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Microbial Composition and Structure of a Rotating Biological Contactor Biofilm Treating Ammonium-Rich Wastewater without Organic Carbon

K. Egli,[1] F. Bosshard,[1] C. Werlen,[1] P. Lais,[2] H. Siegrist,[1] A.J.B. Zehnder,[1] J.R. van der Meer[1]

[1] Swiss Federal Institute for Environmental Science and Technology, CH-8600 Dübendorf, Switzerland
[2] Sondermülldeponie Kölliken, 5742 Kölliken, Switzerland

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Abstract

High nitrogen losses were observed in a rotating biological contactor (RBC) treating ammonium-rich (up to 500 mg NH_4^+-N/L) but organic-carbon-poor leachate from a hazardous waste landfill in Kölliken, Switzerland. The composition and spatial structure of the microbial community in the biofilm on the RBC was analyzed with specific attention for the presence of aerobic ammonium and nitrite oxidizing bacteria and anaerobic ammonium oxidizers. Anaerobic ammonium oxidation (anammox) involves the oxidation of ammonium with nitrite to N_2. First the diversity of the biofilm community was determined from sequencing cloned PCR-amplified 16S rDNA fragments. This revealed the presence of a number of very unusual 16S rDNA sequences, but very few sequences related to known ammonium or nitrite oxidizing bacteria. From analysis of biofilm samples by fluorescence *in situ* hybridization with known phylogenetic probes and by dot-blot hybridization of the same probes to total RNA purified from biofilm samples, the main groups of microorganisms constituting the biofilm were found to be ammonium-oxidizing bacteria from the *Nitrosomonas europaea/eutropha* group, anaerobic ammonium-oxidizing bacteria of the "*Candidatus* Kuenenia stuttgartiensis" type, filamentous bacteria from the phylum Bacteroidetes, and nitrite-oxidizing bacteria from the genus *Nitrospira*. Aerobic and anaerobic ammonium-oxidizing bacteria were present in similar amounts of around 20 to 30% of the biomass, whereas members of the CFB phylum were present at around 7%. Nitrite oxidizing bacteria were only present in relatively low amounts (less than 5% determined with fluorescence *in situ* hybridization). Data from 16S rRNA dot-blot and *in situ* hybridization were not in all cases congruent. FISH analysis of thin-sliced and fixed biofilm samples clearly showed that the aerobic nitrifiers were located at the top of the biofilm in an extremely high density and in alternating clusters. Anammox bacteria were exclusively present in the lower half of the biofilm, whereas CFB-type filamentous bacteria were present throughout the biofilm. The structure and

Correspondence to: J. R. van der Meer; E-mail: vdmeer@eawag.ch

composition of these biofilms correlated very nicely with the proposed physiological functional separations in ammonium conversion.

Introduction

The hazardous (industrial) waste landfill of Kölliken (Canton of Aargau, Switzerland) was constructed during the 1970s and filled with solid industrial waste of various origin. In 1985 the landfill was closed because of environmental concerns. About 250,000 m^3 of industrial waste was stored in this landfill, resulting in several thousand cubic meters of wastewater leachate per year, containing a large variety of soluble organic and inorganic compounds. In the early 1990s a wastewater treatment plant was built on site to treat the wastewater from the hazardous waste landfill. The plant treats up to 160 m^3 per day and consists of two separate rotating biological contactors (RBC) in a row with an extremely high surface area (5728 m^2 for contactor 1 and 7238 m^2 for contactor 2). Among many other compounds, the wastewater flowing into the treatment plant contained a total of 100–400 mg/L dissolved organic carbon and 100–500 mg NH_4^+-N per liter. The first contactor has been used to eliminate 88% of the organic compounds (TOC) in the landfill leachate, and a further 6% of the organic carbon is allowed to adsorb to activated carbon [35]. This ensured an almost total removal of all organic compounds which are typical for the leachate of the hazardous waste landfill (e.g., chlorinated hydrocarbons, phenols and anilines; P. Lais, personal communication). The effluent wastewater from the first contactor therefore mainly only contained the major part of the ammonium (i.e., 100–500 mg/L), which was subsequently treated in a second RBC. The maximum load of the second contactor is 30 kg NH_4^+-N per day (equaling 4 g/m^2·d).

Soon after the treatment plant was running properly (1994), it was found that the total load of inorganic nitrogen (mainly nitrate) coming out of the second compartment was up to 70% lower than the load of inorganic nitrogen (mainly ammonium) in the inlet [35]. This was surprising since nitrogen elimination in wastewater treatment plants was always thought to be due to a combination of nitrification and denitrification, which needs an organic carbon source. However, since the dissolved organic carbon concentration in the influent to the second compartment reactor was lower than 20 mg/L, the observed nitrogen removal could not be satisfactorily explained from nitrification and denitrification.

During the same time and afterward, phenomena of similar nitrogen "loss" were also observed in other locations [13, 31, 40–42]. Further investigations then showed that autotrophic bacteria existed which could oxidize ammonium with nitrite to N_2 (and some nitrate) under strictly anoxic conditions [18, 36, 38]. The physiology and biochemistry of such anaerobic ammonium oxidizing (or anammox) bacteria is now better understood [18, 29, 30, 36, 39], and although the organisms are notoriously difficult to obtain in pure culture, a few highly enriched cultures of various anammox bacteria exist [8, 31, 38]. Their characterization also led to the development of suitable oligonucleotide probes targeting the 16S rRNA [31], and the use of those probes showed that anammox bacteria were present in various wastewater engineering systems [14, 16, 31].

In this work we focused on the characterization of the microbial community in the biofilms of the RBC in the second compartment of the treatment plant in Kölliken, trying to link the community structure and composition with the nitrogen removal process in this unique reactor system. It has become clear from many studies that biofilms provide a substratum for attachment of slow-growing bacteria, produce stratified microenvironments due to gradients of oxygen [10], ammonium, nitrite, or nitrate [10, 33, 34], and protect against predation in the deeper layers. We were wondering if the specific conditions of the Kölliken biofilms would be sufficient for a natural enrichment of anammox bacteria. In fact, very recently anammox bacteria were found to inhabit similar deammonifying biofilms on Kaldness material in a moving- bed reactor treating sludge digester supernatant from a municipal wastewater treatment plant [14]. We also tried to locate the anammox bacteria in the leachate of the landfill, perhaps giving clues to their origin. For this purpose, we combined several tools such as fluorescence *in situ* hybridization (FISH), dot-blot rRNA hybridization, PCR, and 16S rDNA clone library construction.

Materials and Methods

Fluorescence in Situ Hybridization and Microscopy

Fixation of samples was performed by resuspending the cells in 4% (w/v) paraformaldehyde in PBS (consisting of 8 g NaCl, 0.2 g

KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 per litre distilled water at a pH of 7.4) and incubating for 1 h at room temperature. Afterward, the cells were washed twice with PBS and finally resuspended in a 1:1 (v/v) mixture of PBS and ethanol and stored at −20°C. Biomass was recovered by centrifugation, resuspended in a solution of 2.8 g/L sodium pyrophosphate (pH 7.5), and homogenized for 5 min in an ultrasound bath. Glass slides (Huber & Co., Reinach, Switzerland) were coated with gelatin [3]. Between 2 μL and 10 μL of a fixed sample was applied on a well of the coated slide, dried for approximately 1 h at 46°C, and sequentially dehydrated in solutions of 50%, 80%, and 100% ethanol for 3 min each. To start hybridization, 9 μL of hybridization buffer was mixed with 1 μL of fluorescently labeled probe (at a concentration of 50 ng/μL) and added to the sample on the glass slide. The composition of the hybridization buffer was dependent on the melting characteristics of the oligonucleotide probe and consisted of buffer solution, containing 0.9 M NaCl, 20 mM Tris-HCl (pH 8), and 0.01% SDS, mixed with formamide (Table 1). When necessary for increased selectivity, 1 μL of unlabeled competitor probe (at 50 ng/μL) was added to the hybridization mixture. Hybridization was conducted for 90 min at 46°C in a moistured chamber. Following hybridization, a stringent washing step was performed for 10 min at 48°C in a buffer with the appropriate NaCl concentration (Table 1), 20 mM Tris-HCl (pH 8), and 5 mM EDTA. The samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI) at a final concentration of 10 mg/L for 5 min at room temperature [15] and mounted in Citifluor (Citifluor Ltd., London, UK). For quantification with confocal microscopy the EUB-Mix [5] was labeled with Fluoros prime (MWG Biotech, Ebersberg, Germany) and the group-specific probe with the carboxycyanin dye Cy3. In case simultaneous hybridizations with two labeled probes were carried out (e.g., Ntspa662 and NEU on biofilm slices) the probes were labeled with fluorescein isothiocyanate (FITC) and Rhodamin red (both from MWG Biotech).

All oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland) or MWG Biotech. Microscopy was performed on an Olympus BX50 microscope (Olympus Optical AG, Volketswil, Switzerland) equipped with filters HQ-CY3 for Cy3 and Rhodamin red labeled probes, HQ-FITC for FITC labeled, probes and a HQ-filter for DAPI (all filters from AF Analysentechnik, Tübingen, Germany). Digital images were taken with a cooled CCD camera (type Sensys, Photometries Ltd., Tucson, AZ, USA) and acquired in the program METAVIEW (Version 4.1, Universal Imaging Corporation, Visitron Systems, Germany). For image acquisitions with confocal laser scanning microscope, a Zeiss LSM 510 (Zeiss, Jena, Germany) equipped with an Ar ion laser (458 and 488 nm) and a He–Ne laser (543 nm) was used together with the standard software package delivered with the instrument. All confocal laser microscope imaging was carried out at the facility of the Technical University of Münich at Freising, and the authors acknowledge the help of M. Wagner and S. Schmitz-Esser. Electron microscopy was performed with a Philips XL-30 scanning electron microscope (Philips, Eindhoven, The Netherlands) as described elsewhere [44].

Biofilm Slices

Biofilm samples were taken from the secondary stage RBC treating the leachate wastewater from the hazardous waste landfill in Kölliken. Biofilms and support were cut with a sharpknife. An area of 3×5 cm^2 of biofilm was removed from the outer layer of the RBC including the support material. Care was taken to maintain the structure of the biofilm. After fixation with paraformaldehyde (see above, but samples were rinsed with PBS and not centrifuged), a part of the biofilm was embedded in Cryoembedding Compound for low and medium temperatures (Microm Labor AG, Walldorf, Germany) and frozen at −35°C. A small representative piece was selected and cut in slices with a thickness of approximately 10 μm with a MICROM HM 500 OM microtome (Carl Zeiss AG) while remaining at −35°C. Slices were placed flat on gelatin-coated microscope slides, dehydrated, and hybridized as described above.

Nucleic Acid Extraction

For RNA extraction maximally 0.5 g of homogenized biofilm was suspended in 0.9 mL homogenization buffer in a screw-capped vial (homogenization buffer is 200 mM Tris-HCl pH 8.5, 1.5% SDS, 10 mM EDTA, 1% sodiumdesoxycholate, 1% Nonidet-P40). Glass beads (0.4 g, 0.1 mm in diameter) were added and the mixture was shaken two times for 1 min at 4000 rpm in a Braun Cell Homogenizer (Inotech AG, Dottikon, Switzerland) with a 1-min interval on ice. The glass beads were allowed to settle without centrifugation, and the supernatant was transferred to a fresh Eppendorf tube. This sample was mixed 1:1 with sodium acetate-buffered acidic phenol (pH 5.5) and incubated in a water bath at 65°C for 5 min [1], After vortexing and centrifuging for 5 min at 15,000 *g* and 4°C, the water phase was transferred to a new Eppendorf tube and mixed 1:1 (v/v) with Tris-HCl buffered phenol (pH 8.0). After vortexing, an equal volume of chloroform/isoamyl alcohol (24:1) was added, and the mixture was vortexed and centrifuged at 15,000 *g* for 3 min. The water phase was recovered and reextracted with chloroform/isoamyl alcohol, after which the nucleic acids in the water phase were precipitated. Nucleic acids were recovered by centrifugation, washed with 70% ethanol and 30% TE (10 mM Tris/HCl, 1 mM EDTA, and pH 8), and dissolved in RNase-free water. Digestion of DNA was performed for 30 min at 37°C with RNase-free DNase I (10 U/μL, Amersham Biosciences Europe, Dübendorf, Switzerland). RNA was visualized on a 0.8% agarose gel, and the RNA concentration and quality were measured with a spectrophotometer at 260 and 280 nm (using the conversion factor of OD_{1280nm} = 40 μg RNA/mL). RNA independently extracted from three different biofilm pieces was finally pooled.

For DNA extraction the cells were collected from the biofilm sample as described above for RNA isolation and resuspended in 5 mL of TENP solution (TENP is 50 mM Tris-HCl, 20 mM Na_2EDTA, 100 mM NaCl, 1% [w/v] polyvinylpolypyrrolidone, pH 8). Of this suspension, 1.5 mL was removed to a screw-capped Eppendorf tube, containing 0.2 g glass beads. The mixture was

Table 1. Probes used for FISH and dot-blots

Probe	Sequence (5′-3′)	Competitor	Formamide	NaCl concentration in wash buffer (M)	Dot-blot hybridization temperature (°C)	Target position[a]	Reference
EUB338 I	gctgcctcccgtaggagt	—	0%[b]	0.9	54.5	338–355	[3]
EUB338 II	gcagccacccgtaggtgt						[5]
EUB338 III	gctgccacccgtaggtgt						
Amx820	aaaacccctctacttagtg	—	40%	0.056	52	820–839	[31]
Amx1240	tttagcatccctttgtaccaacc	—	60%	0.014		1240–1263	
Kst1275	tcggctttataggtttcgca		25%	0.159		1275–1295	
Nso1225	cgccattgtattacgtgtg	—	35%	0.08	48	1225–1244	[24]
NEU	cccctctgctgcactcta	CTE	40%	0.056	52	653–670	[45]
Ntspa662	ggaattccgcgctcctct	Comp-Ntspa662	35%	0.08		662–679	[6]
Nit3	cctgtgctccatgctccg	CNit3	40%	0.056		1035–1048	[45]
CF319a	tggtccgtgtctcagtac	—	35%	0.08	50	319–356	[23]
Nmo218	cggccgctccaaaagcat	—	35%	0.08		218–235	[10]
Nsv443	ccgtgaccgtttcgttccg	—	30%	0.112		444–462	
Nse1472	accccagtcatgaccccc	—	50%	0.028		1472–1489	[19]
NmII	ttaagacacgttccgatgta	—	25%	0.159		120–139	[27]
NmV	tcctcagagactacgcgg		35%	0.08		174–191	[27]
Pla46	gacttgcatgcctaatcc	—	30%	0.112		46–64	[25]
CTE	ttccatcccccctctgccg		40%	0.056			[45]
Comp-Ntspa662	ggaattccgctctcctct		40%	0.056			[6]
cNit3	cctgtgctccaggctccg		40%	0.056			[46]
AJ224539[c]	attctacttgcctctaa	—	0%	0.9	42	611–628	This study
AJ224540[c]	gcggcactaatctctaag		0%	0.9	51	849–867	This study

[a] Numbers refer to the *Escherichia coli* 16S nucleotide numbering
[b] In case of simultaneous hybridization of the EUB mix with other specific probes, the formamide concentration for the specific probe was used
[c] Numbering: see Table 3

shaken three times for 1 min at 4000 rpm in a Braun Cell Homogenizer with 1-min intervals on ice. The sample was heated to 100°C in a boiling water bath for 2 min and subsequently cooled in liquid nitrogen, twice. After centrifugation for 5 min at 15,000 *g* the water phase was recovered and the DNA was precipitated with sodium acetate (pH 5.5) and isopropanol. DNA was recovered by centrifuging for 15 min at 15,000 *g* and 4°C, washed once, and dissolved in 100 μL of 10 mM Tris-HCl (pH 7.5). The samples were further purified with a BioSpin 30 chromatography column (Bio-Rad Laboratories, Hercules, CA) and subsequently with a MicroSpin S-400 HR column (Amersham Biosciences Europe). Nucleotide concentration and quality were determined from measuring the optical density at 260 and 280 nm.

Leachate was collected from five different monitoring wells at the hazardous waste landfill of Kölliken and from the final wastewater before entering the first RBC. The ammonium and TOC composition and the total DNA concentration isolated from the leachate in the wells is given in Table 2. Four L of leachate was collected by pumping the leachate to the surface. Cells in the leachate (from 2 L) were pelleted by centrifugation and resuspended in a screw-capped vial in 0.9 mL of TENP homogenization buffer. DNA was then extracted as described above.

Polymerase Chain Reaction (PCR)

The PCR was used to detect the presence of anammox bacteria in the landfill leachate and to characterize the 16S rDNA sequence of community members from the Kölliken biofilm. The reaction solution for the PCR contained 5 μL polymerase buffer (from a 10× concentrated stock), 0.25 μL *Tag* DNA polymerase (5 U/μL), 0.4 μL of deoxynucleotide mixture (25 mM each), and 43 μL double–distilled H_2O. Forward (Pla46rc, 5′ gga tta ggc atc caa gtc 3′) and reverse primer (Amx820, 5′ aaa acc cct cta ctt agtg 3′) [31] were each added at 10 ng per reaction. All PCR reagents except oligonucleotides were obtained from Sigma (Buchs, Switzerland). The sequence of the Pla46rc primer is the reverse complement of its published version [25]. An amount of 1 μL of the purified (diluted) sample DNA was finally added to start the PCR. DNA from an enriched culture of '*Candidatus* Kuenenia stuttgartiensis' [8] was used as positive control for the PCR. Amplification was performed in a thermocycler (Eppendorf Mastercycler gradient, Eppendorf-Netheler-Hinz GmbH, Hamburg) under the following conditions: initial denaturing step for 4 min at 95°C, 35 cycles of 1 min at 95°C, annealing at 50°C for 1 min and extension for 1.5 min at 72°C, and a final elongation step of 4 min at 72°C. Optimal annealing temperature for the Amx820/Pla46rc primer set was determined by running a gradient program from 41°C to 54°C. PCR products (10 μL of the reaction) were separated on a 0.8% agarose gel and visualized by staining with ethidium bromide.

16S rDNA fragments from isolated total DNA of the biofilm samples were amplified in the PCR using the following eubacterial primers (modified from [47]): 16S 6F (5′-ggagagttagatcttggc tcag-3′) and 16S 1510R (5′-gtgctgcagggttaccttgttacgact-3′). The 16S rDNA PCR products were cloned in *Escherichia coli* DH5α in

vector pGEM-T-easy (Promega, Wallisellen, Switzerland) by established procedures [28]. Plasmid DNAs for sequencing were isolated by boiling preparation [28]. Plasmid inserts were sequenced on both strands by using a Thermosequenase Kit (Amersham, Little Chalfont, UK) with IRD-800 and IRD-700 labeled primers (MWG Biotech, Ebersberg, Germany). Universal vector-located primer sequences were used, as well as two primers which targeted a conserved region around position 785 of the cloned 16S rDNA fragment (EUB785F, 5′-ggattagatccctggtag-3′ and EUB785R, 5′-ctaccagggtatctaatc-3′). Sequence transcripts were separated and analyzed on a LiCOR 4200L IR2 automated DNA sequencer (LiCOR, Lincoln, NE, USA). Only those clones which had a different "G and T track" were completely sequenced on both strands. The nucleotide sequences of the 16S rRNA gene of the unknown organisms were deposited in the GenBank database under accession numbers AJ250882, AJ224539–224541, and AJ224938–224943. Sequence comparisons were calculated by direct FastA alignments [26].

Dot-Blot Analysis

RNA (1 μg/μL) was serially diluted as follows: 1/3, 1/10, 1/30, 1/60, 1/100, 1/300, 1/1000. RNA dilutions were denatured at 94°C for 2 min and immediately placed on ice until being spotted on the membrane. As quantitative hybridization control, oligonucleotides with a sequence reverse complementary to the hybridization probes (Table 1) were used. A solution of 2.5 ng/μL of each oligonucleotide was then serially diluted to 0.8, 0.25, 0.08, 0.04, 0.025, 0.008, and 0.0025 ng/μL.

Nylon hybridization membrane (Hybond N^+, Amersham Biosciences) was moistened with 1× SSC solution (SSC solution is 150 mM NaCl, 15 mM sodium citrate, pH 7), mounted in a Convertible Filtration Manifold System (Gibco BRL Life Technologies Inc., Gaithersburg, MD; USA), and 20 μL of the diluted RNA and oligonucleotide solutions were pipetted in alternating columns, immediately followed after every pipetting step by vacuum suction. Every well was subsequently washed with 20 μL of 1× SSC solution. After blotting, the membrane was cut into slices, each containing a dilution series of RNA and of the respective oligonucleotide. The RNA and oligos were crosslinked to the membrane using a Stratalinker 1800 (Stratagene, La Jolla, USA) and dried to ambient air.

For hybridization, the membrane slices were each preincubated in a 10-mL polypropylene tube in a water bath at the respective hybridization temperature (Table 1) for 15 min with 2 mL of prehybridization buffer (containing 0.5 M sodium phosphate, 1 mM EDTA, 1% (w/v) BSA (Fraction V Grade), and 7% (w/v) SDS, pH 7.2). Oligonucleotide probes were labeled with biotin 16-ddTUP (Enzo, Roche, Switzerland) by treatment with terminal deoxynucleotidyl transferase for 15 min at 37°C according to specifications of the supplier (Amersham Biosciences). To start hybridization 10 μL of the biotin-labeled probe was added to the prehybridization mixture and the mixture was incubated in a water bath for 90 min at the appropriate hybridization temperature of the probe (Table 1). After hybridization, the membranes were washed twice in 5 mL of a solution of 2× SSC plus 0.1% w/v SDS for 5 min at room temperature, twice in a preheated solution of 1× SSC plus 0.1% SDS for 5 min at hybridization temperature, and twice in 1× SSC solution for 5 min at room temperature, while rotating on a tube roller.

For detection of the biotin label, membranes were processed as described previously [4]. Membranes were exposed to X-ray film (Hyperfilm MP, Amersham Biosciences Europe) for 1 to 30 min and developed with a Kodak X-omat 1000 processor (Eastman Kodak Company, Rochester, NY, USA). Films were scanned and the darkness of each spot quantified by a computing densitometer (Molecular Dynamics) with the program Image Quant (Version 3). Calculation of "copy numbers" of target was done by comparing signal intensities of each RNA dilution to the oligonucleotide standard row. For this purpose, the signal intensities for both dilution series were fitted by hyperbolic curve-fitting with the programs KaleidaGraph and Excel as described elsewhere [22]. Calculated copy numbers were then expressed as the relative percentage of the copy number calculated for the EUB hybridization.

Results

Composition of the Kölliken Rotating Contactor Biofilm

The composition of the microbial community in the Kölliken biofilms was determined by using different independent approaches. In the first instance, a 16S rDNA clone library was constructed from 16S rDNA amplified from total biofilm DNA. The insert DNAs of 26 clones were sequenced, of which 9 were different (Table 3). Two different 16S rDNA sequences (AJ224938 and AJ224940) had 93% nucleotide sequence identity to each other but both at least 97% sequence identity to the 16S rDNA sequence of *Sphingomonas* sp. Another sequence belonged to an organism related to the genus *Staphylococcus* (AJ224939). Furthermore, 16S rDNA of a putative ammonium oxidizer related to the genus *Nitrosomonas* was cloned (AJ224941), which also contained the target sequence for the NEU and Nse1472 probes (Table 1). However, this was the only sequence among the clone library related to the (group of ammonium-oxidizing bacteria. Two sequences showed very low percentages of sequence identity (85% or less) to known species (AJ224539 and AJ224942). Of these, AJ224942 had around 87% identical nucleotide sequence to a 16S rDNA sequence from the Class "Sphingobacteria" within the phylum Bacteroidetes, while the target site for the CF319a-FISH-probe was present in this sequence. AJ224539 grouped within the rather exotic bacterial division OP11, AJ224540 within OP3, and AJ224541 within the phylum Actinobacteria [17]. AJ224943 grouped within the

Phylum Planctomycetes. The phylogenetic position of this 16S rDNA sequence is not very closely related to the anammox bacteria as described elsewhere [32], and the sequence did not match to the general Amx820 probe. The 16S rDNA sequences of the anammox bacteria '*Candidatus* K. stuttgartiensis' or of any putative nitrite-oxidizing bacteria were not detected in the analyzed representation of the biofilm clone library.

With FISH four different bacterial groups comprised the largest populations in the homogenized biofilm samples. Three of those were involved in nitrogen conversion based on their phylogenetic classification. These three were ammonium-oxidizing bacteria from the *Nitrosomonas europaea/eutropha* group (FISH probes NEU and Nse1472, Table 1), annamox bacteria (probe Amx820), and nitrite oxidizers from the genus *Nitrospira* (probe Ntspa662). The fourth main group consisted of filamentous bacteria detected with the probe CF319a, suggesting they belong to the phylum Bacteroidetes and perhaps exemplified by the 16S rDNA clone AJ224942. Ammonium oxidizers from the *Nitrosococcus mobilis* lineage (NmV), *Nitrosospira* spp. (Nsv443), *N. communis* (NmII), and *N. oligotropha/urea* lineage (Nmo218) were not detectable. Around 70% of all cells were stained with the EUB338 probe mixture using epifluorescence microscopy. Ammonium-oxidizing bacteria were present as between 10% (compared to DAPI staining) and 28% (in CLSM-analysis compared to EUB stained biomass) of all cells. CF319a-stained bacteria comprised 7.6% of DAPI-stained cells. Nitrite-oxidizing bacteria were present in lower numbers (less than 5%), which was confirmed with CLSM (2.6%). When trying to quantify the population of anammox bacteria with CLSM it was found that the fluorescent signal of the EUB hybridization on the same aggregates was too weak compared to the Amx820 and Pla46 signals. Therefore, the relative amount of Amx820 stainable biomass compared to EUB could not be determined with CLSM, but manual counting on digital images from epifluorescence microscopy gave a value of 33% Amx820-stainable cells. Judged from the Amx820- and NEU-stained biomass only, aerobic and anaerobic ammonium oxidizers were present in a similar amounts.

The relative abundance of the same phylogenetic groups as in FISH were also investigated with RNA dot blot hybridization (Fig. 1). Compared to the signal intensity of the hybridization of the EUB mixture (set as 100%) the amount of rRNA stained with NEU was around 23% (Table 4). The same relative amount was hybridizable with

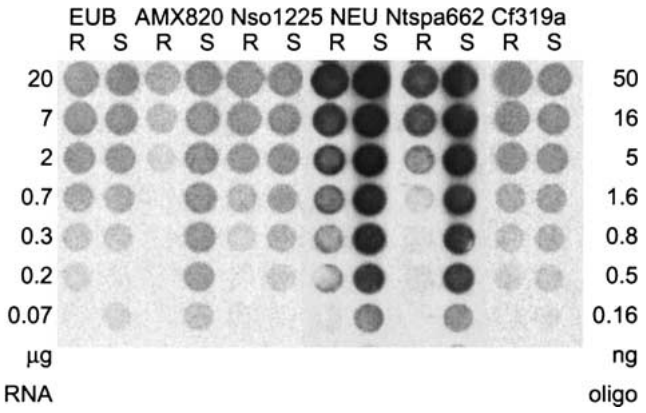

Fig. 1. Example of dot-blot hybridization to RNA extracted from the biofilms. The different hybridization probes are indicated on the top. (R) Columns with (serially diluted) RNA samples (amounts indicated on the left). (S) Columns with serially diluted antisense oligonucleotides (amounts indicated on the right). Picture composed and assembled by using Adobe Photoshop Version 5.0 from images exposed for different times.

the more general probe for ammonium oxidizing bacteria Nso1225. The greatest amount of rRNA hybridized with the probe CF319a detecting the filamentous bacteria from the Bacteroidetes phylum (around 40%). The quantity of nitrite-oxidizing bacteria (detectable by hybridization with the probe Ntspa662) was 14%. The amount of rRNA hybridizing to the Amx820 probe (representative for anammox bacteria) was extremely low (around 1%), although FISH hybridizations had suggested a relative high number of anaerobic ammonium oxidizers in the biofilm. To explain this difference between FISH and rRNA dot-blot hybridizations, hybridizations were carried out at lower temperatures (48°C and 50°C instead of 52°C) to rule out the possibility that the low signal was due to too high a hybridization temperature. However, this did not change the relative hybridization signal intensity. Hybridizations were then performed with extracted rRNA from the anammox enrichment culture which had previously been shown to contain 88 ± 5% anammox bacteria [8]. This resulted in a relative signal intensity of 25.5 ± 3.8% compared to the EUB signal intensity. Assuming that all cells in the enrichment culture contained comparable ribosome numbers, the extraction efficiency of RNA from anammox organisms would therefore only be about 4% (due to the 10% of other bacteria in the enrichment culture with high extraction efficiencies). This would indicate that the Amx820-specific hybridizable population determined by dot-blot hybridization (1% of EUB) would have to be corrected with a factor of 25. The three sequences (i.e.,

AJ224539, AJ224540, and AJ224942) with very low sequence homologies to known species were present in very low numbers among total isolated RNA (less than 1% as determined with rRNA dot-blots). With specific FISH probes targeting these sequences no significant signal was observed at the lowest stringency, indicating that the abundance of microorganisms carrying those 16S rDNA sequences in the biofilm is most likely very low.

A complete balance of all bacterial groups in the biofilm could not be made according to our microscopic and dot-blot analyses. With normal epifluorescence microscopy counting around 71% of all DAPI-stained cells were detected with the EUB probe mixture, and 56% could be accounted for by all other used probes. With rRNA dot-blot analysis, 78% (102% when correcting for the extraction efficiency of anammox RNA) of all bacteria were quantified compared to the EUB mixture. This suggests that some other populations were missed, perhaps those which had been picked up by cloning the 16S rDNA fragments (e.g., α-Proteobacteria and high GC gram-positive organisms). However, the contribution of those populations for the total ammonium conversion by the biofilm is likely to be of minor importance.

Structure of the Biofilm

To elucidate the structural organization of the major populations in the ammonium removing biofilm in Kölliken, thin cross-section biofilm slices (10 μm) were again hybridized with the probes which covered most of the community (aerobic and anaerobic ammonium oxidizing bacteria, nitrite oxidizing bacteria and bacteria from the phylum Bacteroidetes. Typically, the thickness of the biofilm samples was up to 750 μm (±120 μm), varying strongly in the reactors depending on manual cleaning or spontaneous sloughing. Hybridization with the NEU and Nso1225 probes clearly showed a very high abundance of nitrifying bacteria in the top layer of the biofilm (Figs. 2A, 2B). Ammonium oxidizing (NEU-stained) bacteria typically formed dense clusters of around 2 μm to 15 μm in diameter, containing probably up to several hundred cells. Cells hybridizing to the Ntspa662-probe were less abundant than to NEU, but were confined to the top layers and formed clusters as well (Fig. 2I). Simultaneous hybridization of NEU and Ntspa662 with different fluorescent labels showed that ammonium and nitrite oxidizing bacteria were in close proximity to each other (Figs. 2G, 2H, and 2I). In deeper layers a clear upper boundary of Amx820 stainable cells was detectable (not shown), and some aggregates were still visible with DAPI near this boundary. In the lower part of the biofilm no hybridization occurred with the probes NEU and Ntspa662, but very strong hybridization with the Amx820 and Pla46 probes (Figs. 2E, 2F). In contrast, no Amx820 hybridizable signal was obtained in middle and top layers of the biofilm. This suggested the anaerobic ammonium oxidizing bacteria to be condensed to a clear dense layer at the bottom of the biofilm, above which no anammox bacteria sustained (Fig. 2F). Likewise, anammox bacteria formed dense large essentially homogeneous aggregates. In contrast to these three types, bacteria staining with CF319a-probe were present throughout the biofilm (Figs. 2C, 2D).

With electron microscopy the typical cauliflower aggregates were also seen on the surface of the biofilm, which, based on FISH results, consisted of clusters of ammonium and nitrite-oxidizing bacteria (Fig. 3). Observations of cracks in the surface allowed visualization of the filamentous bacteria at the deeper layers. The bottom part of the biofilm consisted of mineral surface (not shown), and the clusters of anammox bacteria could not be discerned by SEM viewing from the outside.

Anammox Amplifiable 16S rDNA at the Kölliken Hazardous Waste Landfill

In order to find a possible source for the anammox bacteria in the biofilms, DNA was extracted from biomass collected from leachate from monitoring wells with the highest ammonium concentrations at the landfill area and from the leachate flowing into the treatment system (Table 2). With the conserved primers Pla46rc and Amx820, which until now target all known anammox bacteria [31], products of the appropriate size of 0.8 kb were obtained for DNA of all sampling sites (Fig. 4). Since DNA from the anammox enrichment culture gave a product of the same size and the negative control with only water gave no product, these results suggest that anammox bacteria were present in the leachate before entering into the RBC and therefore must be native to the groundwater or subsurface at the site or to the industrial waste material filled in the site. From looking at the amount of product in DNA dilutions of the different samples, it appeared that well P8 contained the highest amount of anammox amplifiable material per amount of DNA isolated (Fig. 4). No clear correlation could be observed between ammonium and/or

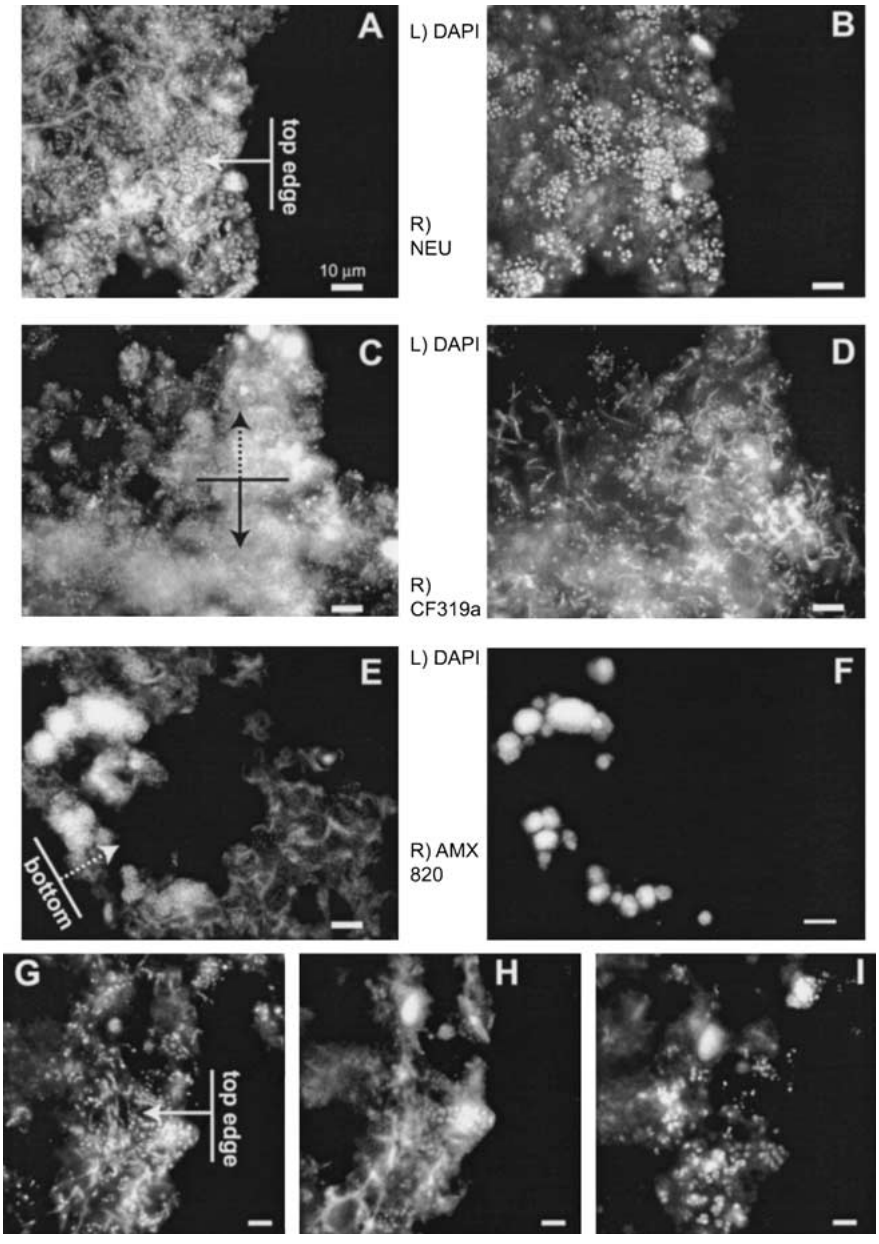

Fig. 2. FISH micrographs of thin-sliced fixed biofilm samples. (A, C, E, and G) DAPI-counterstained images showing all cells. (B) Same area as (A), top layer of the biofilm, specifically stained with NEU. (D) Same area as in (C), middle part of the biofilm, stained with CF319a. (F) Bottom part of the biofilm as in (E), stained with Amx820. (G, H, and I) Top layer, simultaneously stained with DAPI (G), NEU (H), and Ntspa662 (I). In images (B), (D), and (F), probes were labeled with Cy3. In (H) and (I), probes were labeled with Fluos (H) and Rhodamine (I), respectively. Because of limited wavelength separation of fluorescence filters and digital imaging, background illumination is slightly enhanced, resulting in incomplete separation of, for example, NEU-Fluos and Ntspa662-Rho signals in panels H and I. The scale bar indicates a size of 10 µm.

TOC concentration and the abundance of anammox amplifiable material.

Discussion

During the past few years several publications have appeared showing the presence and activity of anammox bacteria in engineered systems, sediments, or enrichment cultures [8, 12, 14, 31, 35, 36, 40, 41, 43]. Here we report on the structure and function of a biofilm spontaneously formed on the surface of an RBC treating an ammonium-rich but organic-carbon-poor leachate from a hazardous waste landfill. A major part (~35% as counted by epifluorescence microscopy) of the biofilm community appeared to consist of anammox bacteria from the type '*Candidatus* K. stuttgartiensis', suggesting that both the operating conditions of the treatment system and the type of leachate were creating optimal conditions for spontaneous inoculation and enrichment of anammox bacteria. Although the natural occurrence of anammox bacteria is still relatively obscure, they have been detected at quite different locations [12, 14, 31, 38, 40, 41]. Evidence could also be obtained by PCR that in the case of the Kölliken biofilm the anammox bacteria were indeed entering the reactors from the leachate of the hazardous waste landfill, suggesting that the subsurface is an environment where they originate.

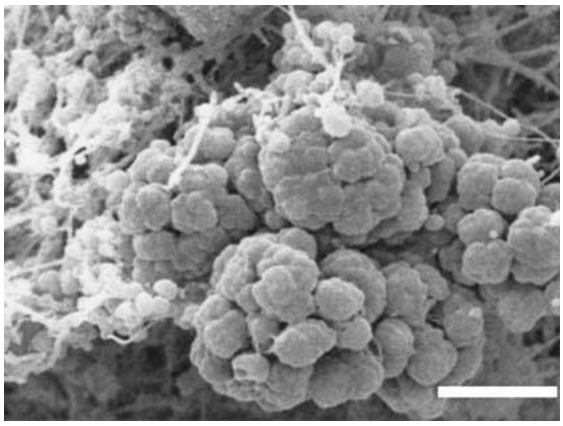

Fig. 3. Scanning electron micrograph of the surface of the biofilm. Scale bar indicates a size of 10 μm.

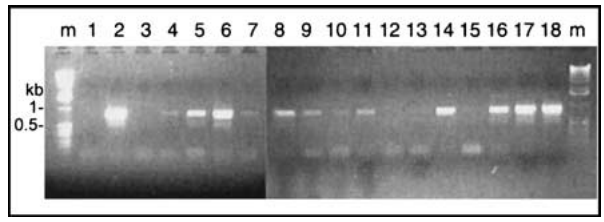

Fig. 4. PCR detection of anammox-amplifiable 16S rDNA in DNA extracted from biomass from leachate at different monitoring wells of the Kölliken hazardous waste landfill site. Lanes: m, 1 kb ladder; 1, water only; 2, anammox enrichment [8]; 3, empty; 4, 5, and 6, well P8 (0.2, 2, and 20 ng, respectively); 7 and 8, well P3 (20 and 200 ng, respectively); 9 and 10, well P2 (25 and 250 ng, respectively); 11 and 12, influent (25 and 250 ng respectively); 13 and 14, well P1 (10 and 100 ng); 15 and 16, well P0 (10 and 100 ng); 17 and 18, anammox enrichment. Inhibition ofthe PCR occurred at the highest DNA amounts in well P2 and the influent.

The biofilm was formed mainly by four different bacterial groups. In the upper part of the biofilm mainly aerobic ammonium oxidizers of the *N. europaea/eutropha* group (which were stained with NEU, Nso1225, and Nse1472) were observed. Data from all three methods (FISH on dispersed biofilm samples, confocal laser scanning with FISH, and quantitative rRNA dot-blotting) agreed more or less on the relative size of the aerobic ammonium oxidizing population (~20%). No ammonium-oxidizing bacteria from other groups were present since the FISH probes NmII, Nsv443, NmV, and Nmo218 [10] did not give any signal. It might be that the high ammonium (up to 35 mM) and salt (10 g/L) concentrations in the influent of the treatment system were selecting for ammonium oxidizing bacteria from the *N. europaea/ eutropha group*, since these are known to resist such conditions better than other ammonium oxidizers, such as those from the *N. oligotropha/urea* lineage [10, 21]. Nitrite-oxidizing bacteria (*Nitrospira*) were clearly detectable, although in a much lower quantity than the aerobic ammonium oxidizers. Their population size estimated by the three methods ranged between 3 and 14%. The population of anaerobic ammonium oxidizers (mainly bacteria related to '*Candidatus* K. stuttgartiensis' since they were stained with Amx820 and Kst1275) as determined by FISH sized around 33%. Although a much lower relative population size (1%) of anammox bacteria was determined by rRNA dot-blot hybridization, this is probably an underestimation of the true relative population size because of a low extraction efficiency of total RNA from the anammox bacteria. It was possible to determine the extraction efficiency for the anammox bacteria by isolating and hybridizing total RNA from an anammox enrichment culture which was characterized previously [8]. When corrected for the extraction efficiency (4%), the relative size of the anammox population determined with FISH (46% of EUB-stained cells) and the corrected value of the rRNA dot-blots (around 25% of the EUB signal) were much better in agreement. This discrepancy indicated that care has to be taken with total RNA-based quantification methods as long as the extraction efficiencies are unknown. Population size determinations of the filamentous-shaped organisms from the Bacteroidetes phylum (CF319a probe) by rRNA hybridization and FISH were also not in good agreement (40% and 8%, respectively). Unfortunately, we could not determine RNA extraction efficiencies for the CF319a-stained bacteria, since we did not possess pure cultures of those. It might be, however, that due to their size (10 μm in length was not unusual), the cells contained higher amounts of rRNA per cell than coccoid or rod-shaped bacteria.

The clone library did not give a good representation of the abundance of the different strains in the biofilm. Although not many clones (i.e., 26) with 16S rDNA fragments were analyzed, no sequence for the anammox or nitrite-oxidizing bacteria was found, despite the large relative sizes of those populations. In contrast, some sequences representative for (probably) minor sized populations, such as *Sphingomonas* sp., were cloned. This clone library showed once more the limitation of this technique [2]. Interestingly, however, two sequences with percentages identity below 87% were detected, which is at the division level for taxonomic positioning. Some of these

Table 2. Total organic carbon (TOC) and NH^+_4 concentrations of the leachate in different monitoring wells

Monitoring well[a]	Ammonium concentration (mM)[b]	TOC (mg C/L)[b]	Isolated DNA concentration (ng/μL)
P0	28.6	504.6	100
P1	32.5	107	100
P2	34	102.6	250
Influent to treatment system	26.2	289.5	250
P3	4.1	85.5	200
P8	8.1	21.7	15

[a] The temperature of the leachate at the sampling time ranged from 14.6 to 16.3°C
[b] Ammonium and TOC concentrations were determined 3 days before sampling for DNA extraction

proposed bacterial strains (such as within the OP3 and OP11 divisions) have never been detected by other means than cloned 16S rDNA sequences [17]. Their detection in the Kölliken biofilm suggested that the organisms belonging to these 16S rDNA sequences might naturally occur in the subsurface groundwater (GenBank entry AF424408, Table 3). However, despite FISH probing, nothing more than a very few single cells could be observed with probes for these sequences, which is not conclusive evidence for their significance or physiological role in the biofilm. It could not be ruled out, however, that these FISH-probes targeted a poor position on the 16S rRNA with respect to the fluorescence signal [9].

Structural analysis demonstrated that the aerobic nitrifiers were at the top and the anammox bacteria in the lower layer of the biofilm. Although we did not probe the oxygen profile in this biofilm, there is good reason to assume that oxygen did not penetrate much further than 100–200 μm below the surface [20] and that the layer where oxygen became absent formed the boundary for the subsistence of the anammox bacteria. Anammox bacteria have been shown to lose their activity [8, 32, 37] when exposed to oxygen for prolonged times, which also may be one reason why no anammox clusters were detected by FISH in the middle of the biofilm, although some clusters of bacteria were seen with DAPI. Nitrifying bacteria formed alternating close homogeneous clusters at the top (oxic) layer of the biofilm. This suggests that not only a vertical decreasing gradient of oxygen, bicarbonate, and ammonium existed in the biofilm, but also smaller hori-

Table 3. Composition of the 16S rDNA clone library

Accession number	Putative microorganism (species/group/phylum)	Percentage identity to other GenBank-entry	Accession number of closest relative	Name of closest related species
AJ224938	*Sphingomonas* sp.	97.2%	D84518	*Sphingomonas* sp. MK329
AJ224940	*Sphingomonas* sp.	99.4%	X85023	*Sphingomonas yanoikuyae*
AJ224939	*Staphylococcus*	99.5%	D83362	*Staphylococcus epidermidis*
AJ224941	*Nitrosomonas*	99.7%	AB079053	*Nitrosomonas* sp. ENI-11
		99%	AF353160	*N. europaea*
AJ224942	"Sphingobacteria"	86.7%	AB015254	*Cytophaga* sp. strain JTB250
AJ224943	*Planctomycetes*	97%	AF314425	Uncultured bacterium PHOS-HE93
AJ224539	OP11 division	82%[a]	AF424439	Uncultured candidate division OP11bacterium MERTZ_2CM_219 16S
AJ224540	OP3 division	93%[a]	AF424408	Uncultured candidate division OP3 bacterium MERTZ_2CM_315 16S
AJ224541	*Actinobacteria*	97%	AB015539	Unidentified Actinomycete BD2-10

[a] Not along the complete 16S rDNA sequence

Table 4. Relative abundance of the major populations of the microbial composition of the Kölliken biofilm as determined with fluorescence microscopy and RNA dot-blot hybridizations

Probe	Representative for group	Epifluorescence[a] (DAPI = 100%)	CLSM[b] (EUB-Mix = 100%)	rRNA dot-blot (EUB-Mix = 100%)
EUB-Mix	Most Bacteria	71.4 ± 19.2 (100)[c]	100	100
NEU	Halophilic and halotolerant members of the genus *Nitrosomonas*	10.3 ± 3.8 (14)	28 ± 8.8	23 ± 4.1
Nso1225	Almost all ammonia-oxidizing β-Proteobacteria	24.7 ± 15.7%	n.a.	23.3 ± 5.5
Amx820	*Candidatus* Kuenenia stuttgartiensis and *Broccadia anammoxidans*	33.4 ± 12.8 (46.8)	n.q.[d]	0.96 ± 0.86 (25)[e]
CF319a	Bacteroidetes	7.6 ± 10 (11.2)	n.q.	39.8 ± 0.8
Ntspa662	*Nitrospira*	<5% (7)	2.6 ± 2	13.7 ± 8.6
Total (NEU, Amx, Cf, and Ntspa)		56.3 ± 26.6	n.q.	78 (102)[e] ± 25

[a] Object counting on dispersed biofilm samples
[b] Biovolume determination on dispersed biofilm samples
[c] Values within brackets indicate percentages compared to the EUB signal
[d] n.a., not analyzed; n.q. not quantifiable
[e] After correcting for RNA extraction efficiency

zontal gradients of nitrite, since the nitrite oxidizer population has to rely on nitrite produced by the aerobic ammonium oxidizers. At present there is also no proven explanation as to the reason why all three nitrogen- converting populations form such dense clusters of individual cells, although such clustering has been observed before [7, 19]. The filamentous bacteria stained with CF319a were present throughout the biofilm, meaning that no specific preference for oxygen existed, although we cannot exclude that actually different strains were targeted by FISH in the different zones of the biofilm. The function of the Bacteroidetes bacteria might lie in providing a structural support for the biofilm (also seen in SEM pictures) as a whole and for other bacteria within the biofilm. Members of the phylum Bacteroidetes have been found in high abundance in various habitats, such as freshwater habitats, soil but also extreme habitats such as Antarctic marine waters [23, 48]. Their physiological repertoire may be correspondingly broad. In the biofilm they might profit from degrading traces of remaining carbon or of carbon released from dead bacteria. The main CF319a-stainable bacterium in the biofilm might be represented by the sequence AJ224942, which, however, showed only low homology to species from the genus *Cytophaga*, although it still contained the FISH-target sequence of the probe CF319a.

The structural composition of the biofilm of the rotating biological contactors (Fig. 5) correlated very nicely to the chemical model of nitrogen conversion in this and other related biofilm systems [11, 14, 20, 35]. The high numbers of aerobic and anaerobic ammonium-oxidizing bacteria in the biofilm, which are both autotrophic bacteria, explains the previously observed nitrogen loss from the influent into the secondary treatment system. The occurrence of nitrate in the effluent from the secondary RBC is the result from nitrate production of anammox and aerobic nitrite-oxidizing bacteria in the biofilms. Anammox bacteria oxidize around 15% of the total amount of consumed nitrite to nitrate to provide electrons for CO_2 reduction and biomass production [8, 18]. Since there were more ammonium than nitrite-oxidizing bacteria in the biofilm, only a part of the produced nitrite was further oxidized to nitrate by nitrite-oxidizing bacteria. Therefore both ammonium and nitrite could diffuse to the lower (anoxic) part of the biofilm where the conversion to N_2 took place. From making some rough assumptions, it can be calculated that the potential activity of the anammox biomass is higher than taking place in the biological contactor. At a measured biofilm thickness of 0.5 mm, a total surface area of the rotating biological contactor of 7238 m^2 and an average biofilm density of 50,000 gCOD $\cdot m^{-3}$ [20], the total biomass equals 181 kg. Assuming that half of the wet biomass is protein and anammox bacteria make up a fraction of 0.35, this would mean that 32 kg anammox protein is present. At a maximal specific activity of anammox bacteria of 25 nmol $N_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ [8], this would indicate that the anammox bacteria in the biofilm are potentially capable of producing 800 mmol N_2 per minute, or 1152 mol per day. During 1.1.97 to 31.12.98,

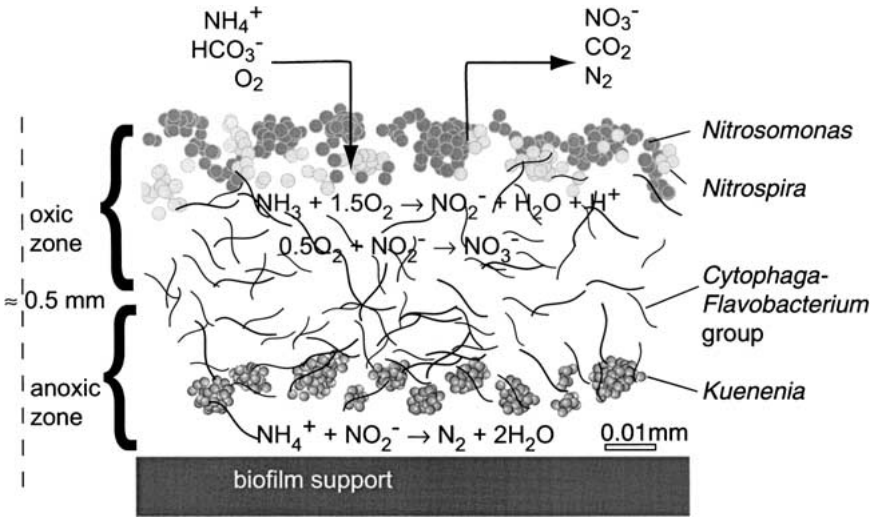

Fig. 5. Schematic drawing of the community structure of the biofilms in the Kölliken RBC and of the main nitrogen conversion reactions (based on information from Koch and Siegriest [20, 35]).

an average of 85 m^3 water containing nearly 15 mM of ammonium was flowing into the second reactor per day. This equals an ammonium load of 1275 mol/day, of which one-third (337 mol) is apparently used by the anammox bacteria for dinitrogen production. Therefore, it appears that the activity of the anammox bacteria in the biofilm is limited by ammonium. Strangely enough, the nitrite oxidizers did not compete very effectively with the anammox bacteria for nitrite and are most likely limited by the available oxygen [20]. Apparently, the redox conditions of the RBC ensured that the anammox population has sufficient competitive advantage to sustain in the biofilm despite their low growth rate.

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