sublineage III were both cut at 66 bp while sequences from the *Nitrospira* sublineage IV gave T-RFs of different sizes (229 bp, 260 bp, 263 bp and 264 bp). *Nitrospira marina* as a representative cultured species of sublineage IV gave the same T-RF (264 bp) as the *Nitrospira*-like sequences that belong to the sublineage I. Interestingly, at 331 bp all sequences included in the tree (including *Leptospirillum ferrooxidans*) contained restriction site for *Hae*III enzyme (i.e. GG|CC; data not shown).

The majority of the sequences from our clone library (36) gave *in-silico* T-RF of 264 bp (and belonged to the *Nitrospira* sublineage I) while only 8 sequences were cut at 331 bp, specific for the *Nitrospira* sublineage II (Table 1). Only one sequence from each sublineage gave different T-RFs (263 bp in sublineage I and 143 bp in sublineage II), although they were very similar (99%) to the other sequences in the two sublineage clusters.

4. Discussion

4.1. Nitrification performance in the reactors

Nitrification activity of activated sludge bacteria as indicated by N-NH_4^+ elimination was high in all bioreactors, independent

Table 1
Distribution of sequences from the four *Nitrospira*-like 16S rRNA gene libraries over different OTUs and *Nitrospira* sublineages. *In-silico* T-RF lengths for the sequences in different OTUs are shown.

Clone library	*Nitrospira* sublineage I		*Nitrospira* sublineage II		Total number
	OTU[a] 1	OTU 2	OTU 3	OTU 4	
R50	5	1	2	3	11
R50P	5	3	0	4	12
R0	8	3	0	0	11
R500	10	2	0	0	12
Total number	28	9	2	7	46
In-silico T-RF[b] (bp)	264	264 (263)	331	331 (143)	

[a] OTU represents a group of sequences with at least 99% of similarity.
[b] Size of terminal restriction fragments as obtained by *in-silico* T-RFLP with restriction enzyme *Hae*III.

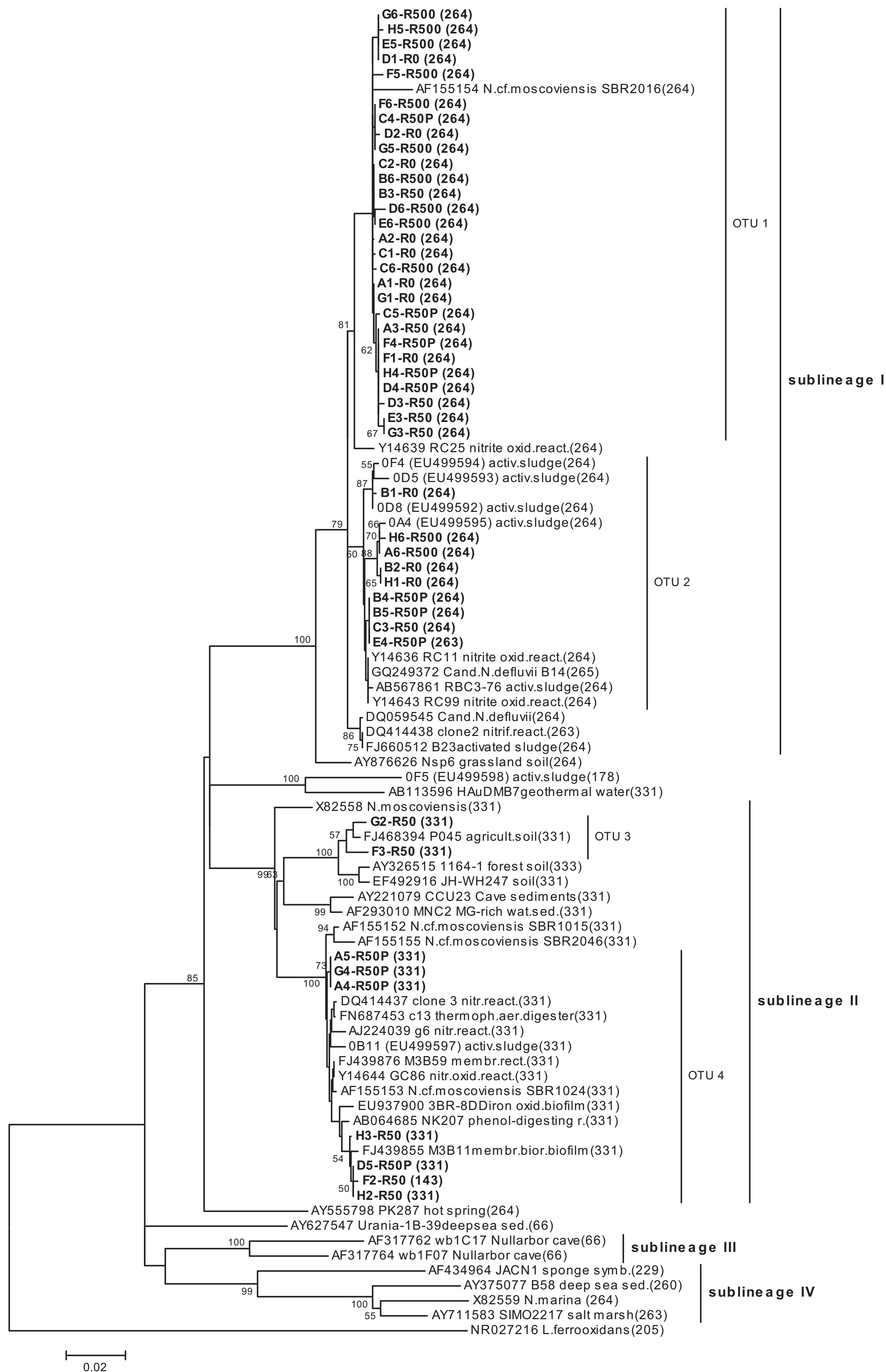

Fig. 4. Phylogenetic tree based on partial (0.7 kb) *Nitrospira*-like 16S rRNA gene sequences from the four libraries (designated in clone names as R0, R50, R50P and R500), and from similar *Nitrospira*-like sequences found by BLAST searches in the GenBank database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown at the nodes (only values over 50 are shown). Sequences obtained in this study are printed bold. *Leptospirillum ferrooxidans* (X86776) was used as the outgroup species. Four sublineages of the *Nitrospira* genus and four different OTUs at a distance of 0.01 are designated. Lengths of the *in-silico* restriction digests with *Hae*III restriction enzyme are given in parentheses.

of concentration of pharmaceuticals in the reactors. Mean effluent concentrations of N-(NO_2^- + NO_3^-) were not reaching the mean influent concentrations of N-NH_4^+. This indicated that denitrification or some other N-(NO_2^- + NO_3^-) removal process occurred in the reactors, especially in the reactors R200 and R500 where relatively low concentrations of N-(NO_2^- + NO_3^-) were detected in the effluents (mean concentrations 9–11 mg L^{-1}), in contrast to the reactors R50 and R50P where concentrations of N-(NO_2^- + NO_3^-) were significantly higher (mean concentrations 56–61 mg L^{-1}).

Although denitrification is generally considered to be an anaerobic process, it may occur under aerobic and microaerophilic conditions [28]. In the previously constructed clone libraries of 16S rRNA genes of total bacteria [17], many *Acidovorax* and *Thauera* spp. have been found, which were also detected in different denitrifying reactors [29–31]. Gentile et al. [32] have found that *Acidovorax*-like isolate reduced nitrate to dinitrogen gas without the accumulation of nitrite or nitrous oxide. These bacteria could perform denitrification in our reactors in the microaerophilic conditions that might develop in activated sludge flocs [33]. Also, nitrifiers (AOB and NOB) can denitrify under O_2-limited conditions [34]. For example, *N. europaea* and *N. eutropha* could nitrify and denitrify simultaneously under O_2 limitation by using nitrite in addition to O_2 as electron acceptor when ammonia and suitable organic compounds were present [35]. Under anoxic conditions, *Nitrospira moscoviensis* could reduce nitrate with hydrogen as electron donor [36]. Considering the above, there were many possibilities for the nitrate removal from the reactors. However, the reason for better removal of nitrate from the reactors with higher concentrations of pharmaceuticals and also from the reactor with no pharmaceuticals (as compared to the reactors R50 and R50P) remains unclear.

4.2. *Nitrospira-like community structure*

According to phylogenetic analyses, the genus *Nitrospira* consists of at least four distinct sublineages [12]. Sublineage I contains only mostly uncultivated organisms from activated sludge, while sublineage II contains the cultivated species *N. moscoviensis*, as well as sequences of uncultivated bacteria retrieved from diverse habitats, including bioreactors [12]. Studies addressing the *Nitrospira* community structure in the activated sludge and *Nitrospira*-specific T-RFs for sublineages I and II are scarce [37].

In-silico T-RFLP of the clone library and database sequences revealed that sublineages I and II could clearly be distinguished using the restriction enzyme *Hae*III. However, the *in-silico* predicted and the experimental T-RF lengths differed for approximately 4 bp for sublineage I and for approximately 2 bp for sublineage II. T-RF drifts of this kind are well known [38] and our results demonstrated that a clone library and your own database for T-RF affiliations should be constructed because public databases for T-RFs can lead to erroneous interpretations of the community composition.

The *Nitrospira* community structure in the reactors was evaluated by T-RFLP and sequence analyses of clone libraries. Influence of pharmaceuticals was detected in the reactors with 50 $\mu g\,L^{-1}$ of selected pharmaceuticals (R50 and R50P), where the T-RF peak height representing *Nitrospira* sublineage II was much higher as compared to the other reactors. Consistent with this, *Nitrospira* sublineage II was detected only in the libraries R50 and R50P, where substantial portions of clones (5 out of 11 and 4 out of 12, respectively; Table 1) were affiliated with this sublineage. The results suggested positive effect of the selected pharmaceuticals at concentration of 50 $\mu g\,L^{-1}$ on the *Nitrospira* sublineage II. A possible explanation for this might be that pharmaceuticals at the observed concentration were used as a source of carbon or were co-metabolized by *Nitrospira* sublineage II, while at higher concentrations sublineage I or some other competitor co-metabolized the pharmaceuticals and the advantage of *Nitrospira* II sublineage was lost. It might also be possible that the pharmaceuticals at concentration of 50 $\mu g\,L^{-1}$ influenced the activity and/or structure of denitrification bacteria, which increased the concentration of nitrate in the reactors R50 and R50P, and that *Nitrospira* community structure was ultimately influenced by the nitrate concentration. However, we did not detect nitrite accumulation in the presence of pharmaceuticals (in all reactors, less than 1 mg L^{-1} was detected; data not shown), which suggests that nitrite oxidation was not inhibited by pharmaceuticals.

Only few studies evaluated effects of different environmental parameters on *Nitrospira* community structure in activated sludge by T-RFLP. For example, Park and Noguera [37] have found that in the reactor operated with high dissolved oxygen a (partial) shift in *Nitrospira* community from sublineage I to sublineage II occurred while this was not the case in the reactor operated with low dissolved oxygen. Siripong and Rittman [39] analyzed the *Nitrospira*-like bacteria in seven different full-scale municipal water reclamation plants (WRPs). T-RF of 277 bp, which was highly abundant in all their samples, represented the sublineage I in our library (i.e. T-RF of 260 bp), and the T-RF of 265-267 bp that was detected only in the samples from Lemont WRP represented the sublineage II in our library (i.e. T-RF of 329 bp). Interestingly, when we checked the conditions in the Lemont WRP (Table 4 in Siripong and Rittmann [39]), especially the winter samples showed higher concentration of N-(NO_2^- + NO_3^-) in the effluent of the Lemont WRP as compared to the other plants (although the removal efficiency of N-NH_4^+ was over 99%). This was consistent with our results, suggesting that the presence of the *Nitrospira* sublineage II could be related to the higher concentration of N-(NO_2^- + NO_3^-) in the effluent. However, further studies should be performed to confirm this observation and to find the reason for this relationship.

First *Nitrospira* sp. from sublineage I was only recently selectively enriched [15] and therefore knowledge about microbiology of these organisms is still very limited. Huang et al. [40] studied the influence of physicochemical and operational parameters on *Nitrobacter* and *Nitrospira* communities in an aerobic activated sludge bioreactor using real-time PCR. *Nitrospira* was negatively correlated to nitrite concentrations and positively to temperature. They have found no correlation to nitrate concentration. However, they have evaluated the quantity of the total *Nitrospira* in the samples by real-time PCR, and differences in the ratio between the two sublineages could not be revealed. It is often presumed that all *Nitrospira* spp. have the same physiological behaviour and are treated as one coherent group when studying different influences. However, as speculated about this already by Schramm et al. [41], indicated in this study, and shown by Maixner et al. [42] and Park and Noguera [37], different physiologies of different *Nitrospira* spp. should be considered when evaluating different effects on *Nitrospira*-like bacteria. By metagenomic studies, Lücker et al. [43] recently revealed that ‘Candidatus *Nitrospira defluvii*’ differs dramatically from other known nitrite oxidizers in the key enzyme nitrite oxidoreductase, in the composition of the respiratory chain, and in the pathway used for autotrophic carbon fixation.

5. Conclusions

Our findings suggest that selected pharmaceuticals often detected in wastewaters may influence the structure of the sensitive nitrite-oxidizing bacterial community in the treatment plants. We have detected that the *Nitrospira* community structure and the effluent N-(NO_2^- + NO_3^-) concentrations in the reactors operated with 50 $\mu g\,L^{-1}$ of pharmaceuticals were different as compared to the reactors operated with higher concentrations of pharmaceuticals or with no pharmaceuticals. *Nitrospira* spp. were present in all reactors, however, the relative abundance of the *Nitrospira*

sublineage II could be related to the effluent N-(NO_2^- + NO_3) concentration. The reason for this observation remains to be clarified by further investigations.

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