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RESEARCH ARTICLE

Colonization of freshwater biofilms by nitrifying bacteria from activated sludge

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Abstract

Effluents from wastewater treatment plants (WWTPs) containing microorganisms and residual nitrogen can stimulate nitrification in freshwater streams. We hypothesized that different ammonia-oxidizing (AOB) and nitrite-oxidizing (NOB) bacteria present in WWTP effluents differ in their potential to colonize biofilms in the receiving streams. In an experimental approach, we monitored biofilm colonization by nitrifiers in ammonium- or nitrite-fed microcosm flumes after inoculation with activated sludge. In a field study, we compared the nitrifier communities in a full-scale WWTP and in epilithic biofilms downstream of the WWTP outlet. Despite substantially different ammonia concentrations in the microcosms and the stream, the same nitrifiers were detected by fluorescence *in situ* hybridization in all biofilms. Of the diverse nitrifiers present in the WWTPs, only AOB of the *Nitrosomonas oligotropha/ureae* lineage and NOB of *Nitrospira* sublineage I colonized the natural biofilms. Analysis of the *amoA* gene encoding the alpha subunit of ammonia monooxygenase of AOB revealed seven identical *amoA* sequence types. Six of these affiliated with the *N. oligotropha/ureae* lineage and were shared between the WWTP and the stream biofilms, but the other shared sequence type grouped with the *N. europaea/eutropha* and *N. communis* lineage. Measured nitrification activities were high in the microcosms and the stream. Our results show that nitrifiers from WWTPs can colonize freshwater biofilms and confirm that WWTP-affected streams are hot spots of nitrification.

Introduction

Effluents from biological wastewater treatment plants (WWTPs) typically contain dissolved organic matter, residual dissolved inorganic nitrogen (DIN), and microorganisms that are not completely retained in the treatment process (Brion & Billen, 2000; Martí *et al.*, 2010). In the receiving lakes or streams, these effluents may affect the microbial community and its biocatalytic activities (Gücker *et al.*, 2006; Ruggiero *et al.*, 2006; Wakelin *et al.*, 2008; Merseburger *et al.*, 2009). In particular, DIN in the form of ammonium can strongly influence nitrification, the microbially catalyzed two-step oxidation of ammonia to nitrate, in the natural water bodies (Merseburger *et al.*, 2005; Ribot *et al.*, 2012).

In habitats affected by WWTP effluents, members of the *Nitrosomonas oligotropha/ureae* lineage often account for the majority of ammonia-oxidizing bacteria (AOB) (de Bie *et al.*, 2001; Cebron *et al.*, 2003, 2004; Nakamura *et al.*, 2006; Dang *et al.*, 2010). This is not unexpected as this lineage constitutes the dominant AOB in many WWTPs (Koops *et al.*, 2006). Therefore, the *N. oligotropha/ureae* lineage was proposed as a potential bio-indicator for pollution due to wastewater effluents (Dang *et al.*, 2010). The communities of nitrite-oxidizing bacteria (NOB) in freshwater biofilms may also be influenced by

WWTP effluents. Members of the genus *Nitrospira* usually prevail in activated sludge (Daims *et al.*, 2001) and were also detected downstream of WWTP outlets (Cebron & Garnier, 2005; Nakamura *et al.*, 2006). However, the nitrifier groups found in WWTPs also occur in pristine environmental samples (Daims *et al.*, 2001; Koops *et al.*, 2006). Thus, the source of these AOB and NOB in WWTP-affected ecosystems remains unclear. They could originate from the freshwater environment (autochthonous nitrifiers) and simply be stimulated by ammonium from the WWTP effluents. Alternatively, they might be introduced into the receiving streams through the effluent discharge (allochthonous nitrifiers) and be able to colonize streambed biofilms (Brion & Billen, 2000; Cebron *et al.*, 2003). Systematic studies demonstrating a successful invasion of freshwater biofilms by AOB and NOB from WWTPs are scarce as molecular data from activated sludge of WWTPs and the respective receiving waters have rarely been screened for identical phylotypes. In an earlier study, identical but only partial 16S rRNA gene sequences from the *N. oligotropha/ureae* lineage were recovered by PCR amplification from WWTP effluents and downstream freshwater biofilms (Cebron *et al.*, 2003). Even though these results may indicate settling of allochthonous AOB in the natural biofilms, alternative explanations could be the PCR-based detection of non-persistent AOB or even naked DNA from the WWTP effluents.

Here, we studied the impact of discharges from WWTPs on AOB and NOB communities in freshwater biofilms, with special focus on the colonization of these biofilms by allochthonous nitrifiers. Ammonia-oxidizing archaea (AOA) were not investigated here, because they most probably do not play a significant role in most WWTP (Mussmann *et al.*, 2011). Known bacterial nitrifiers show ecological niche partitioning with respect to their preferred substrate concentrations (Suwa *et al.*, 1994; Schramm *et al.*, 1999; Bollmann *et al.*, 2002; Koops *et al.*, 2006; Maixner *et al.*, 2006; Sorokin *et al.*, 2012); therefore, we hypothesized that nitrifier populations released from WWTPs differ in their potential to colonize stream biofilms. First, we simulated a discharge of WWTP effluents in microcosm flumes to test whether AOB and NOB from a WWTP were in principle able to colonize, multiply, and nitrify in freshwater biofilms that were previously devoid of nitrifiers. Second, we compared the NOB and AOB populations in the activated sludge of a full-scale municipal WWTP to those in natural streambed biofilms developed along a 850-m reach downstream of the WWTP outlet and estimated whole-reach net nitrification rates. In both settings, the AOB and NOB populations were identified and their presence in the freshwater biofilms was monitored by fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes. In addition, the AOB populations in the streambed biofilms were analyzed by using the *amoA* gene as functional marker that encodes the alpha subunit of ammonia monooxygenase and offers a higher phylogenetic resolution than 16S rRNA gene within the betaproteobacterial (Purkhold *et al.*, 2000). Nitrification activity was corroborated by measuring nitrification rates in both the microcosm and the stream.

Materials and methods

Flume microcosms

Mature freshwater biofilms were grown for 4 weeks on sterile ceramic coupons in eight flumes (length: 130 cm, width: 2 cm, height: 2 cm) that were fed with surface water from the oligotrophic Lake Lunz (Austria). The flume setup to grow biofilms was as described previously (Singer *et al.*, 2006; Augspurger *et al.*, 2010), but the flumes were kept at *c.* 20 °C in the dark. Furthermore, the water was recirculated with a pumping speed of *c.* 30 mL s^{-1}, and *c.* 80% of the water was replaced three times a week. The total water volume per flume was 4 L. The oxygen concentration was around 7 mg L^{-1} in all flumes. The average nutrient concentrations in the lake water were determined before each water exchange and were as follows: 3.2 ± 1.1 μg $P\text{-}PO_4^{3-}$ per L; 2.4 ± 0.8 μg $N\text{-}NO_2^-$ per L; 858 ± 60 μg $N\text{-}NO_3^-$ per L; and 33 ± 25 μg $N\text{-}NH_4^+$ per L. The setup of our experiment is illustrated in Fig. 1. To test the ability of allochthonous nitrifiers to colonize these pregrown freshwater biofilms, diluted activated sludge from a nitrifying sequencing batch reactor of a municipal WWTP (Ingolstadt, Germany, described by Mussmann *et al.* (2011)) was added to four of the flumes. The activated sludge had been sampled 3 days prior to the inoculation. Per flume, 50 mL of activated sludge was mixed with 4 L of lake water for each flume and was recirculated for 3 days to allow nitrifying bacteria to settle on the biofilms. Afterward, the water-containing sludge was removed and two of these four flumes received lake water amended with nitrite (average concentration of 5 mg $N\text{-}NO_2^-$ per L), whereas the other two flumes received lake water supplied with ammonium (average concentration of 65 mg $N\text{-}NH_4^+$ per L). All flumes were incubated for 2–3 days until the lake water was exchanged again and either nitrite or ammonium was replenished. To test for the presence and growth of autochthonous nitrifiers from Lake Lunz, the remaining four flumes were not inoculated with activated sludge but received lake water containing either nitrite or ammonium in the same concentrations as described above. After 13 days of

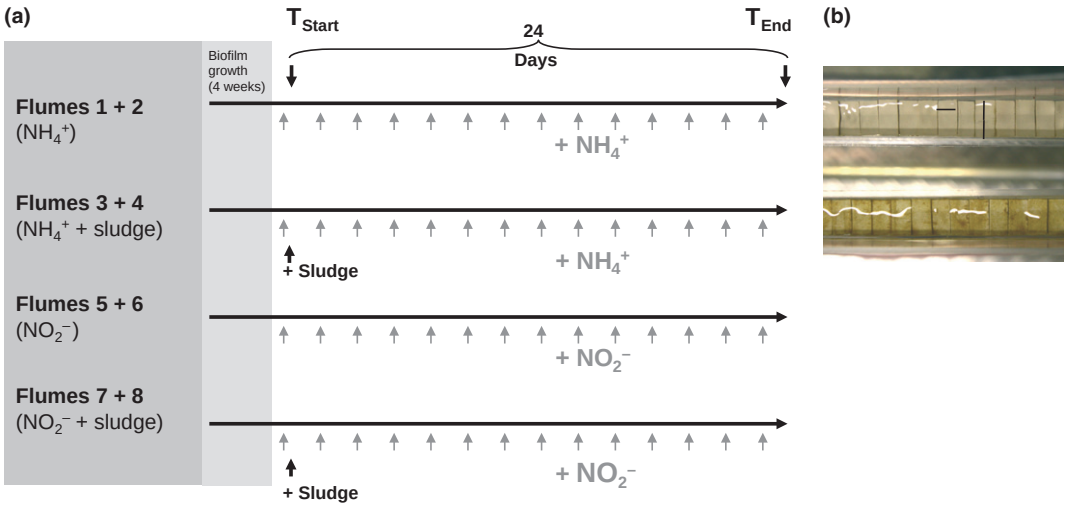

Fig. 1. (a) Experimental setup of the flume experiment. Biofilms were grown on ceramic coupons immersed in water from the oligotrophic mountain Lake Lunz. After mature biofilms had developed, four flumes were inoculated with activated sludge (black arrows). Two inoculated and two noninoculated flumes were supplied with either NH_4^+ or NO_2^- (gray arrows). After 24 days, biofilms were recovered for molecular analysis. Nitrification activity was determined between days 3 and 19 (see also Fig. 4). (b) Photograph of ceramic coupons in NH_4^+-supplied flumes without sludge (upper flume) and inoculated with sludge (lower flume). Size of ceramic coupons was c. 1 × 2 cm.

incubation, a decline from ≥ pH 7 in the lake water to *c.* pH 5 was observed in the flumes that had been inoculated with activated sludge and were being fed with ammonium. Most likely, this pH decrease was due to HNO_3 formation during nitrification. Following the addition of sodium bicarbonate (7.1 mM), the pH in these flumes increased to neutral or slightly alkaline values and was stabilized. No significant pH decline was detected in the other flumes. The eight flumes were operated for 24 days after sludge inoculation (Fig. 1). On day 24, ceramic coupons were removed from different positions in each flume and were preserved for subsequent molecular analysis. Flume waters were sampled to measure DIN transformations on 19 of the 24 days.

Sampling and fixation of biofilms and activated sludge

The field study site was located in the main course of La Tordera River, immediately downstream of the WWTP outlet of the village of Santa Maria de Palautordera (Catalonia, Spain). The WWTP (5808 population equivalents) performs biological secondary treatment with activated sludge. The discharge of the WWTP is relatively constant throughout the year (mean of 27.4 L s^{-1}). In contrast, the stream discharge can vary by several orders of magnitude within and between hydrological years. Thus, the contribution of the WWTP effluent to the total water of the receiving stream ranges from 3% to 100% (Merseburger *et al.*, 2005). The WWTP effluent usually contains 14.9 ± 3.5 mg L^{-1} of DIN, which mainly (> 90%) consists of ammonium. Samples were taken at 11 sites along a 850-m-long reach downstream of the WWTP outlet in order to examine the nitrifier community in the streambed biofilms and to measure net longitudinal changes in the concentrations of ammonium and nitrate. An additional sampling site 50 m upstream of the WWTP outlet was used as a reference to evaluate the effect of the WWTP input. Samples were taken in winter (February 11th) and summer (September 9th) of 2008 to account for seasonal effects. On the day of sampling in summer, the stream was dry upstream of the WWTP outlet.

Biofilms from the flume microcosms were scraped off the ceramic coupons by using sterile coverslips and were pooled in 2-mL plastic vials. Epilithic biofilms from the La Tordera streambed were collected from the shaded bottom side of three randomly selected cobbles per sampling site and date. The cobbles were washed with distilled water, and *c.* 9 cm^2 of biofilm was scraped off with a sterilized knife. The biofilms from the three cobbles per sampling site were pooled. Flume biofilms, streambed biofilms, and activated sludge samples from the WWTPs were fixed immediately in formaldehyde (1.8% for flume biofilms and for samples from WWTP in Ingolstadt, 4% for streambed biofilms and for samples from the WWTP in Santa Maria de Palautordera) dissolved in phosphate-buffered saline (PBS) and were kept cool for up to 12 h. The fixed samples were then washed twice with PBS and stored in a PBS–ethanol solution (1 : 1, vol/vol) at −20 °C.

Fluorescence in situ hybridization (FISH)

For FISH analysis, biofilm samples were transferred to teflon-coated slides and air-dried. FISH was performed using Cy3- or carboxyfluorescein-labeled, rRNA-targeted oligonucleotide probes as previously described (Daims *et al.*, 2005). The applied probes and their target organisms are listed in Supplementary Table S1. Labeled probes and unlabeled competitor oligonucleotides were obtained from Thermo Hybaid and Biomers (both in Ulm, Germany).

Microscopic images of FISH-labeled cells from the flume experiment were acquired using a confocal laser scanning microscope (LSM 510 Meta, Zeiss, Germany) equipped with two HeNe lasers (543 and 633 nm, respectively) and one argon laser (458, 477, 488, and 514 nm). The biovolume fractions of the different AOB and NOB populations were quantified, relative to the total biovolume of all bacteria stained by the EUB338 probe mix (Amann *et al.*, 1990; Daims *et al.*, 1999) by using the software *daime* (Daims *et al.*, 2006) as described by Daims *et al.* (2005). The congruency between the respective specific probe signal and the EUB-mix signal was mostly > 99%, but at least 98%. Quantification by FISH of nitrifiers in the streambed biofilms was not feasible due to a strong autofluorescent background, which apparently was caused by mineral particles.

Table 1. Biovolume fractions of AOB and NOB measured in activated sludge and, after 24 days of incubation, in biofilms from flumes inoculated with the same activated sludge. Experiments were run in duplicates. The experimental setup is illustrated in Fig. 1

	Biovolume fraction (% of EUBI-III)		
Population (FISH probe)	Activated sludge (inoculum) Sample A/B	T_{end} (24 days) NO_2^- flumes Flume 7/8	T_{end} (24 days) NH_4^+ flumes Flume 3/4
Nitrosomonas oligotropha/ureae (Nso192)	0.2/0.2	0	9.4/8.7
Nitrosomonas europaea/eutropha (NEU)	< 0.1/< 0.1	0	0
Nitrospira sublineage I (Ntspa1431)	2.7/2.2	1.0/3.0	9.4/7.8
Nitrospira sublineage II (Ntspa1151)	2.8/4.3	2.1/3.7	3/1.9
Other AOB/NOB (various probes, see Table S1)	0	0	0

amoA gene libraries and phylogenetic analysis

DNA was extracted from formaldehyde-fixed activated sludge from the Santa Maria de Palautordera WWTP (summer 2008) and from La Tordera River biofilms (sampled at 850 m downstream of the WWTP outlet in summer 2008) using the MoBio Soil DNA isolation kit (MoBio, Carlsbad, USA). Betaproteobacterial *amoA* genes were PCR-amplified in three replicates per sample using the primers amoA_1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA 2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) (Rotthauwe *et al.*, 1997). The cycling conditions for PCRs were as follows: initial denaturation at 95 °C for 5 min; 30 cycles consisting of denaturation at 95 °C for 30 s, primer annealing at 53 °C for 30 s, and elongation at 72 °C for 60 s; followed by a final elongation step at 72 °C for 10 min. PCR amplicons were cloned and subsequently Sanger-sequenced at GATC Biotech (Konstanz, Germany). The *amoA* gene sequences were manually refined based on the electropherograms and were aligned and phylogenetically analyzed using the software ARB (Ludwig *et al.*, 2004). A subset of 392 *amoA* sequences (> 400 nucleotides each) were used for tree calculations that considered 455 positions of the *amoA* alignment. For enhanced phylogenetic resolution, the wobble positions were included in the calculations. Phylogenetic trees were computed by maximum-likelihood analysis (PHYLIP version 2.4.5) with a HKY substitution model and by neighbor-joining with the implementation in ARB and Jukes Cantor correction. Multifurcations were inserted manually where the two methods yielded different tree branching patterns. The *amoA* nucleotide sequence data from this study have been deposited at GenBank (accession no. KC193268 – KC193324).

Nitrification activity

To monitor dissolved inorganic nitrogen (DIN) concentrations in the flume experiment, water samples from each flume were collected at times 0, 24, and 48 h after every addition of ammonium or nitrite, respectively. To follow longitudinal changes in DIN concentrations along the WWTP-affected stream reach, we collected water samples at the thalweg of each sampling site in winter and summer. All samples were immediately filtered through precombusted glass-fiber filters (0.7-μm pore size), stored at 4 °C, and analyzed colorimetrically for ammonium, nitrite, and nitrate concentrations within 1 day.

In the flumes, the increase in the nitrate concentration during each 3-day recirculation cycle was used to calculate nitrification rates (h^{-1}). The rates were estimated as the slope of the linear regression between ln-transformed nitrate concentrations and time.

In the WWTP-affected stream, we estimated the longitudinal nitrification rate coefficient (k_{NIT}, m^{-1}) by fitting the longitudinal pattern of nitrate flux along the reach to the two-compartment nitrification model proposed by Bernhardt *et al.* (2002) using the Microsoft Excel Solver tool (Redmond, Washington, USA). The k_{NIT} was estimated for the summer and the winter samplings.

Results

Colonization of the flume microcosms by nitrifiers

During the 4 weeks of preincubation and regular feeding with lake water, mature freshwater biofilms (thickness *c.* 200 μm, not shown) developed on the ceramic coupons in the flume microcosms. After the inoculation with diluted activated sludge, a thin, loosely attached layer of sludge particles settled onto these biofilms (Fig. 1b), while after 2.5 days the particles were tightly bound to the initial biofilms.

To monitor the colonization and growth of allochthonous nitrifiers from activated sludge, we used FISH, confocal microscopy, and image analysis to measure the biovolume fractions of different AOB and NOB populations in the original activated sludge and in the inoculated biofilms. We tested an array of probes specific for AOB and NOB (Supporting Information Table S1) and detected four nitrifier populations (Fig. 2a, Table 1). In the original sludge, the *Nitrosomonas oligotropha/ureae* lineage was the dominant AOB population and accounted for 0.2% of the total bacterial biovolume (Table 2). After 24 days of incubation with lake water and ammonium, the biovolume fraction of this AOB lineage in the freshwater biofilms was as high as 9%, which is a 45 × increase compared to the inoculum. AOB from the *Nitrosomonas europaea/eutropha* lineage were rare in the activated sludge (biovolume fraction < 0.1%) and were not detected in the flume biofilms. No AOB were detected by FISH in the biofilms grown in the flumes supplied with nitrite.

Members of the genus *Nitrospira* were the only NOB detected in the activated sludge inoculum and in flume biofilms. *Nitrospira* sublineage I accounted for *c.* 2.5% of the total bacterial biovolume in the inoculum and increased 3–4 × during 24 days of incubation in the biofilms in the flumes supplied with ammonium (Table 2). The biovolume fraction of *Nitrospira* sublineage II was *c.* 3.5% in the inoculum, but in contrast to sublineage I, this population did not show significant relative increase during the incubation period of 24 days in the biofilms in the flumes supplied with ammonium (Table 1). In the flumes supplied with nitrite, the average biovolume fractions of both *Nitrospira* sublineages I and II remained similar to the inoculum (Table 2). No known nitrifiers were detected by FISH in the control flumes that received either ammonium or nitrite, but were not inoculated with activated sludge.

Nitrifiers in natural streambed biofilms affected by WWTP effluents

To check whether nitrifiers from a WWTP could colonize streambed biofilms in a natural setting, we analyzed the community composition of AOB and NOB in biofilms sampled from the shaded bottom side of benthic cobbles in the La Tordera stream at sites located upstream or downstream of a WWTP outlet. In the activated sludge of the WWTP, AOB of the *N. oligotropha/ureae* and *N. europaea/eutropha* lineages and NOB of the *Nitrospira* sublineages I and II were identified by FISH (Table 2). In streambed biofilms, AOB of the *Nitrosomonas oligotropha/ureae* lineage and NOB of *Nitrospira* sublineage I were detected by FISH throughout a distance of 25–850 m downstream of the WWTP outlet (Fig. 2b, Table 2). No difference in the distribution of these populations was observed, by nonquantitative FISH, in summer and winter (Table 2). AOB of the *N. europaea/eutropha* lineage were found only within 25–50 m downstream of the WWTP outlet in winter, but not in summer (Table 2). NOB of *Nitrospira* sublineage II were not detected in any streambed biofilm sample, although this lineage was present in the WWTP (Table 2). No nitrifiers at all were found by FISH in biofilm sampled upstream of the WWTP outlet in winter (summer data are not available for this sampling site, because the stream was dry upstream of the WWTP).

In addition to the FISH analyses, we compared the sequences of *amoA* genes that were retrieved from the WWTP to *amoA* sequences obtained from streambed biofilm sampled 850 m downstream of the WWTP outlet in summer. In total, 82 *amoA* sequences (38 from the WWTP and 44 from streambed biofilm) were analyzed. Consistent with the FISH results, most of these sequences fell into the *N. oligotropha/ureae* lineage, where they formed five stable phylogenetic subclusters (Nso_1-5, Fig. 3a) with a lowest overall nucleic acid sequence identity of 81%. Interestingly, six completely identical *amoA* sequence types from the *N. oligotropha/ureae* lineage (at least one from each subcluster) were retrieved from the WWTP and the streambed biofilm (Fig. 3a). We also recovered *amoA* sequences that grouped with the *N. communis* and the *N. europaea/eutropha* lineages (subcluster Nso_6, Fig. 3a) and shared one identical sequence type between the sludge and the streambed biofilm. Hence, in total, seven identical *amoA* sequence types were found in

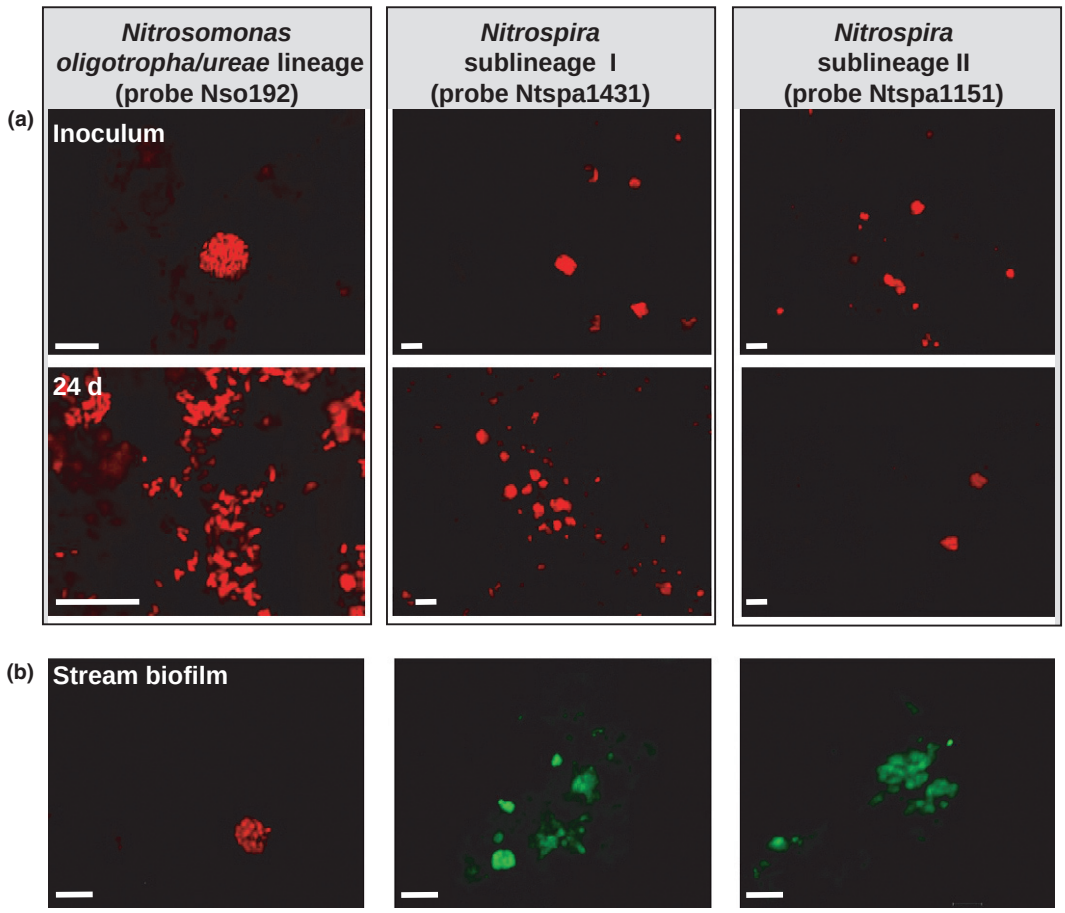

Fig. 2. (a) Upper panels: FISH images (signals in red) of activated sludge (from the Ingolstadt WWTP) that was used as inoculum in the flume experiments. Lower panels: FISH images taken after 24 days of incubation showing biofilms from flumes that were inoculated with activated sludge and supplied with ammonia. Left: probe Nso192 (*Nitrosomonas oligotropha/ureae* lineage). Center: probe Ntspa1431 (*Nitrospira* sublineage I). Right: probe Ntspa1151 (*Nitrospira* sublineage II). Scale bar = 10 μm. (b) FISH images (signals in red or green) of natural streambed biofilms sampled in winter 2008. Left: *Nitrosomonas oligotropha/ureae lineage* (probe Nso192) detected 650 m downstream of the WWTP outlet; center: *Nitrospira* sublineage I (probe Ntspa1431) detected 50 m downstream of the WWTP outlet; right: *Nitrospira* sublineage I (probe Ntspa1431) detected 650 m downstream of the WWTP outlet. Scale bar = 5 μm.

Table 2. Detection by FISH of AOB and NOB in activated sludge and natural streambed biofilms

	Presence/absence of populations				
			Distance downstream of WWTP outlet (winter/summer 2008)		
Population (FISH probe)	Sludge (summer 2008)	Upstream of WWTP outlet (winter 2008)	25/50 m	400 m	650/850 m
Nitrosomonas oligotropha/ureae (Nso192)	+	–	+/+	+/+	+/+
Nitrosomonas europaea/eutropha (NEU)	+	–	+/–	–/–	–/–
Nitrospira sublineage I (Ntspa1431)	+	–	+/+	+/+	+/+
Nitrospira sublineage II (Ntspa1151)	+	–	–/–	–/–	–/–
Other AOB/NOB (various probes, see Table S1)	–	–	–	–	–

the WWTP and the streambed biofilm, and this accounts for *c.* 18% of all detected sequence types ($n = 39$) in the different OTUs (Fig. 3b). Moreover, four additional sequence types found in both the WWTP and the biofilm differed only in a single nucleotide of 455 bp. This low level of sequence dissimilarity cannot be distinguished

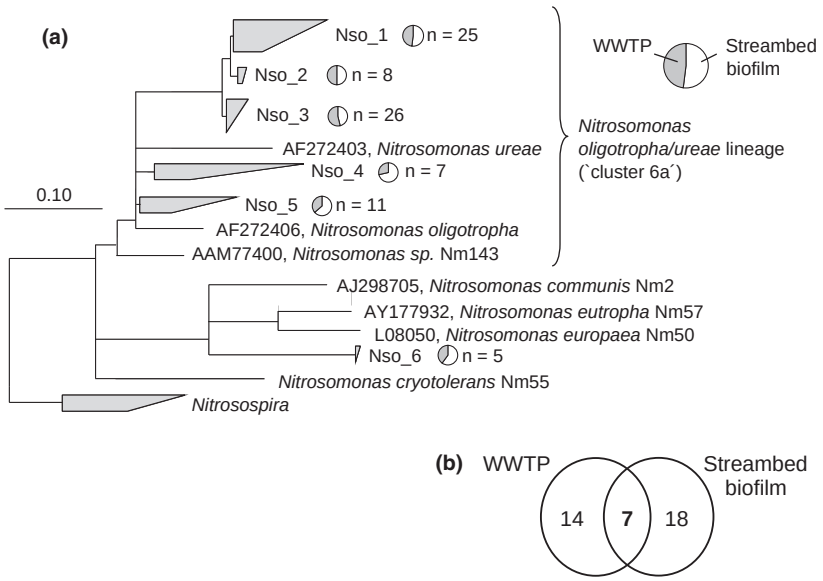

Fig. 3. (a) Phylogenetic analysis of *amoA* sequences recovered from activated sludge of the WWTP (sampled in summer 2008) and from streambed biofilms 850 m downstream of the WWTP outlet. A consensus tree was constructed based on PhyML and NJ trees. Total number of sequences per subcluster is given (n = x). Scale bar corresponds to 10% estimated sequence divergence. (b) Venn diagram showing the number of identical *amoA* sequence types shared between activated sludge and streambed biofilm.

from the error rate of PCR and Sanger sequencing (Keith *et al.*, 1993; Cline *et al.*, 1996; Ewing & Green, 1998; Richter *et al.*, 2008).

Nitrification activities in the flumes and the WWTP-affected stream

Chemical analyses of water samples confirmed active nitrification in the inoculated flumes and in the WWTP-affected stream (Figs 4 and 5). The flumes that had been inoculated with activated sludge and supplied with nitrite showed significantly higher nitrification rates than the control flumes, which had not been inoculated with sludge (Fig. 4). Indeed, the nitrite periodically added to the inoculated flumes was completely transformed to nitrate within 24 h (data not shown). The nitrification rates in these flumes were relatively constant during the whole experiment (Fig. 4). Similarly, the flumes that were inoculated with activated sludge and supplied with ammonium showed nitrification rates that were much higher than the rates measured in the sludge-free control flumes (Fig. 4). Consistent with the sensitivity of many known nitrifiers to low pH (Focht & Verstraete, 1977), buffering with bicarbonate stimulated nitrification in these flumes (Fig. 4). In all flumes, the observed increasing nitrate concentrations correlated with decreasing concentrations of the added ammonium or nitrite.

The WWTP-affected stream showed a high capacity to nitrify the ammonium input from the WWTP as indicated

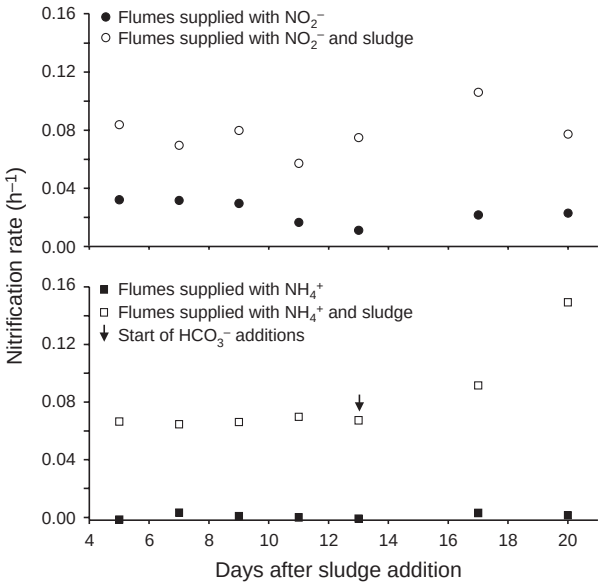

Fig. 4. Nitrification rates in the flumes calculated as NO_3^- production over time in flumes supplied with NO_2^- (upper panel) and flumes supplied with NH_4^+ (lower panel). Black dots and squares, control flumes not inoculated with sludge; white dots and squares, flumes inoculated with sludge. The arrow indicates the start of bicarbonate additions to buffer pH.

by the inverse longitudinal trends in ammonium and nitrate fluxes along the study reach (Fig. 5). However, the k_{NIT} values were higher in summer than in winter,

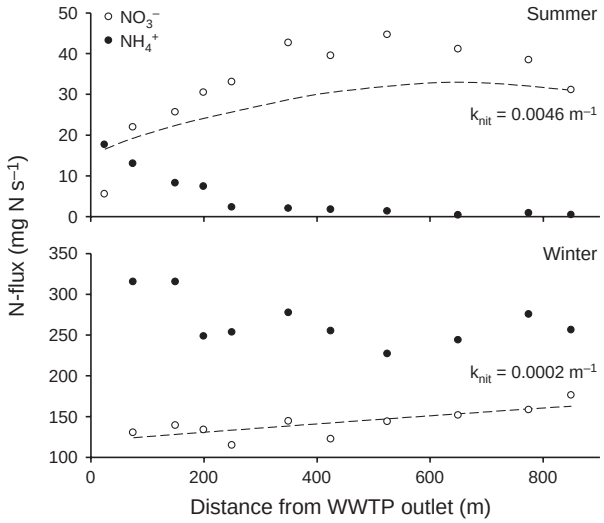

Fig. 5. Longitudinal profiles of observed NO_3^- (white dots) and NH_4^+ (black dots) fluxes along the study reach located below the WWTP outlet of Santa Maria de Palautordera River in summer (upper panel) and winter (lower panel). The modeled NO_3^- flux (dashed line) was used to estimate the longitudinal nitrification rate coefficient (k_{NIT}).

indicating that nitrification was more intense during the summer period (Fig. 5).

Discussion

In this study, we investigated the effects of WWTP discharges on nitrifier populations and nitrification activity in freshwater biofilms in artificial microcosms and in a natural, albeit WWTP-affected stream. In addition to molecular analyses of betaproteobacterial *amoA* genes, FISH was applied and confirmed that AOB and NOB in WWTPs and in WWTP-affected biofilms belonged to the same phylogenetic groups. The use of FISH is an important extension compared to earlier studies of WWTP-affected nitrifier communities (e.g. Cebron *et al.*, 2003, 2004; Nakamura *et al.*, 2006; Dang *et al.*, 2010), because this method unambiguously visualizes the microcolonies formed by the probe-targeted nitrifiers in the biofilms, and thus, it is not affected by possible biases of PCR-based techniques, which include the detection of nonpersistent nitrifier cells or naked DNA. Because a special focus of our study was on the possible biofilm colonization by allochthonous nitrifiers from WWTPs, we restricted our analyses to AOB and did not screen the samples for ammonia-oxidizing archaea (AOA). A previous extensive screening for AOA in municipal and industrial WWTPs had shown that AOA are not key nitrifiers in most engineered systems, including the Ingolstadt plant whose sludge was used as inoculum in our flume microcosm experiment (Mussmann *et al.*, 2011). However, AOA occur as autochthones in the stream investigated in our study (Merbt *et al.*, 2011) and probably also significantly contribute to the observed nitrification to a yet unknown extent.

Biofilm colonization by ammonia-oxidizing bacteria

Both *amoA* sequence analysis and FISH showed that AOB of the *N. oligotropha/ureae* lineage thrived in the examined freshwater biofilms. In the flume experiment, these AOB multiplied in inoculated biofilms that did previously not contain any AOB populations detectable by FISH. The *N. oligotropha/ureae* lineage is widely distributed in natural freshwater environments, but occurs also in activated sludge (Adamczyk *et al.*, 2003; Koops *et al.*, 2006). Cultured representatives from this group display the highest ammonia affinities among known AOB, which allow them to thrive in oligotrophic environments (Bollmann & Laanbroek, 2001; Koops & Pommerening-Röser, 2001). In contrast, the *N. europaea/eutropha* lineage that was also present in the activated sludge used as inoculum did not settle in the flume microcosms. It is well established that members of this lineage have a low affinity for ammonia and thus are restricted to eutrophic environments, where they grow relatively fast and can outcompete *N. oligotropha*-related AOB (Koops & Pommerening-Röser, 2001; Bollmann *et al.*, 2002). Considering that the flumes in our experiment were supplied with a relatively high concentration of ammonium (65 mg N-NH_4^+ per L), the predominance of *N. oligotropha*-related AOB in the microcosms is surprising. However, because local concentrations of DIN within biofilms may strongly differ from the surrounding conditions due to diffusion gradients and consumption by microorganisms (Okabe *et al.*, 1999; Gieseke *et al.*, 2005; Maixner *et al.*, 2006), lower ammonia concentrations in the biofilms than in the supplied water could have favored the *N. oligotropha/ureae* lineage. We also cannot exclude the possibility that yet uncharacterized representatives of the *N. oligotropha/ureae* lineage are adapted to elevated ammonium concentrations. This would be consistent with the previous observation that different ammonium concentrations in nitrifying bioreactors favored different *N. oligotropha*-like populations and that at least one of these organisms was adapted to relatively high levels of ammonium (Lydmark *et al.*, 2007). In addition, other factors such as temperature, oxygen levels, or salinity known to influence AOB communities (Bernhard *et al.*, 2005; Koops *et al.*, 2006; Laanbroek & Speksnijder, 2008; Lage *et al.*, 2010) might have selected for this group in our microcosm experiment.

The ammonium concentrations measured in the WWTP-affected stream were lower than in the flumes and ranged from 1.3 mg $N\text{-}NH_4^+$ per L (summer) to 4.3 mg $N\text{-}NH_4^+$ per L (winter). Consistently, only AOB of the *N. oligotropha/ureae* lineage were detected by FISH in the streambed biofilms and members of this group dominated the *amoA* gene library (Fig. 3a). Although *amoA* genes from *N. europaea-/eutropha-* or *N. communis*-like AOB were retrieved from these biofilms (Fig. 3a), the cell density of these AOB did not exceed the detection limit of FISH (10^3–10^4 cells per mL; Amann, 1995). Interestingly, seven identical *amoA* sequence types were found in the WWTP and the streambed biofilms (Fig. 3b). Considering that these sequences did not differ in a single nucleotide and that *amoA* provides a higher phylogenetic resolution than 16S rRNA gene (Purkhold *et al.*, 2000), this result suggests that at least some of the AOB strains in the biofilm originated from the WWTP. However, as no genome sequences from multiple AOB strains sharing identical *amoA* genes have been published so far, we cannot exclude that AOB with the same *amoA* genes might differ in other genomic regions.

Biofilm colonization by nitrite-oxidizing bacteria

As revealed by FISH, *Nitrospira* from activated sludge were able to colonize freshwater biofilms in flume microcosms (Table 1, Fig. 2a). Both *Nitrospira* sublineages I and II were detected in all flumes supplied with either nitrite or ammonium, but a strong increase in the biovolume fraction was observed only for sublineage I and only in the flumes supplied with ammonium (Table 1). Past research demonstrated ecological niche partitioning of these two *Nitrospira* sublineages with respect to nitrite concentrations, where sublineage I prefers higher levels of nitrite than sublineage II (Maixner *et al.*, 2006). Moreover, the local nitrite concentration within biofilms likely is highest in the close vicinity of AOB microcolonies (Maixner *et al.*, 2006). As the flumes received high ammonium concentrations (65 mg $N\text{-}NH_4^+$ per L), it appears possible that relatively high local nitrite concentrations occurred in the biofilm and favored sublineage I *Nitrospira*. In contrast, the lack of an increase in *Nitrospira* in the flumes supplied with nitrite is surprising. Here, we cannot exclude that other yet unknown NOB were present, which competed with *Nitrospira* for nitrite. The added nitrite concentration (5 mg $N\text{-}NO_2^-$ per L) was previously shown to support the growth of sublineage I *Nitrospira* in a long-term experiment (Maixner *et al.*, 2006). The results obtained here indicate that, at least under the conditions in the flume biofilms, other factors aside from nitrite may be important for efficient growth of these NOB. Because the addition of either ammonium or nitrite was the only difference in the treatment of the flumes, it appears that the presence of active AOB in the flumes supplied with ammonium had a strong positive influence on *Nitrospira*. It remains unknown whether AOB just provide nitrite at optimal rates or are involved in further molecular interactions with *Nitrospira*, but the answer would likely be important for our understanding of the interactions between nitrifiers in WWTPs and natural ecosystems.

Assuming that 0.5% of the total DIN is present as nitrite (Merseburger *et al.*, 2005; Ribot *et al.*, 2012), the estimated nitrite concentrations in the WWTP-affected stream were low and ranged from 0.018 mg $N\text{-}NO_2^-$ per L in summer to 0.3 mg $N\text{-}NO_2^-$ per L in winter. Concentrations in this range could have favored *Nitrospira* sublineage II (Maixner *et al.*, 2006), but we did not detect sublineage II by FISH in the streambed biofilms (Table 2), although these NOB were clearly present in the activated sludge in the WWTP. Instead, the only *Nitrospira* found *in situ* in the streambed biofilms belonged to sublineage I (Table 2). The reason might be that, in analogy to biofilms in nitrifying reactors (Schramm *et al.*, 2000; Maixner *et al.*, 2006), the nitrite concentrations were higher in the close vicinity of AOB than in the ambient stream waters and thus selected for sublineage I *Nitrospira*. However, attempts to explain the observed colonization patterns merely based on the nitrite concentrations may be too simplistic. Other factors including oxygen, certain organic substrates, temperature, and resistance to xenologous compounds such as chlorite may also influence the distribution of *Nitrospira* sublineages (Daims *et al.*, 2001; Maixner *et al.*, 2008; Lücker *et al.*, 2010; Off *et al.*, 2010; Lebedeva *et al.*, 2011). The colonization of the streambed biofilms by sublineage I *Nitrospira* is intriguing, because sublineage I has almost exclusively been found in WWTPs (Daims *et al.*, 2001) and has previously not been detected by FISH in natural ecosystems.

Nitrification activities in colonized biofilms

Nitrifying bacteria maintain a high cellular ribosome content during periods of starvation, and thus, even inactive nitrifiers can be detectable by FISH (Morgenroth *et al.*, 2000). However, the nitrification rates measured in the inoculated flumes were clearly higher than in the control microcosms free of sludge (Fig. 4). Likewise, remarkable longitudinal patterns of ammonium and nitrate fluxes were detected along the 850 m downstream of the WWTP outlet in the natural stream. Previously observed longitudinal changes in the ^{15}N isotopic signature of ammonium and nitrate in the same reach of this stream provide further support that changes in DIN concentrations are mostly

driven by nitrification (Ribot *et al.*, 2012). These results show that the nitrifiers not only were present in the different biofilms, but also were metabolically highly active and influenced nitrogen cycling in the flumes and in the stream ecosystem.

Conclusions

The results of this study strongly suggest that WWTP discharges influence nitrification activity and nitrifier communities in biofilms in the receiving waters. Such impact may be the colonization by allochthonous nitrifiers released from WWTPs or the stimulation of autochthonous nitrifier populations by the additional nitrogen. The presence of AOB with identical *amoA* genes in a WWTP and in downstream epilithic biofilms, as well as the presence in these biofilms of sublineage I *Nitrospira* found so far only in WWTPs, suggests that at least some nitrifiers from WWTPs do successfully colonize natural freshwater biofilms. Even though the setup was artificial, the experiments with flume microcosms provide further evidence that such invasion is possible. The absence of *in situ* detectable bacterial nitrifiers upstream of the WWTP in the studied freshwater stream further indicates that the higher abundance of nitrifiers downstream was caused by the WWTP. In any case, it seems likely that both effects – invasion and stimulation – overlap and may be differently pronounced with increasing distance from a WWTP outlet. The ecological consequences of this influence in particular on archaeal nitrifiers present in this stream (Merbt *et al.*, 2011) remain to be elucidated, but our results confirm the hypothesis that WWTP-affected streams are hot spots of nitrification (Merseburger *et al.*, 2005). The enhanced activity and growth of nitrifiers caused by WWTPs also contribute to the recovery of streambed biofilms after disturbances such as floods (Merbt *et al.*, 2011). On the other hand, a high invasion pressure by allochthonous organisms may reduce the microbial diversity of receiving biofilm assemblages (Urakawa *et al.*, 2006).

Interestingly, among all the nitrifier clades detected in the two WWTPs, only the *N. oligotropha/ureae* lineage (AOB) and *Nitrospira* sublineage I (NOB) were detectable in both WWTP-affected freshwater biofilms. Substrate affinity and availability likely are key factors selecting for specific ecotypes of nitrifiers; however, other important selective forces especially in streambed biofilms may include flow regimes, light, sedimentology, and biofilm architecture (Battin *et al.*, 2007; Augspurger *et al.*, 2010; Dang *et al.*, 2010; Merbt *et al.*, 2012). Disentangling the complexity of abiotic and biotic factors, which may determine the ecological success of invading microorganisms in multispecies biofilms, will be a challenging task for future research. Ammonia and nitrite oxidizers are clearly defined functional groups, whose members play key roles in the nitrogen cycle, display ecological niche partitioning, and are detectable in the environment by a battery of established molecular tools. Thus, nitrifiers in WWTP-affected habitats are well-suited model organisms for studying phenomena of microbial invasion and the consequences of anthropogenic impact for ecosystem functioning.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotide probes used in this study.