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Biofilm Formation on Reverse Osmosis Membranes Is Initiated and Dominated by Sphingomonas spp.†‡

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In the water production industry, reverse osmosis (RO) membrane technology is a durable, promising, and much-used separation method. Its application enables the efficient removal of a wide variety of contaminants (i.e., microbial constituents, total dissolved solids, and organic compounds). Feed streams of different qualities (e.g., raw, natural, chemically contaminated or brackish, and seawater) are used to produce high-purity water that is microbiologically safe and biologically stable (15, 25). However, the widespread application of this technology is limited because the current generation of RO filtration units experience biofouling problems (14). The design of so-called “spiral wound” membrane elements and the conditions at the membrane, feed-side spacer, and other internal surfaces within these RO filters make them prone to microbial attachment and the subsequent formation of biofilm layers. A variety of microorganisms are involved in the development of these surface-attached complex structures after prolonged operation of the RO system, depending on the type and concentration of contaminants in the feed water and the type of pretreatment (5, 6, 7, 32, 38). The biofilm occurrence is a principal problem for proper RO system performance. It can lead to blocking of the feed concentrate channel and to clogging of the membrane. Biofilm formation results in an increased energy requirement of the feed water pumps, a lower flux, and a decrease of permeate quality (14). Conventional prevention and/or management strategies of biofouling-caused problems require more frequent chemical cleanings, thereby leading to a shortened membrane life and, ultimately, to a loss of capacity of the water supply plant (3, 14). Finding more effective ways to deal with biofouling problems in the current RO systems still needs more fundamental investigations of all aspects of biofilm formation. Little is known about the microbial community that makes up the biofilm on the membranes. To diagnose biofouling and to choose the most appropriate pretreatment and cleaning strategies, the pressure difference between the inlet and outlet channels and microbial biomass concentrations can be determined (48). Additional microbiological research, such as total cell and heterotrophic plate counts, provides some basic information (12, 23). However, such experiments do not allow for a reliable evaluation of microbial abundance and diversity of species, because the majority of the microorganisms in ecosystems cannot be cultured (21). While knowledge of real biofilm microbial composition is essential in identifying the most effective cleaning protocols, only a few molecular-based microbial diversity studies on RO membrane surfaces are reported (5, 6, 7, 32). In addition, limited data about the formation and development of biofilms over time are available. What little is known comes from laboratory-controlled biofilm monitoring studies using one or a few bacterial strains for biofilm formation (18, 19). These studies, therefore, may not provide a true representation of the RO biofilm problem in situ.

In this study, we investigated microbial biofilm formation in an experimental setup similar to an authentic RO system. Using stainless steel flow cells connected in parallel to the reverse osmosis system of a full-scale water treatment plant, the spatiotemporal development of microbial biofilms on the surfaces of new and clean reverse osmosis membranes and feed-side spacers was monitored. The bacteria responsible for the initial colonization and development of the biofilms were identified by various molecular and microscopic techniques.

† Supplemental material for this article may be found at http://aem.asm.org/.
‡ Published ahead of print on 26 February 2010.
MATERIALS AND METHODS

Experimental setup. Four high-pressure (12 bar) flow cells (design of the University of Twente, Netherlands) made of stainless steel units (AISI 316) were used to monitor the initial formation and temporal progression of biofilms. The biofilms were developed under cross-flow conditions on flat-sheet reverse osmosis membranes (19.8 by 12.7 cm; ± 0.85% porosity) and feed-side spacers (0.7 mm thick) excised from a commercial spiral-wound ESPA (energy-saving polyamide) membrane element (ESPA 2; Hydronautics, CA). To mimic the authentic environment of a conventional RO system, the flow cells were connected (Fig. 1) in parallel with RO systems of a full-scale RO water purification plant in Veenendaal (Netherlands). In continuous-flow mode, the RO feed water—fresh surface water treated by the sequential application of coagulation, flocculation, and sand filtration (CSF), the ultrafiltration (UF), and the cartridge filtration (CF) processes—and used as the feed to the 2-stage RO system and to the connected flow cells. The plant produced process quality water (P).

Sampling procedures. At the end of each experiment, the RO membrane and the feed-side spacer were removed from the flow cell. Different small sections from randomly selected positions on the membrane and spacer along the length of the feed channel were carefully cut out and immediately fixed. For the total-DNA extractions, the samples (1.5 by 2.0 cm) were transferred into sterile 1× phosphate-buffered saline (PBS) (0.5 ml) and kept on ice. For use in field emission scanning electron microscopy (FESEM), the samples (0.5 by 0.5 cm) were immersed in a solution of 2.5% glutaraldehyde in 1× PBS (pH 7.0). For epifluorescence and confocal laser scanning microscopy (CLSM), the samples (0.5 by 2.0 cm) were fixed with 4% paraformaldehyde or 50% ethanol. Samples were transported to a laboratory for further processing.

DNA extractions, PCR, DGGE, cloning, and sequencing analysis. The extraction of the total community DNA from the collected biofilm samples, PCR amplification of bacterial 16S rRNA gene fragments, denaturing gradient gel electrophoresis (DGGE) separation of the generated amplicons, and construction and analysis of the 16S rRNA gene clone libraries were carried out as previously described (5). Using BioNumerics software (version 4.0; Applied Maths, Belgium), a similarity dendrogram was constructed from the normalized correlation coefficient (47) and by the unweighted pair group method with arithmetic average (UPGMA) clustering (41).

Scanning electron microscopy. After 2 h in fixative, the samples were gently washed three times for 15 min with 1× PBS, postfixed for 15 min with a solution of 1% OsO4 in 1× PBS, and rinsed twice with MilliQ water. Subsequently, they were dehydrated by sequential immersing in an ethanol series (10, 30, 50, 70, 90, and 100%) and critical-point dried with carbon dioxide. The dried samples were sputter coated with 10 nm platinum in a dedicated cryopreparation chamber (CT 1500 HF; Oxford Instruments, United Kingdom) and examined with a FESEM (JEOL JSM-6300F; JEOL, Japan) at a working distance of 8 mm and with an accelerating voltage of 5 kV. Optimization of the digitally recorded images was done using Adobe Photoshop (Adobe Systems, Inc., CA).

FISH. Fluorescence in situ hybridization (FISH) analysis was conducted using a modification of the previously described methods (26, 44). Following fixation (1 h), the samples were gently rinsed twice with sterile 1× PBS, dehydrated by sequential immersions in an ethanol series (50%, 80%, and 96%, for 3 min each), and incubated for 20 min at 46°C in 2 ml of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], and 0.01% SDS). Then, the oligonucleotide probes (Eurogentec, Netherlands) EUB338-l, -II, and -III (EUB338-l/III), ALF986, BET42a, GAM42a, CF319a, HOC69a, SPH120, Burkho, and ARCH1915 were added to each sample individually or in combinations of two different probes simultaneously. Probe NON338 was used as a negative control (49). The probes were labeled with cyanine (Cy3/Cy5) or fluorescein isothiocyanate (FITC) at the 5′ end. The hybridization was performed for 3 h at 46°C under stringency conditions appropriate for each probe. The specific details about the hybridization conditions for each of the probes used and literature references can be found in probeBase (24). After hybridization, each sample was transferred to a vial containing 20 ml of prewarmed (48°C) washing solution (20 mM Tris-HCl [pH 7.4], 5 mM EDTA, 0.01% SDS, and a concentration of NaCl appropriate for each probe combination) and then incubated at 48°C for 20 min. Following hybridization, the samples were briefly rinsed in MilliQ water and counterstained for 30 min at 4°C with 20 μM Syto13 (Molecular Probes, Netherlands) or 10 μg/ml DAPI (4′,6-diamidino-2-phenylindole). Each sample was also stained with 10 μg/ml Calcofluor white or 10 μg/ml FITC-labeled concanavalin A (ConA), both purchased from Sigma-Aldrich. After 30 min, the stained samples were rinsed with MilliQ water, air dried in the dark, and mounted in a Vectashield medium. Immediately after staining, the samples were examined by epifluorescence microscopy and, the next day, by confocal laser scanning microscopy.

Epifluorescence microscopy. Hybridized/stained bacterial cells and their extracellular polymeric substances were visualized with a Leica DM6000 epifluorescence microscope equipped with four filter sets (Table 1). The numbers of DAPI-stained cells were determined in 20 randomly chosen microscopic viewing fields. All counts were done in triplicate. The images were captured with a Leica DFC350FXR2 digital camera and analyzed with Leica Application Suite (LAS) software. The microphotographs obtained, stored as separate digital files, were optimized using Adobe Photoshop.

Confocal laser scanning microscopy. Biofilm samples were examined on an LSM 510 META laser scanning microscope (Carl Zeiss, Germany), using a Plan-Apochromat 63×/1.4 oil (differential interference contrast [DIC]) lens. Images of samples labeled with three multiple fluorochromes were visualized simultaneously using a multitrack mode. The optimum setting was determined in a preexperiment and subsequently used for all the samples. Series of horizontal (x-y) optical sections were taken throughout the length of each sample at regular intervals (1 μm) across the z axis. At least three different regions were scanned at the surface of each biofilm. The captured image stacks were evaluated afterwards with LSM5 Image Examiner (Zeiss, Germany). The total biomass area and probe-stained area were measured from CLSM projection images using image analysis software provided by Zeiss. The reconstructed three-dimensional representations and in situ visualizations of biofilms were further processed with Adobe Photoshop.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this study were submitted to GenBank under the accession numbers GQ385249 to GQ385296.

RESULTS

General observations during autopsy. Four reverse osmosis (RO) test flow cells were operated for 4 to 32 days parallel to a full-scale RO installation. They were fed with the same water.

FIG. 1. Schematic outline of the reverse osmosis (RO) system of a full-scale water purification plant. The fresh surface water (F) was extensively treated by the sequential application of coagulation, flocculation, and sand filtration (CSF), the ultrafiltration (UF), and the cartridge filtration (CF) processes and used as the feed to the 2-stage RO system and to the connected flow cells. The plant produced process quality water (P).
at the same linear flow velocities as the first 20 cm of the full-scale installation. After several days, the test flow cells were opened and the fouling at the surfaces of the RO membranes (Fig. 2A) and their feed-side spacers (Fig. 2B) was visually examined. After 4 days of flow cell operation, a fouling layer and deposits of the rejected feed water components were already visible at the entrance of the flow cell (Fig. 2, 4d). During the experiment, the fouling gradually expanded over the surfaces (Fig. 2, 8d, 16d, and 32d). A muculent light-brown fouling layer was quite homogeneously distributed over the surface, while dark-brown-colored deposits were spread rather irregularly. The dark-brown deposits were most numerous on the membrane and spacer surfaces at the entrance of the flow cell. In general, all the membrane surfaces examined were more intensely fouled than their associated spacer surfaces.

**SEM imaging of biofilms.** The structure of the initial fouling layer was observed by scanning electron microscopy of a membrane sample from the flow cell that was operated for 4 days. It revealed the presence of both single cells and cells embedded in a polymeric gel layer. Cocci, spirilla, and (mainly) rod-shaped bacteria were observed on the RO membrane and on the spacer. Various mineral-like deposits were present on the membrane surface. Many single bacteria were spread irregularly over the entire membrane surface and showed no specialized structures around their cells. Rod-shaped bacterial cells with an average size of 0.3 to 0.8 by 1 to 2 μm were clearly involved in the formation of typical biofilm layers attached to the membrane and feed-side spacer surfaces. These cells were present in the form of microcolonies embedded in a common extracellular polymeric substance (EPS) matrix on the membrane, with a 2- to 10-μm cell-to-cell separation. The matrix showed two clearly distinguishable structures: a thin regular layer, presumably exopolysaccharide, stretched out directly around the bacterial cells and a compact, irregular layer of granular matter distributed randomly on top of the first layer (Fig. 3A). Some of the bacterial cells with an average size of 0.5 to 1 by 1.5 to 5 μm started to form compact aggregates of 3 to 9 cells embedded in a thin (<0.5 μm) exopolymeric matrix. The remaining foulants were associated with solid components (such as colloidal or particulate matter, pieces of a loose network of extracellular polymeric fibrils, and flocks [clumps of bacterial cells and EPS matrix]). Most of these foulants were distributed randomly over the entire membrane surface, whereas the aggregates were primarily observed at the entrance of the flow cells.

Within days, the preliminary biofilm layers, the microcolonies, and the aggregates increased considerably in size and amount (Fig. 3B). The mature biofilm that formed subsequently (at day 16) displayed a complex heterogeneous structure and was spread uniformly over the entire membrane surface. Various microcolonies and single cells were positioned on top of a surface-covering monolayer of rod-shaped cells. This monolayer increased in cell density over time, and at day 32, an even more complex and thicker biofilm structure was observed.

<table>
<thead>
<tr>
<th>Target</th>
<th>Staining</th>
<th>Filter cube</th>
<th>Excitation filter (nm)</th>
<th>Suppression filter (nm)</th>
<th>Dichromatic mirror (nm)</th>
<th>Laser Line (nm)</th>
<th>Emissions (nm)</th>
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<tr>
<td>Total cells</td>
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<td>I3</td>
<td>BP 450–490</td>
<td>LP 515</td>
<td>510</td>
<td>Ar</td>
<td>488</td>
<td>BP 505–550</td>
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<tr>
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<td></td>
<td>A</td>
<td>BP 340–380</td>
<td>LP 425</td>
<td>400</td>
<td>Diode</td>
<td>405</td>
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<tr>
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<td>FITC</td>
<td>I3</td>
<td>BP 450–490</td>
<td>LP 515</td>
<td>510</td>
<td>Ar</td>
<td>488</td>
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<td>HeNe</td>
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<tr>
<td>EPS component</td>
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<td>EPS component</td>
<td>Calcofluor white</td>
<td>D</td>
<td>BP 355–425</td>
<td>LP 470</td>
<td>455</td>
<td>Diode</td>
<td>405</td>
<td>BP 420–480 IR</td>
</tr>
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* a-α-Mannopyranosyl and glucopyranosyl sugars of the biofilm EPS matrix.

* b-1,4 and b-1,3 polysaccharides of the biofilm EPS matrix.

**FIG. 2.** Photographs of fouled reverse osmosis membranes (A) and their feed-side spacers (B). The membranes and spacers were removed from the flow cells after 4 (column 4d), 8 (column 8d), 16 (column 16d), and 32 (column 32d) days of operation. The direction of the feed water flow along the length of each flow cell was from left to right.
A small number of unicellular eukaryotes, e.g., diatoms and protozoa, was occasionally observed on top of the biofilm (see Fig. S1 in the supplemental material).

**Biofilm community structure as revealed by 16S rRNA gene clone libraries.** In total, three 16S rRNA gene clone libraries were constructed with a *Bacteria* primer set (7-f and 1510-r), using total genomic DNA isolated from the 4-, 8-, and 16-day RO membrane biofilm samples, respectively. All clones in the libraries were subjected to restriction fragment length polymorphism (RFLP) analysis, and clones with identical RFLP patterns were grouped together into clone families. One representative clone from each clone family was partially sequenced. Subsequently, the full sequence of the 16S rRNA gene of those clones that contained a unique sequence and corresponded with a dominant band in the community DGGE fingerprints was determined. The nonchimeric nucleotide sequences of the 272 clones (90 4-day, 87 8-day, and 95 16-day clones) were further analyzed for their phylogenetic affiliation. The nonchimeric nucleotide sequences of the 272 clones (90 4-day, 87 8-day, and 95 16-day clones) were further analyzed for their phylogenetic affiliation and identification of their closest relatives. Different sequence types (operational taxonomic units [OTUs]) affiliated with various phylogenetic lineages of the domain *Bacteria* (with sequence similarities of $\geq 0.90$) were obtained from the clone libraries (see Table S1 and Fig. S2 in the supplemental material).

Phylogenetic analysis indicated that the *Proteobacteria* division dominated all clone libraries in this study (at 4 and 8 days, 100% of the total clones, and at 16 days, 95%). The *Alphaproteobacteria* subdivision was the largest bacterial group found in the 16-day-old biofilm sample (44% of the total clones) and the second-most-abundant fraction in the 4- (27% of the total clones) and 8- (31% of the total clones) day-old biofilms. The *Betaproteobacteria* subdivision was most frequently encountered in the 4- (67% of the total clones) and 8- (66% of the total clones) day-old biofilms and was the second largest fraction in the library after 16 days (30% of the total clones). All biofilm samples further comprised OTUs from the *Gammaproteobacteria* division (at 4 days, 7%, at 8 days, 2%, and at 16 days, 20% of the total clones). The majority of the *Alphaproteobacteria* OTUs found in all samples were affiliated with the *Sphingomonas* genus. The *Sphingomonas* genus was the most frequently encountered bacterial genus in the 16-day library (31% of the total clones). The remaining clones in this group were closely related to other members of the *Alphaproteobacteria* subdivision, including *Sphingopyxis* spp. (3 to 10% of the total clones), *Azospirillum* sp. (2 to 3% of the total clones), and *Hyphomicrobiun* spp. (at 4 days, 3% of the total clones) and *Nitrosomonas* spp. (38% of the total clones). The members of the *Betaproteobacteria* division, *Candidatus* “*Nitrotoga arctica*,” *Nitrosomonas* spp., and members of the order *Burkholderiales* were common in all samples. *Candidatus* “*Nitrotoga arctica*” represented the largest fraction in the 4-day library (27% of the total clones), and the most dominant bacterial genus in the 8-day biofilm was related to *Nitrosomonas* spp. (38% of the total clones). The members of the *Burkholderiales* group found in the biofilms (at 4 days, 6%, at 8 days, 21%, and at 16 days, 15% of the total clones) consisted mainly of *Acidovorax*, *Aquaomonas*, *Aquaspirillum*, *Polaromonas*, *Variovorax*, and *Xylephilus* species and bacteria belonging to the family *Comamonadaceae*. The bacteria related to the *Aquaspirillum* genus and the *Comamonadaceae* family were common in all biofilm samples. Only the 4-day sample contained clones related to *Nitrosospira* spp. (3% of the total clones). In the 4-day and 16-day biofilms, 1 to 7% of the total clones were related to uncultured *Betaproteobacteria* species. The remaining sequences (at 4 days, 1%, and at 8 days and 16 days, 2% of the total clones) identified as belonging to the *Betaproteobacteria* subdivision were related to betaproteobacterium HIBAF011 (97% similarity) or to betaproteobacterium A0637 (95% similarity). Within the *Gammaproteobacteria* lineage, the most frequently encountered OTUs from all biofilm samples were closely related to the *Pseudomonas* genus (at 4 days, 7%, at 8 days, 2%, and at 16 days, 19% of the total clones). In the 16-day biofilm, 1% of the total clones showed 93% similarity.
with *Aquicella* spp. and 5% of the total clones were related to *Nitrosira* spp.

**Fingerprinting the biofilm communities by DGGE.** The DGGE analysis of the PCR-amplified fragments (415 bp) of the hypervariable V6 to V8 region of the bacterial 16S rRNA genes, retrieved from biofilm samples, revealed discriminative "fingerprints" (see Fig. S3 in the supplemental material) of the bacterial communities that had developed on the RO membranes in 4, 8, 16, and 32 days (Fig. S3, lanes 1M1, 1M2, 1M3, and 1M4, respectively). About 5 to 9 sharp DGGE bands and 8 to 14 vague bands were observed in each community pattern. In total, 19 distinct DGGE bands could be associated with at least one of the identified clones in the constructed clone libraries (see Table S1 in the supplemental material). Five of these bands (3 (98% similar to a bacterium belonging to the *Comamonadaceae*), 6 (96 to 98% similar to *Pseudomonas* spp.), 10 (99% similar to *Sphingopyxis* spp. and 96 to 97% similar to *Sphingomonas* spp.), 11 (98% similar to *Nitrosomonas* spp.), and 16 (97% similar to *Sphingomonas* spp.) were observed in all biofilm fingerprints but with various band intensities. The remaining distinct bands showed an infrequent pattern of occurrence and abundance in the fingerprints examined. Overall, the community DGGE profile derived from the youngest biofilm appeared to be less complex (fewer dominant bands were apparent) than those derived from the mature biofilms. The similarity dendrogram revealed that the DGGE pattern of the 4-day biofilm was relatively similar (41%) to the 8-day fingerprint but clearly different from the profiles at 16 days (16% similarity) and 32 days (19% similarity). The 16-day biofilm fingerprint clustered with the 32-day biofilm pattern (46% similarity), though each of them appears to have several unique bands.

**Community composition as revealed by FISH.** The number of DAPI-stained bacterial cells recovered from the RO membranes after 4, 8, 16, and 32 days increased exponentially (3.8 × 10^7 cells/cm^2, 3.6 × 10^8 cells/cm^2, 4.1 × 10^8 cells/cm^2, and 3.2 × 10^8 cells/cm^2, respectively) Approximately 0.8% of the cells were attached to the 32-day feed-side spacer (2.4 × 10^8 cells/cm^2). Fluorescence in situ hybridization analysis of biofilm-forming communities in membrane samples showed that more than 95% of the DAPI-stained cells were detectable with the EUB338-I/II/III probe (10). The majority (~95%) of those hybridized with probes targeting members of the *Alphaproteobacteria* (at 4 days, 25%, at 8 days, 30%, at 16 days, 45%, and at 32 days, 50%), *Betaproteobacteria* (at 4 days, 65%, at 8 days, 60%, at 16 days, 30%, and at 32 days, 25%), and *Gammaproteobacteria* (at 4 and 32 days, 5 to 10%, at 8 days, 1 to 5%, and at 16 days, 15 to 20%) (Fig. 4C). The application of two species-specific probes (SPHI20 [28] and Burkho [29]) resulted in the identification of members of the genera *Sphingomonas* (dominant *Alphaproteobacteria* genus) (Fig. 4A) and *Burkholderiales* (common *Betaproteobacteria* genus) (Fig. 4B) in all biofilms. High FISH detection rates of these groups were consistent with the results obtained by the cloning method (see Table S1 in the supplemental material). In contrast with the data obtained from the clone libraries, members of the *Cytophaga, Flexibacter, and Bacteroidales* (CFB) division were discovered in all biofilms (at 4 days and 8 days, 1 to 2%, at 16 days, 3 to 5%, and at 32 days, 5 to 10%) (Fig. 4D). In addition, 1 to 2% of the bacteria in all samples hybridized with probes targeting *Planctomycetes* (EUB338-II) and *Verrucomicrobiales* (EUB338-III) cells. For all biofilm samples examined, FISH analyses did not show autofluorescence or hybridization with the ARCH (42) or the NONEUB probe (49). Extracellular polysaccharides associated with bacterial cells were detectable with both FITC-ConA (*Sphingomonas* and *Gammaproteobacteria*) and Calcofluor white (*Betaproteobacteria, Gammaproteobacteria, CFB,* and *Verrucomicrobia*).

**Biofilm architecture.** Epifluorescence microscopy revealed a random distribution of *Alpha-, Beta-, and Gammaproteobacteria* cells over the entire RO membrane surface after four days of flow cell operation. Some of the *Beta- and Gammaproteobacteria* (Fig. 4B1 and C1) form initial microcolonies (3 to 20 cells) with average sizes of 2 to 10 μm. Around some of these microcolonies, a thin EPS layer was observed. Primarily at the entrance of the flow cell, mixed-species clusters (up to ~10 μm thick and ~20 μm wide) of diverse and overlapping *Beta- and Gammaproteobacteria* microcolonies (20 to 50 cells) were observed covering about 10% of the membrane surface. On the surface of these clusters, single cells of bacteria related to *Sphingomonas*, CFB, *Planctomycetes*, and *Verrucomicrobia* were randomly attached. Around some of the microcolonies, a relatively thin EPS matrix was present. In contrast, most of the dominant *Alphaproteobacteria* (*Sphingomonas*) cells were observed in groups (7 to 24 cells) within an EPS matrix, up to 30 μm wide and stretched in the flow direction over the membrane surface area (Fig. 4A1). Around 20% of the total membrane surface area was covered with a 1-μm-thick monolayer of *Sphingomonas* cells.

At day 8 (Fig. 4A2), the *Sphingomonas* monolayer covered ~40% of the total membrane surface area and the first microcolonies of CFB (Fig. 4D2) and *Alphaproteobacteria* emerged on the membrane surface. The beta- and gammad proteobacterial microcolonies (Fig. 4B2 and C2) were larger and more abundant. At days 16 and 32, the biofilm appeared as a dense heterogeneous structure in the epifluorescence images (Fig. 4, columns 3 and 4) and scanning electron microscopy images. Only the top of this highly complex structure was visible. The CLSM examinations of the biofilms provided images of complex multispecies biofilm layers (Fig. 5) with thicknesses of 4 to 8 μm at day 16 (data not shown) and 5 to 10 μm at day 32. Both biofilms exhibited similar architecture. The uniform layer of *Sphingomonas* cells, embedded in a common 2-μm- to 3-μm-thick EPS matrix, was stretched directly over the membrane surface and covered 70 to 100% of the total area. The maximal cell density was observed near the top of the layer. On top of the *Sphingomonas* layer, a heterogeneous layer with average thicknesses of 2 μm (16-day biofilm) and 3 μm (32-day biofilm) was observed. This second biofilm layer consisted of a mixture of different *Alpha-, Beta-, and Gammad proteobacteria, CFB, Planctomycetes,* and *Verrucomicrobia* cells and their microcolonies. The single cells of *Sphingomonas* spp. were quite uniformly spread within the EPS matrix of the layer, while the distribution of the remaining community members was rather variable. The maximal cell distribution of the *Verrucomicrobia* cells was observed on top of the layer, while most *Planctomycetes* cells colonized the base. Most of the *Betaproteobacteria* microcolonies clustered together as tower-like structures that were 3 to 5 μm high at day 16 and 4 to 7 μm high at day 32, which obviously rose above the surface of the layer. On these
structures, single cells of *Sphingomonas* and *Verrucomicrobia*, combined with various CFB and *Gammaproteobacteria* microcolonies, were frequently detected.

Examination of the distribution of the EPS matrix in the confocal images revealed that the majority of EPS (~80%) within the mature (16 and 32 day old) biofilms was localized directly on top of the RO membrane surface and around *Sphingomonas* cells. The other members of the biofilm community displayed limited EPS development. In the confocal sections, they usually appeared as dense compact clusters of cells (microcolonies) with an EPS matrix just around the cells. The biofilm density and EPS concentration increased over the period of flow cell operation. The highest values for both were recorded after 32 days.

**DISCUSSION**

**Experimental approach.** In the current field of RO biofouling research, most biofilm-monitoring studies have been carried out in simplified laboratory systems with one (18, 19) or a few (34) bacterial strains. Though these model systems contribute to our fundamental understanding of bacterial biofilms, they may not provide a true representation of the biofilm problem *in situ*. The uncertainties with the extrapolation of the results obtained to the natural system are a principal drawback of this approach. The impact of general environmental conditions (e.g., flow properties, osmolarity, temperature, pH, etc.) on these complicated processes was already recognized some time ago (50). Direct observation of microbial processes in

**FIG. 4.** Epifluorescence micrographs depicting mode of initial formation and spatiotemporal development of biofilm structures by pioneer colonizers of RO membrane surfaces. Horizontal panels (A to D) represent images of microcolonies (red and pink fluorescence) as follows: “carpets” of *Sphingomonas* spp. (A) and “patches” of members of the *Betaproteobacteria* (B), *Gammaproteobacteria* (C), and CFB (D). The ages of the biofilms are represented in the vertical columns, with columns 1 to 4 showing images from 4, 8, 16, and 32 days, respectively. Red fluorescence in the images was acquired from the Cy3-labeled probes (SPH120, BET42a, GAM42a, and CF319a), while blue is from the DAPI-stained cells or from Calcofluor white-stained β-1,4-linked polymers of the biofilm EPS matrix, and green is from the positive interaction of FITC-ConA with α-D-glucose and α-D-mannose. Bars, 5 μm (C1) and 10 μm (the other images).
spiral-wound RO modules is only possible after autopsy of the membrane unit or with the recently published magnetic resonance imaging (MRI) methods (17). Autopsy is done rarely and only in cases of severe fouling, and MRI is limited to small membrane modules that fit in the MRI sampling tube. In this study, we used stainless steel flow cells connected in parallel to a full-scale reverse osmosis system to monitor microbial biofilm formation on the surfaces of new and clean reverse osmosis membranes and feed-side spacers. This approach allowed the investigation of microbial biofilms under conditions similar to those in the full-scale RO system with respect to the membrane, feed-side spacer, feed water, temperature, pH, nutrient conditions, pretreatment, microbial population, and operation mode used. With multiple flow cells, we were able to study the development of biofilm formation over time in situ with different molecular and microscopic techniques. We propose that representative flow cells integrated in a full-scale membrane installation are the preferred experimental tools to increase our understanding of fouling phenomena in (spiral wound) membrane systems.

Colonization of new surfaces. The sequence observed in the colonization of new RO membrane and spacer surfaces is similar to biofilm formation on solid surfaces (9, 11, 22). The process consists of the following events: (i) the transport of biological material to the surfaces, (ii) the attachment of primary colonizers, (iii) the initiation of early biofilm structures, and (iv) a spatiotemporal development into a multispecies slime layer with a complex three-dimensional architecture. In our experiments, we clearly observed two additional aspects: cells that mainly adhered in clumps and grew out as such (Fig. 3B and 4B and C) and cells that mainly adhered as single cells and colonized the surface almost as a monolayer (Fig. 3A and 4A and D).

In previous studies (5, 6), we showed that the feed water of the RO system (UF permeate passed through cartridge filtration) contained a broad diversity of typical freshwater phylotypes (51) (Alpha-, Beta-, and Gammaproteobacteria, Planctomycetes, Verrucomicrobia, and members of the Cytophaga-Flexibacter-Bacteroides group). In this study, we observed that not all of these feed water bacteria were capable of active colonization of the membrane and spacer surfaces.

Most of the early biofilm structures were found at the flow cell entrance. These structures were composed of different members of the Betaproteobacteria subdivision and Pseudomonas genus. The pioneering success of the Betaproteobacteria and Pseudomonas (Gammaproteobacteria) cells was related to their prevalent existence as clumps, i.e., free-floating feed water aggregates of EPS-embedded bacterial cells (Fig. 3B). Most likely the clumps were detached parts of biofilms that were present upstream in the production plant. The dominance of these organisms in the aggregates may indicate their prevalence in the upper layers of the mature biofilms in the upstream compartments of the plant (see also the discussion on mature biofilms below). This may be especially the case for the nitrifiers, a dominant (53%) fraction of the membrane-associated population (see Table S1, 1M1, in the supplemental material), given that they usually represent a negligible fraction (~1%) of the bacterial population in the feed (fresh surface) water of the plant (5). According to the results of epifluorescence and scanning electron microscopy, the extracellular polymeric substances of the aggregates facilitated attachment of the indigenous bacteria to both rough (membrane) and smooth (spacer) surfaces. In contrast, single, nonaggregated cells were not found at the spacer surface within the first 4 days of flow cell operation. A few individual Betaproteobacteria and Pseudomonas cells were present on the rougher membrane surface. Studies of attachment have shown that surface physicochemical characteristics influence bacterial adhesion and biofilm formation to only a minor extent (16, 46). The presence of bacterial external appendages (e.g., flagella and type IV pilus) and extracellular polymers (i.e., polysaccharides) (8, 29) were the key determinants of colonization efficacy (31, 45). The bacteria within the aggregates proliferated after attachment, whereas their single-cell counterparts remained small or showed reduced cell division, indicating starvation (27). Under conditions of substrate-limited growth on the initially clean membrane surface, the growth of the aggregated organisms and their subsequently formed biofilm structures may be supported by accumulation of feed water nutrients in the indigenous EPS matrix. The clumps were more abundant on the membranes and spacers at the entrance of the flow cell, showing that their transport along the surfaces was evidently constrained by the stickiness of the EPS structures (30) and by the filtration effect.
of the membrane/spacer configuration, similar to that of commercial spiral-wound RO modules (Fig. 2).

The members of the Alphaproteobacteria subdivision in the biofilm presumably also originated from the mature biofilms of the upstream compartments of the plant. In the previous study (5), the genus Sphingomonas represented a major fraction (~25%) of the sessile communities in the cartridge filter and ultrafiltration storage tank but was less dominant (~7%) in the planktonic community of the RO plant feed water. In contrast to the other pioneers, the majority of the Alphaproteobacteria colonizers, consisting of various Sphingomonas spp., were present as dispersed cells in the feed water of the RO system. Planktonic Sphingomonas cells have been reported to indicate depletion of suitable carbon sources and/or oxygen in the environment, i.e., oligotrophic conditions (36). Through the change from biofilm mode to planktonic mode, these bacteria are able to colonize new suitable environments. Traces of a broad range of naturally occurring organic compounds are supposed to be sufficient for growth, since sphingomonads are metabolically versatile organisms and have high-affinity uptake systems under nutrient-limiting conditions (4, 13, 39). It is postulated that after finding a suitable microenvironment, the Sphingomonas-like bacteria irreversibly attach by producing exopolysaccharides around their cells (2, 36, 37). This behavior leads to a relatively fast spreading of the cells over the membrane and spacer surfaces and make them the real colonizers of the membrane area. The wide spreading of the Sphingomonas EPS matrix over the membrane surface (Fig. 3A and 4A) could well be due to the shear stress caused by the fluid flow (43). Surface spreading also leads to enhanced substrate availability per cell compared to the availability of substrate to a dense packing and is advantageous in oligotrophic systems. The observed rapid spreading of the sphingomonads, concomitantly producing a layer of EPS on the surface, makes them a prime target for potential biofouling control approaches. They might not be the dominant organism in the fouling layer (7, 33), but their almost unicellular layer and high level of EPS production likely gives them a more substantial contribution to membrane biofouling than aggregate-forming bacteria.

**Mature biofilm architecture.** It is remarkable that within a relatively short operational time (approximately 1 month), the biofilm reached a structure similar to that of a 5-year-old fouling layer that was observed in a previous study in a membrane module from the same water production plant (6). This general biofilm structure is shown schematically in Fig. 6. We observed a 2- to 3-μm-thick base layer dominated by the Sphingomonas-like bacteria on which towers of other microbial species grew. This is very similar to the observations on biofilm formation by motile and nonmotile cells reported by Siebel and Characklis (40) and Picoreanu et al. (35). It seems that the biofilm-associated sphingomonads have a different ecology than most of the other observed bacteria. The members of the Sphingomonas genus appear to leave the biofilm as individual cells, which enables them to colonize new surfaces and efficiently spread over the entire surface. The other main colonizers (i.e., Beta- and Gammaproteobacteria) appear to grow in microcolonies that detach at a certain moment from a mature biofilm and adhere as an aggregate somewhere else. This is in line with the observations of the microbial population in the feed water. On top of the initial biofilm, a secondary group of bacterial colonizers occurs in time. These secondary colonizers (mainly present in the feed water as individual cells) consist of the Cytophaga-Flexibacter-Bacteroides group and Verrucomicrobia, Burkholderiales, and Planctomycetales representatives. These bacteria appear to grow on microbial or decay products from the primary colonizers. They are observed as dispersed cells in and on the secondary Sphingomonas layer and on the towering microcolonies. The postulated growth on decay and microbial products explains why, in the first stages of the colonization, these bacteria do not grow in the initial biofilm despite their presence in the feed water. When the biofilm is observed by microscopy from the top of the film, it appears as if sphingomonads are not an important population in the biofilm system. This could also appear to be the case from the cloning and DGGE data. However, in reality, they form a thin base layer on which other types of bacteria develop. Their EPS matrix appears to form the basic layer leading to extra concentration polarization in reverse osmosis systems.

**Conclusion.** Many bacteria play a role in biofilm formation on RO membranes, but from the results of this study, it appears that sphingomonads are the key biofouling organisms. They rapidly colonize the entire membrane and spacer surfaces and cover them with their EPS. It is likely that sphingomonads are also responsible for the initial biofilm formation in other systems where fresh surface water is exposed to surfaces. The extensive EPS synthesis by these organisms results in modified surfaces onto which other microorganisms are able to attach and proliferate. This study is therefore also relevant to other technical systems where biofouling occurs under oligotrophic conditions (e.g., heat exchangers and drinking water distribution systems) and natural systems. In biofouling control experiments, sphingomonads might be good model organisms to study in detail the initial attachment and growth of biofilms on various wet surfaces.

![FIG. 6. A schematic representation of the observed biofilm structure in a mature RO membrane biofouling layer. Single planktonic cells of Sphingomonas spp. and clumps of Beta- and Gammaproteobacteria present in the feed water colonize surfaces.](image-url)
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