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Nitrification in a Biofilm at Low pH Values: Role of In Situ Microenvironments and Acid Tolerance

Armin Gieseke,[1]* Sheldon Tarre,[2] Michal Green,[2] and Dirk de Beer[1]

Microsensor Group, Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany,[1] and Faculty of Civil and Environmental Engineering, Technion—Israel Institute of Technology, Technion City, Haifa 32000, Israel[2]

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The sensitivity of nitrifying bacteria to acidic conditions is a well-known phenomenon and generally attributed to the lack and/or toxicity of substrates (NH_3 and HNO_2) with decreasing pHs. In contrast, we observed strong nitrification at a pH around 4 in biofilms grown on chalk particles and investigated the following hypotheses: the presence of less acidic microenvironments and/or the existence of acid-tolerant nitrifiers. Microelectrode measurements (in situ and under various experimental conditions) showed no evidence of a neutral microenvironment, either within the highly active biofilm colonizing the chalk surface or within a control biofilm grown on a nonbuffering (i.e., sintered glass) surface under acidic pH. A 16S rRNA approach (clone libraries and fluorescence in situ hybridizations) did not reveal uncommon nitrifying (potentially acid-tolerant) strains. Instead, we found a strongly acidic microenvironment, evidence for a clear adaptation to the low pH in situ, and the presence of nitrifying populations related to subgroups with low K_ms for ammonia (*Nitrosopira* spp., *Nitrosomonas oligotropha*, and *Nitrospira* spp.). Acid-consuming (chalk dissolution) and acid-producing (ammonia oxidation) processes are equilibrated on a low-pH steady state that is controlled by mass transfer limitation through the biofilm. Strong affinity to ammonia and possibly the expression of additional functions, e.g., ammonium transporters, are adaptations that allow nitrifiers to cope with acidic conditions in biofilms and other habitats.

Chemolithoautotrophic nitrifying bacteria, i.e., ammonia-oxidizing bacteria (AOB), catalyzing the first oxidation step of ammonia to nitrite and nitrite-oxidizing bacteria (NOB) completing the oxidation of the intermediate nitrite to nitrate are known to be sensitive to low pHs. Optimum growth occurs under neutral to moderately alkaline conditions (pH 7.5 to 8.0). In liquid pure culture, growth is usually restricted to a lower pH of 5.8 (AOB) or 6.5 (NOB) (62) and activity ceases typically below pH 5.5 (28, 31). The failure of AOB to cope with acidic conditions is thought to be primarily based on the unavailability of a substrate: with decreasing pHs, ammonia, the substrate of AOB (58), is increasingly protonated. Nitrite, the substrate of NOB, undergoes protonation to nitric acid, which disproportionates to nitrate and gaseous nitric oxide at low pHs (6). Furthermore, when present at elevated concentrations under low pHs, free nitric acid negatively affects the growth and activity of nitrifying bacteria (4).

Despite these limitations, autotrophic nitrifying bacteria have been isolated from, or nitrifying activity has been demonstrated in, acidic environments, such as soils, activated sludge, and biofilms. Numerous nitrifying isolates have been obtained from soils with pHs around 4 (for a review, see reference 18 and references therein) to even as low as 2.5 (45). However, the majority of such isolates do not show nitrifying activity in acidic mineral medium (18). In contrast, autotrophic nitrifying activity in soil samples could be maintained at a pH as low as 4 (18). The inhibition of nitrification is commonly observed in activated sludge and dedicated suspended nitrifying biomass systems below pH 6 (41). Exceptions down to a bulk pH below 5, however, have been reported for highly nitrogenous wastes (poultry manure) and when using suspended marble particles (37, 44). In biofilm-based systems, stable but low rates of nitrification could be achieved at pH 5 or below (2, 17, 33). More recently, a high rate of nitrification using chalk particles as the biofilm carrier was demonstrated with an average pH of 5 (24) or even as low as pH 3.8 using sintered glass particles as the carrier and in suspended systems (60).

The prevailing major mechanisms explaining nitrifying activity at low pHs are the use of urea as an alternative substrate (1, 9, 18, 43); the restriction of activity and growth to microsites with pHs conducive to nitrification (18, 37, 45, 50) (such microniches are hypothesized to originate from coexistence with ammonifying microorganisms that increase the pHs via alkaline reactions of excreted ammonia [for a review, see reference 18 and references therein] and/or from existing locally buffering microparticles/surfaces like calcium carbonate [27, 37]); and the presence of acid-tolerant or even acidophilic nitrifying bacteria (for a review, see reference 18 and references therein). The hydrolysis and use of urea by AOB has been investigated in detail, in particular in the field of soil microbiology (18). Much less, however, is known about the role of the other two hypothetical mechanisms. Conducive microsites have been suggested frequently but have been investigated only scarcely and by indirect means. With the exception of a few strains of AOB (17, 29) and one NOB strain (28), no isolates exist so far to provide direct support for the hypothesis of acid tolerance in nitrifying bacteria.

In situ methodology is suitable for obtaining evidence for the conducive microenvironment and the acid tolerance hypotheses. Microsensors are adequate methods for the characterization of microenvironments in immobilized microbial biomass

* Corresponding author. Mailing address: Microsensor Group, Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany. Phone: 49-(0)421-2028-836. Fax: 49-(0)421-2028-690. E-mail: agieseke@mpi-bremen.de.

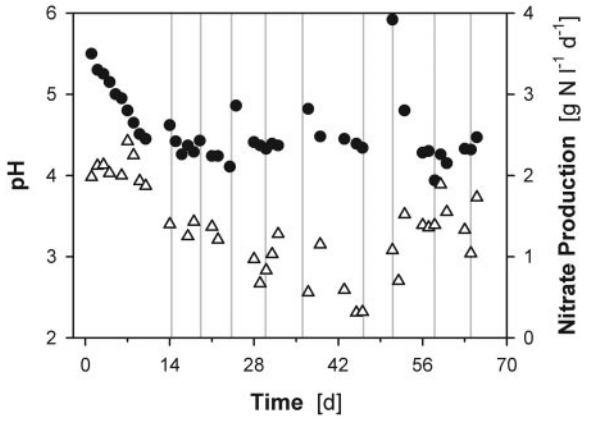

FIG. 1. Performance of the chalk reactor during the experimental period. Black circles, pH; white triangles, nitrate.

(14, 21, 49). Molecular tools based on 16S rRNA sequence analysis and fluorescence in situ hybridization (FISH) allow the diversity and structure of a microbial community (3) formed under acidic conditions to be revealed.

In this study, we applied these tools to investigate the microenvironment, local activity, and community structure of a highly active nitrifying biofilm grown under acidic conditions on chalk particles (24, 59). The following two hypotheses were addressed: the chalk surface, by concomitant dissolution, provides a microenvironment with a locally elevated pH conducive to nitrification, and the conditions in the system select for the presence of (unknown) acid-tolerant strains of nitrifying bacteria.

MATERIALS AND METHODS

Experimental reactor. A smaller version of a two-column fluidized-bed reactor system with chalk particles as a carrier material was operated as described earlier (59). The total volume of the system was 2 liters (column inside diameter, 34 mm), and the fluidized bed consisted of 800 g of chalk taken from a larger reactor with a similar operation (59). Oxygen and pH levels in the reactor medium were measured daily with conventional electrochemical sensors (Oxi 92; WTW, Weilheim, Germany, and MA130; Mettler-Toledo AG, Schwerzenbach, Switzerland). The nitrate concentration was determined from 0.2-μm filtered and diluted water samples by UV absorption (25) on a UV spectrometer (DU 530; Beckman, Fullerton, CA). The half-life of chalk (at a nitrification rate of 1 g N per liter per day) was around 45 days, and consumed chalk was replenished every 2 weeks. The reactor performance is shown in Fig. 1. Overall activity and growth parameters were comparable to those described earlier (59).

Experimental setup. The local microenvironment within the biofilm was investigated under reactor conditions and in a series of experimental incubations. A small flow chamber was supplied directly with water from the reactor or was connected to an external reservoir with 6 liters of artificial medium, respectively. Biofilm-covered particles were sampled from the reactor column daily through a lateral outlet, and single specimens were carefully placed in the flow chamber. Microfunnels (upper diameter, ~1 mm) made of glass were used as a support. The particles were positioned in the laminar flow field of the medium about 15 mm above the bottom. A flow of 10 mm s^{-1} was adjusted in the flow chamber.

The medium used for experimental incubations was composed of tap water with 0.2 mM KH_2PO_4 and 1 mM NH_4Cl and adjusted to the appropriate pH by the addition of acid. The reservoir was covered with hollow plastic spheres to prevent evaporation. Oxygen and pH in the reservoir were monitored with respective electrochemical sensors and regulated automatically with a controller system (Aquastar; IKS Computersysteme GmbH, Karlsbad, Germany) by the addition of 0.1 M hydrochloric acid and aeration with oxygen where necessary.

A series of incubations were performed ranging from pH 4 to 7.

Microsensor measurements. Local activities and microenvironments within the biofilm were investigated with microsensors for oxygen (48) and pH (13). Measurements were performed with a semiautomatic setup that consisted of a three-axis micromanipulator (MM 33; Märzhäuser, Wetzlar, Germany) mounted to a motor system (VT-80 100-BL; MICOS GmbH, Eschberg, Germany). Signals for oxygen and pH were measured with a high-precision picoammeter or a millivolt meter, respectively, and recorded with a data acquisition system (DAQCard-AI-16XE-50; National Instruments, Austin, TX). Sensor positioning and data acquisition were controlled via personal computer by custom-programmed software.

Rate calculations. Areal oxygen uptake rates were calculated from the linear concentration gradient through the diffusive boundary layer above the biofilm surface. A molecular diffusion coefficient of oxygen in water at 26°C of 2.4903×10^{-5} cm^2 s^{-1} was used (8). The Tukey-Kramer method was applied to test the significance of differences between oxygen uptake rates and pH gradients from different treatments (pH) at a significance level of 0.05.

Cloning and sequencing of 16S rRNA. Samples for molecular studies were obtained as described above. Tubes containing particles for 16S rRNA cloning and sequencing analysis were frozen immediately after sampling in liquid N_2 and stored at −80°C until further processing. Tubes containing about 30 ml of reactor suspension with particles and biomass were rigorously shaken, and the supernatant was decanted and centrifuged for 1 min at 4,200 × *g*. Total community DNA was extracted from the pellet of 0.2 g (wet weight) of biomass by use of an extraction kit (Fast DNA spin kit for soil; Bio101, Carlsbad, CA) according to the procedure described by the manufacturer. The two primer sets used for subsequent PCR amplification of 16S rRNA sequences from extracted chromosomal DNA were GM3 and GM4 (40), producing a 1,500-bp product, and βAMOf and βAMOr (46) that result in a ca. 1,180-bp product. A reaction mixture of 10 pmol of each primer, 50 nmol of each deoxyribonucleoside triphosphate, 6 μg of bovine serum albumin, 1× PCR buffer, 0.4 U of *Taq* DNA polymerase (Cambrex Corp., East Rutherford, NJ), and 10 to 15 ng of template DNA was adjusted to a final volume of 20 μl with sterile water. The following thermocycles were applied for both sets of primers: 1 cycle at 94°C for 4 min; 33 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min; and 1 final cycle at 94°C for 1 min, 60°C for 1 min, and 72°C for 10 min. Products were subsequently purified with a DNA purification kit (QIAquick PCR purification kit; QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Clone libraries were constructed by use of the pGEM-T-Easy (Promega, Madison, WI) or the TOPO pCR4 vector system (Invitrogen, Karlsruhe, Germany) and *Escherichia coli* TOP10 (Invitrogen, Karlsruhe, Germany) as described in the manufacturers' protocols. Plasmids were prepared by using a QIAprep plasmid kit (QIAGEN, Hilden, Germany), and inserts were screened by partial sequencing with the vector primer M13uni. Sequencing was performed by use of ABI BigDye 3.0 terminator cycle sequencing reagent on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences for distinct clones were obtained by use of primers M13uni, M13rev, and p1 (39).

Phylogenetic analysis. The 16S rRNA sequences were added to a published data set of sequences (spring 2001 release of the ARB project with additional sequences added individually) by use of the ARB software package (38). Sequences were aligned by use of the FastAligner tool with additional manual corrections and added to the global tree by a parsimony procedure. Tree construction for AOB and NOB was based on published sequences with lengths of >1,300 bp and >1,400 bp, respectively, and a maximum likelihood procedure ("FastDNAml") by use of the ARB software package. Sequences obtained in this study were added by a parsimony procedure without changing the tree topology.

Visualization of microorganisms. Particles for general DNA staining and FISH were fixed in fresh 4% paraformaldehyde for 3 h at 4°C, washed twice with phosphate-buffered saline, and stored in phosphate-buffered saline–ethanol (1:1, vol/vol) at −20°C until further processing. Biofilm adhering to chalk particles was stained with 4′-6-diamidine-2-phenyl indole (DAPI) (0.5 μg ml^{-1}) for 15 min at 4°C. For FISH, biofilm material was subjected to a procedure described earlier (23) with the probes listed in Table 1. Hybridization conditions were used as described earlier (20, 23). Samples were embedded in fluorescence-preserving mounting medium (VectaShield H-1000; Vector Laboratories, Burlingame, CA) and analyzed by epifluorescence microscopy or confocal laser scanning microscopy (Zeiss LSM 510; Carl Zeiss AG, Oberkochen, Germany).

Nucleotide sequence accession numbers. The 16S rRNA sequences retrieved in this study were deposited in the EMBL nucleotide sequence database under accession nos. AM182857 to AM182872.

RESULTS

Reactor operation. The reactor operation was similar to the performance described earlier for such systems (28) (Fig. 1). The pH of the medium stabilized around 4.3 ± 0.13 (mean ±

TABLE 1. Probe set used for the detection of AOB and NOB on chalk carrier and results

Probe	Target organism(s)	Result[a]
EUB338	Domain *Bacteria*	+
ALF968	Significant part of α-proteobacteria and other bacteria	−
BET42a	β-Proteobacteria	+
GAM42a	γ-Proteobacteria	−
Nso1225	Ammonia-oxidizing subgroup of β-proteobacteria	+
Nso190	Ammonia-oxidizing subgroup of β-proteobacteria	+
Nsv443	*Nitrosospira* spp.	+
NmII	*Nitrosomonas communis* lineage	−
NmIV	*Nitrosomonas cryotolerans* lineage	−
NEU	Halophilic and halotolerant species of the genus *Nitrosomonas*	+
Nse1472	*N. europaea*	○
NmV	*Nitrosococcus mobilis* lineage	−
NOLI191	Various species of *Nitrosomonas oligotropha* lineage	+
Nmo218	*Nitrosomonas oligotropha* lineage	+
NIT3	*Nitrobacter* spp.	−
NSR1156	Various *Nitrospira* spp.	+
Ntspa712	Phylum *Nitrospira*	+
Ntspa662	Genus *Nitrospira*	+

[a] +, positive (hybridized cells detected); −, negative (no hybridized cells detected); ○, few hybridized cells detected.

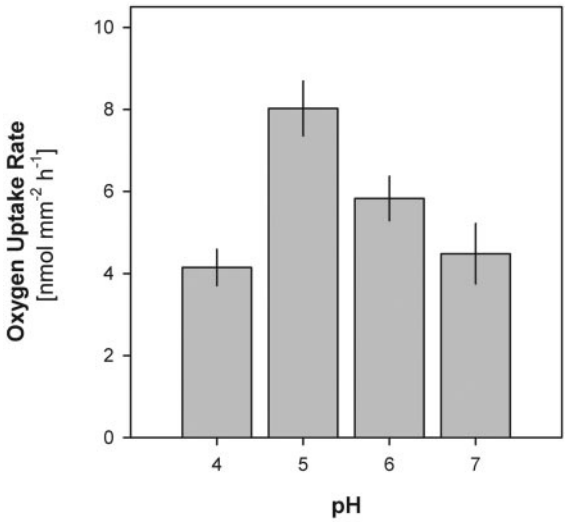

FIG. 3. Averaged areal oxygen uptake rates of biofilm-covered chalk particles incubated at pHs 4 ($n = 32$), 5 ($n = 27$), 6 ($n = 30$), and 7 ($n = 24$). Error bars indicate standard errors.

standard deviation; $n = 29$) between days 7 and 70 with a few peaks due to chalk addition. During most of the time, the nitrate production rate was around 1 g N per liter per day. Sampling for microsensor measurements and molecular analyses was done during the period of stable pH (day 7 to 70).

Microsensor experiments. (i) In situ measurements. A dissolution of chalk in an acidic medium leads to a local pH increase near the surface. This was observed when bare chalk particles were incubated in the reactor medium. The pH went up from about 4.6 to 4.9 towards the chalk surface (Fig. 2, left panel). In contrast, acidic conditions were measured on biofilm-covered surfaces: the pH at the biofilm base went down to about 4.2. Differences in acidification between smaller particles sampled from the top of the reactor column and larger ones from the bottom were insignificant.

Despite the limited thickness of biofilms formed on the chalk surface (75 to 125 μm), their activity was very high. Oxygen penetrated to the bottom but was consumed to a level of about one-third of air saturation (76 ± 39.6 μM; $n = 13$) (Fig. 2, right panel). The oxygen uptake rate under in situ conditions was 13 ± 5.8 nmol mm^{-2} h^{-1} ($n = 42$).

(ii) Incubation experiments. Particles from the reactor (at pH around 5) incubated at different pHs showed clear responses to the treatment, with highest activities at pHs close to in situ conditions. Areal oxygen uptake rates were highest at

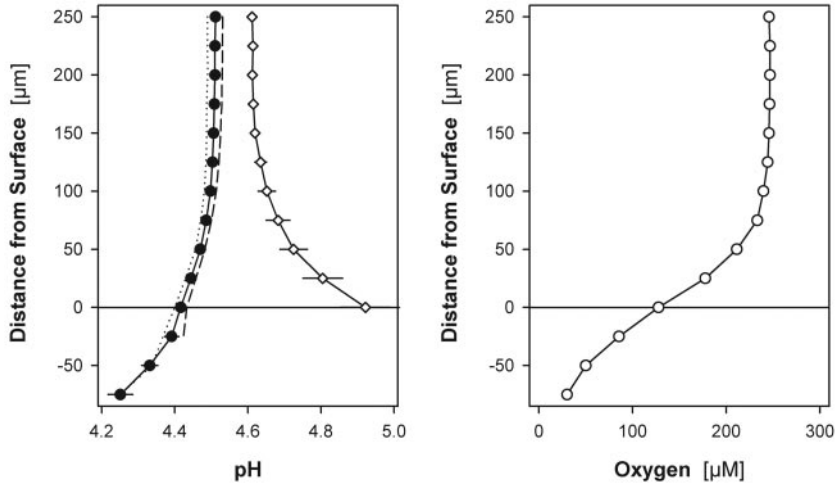

FIG. 2. Averaged microprofiles (pH/concentration versus depth) of pH (left panel; $n = 22$) and oxygen (right panel; $n = 13$) on biofilms grown on chalk particles under reactor conditions (see Fig. 1). The left panel shows data measured on both bare chalk surface (white diamonds) and biofilm-covered chalk particles (black circles). Subsets of data for biofilm-covered particles that originated from the top (dashed line; $n = 11$) and bottom (dotted line; $n = 11$) of the reactor column are shown separately. Error bars indicate standard errors.

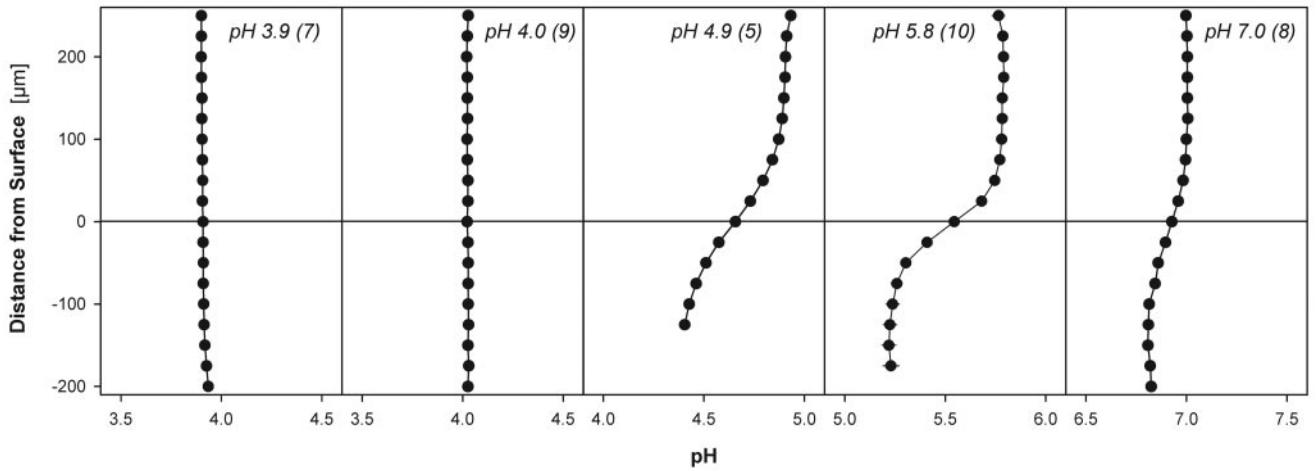

FIG. 4. Averaged pH profiles on biofilm-covered chalk particles (from the reactor running at a pH ~5) incubated at different pHs (the incubation pH and number of profiles are shown in italics). Error bars indicate standard errors.

pH 5 and significantly different from rates measured at all other incubations. Rates decreased towards both lower and higher pHs (Fig. 3). Comparable oxygen uptake rates occurred at pH 4, 6, and 7 (no significant differences). A similar response was found in acidification activity, illustrated by the pH difference between medium and biofilm base (Fig. 4). A strong change in pH as high as 0.6 units was obtained at incubation with a medium pH of 5.8 and 4.9 (no significant difference). Gradients were significantly lower at pH 4 and 3.9 (no significant difference) and at pH 7.

(iii) Biofilm on sintered glass beads. The profiles of oxygen and pH on a biofilm grown on nonbuffering carrier (sintered glass) revealed behaviors in situ (reactor conditions) similar to that of chalk-bound biofilm. High activity, however, was concentrated on individual thin patches of biofilm growing locally on the bead (Fig. 5).

16S rRNA cloning analysis. Clones retrieved from the chalk biofilm material affiliated to two groups within the β subclass of *Proteobacteria* (Fig. 6). One group of 12 clones showed high similarity to *Nitrosospira* cluster 3 (47). The closest described relative is *Nitrosospira multiformis* (*Nitrosolobus multiformis*), however, with a sequence similarity of less than 97%. Further, two clones affiliated to the *Nitrosomonas oligotropha* lineage and showed high similarity with *Nitrosomonas* sp. strain Nm47. The clones with similarity to the 16S rRNA sequence of NOB were grouped exclusively within the phylum *Nitrospira* (Fig. 7) with high similarity to sequences of the *Nitrospira* cluster II (12). *Nitrospira moscoviensis* is the only cultivated member of this cluster. *Nitrobacter*-associated sequences were not detected. The application of general primers produced further clones not affiliated to any of the functional guilds mentioned above. These encompassed various sequences related to *Rhodococcus* isolates (*Rhodococcus rhodochrous* strain 372, eight sequences) and to *Geothrix* isolates (*Geothrix fermentans* strain H5, three sequences).

Microscopic analysis. Analysis of particles from an early stage of the reactor operation showed numerous microcolonies (Fig. 8A) that occurred in small cavities at the surfaces of the

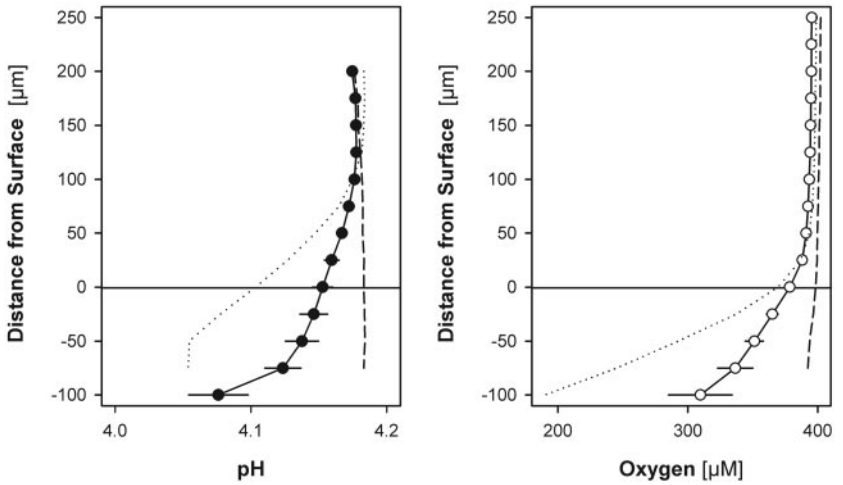

FIG. 5. Averaged microprofiles (pH/concentration versus depth) and extreme example profiles (dashed and dotted lines) of pH (left panel; $n = 16$) and oxygen (right panel; $n = 17$) on biofilms grown on sintered glass particles under reactor conditions. Error bars indicate standard errors.

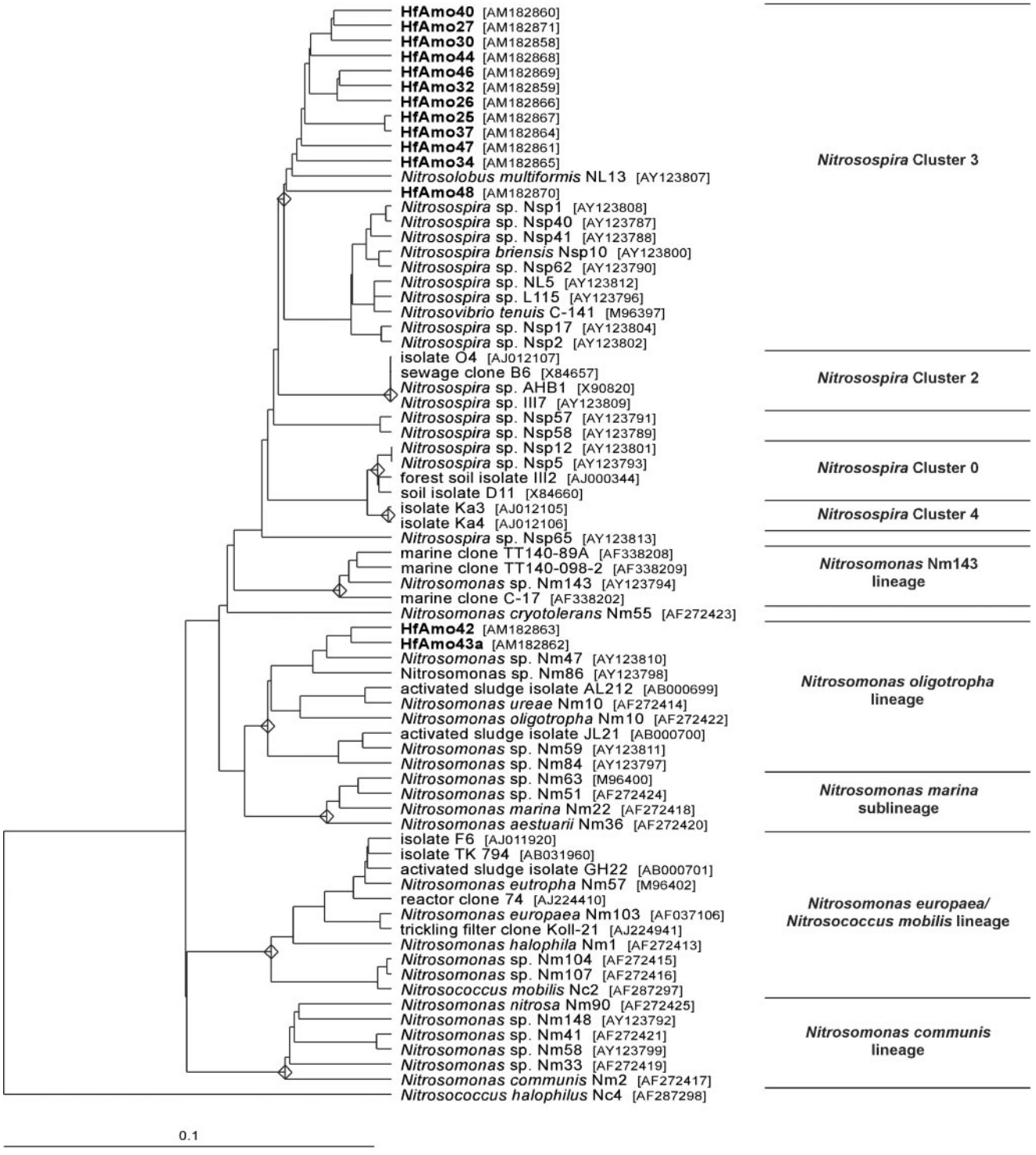

FIG. 6. Phylogenetic tree of AOB of the β subclass of *Proteobacteria* and affiliation of AOB-related clones retrieved from the low-pH chalk reactor (in bold). The tree calculation is based on published 16S rRNA sequences (>1,300 bp) and the maximum likelihood method (FastDNAml). Clones retrieved in this study have been added to the tree by the maximum parsimony method without changing the tree topology. Branches with diamond symbols refer to commonly accepted phylogenetic groups (47).

particles. Particles from a later stage that were used for the analysis described above showed a homogeneous biofilm (thickness, 75 to 125 μm) covering the surface. Eventually, particles were completely interfused with biofilm.

Fluorescence in situ hybridization of biofilm samples with various oligonucleotide probes for different subgroups of AOB and NOB supported the results obtained by 16S rRNA sequence analysis. Signals were obtained with probes Nso190, Nso1225, and Nsv443 indicative for *Nitrosospira* spp. (Fig. 8B) and with Nso190, Nso1225, Nmo218, and

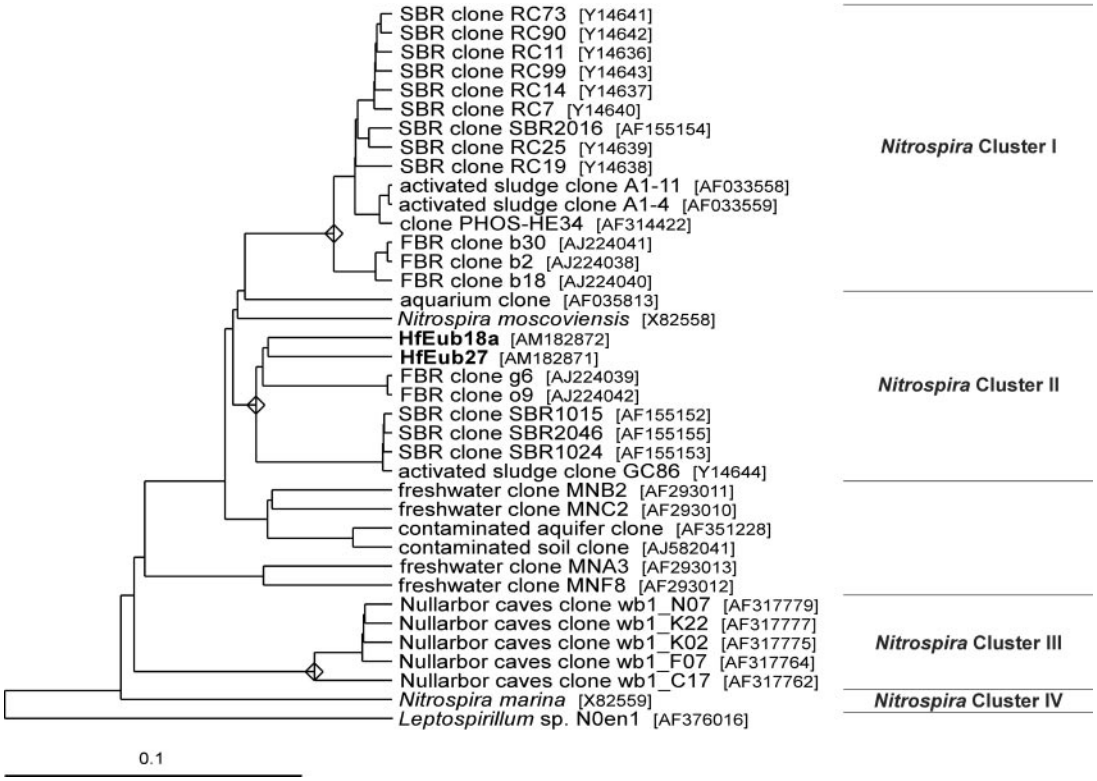

FIG. 7. Phylogenetic tree of NOB of the phylum *Nitrospira* and affiliation of NOB-related clones retrieved from the low-pH chalk reactor (in bold). Tree calculation is based on published 16S rRNA sequences (>1,400 bp) and the maximum likelihood method (FastDNAml). Clones retrieved in this study have been added to the tree by the maximum parsimony method without changing the tree topology. Branches with diamond symbols refer to commonly accepted phylogenetic groups (12).

NOLI191 indicative for cells affiliated to the *Nitrosomonas oligotropha* lineage (Fig. 8C). Cells of both groups occurred as typical microcolonies. While colonies of *Nitrosospira* spp. were large (and more numerous), those of the *Nitrosomonas oligotropha* lineage were rather small. Distinct amounts of extracellular polymeric substances (EPS), particularly around *Nitrosospira* spp. microcolonies but also around those of the *Nitrosomonas oligotropha* lineage, were visible. A certain smaller number of cells hybridized with probe NEU but not Nsm156 or Nse1472. This group of cells was also not detected in the cloning approach. Therefore, its phylogenetic status remains unclear.

Furthermore, indicative of NOB of the genus *Nitrospira*, a large number of cells was found to hybridize with probes NSR1156, Ntspa712, and Ntspa662 (Fig. 8D). The respective microcolonies showed the typical irregular shape, and the abundance of the NOB was visually larger than that of the AOB.

DISCUSSION

Several hypotheses have been proposed in the last decades to explain the activity and growth of chemolithoautotrophic nitrifiers, which are generally known as acid sensitive, in acidic environments (for a review, see reference 18). In the past, emphasis (in particular in soil microbiology) has been put on the hypothesis of urea as an alternative substrate for AOB. Urea may passively enter the cell and is hydrolized internally, thereby providing ammonia and contributing to a higher internal pH (1, 9, 18, 43). Our aim was to find evidence for two alternative major hypotheses frequently suggested to explain nitrification at low pHs: (i) the existence of less-acidic microenvironments that are conducive to nitrification and (ii) the existence of acid-tolerant strains among nitrifying bacteria (i.e., strains active at low pHs without the need for special ammonia-generating mechanisms).

A fluidized-bed reactor system with chalk as a carrier material was shown earlier to provide conditions leading to reliable and high nitrification rates at pHs as low as 3.8 (24, 60) and, therefore, was chosen as a model system for appropriate sample material. Besides the fact that similar system performance is obtained when urea is provided as a substrate in this reactor, its use was excluded under the operation conditions applied here.

The "conducive microenvironment" hypothesis. The dissolution of solids with buffering capacities in acidic media causes an elevated pH in the immediate vicinity of the particle surface (for an example, see reference 53). The hypothesis of "conducive microenvironment" is based on this fact. The presence of neutral or less-acidic microenvironments has been postulated to explain nitrification in acidic soils (for details, see reference 18 and references therein) and in biofilms grown on buffering solids (24, 37). To the best of our knowledge, the existence of such microenvironments, however, has not been directly dem-

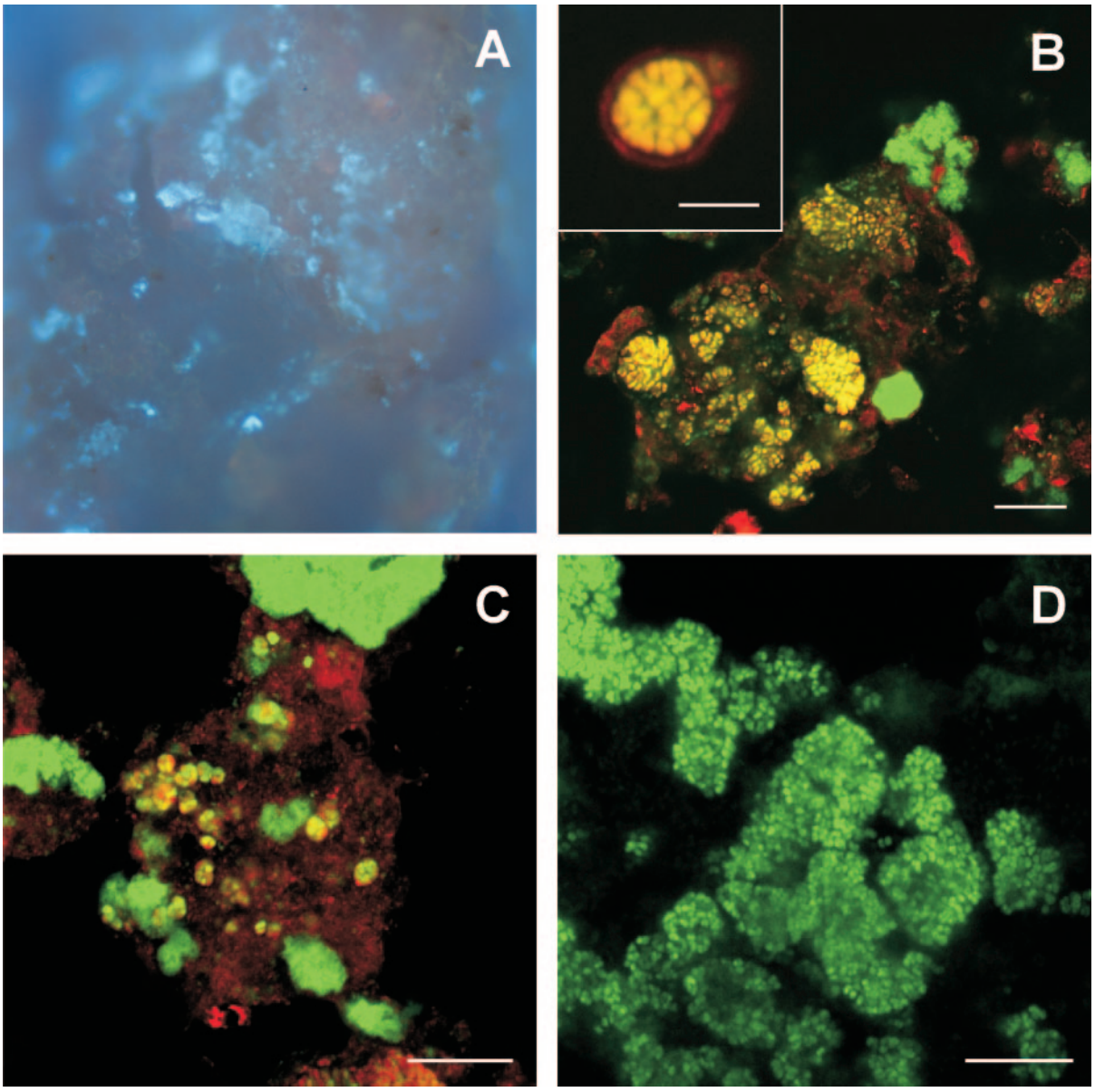

FIG. 8. Epifluorescence and confocal laser scanning micrographs of biofilm samples from chalk after staining with DAPI (A) and after FISH (B through D). (A) Surface of chalk particle from the early process period after DAPI staining. (B) Microcolonies of *Nitrosospira* spp. and other bacteria (red, Nsv443 probe; green, EUB338 probe). The insert shows EPS around the *Nitrosospira* spp. microcolony. (C) Small microcolonies of *Nitrosomonas oligotropha*-affiliated cells (red, Nmo218 probe; green, EUB338 probe). (D) Extended microcolonies of the NOB *Nitrospira* spp. (green, Ntspa662 probe). Yellow indicates the colocalization of two probes. Scale bars, 20 μm (B to C), 10 μm (D), and 5 μm (insert).

onstrated until now. Bare chalk particles indeed provide a surface-near zone of elevated pH when exposed to acidic medium with a strong increase of pH in a thin layer close to the surface (53). While the pH profiles shown here (Fig. 2) are in agreement with these facts, they lack the thin layer (a few micrometers) of strongly increased pH close to the chalk surface. This deviation from theoretical considerations is explained by the fact that each data point obtained with microsensors is a single measurement that integrates over a certain spatial volume with a dimension of about two times the tip diameter. With the sensors used here, the integration range can be considered to be ≥10 μm. In the acidic pH range of our system, the dissolution rate of the bare chalk is proportional to the pH and transport controlled with respect to the end members of the dissolution reaction sequence (42, 53).

Nevertheless, in the nitrifying biofilm covering the chalk particle, no evidence for a less-acidic microenvironment was found. In contrast to bare chalk, we measured an even lower pH close to the chalk surface when the surface was covered with a biofilm relative to the bulk pH. Interestingly, the lowest local pH found in all experiments was around 3.9, which is close to what is reported to be the lower limit for nitrification (50) and reactor performance (60). Future studies of the phys-

iological mechanisms of adaptation might help to find additional evidence and explanations for this empirical limit.

Our microprofiles show that under conditions of low bulk pH, oxygen consumption (as indicated by the curvature of profiles) and nitrifying activity generating protons were found throughout the whole biofilm; thus, all of the active biomass was exposed to strongly acidic conditions. Produced protons are subject to mass transport limitations within the biofilm, which causes it to be even more acidic than the bulk pH. Under these conditions, chalk is dissolved in stoichiometric amounts to the ammonium consumed and acid produced (24). Consequently, and in contrast to the scenario on bare chalk, a steady state with a very low biofilm pH is reached. The chalk dissolution rate is then controlled by the nitrification rate, which in turn is determined by nitrifier abundance and substrate (ammonium or oxygen) availability. The maintenance of a low-pH steady state with high activity was also demonstrated in a system using nonbuffering carrier material, i.e., sintered glass beads. The system was supplied with bicarbonate in stoichiometric amounts to the nitrification rate (60). No difference in pH or oxygen microprofiles or in local conversion rates was found between biofilms grown on both materials. This similarity strongly supports our hypothesis that pH, within wide limits, does not primarily control growth and activity of nitrifiers. Our findings are in line with the observation that the increased addition of calcium carbonate to acidic tea soils does not affect nitrification rate (30).

Mass transfer limitation and a low-pH steady state might occur similarly in nitrifying communities in other acidic environments like, e.g., acidic soils. The observation that nitrification in acidic soils (not limited in ammonium) is coupled to N mineralization (15) does not necessarily disprove the existence of acidic microenvironments. In fact, ammonification provides a buffer capacity and its availability has been suggested to control the nitrification rate in acidic soils (15). If the production and consumption of buffer capacity equilibrate, a low-pH steady state could be maintained even in close associations of ammonifiers and nitrifiers, which is analogous to our observations of the biofilm grown on chalk. Consequently, there is neither theoretical nor direct experimental support for the conducive microenvironment hypothesis and, therefore, it is insufficient to explain the presence and activity of nitrifying bacteria in such environments.

The "acid-tolerant nitrifiers" hypothesis. Various studies indicated the existence of acid-tolerant nitrifiers (for a review, see reference 18 and references therein). However, it is unclear whether such nitrifiers comprise either unknown species or common acid-sensitive ones that are more or less permanently adapted to low pHs (18). A 16S rRNA approach with general 16S rRNA primers has been used here to elucidate the composition and structure of the nitrifying biofilm grown under acidic conditions. For the AOB guild, the majority of clones were affiliated to *Nitrosospira* cluster 3 (47). Isolation approaches used on samples from acidic terrestrial environments, such as soils from forests, tea plantations, and heaths, typically lead to cultures of *Nitrosospira* spp. (1, 5, 18, 31). Molecular techniques based on 16S rRNA detection preferentially recover sequences affiliated to the *Nitrosospira* cluster 2 (10, 18, 36, 56, 57) from such habitats. Representatives of the *Nitrosomonas oligotropha* lineage, the second group of AOB detected in this biofilm, have been reported from a wide range of habitats, indicating its physiological versatility (36, 46, 55, 57). These organisms are characterized by their ability to cope with low substrate concentrations (7, 22, 54, 55). Both *N. oligotropha* and *Nitrosospira*-related strains have a lower K_m for ammonia than do other AOB: pure cultures affiliated to the *Nitrosomonas europaea*/*Nitrosococcus mobilis* lineage show a $K_m(NH_3)$ of 18 to 70 μM (35, 58), whereas those of isolates affiliated to *Nitrosospira* clusters 2 and 3 and to the *N. oligotropha* lineage have been found at 6 to 11 μM (31) and around 3 μM (31, 55), respectively. Estimates based on data measured in situ on an aggregated nitrifying community lead to a $K_m(NH_3)$ for *Nitrosospira* spp. of 1.4 μM (52). This strong affinity might represent an adaptive advantage under acidic conditions. The concentration of ammonia in our system below a pH of 5, however, is virtually zero, and additional mechanisms have to exist to allow persistence and activity under these conditions (see below).

Isolation efforts for NOB from acidic habitats have led to *Nitrobacter* strains (16, 28). The NOB guild in the system studied, however, consisted exclusively of *Nitrospira* spp. This is analogous to the finding that *Nitrospira* spp. are the most abundant strains in wastewater treatment systems and aquarium filters (6). *Nitrospira* spp. affiliated to sublineage II originate mostly from wastewater treatment systems and various other habitats (12). The large abundance of *Nitrospira* spp. in the studied biofilm is in line with observations in other biofilm-based wastewater treatment systems (23, 51). The knowledge regarding the ecophysiology of members of this genus is limited, as is the number of studied isolates. In situ measurements on nitrifying sludge flocs, however, suggest a rather low K_m as well that might represent an adaptive advantage at low substrate concentrations (20, 51, 52). Recent findings indicate that members of the phylum *Nitrospira* can persist in rather extreme environments with high concentrations of dissolved sulfide and low pHs (around 4) (32).

It has been shown that slime production and surface attachment are common features of AOB and NOB thriving at low pH (33). Furthermore, aggregates, in contrast to single cells, are able to nitrify at low pHs (18). Conspicuous EPS formation of nitrifying microcolonies also occurred in the system studied here (Fig. 8). In the literature, these phenomena are discussed as protective mechanisms against acidic pHs. If nitrification is the source of acidity, however, the lowest pH is found inside such aggregates, namely in the periplasmic space (26), and both aggregation and extended EPS production hamper neutralization of the respective microenvironment. Furthermore, the diffusivity of substrates and products along with protons will be reduced and thus negatively affect the activity and growth of cells in such microcolonies. The role of these observations remains unclear, and further studies focusing on the composition of the EPS might help clarify its significance for an adaptation to acidic conditions.

Physiological mechanisms. Both experimental observations (19, 31, 58) and the hypothetical mechanism of the reaction (61) show that ammonia (not ammonium) is the substrate for the first step of ammonia oxidation catalyzed by ammonia monooxygenase. How is ammonia provided to AMO under such acidic conditions? It is not clear whether the active site of the membrane-bound AMO faces the periplasmic space or the

cytoplasm. Ammonia is transported into the cytoplasm by diffusion (34). Despite poor pH regulation properties reported for *N. europaea*, the internal pH under acidic conditions is still higher that that of the periplasm (19). Consequently, an active site facing the cytoplasm would be advantageous. At pH 4, however, ammonia is virtually absent, and its protonated form, ammonium, would require specific ammonium transporters to enter the cell. Such transporters have been found in the genome of *N. europaea* (11). Their expression would be indispensable if cells had to cope with acidic conditions. De novo synthesis of such transporters, however, might take considerable time and an additional expense of energy for slow-growing cells of AOB. This is in line with the observation made when cells were shifted from neutral pH to around pH 4 conditions in nitrifying reactors containing biofilms or nitrifying flocs: a few weeks were necessary for nitrification rates to recover to original levels, and lower biomass yields were obtained (60). The dilatoriness of this adaptation also explains why pure cultures of AOB typically do not grow instantly in an acidic medium. A slow shift to acidic conditions is a seemingly more successful strategy.

Conclusions. There is no evidence for either the conducive microenvironment hypothesis or the common existence of specific groups of acid-tolerant nitrifying bacteria. Instead, physiological adaptations to low pH could be shown for various AOB and NOB. Under acidic conditions, subgroups of nitrifiers, such as *Nitrosospira* spp., *N. oligotropha*, and *Nitrospira* spp., seem to profit from their strong substrate affinity. The adaptation process is slow and possibly linked to the expression of additional cellular functions, e.g., ammonium transporters.

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