Ammonium Availability Affects the Ratio of Ammonia-Oxidizing Bacteria to Ammonia-Oxidizing Archaea in Simulated Creek Ecosystems^{∇†}

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The ammonia-oxidizing microbial community colonizing clay tiles in flow channels changed in favor of ammonia-oxidizing bacteria during a 12-week incubation period even at originally high ratios of ammonia-oxidizing archaea to ammonia-oxidizing bacteria (AOB). AOB predominance was established more rapidly in flow channels incubated at 350 μM NH_4^+ than in those incubated at 50 or 20 μM NH_4^+ . Biofilm-associated potential nitrification activity was first detected after 28 days and was positively correlated with bacterial but not archaeal *amoA* gene copy numbers.

Nitrogen transformation processes in small-river ecosystems are primarily associated with the sediment or epilithic biofilms (4, 5, 18). Here, nitrification as the first step in coupled nitrification-denitrification is a key step in the removal of the allochthonous nitrogen load, preventing the eutrophication of downstream ecosystems (1, 5, 29, 39). The activity and community composition of biofilm-associated nitrifying microorganisms have been investigated extensively in wastewater treatment systems (27, 36), in drinking water distribution systems (17, 41), and in lakes, large rivers, and estuaries (7, 19, 21, 39) but only rarely in small-creek ecosystems. The first and rate-limiting step in nitrification, oxidation of ammonia, is carried out by ammonia-oxidizing archaea (AOA) (14) and ammonia-oxidizing bacteria (AOB) (16). Molecular surveys targeting the *amoA* gene, which encodes catalytic subunit A of ammonia monooxygenase, the key enzyme of ammonia oxidation (33), as well as cultivation-based studies, indicated that pH (26), salinity (25, 34), and especially ammonium availability (6, 22) could play important roles in the ecological niche differentiation of AOA and AOB. However, only little is known about archaeal versus bacterial ammonia oxidizers in creek or river ecosystems or about the association of AOA with biofilms (37, 43). In this study, we used flow channels (FC) as simulated creek ecosystems to investigate the influence of ammonium availability (i) on the establishment of biofilm-associated nitrification activity, (ii) on the community composition of biofilm-associated AOA

and AOB, and (iii) on temporal patterns of the abundance of AOA and AOB during a 12-week incubation period.

FC experiment. Water for FC was obtained from three shell limestone creeks (Ammerbach, Leutra South, and Leutra North) located near the city of Jena (Thuringia, Germany). The creeks differed in their nutrient concentrations, with the highest nutrient load at Ammerbach resulting from diffuse wastewater inputs (Table 1). Flow velocities ranged from 0.105 ± 0.092 to 0.230 ± 0.224 m s^{-1} . Plexiglas FC measuring 160 by 10 by 18 cm (see Fig. S1 in the supplemental material) were set up in triplicate for each creek. Concentrations of NH_4^+ in the water phase of the FC were adjusted to 350 $\mu\text{mol liter}^{-1}$ (Ammerbach; FC 1 to 3), 50 $\mu\text{mol liter}^{-1}$ (Leutra South, FC 4 to 6), and 20 $\mu\text{mol liter}^{-1}$ (Leutra North, FC 7 to 9) to run the experiment at three distinct levels of NH_4^+ . pHs and concentrations of NH_4^+ were monitored during the experiment (see Fig. S2 in the supplemental material). Clay tiles measuring 4.7 by 4.7 cm were used to provide surfaces for biofilm development. The FC were run at 13°C in the dark at a circulation velocity of 0.3 m s^{-1} for 12 weeks, starting on 8 August 2006. A period of 12 weeks was considered representative of biofilm development under natural conditions, where colonization of new substrata occurs rapidly (30) and, in turn, high flow velocities, i.e., during flood events, may cause significant removal of epilithic biofilms (23), thus keeping them in early developmental stages. The water in the FC was replaced by freshly sampled water from the corresponding creeks on days 1, 15, 30, 51, and 72 of the experiment (see Fig. S2 in the supplemental material). Chemical analysis of the water (9, 42) was performed at each sampling time. Due to leakage problems, FC 2 and 9 had to be terminated at an early stage of the experiment.

PNA. Duplicate clay tiles from each FC were incubated with phosphate buffer (0.001 M, pH 7.4) and 0.5 mM NH_4^+ at 13°C with constant agitation (100 rpm) for 8 h, and rates of potential nitrification activity (PNA) were determined by linear regression of the cumulative increase in NO_2^- and NO_3^- concentrations over time. PNA remained at a very low level in the FC run at low ammonium concentrations, with maximum rates of 2.5 to 4.5 $\text{nmol NO}_2^- + \text{NO}_3^- \text{ cm}^{-2} \text{ h}^{-1}$ (Leutra South) and

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TABLE 1. Hydrological and physicochemical characteristics of the three creeks in this study^a

Site	Width (m)	Depth (m)	pH	Concn ($\mu\text{mol liter}^{-1}$) of:			
				NH_4^+	NH_3	NO_3^-	PO_4^{3-}
Ammerbach	1.8	0.24 ± 0.05	7.8 ± 0.3	674.4 ± 583.5	16.94 ± 14.66	$1,028 \pm 219$	35.6 ± 13.3
Leutra South	2.4	0.21 ± 0.03	8.3 ± 0.3	14.7 ± 6.7	0.79 ± 0.36	817 ± 88.0	0.8 ± 0.5
Leutra North	2	0.18 ± 0.04	7.8 ± 0.1	7.4 ± 5.4	0.13 ± 0.09	616 ± 59.7	3.0 ± 2.9

^a Values are mean results $\pm$ standard deviations obtained at five sampling times (except for width).

$0.9 \text{ nmol NO}_2^- + \text{NO}_3^- \text{ cm}^{-2} \text{ h}^{-1}$ (Leutra North) after 83 days of incubation (Fig. 1). In contrast, PNA was $28.1 \text{ nmol NO}_2^- + \text{NO}_3^- \text{ cm}^{-2} \text{ h}^{-1}$ already after 55 days of incubation at $350 \mu\text{mol liter}^{-1} \text{ NH}_4^+$ (Fig. 1), indicating that ammonium availability had a stimulating effect on both the establishment and the intensity of biofilm-associated nitrification activity, as previously reported (3). Maximum rates were similar to activities measured at the oxic/anoxic interface of river sediments (18 to $45 \text{ nmol cm}^{-2} \text{ h}^{-1}$) (7) but in the lower range of activities reported from river and estuary biofilms (10 to $171 \text{ nmol cm}^{-2} \text{ h}^{-1}$) (13, 19, 20, 38).

Community composition and abundance of ammonia oxidizers. Water samples (500 ml) for molecular analysis were obtained from the FC on days 58, 65, and 72 of the experiment before the water was exchanged and from the creeks on days 30, 51, and 72 (see Fig. S2 in the supplemental material) and filtered through $0.2\text{-}\mu\text{m}$ cellulose acetate filters (Sartorius AG, Goettingen, Germany). Biofilm was sampled from clay tiles of each FC on days 37, 49, and 85 (FC 5, day 71; see Fig. S2 in the supplemental material) by scraping off the biofilm into 50 ml of sterile double-distilled water, followed by filtration over cellulose acetate filters. DNA was extracted from the filters by phenol-chloroform extraction as described elsewhere (24).

Archaeal and bacterial *amoA* genes were amplified from the creek water and from water phase and biofilm samples from all FC and time points (see supplemental material). Analysis of the PCR products by denaturing gradient gel electrophoresis suggested a clear effect of ammonium availability on the community composition of AOA and AOB but only small differences between water phase- and biofilm-associated communi-

ties (see Fig. S3 in the supplemental material) or between different sampling times (data not shown). For a more detailed analysis of the biofilm-associated communities, three clone libraries for AOA *amoA* (Abf-AOA, LSbf-AOA, and LNbf-AOA) or AOB *amoA* (Abf-AOB, LSbf-AOB, and LNbf-AOB) were generated (see supplemental material).

The majority of the archaeal *AmoA* sequences affiliated with the “soil and other environments” cluster as proposed by Francis et al. (8) and Prosser and Nicol (31), while only a few sequences affiliated with the “marine water and other environments” cluster (31) (see Fig. S4 in the supplemental material). Here, sequences formed a cluster together with sequences obtained from groundwater (32) and from a drinking water distribution system (41), which may point to a special affiliation of this group with freshwater environments. However, we did not detect novel AOA *AmoA* clusters that would indicate the existence of special creek-associated AOA. Bacterial *AmoA* sequences obtained from the biofilms of the Leutra South and Leutra North FC were affiliated exclusively with *Nitrosospira*-related sequences (see Fig. S5 in the supplemental material), while about half of the clones from the Ammerbach FC (19 out of 34) were affiliated with the *Nitrosomonas europaea* lineage (see Fig. S5 in the supplemental material). These findings agree well with the previously reported adaptation of members of this lineage to high NH_4^+ concentrations (2, 15), while members of the *Nitrosospira* clusters are often better competitors in low-substrate environments (35).

Copy numbers of bacterial and archaeal *amoA* genes in association with the water phase and the biofilms of the FC

FIG. 1. Biofilm-associated PNA ($\text{nmol NO}_3^- + \text{NO}_2^- \text{ cm}^{-2} \text{ h}^{-1}$) of the different FC with ongoing incubation. FC 1 and 3, Ammerbach; FC 4 to 6, Leutra South; FC 7 and 8, Leutra North.

TABLE 2. Correlation^a between AOB *amoA* and AOA *amoA* gene copy numbers or AOB *amoA*/AOA *amoA* gene ratios and concentrations of NH_4^+ or calculated concentrations of NH_3 in original creek water^b or biofilm-associated PNA in FC^c

Parameter	Correlation with creek water characteristic:		Correlation with biofilm-associated PNA in FC
	NH_4^+ concn ($\mu\text{mol liter}^{-1}$)	Calculated NH_3 concn ($\mu\text{mol liter}^{-1}$)	
AOA <i>amoA</i> gene copy no.	0.87 ^e	0.92 ^d	0.43
AOB <i>amoA</i> gene copy no.	0.94 ^d	0.96 ^d	0.72 ^d
AOB <i>amoA</i> /AOA <i>amoA</i> gene ratio	0.87 ^e	0.87 ^e	0.70 ^d

^a Spearman rank correlation coefficient.

^b $n = 9$.

^c $n = 21$.

^d $P \leq 0.001$.

^e $P \leq 0.01$.

FIG. 2. AOB *amoA* and AOA *amoA* gene copy numbers per liter in the water phase (A) or per cm^{-2} in the biofilm-associated communities (B) of FC. Field samples (creek water from Ammerbach, Leutra South, and Leutra North) were obtained on days 30, 51, and 72 of the experiment. The FC water phase was sampled on days 58, 65, and 72 of the experiment (7, 14, and 21 days after replacement of the FC water with freshly sampled creek water). Biofilm samples were obtained after 37, 49, and 85 days of incubation (FC 5, last sampling time after 71 days). Columns represent means and standard deviations of triplicate qPCR measurements. The water phase sample from FC 1 after 65 days was excluded from quantitative analysis because the yield of environmental DNA was too low to obtain reliable results. Archaeal and bacterial *amoA* gene copy numbers were below the detection limit of the qPCR for the FC 7 biofilm sample after 85 days of incubation.

were determined by quantitative PCR (qPCR) using primers Arch-AmoAF/Arch-AmoAR (8) and AmoA-1F/AmoA-2R (33), respectively, with SensiMix SYBR Low-ROX Mastermix (Quantace) on an Mx3000P instrument (Agilent) with cycling conditions as described previously (10) and in the supplemental material. Numbers of archaeal and bacterial *amoA* gene copies, as well as AOB *amoA*/AOA *amoA* gene ratios, in the original creek water were positively correlated with the ammonium concentrations and the calculated concentrations of free NH_3 in the water phase (Table 2), suggesting a positive effect of substrate availability on the population size of ammonia oxidizers (28) and an increasing importance of AOB under conditions of higher substrate availability. The observed numerical predominance of AOA at low ammonium concentrations (Leutra South and Leutra North field samples, Fig. 2A) agrees with previous findings on freshwater sediments or soils (6, 11, 12) and is likely to be based on an extremely high substrate affinity of archaeal ammonia oxidizers (22, 40).

AOB *amoA*/AOA *amoA* gene ratios changed in favor of AOB during the 12-week incubation period in both the water phase- and biofilm-associated communities of all FC. In the water phase, the AOB *amoA*/AOA *amoA* ratios increased by more than 2 orders of magnitude within only 3 weeks (Fig. 2A). In the biofilm-associated communities, AOB *amoA* genes outnumbered AOA *amoA* genes by more than 1 order of magnitude after 49 or 85 days of incubation at 50 or 20 $\mu\text{mol liter}^{-1}$ NH_4^+ (Leutra South, Leutra North). In contrast, a clear predominance of AOB was already established after 37 days of incubation at 350 $\mu\text{mol liter}^{-1}$ NH_4^+ (Ammerbach, Fig. 2B), probably due to higher growth rates of AOB at increased NH_4^+ concentrations. Interestingly, a predominance of AOB was established in all of the FC even though the FC water was replaced with freshly sampled creek water five times during the incubation period, each time reintroducing ammonia-oxidizing populations where AOA were about equally abundant or more numerous than AOB.

Biofilm-associated PNA was positively correlated with AOB *amoA*/AOA *amoA* gene ratios and with the numbers of bacterial *amoA* gene copies but not with archaeal *amoA* gene copies (Table 2), suggesting that the enrichment of AOB in the biofilm-associated communities contributed to the observed increase in nitrification potential with ongoing incubation. High ammonium concentrations, such as in the Ammerbach FC, a constant supply of ammonium, avoiding periods of NH_4^+ deficiency, compared to field conditions obviously favored bacterial over archaeal ammonia oxidizers under the experimental conditions of this study. Similarly, a stimulating effect of substrate availability on the growth of AOB but not AOA has recently been observed in grassland soil mesocosms incubated with animal urine (6). The results obtained in our study provide clear experimental evidence that ammonium availability is an important factor influencing AOB/AOA ratios in aquatic ecosystems.

Nucleotide sequence accession numbers. Sequences obtained in this study have been deposited in GenBank under accession numbers HQ401373 to HQ401474 (archaeal *amoA*) and HQ401475 to HQ401562 (bacterial *amoA*).

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