BIOFILM DEVELOPMENT

ON NEW AND CLEANED MEMBRANE SURFACES

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Ludmila Anatoljevna Bereschenko

Thesis

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Dedicated to my kids,
Artem, Arizo en Fawad

PREFACE

This thesis is a result of a PhD study, carried out between 2004 and 2009 in a close and inspiring collaboration with many professionals from Wetsus, Waterlaboratorium Noord (WLN), Evides, Global Membrains, Vitens, Norit, Shell Global Solutions, X-flow, Water companies from Groningen (WbG), Drenthe (WMD) and Noord-Holland (PWN) and researchers from the Wageningen (WUR) and Delft (TUD) universities, with Prof. dr. ir. A. J. M. Stams and Prof. dr. ir. M. C. M. van Loosdrecht as the supervisors and Dr. Gert-Jan Euverink as a co-supervisor. Many of the participants have to be acknowledged for their commitment in realization of this thesis.

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Secondly, I would like to express my gratitude to all the participants of the Wetsus, WLN and Norit “Biofouling team” meetings and the various PhD- and/or research meetings at Wetsus, SENSE, WUR and TUD. Expertise, advice and inspiring enthusiasm of many of you have helped me to understand what is important and true for doing this study in a scientifically proper way. Cooperation, friendship and/or stimulating personal conversations with many of you (Astrid Roosjen, Jan Post, Johannes Boonstra, Guillo Schrader, Hans-Curt Flemming, Hans Vrouwenvelder, Hardy Temmink, Henry van Straten, Koos Baas, Michel Saakes, René Rozendal and Sybrand Metz) have changed my perspective on some aspects of life and helped me to function as a professional. Charming personality of many of you (Agata, Aleid, Adriaan, Bart, Ingo, Joost, Elsemiek, Helena, Hellen, Lucia, Loes, Luewton, Marko, Marthe, Maxime, Nelleke, Natasja, Nienke, Petra, Paula, Piotr, Phillip, Ronald, Sandra, Tim, Trienke and Urania) have inspired my optimism and perseverance at some of the critical moments of this development.

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Last but not least, I would like to thank my family and friends for finding a thousand and one manner to support me during this exciting period of my life. I admire their courage and altruism and am grateful for their continuous understanding, trust, loyalty, encouragement and/or cooperation in everything. Frits and Ger, thank you both for the trust in me and helping me out when I needed it most. Antoon, how I wish you could see me now.

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Ludmila A. Bereschenko

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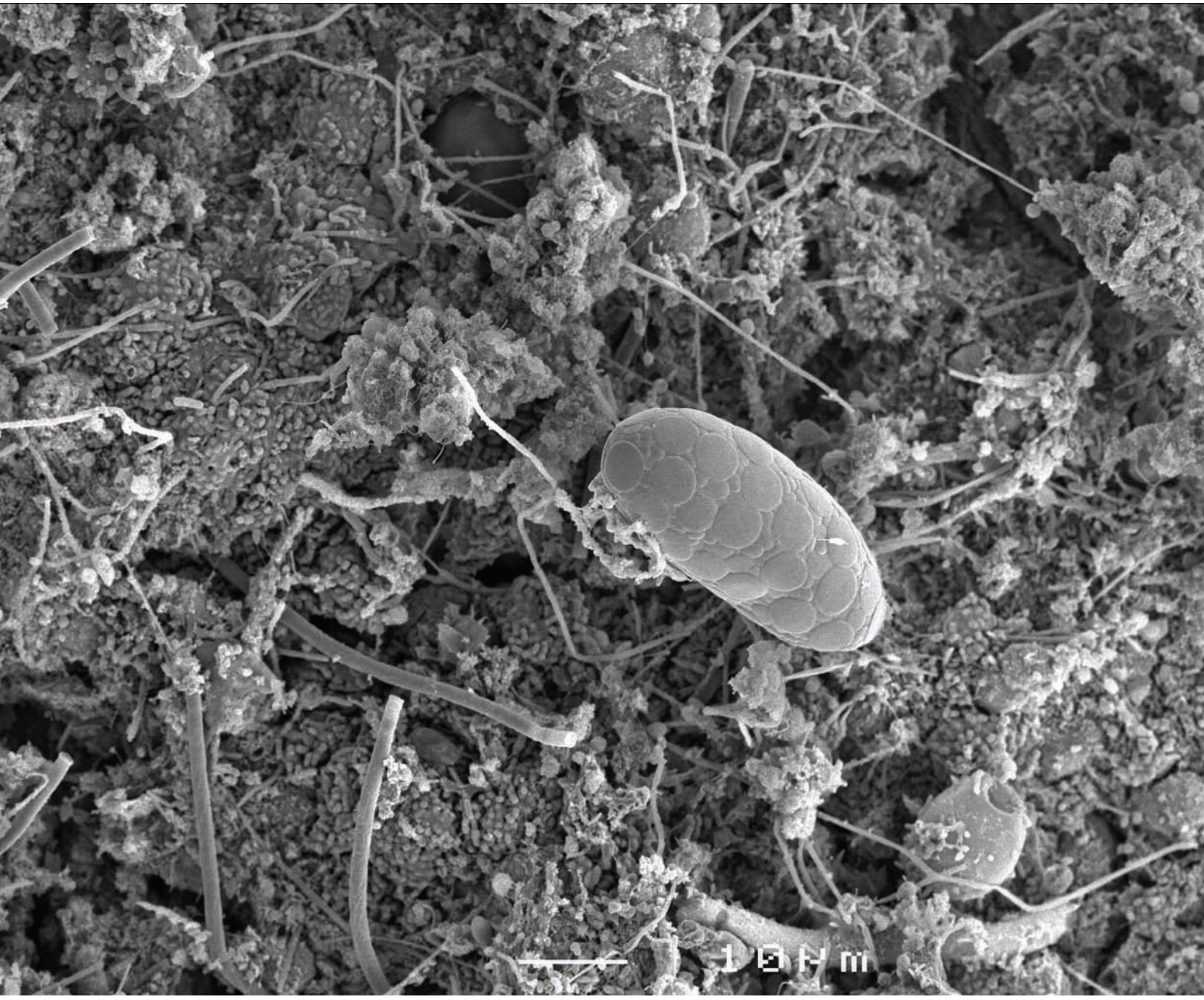
1 General Introduction



Biofouling: challenge for membrane treatment processes

Membrane technology based water treatment systems have gained considerable interests in the past decades due to the ability to remove a large number of compounds in a single purification step. It can contribute considerably to the availability of pure and healthy freshwater for personal and industrial use. Membrane systems are designed to remove a wide variety of substances (pathogens, toxic compounds, salts, humic acids, metals, etc.) from e.g. groundwater and (fresh and sea) surface water. Reuse of industrial and municipal wastewater becomes feasible if membranes are used in the purification process. However, all membrane systems eventually foul during operation and need to be cleaned on a regular basis. From the different types of fouling, biofouling is the most persistent and difficult to control (1). Biofouling is a process in which deposition and growth of microorganisms on available surfaces in a water treatment system ultimately result in significant reduction in the quality and amount of produced water (2, 3). If left unattended (or too strongly treated with cleaning chemicals), system performance and lifetime of the membranes will be reduced (4).

Biofouling in reverse osmosis (RO) spiral wound modules is seen as an operational defined problem (5). Microorganisms growing in biofilms (figure 1) cause such a problem in the water production plant (6).

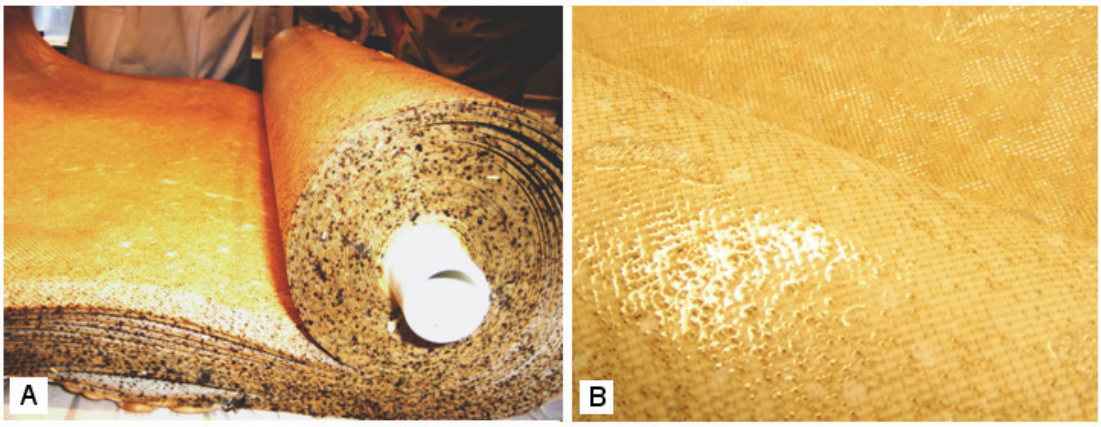


Figure 1. Photographs depicting membrane fouling within the dissected RO membrane element (Hydranautics, ESPA-2, USA). The element (**A**) was used for about 5.4 years in a full-scale RO system for production of process water from extensively pre-treated surface water. Microbial growth is visible as a yellow slimy layer with dark-brown spots on RO membrane and feed-side spacer surfaces (**B**). The highest concentration of biomass is located at the inlet site of the element (in front of the picture **A**).

Over time their presence and development result in an increased operating pressure (Fig. 2), whereby membrane water flux and rejection of salts are decreased (7, 8).

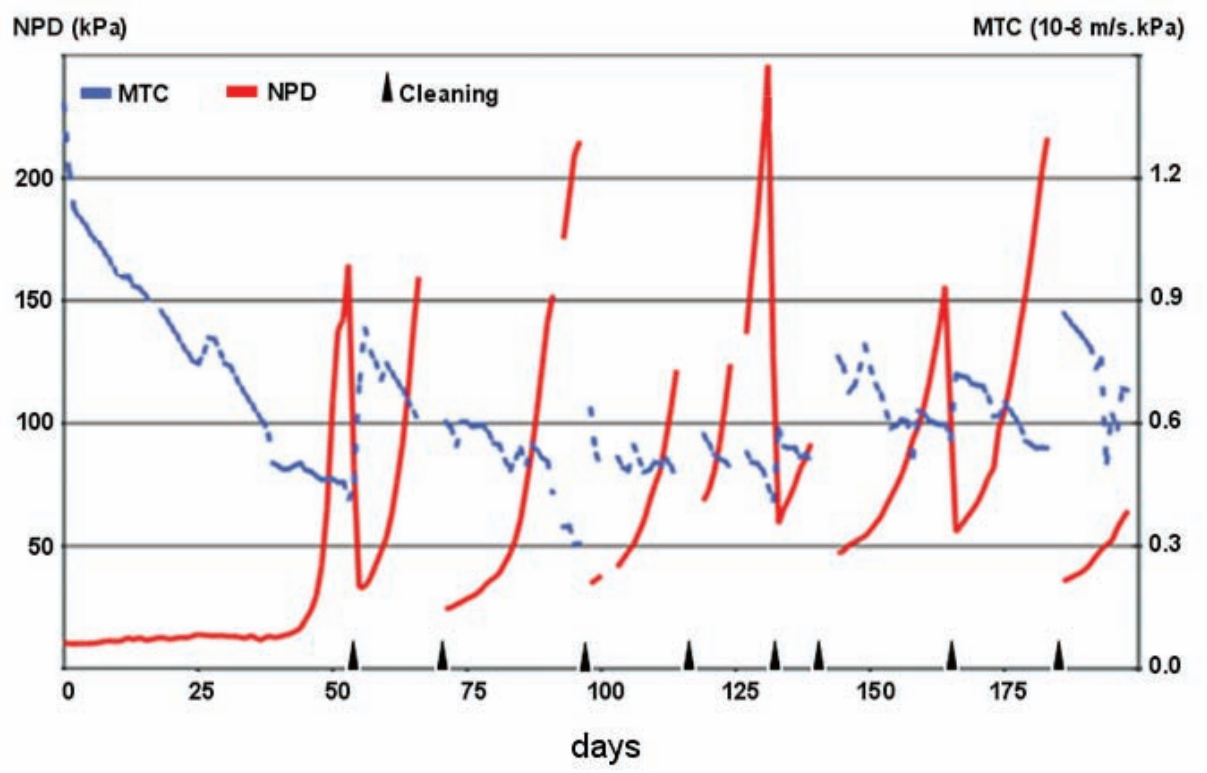


Figure 2. Effects of development of biofouling in a full-scale RO membrane system. The system produced process water from extensively pre-treated surface water. Failure of system performance takes place, when the normalized pressure drop (NPD) over the feed-concentrate channel increase and/or the normalized flux (MTC) decrease exceed 15% of the start-up values. This is clearly the case after approximately 50 days. Due to biofouling, the membrane elements were frequently treated by chemical procedures to maintain an acceptable pressure drop and reasonable MTC (Mass Transfer Coefficient). The graphs are unpublished results from a pilot study performed in 2004 by Vitens, WMD, WbG and WLN and kindly supplied by Waterlaboratorium Noord (Glimmen, The Netherlands).

Solution and/or management of these problems are a challenge for operators, wherever an RO system is used: for desalination of sea water, treatment of wastewater, production of ultra pure water, treatment of purified water for households, and the like. It appears that despite much research and advances that have been made over last decennia in design of suitable anti-

fouling strategies for high technology membrane applications (described in 5, 9-31), the anti-fouling measures used thus far are not efficient in the biofouling prevention or control (5, 32-34). Although different pre-treatment approaches (i.e., coagulation, flocculation, sand filtration, granular activated carbon filtration, microfiltration, ultrafiltration, cartridge filtration, chemical dosage, ozone and ultraviolet radiation) have been exploited (5, 12, 29, 35, 36), each of them (as well as combinations of them) falls short of totally removing of organic and biological matter in a feed water of RO systems (37-39). As a consequence, depending on the feed water quality and type and efficiency of the pre-treatment, a variety of microorganisms and organic substances will foul different parts of an RO system (33, 40-42).

According to Flemming (5), even if 99.9–99.99% of all bacteria are eliminated by pretreatment, a few will enter the system and adhere to surfaces and multiply at the expense of biodegradable substances. So, during the first hours of operation, microorganisms may settle at the membrane surface (43) and about 3 (44) to 16 (45) days later, the membrane surface may be completely covered by a fixed (i.e., surface-attached), gelatinous or slimy (glue-like) layer known as “biofilm” (46). Given sufficient resources for growth, such a film will quickly develop into a mature biofilm (Fig. 3).

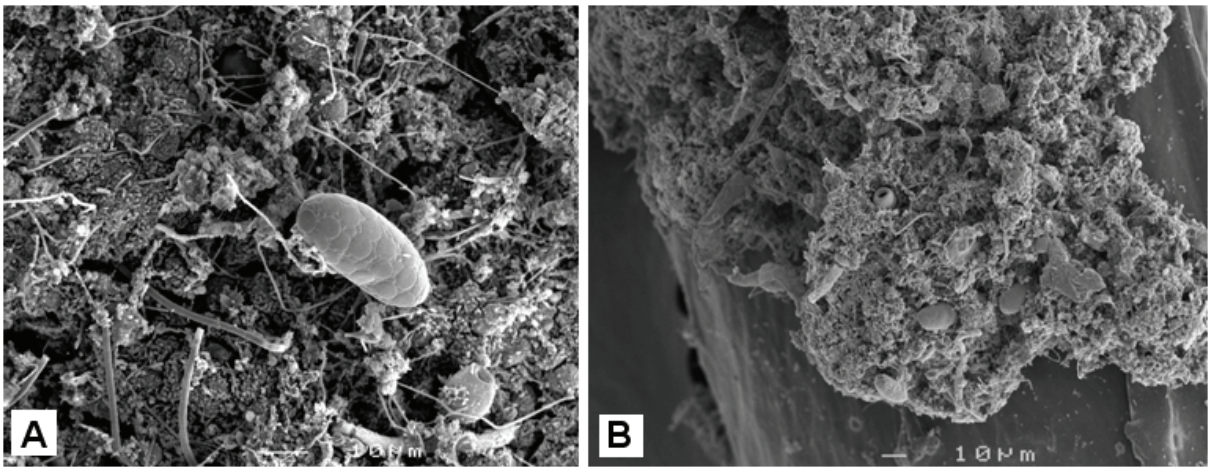


Figure 3. Field emission scanning electron micrographs of a mature biofilm on a fouled RO membrane (**A**) and feed-side spacer (**B**). On both surfaces, the biofilm is visible as a complex heterogeneous microbial population with extracellular fibrillar material structures. Typical bacterial macrocolonies, consisting of microcolonies and single bacterial cells (rods, cocci and spirilla) and a small number of the unicellular eukaryotes (protozoa) are visible within the biofilm. Bar, 10 μm.

Destroying and removal of biofilms from RO membranes and/or spacer surfaces by conventional cleaning approaches appear to be difficult (5, 47). Apparently, microorganisms in a biofilm can persist and survive disinfectants and chemical cleaning agents (48-53). The biofilm structure may not only protect bacteria from disinfection procedures but also provide an environment where disinfectant injured cells can repair cellular damage and grow (54). In addition, removal of the affected biofilms is suggested to be hampered by the design of spiral-wound membrane modules (33), with a large membrane surface area and narrow flow channels containing feed spacers (Fig. 4).

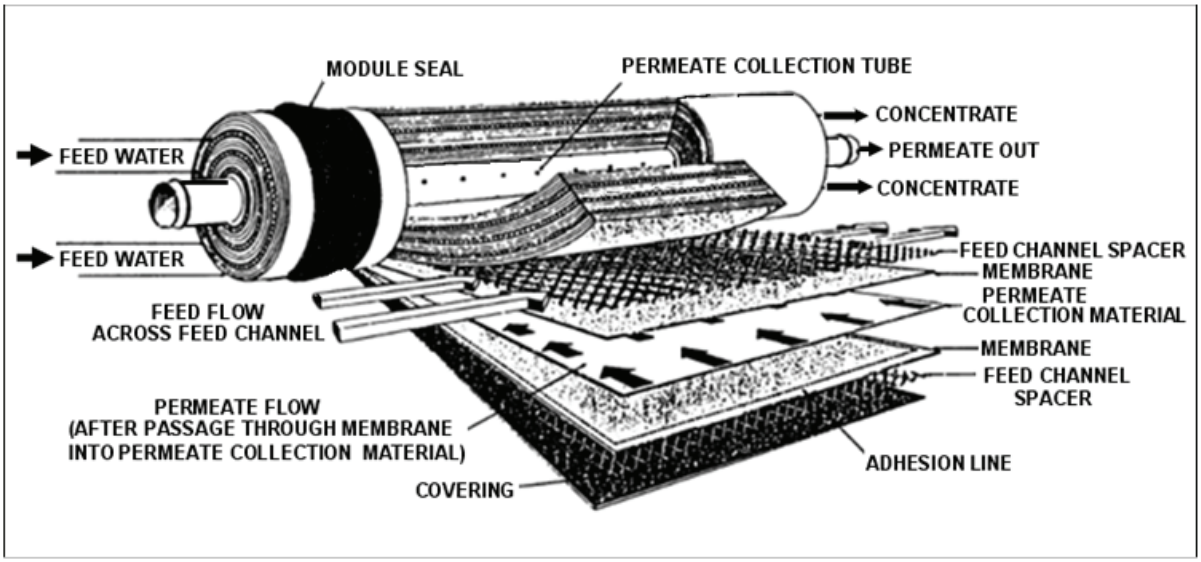


Figure 4. Schematic illustration of the construction details of a spiral-wound RO membrane element (adopted from ref. 42). The module is composed of a number of flat-sheet membrane envelopes, separated from each other on the feed-side by a plastic spacer and wound along a perforated permeate collection tube. In each envelope, two flat-sheet membranes are separated by a porous product spacer and are glued together on the inside at three of its edges. The remaining open edge is connected to the permeate collection tube. Each of the flat-sheet membranes is composed of a selective material that is capable of separating feed substances as a function of their physical and chemical properties when a driving force is applied across the membranes. There are therefore two simultaneously presented streams by operation of the module: the feed stream, flowing in parallel to the feed-side of membrane walls and the permeate stream, passing the membrane sheets and flowing through the product spacer channels to the permeate collection tube (55). The concentrate is a fraction of the feed that does not go through the membrane.

The survived biofilm organisms as well as biomass in the feed water can quickly re-colonize available surfaces within an RO membrane module by growing on inactivated and/or dead microbial cells and remainders of biodegradable cleaning agents (5). As a result, improvement of the RO system performance (i.e., a pressure drop decrease and/or water flux increase) after each particular cleaning procedure is temporary (Fig. 2). The quick biofilm re-growth results in new cycles of biofouling-induced system failure.

The current solution to solve biofouling is to increase the cleaning frequency, but this leads to an increased usage of cleaning chemicals, increased production of wastewater and decreased membrane life-time, which is environmentally unacceptable. In addition, after a number of cleaning cycles the membrane modules become irreversibly fouled (Fig. 1) and need to be replaced to restore water production levels. This results in a loss of the water supply plant capacity (5, 22, 33). A large part of the operation costs in current RO plants are therefore due to repair damage caused by biofouling and in determination and prevention of such fouling. This imposes a large economic burden on the plants (5) and limits the widespread application of RO membrane separation technology (1, 55).

Thus, there is a widely recognized (5, 56) need for the identification of robust and sustainable methods to manage biofouling problems at a lower cost and with less energy, while at the same time minimizing the use of chemicals and impact on the environment. This requires a thorough understanding of the mechanisms responsible for the occurrence and survival of microbial populations in biofilms in such environment (5).

Biofilms are ubiquitous

Biofilms are thought to be the first records of life on Earth (57). They develop and persist under an extremely wide range of conditions, including environments such as acid mine drainages, hot springs, frozen glaciers, space stations and highly irradiated areas of nuclear power plants (5, 58-62).

Environmental scientists have long recognized that biofilms in nature are conducting a variety of biological processes, such as photosynthesis, production and degradation of organic matter, degradation of many environmental pollutants and cycling of carbon, hydrogen, nitrogen, sulphur, phosphorus and many metals (58, 63). This feature of a biofilm is profitably harnessed for constructive purposes, such as biomineralization and bioremediation applications, industrial biotechnology and water and wastewater industry (64-68). However, biofilm formation can also be undesired, especially when it occurs “at the wrong place” and

"at the wrong times" (5). This is the case when biofilm occurrence causes problems in medical, food and process industries or in industrial water systems (2, 69-72). Besides causing problems in cleaning and hygiene, the biofilm-associated microbial activity may cause corrosion and/or blockages in condenser tubes, sensors, cooling tower fill materials, water and wastewater circuits, membrane modules, heat exchange tubes and on ship hulls (73-76). Biofilm can also present microbial risks due to the release of pathogens (e.g., *Legionella*) from biofilms in cooling towers, shower curtains, dental unit waterlines or by reducing water quality in drinking water distribution systems (48, 77-80).

Given that the preference for bacteria to become attached to surfaces is so ubiquitous in diverse ecosystems, biofilm investigators are convinced that the majority of all microorganisms in natural, industrial and hospital settings reside in such aggregates (5, 46, 81, 82). It is also widely believed that this mode of microbial life develops and persists wherever dissolved nutrients are available (5, 83, 84). Hence, it is inevitable that virtually every non-shedding surface in a non-sterile aqueous environment can and will be colonized by microbial layers, causing biofouling if given the right conditions (5).

Biofilms are complex and highly developed structures

A biofilm is a structurally and spatially well-organized biological formation (Fig. 3) that develops and persists at solid surfaces or at phase interfaces in aqueous environments (43, 46, 83). Such formation is an aggregate of extracellular polymeric substances (EPSs) and microorganisms (Fig. 5-A) (81, 85).

The EPS matrix is suggested to be a polymeric conglomeration that is in part secreted by microorganisms (during growth or cell lysis, [86]). The underlying mechanism of EPS synthesis as well as the distribution and chemical nature of produced EPS molecules are largely unknown. Overall, 75% to 95% of the volume in a commonly highly heterogeneous (87) mature biofilm appears to be occupied by EPS matrix (88, 89), but much of that volume may be water channels (90, 91). After water, polysaccharides and proteins which form hydro gel matrices (86) are the major components in a mature biofilm (92-95). Such matrices may involve also exogenous deoxyribonucleic acids (96), uronic acids (97), lipids (98), humic acids (99) and minerals, nutrients, etc., entrapped from the local environment (100). The polysaccharide fraction consists of glucose, fucose, mannose, galactose, fructose, pyruvate and mannuronic or glucoronic acid-base complexes (101) in various configurations (102).

Such structural complexity provides a slimy glue that holds biofilm to the surface and allows the involved organisms to establish stable microconsortia (86).

While a variety of microorganisms (e.g., bacteria, archaea, algae, fungi and protozoa) can collectively proliferate within an adhesive network of extracellular polymers (81), the biofilms grown in nature and on industrial equipment are usually dominated by bacteria (103). Depending on the environmental conditions biofilms may be formed by either single or multiple microbial species. Under natural conditions, the monospecies biofilms are rare (83, 104). They are found mainly in the laboratory and certain clinical settings. The multispecies microconsortia can result from an association between the metabolically cooperative organisms. Their close proximity in biofilms facilitates interspecies substrate exchange and the removal or distribution of metabolic products (105). This adds to the general complexity of the macromolecular mixture present (102).

Although every biofilm community is a unique reflection of the combination of traits of involved bacteria, external physiochemical environment and overall ecosystem functioning, some structural attributes of an established biofilm appear to be universal. The basic structural unit of each biofilm is the microcolony (103) – an EPS matrix-encased aggregation from one or more bacterial species (106). Under certain conditions microcolonies can develop into a macroscopic structure several millimeters or centimeters in thickness (Fig. 3) and can cover large surface areas (Fig. 1). The complexity of this structure can be different: ranging from a single-cell layered, more or less confluent aggregate with a high degree of patchiness, low cell numbers and limited presence of polymeric compounds (Fig. 5-1) to a multilayered, highly-organized, three-dimensional formation with non-uniform, mushroom-shaped or finger-like columns (Fig. 3 and 5-2) surrounded by fluid-filled channels and pores, multiple microbial species and different polymer compositions, different densities of active cells, etc. (79, 106, 107).

Often, particularly in aerobic heterotrophic biofilms, filamentous structures (known as “streamers” [108]) protrude out of the film into the external liquid (104). In general, spatiotemporal distribution of cells and exopolymer secretions manifest in a complex physical, chemical and biological organization of the biofilm. Consequently, architecture of an established biofilm may display dynamic behaviour in changing environments (109, 110).

Being part of such complex and dynamic formations, the bacterial cells show a variety of phenotypic differences when compared to their planktonic (i.e., free-floating) counterparts in aqueous medium (111-113). These differences are related to growth rate, respiration,

substrate uptake and breakdown, motility, synthesis of extracellular polymers, heat production and response to disinfectants, biocides and grazing by protozoa (114-116).

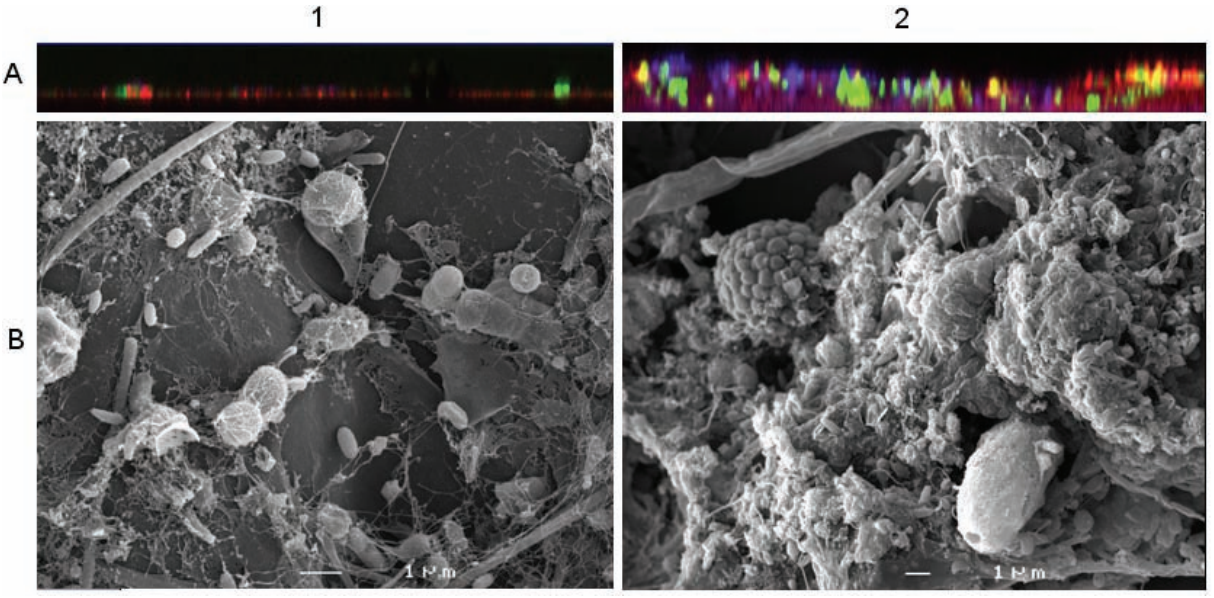


Figure 5. Confocal laser scanning (panel **A**) and field emission scanning electron (panel **B**) micrographs showing complexity of a macromolecular biofilm mixture present on a feed-side spacer surface of a spiral wound RO membrane element (Hydranautics, ESPA-2, USA) after 5 (vertical column **1**) and 17 (vertical columns **2**) days of operation in an RO test flow cell. The test flow cell was used in parallel to a full-scale RO system for production of a process water from extensively pre-treated surface water. **Red fluorescence** in the representative sagittal (*x*-*z*) sections of a biofilm (panel **A**) is from the (SPH120-Cy3-positive) *Sphingomonas* cells, **green** – from the FITC-labeled BET42a probe (*Betaproteobacteria*) and **blue** – from the Calcofluor white-stained polymers of the biofilm EPS matrix. Bars: 1 μm.

Overall, sessile (surface-attached) and EPS embedded bacteria appear to be more adapted to a variety of environmental stress conditions than the associated planktonic organisms. This is because the EPS matrix is not only a structural component, but also a functional component of biofilms. The EPS matrix contributes significantly to biofilm activity and performance, providing stability and shear force resistance (110). The viscous structure facilitates

communication among the microorganisms through biochemical signals (117, 118). The physical stability of the EPS layer contributes to the protection of the cells from potentially toxic agents, UV radiation, pH shifts, and osmotic shock, (110). The EPS can act as a carbon and energy source at times of nutrient deprivation (106, 119). In addition, the EPS matrix is capable of entrapment of essential growth factors and non-cellular materials from the surrounding environment, thereby contributing to the enhanced development of the resident organisms (120). Especially the nutrient entrapment and reuse of the EPS as carbon and energy source is part of a general microbial strategy for survival under oligotrophic (nutrient poor) conditions (59, 121).

Living as a part of a collective (107) within a dense and protected environment, biofilm organisms are able to establish synergistic relationships (86), wherein each group performs specialized metabolic functions. They obtain an additional benefit of the phenotypic versatility of their neighbours (104, 111).

Biofilm establishment is a sequential process

One way to study complex biofilm systems is to develop realistic models of natural communities in the laboratory (81). The process of biofilm formation was examined by a variety of approaches - i.e., cultivation (122), monitoring in laboratory-scale units (123-126) and detailed analysis of the biofilm-forming organisms (91, 124-132).

The key processes in regulation of the biofilm formation were not completely understood from the results of these experiments (81, 133). However, the general features and three-dimensional complexity achieved in some biofilms led to the idea that biofilm formation occurs through the same key steps in various environments (134, 135). The phases consist of an orderly sequence of several developmental events (136, 137), each of which is required to ultimately reach a level of maturity (103, 135). Two general models have been proposed to explain the establishment of each developmental stage.

The ***first*** model (Fig. 6) is based on the idea that biofilm development is a fixed program that could be explained and controlled through genetic analyses (138, 139). Accordingly, each step of biofilm formation (i.e., attachment, microcolony formation, biofilm maturation and dispersion) is mediated by changes in gene expression that regulate synthesis of specific bacterial factors, including flagella and fimbriae (or pili), outer membrane proteins, wall polysaccharides (capsules), lipopolysaccharide, cellulose, cell surface agglutinin and curli (98, 140-147).

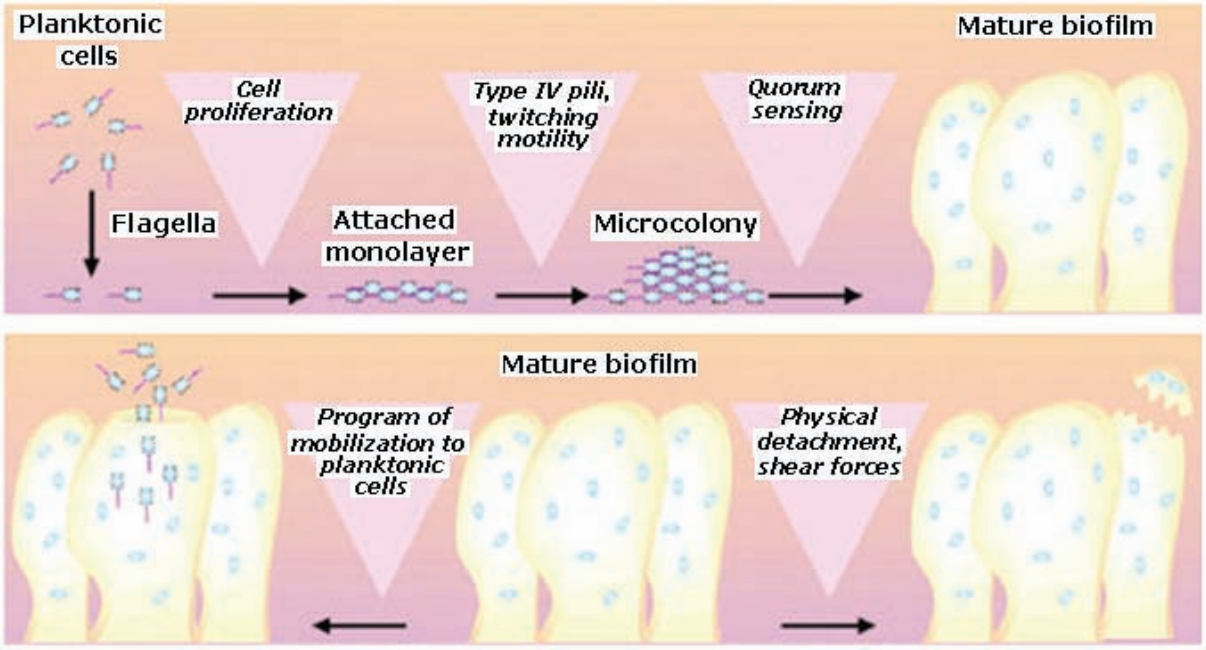


Figure 6. Schematic illustration (ref. 103) of steps involved in biofilm formation on a virgin surface. The free-floating bacteria swim towards the surface using polar flagella, form random loose attachments and migrate over the surface to form a microcolony and, produce exopolysaccharides to form a three-dimensional structure. When environmental conditions become unfavourable, the bacterial cells may detach from the biofilm and swim away to find a new surface in a more suitable environment.

The flagellum-driven motility is suggested to be required for bringing the bacterium into proximity of the surface, moving across the surface or within a biofilm matrix and to spread out to colonize new surfaces (137, 148). It is also supposed that flagella play a key role in the formation of loose protruding biofilm structures (149). However, in membrane filtration the presence of a convective flow reduces the dependence of bacterial cells on flagella-mediated motility in establishing the initial cell-to-surface contact (150, 151).

The twitching and swarming motility were found to be important for cell aggregation and creation of initial microcolonies in a static system (140, 152, 153). However, in a constantly flowing system, cells exposed to shear forces have limited surface movement via type IV pili (twitching motility). The initial microcolony formation is affected mostly by other mechanisms, such as cellular division (primarily) in combination with cell clustering (149).

When biofilm-associated microbial cells start to grow certain metabolites accumulate in the biofilm. It is suggested that some of these chemicals have a function in cell-to-cell

communication, known as “quorum sensing” (154). Quorum sensing has been shown to be important for the formation of a mature biofilm (103).

EPS production plays a key role in the attachment of cells (155, 156) and is required for development of architecture during biofilm formation (94, 110, 132, 157). Once EPS production starts, the biofilm grows through a combination of cell division and recruitment of other microorganisms within the embedded exopolysaccharide matrix, giving rise to the formation of microcolonies. The quorum sensing signals are suggested to be in part responsible for biofilm development by regulating the differentiation from microcolonies into much more sophisticated stacks of bacterial cells (“mushrooms” and/or “towers”) by population density-dependent gene expression (158, 159).

In the final stage of the biofilm lifecycle, some of the biofilm organisms return to the planktonic state. This is presumably achieved by coordinating the degradation of the surrounding extracellular matrix by secreted or cell surface-associated enzymes (160, 161) in combination with activation of motility functions (103).

The ***second*** model is derived from the assumption that exact structure of any biofilm is probably the result of the environment in which it develops. This means that it is a predictable consequence of the physiochemical conditions in this environment. As pointed out by Flemming (5), a change in nutrient concentration, shear forces, temperature or other factors can cause either biomass production or sloughing of biofilms. According to Stoodley and co-workers (108), hydrodynamics and nutrient concentration greatly affect the nature of laboratory developed biofilms and this is supposed to be equally true for all biofilm types. Wimpenny and Colasanti (162) have also suggested that biofilm structure is largely determined by the substrate concentration. They further postulated that differences in substrate concentration also validated at least three conceptual biofilm models: (i) isolated colonies scattered on the surface as towers (at low substrate concentration); (ii) structured biofilms with “mushrooms” surrounded by water channels (at intermediate substrate concentration) and (ii) a thick microbial mat (at high substrate concentration). Other researchers (163, 164) proceeded using a combination of a discrete and continuum model, in which “real” biological parameters were in use.

According to Wimpenny *et al*. (165), mathematical simulations based on simple assumptions regarding growth, nutrients, mass-transport and detachment were able to predict a variety of biofilm morphologies as can be seen in nature. However, more recent research (reviewed in ref. 166) indicated that biofilm development is a dynamic multifactor phenomenon. The biofilms constantly fluctuate both in time and space. It is important to keep

in mind that all of the identified and yet-to-be-discovered characteristics are part of a dynamic biofilm development and that there will be no single global regulating pathway to control this process. In view of this, the suggested "integrated approach" seems to be one of the most promising strategies known today for a sustainable anti-fouling management in full-scale scale water treatment membrane systems (5).

How do biofilms form on RO membrane and spacer surfaces?

The exact mechanism of biofilm formation in RO membrane systems is not entirely clear (4). A generalized understanding of the formation of microbial layers within a full-scale RO system can be deduced from the general understanding of biofilm properties and biofilm formation events as described above.

The first stage in biofilm formation is when feed water comes in contact with the membrane and feed-spacer surfaces within an RO membrane element. The bulk feed water with dissolved and/or suspended particles, colloids and nutrients (5) comes in contact with the membrane and feed-side spacer surfaces when it is forced into the feed channel between two sheets of membrane (Fig. 4) by a high pressure pump. As a result, during the first few hours of operation not only the bacterial cells, but also a variety of inorganic and organic compounds remain deposited at the water/solid interface, because they do not diffuse through the semi-permeable RO membrane readily (22). The attachment of bacteria to the surface is supposed to be mediated by the convective transport of bacterial cells to the solid–liquid interface (150, 151). Random (Brownian) motion, gravitational settling and chemotactic, aerotactic or phototactic responses, which are claimed to be important in the static systems (103, 137, 142; 167, 168) are less likely to occur.

"Conditioning" of the initially clean surfaces with the adsorbed (within seconds) macromolecules is suggested to change surface chemistry (169, 170), thereby increasing the potential of bacterial cells for the attachment, augmentation and cell-to-cell aggregation (70, 171, 172). Ridgway (173) observed that only certain bacteria can attach at this stage of biofilm development and that there are a limited number of attachment sites. It appears, that the properties of the feed water (e.g., pH, concentration of nutrients, temperature, ionic strength, presence of multivalent cations, flow velocity, etc.) as well as the properties of membrane, feed-side spacer (e.g., material, area, charge, roughness, hydrophobicity, molecular weight cut off, etc.) promote or discourage microbial adhesion (172, 174, 175).

These properties vary during membrane system operation because biofilm development starts immediately and the surface properties are changed (142, 143, 176, 177). Also various physiochemical forces (i.e., electrostatic and hydrodynamic forces, hydrophobic interactions, steric hindrance, etc. [172, 175, 178]) contribute to the dynamic development of biofilms on membranes.

At this stage the adhesion is still a weak interaction of bacterial cells with the surface. The attachment of bacteria is still reversible and can be easily removed by application of mild shear forces. When the bacteria are attached to the membrane they start to grow at a rate that is related to the availability of nutrients (C, N, O, P, and trace elements) (5). Immediately after primary adhesion and initial growth, bacteria begin to produce extracellular polymeric substances, leading to irreversible surface colonization (49, 179). According to some researchers (180, 181), the first irreversible attachment of cells occurs after a few minutes of contact between the RO membrane and raw water. Removal of such cells is difficult and requires intense scrubbing or scraping to break the attachment forces.

The appearance of a slimy biofilm matrix significantly changes the properties of the RO membrane and feed-side spacer surfaces and promotes the development of an organized biofilm structure. Growth and differentiation of the resident (sessile) cells into microcolonies and simultaneous attachment of free-floating (similar or different) feed water bacterial species to the EPS matrix - a phenomenon known as the “co-adhesion” (49) – result in the onset of biofilm maturation.

As the biofilm matures, the structure of the EPS matrix changes according to the properties of the bacteria that are associated with it. As a result, the complexity, density, thickness and stability of the biofilm significantly increase. Within the maturing biofilm water channels may be formed (85). The channels function as a primitive circulatory system, delivering fresh supplies of nutrients and oxygen and removing potentially toxic metabolites (46, 83, 107). The capacity of the water channels however, ultimately limits the degree of biofilm maturation (182). Other factors that influence biofilm maturation include internal pH, osmolarity, nutrient availability and oxygen perfusion (49). Overall, this stage of biofilm development may take several hours to several weeks to fully develop if environmental conditions are suitable for sufficient bacterial growth (183). It may result in coating of the entire surface area with several layers of bacterial cells (10 to 20 μm thick). The bacteria appear to be firmly attached to the membrane surface by an extensive network of extracellular polymeric fibrils (42, 183).

The mature status of a biofilm is a dynamic equilibrium, in which the surface and dead cells are replaced by newly adsorbing cells and proliferating cells within the biofilm (182). In this final stage the biofilm is a complex microbial community of primary colonizers that created an environment with sufficient available nutrients and conditions to support growth of secondary colonizers (81).

Once the biofilm has been fully developed, dispersal of cells from the biomass may occur (184). The mode of dispersal affects the phenotypic characteristics of the released organisms. Eroded or sloughed aggregates from the biofilm are likely to retain certain biofilm characteristics, such as increased antimicrobial resistance. Cells that have been shed as a result of growth may revert quickly to the planktonic phenotype (5). Both phenomena lead to increasing contamination of the feed water and a higher potential for effective re-adhesion and additional biofilm formation on different surfaces within spiral-wound RO membrane elements (5).

Knowledge on biofouling in RO systems is not complete

The information presented above clearly shows that basic understanding of the microbiological processes is necessary for an efficient and sustainable control of biofouling. Nevertheless, virtually all reports in the field do not pay attention to the microbial communities in biofouled membrane systems.

Biofouling is often diagnosed as pressure drop measurements over the feed channel during operation (185). Afterwards biofouling is usually quantitatively analyzed, by i.e. determination of adenosine triphosphate or active biomass concentration (7). Additional microbiological research on fouling substances from membrane surface (i.e., total cell and heterotrophic plate counts) is also informative for this purpose (33, 42). However, these experiments do not allow a reliable evaluation of microbial abundance and diversity of species. Thus far, only a small fraction of the total bacterial population can be cultivated under standard laboratory conditions (186-189). It is expected that this is also true for biofilms on membranes. Agar plate counts are therefore not very valuable to determine the microbial diversity. This method is now routinely replaced by cultivation-independent techniques based on DNA detection.

While knowledge of microbial composition is very useful in identifying the most effective cleaning protocols, only a few DNA-based microbial diversity studies on RO membrane surfaces are reported (40, 41). However, also these studies required removal of

fouled membrane elements from the system and dissection or “autopsy” (Fig. 7) of an already established biofilm (33, 190). These studies do not provide sufficient data on the formation and development of biofilms *in situ* as only the end point is analyzed.

Figure 7. Photographs of the autopsy of a fouled RO membrane module (Hydranautics, ESPA-2, USA). The module was removed from a full-scale RO plant after 6.4 years of operation because of biofouling and transported to a laboratory for dissection, visual analysis and collection of biofilm samples.

How species recognize and respond to each other is a key question in any multispecies system. Although this question is widely accepted, most of the biofilm monitoring studies in the field today involve pure-culture systems. In addition, they are mainly done in simplified laboratory systems (*in vitro*) with one (150, 191-193) or a few (151) bacterial strains. The results of these studies are of limited use to describe multispecies biofilm development in fluctuating environments like membrane systems. The impact of environmental conditions (e.g., flow properties, osmolarity, temperature, pH, etc.) on these processes is already recognized (87, 108, 109) and may not be ignored. A full-scale RO system may also show additional or unrecognized selection criteria affecting microbial growth and survival that are not taken into account in laboratory experiments. It is possible that biofilms in experimental *in vitro* settings and those produced in nature (*in situ*) by mixed species consortia express different genetic pathways, even if they exhibit similar overall structural features (46, 137,

157). The development of biofilm structures is, to a certain extent, a stochastic and multifactor process and independent rounds of biofilm experiments never result in exact structural copies, even if the experimental conditions are kept constant (83). This may be a major cause of some of the discrepancies in scientific reports.

Currently we are able to describe the formation of biofilms with a reasonable degree of detail. We can detect the microbial composition and measure the synthesis of polysaccharides. We have however limited information on the metabolic pathways and how organisms interact with each other. Much research is required before we understand and possibly manage the complex processes in biofilm microbial communities in full-scale water treatment membrane applications. To completely understand the complex processes that dictate biofilm formation and biofouling development within current RO systems we need to know much more about the origin, succession, true composition, spatiotemporal structure, specificity and stability of the bacterial biofilm communities. Whole community sequencing and expression studies using DNA-chips, proteomics and metabolomics are the future methods in the analysis of biofilms and biofouling-associated troubles in full-scale RO membrane facilities. We also need to understand the impact of biofilm architecture on the occurrence and development of undesired operational troubles in the full-scale RO systems. Furthermore, if we know the type of organisms that play a major role in biofilm development at various stages we can come up with more efficient chemical cleaning strategies to manage biofouling. The effect of chemical cleaning of membranes on biofilm removal is described in only a limited number of papers (16, 21, 152, 194-197). Monitoring the pressure drop decrease and membrane flux increase determines the effect of cleaning procedures. Finally, there is a lack of detailed description of the specific microbiological features of biofilms associated with the RO feed-side spacer surface. Attachment and growth of microorganisms on feed side spacers that are located between two membrane sheets in a spiral-wound RO membrane element is suggested to play a role in development of biofouling-induced system failure in RO plants (198).

Research objectives of this PhD study

The aim of the present investigation was to generate knowledge on the microbiological processes associated with microbial biofilm formation and development of biofouling. The origin, succession and spatiotemporal development of microbial biofilms in full-scale reverse osmosis systems, in particular in relation to the operational performance of the RO system are addressed. The key question to answer is, if a general pattern can be discovered in biofilm

development in different RO membrane installations. In other words, does biofouling always develop along similar pathways or is every case unique? Not only is this information of fundamental interest, but it is also extremely useful in the development of control strategies to minimize membrane fouling. It is also valuable information to further improve membrane technology and plant design and operation to prevent or reduce the rate of biofouling.

Thesis outline

This thesis presents a comprehensive research report on microbiological aspects of biofouling occurrence in full-scale reverse osmosis systems. In the first study (***Chapter 2***), biofilms from a number of severely fouled RO membrane elements were investigated. The RO system was used to produce process water from extensively pre-treated surface water. The abundance and species diversity of the biofilm microorganisms, responsible for biofouling in the full-scale RO membrane system were determined. The biofilm communities were analyzed by 16S rRNA-gene-targeted denaturing gradient gel electrophoresis (DGGE) and their phylogenetic affiliations were determined by sequence analyses of individual 16S rRNA genes cloned in *E. coli*. It was also discovered that conventional cleaning does not remove biofilms from the surfaces of RO membranes and feed-side spacers in the spiral-wound membrane elements. Cleaning restored the performance of the membrane elements, but the effect was quickly reduced after continued operation. Five distinct bacterial genotypes (i.e., *Sphingomonas, Beta proteobacterium proteobacterium* [AF236004], *Flavobacterium, Nitrosomonas* and *Sphingobacterium*) were found to be dominant on surfaces of fouled RO membranes and feed-side spacers at the end of their operational lifetime (i.e., after five and half years). The finding that all five “key players” could also be recovered from cartridge filters positioned in front of this RO system suggested that these microorganisms originate from the feed water rather than from the RO system itself despite the fact that the feed water passed an ultrafiltration membrane (pore size approximately 40 nm).

The second study (***Chapter 3***) aimed to get insight in an intermediate time point of the biofouling development in the full-scale reverse osmosis systems. For this purpose, a first module from the first stage of the same full-scale RO system was analyzed after one year of operation. Also various water samples were analyzed after different pre-treatment steps and material surfaces at different locations. The presence, diversity and community structure of potential and known biofilm-formers in the upstream parts of the main RO system were studied. The investigation showed that the bacterial community of the RO membrane biofilm

was clearly different from the bacterial community present at other locations in the RO plant. This indicates the development of a specialized bacterial community on the RO membranes. The typical freshwater phylotypes in the RO membrane biofilm were also present in the water sample fed to the plant, suggesting that the biofilm bacteria are of feed water origin. However, the relative abundances of the different species in the mature biofilm were different from those in the feed water, indicating that the biofilm was actively formed on the RO membrane sheets and was not the result of a concentration of bacteria present in the feed water. The majority of the microorganisms (59% of the total number of clones) in the biofilm were related to the class *Proteobacteria*, with a dominance of *Sphingomonas* spp. (27% of all clones), indicating that members of this genus are associated with the membrane biofouling in the full-scale RO installations.

In ***Chapter 4*** a study is presented on biofilms in an environment similar to that of a full-scale RO system. The dynamics and biological succession involved in biofilm formation on reverse osmosis membranes and feed-side spacers were monitored *in situ* using flow cells placed in parallel to the main RO system of the same full-scale water treatment plant. The abundance, composition, architecture and three-dimensional structure of the microbial community at different stages of *in situ* biofilm development was determined by several well established complementary techniques (i.e., fluorescence *in situ* hybridization, DGGE, cloning and field emission scanning electron, epifluorescence and confocal laser scanning microscopy). This provided an accurate description of the changing community structure during biofilm maturation. A comparison of the results obtained after 1-month with results described in chapter 2 indicated that the same community persisted for a 5-year period. This showed that the primary changes in biofilm structure occur within the first month of formation. The study also pointed out the unique role of the *Sphingomonas* spp. in the initial formation and subsequent maturation of biofilms on RO membrane and feed-side spacer surfaces.

In an effort to obtain more knowledge of the microbiological processes associated with biofouling, the establishment and spatiotemporal development of microbial film layers was monitored during a period of one year *in situ*, on the surfaces of fresh and chemically cleaned RO membranes and feed-side spacers (***Chapter 5***). In this way, the development of microbial biofilms to a level of “biofouling” - determined by the pressure drop increase – and impact of conventional cleaning procedures on biofouling were assessed in detail. The study demonstrated that conventional treatment with toxic chemicals was not effective in cleaning the RO system. It became obvious that biofouling control is only possible if the cleaning

procedures are adapted to effectively remove the (dead) biomass from the RO membrane and spacer surfaces. It became also clear, that special attention must be paid to the sphingomonads in the development of new approaches to control or prevent biofouling. These bacteria contributed a lot to the cleaning-associated stability of bacterial biofilms, even when they were not the dominant group in the surface-attached biofilm communities.

Finally, the general conclusions from this study are discussed in ***Chapter 6*** in relation to the significance in the design of appropriate anti-fouling strategies and suggestions for further research are given.

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2 Investigation of microbial communities on reverse osmosis membranes used for process water production

This chapter is identical to the paper:
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Abstract

In the present study, the diversity and the phylogenetic affiliation of bacteria in a biofouling layer on reverse osmosis (RO) membranes were determined. Fresh surface water was used as a feed in a membrane-based water purification process. Total DNA was extracted from attached cells from feed spacer, RO membrane and product spacer. Universal primers were used to amplify the bacterial 16S rRNA genes. The biofilm community was analysed by 16S rRNA-gene-targeted denaturing gradient gel electrophoresis (DGGE) and the phylogenetic affiliation was determined by sequence analyses of individual 16S rDNA clones. Using this approach, we found that five distinct bacterial genotypes (*Sphingomonas*, *Beta proteobacterium* [AF236004], *Flavobacterium*, *Nitrosomonas* and *Sphingobacterium*) were dominant genera on surfaces of fouled RO membranes. Moreover, the finding that all five "key players" could be recovered from the cartridge filters of this RO system, which cartridge filters are positioned before the RO membrane, together with literature information where these bacteria are normally encountered, suggests that these microorganisms originate from the feed water rather than from the RO system itself, and represent the fresh water bacteria present in the feed water, despite the fact that the feed water passes an ultrafiltration (UF) membrane (pore size approximately 40 nm), which is able to remove microorganisms to a large extent.

Introduction

Biological fouling (biofouling) is one of the most serious problems in the water treatment industry where reverse osmosis (RO) technology is used (Flemming et al., 1997; Al-Ahmad et al., 2000; Flemming, 2002). The formation of laminar microbial networks (biofilms) of low permeability in the current generation of spiral wound RO filtration units cause harmful and often irreversible effects on the RO system performance. Drastic long-term membrane flux decline of the RO plant and/or significant increase in the differential pressure and the feed pressure of the RO modules (Ridgway et al., 1984; Wiesner and Aptel, 1996; Vrouwenvelder and van der Kooij, 2001), are typical problems, identified as "biofouling" in the actual practice of RO plants. The capacity of the RO plant to produce microbiologically safe and biologically stable water by removing inorganic/organic compounds and microorganisms from the filtered feed water (Ridgway et al., 1983) may be lost due to this phenomenon.

To overcome problems associated with fouling, frequent chemical cleanings and pre-treatment of the feed water is used (Flemming, 1996, 2002). However, the periodical chemical cleaning reduces membrane service life, increases operational costs and is environmentally unacceptable (Ridgway and Flemming, 1996; Al-Ahmad et al., 2000). Moreover, the procedures are not always effective in removing the biofilm from RO membranes. As a consequence, depending on the feed water quality and the type and efficiency of the pre-treatment, microbes will always invade the RO system.

Although evidence of biofilm formation in RO systems is nowadays generally accepted, little is known about the diversity and complexity of the microorganisms, responsible for the formation of these biofilms. Our current understanding of diversity in biofilm microbial communities in oligotrophic RO systems is mainly based on organisms, identified by cultivation methods. However, only a small fraction (0.1–3%, Amann et al., 1995; Kalmbach et al., 1997) of the total bacterial population can be cultivated under standard laboratory conditions. Therefore, the composition, structure, specificity and stability of the bacterial biofilm communities on RO membranes remain for the most part unexplored and to date, only a few molecular studies of these microbial communities have been performed (Chen et al., 2004a, b). Consequently, our general understanding of the biofouling process is far from complete. In fact, the key processes regulating biofilm formation and biofouling development on RO membranes are poorly understood. A better understanding of these processes may help in devising novel strategies for biofilm control and to provide improvements in membrane technology to prevent or reduce the rate of biofouling.

In the present study, the microbial diversity and the phylogenetic affiliation of the bacteria in a biofouling layer of RO membranes were explored using a PCR-DGGE approach (Muyzer et al., 1993) combined with the analysis of constructed clone libraries containing larger fragments of the amplified 16S ribosomal RNA gene (Ward et al., 1990) and the DGGE screening of the isolated clones. The main aim was to investigate which species are most frequently encountered in the membrane biofilm microbial communities and are therefore assumed to play a role throughout the development of the biofilm (the potential key players).

Materials and methods

Sampling

The biofilm samples were collected in May 2005 during autopsy of spiral-wound RO membrane elements, removed from a full-scale water treatment plant (Figure 1).

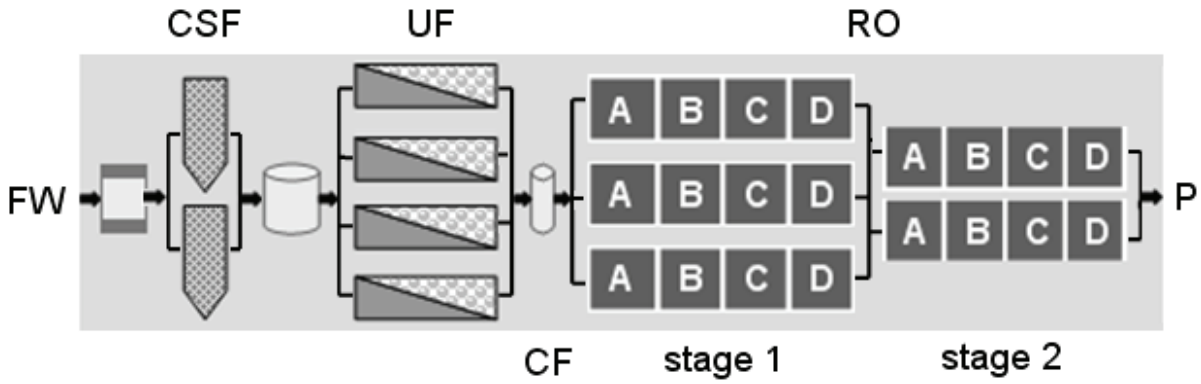

Figure 1. RO plant outline. Fresh surface water, pumped in a full-scale water treatment plant, was initially pre-treated using the continuous sand filtration (CSF), followed by ultrafiltration (UF) and treated using cartridge filtration (CF) and two-stage RO system, in which membrane elements were installed in series inside pressure vessels. FW – feed water, P – permeate.

In total, four fouled RO membrane elements were removed from the RO system and subjected to autopsy: the first (A) and last (D) element from the first stage and the first (Ac) and the last (Dc) element from the second stage of an RO unit. The two elements from the second stage were cleaned by chemical treatment prior to removal. The samples of tightly associated biofilms were taken directly from different locations in the fouled membrane (Hydranautics ESPA 2), feed spacer and product spacer. For microbial community analysis, microbial biomass was collected from these biofilm samples by mechanical beating with a Mini-bead beater (Biospec Products). The loose biofilm samples were collected by manual scraping from a known membrane surface area. Additionally, samples from the cartridge filter (pore size approximately 100-mm) were collected approximately 2 months before the element autopsy was carried out. Samples were cooled on ice after sampling and frozen at -70° C.

Total DNA extraction

Each biofilm sample was defrosted, suspended in 1mL of phosphate-buffered saline (PBS) and homogenised using vortex. The total community DNA was extracted from 0.5 mL of biofilm homogenate using the Mini-bead beater with Fast DNA Spin kit for soil (Bio 101) in accordance with the manufacturer's instructions. Aliquots of each DNA extract was further purified and concentrated with DNA Clean & Concentrator-5 Kit (Zymo Research) according to the manufacturer's directions. Amplification of 16S rRNA genes for DGGE analysis Fragments (456 bp) of bacterial 16S rRNA genes were amplified from total genomic DNA by PCR using Go *Taq* ^{R} DNA polymerase (Promega) with 954-f and 1369-r primers (MWG-Biotech AG) targeting hypervariable V6-V8 region, as was described by Zhongtang and Morrison (2004). A 40-base GC clamp (5´-CGCCGGGGGCGCGCCCCGGGCGGGGCGG-GGGCACGGGGGG-3´) was attached to the forward primer at the 5´ end. Each 50-µL reaction mixture contained 10 ng template DNA, each deoxynucleoside triphosphate (dNTP) at 200 µM (Invitrogen), each primer at 0.5 µM, 1.25 U of Go *Taq* ^{R} DNA polymerase, and 1x PCR buffer containing $MgCl_2$ at 3mM (Promega). Reactions were performed in a I-Cycler (BioRad) applying predenaturation at 94° C for 2 min; 35 cycles of denaturation at 94° C for 30 s, annealing at 56° C for 30 s, and extension at 72° C for 60 s. Cycling was completed by a final extension step of 72° C for 7min.

DGGE analysis of amplified 16S rRNA genes

DGGE analysis of the generated amplicons of the total bacterial communities was performed using a DCode™ System (BioRad) as was described by Heilig et al. (2002). The number of operational taxonomic units (OTU's) in each sample was defined as the number of DGGE bands with distinct electrophoretic mobilities. A mixture of the DGGE-PCR products from nine bacterial species was applied at the extremities of the gels as a marker to check the electrophoresis run and to compare fragment migration between gels. Cloning of PCR-amplified products, sequence, and phylogenetic analyses The almost full-length bacterial 16S rRNA gene fragments were amplified using universal bacterial primers 7-f (5´-AGAGTTTGATYMTGGCTCAG-3´) and 1510-r (5´-TACGGYTACCTTGTTACGACTT-3´) (Lane, 1991). Reaction mixtures of 50 µL contained 10 ng total bacterial community DNA of the biofilm samples as template, 10x PCR reaction buffer at final concentrations of 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3 (Invitrogen), and 3 mM $MgCl_2$, each dNTP at 200 µM (Invitrogen), each primer at 0.2 µM (MWG-Biotech AG), and 1.25 U *Taq* DNA polymerase (Invitrogen). Amplification was performed with the I-Cycler as follows: predenaturation at 92° C for 2 min; 35 cycles of denaturation at 95° C for 30 s, annealing at 52° C for 40 s, extension at 72° C for 1.5 min; and a final 72° C extension step for 5 min. The obtained amplicons were purified with the DNA Clean & Concentrator-5 Kit and subsequently cloned into *E. coli* XL-1 Blue by using the pGEM-T-Easy vector system cloning kit (Promega) according to the manufacturer's instructions. Colonies of ampicillin-resistant transformants were transferred with a sterile toothpick to 50 µL of Tris-EDTA and were incubated at 95° C for 15 min to lyse the cells. PCRs were performed with cell lysates using pGEM-T-specific primers T7 (5´-TAATACGACTCACTATAGGG-3´) and Sp6 (5´-ATTTAGGTGACACTATAGAATAC-3´) to check the sizes of the inserts as described before (Heilig et al., 2002). To establish the diversity within the group of selected clones of each origin, positive amplification products of the correct size were subjected to restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme cocktail of *Msp*I, *Cfo*I and *Alu*I. Individual clones with a unique RFLP pattern were selected and screened by DGGE analysis with the V6-V8 primers (GC-954-f and 1369-r). Their DGGE bands were detected in the original DGGE fingerprints profile of the biofilm community using the BioNumerics software (BioSystematica). Unique inserts were bi-directionally sequenced with T7 and Sp6 primers (BaseClear, Leiden, The Netherlands). Chimeric sequences were removed after applying a Chimera Check program at http://www.cme.msu.

edu/RDP/html/index.html (Maidak et al., 2001), and the sequence similarity was analysed using the NCBI BLAST search tool at http://www.ncbi.nlm.nih.gov/BLAST (Altschul et al., 1997). Alignment with FastAligner and further phylogenetic analysis of the sequences were performed using the ARB software package (http://www.arb-home.de). The topology of the generated phylogenetic trees was confirmed by applying neighborjoining method (Saitou and Nei, 1987) with the Felsenstein correction.

Results and discussion

Autopsy was performed on selected membrane modules taken from different locations in the RO system (Figure 1) because of suspected biofouling. These membrane elements were used in the plant for a period of approximately five and half years to produce process water from the pre-treated fresh surface water. The plant suffered significant increases in the pressure drop and dramatic flux declines due to fouling. The visual inspection of examined membrane elements showed the presence of a slimy, opaque, light brown coloured deposit on the surfaces of the feed side of the membrane and feed spacer, except on the surfaces of the product side of the membrane and spacer. The absence of fouling on the product spacers and the accumulation of rejected feed water components at the feed side of the membrane surface is in accordance with the RO system performance, however, the degree in which the deposit was present was significant and could indicate biofouling. It was also noted that the visible deposit was uniformly spread over the whole membrane and feed spacer surfaces in all examined RO modules, which was unexpected, since it is generally believed that biological fouling is most severe in those parts that come in first contact with the feed water and should therefore be confined to the first elements of the first stage of an RO unit. Apparently, the biofouling had expanded throughout the RO system in the course of years.

Furthermore, the visual examination showed no significant differences between the cleaned and not cleaned RO modules, clearly demonstrating the failure of applied cleaning strategies (alkaline and acid treatment). Fouling regularly covered the feed side of all membranes in the shape of the feed spacer. The fouling layer was loosely attached to the surfaces of the cleaned membranes and could easily be removed from the surfaces in contrast to the biofilm on the untreated membrane surfaces. It is assumed that the treatment of modules may have affected the structure of extracellular polymeric substances (EPS) matrix of bacterial biofilms, by which bacterial cells were attached to the membrane surface.

Although the biofilm is loosely attached to the membranes surface it is not removed during the flushing steps of the cleaning procedure. The design of these spiral-wound membrane elements (spacers, narrow feed channels) may hamper the removal of the fouling. Cleaning of the membranes by chemical treatment kills most of the bacteria as was shown by ATP measurements (data not shown). The remaining bacteria within the complex biofilm community which have survived the cleaning process will quickly build up a new biofilm (Flemming, 2002). The dead bacterial cells in the unremoved fouling layer serves as a food source for the growing biofilm. It can also not be excluded that the spiral-wound design of RO membrane elements actually supports the accumulation and growth of bacterial biofilms in these environments (Ridgway and Safarik, 1991; Baker and Dudley, 1998).

DGGE analysis of the biofilm DNA samples

PCR-amplified bacterial 16S rRNA gene fragments from each of the four examinated RO membrane elements and from the cartridge filter (Figure 1) were analysed by DGGE for an initial determination of microbial diversity throughout the RO system. The obtained fingerprints of the bacterial community (Figure 2) showed many bands in all samples tested, indicating that multiple species were present. Generally, approximately 10 OTU's could be determined within each single biofilm sample, of which at least five DGGE bands could be clearly discriminated as dominant (intense bands).

It can be seen from Figure 2 that each biofilm sample produced an analogous DGGE biofilm community pattern, representing the presence of the same bacteria, however, in relatively different amounts (according to the band intensity in the gel). The DGGE profiles obtained with samples from cleaned membrane elements showed no significant differences in the biofilm community structure compared to those of the untreated elements. Ten bands, including five dominant bands, are found in both profiles. These results support the findings of the visual observations of fouled membrane elements, suggesting that the applied cleaning strategies were not effective in removing biofilms from RO membranes in spiral-wound membrane elements.

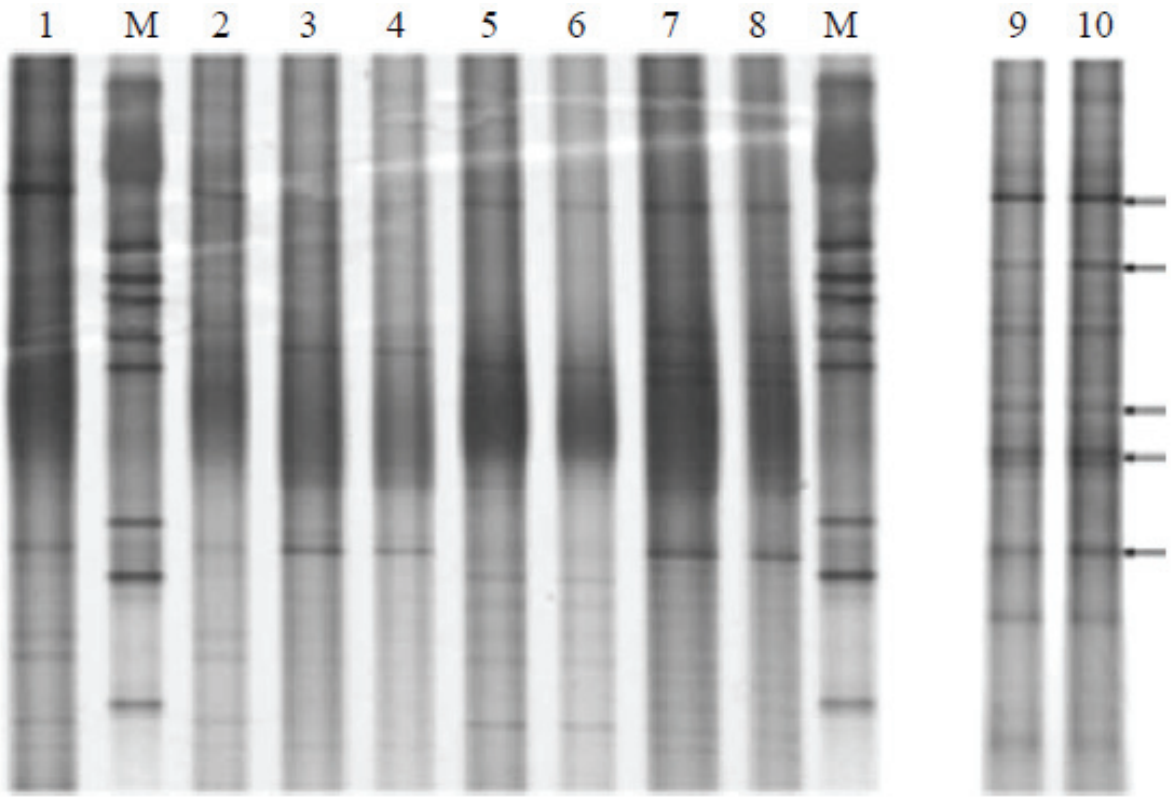

Figure 2. DGGE fingerprinting of RO biofilm samples collected from a full-scale water treatment plant. Lanes 1 and 2 – the first (A), and lanes 3 and 4 – the last (D) elements from the first stage; lanes 5 and 6 – the first (Ac), and lanes 7 and 8 – the last (Dc) cleaned elements from the second stage of the system. M corresponds to a synthetic marker (see Materials and methods). Arrows at the right indicate positions of the five dominant bands. Lanes 1 to 8 – scrapings from membranes of examined RO elements. Lanes 9 and 10 – the tightly associated membrane sample from the element A.

In addition, the DNA from the samples of tightly associated biofilms (Figure 3), collected from the surfaces of RO membranes and feed spacers, yielded a greater number of DGGE bands than DNA from the loosely associated biofilms (the scrapings from a membrane surface area). The samples of the tested product spacers were DNA free, however, in some cases a vaguely visible DNA bands were present, probably due to contamination during the sampling procedures. These bands were consistent with the bands on the feed side of tested membranes.

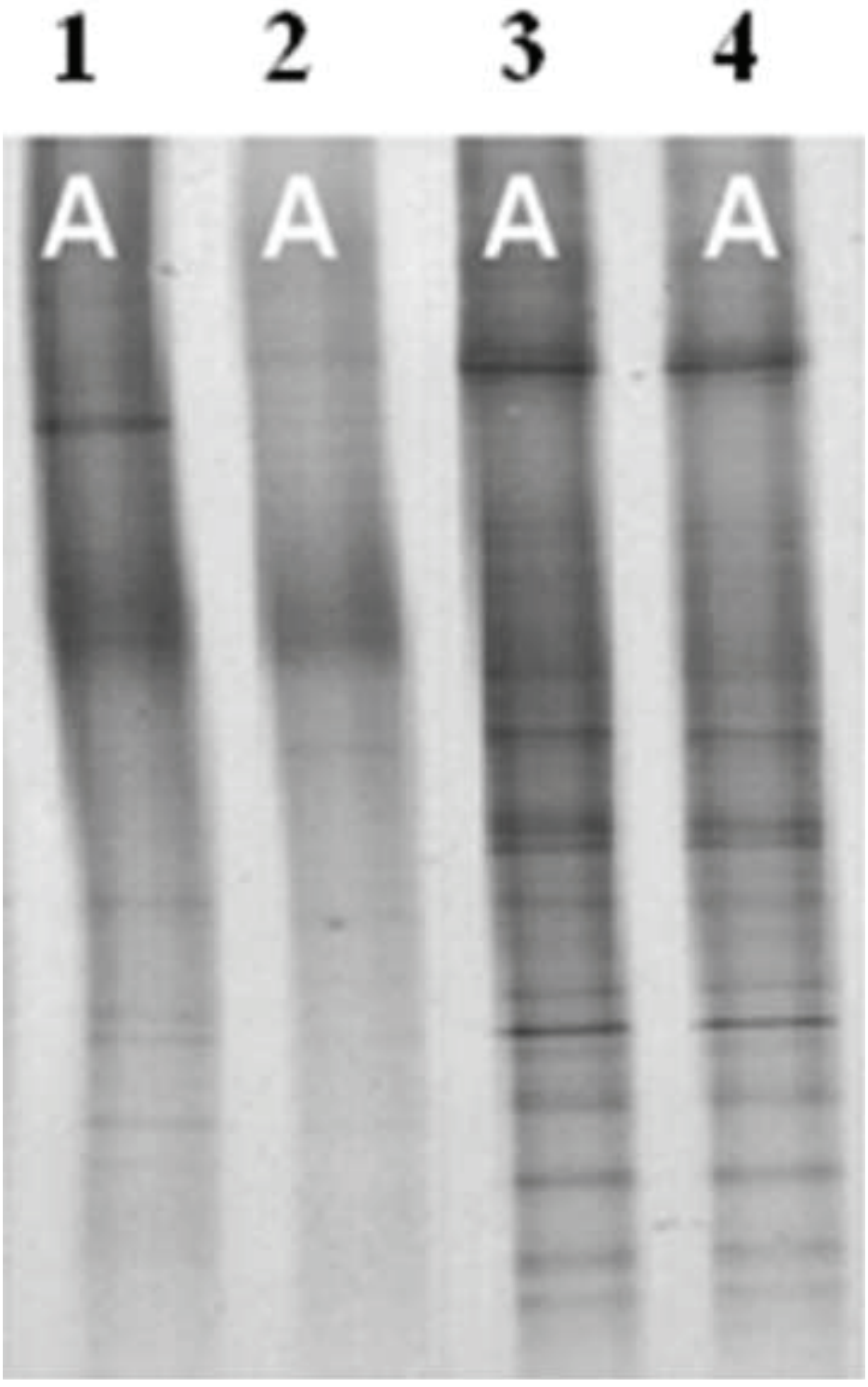

Figure 3. DGGE analysis of bacterial 16S rDNA amplicons comparing the banding patterns from different locations of the RO membrane element (A): lane 1 – scrapings from a membrane surface area with loosely associated biofilm; lanes 2 to 4 – biofilm sample from surfaces of product spacer, membrane and feed spacer, respectively.

Clone library construction

In this study, five RO biofilm 16S rRNA gene libraries, containing a total of 356 clones, were constructed with Bacteria universal primers set (7-f and 1510-r): library I (module A) contained 85 clones, library II (module D) – 74, library III (module Ac) – 58, library IV (module Dc) – 61 and library V (cartridge filter) – 64 clones. The libraries were subjected to RFLP analysis and clones with identical RFLP patterns were grouped together into clone families. To achieve a reliable identification of the microbial species, representatives from each of the clone families (unique sequences) were initially partially sequenced using the T7 primer and then the unique inserts, corresponding to the dominant bands in the original community DGGE fingerprints, were additionally sequenced in the reverse direction with the Sp6 primer.

Clone library analysis

Sequence analysis of the 356 clones revealed that 51 clones were putative chimeric clones. These clones were excluded from further community analysis. Phylogenetic analysis of the remaining 305 sequences revealed ten major phylogenetic lineages of the domain Bacteria (Table 1). The majority (77.9%) of the *Alphaproteobacteria*, which dominated the clone libraries in this study, were closely related to the *Sphingomonas* genus, which was numerically a most frequent encountered (34.1% of all bacteria in the library) bacterial genus in all biofilm samples tested. *Beta proteobacterium* (AF236004) was the second largest genus in the library (15.1%), followed by the *Flavobacterium* (14.4%), *Nitrosomonas* (9.2%), and *Sphingobacterium* (5.0%) genera. The remaining (29.8%) sequences in the library belonged to many other bacterial genera, which were present in a much lower proportion (0–4%) in all examined biofilm samples. The five 16S rRNA clone libraries were constructed using DNA isolated from different points in the RO system (Figure 1). Analysis of these different libraries showed that the dominant sequences from the *Sphingomonas*, *Beta proteobacterium* (AF236004), *Flavobacterium*, *Nitrosomonas* and *Sphingobacterium* genera were present throughout the whole RO system in equal ratios.

Table 1. Phylogenetic affiliations and frequency of cloned bacterial 16S rRNA gene amplicons*

Phylogenetic lineages (% of all bacteria in the library)	**No.** of clones	**Closest relative in GenBank** (taxon, accession no.)	**%** **
34.1% *Alphaproteobacteria*	70	*Sphingomonas sp*. (AB074191.1)	95–97
	1	*Sphingomonas sp*. (AF410927.1)	98
	1	*Sphingomonas sp*. (AF385529.1)	99
	8	Uncultured bacterium (DQ190175.1)	99
	1	Uncultured bacterium (DQ088792.1)	99
	6	*Sphingopyxis sp*. (DQ177493.1)	97–99
	5	Uncultured bacterium (DQ158105.1)	99
	7	*Hyphomicrobium zavarzinii* (Y14306.1)	94
	3	Uncultured bacterium (AY957940.1)	91–94
	2	Uncultured bacterium (DQ395494.1)	90
33.4% *Betaproteobacteria*	46	*Beta proteobacterium* (AF236004)	96–98
	28	*Nitrosomonas sp*. (AY123797.1)	98–99
	5	Uncultured bacterium (AJ867754.1)	99
	2	Uncultured bacterium clone (DQ017942.1)	99
	11	Uncultured bacterium (AB247475.1)	99
	7	Uncultured bacterium (AF407411.1	98
	3	Uncultured bacterium (AF351219.1)	97
3.3% *Gammaproteobacteria*	1	*Legionella sp.* (Z49717.1)	97
	6	*Pseudoxanthomonas yeongjuensis* (DQ438977.1)	99
	2	*Pseudomonas sp.* (AF321239)	99
	1	*Escherichia coli* O157:H7 (AE005174.2)	99
19.3% *Cytophaga-Flexibacter-Bacteroides*	20	*Flavobacterium sp.* (AM177636.1)	97
	1	*Flavobacterium sp.* (AM167556.1)	98
	9	Uncultured *Bacteroidetes* (AY948027.1)	94
	9	Uncultured bacterium (DQ178976.1)	94
	4	Uncultured bacterium (AY212620.1)	99
	1	Uncultured bacterium (AY187355.1)	98
	5	Uncultured bacterium (AJ575723.1)	92
	5	Uncultured *Sphingobacteriaceae* (AY509378.1)	98
	5	*Glacier bacterium* (AY315161.1)	99
1.6% *Nitrospira*	2	*Nitrospira sp.* (PY14639)	99
	3	Uncultured bacterium (AY532585.1)	98–99
0.7% *Firmicutes*	2	Uncultured *Clostridiales* (AY360624.1)	98
2.6% *Planctomycetales*	8	Uncultured bacterium (AY917697.1)	95
0.3% *Verrucomicrobia*	1	Uncultured bacterium (AJ401108.1)	95
0.3% *Candidate division* OD1	1	Uncultured bacterium (AY193203.1)	88
4.3% *Firbrobacteria/Acidobacteria*	12	*Acidobacteria* bacterium (AM162405.1)	97
	1	Uncultured bacterium (AB240484.1)	96

* Amplicons were ~ 1.45 kb and retrieved from biofilm samples from a full-scale water treatment RO plant

** Percentage of similarity between cloned 16S rRNA gene and the closest relative in the NCBI database

DGGE identification of 16S rRNA bands

The bacterial species representing the various bands of the DGGE profiles were identified by comparing the PCR amplicon migration profiles in the original DGGE profiles with those of 16S rRNA bands of identified clones. Using this approach all intense bands in the DGGE profile, together reflecting the complex microbial community in each RO biofilm sample, could be identified (Figure 4).

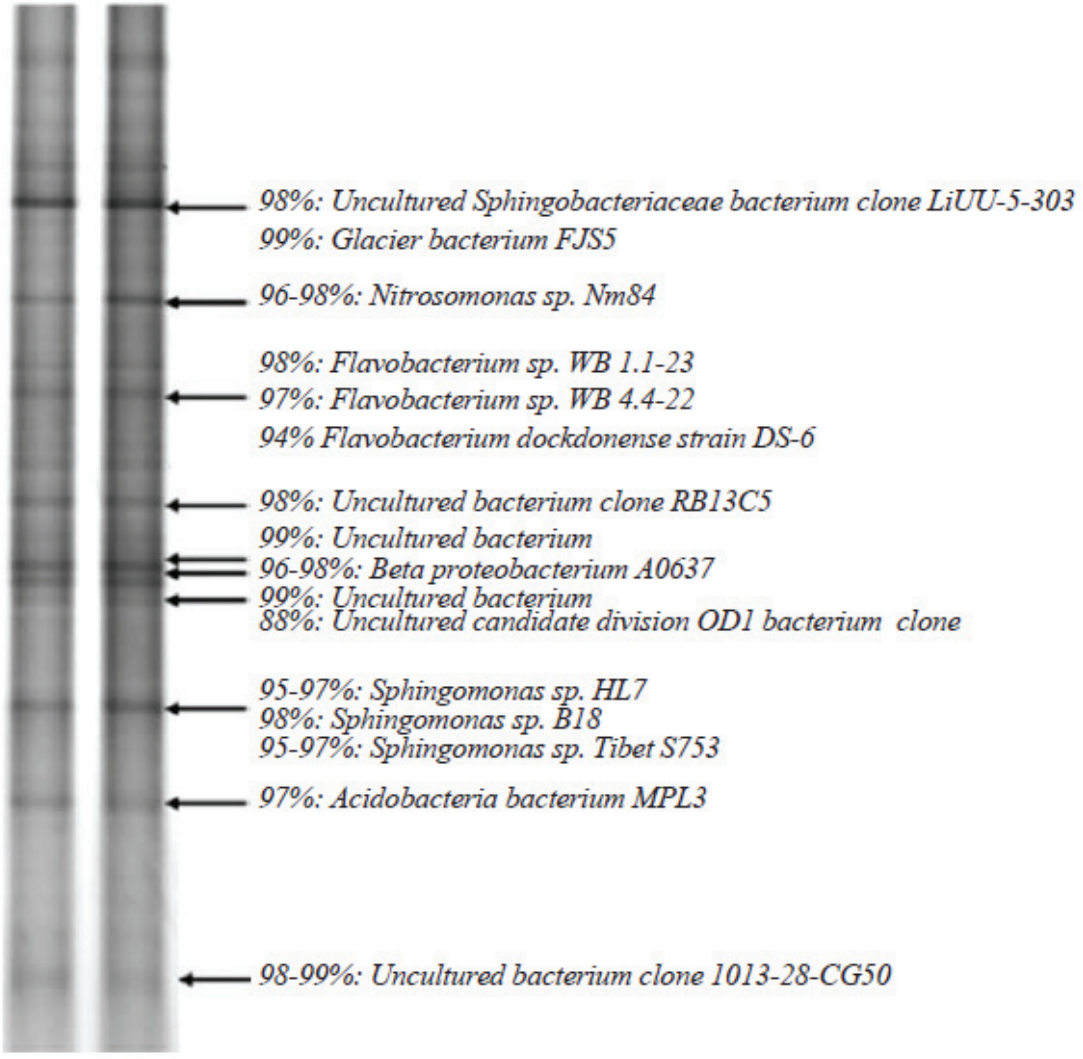

Figure 4. Identification of 16S rRNA bands of cloned sequences in the bacterial DGGE pattern of RO biofilm samples from a full-scale water treatment plant. Percentage values indicate sequence similarity with closest relative present in the GenBank.

According to matching of the migration profiles, five major (intense) DGGE bands were identified as members of *Sphingomonas*, *Beta proteobacterium* (AF236004), *Flavobacterium*, *Nitrosomonas* and *Sphingobacterium* genera. The minor constituents detected by the cloning library approach could not be associated with any of the visible bands in the original bandpatterns of biofilm communities. Some of the vague and barely visible bands on a DGGE gel were probably known artefacts associated with PCR-based methods (Suzuki and Giovannoni, 1996). In general, it was found that the average diversity of clones in the PCR library was higher than the diversity detected by DGGE analysis.

Concluding remarks

Our results show that conventional cleaning does not remove biofouling from the surfaces of RO membranes in the spiral-wound membrane elements although cleaning restores the performance of the membranes but decreases shortly after the cleaning. The biofilm community in the RO system was characterised by 16S rRNA-gene-targeted DGGE and phylogenetic sequence analyses of individual 16S rRNA clones. Using this approach, five distinct bacterial genotypes (*Sphingomonas*, *Beta proteobacterium* [AF236004], *Flavobacterium*, *Nitrosomonas* and *Sphingobacterium*) were found to dominate the surfaces of contaminated RO membranes. These genera are common species in freshwater (Zwart et al., 2002). Therefore, it is unlikely that these bacteria are contaminants incorporated in the RO unit during its manufacturing. Most likely they originate from the feed water, since they could also be recovered from the upstream cartridge filters of the RO system. This indicates that the UF membranes (pore size < 40 nm) do not effectively prevent their entry into the RO system. By using the five dominant members of RO biofilms identified in the present study, more detailed investigations are possible into the complex microbiological processes associated with the early establishment and subsequent development of the biofilm. As a result, the process of fouling of RO membranes and possible strategies for antifouling can be studied in greater detail.

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3 Molecular characterization of the bacterial communities in the different compartments of a full-scale reverse-osmosis water purification plant

This chapter is identical to the paper:
Bereschenko, L. A., G. H. J. Heilig, M. M. Nederlof, M. C. M. van Loosdrecht, A. J. M. Stams, and G. J. W. Euverink (2008) in *Appl. Environ. Microbiol.* 74: 5297–5304.

Abstract

The origin, structure and composition of biofilms in various compartments of an industrial full-scale reverse-osmosis (RO) membrane water purification plant were analyzed by molecular biological methods. Samples were taken when the RO installation suffered from a substantial pressure drop and decreased production. The bacterial community of the RO membrane biofilm was clearly different from the bacterial community present at other locations in the RO plant, indicating the development of a specialized bacterial community on the RO membranes. The typical freshwater phylotypes in the RO membrane biofilm (i.e., *Proteobacteria, Cytophaga-Flexibacter-Bacteroides* group and *Firmicutes*) were also present in the water sample fed to the plant, suggesting a feed water origin. However, the relative abundances of the different species in the mature biofilm were different from those in the feed water, indicating that the biofilm was actively formed on the RO membrane sheets and was not the result of a concentration of bacteria present in the feed water. The majority of the microorganisms (59% of the total number of clones) in the biofilm were related to the class *Proteobacteria*, with a dominance of *Sphingomonas* spp. (27% of all clones). Members of the genus *Sphingomonas* seem to be responsible for the biofouling of the membranes in the RO installation.

Introduction

Membrane biofouling is an important problem for reverse osmosis (RO) systems, in particular for RO membranes (13, 14, 17). The attachment of bacteria to membrane surfaces and subsequent biofilm growth in the spiral-wound RO membrane elements strongly influence RO system performance and RO plant productivity. Problems are due primarily to an increase in the differential pressures of the RO modules, the long-term membrane flux reduction of the RO plant and the deterioration of product water quality as a result of high levels of biomass accumulation on RO membrane surfaces (37, 43, 45). Once in progress, biofouling regularly and persistently hampers the RO water treatment process (13, 15).

Presently, adequate measures to prevent or reduce biofouling are lacking. The microbiological and physical processes associated with biofilm formation and biofouling in these dynamic and high-pressure environments are poorly understood. The conditions change from an oligotrophic environment in the beginning to a heterotrophic environment when the biofilm is mature. The first indications that a variety of different microorganisms participate in biofilm development on RO membranes were obtained by traditional dissections of fouled RO membrane elements (autopsies) and the subsequent analysis of the membrane surface-fouling layers. The conventional plating and colony isolation methods showed the presence of a wide variety of species on the feed and permeative surfaces of biofouled cellulose acetate, polyetherurea thin-film composite or polyamide thin-film-composite membranes (4, 9, 17, 19, 28, 38, 39). However, by cultivation-dependent methods, information about only 0.01 to 3% of the population in natural environments is obtained (2, 20, 23). In recent years, the microbial community structure in RO membrane samples obtained from full-scale membrane-based water purification processes was examined using 16S rRNA gene clone libraries and fluorescence in situ hybridization methods (7) and using PCR-denaturing gradient gel electrophoresis (DGGE) and sequence analysis of constructed clone libraries containing larger PCR fragments of the 16S rRNA gene (6). Pang and Liu (33) investigated the microbial-community composition of a biofilm retrieved from a lab-scale RO membrane module by applying a 16S rRNA gene-based clone library and terminal restriction fragment length polymorphism (RFLP) analysis. Nevertheless, a complete picture of the bacterial population responsible for the biofouling of RO systems is still lacking. A molecular study of microbial populations in all compartments of a full-scale RO water purification plant had not yet been performed.

This study aims to gain insight into the origins and compositions of the biofilms in full-scale RO systems by investigating the bacterial communities in terms of species composition and species diversity, as part of the free-living communities in the feed and product water, and as part of the film-forming communities attached to surfaces. The bacterial-community structure in various compartments of a full-scale RO water purification plant, including the RO feed water (F) (fresh surface water), the wall of the ultrafiltration storage tank (UF), a cartridge filter (CF), a biofouled RO membrane (M) and RO product water (P) (process water) was determined by molecular techniques. A PCR-DGGE approach (31) combined with the analysis of constructed clone libraries containing larger PCR fragments of the 16S rRNA gene (44) and DGGE screening of the isolated clones were used to reveal the differences between the bacterial community of the RO membrane biofilm and the other different locations of an RO plant.

Materials and methods

Sampling locations and procedures

Samples were collected in May 2006 from a full-scale RO water purification plant located in Veendam, The Netherlands. The plant used energy-saving polyamide (ESPA) RO membrane elements (ESPA 2; Hydranautics, CA) to produce process water. The F fed to the RO system of the plant was extensively treated by the sequential application of coagulation, flocculation, sand filtration, ultrafiltration and cartridge filtration processes. An additional chemical treatment of the RO membrane elements with an acid-alkaline solution was applied to this system once a week to maintain a reasonable flux. The samples were taken from the F, UF, CF, M and P when the RO installation suffered from a substantial pressure drop and decreased production. The F, P and UF samples were obtained prior to plant shutdown and RO membrane element removal. The UF sample was scraped from the walls of the ultrafiltration storage tank. For the collection of the RO membrane samples, the first membrane element from the first stage of the investigated RO system was selected. The element, used for about 1 year in the water purification process, was retrieved from the RO unit after plant shutdown, wrapped in plastic sheeting and transferred to the laboratory for an autopsy on the same day. The samples were taken directly after physical dissection and during the autopsy of the RO membrane by excising small sections from different locations in the

membranes (the tightly associated membrane samples) or by scraping material from a known area on the surfaces of the membranes (the loose biofilm samples). All samples were collected in sterile tubes and kept on ice until further processing within 1 day.

Total DNA extraction.

The microbial biomass from the water samples (10 ml) was collected by centrifugation at 10,000 x *g* for 10 min and suspended in 0.5 ml of phosphate-buffered saline (pH 7.0). Approximately 0.5 mg material was transferred from the biofilm samples to a clean tube, mixed with 0.5 ml of phosphate-buffered saline (pH 7.0) and homogenized using a vortex. All samples were subjected to 20 min of sonication and the total community DNA was extracted from 0.5 ml of homogenate using a minibead beater with the Fast DNA spin kit for soil (MP Biomedicals) in accordance with the manufacturer's instructions. The quality of the DNA was checked by agarose gel electrophoresis. Aliquots of each DNA extract were further purified and concentrated with a DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's instructions.

PCR amplification and DGGE analysis of amplified 16S rRNA genes

PCR amplification of bacterial 16S rRNA genes from total genomic DNA was performed using Go *Taq* DNA polymerase (Promega) with primers 954-f and 1369-r (MWG-Biotech AG), targeting the hypervariable V6-V8 region, as previously described by Zhongtang and Morrison (46). A 40-base GC clamp (5´-CGCCGGGGGCGCGCCCCGGGCGGGGCGGGG GCACGGGGGG-3´) was attached to the forward primer at the 5´ end. A typical PCR mixture (50 µl) contained 10 ng template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM (Invitrogen), each primer at 0.5 µM, 1.25 U of Go *Taq* DNA polymerase and 1x PCR buffer containing 3 mM $MgCl_2$ (Promega). The reactions were performed in an iCycler (Bio-Rad) with predenaturation at 94°C for 2 min, followed by 35 cycles consisting of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. The cycles were completed with a final extension step of 7 min at 72°C. DGGE analysis of the generated amplicons was performed using a DCode System (Bio-Rad) as previously described by Heilig et al. (18). A mixture of the DGGE-PCR products from nine bacterial species was applied as a marker. The number of operational

taxonomic units (OTUs) in each sample was defined as the number of DGGE bands with distinct electrophoretic mobilities.

Cloning of PCR-amplified products and sequence analyses

Amplification of the almost-full-length bacterial 16S rRNA gene fragments with the 7-f and 1510-r universal bacterial primers (27) was performed with the iCycler as described previously (6). Amplified fragments were purified with the DNA Clean & Concentrator-5 kit, ligated into the pGEM-T easy vector (Promega kit) and cloned into *Escherichia coli* XL1-Blue according to the manufacturer's instructions. Vector-harboring clones were transferred with a sterile toothpick into 50 µl of Tris-EDTA buffer and were incubated at 95°C for 15 min to lyse the cells. The PCR amplification of cell lysates with T7 and Sp6 pGEM-T-specific primers and the selection of clones containing insertions of the appropriate sizes by the RFLP analysis were performed as described previously (6). Individual clones with a unique RFLP pattern were selected and screened by DGGE analysis with the V6-V8 primers (GC-954-f and 1369-r). Their DGGE bands were detected in the original DGGE fingerprint profile of the biofilm community by using Bio-Numerics software (BioSystematica). Unique inserts were bidirectionally sequenced with T7 and Sp6 primers (BaseClear, Leiden, The Netherlands). Checks for chimeric sequences were conducted by using the Chimera Check program at http://www.cme.msu.edu/RDP/html/index.html (29) and sequence similarity was analyzed by using the NCBI BLAST search tool at http://www.ncbi.nlm.nih.gov/BLAST (1) and the GenBank database.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this study were deposited in GenBank under accession numbers EU428849 to EU428950.

Results

Observations during autopsy

At the laboratory, the removed RO membrane element was unpacked and cut open, and the membrane packs were unfolded and visually examined. The visual inspection of the element showed the presence of a slimy, opaque, light-brown deposit on the surfaces of all membrane sheets and feed spacers (Fig. 1), indicating that the fouling layers were not eliminated by the routine (once-a-week) cleaning procedures of the RO units in this system.

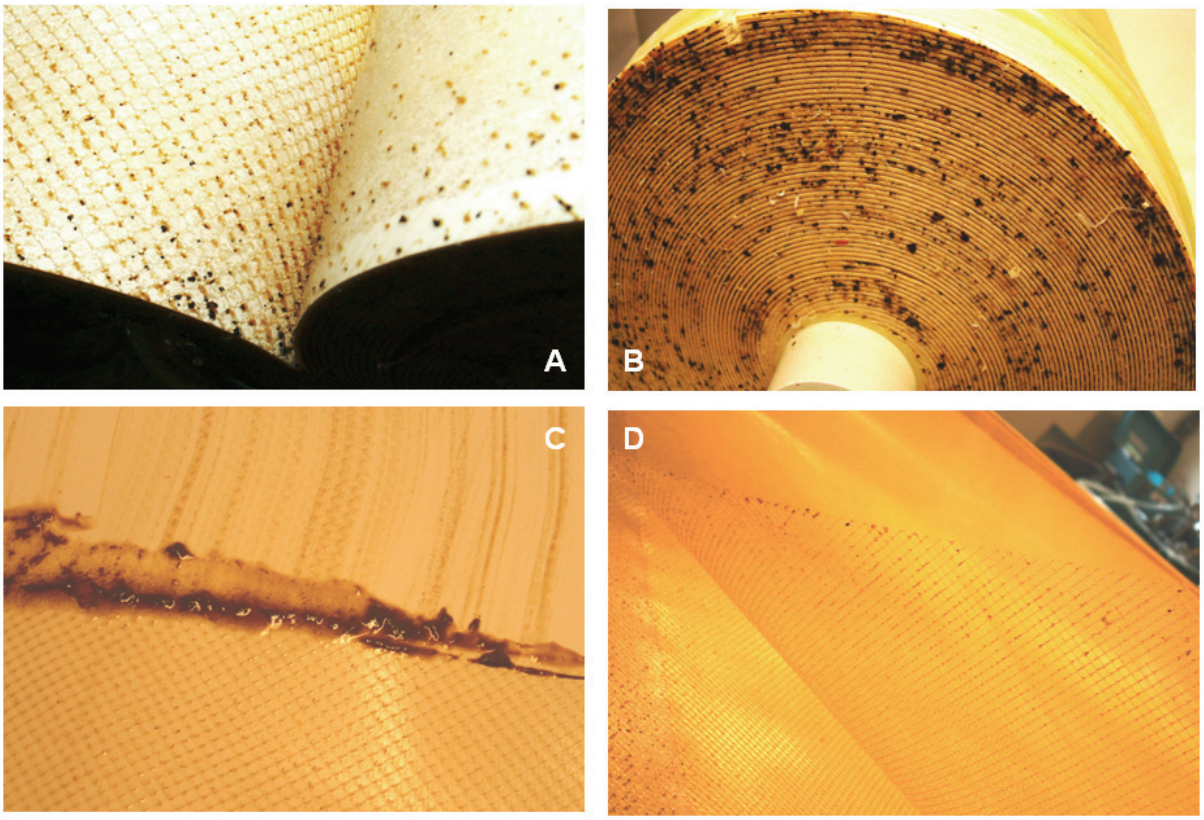

FIG. 1. Photographs of an autopsy of fouled RO ESPA-2 spiral-wound M elements (Hydranautics ESPA). The feed side of the membrane element (**A** and **B**), the feed side of the fouled membrane (**C**) and the fouled plastic feed channel spacer (**D**) are shown. The surfaces of the membrane and spacer were completely covered with fouling layers. The fouling layer could be relatively easily scraped from the membrane surfaces (**C**).

After 1 year of operation, the fouling layer was spread over the complete membrane and the feed spacer surfaces in the module. This fouling layer was quite loosely attached to the RO membrane and could be relatively easily scraped from the surface (Fig. 1-C). It was also noted that the membrane surfaces were more intensely fouled than the feed spacer surfaces and no visible fouling was observed on the surfaces of the product spacers.

Clone library construction and analysis.

In total, five 16S rRNA gene clone libraries, containing a total of 635 clones, were constructed with the *Bacteria* primer set (7-f and 1510-r) by using total genomic DNA isolated from the F, UF, CF, M and P. All the clones in the libraries were subjected to 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 was determined from those clones that contained a unique sequence and that corresponded with a dominant band in the DGGE community fingerprints.

The nucleotide sequences of a total of 635 clones were determined. A total of 35 clones were detected as possible chimeras and were excluded from further community analysis. The nucleotide sequences of the remaining clones, which included 179, 67, 90, 152 and 112 clones from the F, UF, CF, M and P libraries, respectively, were further analyzed for their phylogenetic affiliations and closest relatives by searching the GenBank database with the NCBI BLAST search tool. Different sequence types (or OTUs) affiliated with various phylogenetic lineages of the domain *Bacteria* (with a sequence similarity of > 0.90) were obtained from the clone libraries (Table 1).

Phylogenetic analysis indicated that the *Proteobacteria* division dominated all clone libraries in this study, in which the *Betaproteobacteria* subdivision was the largest bacterial group found in water samples of the F and P (85% and 65% of total clones, respectively) and members of the *Alphaproteobacteria* subdivision were numerically the most frequently encountered in the biofilms of the samples of the UF, CF and M (28%, 29% and 35% of total clones, respectively). Furthermore, members of the *Betaproteobacteria* subdivision made up the second-largest fraction in the UF, CF and M (24%, 20% and 14% of total clones, respectively), whereas members of the *Alphaproteobacteria* subdivision made up the second-largest fraction in the F and P (8 and 9% of total clones).

TABLE 1. Phylogenetic affiliations and frequencies of cloned bacterial 16S rRNA gene amplicons[a] retrieved from RO samples from the full-scale RO water treatment plant.

Closest relative in GenBank (accession no.)	Similarity (%)[b]	% of clones in indicated clone library[c]				
		F	UF	CF	M	P
Sphingomonas sp. MTR-71 DQ898300.1	95		16.4	17.7	7.9	2.7
Sphingomonas subterranea AB025014.1	98	1.7	1.5	2.2	3.3	0.9
Sphingomonas sp. ORS 1497 AJ968701.1	96		1.5	1.1	0.7	
Sphingomonas sp. DB-1 AY947554.1	98		1.5	2.2	0.7	
Sphingomonas sp. BAC151 EU131005.1	97	5.6	1.5	1.1	9.2	2.7
Sphingomonas sp. HI-K4 DQ205308.1	98		3.0		1.3	0.9
Sphingomonas sp. BAC13P EU131003.1	97			1.1	0.7	0.9
Sphingomonas oligophenolica AB365794.1	98				0.7	
Sphingomonas sp. HTCC500 AY584571.1	98				0.7	
Sphingomonas suberifaciens D13737.1	97				0.7	
Sphingomonas sp. P2 AB091683.1	97				0.7	
Other *α-Proteobacteria* AY029562.1, AB271055.1, AM411913.1, DQ414680.1, AM286550.1, X97691.1, DQ177493.1, AY921677.1, EU050759.1	94-99	1.1	3.0	3.3	8.6	0.9
Acidovorax sp. AM262110.1, Y18617.1, EF540489.1, AF235013.1	99	1.1	1.5	1.1	2.1	0.9
Burkholderia sp. AB232330.1, AY752954.1, AB212237.1, DQ156083.1	99	78.3	1.5	1.1	1.4	41.2
*Janthinobacterium sp.*AF174648.1, EF422171.1 , AJ551147.1	98-99	5.1	14.9	14.4	1.4	6.3
Nitrosomonas sp. AB000700.1, AY123811.1, AY123797.1	95-99	0.6	1.5	1.1	4.6	
Other *β-Proteobacteria* AB120966.1, AB195750.1, AF236004.1, AJ556799.1, DQ413154.1, EU130968.1, AM236310.1, AF351219.1, AJ575695.1, AF204252.1, AB265946.2, AF078758.1	93-99		4.5	2.2	4.7	16.2
*Pseudomonas sp.*AM157452.1, DQ178233.1	97-99	1.1	20.9	15.5	2.0	
Lysobacter sp. AB161360.1, AB249682.1	95-96	0.6			6.0	0.9
Legionella sp. X73406.1,, AM747393.1	93-97	0.6		1.1	1.3	2.7
Other *γ-Proteobacteria* AM396494.1, EF191354.1, AJ583181.1, AM229325.1	97-99	1.7	1.5	2.2		
δ-Proteobacteria AY921696.1, AF418174.1, U41561.1	98				0.7	1.8
Flavobacterium sp. AM230485.1, DQ628949.1, EF520552.1, EF540472.1	97-100	2.2	1.5	3.3		2.7
Other CFB AY780553.1, AB074940.1, DQ640688.1, AY910857.1	92-98		1.5	1.1	2.0	4.5
Clostridium sp. AY360624.1, X75909.1, AJ506120.1, AB288643.1, and AY935674.1	92-99		10.5	13.3	2.0	4.5
Other *Firmicutes* EF033503.1, AM745263.1, AY766466.1	91-97	0.6	1.5	3.3		
Actinobacteria AB271048.1, AY368456.1, AM410685.1	98				3.9	
Chlorobium phaeobacteroides AM050128.1	98					0.9
Uncultured *candidate division* OP11 AF047573.1	92		1.5		2.0	
Acidobacteria DQ513986.1, AY921727.1, EF032752.1	96-97		6.0	7.8	3.3	0.9
Planctomycetales AY942960.1, AY500064.1, AB116499.1, DQ676396.1, AJ290177.1, EF221226.1, X81950.1	91-98				14.5	4.5
Verrucomicrobia AB305640.1, AB288576.1, AB288579.1	92-97		3.0	4.4	2.1	0..9
Uncultured AY917428.1, EF220517.1, EF663458.1, EU273223.1, AB288666.1, EF506959.1, EF688335.1, AB290357.1, DQ241389.1	84-98				10.8	2.7

a **-** Amplicons were approximately 1.45 kb in size; ***b*** **-** percentage of similarity between the cloned 16S rRNA gene and its closest relative in the NCBI database; ***c*** **-** F clone library (179 clones), UF clone library (67 clones), CF clone library (90 clones), M clone library (152 clones), P clone library (112 clones); ***d*** **-** CFB, *Cytophaga-Flexibacter-Bacteroides* spp.

The majority of the *Alphaproteobacteria* found in all samples were primarily affiliated with the genus *Sphingomonas*; 2 to 7% of the total number of clones in these libraries were related to known *Sphingomonas* species (>97% similarity) (Table 1). The remaining clones in this group were closely related to other known *Alphaproteobacteria*, like *Afipia massiliensis* and *Hyphomicrobium* sp. (present in three of the five samples), "*Caulobacter ginsengisoli*," *Mesorhizobium* sp., uncultured "*Nordella*" sp., *Pedomicrobium manganicum* and *Sphingopyxis* sp. Two OTUs (3% of the total number of clones) from the M sample were related to two uncultured species of *Alphaproteobacteria*.

Within the *Betaproteobacteria* lineage, *Acidovorax*, *Burkholderia* and *Janthinobacterium* were the common bacterial genera in all samples. In this study, the genus *Burkholderia* represented the largest fraction in the *Betaproteobacteria* subgroup (Fig. 2) of the F and P libraries (78% and 41% of total clones, respectively).

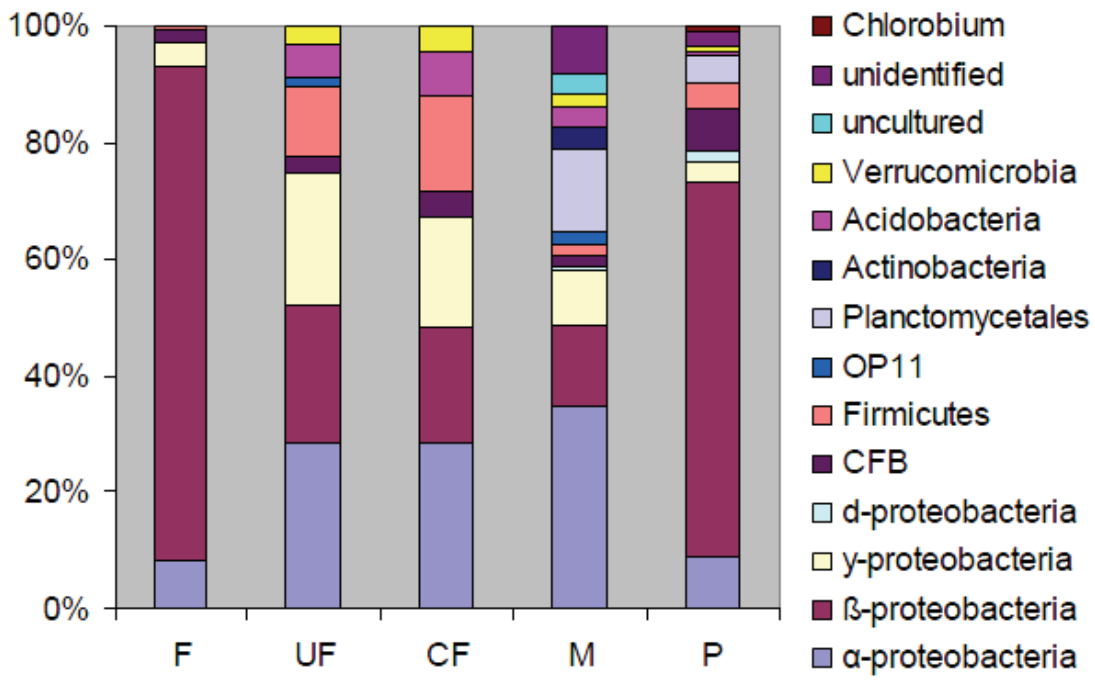

FIG. 2. Schematic diagram depicting the results of the clone library analyses performed on the samples obtained from the different functional parts of a full-scale RO plant. F was initially pre-treated by the sequential application of coagulation, flocculation, sand filtration, UF and CF processes. The two-stage RO system integrated ESPA-2 spirally wound M elements (Hydranautics ESPA) which were installed in a series inside pressure vessels.

Janthinobacterium spp. were found mainly in the UF and CF samples (15% and 14% of total clones, respectively). The most dominant betaproteobacterium in the M sample was related to *Nitrosomona*s sp. strain Nm59 (4%). The remaining sequences identified as *Betaproteobacteria* were closely related to known species, such as "*Aquamonas fontana*," the aquatic bacterium R1-B18, the betaproteobacterium A0637, *Comamonadaceae*, *Hydrogenophaga* spp. and *Simplicispira* spp. However, in P samples, 11% of the total clones were related to uncultured betaproteobacterium species.

All biofilm samples (UF, CF and M) further comprised OTUs from the *Gammaproteobacteria* division (22%, 19% and 9% of the total clones, respectively). Members of the phylum *Firmicutes* were found mostly in UF and CF biofilms (12% and 17% of the total clones, respectively) and consisted mainly of *Clostridium* species (UF: 11% of the total clones, CF: 13%). From the *Gammaproteobacteria* division, the most frequently encountered OTUs from the biofilm samples were closely related to *Pseudomonas* spp. (UF: 21% of the total clones, CF: 16%) or showed 95 to 96% similarity with *Lysobacter* spp. (M: 6% of the total clones). Bacteria related to members of the *Cytophaga-Flexibacter-Bacteroides* group were found in all samples in similar percentages. Furthermore, in biofilms of the UF, CF and M, bacteria related to uncultured environmental clones from the *Acidobacteria* (6%, 8% and 3% of total clones, respectively) and *Verrucomicrobiae* groups (3%, 4% and 2% of total clones, respectively) were found. Species related to uncultured environmental clones from the *Planctomycetacea* group were found in the M and P samples only (15% and 5% of all clones, respectively). Differing from the rest of the biofilm samples, the M sample further comprised clones related to *Actinobacteria* (4% of the total clones). Nine other OTUs from M samples (11% of the total clones) were related to unknown uncultured bacteria, some with a homology of less than 95%, or showed no exact match (<90% similarity) with any of the known bacterial sequences found in the databases. Similarly, no exact match was found for 3% of the total clones in the P sample.

Fingerprinting of RO biofilm communities by DGGE

DGGE analysis of PCR-amplified fragments of the hypervariable V6-V8 region of the bacterial 16S rRNA gene (approximately 415 bp) obtained from the F, UF, CF, M and P

samples revealed clearly discriminative “fingerprints” of bacterial communities from various compartments of the investigated RO plant (Fig. 3).

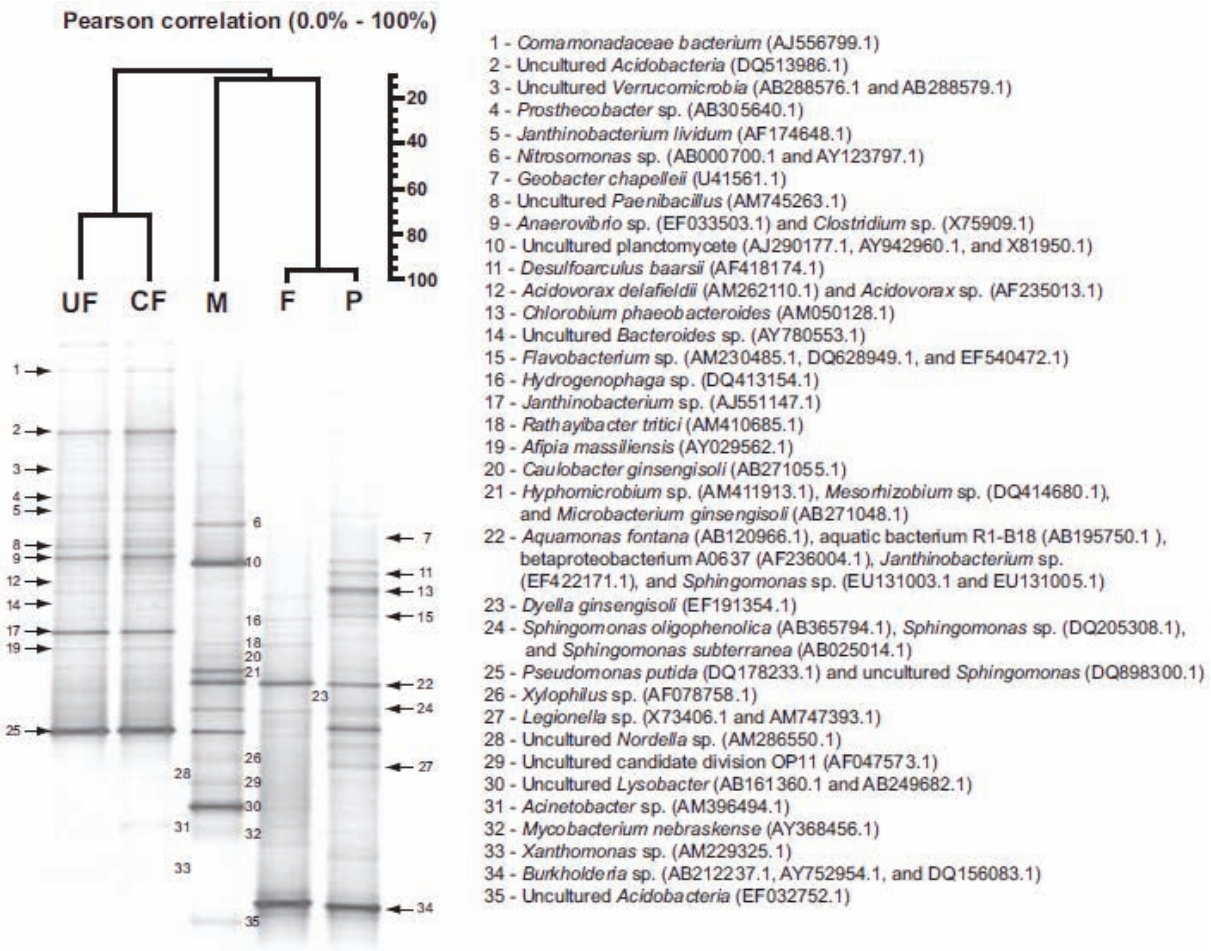

FIG. 3. DGGE fingerprinting of RO biofilm samples collected from a full-scale water treatment plant. Arrows with numbers indicate the positions of the identified bands.

The gel image shows distinct bands (or OTUs), indicating the presence of multiple species in all of the samples tested. At least two DGGE bands could clearly be discriminated as dominant (intense bands) within each single biofilm sample. The highest number of dominant bands was present in the M sample (seven bands) and the lowest in the F sample (two bands). The remainder of the samples each contained four dominant bands.

The complexity of the DGGE profiles (Fig. 3) of the microbial community in the F sample (lane F) is less than that of the free-living community in the P sample (lane P) and that of the biofilm-forming communities attached to the surfaces of the UF (lane UF), CF (lane CF) and M (lane M). In general, the bacterial communities from the F and P samples had similar community fingerprints (71% similarity) but were markedly different from those of the other three samples. The DGGE profiles obtained from the samples from the CF and UF showed similar community fingerprints (96% similarity), but these were also different from the fingerprints from the other three samples. The M sample had a unique fingerprint compared to those of the other samples. The feed spacer sample of the investigated RO membrane module had a very similar DGGE pattern (data not shown). Moreover, the samples of the tested product spacer showed the presence of bacteria that were also found in the RO membrane sample but in relatively low numbers according to their band intensities in the gel (vaguely visible bands [data not shown]). The bacterial species representing the visible bands of the DGGE profiles of the F, UF, CF, M and P samples were identified by comparing the migration profiles of the PCR amplicons in the original biofilm community fingerprints with the migration profiles of the DGGE-PCR products from the individual clones. Except for the minor constituents, most members of biofilm communities detected by the cloning library approach could be associated with one of the visible bands in the DGGE profiles. In total, 35 distinct DGGE bands could be associated with at least one of the identified clones in the constructed libraries.

Discussion

In this study, we describe the complex and diverse bacterial communities in various compartments of a full-scale RO water purification plant by using two culture-independent methods. The bacterial communities were investigated in terms of their species diversity and the relative abundances of the free-living communities in the F and P samples and of the film-forming communities attached to the UF, CF and M. The biodiversity of these communities, as revealed by analysis of 16S rRNA genes from the total biofilm community, was larger than that found by the DGGE approach. The cloning method was more powerful than DGGE in evaluating the complexity and composition especially of biofilms on M, because 2.5-fold-more genetically different bacteria were identified than in the DGGE analysis. The DGGE fingerprints underestimated the diversity of the communities due to the comigration of several

different 16S rRNA gene fragments observed in experiments where individual clones from the libraries were subjected to DGGE profiling. All OTUs from the species that were present in relative high numbers in the sample communities (Table 1, Fig. 2) were detected as visible DGGE bands (Fig. 3). Although DGGE analysis in this study did not visualize all the members of a complex microbial community as separate bands, both methods could detect the same dominant species in the communities. The inability to detect populations of low abundance and overlapping DGGE bands was also shown by Muyzer et al. (31) and Murray et al. (30).

A large difference exists between the bacterial-community composition of the M biofilm and the bacterial-community compositions at other locations in the RO plant. The M biofilm community was more complex than the bacterial populations in the other compartments of the RO plant (Table 1, Fig. 2 and 3), indicating the occurrence of different selection mechanisms at different compartments in the full-scale plant. These differences indicate that the biofilm was actively formed on the M surfaces and was not the result of a simple concentration of bacteria present in the F. Undoubtedly, a bacterial community adapted to this environment was present in the form of a biofilm on the M surface at the moment of sampling, when changes in plant performance were noted (an increased pressure drop over the RO module). This complex community was represented by bacterial species with different physiological traits, most likely selectively promoted under changing physical-chemical and microbiological conditions in the dynamic and high-pressure (12-bar) operating environment of the RO system. Apparently, the predominant bacterial species capable of handling these conditions were related to the genus *Sphingomonas* (27% of all clones), which is known to thrive in biofilms (7, 21, 24, 25). The *Planctomycetacea*, the second largest group associated with the M biofilm (15% of all clones), are free-living aquatic oligotrophs that feed on algae or on their degradation products (16). Some of them contain a large number of open reading frames coding for enzymes necessary for polysaccharide degradation (www.regx.de), which are present in large amounts in biofilms (22, 26). A relatively low abundance of other different species in the M biofilm community found in this study (Table 1) cannot be interpreted as evidence that these minority populations are of little importance to the community as a whole. Even very low levels of bacterial species can maintain community activity (11).

The discovery of the typical freshwater phylotypes (47) in the M biofilm, as well as the detection of most of them (i.e., *Proteobacteria, Cytophaga-Flexibacter-Bacteroides* and *Firmicutes*) in the F sample, suggests a feed water origin rather than a manufacturing

contamination in the RO unit. Although a relatively small number of bacterial genera (approximately 12) appears to dominate the F community at the moment of the sampling, all of them, except *Dyella*, were also found in the film-forming communities on the UF, CF or M surfaces in addition to the P community (Table 1). The observed dominance of the genus *Sphingomonas* in the UF, CF and M samples (Table 1, Fig. 2 and 3) may be explained by the strong association of these organisms with surfaces (34), while the prevalence of the *Betaproteobacteria* in the water samples (the F and P samples) was consistent with their abundance in the freshwater as plankton (47). On the other hand, the absence of bacteria related to *Acidobacteria*, *Actinobacteria*, *Deltaproteobacteria*, *Chlorobium*, *Planctomycetacea*, *Verrucomicrobiae* and some other bacterial groups in the F sample and their presence in the other samples suggest that these organisms entered the plant prior to the sampling. The logical explanation of the exclusive presence of some bacterial species at different locations is that the conditions inside the RO plant were optimal.

The detection of different bacterial sequences in the P sample was rather unexpected, since the passage of the bacteria through the RO membrane (8-in. Hydranautics ESPA 2) is theoretically impossible. Also, it is not clear why bacteria such as *Aquamonas*, *Chlorobium*, *Desulfarculus*, *Geobacter* and *Mesorhizobium* were found in the P sample, since they were not detected in the other samples. The reason might be that these organisms are involved in the biofouling of the pipelines connecting the RO system with the permeate storage tank. The presence of *Geobacter*, an anaerobe involved in the reduction of Fe(III) (8), could indicate the corrosion processes of these pipelines on metallic surfaces. Also, the detection of the green sulfur bacteria from the genus *Chlorobium* indicates that the environment is anaerobic, because their photosynthesis can occur only in the complete absence of oxygen (32). However, it is not clear how these bacteria can survive and possibly even grow without light.

This is the first molecular study of microbial populations that has been performed on all units of a full-scale RO water purification plant. This approach allowed for the understanding of how bacterial communities are distributed throughout the RO plant and where they originate. The investigations suggest an important role of *Sphingomonas* in the biological membrane fouling of spiral-wound membrane elements applied in the RO water purification processes. The members of this genus were the most prevalent organisms in the M biofilms in this study but also in our previously reported investigations, in which bacterial biofilms that developed on RO membranes of ~5.5 year-old M elements were investigated (6). As the RO plant location, process configuration, cleaning type and frequency, membrane surface material, feed water and the sampling time (May) of the M samples were the same as

in our earlier study, the presence of *Sphingomonas* in all membrane biofilm communities confirms that these organisms are positively selected because of their competitive advantages for survival in this environment. As facultative oligotrophs, they are metabolically well adapted to a low-carbon environment (10, 41) and can proliferate under conditions of limited substrates for bacterial growth in the initially clean RO system. *Sphingomonas* organisms are able to utilize a broad range of naturally occurring organic compounds as well as many types of environmental contaminants (5). Apparently, they are also able to survive at high nutrient concentrations that occur close to the membrane surface in the RO units due to the concentration polarization effect in membrane separation processes and the accumulation of nutrients in the biofilm matrix. Furthermore, *Sphingomonas* species can change their planktonic state to sessile when the culture conditions, such as the level of aeration, are changed (35). Hence, a low-oxygen concentration, generally typical for the M modules, could stimulate their potential ability to form M biofilms. The transport of *Sphingomonas* to the membrane surfaces under continuous-flow conditions in spiral-wound M elements could be facilitated by their twitching and swarming motility (34). Their ability to produce different kinds of extracellular polysaccharides (12, 22, 35, 36) can help to initiate biofilm formation and to keep them attached to the membranes (3, 34). Moreover, the slimy extracellular polysaccharide matrix may protect the cells inside a biofilm matrix against the regular chemical cleaning procedures by acting as a chemically reactive barrier that inactivates the cleaning chemicals (40). Pang and coworkers (34) observed that one of the most dominant bacterial isolates previously retrieved by Chen et al. (7) from a biofouled M sample treating potable water, *Sphingomonas* sp. strain RO2, effectively colonized different RO membranes in continuous-flow cell systems regardless of their surface properties. Hence, *Sphingomonas* and other biofilm-associated slime producers, like *Rhizobiales* bacteria (33), are responsible for membrane surface colonization that facilitates the attachment of other bacteria and encourages the maturation of the biofilm. The formation and accumulation of exopolymeric substances, characteristic of growing biofilms (42), substantially decrease the water flux through membranes (21), one of the typical problems associated with biofouling in the actual practice of RO systems. Research is in progress to identify the nature of exopolymers formed by sphingomonas strains isolated from RO membranes.

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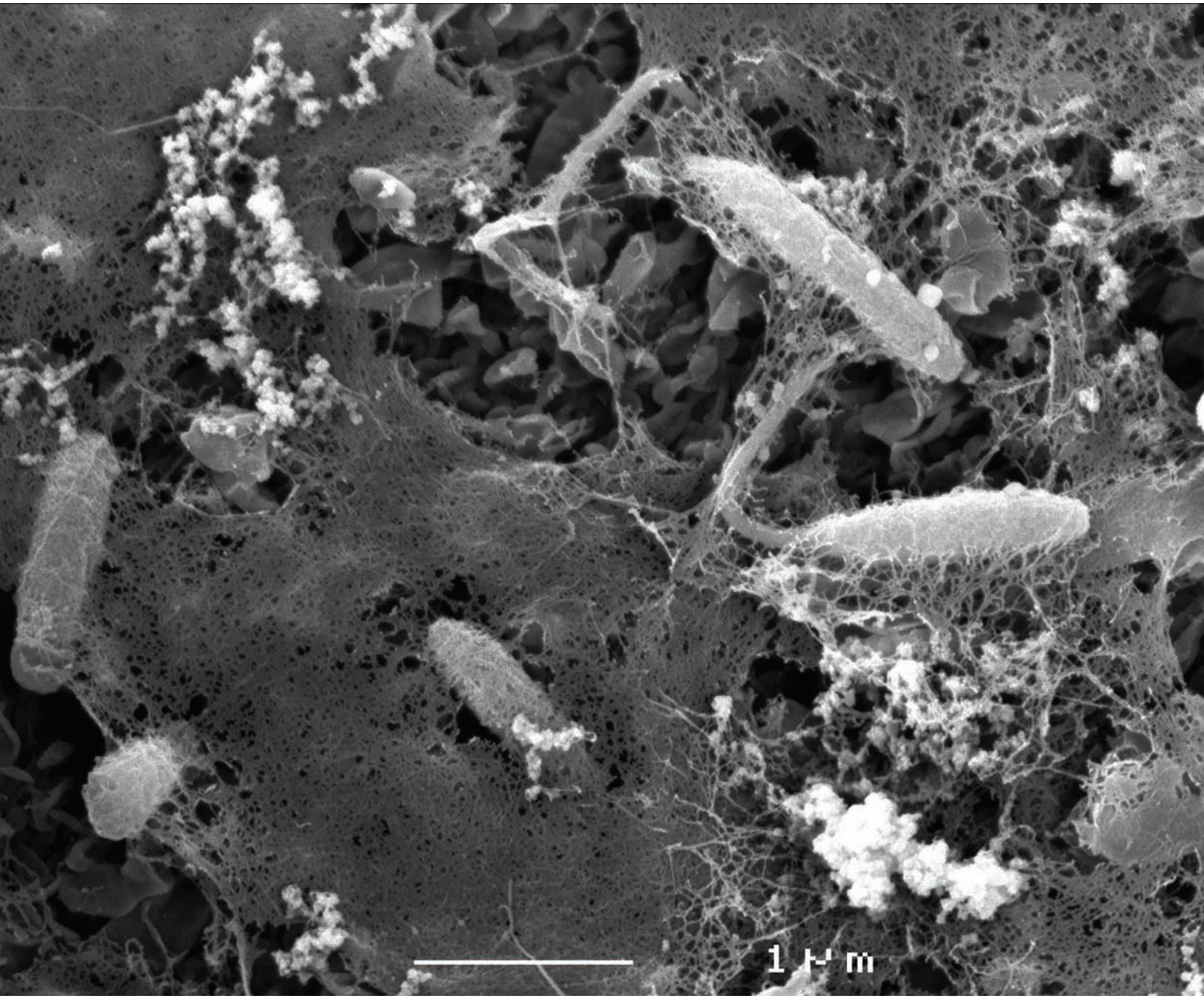
4 Biofilm formation on reverse osmosis membranes is initiated and dominated by *Sphingomonas* spp.



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Abstract

The initial formation and spatiotemporal development of microbial biofilm layers on surfaces of new and clean reverse osmosis (RO) membranes and feed-side spacers were monitored *in situ* using flow cells placed in parallel with the RO system of a full-scale water treatment plant. The feed water of the RO system had been treated by the sequential application of coagulation, flocculation, sand filtration, ultrafiltration and cartridge filtration processes. The design of the flow cells permitted the production of permeate under cross-flow conditions similar to those in spiral-wound RO membrane elements of the full-scale system. Membrane autopsies were done after 4, 8, 16 and 32 days of flow-cell operation. A combination of molecular (fluorescence *in situ* hybridization [FISH], denaturing gradient gel electrophoresis [DGGE] and cloning) and microscopic (field emission scanning electron, epifluorescence and confocal laser scanning microscopy) techniques was applied to analyze the abundance, composition, architecture and three-dimensional structure of biofilm communities. The results of the study point out the unique role of *Sphingomonas* spp. in the initial formation and subsequent maturation of biofilms on the RO membrane and feed-side spacer surfaces.

Introduction

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.

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; Hydranautics, 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 Veendam (Netherlands). In continuous-flow mode, the RO feed water - fresh surface water pre-treated by the sequential application of coagulation, flocculation and sand filtration (CSF), ultrafiltration (UF) and cartridge filtration (CF) processes - entered the flow chambers (size, 19.8 cm by 12.7 cm by 3.0 mm) at a rate of 75 liter/h. Operated with 0.05 m/s linear cross-flow water velocity and recovery (permeate/feed ratio) of 1 to 1.2 %, the flow cells produced 32 liter/$m^2 \cdot$ h process water. The experiment was run from March to April 2008 at an ambient temperature of 5°C to 10°C. The flow cells were initiated simultaneously but sacrificed sequentially after 4, 8, 16 and 32 days.

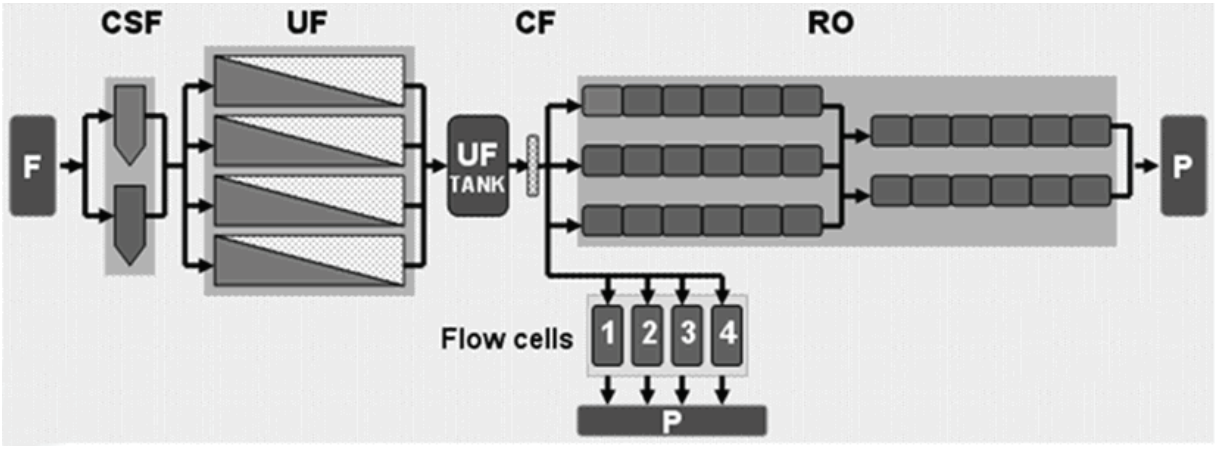

Figure 1. Schematic outline of the reverse osmosis (**RO**) system of a full-scale water purification plant. The fresh surface water (**F**) was extensively treated by the sequential application of coagulation, flocculation and sand filtration (**CSF**), the ultrafiltration (**UF**) and the cartridge 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**).

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 1x 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 1x 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 banding pattern of the DGGE data by calculating the Pearson product moment 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 1x PBS, postfixed for 15 min with a solution of 1% OsO_4 in 1x 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 two times with sterile 1x 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-I, -II and -III (EUB338-I/II/III),

ALF968, BET42a, GAM42a, CF319a, HGC69a, SPH120, Burkho and ARCH915 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 (Cy3/5) 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.

TABLE 1.

Targets, staining, filters, and lasers for epifluorescence and confocal laser scanning microscopy.

Target	Staining	Detection by EPIM				Detection by CLSM				Fluorescence signal
		Filter cube	Excitation filter (nm)	Suppression filter (nm)	Dichromatic mirror (nm)	Laser	Line (nm)	Emissions (nm)	Detection channels	
Total cells	SYTO13	I3	BP 450-490	LP 515	510	Ar	488	BP 505-550	Green	Green
	DAPI	A	BP 340-380	LP 425	400	Diode	405	BP 420-480 IR	Blue	Blue
16S rRNA	FITC2	I3	BP 450-490	LP 515	510	Ar	488	BP 505-550	Green	Green
	CY52					HeNe	633	LP 650	Blue	Blue
	CY32	N2.1	BP 515-560	LP 590	580	HeNe	543	BP 561-625	Red	Red
EPS component[a]	FITC-ConA	I3	BP 450-490	LP 515	510	Ar	488	BP 505-550	Green	Green
EPS component[b]	Calcofluor white	D	BP 355-425	LP 470	455	Diode	405	BP 420-480 IR	Blue	Blue

[a] α-D-Mannopyranosyl and glucopyranosyl sugars of the biofilm EPS matrix.

[b] β-1,4 and β-1,3 polysaccharides of the biofilm EPS matrix.

Confocal laser scanning microscopy

Biofilm samples were examined on an LSM 510 META laser scanning microscope (Carl Zeiss, Germany), using a Plan-Apochromat 63x/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 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.

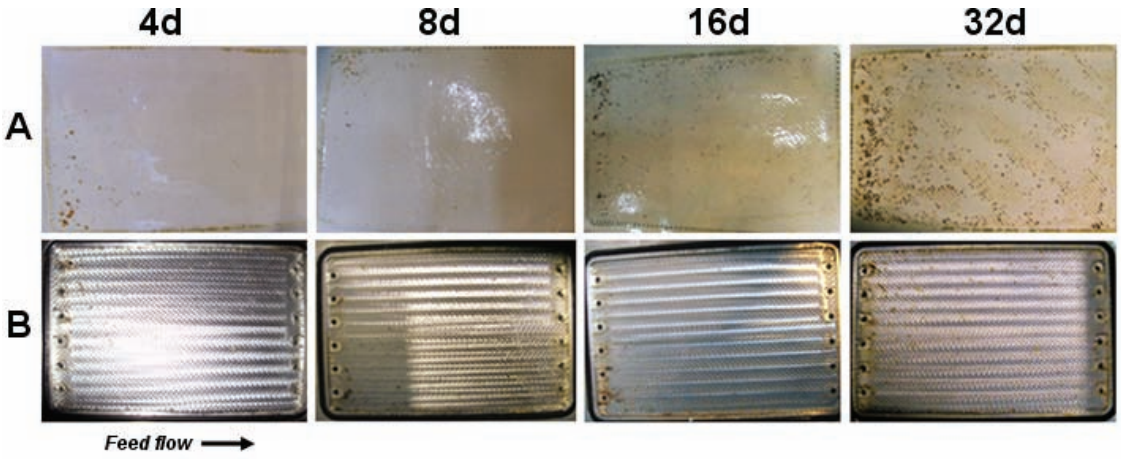

Figure 2. Photographs of fouled reverse osmosis membranes (**A**) and their feed-side spacers (**B**). The membranes and spacers were removed from the flow cells after 4 (**column 4d**), 8 (**column 8d**), 16 (**column 16d**), and 32 (**column 32d**) days of operation. The direction of the feed water flow along the length of each flow cell was from left to right.

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) rodshaped 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).

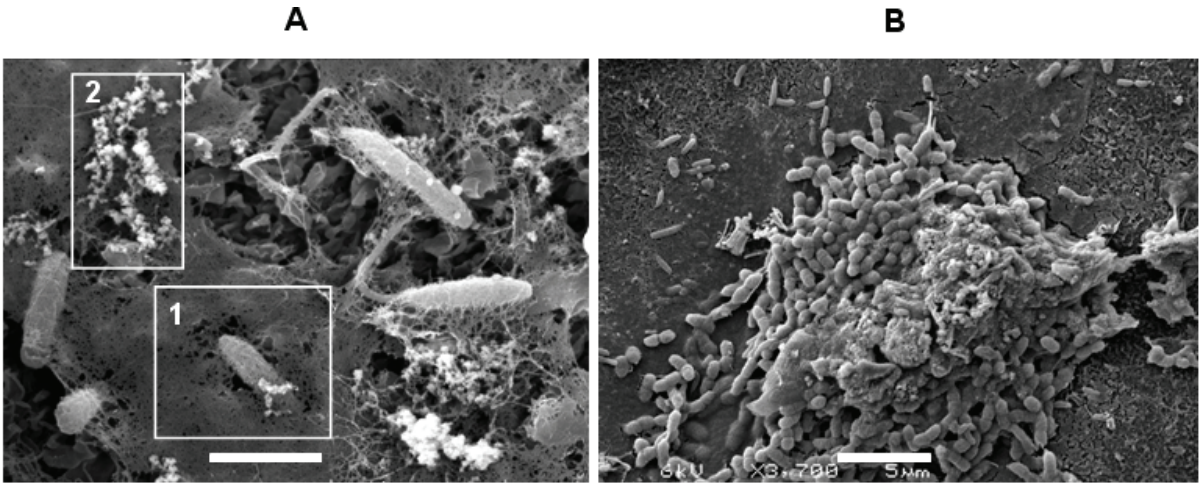

Figure 3. Scanning electron micrographs of the surface of the RO membrane after 4 and 16 days. (**A**) Rod-shaped cells embedded in an extracellular fibrillar material structure (**square 1**). Compact aggregates are visible on top of this biofilm (**square 2**). The RO membrane surface is visible at the bottom (under the biofilm layer) as a rough-appearing texture. Bar, 1 μm. (**B**) Typical microcolony formed on the surface of the RO membrane after 16 days. Bar, 5 μm.

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. 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 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 >0.90) were obtained from the clone libraries (see Fig. S2 and Table S1 in the supplemental material).

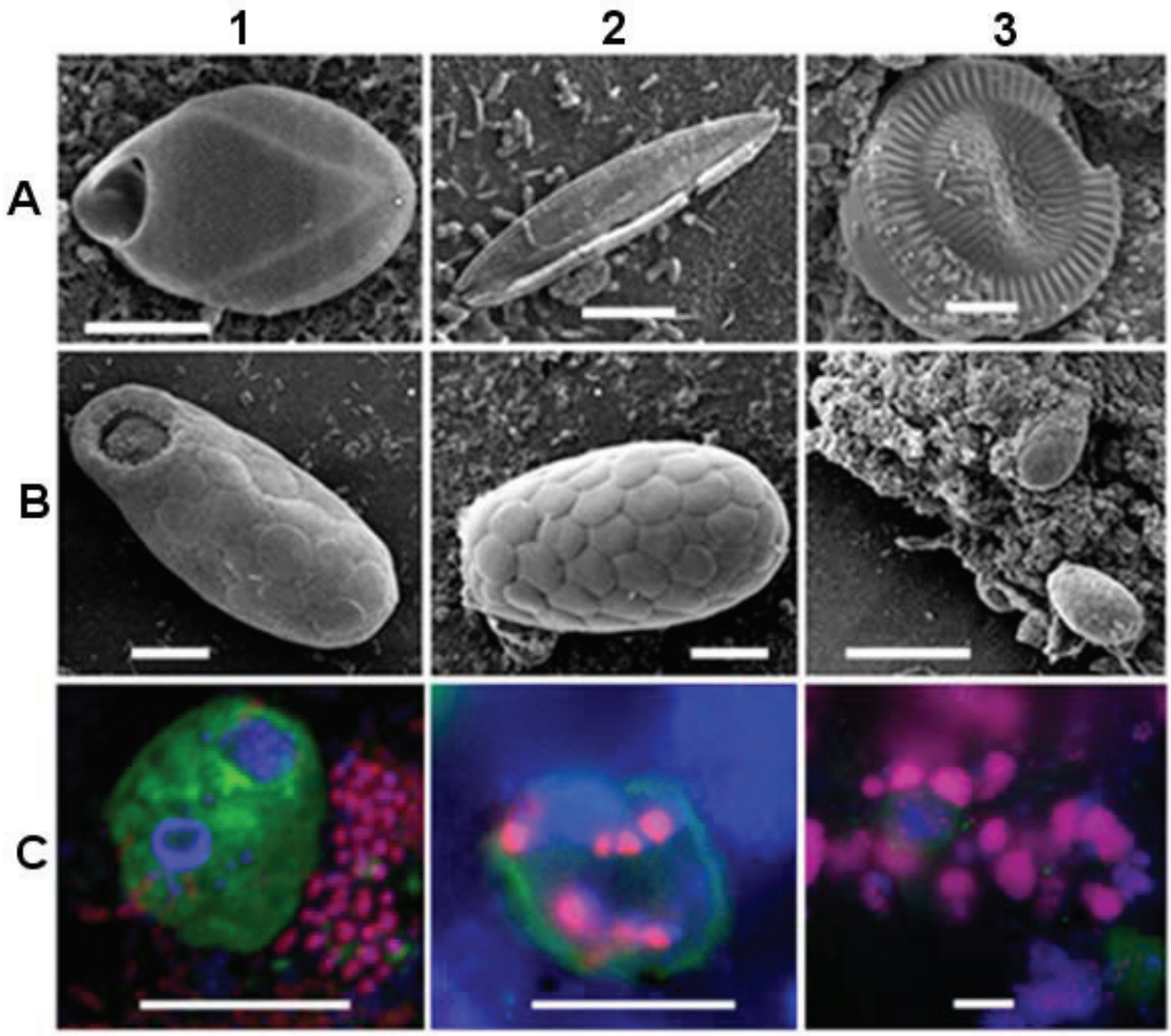

Figure S1. Micrographs showing involvement of unicellular eukaryotes in biofouling layers of RO membranes. SEM images represent an unknown eukaryotic cell (**A**-1), two unknown diatoms (**A**-2, 3) and four *Trinema* (family *Euglyphidae*) (**B**-1-3) on surfaces of the 4-(**B**-1), 16- (**A**-1-3) and 32-(**B**-2, 3) days-old RO membrane biofilms. Panel **C** views CLSM (1) and epifluorescence (2-3) images of free-floating (along the upper surface of 32-days-old RO membrane biofilm) trophozoites of *Acanthamoeba* sp. In their ConA-stained cytoplasm (green fluorescence) is visible DAPI-stained nucleus (blue fluorescence) and bacterial cells (blue or red fluorescence). Bars: 2 μm (**A**-1), 5 μm (**A**-2, 3), 10 μm (**B**-1-2 and **C**-1-3) and 50 μm (**B**-3).

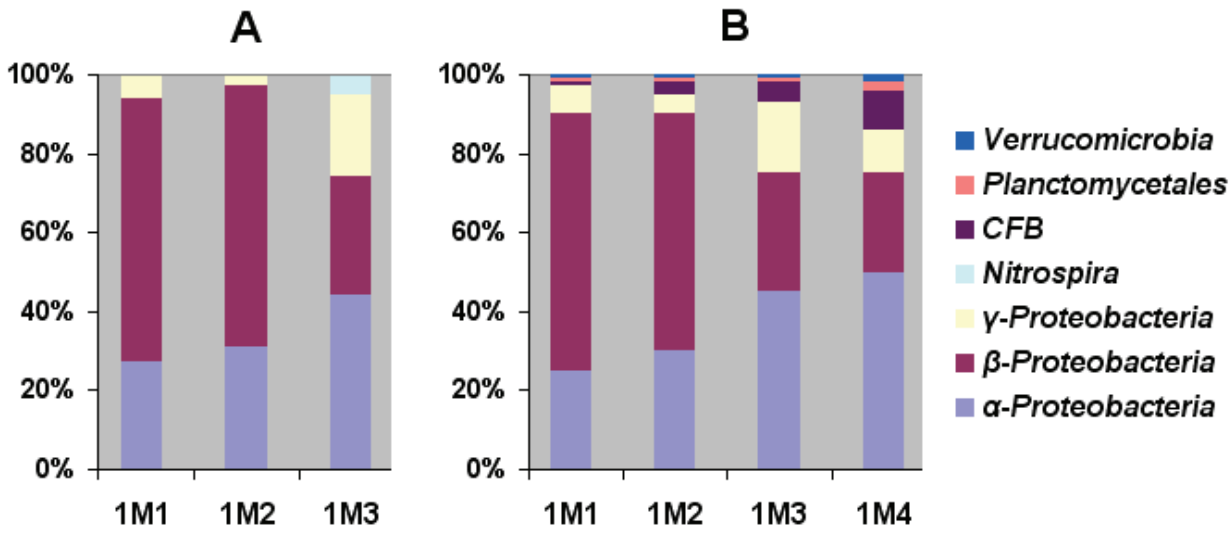

Figure S2. Schematic diagram illustrations depicting results of the clone libraries (**A**) and FISH (**B**) analyses of biofouling layers in RO membrane flow cells. **1M1, 1M2, 1M3** and **1M4** – the biofilms developed in 4, 8, 16 and 32 days, respectively, on RO membranes (type Hydranautics ESPA, USA) in flow cells connected in parallel with the RO system of a full-scale water treatment plant. The biovolume obtained for each taxonomic group in **B** was expressed as a percentage of the total biovolume obtained by DAPI staining.

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).

TABLE S1.

Phylogenetic affiliations and frequencies of cloned bacterial 16S rRNA gene amplicons[a] retrieved from RO membrane samples.

Phylogenetic lineages	**Closest relative in GenBank**		**Clone library[c]**		
	Accession no., taxon	**Similarity (%)[b]**	**1M1**	**1M2**	**1M3**
α-*Proteobacteria*	AY118225.1 *Azospirillum* sp.	91	3.3	2.3	2.1
	AF408954.1 *Hyphomicrobium* sp.	94	2.2		
	Y14306.1 Uncultured *Hyphomicrobiaceae*	94	1.1		
	EF140635.1 *Endosymbiont of Acanthamoeba* sp.	93		2.3	
	Y09639.1 *Sphingomonas* sp.	96			2.1
	Z23157.1 *Sphingomonas* sp.	98			2.1
	EF462462.1 *Sphingomonas* sp.	94	4.4	4.6	3.2
	AB023290.1 *Sphingomonas* sp.	99		2.3	1.1
	DQ789172.1 *Sphingomonas* sp.	97	5.6	8.1	6.3
	AB365794.1 *Sphingomonas oligophenolica*	96		2.3	3.2
	AJ968701.1 *Sphingomonas* sp.	96	1.1		2.1
	AM900788.1 *Sphingomonas* sp.	96	1.1		2.1
	AY521009.2 *Sphingomonas suberifaciens*	96	1.1		
	AF385533.1 *Sphingomonas* sp.	96			2.1
	AB426571.1 *Sphingomonas* sp.	98	3.3		4.2
	EF540471.1 *Sphingomonas* sp.	96			2.1
	EF018845.1 Uncultured *Sphingomonadaceae*	92			1.1
	EF540445.1 *Sphingopyxis* sp.	96			1.1
	EF540479.1 *Sphingopyxis* sp.	99	2.2	4.6	4.2
	DQ177493.1 *Sphingopyxis* sp.	99	1.1	4.6	4.2
	AY139005.1 Uncultured *alpha proteobacterium*	93			1.1
β-*Proteobacteria*	AM943035.1 *Acidovorax defluvii*	99		2.3	2.1
	AB120965.1 *Aquamonas fontana*	99			2.1
	AB074524.1 *Aquaspirillum* sp.	96	2.2	3.4	3.2
	AF078756.1 *Aquaspirillum* sp.	96	1.1	2.3	2.1
	AJ556799.1 *Comamonadaceae bacterium*	98	1.1	1.1	
	DQ234222.2 Uncultured *Comamonadaceae*	91			1.1
	EF127651.1 *Polaromonas rhizosphaerae*	97-98	1.1	1.1	
	AY571831.1 *Variovorax* sp.	96		1.1	
	AF078758.1 *Xylophilus ampelinus*	97			2.1
	EF667920.1 Uncultured *Burkholderiales*	97		9.2	2.1

	AF236004.1 *Beta proteobacterium* A0637	95	1.1	2.3	1.1
	AB452986.1 *Beta proteobacterium* HIBAF011	97			1.1
	AJ621027.1 *Nitrosomonas* sp.	94		2.3	
	AJ621026.1 *Nitrosomonas* sp.	98	4.4	5.7	2.1
	AY123811.1 *Nitrosomonas* sp.	94-95	2.2	5.7	
	AY123797.1 *Nitrosomonas* sp.	96-99	6.7	8.1	2.1
	AY123798.1 *Nitrosomonas* sp.	95-97	2.2	1.1	
	AF272422.1 *Nitrosomonas oligotropha*	98	7.8	14.9	5.3
	AY635573.1 *Nitrosospira* sp.	99	3.3		
	DQ839562.1 Candidatus *Nitrotoga arctica*	98	26.7	5.7	2.1
	AY345556.1 Uncultured *beta proteobacterium*	98	6.7		
	AJ964895.1 Uncultured *beta proteobacterium*	98			1.1
γ-*Proteobacteria*	AB031277.1 *Pseudomonas* sp.	96-98	4.4	1.1	6.3
	AM184223.1 *Pseudomonas* sp.	98	2.2		
	AY942995.1 *Pseudomonas* sp.	91			1.1
	EF442067.1 *Pseudomonas* sp.	99		1.1	
	EF540467.1 *Pseudomonas* sp.	96			1.1
	AM689949.1 *Pseudomonas* sp.	98			1.1
	EU275166.1 *Pseudomonas* sp.	98			8.4
	AM184269.1 *Pseudomonas* sp.	98			1.1
	AY359282.1 *Aquicella* sp.	93			2.1
Nitrospira	Y14639.1 *Nitrospira* sp.	98			5.3

a Amplicons were approximately 1.45 kb in size.

b Percentage of similarity between the cloned 16S rRNA gene and its closest relative in the NCBI database.

c 1M1clone library, 90 clones; 1M2 clone library, 87 clones; 1M3 clone library, 95 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), an endosymbiont of *Acanthamoeba* spp. (at 8 days, 2% of the total clones) and *Hyphomicrobium* spp. (at 4 days, 3% of the total clones). Two OTUs (2% of the total clones) from the 16-day biofilm were related to an uncultured alphaproteobacterium.

In 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*, *Aquamonas*, *Aquaspirillum*, *Polaromonas*, *Variovorax* and *Xylophilus* 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 *Nitrospira* 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 them, 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.

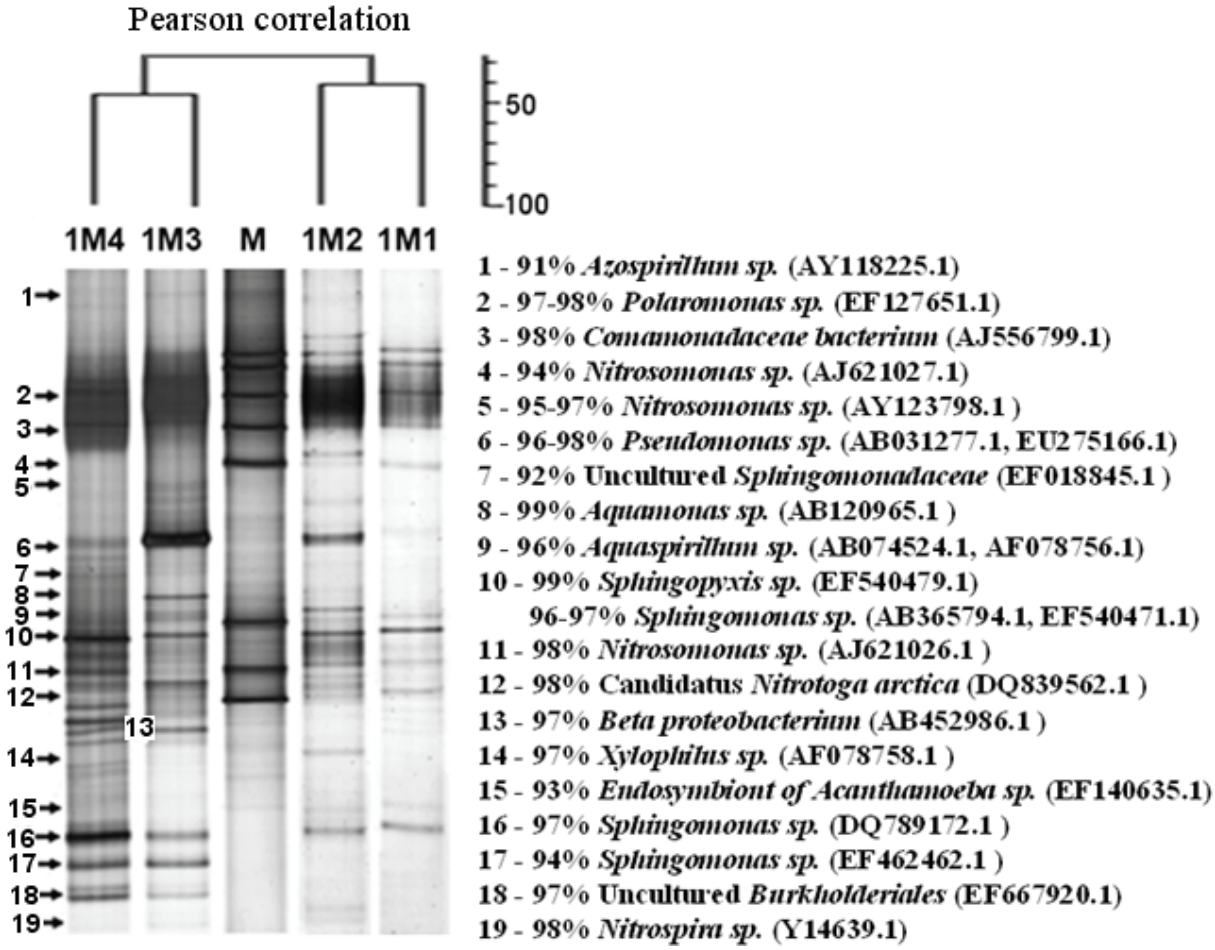

Figure S3. DGGE fingerprints of RO membrane biofilm samples. Lanes **1M1, 1M2, 1M3** and **1M4** – the fingerprints of bacterial communities, developed on RO membrane surfaces in 4, 8, 16 and 32 days, respectively. **M** corresponds to a synthetic marker - a mixture of the DGGE-PCR products from 8 bacterial species. Arrows at the left indicate positions of the identified bands. Simplified dendrogram (above the fingerprints) depicts the degree (%) of the similarity of the digitized PCR-DGGE profiles.

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 (3.8×10^4 cells/cm^2, 3.6×10^5 cells/cm^2, 4.1×10^6 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 (2.4×10^6 cells/cm^2).

Fluorescence *in situ* hybridization analysis of biofilm-forming communities in membrane samples showed that more than 95% of DAPI-stained cells were detectable with the EUB338-I/II/III probe (1, 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 days 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 (SPH120 [28] and Burkho [20]) 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 *Bacteroides* (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 *Planctomycetales* (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).

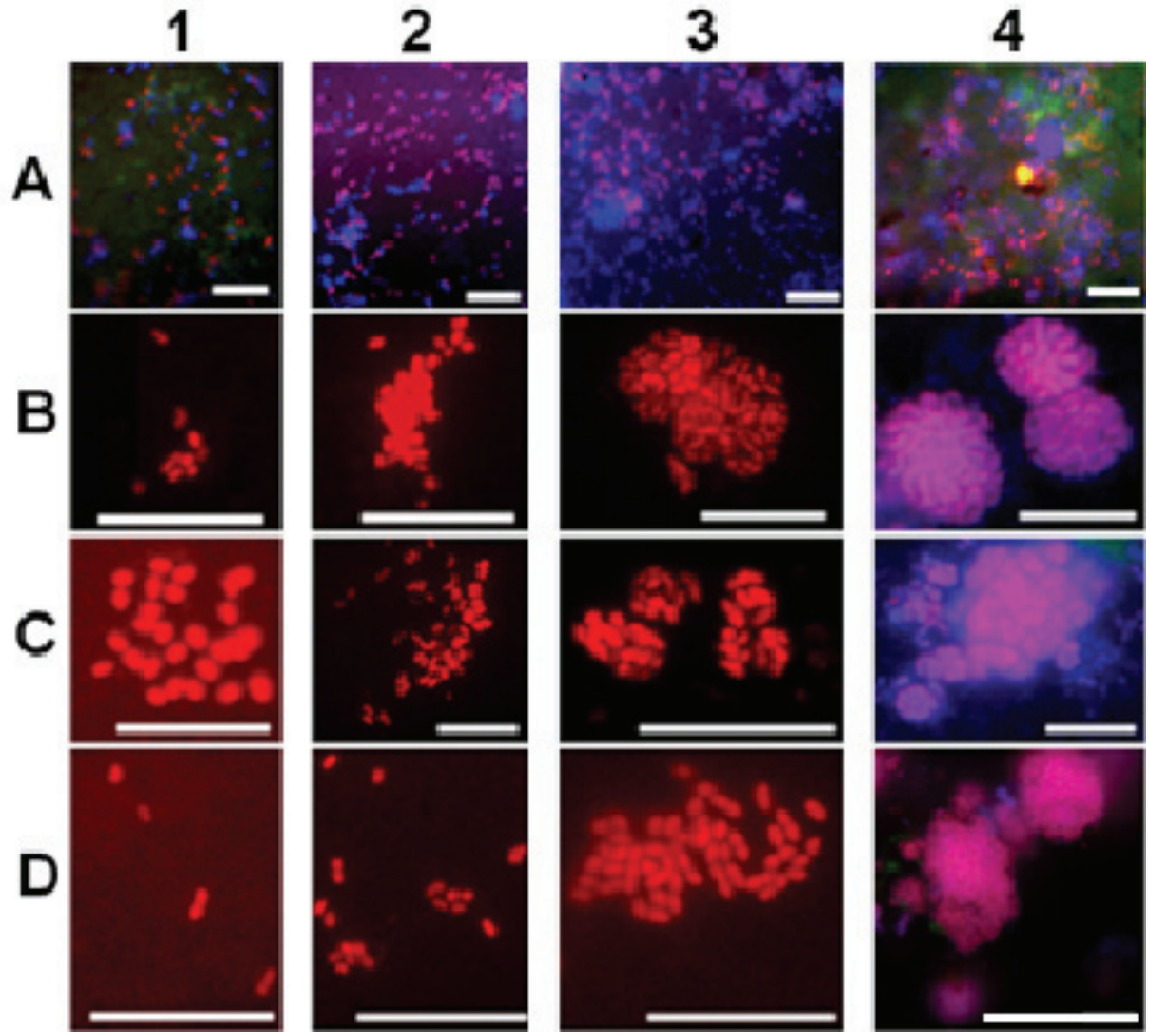

Figure 4. Epifluorescence micrographs depicting mode of initial formation and spatiotemporal development of biofilm structures by pioneer colonizers of RO membrane surfaces. Horizontal panels (**A** to **D**) represent images of microcolonies (red and pink fluorescence) as follows: "carpets" of *Sphingomonas* 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).

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, *Planctomycetales* 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). About 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 gammaproteobacterial 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.

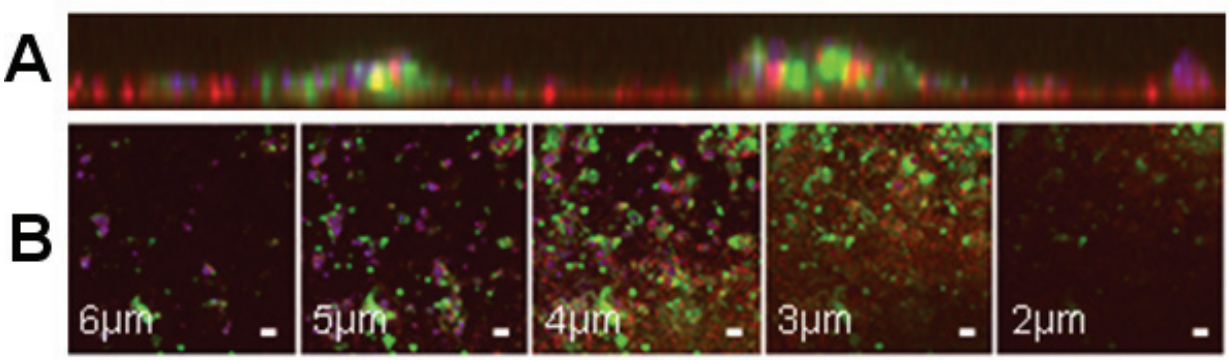

Figure 5. Representative CLSM images of RO membrane biofilms depicting complex architecture of mature microbial communities after 32 days of operation. Series of horizontal (*x*-*y*) (**A**) and sagittal (*x*-*z*) (**B**) optical sections were taken at 1-µm intervals across the *z* axis of the biofilm. The sections show shapes and spatial arrangements of bacterial cells and EPS matrix within mixed-species biofilm communities. The main distribution of cells and polysaccharides was at the top of the RO membrane surface. Cells of *Sphingomonas* spp. were stained with Cy3-labeled SPH120 probe (red fluorescence), and cells of remaining community members with DAPI (blue fluorescence). α-Polysaccharides of biofilm EPS matrix were stained with FITC-ConA (green fluorescence). *Z*-scan positions in µm from the top of the RO membrane surface are indicated in each image. Bars, 10 µm.

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 *Gammaproteobacteria*, CFB, *Planctomycetales* 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 *Planctomycetales* 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 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 pili) 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 behaviour 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.

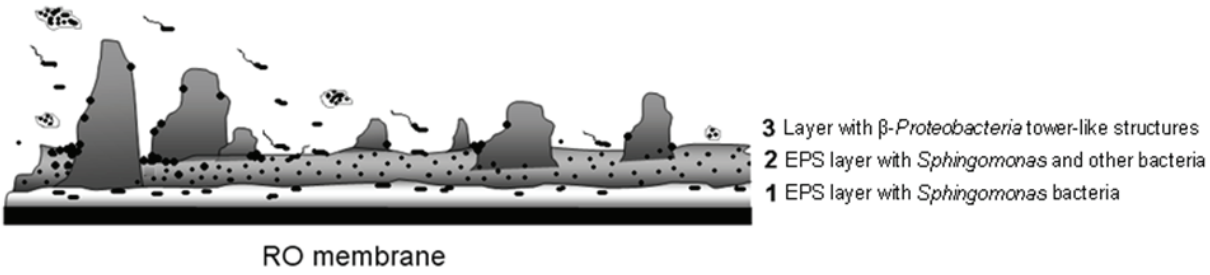

Figure 6. A schematic representation of the observed biofilm structure in a mature RO membrane biofouling layer. Single planktonic cells of *Sphingomonas* spp. and clumps of *Beta-* and *Gammaproteobacteria* present in the feed water colonize surfaces.

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 Picioreanu 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.

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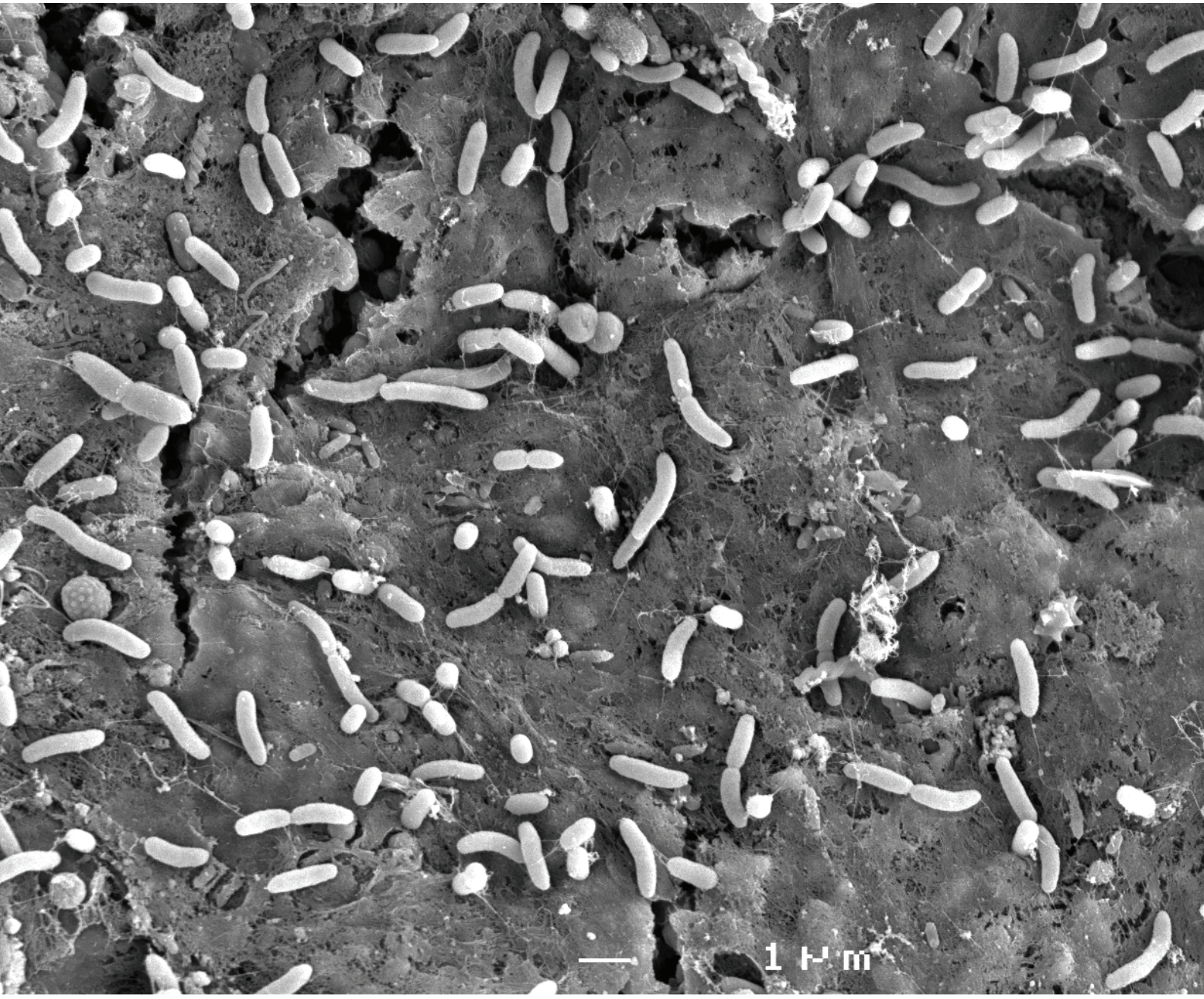
5 Effect of conventional chemical treatment on the microbial population in a biofouling layer of reverse osmosis systems



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Abstract

The impact of conventional chemical treatment on initiation and spatiotemporal development of biofilms on reverse osmosis (RO) membranes was investigated in situ using flow cells placed in parallel with the RO system of a full-scale water treatment plant. The flow cells got the same feed (extensively pre-treated fresh surface water) and operational conditions (temperature, pressure and membrane flux) as the full-scale installation. With regular intervals both the full-scale RO membrane modules and the flow cells were cleaned using conventional chemical treatment. For comparison some flow cells were not cleaned. Sampling was done at different time periods of flow cell operation (i.e., 1, 5, 10 and 17 days and 1, 3, 6 and 12 months). The combination of molecular (FISH, DGGE, clone libraries and sequencing) and microscopic (field emission scanning electron, epifluorescence and confocal laser scanning microscopy) techniques made it possible to thoroughly analyze the abundance, composition and 3D architecture of the emerged microbial layers. The results suggest that chemical treatment facilitates initiation and subsequent maturation of biofilm structures on the RO membrane and feed-side spacer surfaces. Biofouling control might be possible only if the cleaning procedures are adapted to effectively remove the (dead) biomass from the RO modules after chemical treatment.

Introduction

In current full-scale reverse osmosis (RO) water treatment plants drastic changes in system performance (i.e., significant increase in the feed pressure of RO membrane units and/or long-term membrane flux decline) indicate fouling of membrane surfaces within RO membrane units (Wiesner and Aptel, 1996; Vrouwenvelder and van der Kooij, 2001; Bishop, 2007). Fouling by precipitation and abundance of membrane-rejected feed water dissolved solids and organic compounds (i.e., organic and/or inorganic fouling) are usually manageable by application of conventional cleaning agents. Prevention and control of attachment and proliferation of feed water bacteria on the membrane, feed-side spacer and other internals within the RO units are still difficult (Ridgway and Safarik, 1991; Flemming et al., 1997; Baker and Dudley, 1998; Al-Ahmad et al., 2000). The common techniques to reduce membrane fouling comprise dosing of chemical agents and pre-treatment of the feed water. These treatments generally only have a temporary effect. Microorganisms may survive pre-treatment processes like coagulation, flocculation, sand filtration, ultra filtration and cartridge filtration. With time they will colonize a variety of surfaces within the plant (Bereschenko et al., 2008). On the surface of new and clean RO membranes, fed with extensively pre-treated water, early biofilm structures occur within the first 4 days of the system operation (Bereschenko et al., 2010). Within the following 12 days, the biofilm spreads over the entire surface area and forms a mature heterogeneous layer (Bereschenko et al., 2010). When living within the complex, three-dimensional structures of a self-produced organic polymer matrix (Davey and O'Toole, 2000; Tolker-Nielsen and Molin, 2000; Watnick and Kolter, 2000), the microbial communities are less sensitive to chemical cleaning (Nichols, 1989; Anwar et al., 1992; Davies et al., 1998; LeChevalier et al., 1988; Branda et al., 2005). As a result, chemical treatment of biofouled RO membrane units is generally not effective in removing and/or completely destroying the complex multicellular structures (Flemming, 2002). Re-growth of the membrane surface-attached microbial layer quickly results in a repetition of the biofouling-related system failure. The cleaning-related improvement of the RO system performance is commonly associated with a decline of the pressure drop and increase of water flux, but is of temporary nature. Periodic and more frequent chemical cleanings are, therefore, unavoidable for membrane filtration installations but lead to an increased usage of cleaning chemicals and increased production of waste water. Frequent cleaning procedures also result in a shortened membrane life and ultimately in a loss of capacity of the water supply plant (Baker and Dudley, 1998; Flemming, 2002).

The effect of chemical cleaning on the microorganisms in fouling layers is hardly investigated. Often, only the change in pressure drop and membrane flux is measured to determine the effect of cleaning procedures. The development of more effective strategies for biofouling control requires research directed to determine the effect on the microorganisms and the structure of the biofouling layer on the RO membranes. Insight into processes that are of important for membrane biofilm formation and development may help to find ways to prevent biofouling. Nevertheless, a proper assessment of the in situ biofilm formation and development is rarely done in RO biofouling research (Bereschenko et al., 2010). In addition, biofilm monitoring studies that were done previously may not provide a true representation of the RO biofilm problem in situ. These experiments were performed using simplified laboratory systems with one or a few bacterial strains (Pang et al., 2005; Eshed et al., 2008; Herzberg and Elimelech, 2007, 2008) or ignored the impact of prevailing environmental conditions (Pang and Liu, 2006).

In this study, we monitored in situ initiation and spatiotemporal development of microbial biofilm layers on the surfaces of fresh and chemically cleaned reverse osmosis membranes and feed-side spacers. This was done by using stainless steel flow cells connected in parallel to the reverse osmosis system of a full-scale water treatment plant. Members of a feed water microbial community, responsible for initial colonization of the membrane and feed-side spacer surfaces were identified by molecular biological techniques. Their abundance and spatial organization during the temporal development of the biofilm was studied by microscopic techniques. The development of membrane-attached biofilms to a level of “biofouling” e recognized by the pressure drop increase e and the impact of chemical cleaning was assessed over a 1-year period.

Materials and methods

Sampling

Four high-pressure (12 bar) test flow cells of stainless steel were operated from March 2007 to March 2008 (experimental phase I) and from 11 April to 11 May 2008 (experimental phase II) parallel to a full-scale RO installation (Fig. S1, for more details see Bereschenko et al. [2010]).

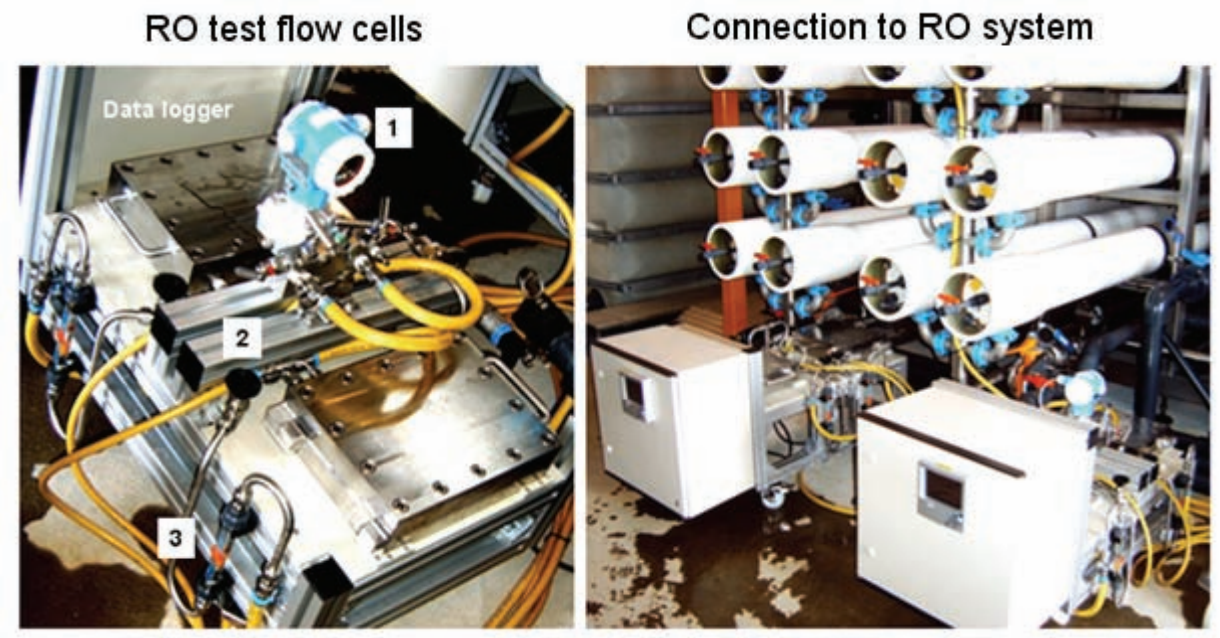

Figure S1. Photographs showing experimental setup. RO test flow cells equipped with differential pressure transmitters (**1**), digital data loggers, high pressure taps (**2**) and flow meters (**3**) were connected in parallel to a reverse osmosis system of a full-scale water purification plant. In continuous-flow mode, the RO feed water (i.e., the pre-treated by coagulation, flocculation, sand filtration, ultra filtration (UF) and cartridge filtration processes fresh surface water) entered flow chambers (size: 19.8 cm x 12.7 cm x 3.0 mm) of the flow cells at a rate of 75 L/h. Operated with 0.05 m/s linear cross flow water velocity and recovery (i.e., permeate/feed ratio) of 1 to 1.2%, the flow cells produced 32 L/m^2.h process water. With time, under cross flow conditions on pieces (19.8 by 12.7 cm) of flat sheet reverse osmosis membranes (±0.85% porosity, effective area 450 cm^2) and feed-side spacers (0.7 mm thick) occurred formation of microbial biofilms (see Fig. 3).

Chemical cleaning of RO membranes and feed-side spacers e excised from a commercial spiral-wound ESPA membrane element (Hydranautics ESPA 2, CA, USA) and placed in the flow chambers of the flow cells e occurred during a routine chemical treatment of the full-scale RO membrane units, used to maintain a reasonable flux in the system. The treatment consisted of sequentially applied washing steps: RO permeate (20-25°C), biocide (30% sodium bisulfite solution, 30-40°C, pH 10-11, for 2-3 h) and mixed acid detergent descaler (Divos 2 [JohnsonDiversey, UK], 20-25°C, pH 2.6, for 2 h). After each step, the chemical

compounds were washed away with RO permeate of ambient temperature. The development of pressure drop (i.e., pressure drop is defined as the difference between the feed pressure and the concentrate pressure) over the flow cell feed channels during each particular experiment was monitored using a differential pressure transmitter (Deltabar S PMD70 [Endress & Hauser Inc., CA], range: 0.05-500 mbar), with accuracy of 0.1 mbar. The measurements were recorded automatically every 30 min by a data logger device and the acquired data were read out with the READWIN 2000 software (Endress & Hauser Inc.). At the end of each experiment, the membranes and spacers were removed from the sacrificed flow cells. Small sections from randomly selected positions on their surfaces along the length of the feed channel were carefully cut out and processed for total DNA extraction and microscopical analysis (fluorescence in situ hybridization [FISH] and epifluorescence [EPIM], confocal laser scanning [CLSM] and field emission scanning electron [FESEM] microscopy) as previously described (Bereschenko et al., 2010). The simultaneously collected water samples (i.e., fresh surface water fed to the plant and permeate from the flow cells and ultra filtration and RO systems) were kept on ice and transferred to a laboratory for further processing.

Processing of water samples

Each water sample (100 ml) was mixed with 3 volumes of freshly prepared 4% formaldehyde, incubated for 1 h and filtered through a black polycarbonate filter (pore size, 0.2 μm; type GTTP 4700, Millipore, Germany). The filters were processed further using FISH of bacteria. The determination of the total number of bacteria was done by incubating the preserved filters with DAPI (4´,6-diamidino-2-phenylindole) solution (2 μg/ml, Sigma-Aldrich) in the dark at 4°C. After 10 min the membranes were gently rinsed with MilliQ water, air-dried and mounted in a Vectashield medium (Vector Laboratories, UK). The stained cells were counted (in triplicate) in 20 randomly chosen EPIM viewing fields. For FESEM, microbial biomass from 1 L of each water sample was concentrated by filtration on the 0.2-μm filter. The cells on the filter were fixed by submerging the filter in a 2.5% (v/v) glutaraldehyde solution and processed further as described previously (Bereschenko et al., 2010). For total DNA extractions, 10 ml of each water sample was centrifuged at 10,000 x g for 10 min and the pellet was resuspended in 0.5 ml of 1x phosphate-buffered saline (PBS) solution (pH 7.0).

Microbial community analysis

The samples from the biofilms and the water were analyzed using denaturation gradient gel electrophoresis (DGGE) and clone library analysis of 16S rRNA genes. The procedures to extract the total community DNA, PCR amplifications of bacterial 16S rRNA gene fragments, DGGE separations of the generated amplicons, construction and analysis of the 16S rRNA gene clone libraries were done as previously described (Bereschenko et al., 2010).

The nucleotide sequence data reported in this study were submitted to the GenBank under the accession numbers GQ385250, GQ385251, GQ385256, GQ385260, GQ385262, GQ385264-GQ385269, GQ385276, GQ385277, GQ385280, GQ385282, GQ385286, GQ385287, GQ385290-GQ385292, GQ385294, GQ385295 and GU585911-GU585936.

Results

Four reverse osmosis test flow cells were operated for 3-12 months (experimental phase I) and 1-32 days (experimental phase II) parallel to a full-scale RO installation (Fig. S1). Chemical cleaning of RO membranes and feed-side spacers within the flow cells occurred during the routine cleaning of the full-scale system with sodium bisulfite and Divos 2 (mixed acid detergent descaler). In phase I, the cleaning was applied weekly and in the phase II - after 11 days of the start of the flow cell operation (Fig. 1). For comparison, some RO membranes and their feed-side spacers were not cleaned. At the end of each experiment, the chemically cleaned and non-cleaned flow cells were opened and their membrane and spacer surfaces were examined visually (Fig. 2 and 3) and microscopically (Fig. 2, 6, 7 and S4) on the presence, intensity, distribution and nature of fouling. Diversity, abundance and distribution of bacterial species during different stages of biofilm community development at these surfaces were evaluated by clone libraries and sequencing (Table 1), DGGE fingerprinting (Fig. S3) and FISH microscopy (Fig. 4) analyses. Three-dimensional (3-D) distribution of microbial organisms - with respect to each other and to exopolysaccharides - was examined using CLSM and image analysis (Fig. 2, 5-7 and S2). Presence, abundance and diversity of planktonic bacterial communities in the collected water samples were investigated by the FESEM (Fig. S4), DGGE (Fig. S5), and FISH microscopy (Fig. 4). Below we describe the

effect of cleaning on the occurrence and proliferation of microbial population in the surface-attached fouling layer.

Development of fouling in membrane systems

Fouling in RO systems is in practice often recognized as a long-term membrane flux decline of the RO plant and/or significant increase in the feed pressure of the RO module to maintain constant permeate production (Bishop, 2007; Vrouwenvelder and van der Kooij, 2001; Wiesner and Aptel, 1996). This is in the case of biofouling the result of the formation of a "critical level biofilm" in the spiral-wound RO filtration units that lead to the arbitrary threshold of interference of the pressure drop (Flemming, 2002). In the present study, establishment of the "critical level biofilm" was indeed associated with significant changes in pressure drop over the feed channels of the test RO flow cells, operated parallel to a full-scale RO installation. The pressure drop measurements indicated that overall the development of a "critical level biofilm" was not very different for cleaned and non-cleaned surfaces in the short-term (1 month) experiment (Fig. 1-A and B).

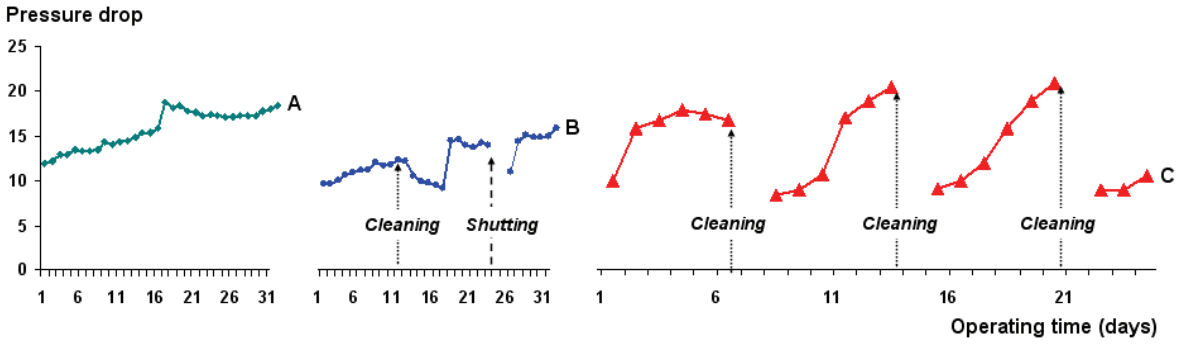

Figure 1. Pressure drop development in the RO flow cells as function of time. The graphs show the development of pressure drop (in mbar) over the feed channels of the non-cleaned (**A**) and cleaned (after 11 days [**B**] and weekly [**C**]) flow cells, operated in parallel with RO systems of a full-scale RO water purification plant. Feed water (UF permeate) was supplied to the flow cells at a pressure of 8-11 bar. "**Cleaning**" indicates application of chemical treatment to the membranes and spacers within the flow cells. "**Shutting**" point to the occurrence of an unexpected (two days) shut-down of the full-scale RO installation.

Cleaning leads to a temporary decline in pressure drop, but very rapidly the fouling layer grew again leading to a quick increase in pressure drop after the cleaning event. When the flow cells operation time was prolonged for 3-12 months and the cleaning occurred weekly, the chemical treatment was effective in decreasing the pressure drop over the system. A quite abrupt and significant (9-13 mbar) decrease in the pressure drop value was observed after each of the cleaning steps (Fig. 1-C), indicating that the weekly treatment could be used to control the pressure drop during long-term operation. The long-term (12 months) system operation without chemical treatment resulted in a slow but sure pressure drop increase (data not shown) to a value of 47 mbar, indeed much higher then for the cleaned system, being 21 mbar.

Biofilm structure after cleaning

The direct impact of the weekly applied chemical cleaning procedures on the established biofilm structures at the RO membrane and feed-side spacer surfaces was evaluated using samples collected the day after the treatment. Visual inspection of the membranes revealed the presence of moist, slimy, yellow and light to dark-brown coloured deposits, distributed irregularly (1-10 days old samples) or uniformly (samples from long-term operated membranes) over the surface of the cleaned membranes and spacers. Compared to the fouling layers in the samples collected the day before the cleaning they were slightly less intense in colour and density (Fig. 2-A and 3). In addition, they could be much easier scraped from the membrane and/or spacer surfaces. Microscopic examinations showed the presence of damaged protozoa (e.g., the *Trinema*, Fig. 2-B), deformed bacterial microcolonies (Fig. 2-C) and squashed (to 1-2 mm of the overall thickness) EPS biofilm matrix (Fig. 2-D and 5) on membrane and/or spacer surface the day after the treatment. The observations were similar for membranes examined after short-term and long-term operation. No intact protozoa were present on the top of the collapsed biofilm structures, while a variety of growing and dividing bacteria (Fig. 2-C and D) of the *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Cytophaga-Flavobacter-Bacteroidetes* (CFB), *Verrucomicrobia* and *Planctomycetes* were abundantly present as detected by FISH analysis (Fig. 5 [*Betaproteobacteria*, other bacteria: data not shown]). In both CLSM and SEM images no EPS layers were visible around their cells (Fig. 2-D and 5).

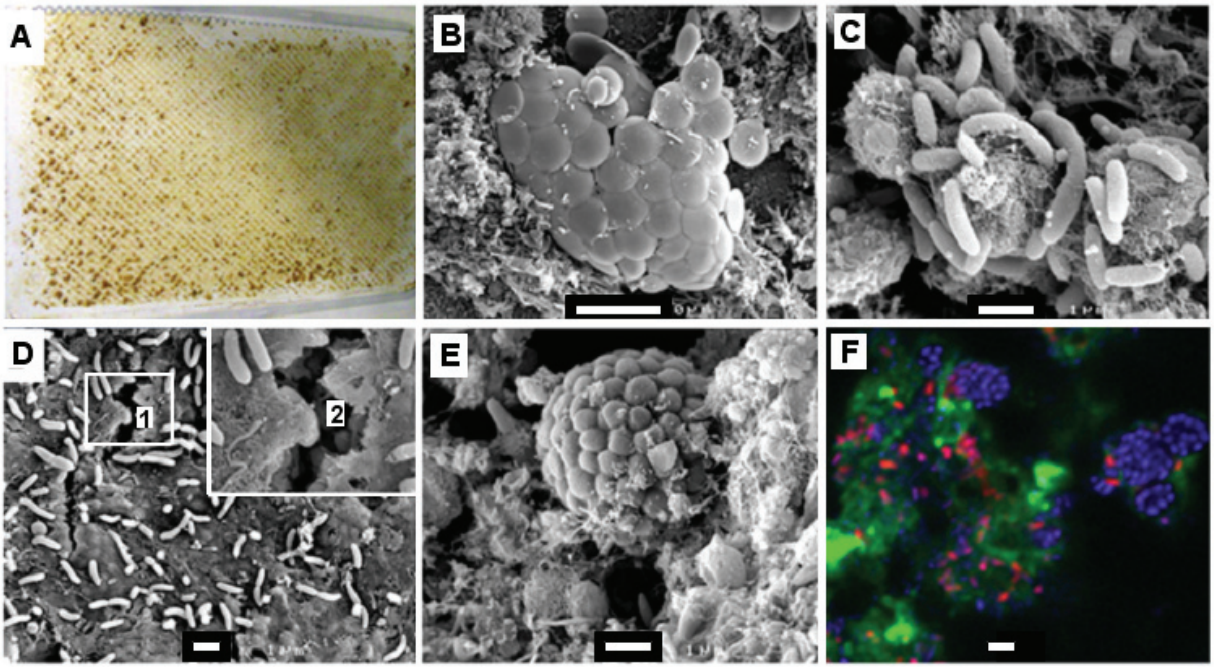

Figure 2. Effect of conventional chemical treatment on biofilm occurrence. The photograph (**A**) shows the appearance of the fouling deposits at the RO membrane surface, operated for one year with the weekly applied cleaning procedures and removed from the test flow cells the day after the cleaning. The SEM images (**B-D**) represent the associated with the treated fouling layer damaged protozoa (i.e., *Trinema*, **B**), bacterial microcolonies (**C**) and EPS network (**D**). Note: the presence of freshly deposited feed water bacteria on the collapsed biofilm structures in (**B-D**) and the presence of intact bacterial cells (**2**) within and/or under the collapsed biofilm matrix (**1**) in (**D**). SEM (**E**) and CLSM (**F**) images represent surface of the re-grown [within 6 days after the chemical treatment application] biofilm. Green fluorescence is from the ConA-positive bacterial EPSs, red - from the (SPH120-Cy3-positive) *Sphingomonas* cells and blue - from the DAPI-stained remainder of the biofilm community members. Bars: 1 μm (**C-F**) and 10 μm (**B**).

In contrast, many of the intact bacterial cells (9-3700 cells/cm^2 membrane surface) within the collapsed biofilm matrix were EPS-embedded (Fig. 2-D and 5). These cells hybridized with the SPH120 probe, indicating the presence of the *Sphingomonas* species (Neef et al., 1999). The diffused fluorescence from the FITC-labeled Concanavalin A (ConA) around their cells indicated the presence of β-1,4-linked sugar polymers (Johnson et al., 2000). However, the specificity of these probes for polysaccharides is not 100%. It cannot be excluded that the

matrix around the cells consisted of other molecules that also interacted with the fluorescent probes. Irrespectively of the cleaning frequency (weekly or after 11 days of the flow cell operation), within 6-7 days after the treatment the collapsed biofilm structures appeared to be completely covered by a fresh layer of EPS-embedded bacterial cells and (single or clustered) microcolonies (Fig. 2-E and F, Fig. 5 and 7 [cleaned: 3-6 months]). In all the examined microscopic images, the re-grown biofilms appeared to be, in general, more uniformly stretched at the membranes than at the associated feed-side spacer surfaces. The overall thickness of this re-grown layer was also different (e.g., in the 17 days old samples: 3-6 μm [membrane] versus 1-3 μm [spacer]; in the 3-6 months old samples: 4-9 μm [membrane] versus 1-7 μm [spacer]). This observation correlated with the visual inspection of the routinely treated membranes and spacers, where all the examined membrane surfaces appeared to be more severely fouled than their feed-side spacers (e.g., see the non-cleaned 6 months and cleaned 1-year old samples in Fig. 3).

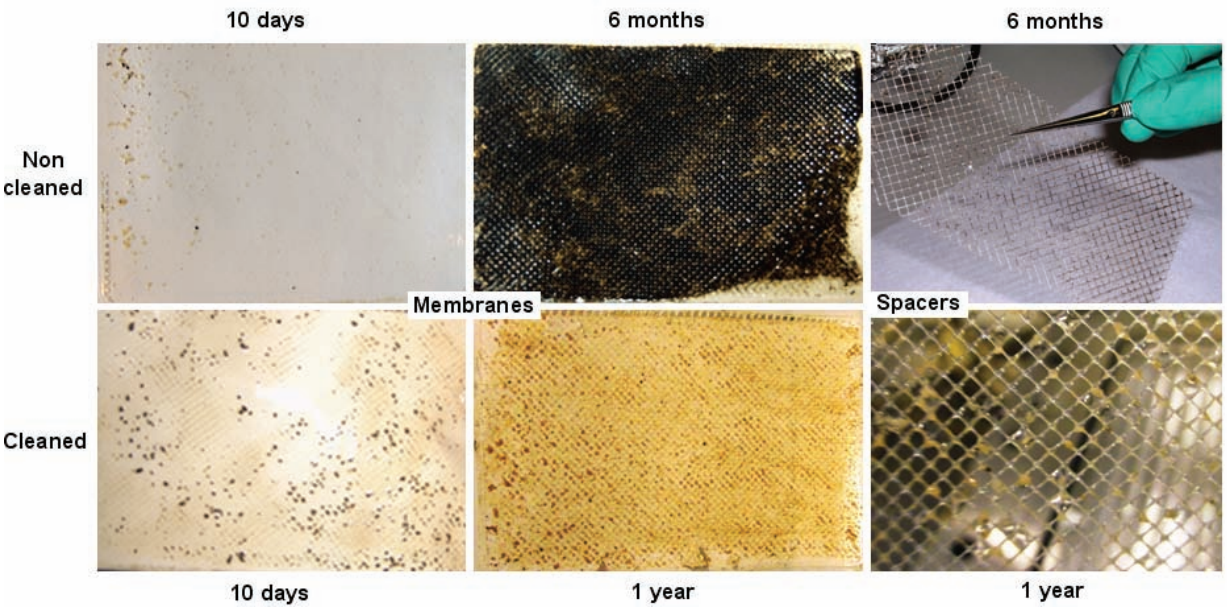

Figure 3. Photographs depicting the failure of the weekly applied chemical treatment to prevent accumulation of fouling deposits on the surfaces of RO membranes and their feed-side spacers. The photographs were taken during autopsy of the RO test flow cells, operated for 10 days, 6 months and 1 year with or without a routine (once a week) cleaning application and are representatives of a series of observations. The direction of the feed water flow along the length of each flow cell was from left to right.

The phylogenetic analysis of the sequences obtained from the clone libraries (Table 1), constructed for the biofilms from the cleaned membranes, revealed dominance of the *Actinobacteria* in the clone library from the weekly cleaned 6 months old membrane sample (50% of the total clones). In the younger samples (17 days - cleaned once; 3 months - cleaned weekly) there was prevalence of the *Proteobacteria* division in the clone libraries. In the cleaned 17 days old membrane sample, the largest bacterial group within the *Proteobacteria* was represented by the *Betaproteobacteria* subdivision (39% of the total clones). This group was also dominating the planktonic community in the fresh surface water fed to the RO plant and in the plant cartridge-treated ultrafiltration permeate fed to the flow cells and RO systems (Fig. 4).

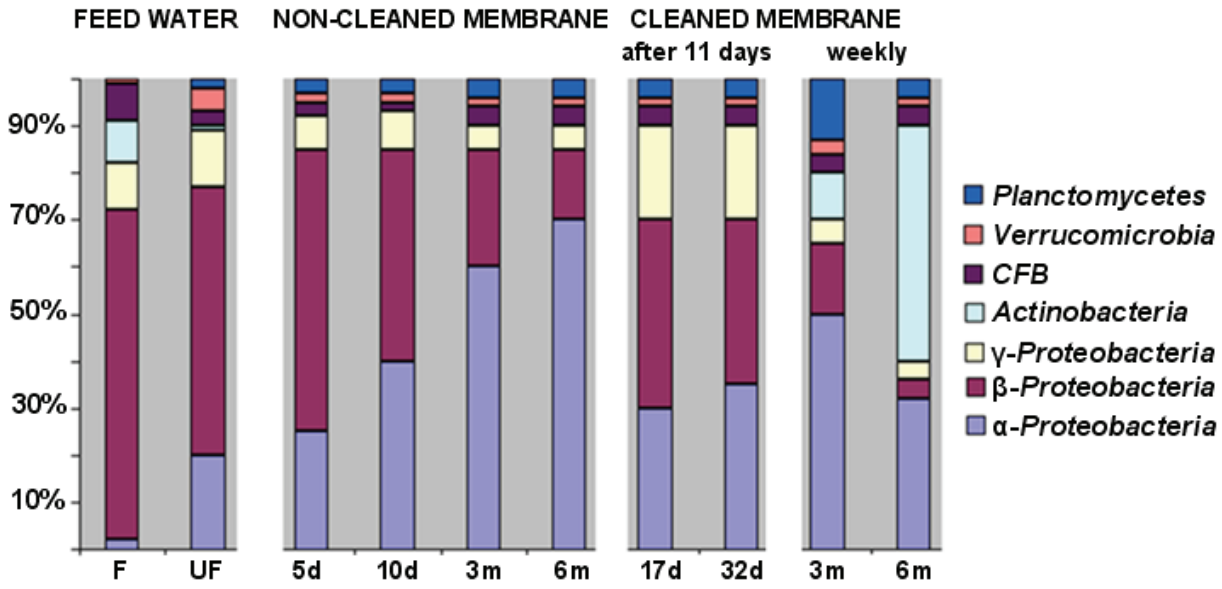

Figure 4. Composition of microbial populations in two water samples and membranes obtained from the flow cells after different operating times determined by FISH analyses. The membranes were removed from the flow cells after 5 (**5d**), 10 (**10d**), 17 (**17d**), 32 (**32d**) days, 3 (**3 m**) and 6 (**6 m**) months of operation with or without the chemical treatment application. The F and UF represent patterns of the planktonic bacterial communities in the RO plant feed water (surface water) and UF permeate (RO system feed water). The biovolume obtained for each taxonomic group was expressed as a percentage of the total biovolume obtained by DAPI staining.

Table 1. Phylogenetic affiliations and frequencies of cloned bacterial 16S rRNA gene amplicons[a] retrieved from RO membrane samples.

Closest relative in GenBank		Clone library						
		Cleaned				Non-cleaned		
Accession no., taxon	**(%)[b]**	**17 days**	**32 days**	**3 months**	**6 months**	**5 days**	**3 months**	**6 months**
EF140635.1 *Endosymb. of Acanthamoeba* sp.	93		2.2	2.2				
AY118225.1 *Azospirillum* sp.	91	1.1	2.2	2.2	2.3	1.1	1.6	
FJ711209.1 *Hyphomicrobium* sp.	96	1.1	1.1	2.2	2.3		4.7	1.2
EF012357.1 *Devosia insulae*	99			3.3	4.5		6.3	3.7
AY689051.1 *Mycoplana* sp.	99				6.8		3.1	3.7
DQ303329.1 Uncultured *Bradyrhizobium* sp.	98			6.7			6.3	7.4
DQ303345.1 Uncultured *Bradyrhizobium* sp.	99						3.1	1.2
AM403722.1 *Microbacterium* sp.	99				9.1			
AY162029.1 *Mycobacterium* sp.	96			3.3	9.1			
AM921641.1 *Nocardiaceae bacterium*	99			3.3	32.0			
EU440981.1 *Novosphingobium* sp.	96	2.2	1.1					3.7
FJ193529.1 Uncultured *Sphingobium* sp.	93	1.1	4.3	6.7	2.3		3.1	2.5
Z23157.1 *Sphingomonas* sp.	98	3.2	4.3				3.1	1.2
AB365794.1 *Sphingomonas oligophenolica*	96	2.2	2.2	6.7		3.2		1.2
AY521009.2 *Sphingomonas suberifaciens*	96	2.2	2.2			2.1		2.5
CP000699.1 *Sphingomonas wittichii*	97						1.6	3.7
EU591707.1 *Sphingomonas* sp.	92	1.1		6.7			4.7	1.2
GQ161989.1 *Sphingomonas* sp.	97			10.0	2.3		3.1	6.2
AB362260.1 *Sphingomonas* sp.	95						4.7	3.7
AF410927.1 *Sphingomonas* sp.	95	2.2	3.2			2.1		
AY162145.1 *Sphingomonas* sp.	94						3.1	2.5
DQ789172.1 *Sphingomonas sanxanigenens*	94	11.8	9.7			8.5		6.2
AY599670.1 Uncultured *Sphingomonas* sp.	97						4.7	2.5
DQ177493.1 *Sphingopyxis* sp.	98	3.2		3.3		2.1	1.6	4.9
EU703439.1 Uncultured *Sphingopyxis* sp.	98	1.1			16		6.3	11.1
EF540479.1 *Sphingopyxis* sp.	99	2.2				2.1	3.1	4.9
EU304287.1 *Acidovorax* sp.	99	2.2	4.3		2.3		1.6	
AB120965.1 *Aquamonas fontana*	92	1.1	2.2					
AB074524.1 *Aquaspirillum autotrophicum*	96	4.3	5.4	6.7			3.1	
EU817490.1 *Hydrogenophaga* sp.	92	2.2	2.2				3.1	
AJ556799.1 *Comamonadaceae bacterium*	99	2.2	2.2	3.3		1.1	1.6	
EF127651.1 *Polaromonas rhizosphaerae*	98	3.2	2.2			1.1	1.6	
AB504747.1 *Xylophilus* sp.	97	3.2	5.4				6.3	1.2
EF667920.1 Uncultured *Burkholderiales*	91	2.2	4.3					
AF236004.1 *Beta proteobacterium*	95	1.1	2.2	3.3			1.6	
AB452986.1 *Beta proteobacterium*	95	2.2	1.1	3.3				1.2
AJ621027.1 *Nitrosomonas* sp.	96	3.2	1.1			6.4		1.2
AY123811.1 *Nitrosomonas* sp.	94	3.2				3.2		2.5
AY123797.1 *Nitrosomonas* sp.	99	4.3	2.2			9.6		3.7
AY123798.1 *Nitrosomonas* sp.	95	4.3	3.2			6.4	1.6	
DQ839562.1 Candidatus *Nitrotoga arctica*	98					27.7		
EF540467.1 *Pseudomonas* sp.	96	5.4	2.2			5.3	1.6	1.2
AM689949.1 *Pseudomonas* sp.	98	5.4	7.5			4.2		
EU275166.1 *Pseudomonas* sp.	98	9.7	13.0			7.4		
EU034540.1 *Stenotrophomonas maltophilia*	99			3.3	4.5		3.1	2.5
AM230485.1 Flavobacterium aquatile	97	1.1	2.2	3.3	2.3	2.1	3.1	2.5
AB252939.1 Uncultured *Nitrospirae*	99	3.2	2.2	6.7	2.3	4.2	1.6	6.2
AF239693.1 *Gemmata*-like str.	95	1.1	2.2	13.3	2.3		6.3	2.5
Total clones in the library		**93**	**93**	**90**	**88**	**96**	**128**	**81**

[a] Amplicons were approximately 1.45 kb in size

[b] Percentage of similarity between the cloned 16S rRNA gene and its closest relative in the NCBI database

The *Alphaproteobacteria* subdivision members were numerically the most frequently encountered in the weekly-cleaned 3-6 months old samples (50% and 37% of the total clones, respectively). Within the *Alphaproteobacteria*, the family *Sphingomonadaceae* dominated all the three clone libraries. Within the family, *Sphingopyxis* was numerically most abundant in the weekly-cleaned 6 months old membrane sample, while *Sphingomonas* was most abundant in the other two biofilms. The same phylogenetic groups within the cleaned membrane samples were identified by the FISH approach (Fig. 4).

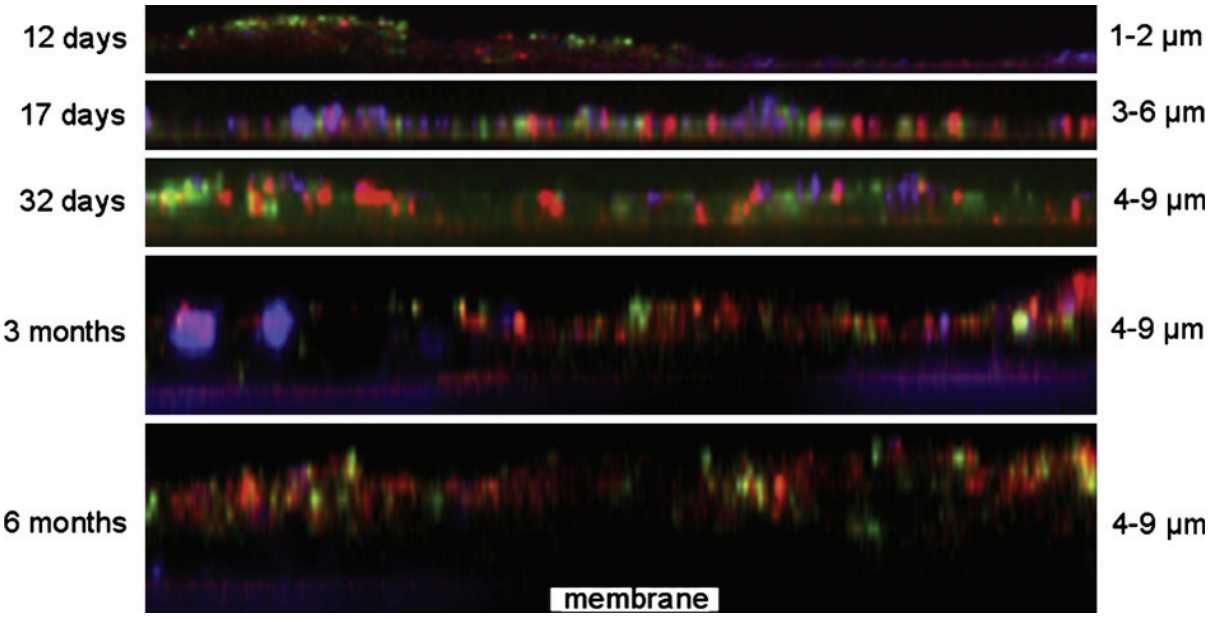

Figure 5. Representative sagittal (***x-z***) sections of biofilms on cleaned RO membranes. The sections were taken at 1 µm intervals across the ***z*** axis of biofilm samples and show the form and spatial arrangement of EPSs, cells and microcolonies in vertical cross sections. The flow cells were operated during 32 days. After 11 days the membranes were cleaned and samples were taken at day 12, 17 and 32 days of operation. Red - *Sphingomonas* (SPH120-Cy3 probe), blue - *Betaproteobacteria* (BET42-Cy5 probe) and green - FITC-ConA-positive EPSs. In the 3-6 months operation the membranes were cleaned once a week. Red - *Alphaproteobacteria* (ALF968-Cy3 probe), green - *Betaproteobacteria* (BET42-FITC probe) and blue - Calcofluor white-positive EPSs.

Compared to the associated feed-side spacers, the membranes showed larger *Alphaproteobacteria* (e.g., cleaned 3 months old biofilm sample: 50% [membrane] versus 38% [spacer]) and smaller *Betaproteobacteria* (17% [membrane] versus 29% [spacer]) fractions in the biofilm-forming communities at their surfaces. Nevertheless, the 3-D structural organization of the re-grown biofilms (Fig. 5 and S2) was similar at both surfaces.

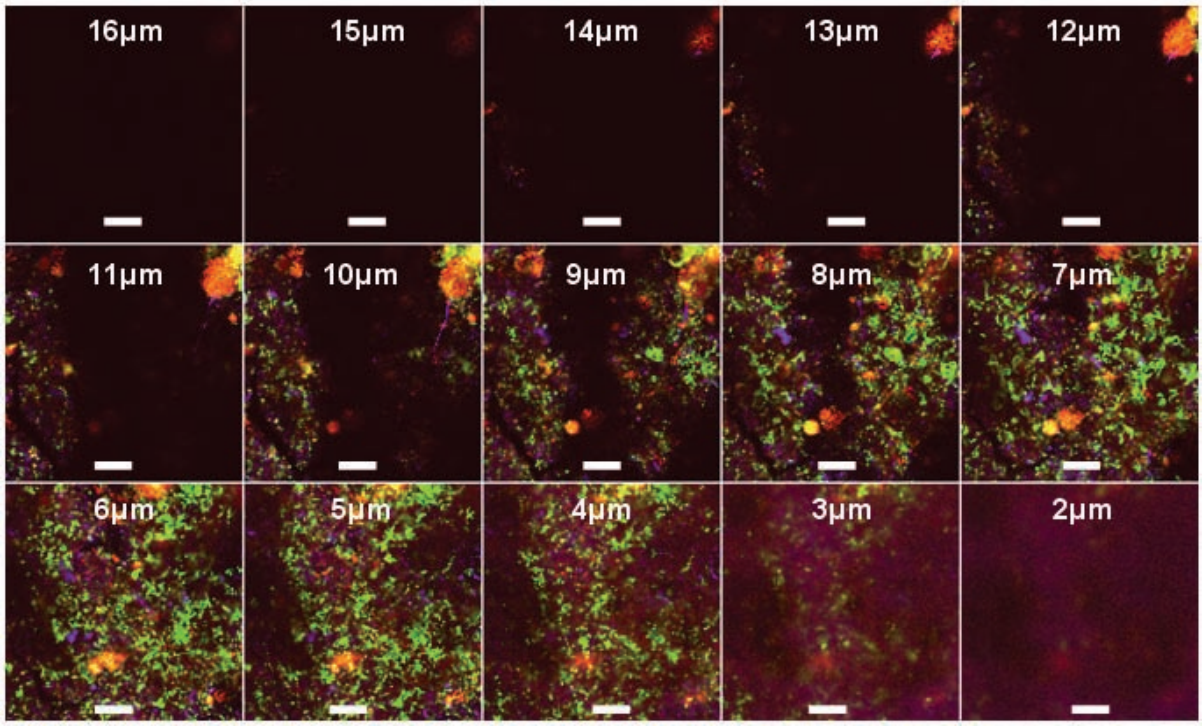

Figure S2. Representative CLSM images depicting architecture of microbial communities developed on weekly cleaned RO membranes. Series of horizontal (***x-y***) optical sections were taken at 1 µm intervals across the ***z*** axis of 6 months-old RO membrane biofilm sample. The sections show shape and spatial arrangement of bacterial cells and EPS matrix within mixed species biofilm communities. The main distribution of cells and polysaccharides were at the top of the RO membrane surface. Cells of *Alphaproteobacteria* were visualized with Cy3-labeled ALF968 probe (red fluorescence), while cells of remaining community members - with DAPI (blue fluorescence) and α-polysaccharides of biofilm EPS matrix – with FITC-Con A (green fluorescence). ***Z***-scan positions are indicated in each image as a number of µm from the top of the RO membrane surface. Bars: 10 µm.

In all the examined CLSM sections, the cleaned 17 days and 3-6 months old membrane and spacer samples possessed a layer of the *Sphingomonas* cells at the dark areas of 1-2 μm (17 days) or 2-3 μm (3-6 months). The areas showed no fluorescence signal with the applied probes or staining dyes (Bereschenko et al., 2010) and filled the space between the *Sphingomonas* cell monolayer (at the biofilm bottom) and the membrane or spacer surface.

In the *Sphingomonas* layer, individual cells were sporadically distributed near the top of a uniformly spread EPS matrix of 1 μm (17 days) or 2-3 μm (3-6 months) thick.

On top of the *Sphingomonas* layer, a second film with heterogeneous EPS and cellular biomass was present. The majority (>80%) of the EPS network appeared within the first 4-8 μm of this layer and was detectable with ConA and Calcofluore white, indicating the presence of the *β*-1,4-linked and *α*-D-glucose and *α*-D-mannose polymers (Johnson et al., 2000). The β-1,4-linked polymers were quite uniformly spread, while the *α*-D-glucose and *α*-D-mannose polymers were scattered irregularly. Most of the detected bacteria were dispersed as individual cells and/or microcolonies within the basal 4 μm (17 days) or 2-6 μm (3-6 months) thick fraction. The *Sphingomonas* cells were uniformly spread over the entire EPS-matrix of this fraction, while the other *Alphaproteobacteria*, *CFB*, *Betaproteobacteria* and *Actinobacteria* colonized its upper and the *Gammaproteobacteria* the middle part. The *Planctomycetales* were mostly present in the basis and the *Verrucomicrobia* - on top of the biofilm. The cylindrical and/or mushroom shaped microcolonies were associated with the *Alphaproteobacteria*, while the microcolonies of *Beta*- and/or *Gammaproteobacteria* were round shaped. Irregularly shaped microcolonies consisted of members of the *Burkholderiales, CFB* and/or *Verrucomicrobia*. Most of the *Betaproteobacteria* microcolonies stuck together in the EPS-associated stacks and extended at irregular intervals from the surface of the basal fraction into the bulk aqueous phase. In all three samples, the stacks were up to 6 μm high and showed the presence of irregularly scattered single *Sphingomonas* and/or *Verrucomicrobia* cells and/or microcolonies of the *Gammaproteobacteria* and/or *CFB* origin.

In some SEM images of biofilms eukaryotes were also visible (Fig. 6). Overall, up to 2.0 x 10^6 bacterial cells/cm^2 were recovered from the membrane surface.

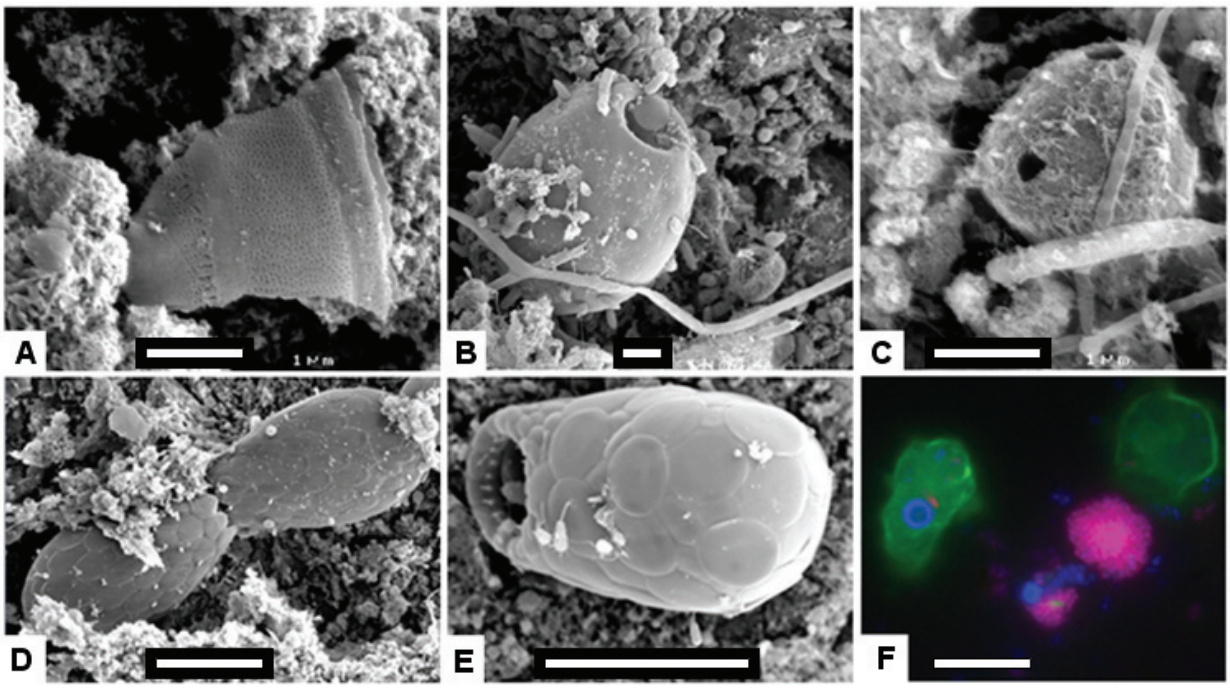

Figure 6. Scanning electron and epifluorescence micrographs showing presence of unicellular eukaryotes in biofilms from chemically cleaned and non-cleaned RO membranes. (**A-C**) - SEM images of unknown unicellular eukaryotes on top of the biofilms from the non-cleaned 3 (**A**-**B**) and 6 (**C**) months old membranes. (**D**) - SEM image of the *Euglypha* in the biofilm from the weekly cleaned 6 months old membrane. (**E**) - SEM image of the *Trinema* on the biofilm from the weekly cleaned 3 months old membrane. Various single and EPS-embedded bacterial cells on the surface of the eukaryotes and within the biofilms can be observed. (**F**) - EPIM image of two trophozoites of the *Acanthamoeba sp.* on the surface of the non-cleaned 3 months old membrane biofilm. Note cell wall (FITC-ConA-stained, green fluorescence) and nucleus (DAPI-stained, blue fluorescence) of the eukaryotes and microcolonies of the *Betaproteobacteria* (red fluorescence from positive hybridization with the Cy-3-labeled BET42a probe). Bars: 1 μm (A-C) and 10 μm (D-F).

No significant changes in the structure of RO membrane and spacer-associated biofilm layers were observed within the next 15 days of the flow cell operation without cleaning (see the 32 days old sample in Table 1 and Fig. 4, 5 and S3), however the layers increased in thickness (6-9 μm [membrane] and 2-5 μm [spacer]), cell density (1.2 x 10^9 cells/ cm^2 membrane) and diversity (e.g., occurrence of the *Actinobacteria*, *Euglypha* and trophozoites of *Acanthamoeba sp.*).

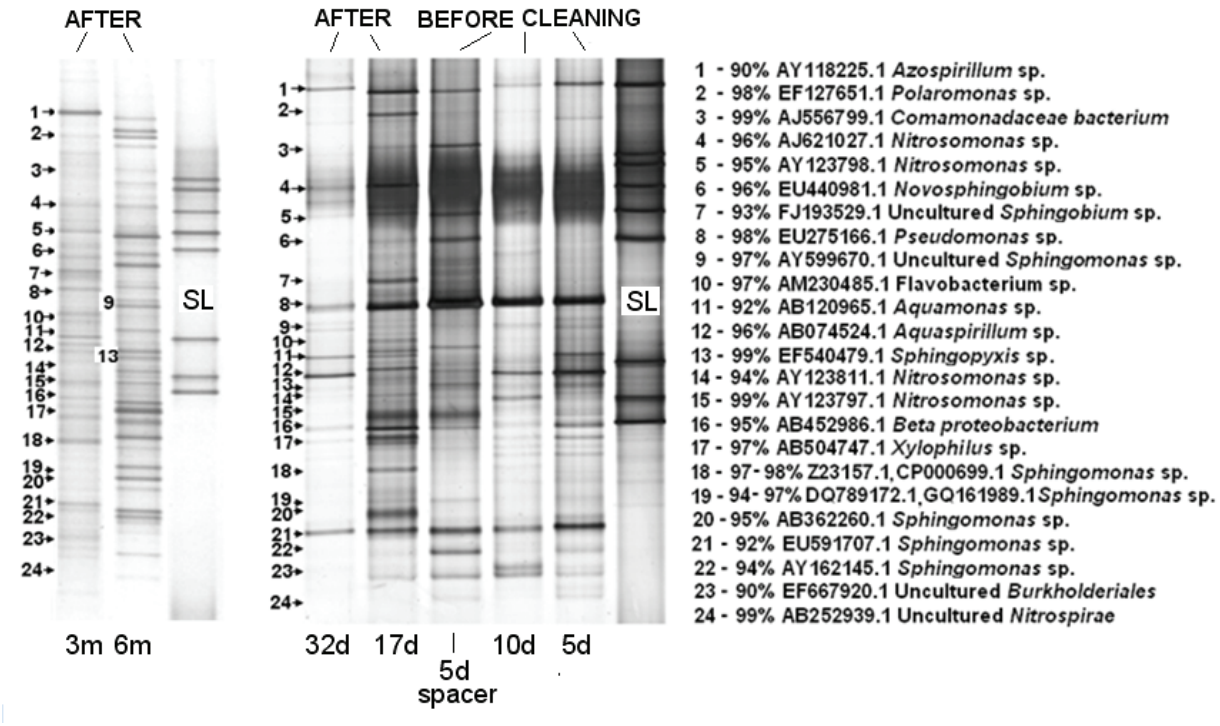

Figure S3. DGGE fingerprints of RO membrane biofilm samples. Lanes represents the fingerprints of bacterial communities, developed on RO membrane surfaces in 3 (**3m**) - 6 (**6m**) months and 5-32 days with or without chemical treatment of flow cells. Lane **5d** spacer - the DGGE patterns of bacterial communities, developed in 5 days without cleaning of the flow cells on the feed-side spacer. **SL** corresponds to a synthetic marker. Arrows at the left indicate positions of the identified bands.

Biofouling on cleaned versus non-cleaned membranes

Compared to the biofouling rate of the weekly cleaned RO membrane and/or feed-side spacer surfaces, the biofilm initiation at the new membrane and/or spacer surfaces occurred slower, but its spatiotemporal development resulted in an evidently higher severity of the fouling (Fig. 3). Without cleaning, the appearance of single and EPS-embedded bacterial cells was observed within the first 5 days of the flow cell operation (Fig. S4, panel E and F).

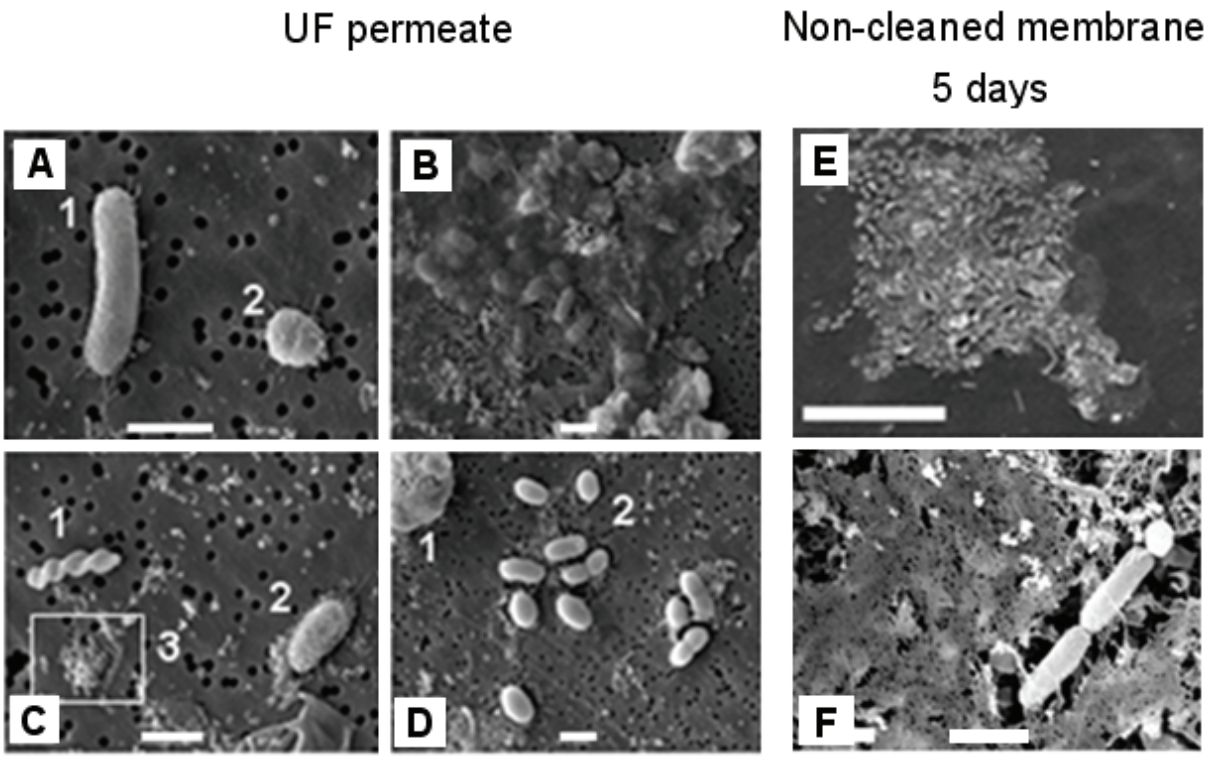

Figure S4. Scanning electron micrographs showing diversity of fouling substances in the ultrafiltration permeate fed to the RO system of a full-scale water treatment plant (**A-D**) and presence of the nascent biofilm structures in the non-cleaned 5 days old membrane samples (**E-F**). At the surface of a black polycarbonate filter (pore size, 0.2 µm; type GTTP 4700, Millipore, Germany): rod-shaped (**A**-1 and **C**-2), spherical (**A**-2) and spirillum-like (**C**-1) bacterial cells, flocks (**B** and **D**-2) and pieces of a loose network of extracellular polymeric fibrils (**C**-3). At the RO membrane surface: attached flock (**E**) and 5 days old bacterial cell monolayer. Bars: 1 µm (A, B, C, D and F) and 10 µm (E).

Their accumulation was associated with the presence of pieces of floating biofilms (flocks) and single bacterial cells in the RO feed water (i.e., a cartridge-treated ultrafiltration permeate), as detected by the FESEM (Fig. S4, panel A-D), FISH (Fig. 4) and DGGE analyses (Fig. S5).

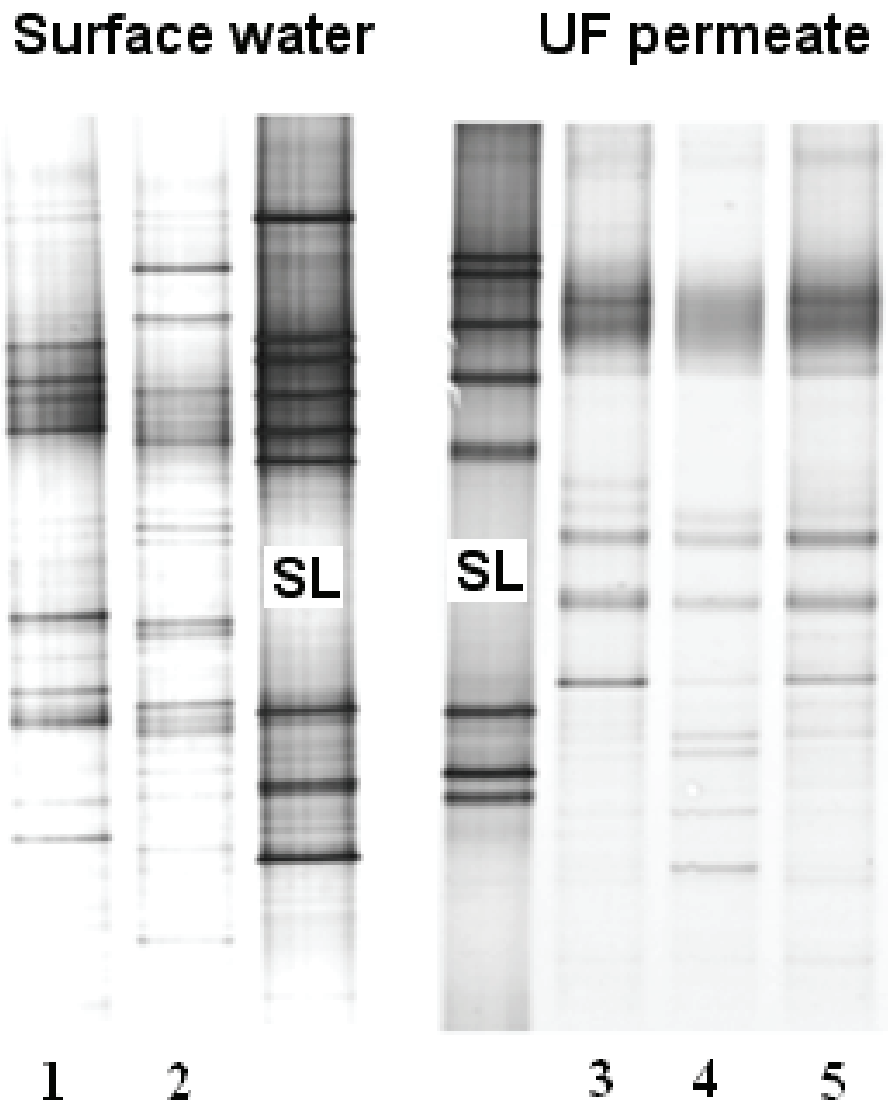

Figure S5. DGGE fingerprinting of bacterioplanktonic communities from a full-scale RO water treatment plant. **Lanes 1** and **2** – the DGGE profiles of the bacterial communities from the RO feed water (fresh surface water), collected in March and April, respectively. **Lanes 3, 4** and **5** – the fingerprints of the bacterioplankton from the ultrafiltration permeate, fed to the RO system of the plant in March, April and May, respectively. In both panels, lane **SL** corresponds to a synthetic marker.

Based on total bacterial cell number (DAPI) determinations, from11 April to 11 May 2008 approximately 2.3 x 10^6 - 1.5 x 10^7 cells/L were present in the fresh surface water that was fed into the full-scale RO plant. About 1.5 x 10^3 to 7.0 x 10^4 cells/L were present in the ultrafiltration permeate that was fed into the RO membrane modules and test flow cell units. Surprisingly, 6.1 x 10^2 to 2.0 x 10^4 cells/L were detected in the RO permeate from the full-scale RO. In contrast, no bacterial cells were detected in permeate from the test flow cells.

SEM and CLSM examinations of the emerging biofilms on the non-cleaned 5 and 10 days old membrane and feed-side spacer surfaces revealed differences in their spatial organization. In the flocks, cells of the b or *Gammaproteobacteria* were uniformly distributed within a common (<0.5 μm thick) EPS matrix. The *Beta-* and *Gammaproteobacteria* also emerged in the close proximity to each other. The uniform species clusters were small (~1 x 3 μm) and occurred at irregular intervals over the entire feed side of the membrane and in the corners of the associated spacer. The mixed species aggregates (Fig. S4-E) were large (~10 x 20 μm) and appeared primarily at the flow cell entrance. Their accumulation was also visible by the naked eye (Fig. 3). At the surfaces of these aggregates cells of the *Alphaproteobacteria, CFB, Verrucomicrobia* and/or *Planctomycetes* were randomly distributed. In the Sphingomonas monolayers, individual cells were embedded in a 1 μm thick EPS matrix that filled the 2-10 μm spaces between the cells (Fig. S4-F). In the 10 days old samples, these layers were stretched up to 60 μm wide and covered (at irregular intervals) up to 50% (membrane) and 20% (spacer) of the total surface area. According to the clone libraries analysis (Table 1), the *Betaproteobacteria* subdivision was the largest bacterial group in the libraries from the non-cleaned 5 days old membrane sample (62% on the total clones). Within the group, the genera "*Candidatus*" Nitrotoga arctica and *Nitrosomonas* dominated (36% and 24% of total clones) the non-cleaned 5 days old membrane library.

In the longer (17 days-6 months) operated systems, the arrangement of biomass and biogenic extracellular material at the non-cleaned membranes and/or spacers was similar to the 3-D biofilm organization on the weekly cleaned and long-term (3e12 months) operated surfaces. However, the presence of a dark (no fluorescent) area between the biofilm bottom and membrane or spacer surface was not observed. The second fraction of the biofilm (on the top of the basal, *Sphingomonas* biofilm) was 4-5 μm thicker and the *Gammaproteobacteria* emerged in the upper part of this fraction. The *Betaproteobacteria* stacks were 6 μm higher and the majority (>80%) of the bacterial EPS appeared within the first 10-13 μm (from the biofilm bottom). The *Actinobacteria* were not detected in the biofilms that were present on the non-cleaned membrane and spacer surfaces.

Observed from the top, the biofilms appeared as lumpy establishments on the non-cleaned surfaces and as relatively flat carpets on the cleaned surfaces (Fig. 7).

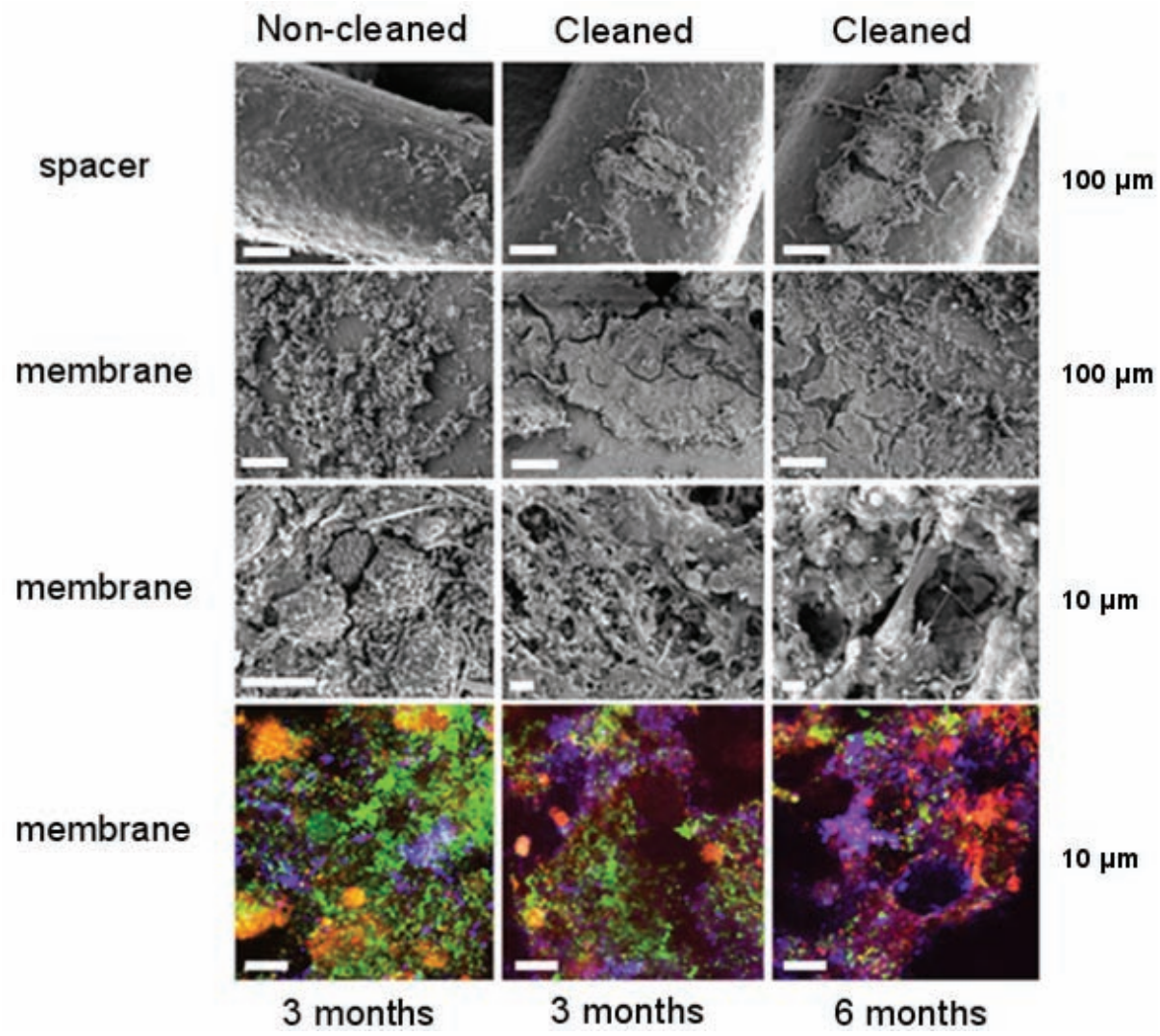

Figure 7. Scanning electron and CLSM micrographs demonstrating the effect of weekly chemical cleaning procedures on the structure and complexity of RO membrane and feed-side spacer fouling layers. **Vertical columns** represent images from the not-cleaned 3 months old and cleaned 3-6 months old samples. **Horizontal panels** represent SEM and CLSM images of biofilms at the RO membrane and feed-side spacer surfaces. Note presence of water channels in the images. Red fluorescence in the CLSM images was acquired from the Cy3-labeled BET42a probe, while green - from the FAM-labeled SPH120 probe and blue - from the Calcofluor white-stained α-D-glucose and α-D-mannose of the biofilm EPS matrix. Bars: 10 and 100 µm.

Separated microcolonies were more abundant and larger in size (10-15 μm) on the non-cleaned surfaces compared to the size (<5 μm) of the microcolonies on the cleaned surfaces (Fig. 7). Voids larger than 5 μm occurred only within the biofilm matrix on the cleaned surfaces (Fig. 7). The number of total bacteria was higher and increased with the operating time at the non-cleaned membrane surface: 6.3×10^4 (5 days), 9.7×10^5 (10 days), 6.1×10^8 (3 months) and 2.1×10^9 (6 months) cells/cm2. On the cleaned membrane surface lower numbers of bacteria were detected after 3 (8.2×10^7 cells/cm^2) and 6 months (3.7×10^7 cells/cm^2).

Discussion

During a period of one year we have studied the effect of conventional chemical treatment on occurrence and development of biofouling in reverse osmosis (RO) membrane units. A comprehensive evaluation of the cleaning impact was achieved by monitoring microbial populations on the surfaces of cleaned and non-cleaned RO membranes and feed-side spacers and correlating the outcomes with pressure drop measurements over the feed channel of the test flow cells during one year. The test flow cells were connected in parallel to an RO system of a full-scale water treatment plant that produced process water from extensively pre-treated surface water (Bereschenko et al., 2010).

The result of this study describes the dynamics of biofouling under real field conditions and may be important for the development of new anti-fouling strategies in membrane separation processes.

Chemical treatment is not cleaning

This research confirms previous (Baker and Dudley, 1998; Flemming, 2002) suggestions that the failure in removing established biofilms from RO membrane unit surfaces is the main reason for the limited effect of conventional chemical treatment on prevention and/or elimination of biofouling in full-scale RO water purification plants. The biofilm layers are often still present on the RO membrane and feed-side spacer surfaces within the RO test flow cells after the weekly applied chemical cleaning procedures (Bereschenko et al., 2010, 2007,

2008, this study). However, their structures were drastically affected (Fig. 2 and 5) and became more loosely attached (i.e., could be more easily scraped than the biofilms on the non-cleaned surfaces). This indeed results in a lower pressure drop over the feed channel (Fig. 1). The loosely attached biofilm is not completely removed, most likely because the flow inside the membrane module cannot exert sufficient friction to flush the biomass away due to the presence of the feed spacer. Similar phenomena were observed in our previous studies (Bereschenko et al., 2007, 2008), on the surfaces of the industrially used (for 1 and 5.5 years) bi-weekly cleaned (by a similar cleaning procedure) RO membrane from the same RO system. It appears that factors as surface texture (rough: membrane or smooth: spacer), system configuration (flat-sheet: test flow cell or spiral wound: commercial RO module), operation time (days, months or years) and frequency of conventional cleaning do not have a significant influence on the stability of microbial biofilms. Apparently, the inherent properties of the biofilm-associated bacterial cells and extracellular polymeric substances play a role. From the microscopic examinations, it is evident that the network of biofilm-associated EPSs appeared to be remarkably stable to the chemical cleaning procedures, whereas the majority (67%-79% of the total clones, Table 1) of the associated bacterial population disappeared due to toxic effect of the chemicals. Consequently, each single chemical treatment resulted in the collapse of the established three-dimensional biofilm structure and not in biofilm removal from the different surfaces as was expected. In the CLSM and SEM images, only the upper RO biofilm layer was usually affected (i.e., collapsed or disappeared), while the structural integrity of the base layer was hardly changed (Fig. 2 and 5). Only *Sphingomonas* species - typically localized at the biofilm base, according to this and previous study (Bereschenko et al., 2010) - were able to survive the chemical cleaning procedures (Fig. 5). There are two options that might lead to their resistance to cleaning. Firstly, by being present in the base of the biofilm they might be protected from the biocide (sodium bisulphite). The biocide will react with the organic matter in the top-layer of the biofilm and most likely will not reach the lower localized *Sphingomonas* cells. Increase in applied concentration would be an option to circumvent this problem, but there is a delicate balance between disinfection efficiency and protection of the membrane (certainly on places without biofouling) from the adverse effects of the biocide. It might also be that the specific properties of sphingomonads EPS offer additional protection against chemical attack. The sphingomonads are producers of various extracellular biopolymers (sphingans), including gellan-like polysaccharides (Pollock, 1993; Lobas et al., 1994; Pollock and Armentrout, 1999), which are known for their relative stability to many environmental conditions (i.e., extremes of pH, temperature, salinity and autoclaving

[Ashtaputre and Shah, 1995]). Microorganisms that are present in these EPS layers are much more resistant to many antibiotics (Smalley et al., 1983). There is however no literature data on the effect of bisulphite on these gellans and microorganisms that are embedded in these polysaccharides. A large amount of EPS structures was visible in the CSLM images compared to the amount of cells (Fig. 5). Newly produced EPS will require a lot of space and push newly divided cells wide apart preventing the formation of microcolonies in the biofilm (Picioreanu et al., 2004). The sphingans are localized at the base (Fig. 5 and S2) and take up a major part (up to 80% of the volume) of the biofilm matrix in the chemically treated samples. It can, therefore, be assumed that the sphingans are the most important contributors to the cohesive strength of the fouling layer on the membrane surface. Furthermore, the presence of glycosphingolipids in the cell envelopes of the sphingomonads, which is unique and clearly distinguish them from other bacteria (Kawasaki et al., 1994; Balkwill et al., 2006), may give them a more substantial protection to chemically active agents than the lipopolysaccharides that are present in the cell envelopes of other bacteria (Smalley et al., 1983). Additional experiments with *Spinghomonas* spp. will be necessary to prove this hypothesis. Current cleaning procedures with surfactants and chelators are often tested on non-sphingomonads biofilms. Apparently, they are not effective on *Sphingomonas sp.* and their EPSs as might be expected from the physicochemical properties of the components involved (Balkwill et al., 2006; Denner et al., 2001; Pollock, 2002). The study of the unique EPSs and glycosphingolipids of sphingomonads species might result in the development of more effective and directed cleaning methods to control biofouling.

Rapid re-growth of biofouling layers

The results indicate that microbial colonization of the collapsed biofilm layers starts directly after chemical cleaning. Two clearly different features were hereby observed: attachment and growth of primary colonizers (single cells and cells in clumps, Fig. 2, 5, and S4) transported by the RO feed water to the surfaces and proliferation of organisms that survived the chemical cleaning within the collapsed biofilm layer (Fig. 2). The colonization process consists of similar events as described previously for clean surfaces (Bereschenko et al., 2010): the initiation of early biofilm structures and a spatiotemporal development into a multispecies slime layer with a complex three-dimensional architecture (Fig. 5 and S2). The re-growth of the bacterial biofilms attached to the membrane and feed-side spacer surface results in the

same biofouling-related system failure as before the cleaning and occurs within a relatively short operational time (approx. 1 week). In contrast, the development of a "critical level biofilm" on fresh (non-cleaned) RO membrane and feed-side spacer surfaces take approximately 16-17 days (Fig. 1-A). Factors that facilitates this rapid biofilm re-growth on the treated surfaces may be: (i) presence of attractive attachment surfaces (i.e., clearly rough surface with, possibly, adhesive EPSs), (ii) abundance of nutrients (i.e., damaged EPSs, proteins and other macromolecules from lysed cells) trapped in the EPS matrix and (iii) presence of viable cells under the collapsed top of treated biofilm layer. The microbial communities within the re-grown biofilm layers are usually more complex in structure and composition (Table 1 and Fig. 5-7 and S3), compared to the communities on the fresh RO surfaces. However, the general biofilm architecture was the same in both cases (i.e., the mixed species layer on top of the *Sphingomonas* monolayer at the basis, Fig. 5 and S2).

The observed biofilm removal failure and subsequent rapid biofilm layer re-growth were observed after each scheduled treatment. From a microbiological point of view, the re-growth process remains the same, with some small shifts in the structure and composition of the involved microbial community, more related to seasonal changes (Fig. S3) than to the operating and cleaning procedures. Remarkably is, however, that within 6-7 days after cleaning the biofilm reached already a structure similar to a five years old fouling layer as observed in a previous study (Bereschenko et al., 2008) on a membrane module from the same water production plant. This emphasizes the need for radical new biofouling control methods, potentially based on the properties of the sphingomonads and their EPSs.

Conclusions

This microbial molecular ecology study clearly demonstrates that conventional cleaning with toxic chemicals has an effect on the occurrence of biofouling in RO systems, but is not effective in really cleaning the RO system. For development of new approaches to control biofouling in membrane-based water treatment systems special attention has to be paid to the sphingomonads. These versatile bacteria are widely spread in natural water environments and man-made water systems (Chen et al., 2004; Koskinen et al., 2000; Pang and Liu, 2006). They are strong competitors in scavenging a variety of nutrient sources under oligotrophic conditions. They contribute a lot to the cleaning-associated stability of bacterial biofilms,

even if they are number wise not the dominant group in the surface-attached biofilm communities.

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6 General Discussion

Research purpose and questions

Reverse osmosis systems play an important role in removal of a wide variety of contaminants from water streams of different quality. Maintenance and operational costs of such systems are affected by biofouling, a process in which microorganisms attach to membranes and develop into a film that can disturb the entire RO system. Management of this problem requires basic understanding of the mechanism of this biological phenomenon. Essential questions that need to be answered are: which organisms are involved, how do they interact to form the biofilms, can anything be done to avoid attachment of such organisms and establishment of the biofilms. If biofilm formation cannot be avoided, then we should try to manage the system such that biofilm formation has a minimal impact? The basic questions of this PhD research program therefore addressed the origin, succession and spatiotemporal development of microbial biofilms in full-scale reverse osmosis systems, in particular in relation to operational aspects of the RO system, that is, when biofouling affects the engineering aspect of the system, by influencing performance parameters.

Research strategy and methods

The pivotal problem in answering the basic research questions resided in the unavailability of representative samples. Sampling from industrially used RO membrane units is only possible after their removal from the system and autopsy. For current full-scale RO systems this is rarely done and only in case of severe biofouling they are replaced and can be investigated (chapter 2). Hence, provision of appropriate samples of the biofilm at various stages of development was not a realistic option and only end-stage fully fouled samples could be obtained. A solution for this problem was achieved by the design of an experimental system consisting of parallel operated flow cells (Fig. 1 [chapter 4] and S1 [chapter 5]). These flow cells reflected the main RO membrane modules very closely in terms of geometry of the feed channel, flow velocity, temperature, pH and composition of feed water. As a result, the flow cells are operated similar as the full-scale system, but they could be opened daily for sampling of the membranes and analysis of biofilm structures. In addition to investigating the flow cells, modules from different locations in the main RO system containing the main membranes were investigated at an intermediate time point (chapter 3) and at the end of their operational lifetime (chapter 2) in order to compare results between full scale membrane modules and flow cell systems. Liquid (including feed water, chapters 3 and 5) and material

surfaces at different locations in the RO plant (chapters 2 and 3) were also sampled to study the presence, diversity and community structure of potential and known biofilm-formers in the upstream parts of the main RO system.

A second characteristic of the research strategy presented in this thesis was the application of different techniques to examine biofilm samples. A biofilm is an aggregate of surface-adhered microbial communities mostly embedded in a self-produced slimy matrix of extracellular polymeric substances (EPSs). The EPSs are a polymeric conglomeration of nucleic acids, lipids, proteins and polysaccharides. The microbial community consist of different microbial species, each living in a customized microniche. Study of the biofilm thus requires deployment of combinations of a number of well-established and complementary methods for a proper assessment of its structural and spatial complexity. With respect to the origin, succession and spatiotemporal development of microbial biofilms, knowledge of the various taxonomic groups of microorganisms that play a role in full-scale RO systems must be obtained, and a detailed assessment of the population composition in time and space is required. Classical cultivation techniques for studying microorganisms are biased by those microorganisms that grow in the cultivation media provided, and are therefore less suitable for systems that are known to harbour many yet unculturable microorganisms. Hence, alternative methods should be used. A wide array of molecular tools is, in principle, available, but the application of each requires inclusion of proper controls and careful evaluation of results. After all, DNA sequence analysis alone cannot provide the answers to intricate ecological questions. As a consequence, various molecular microbiological tools were used in the presented PhD research for studying the *in situ* microbial ecology of the RO systems. Presence and distribution of general fouling at the RO membrane and structures was evaluated by visual inspections. Origin, abundance, distribution and morphology of the bacteria was further explored by Field Emission Scanning Electron Microscopy (FESEM). In the film-forming communities, species diversity and relative abundance was determined by Fluorescence *in situ* Hybridization (FISH) and/or cloning and sequence analysis of the 16S ribosomal RNA gene of the bacteria. The presence and structure of the EPS matrix was evaluated by the FISH and/or FESEM. Detailed examination of spatiotemporal arrangement of the species within the biofilm matrix - with respect to each other and to exopolysaccharides - was performed using histological staining with DAPI, Calcofluor white, fluorescently labelled lectin Con A and FISH probes of various specificity and evaluation of the staining with epifluorescence microscopy and confocal laser scanning microscopy (CLSM). Finally, sequential changes in community composition during different developmental stages were

assessed using the Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting technology.

Things learned

About collection and processing of representative biofilm samples

The results obtained show that the sampling strategy is a crucial factor in understanding the elemental aspects of the biofouling phenomenon within the full-scale water treatment plant. It appears that the sampling from industrially used RO membrane units by removal from the system and subsequent autopsy provides only a snapshot of surface-associated microbial communities. For example, the observed dominance of some bacterial groups in the biofilms at specific moments of sampling (e.g., the succession in species from Candidatus *Nitrotoga arctica* and *Nitrosomonas* in 4 days old membranes, via *Actinobacteria* and *Sphingomonas* with *Planctomyces* and ultimately into *Sphingomonas, Beta proteobacteria* A0637, *Flavobacterium*, *Nitrosomonas* and *Sphingobacterium in* RO membranes of 5.5 years) was only possible by the sampling possibilities. The operation of flow cells in parallel to the RO system made it possible to study the bacterial activity in the system at different stages of the membrane operational lifetime. As a result, a more comprehensive understanding of the species abundance and species diversity involved in the initiation and spatiotemporal development of microbial layers on RO membrane and feed-side spacer surfaces could be attained. Accordingly, the prevalence in numbers at a certain moment of sampling is not always a direct indication of the presence of specific phenotypic or metabolic traits that provide these bacteria with a selective advantage in sustaining biofilm formation processes or in providing biofilm stability during chemical cleaning procedures. For example, it appears that sphingomonads contribute a lot to the early establishment of a biofilm on new membrane and feed-side spacer surfaces (chapters 4) as well as to the cleaning-associated stability of the established biofilms (chapter 5), even if they are number wise not the main group in the associated planktonic or biofilm populations. In contrast, this was not observed for the remaining bacteria, recognized as the members of biofilm community at different stages of biofouling development (chapters 2-5). All of them appear to live a biofilm packed 3-D formations of aggregated single cells and/or microcolonies (Fig. 4, chapter 4), with a relatively thin (< 0.5 µm) EPS matrix. Being the prevalent community members at a specific moment of biofilm development but existing in such formations (chapter 4), these organisms

disappear during a regular (e.g., weekly applied) chemical cleaning, whereas many sphingomonads and the associated EPS matrix (Fig. 2 and 5, chapter 5) are still present on the treated surfaces (chapter 5). Hence, the dominance of certain groups of bacteria within an RO biofilm community at a certain moment of biofilm existence just reflects the prevailing environmental conditions in the system prior to the sampling. Such conditions, that can be brought about by cleaning procedures (chapters 2 and 5) but also by flow properties, nutrient levels, osmolarity, temperature, pH and/or other factors, may result in selection of microorganisms by type and frequency (5).

The second know-how with respect to the sampling was gained from the observation that biofilms collected from the membrane surfaces as a "tightly associated" bacterial community (chapter 2) were not always equivalent in the species abundance and diversity to the "loose" biofilms (collected as the scrapings from a membrane surface area, Fig. 1-C [chapter3]) and the "tightly attached" biofilms on the associated feed-side spacers (for examples see figures 3 [chapter 2] and S3 [chapter 5])**.** This discrepancy became clear by the *in situ* monitoring of the 3-D biofilm structural organization (chapters 4 and 5). It appears that the mature biofilms on both the membrane and feed-side spacer surfaces consist of two different layers: the basically located and firmly attached almost uniform *Sphingomonas* cells and *Sphingomonas* EPSs layer with an average thickness of 1 to 3 μm and a second, heterogeneous in species and EPSs abundance and diversity layer (~2 to 19 μm thick) on the top of it (figures 5, 6 [chapter 4] and 5 [chapter 5]). By the scrapping from the membrane surfaces firmly attached sphingomonads are not properly sampled, which results in the underestimation of their contribution to the structural complexity of RO membrane and/or spacer biofilms. At the same time, the remarkable dominance of *Betaproteobacteria* in the RO feed water (chapters 3 and 5) and their prevalent accumulation at the feed-side spacer surface and at the entrance of the membrane element (chapters 4 and 5), may result in their numerical prevalence within the associated biofilm communities after a prolonged time of system operation and at a certain point of the biofilm development (as discussed above). Therefore, to avoid a misinterpretation of the biofouling process in a full-scale RO system, the biofilm samples should be collected from membrane and spacer surfaces in parallel and from different locations through a total surface area, avoiding collection of the "loose" biofilm communities.

In the current biofilm and biofouling research, significance of these aspects for a proper understanding of the biofilm and/or biofouling development in a full-scale membrane system are not yet fully recognized. The collection of biofilms as scrapings from the

industrially used and operationally failed membrane elements is still a widely accepted sampling strategy.

About analysis of biofilm structural complexity and development

The multifaceted technological approach involving the use of many molecular and microscopic analysis techniques in parallel has enabled a comprehensive evaluation of representative water and biofilm samples. In this way some of the known (6-13) drawbacks with respect to the applied techniques could be overcome and the complexity of the formation and spatiotemporal development of microbial biofilms in RO membrane systems could be understood. For instance, in Chapter 4 the role of the *Planctomycetes*, *Verrucomicrobia* and *CFB* bacteria in all stages of biofilm formation was uncovered by FISH, not by the cloning or DGGE methods. This discrepancy may be a consequence of the formation of "chimeras" (13) during PCR amplification of bacterial 16S rRNA gene fragments. Some of the "chimerical" PCR products in this study (data not shown), which were excluded from the 16S rRNA gene clone libraries were actually up to 97% similar to some of the *Planctomycetes*, *Verrucomicrobia* and *CFB* species. Hence, some of the unidentified bands in the DGGE fingerprints could be from these sequences. Similarly, significance of the *Sphingomonas spp*. in the RO biofouling became clear from scrupulous CLSM examinations of the *in situ* monitored biofilms, not by the epifluorescence and/or FESEM microscopy, clone library or DGGE studies of the same samples. Furthermore, the involvement of diverse unicellular eukaryotes became clear from the outcome of applying different techniques. The trophozoites of *Acanthamoeba* sp. (figures S1-C [chapter 4] and 6-F [chapter 5]) were recognized by the examination of the epifluorescence and CLSM images of biofilms, while other protozoa, as well as diatoms (figures S1-A, B [chapter 4] and 6, A-E [chapter 5]), were discovered by FESEM. The identification of the endosymbiont of *Acanthamoeba* spp. by the cloning method (Tab. S1, chapter 4) revealed an additional indication of the participation of the *Acanthamoeba* spp. in the RO biofilms. Such aspects reveal the complexity of the system, which would not have been noticed when using the various techniques of isolation. Recent studies in the biofouling field (14, 15) also point to the need to combine molecular ecology tools with microscopic tools to comprehensively describe and understand the complexity of microbial population dynamics in fouling of the industrially used membrane systems.

About the origin and development of biofouling layers

The results obtained from the comprehensive evaluation of the representative samples clearly indicate that the microorganisms that form a biofouling layer in a full-scale RO system originated from the water fed to the system (chapters 2, 3 and 5). This observation contrast with previously emerged (16, 17) and currently still believed (personal communication with operators, scientists and engineers) statements that formation of a biofouling layer in the systems with an extensively pre-treated feed water may be a result of an at random process, caused by the microbial contaminants incorporated accidentally (e.g., during manufacturing of the RO units, their placement in the system, repair and/or cleaning operations in the system, etc.). The feed water origin of the pioneering and secondary biofilm-formers (chapters 4 and 5) indicates that the biofilm mode of existence may be an evolutionary promoted adaptation of the intrinsically present (in this case, the freshwater) bacteria to live or survive under conditions of flowing, changing and/or nutrient-deficient environments. In a sequence of separation and filtration steps before the system, they may therefore be selected rather than microbial contaminants incorporated accidentally for the formation of surface-associated communities over free-floating (planktonic) cells, which would be easily transported by the flowing water to potentially hostile environments downstream. Indeed, the biofilms of a typically fresh water phylotypes (18) grow on any surface in an RO water treatment facility (chapter 3). This gives local niches for growth and detachment of biomass either as single cells or cell clumps, spreading bacteria to the further stages of the RO plant. In the membrane modules of RO system, the enriched bacteria might more easily colonise the surfaces since they will be better adapted to growth in the system than bacteria present in the feed water or than microbial contaminants incorporated accidentally. It can be argued that even though the external bacterial contaminants might be expected to employ similar strategies for survival under conditions encountered in an unfavourable environment, they may be less strong competitors in scavenging available nutrient sources under (oligotrophic) RO conditions. Hence, they may be outcompeted by organisms that are well adapted to nutrient depletions. The absence of e.g., *Stenotrophomonas* and *Corynebacterium spp*. (respectively: opportunistic human pathogens and common commensals on human skin, and therefore often recovered in bacterial air samples) in early biofilms at fresh (virgin) surfaces (chapters 4) and their potential proliferation in biofilms (e.g., directly after chemical treatment, chapter 5) - likely by availability of extra substrates in the system (19) - supports this conclusion. Given

this and previously reported (5, 15, 19) considerations, it became clear that the feed water quality is the most important determinant in the occurrence of membrane biofouling.

It is also evident that a "critical level biofilm" (19) was actively formed on the RO membrane sheets and their feed-side spacer surfaces and was not the result of a simple concentration of bacteria present in the feed water (chapters 3, 4 and 5). So, only certain feed water bacteria appear to be capable in the initiation and spatiotemporal development of early biofilm structures at these surfaces (chapters 4 and 5). Correspondingly, Ridgway (20) observed that only certain bacteria can attach at this stage of biofilm development and that there are a limited number of attachment sites.

Our data further show that in the water phase of different stages of a full-scale RO plant, bacteria occur as clumps or single cells (chapters 4 and 5). A large fraction of these cells appear to be dormant and are just transported by the flowing water and end up resting at available surfaces (chapter 4, ref. 21, 22). This observation suggests that the previously proposed mechanism – i.e., formation of "conditioning film" (19) - may not be necessary a prerequisite for microbial attachment at RO membrane and feed-side spacer surfaces. However, its ubiquitous presence (4) and patterns observed in the bacterial mode of existence in the water (chapter 5) and at the fresh membrane and spacer surfaces (chapter 4) indicate that the mode and efficiency of primary colonisers in the establishment of early RO biofilm structures is inevitably influenced by the nature of these films.

Although the specific role of each type of planktonic or sessile bacteria on the structure and activity of the RO membrane and feed-side spacer biofilms still remains unclear, a generic pattern of their contribution to the occurrence of biofouling layers in a full-scale RO system can be established. Accordingly, the formation of biofilms on fresh RO membrane and feed-side spacer surfaces involves four basic steps: (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 (chapters 4 and 5). In contrast to biofilm formation on other solid surfaces (23, 24), in our experiments we clearly observed two additional aspects: cells (mainly *Beta*- and *Gammaproteobacteria*) that mainly adhered in clumps and grew out as such and cells (sphingomonads) that mainly adhered as single cells and colonized the surface almost as a monolayer (Fig. 6, chapter 4). The sphingomonads quickly spread over the entire membrane and feed-side spacer surfaces. Within 4-5 days of system operation, they mainly develop in a flat monolayer of the relatively wide dispersed and abundantly EPS-embedded cell monolayers (Fig. 4 [chapter 4] and S4 [chapter 5]). This mode of growth

makes them rapid and efficient colonisers of virgin surfaces. In contrast, the clumps-associated pioneering organisms (Fig. 3-B [chapter 4] and S4 [chapter 5]) appear to be trapped mainly in the first part (Fig. 2, chapter 4]) of the RO membrane module, most likely due to filtering of the spacer, as discussed in chapter 4. In time, these microorganisms grow in pillar-like structures and slowly spread throughout the whole membrane module on top of the established sphingomonads film (Fig. 4-6, chapter 4). During this process it was observed that the secondary colonisers - bacteria (i.e., *Deltaproteobacteria, Firmicutes, Nitrospira, Planctomycetales, Verrucomicrobia, Cytophaga-Flexibacter-Bacteroides* and *Alpha-, Beta-* and *Gammaproteobacteria*) and eukaryotes (Fig. S1 [chapter 4] and 6 [chapter 5]) - occur in the resulting biofilm structures.

The proliferation of *Proteobacteria* at the fresh and cleaned RO membrane and feed-side spacer surfaces and their prevalence in the free-floating as well as in the biofilm-associated microbial communities in different compartments of a full-scale RO plant is not surprising. *Betaproteobacteria* appear to be dominant in freshwater systems and *Alphaproteobacteria* – in marine waters as a free-floating population (25). In contrast, *Cytophaga, Planctomyces* and *Gammaproteobacteria* appear to be associated with the macroaggregates in the marine environment (26). The remarkable dominance of *Proteobacteria* in RO systems suggests that RO conditions exert a selection pressure that favours the growth of organisms physiologically adapted for survival under low-nutrient conditions. Accordingly, the predominant bacterial species capable of handling these conditions appear to be members of the genus *Sphingomonas* (chapters 2 – 5, ref. 5), which are known to thrive in low-nutrient habitats, biofilms and on different surfaces (13-15, 27-30). According to the research reported to date, these organisms are exceedingly widespread and numerically abundant in nature primarily due to their physiological and metabolic versatility. Results of this (chapters 4 and 5) and previously reported (31, 32) studies indicate that the widespread distribution of sphingomonads in natural and engineered systems may also be the result of their remarkable ability for producing large amounts of EPSs under oligotrophic conditions. Formation and accumulation of exopolymeric substances is currently recognized as the major cause of disturbed performance in membrane systems. This requires further research on sphingomonads cell wall and EPSs (chapter 5, ref. 15, 33-35).

Although the composition of the biofilm microbial community clearly undergoes a succession in time, the architecture of an established mature biofouling layer appears to be rather stable (chapters 4 and 5). Cleaning of the RO membrane modules with chemicals did not lead to large changes in the biofilm morphology; a thin sphingomonads layer remained,

which forms the basis for other bacteria to easily attach and grow (chapter 5). The conventional cleaning agents appeared to mainly affect the bacteria in the top layers of the biofouling layer. The top layer disappeared from the film and only the deeper laying sphingomonads cells can be detected under the collapsed but obviously not removed biofilm EPS matrix (Fig. 2 and 5, chapter 5). Due to the killing of cells in the biofilm matrix and re-growth of a new microbial layer on its top, the basal EPS layer increases somewhat in time. The killing of cells during cleaning leads to the availability of substrates in the system and, consequently, the growth of a wider diversity of bacteria. Especially, the *Actinobacteria* seemed to profit from such additional substrates (Fig. 4, chapter 5). After cleaning the biofouling layer seemed to grow faster than a fresh biofilm. Without cleaning it takes around 16 days before biofilm becomes spread over the entire membrane surface (chapter 4), whereas after cleaning this happens already within 6 days of the system operation (chapter 5). Likely, this is related to the extra substrate supply derived from the dead cells and/or from chemicals, used in cleaning procedures (9). This observation appears to reflect a mechanism of survival adaptation of the oligotrophs and mesotrophs, with species variation as well as variation in characteristics being related to environmental conditions (e.g., degree of preventative maintenance which is being applied during the operation of the module, presence of spacer in a spiral wound module).

To conclude, the RO biofouling is a complex phenomenon with two appearances: a fouling layer on the membrane limiting the water flux through the membrane and a fouling layer on the spacer limiting the water flow through the spacer channel and giving an increased pressure drop over the membrane module. The fouling layer on the spacer might also lead to a non-uniform flow through the module resulting in parts of the membrane being a dead zone (i.e., no flow), which also may result in the decreased water fluxes.

Conclusions and Future prospects

The need for identification of robust and sustainable methods to manage biofouling problems in current generation of full-scale RO water treatment systems has led to renewed interest in the ways that microorganisms protect themselves against chemical cleaning and facilitate successful colonisation and establishment of biofouling layers on active surfaces within the associated RO membrane modules. The previously reported work in the field revealed evidence on presence of a variety of microbial organisms in RO membrane biofilms, however a thorough understanding of mechanisms associated with the occurrence, development and

survival of microbial populations in biofilms in such environment has not been emerged (chapter 1).

The data obtained in the present PhD study provide a consensus of knowledge and therefore - a good understanding of some microbiological features and principals involved in the formation and development of biofouling layers at available surfaces in a full-scale RO plant. New insight is obtained on the origin, succession and complexity of spatiotemporal development of biofouling layers under oligotrophic (fresh surfaces) and mesotrophic (chemically cleaned surfaces) conditions in a flowing, changing and high-pressure environment of a full-scale RO system. By monitoring of biofilm formation *in situ*, the impact of chemical cleaning on these processes could be elucidated to a great detail. Moreover, the correlation between microbial community composition, architecture and occurrence of biofouling problems (i.e., pressure drop increase) could be established.

The information obtained contribute essentially to our fundamental understanding of *in situ* bacterial biofilm survival. The true representation of the phenomenon forms essential foundation for development of new biofilm and/or biofouling studies. The multifaceted evaluation of the ecologically relevant samples by use a combination of complementary and well-established molecular and microscopic analyses and monitoring of biofilm appearance *in situ*, as it was applied in the present study, may be a very usable approach also for the examination of biofilms and biofouling in other membrane-based water treatment systems.

The knowledge emerged is also of significance for the development of new, robust en effective strategies for prevention, control and/or management of biofouling occurrence in membrane-based water treatment facilities. Accordingly, it became clear that cleaning strategies should focus more on the removal of the accumulated biomass and not only on killing it. Moreover, the basal *Sphingomonas* layer requires further research to appropriately control biofouling in RO systems, while the feed water pretreatment measures have to be focused on the improvement of flocks removal. It might also be possible to design the RO - membrane module in a different manner, leading to a different biofilm morphology which gives less rise to operational problems. It must be remembered, however, that bacteria are opportunistic organisms and controlling one bacterial species or even one bacterial group may open up niches for successful colonisation by less well known and/or examined species and/or groups.

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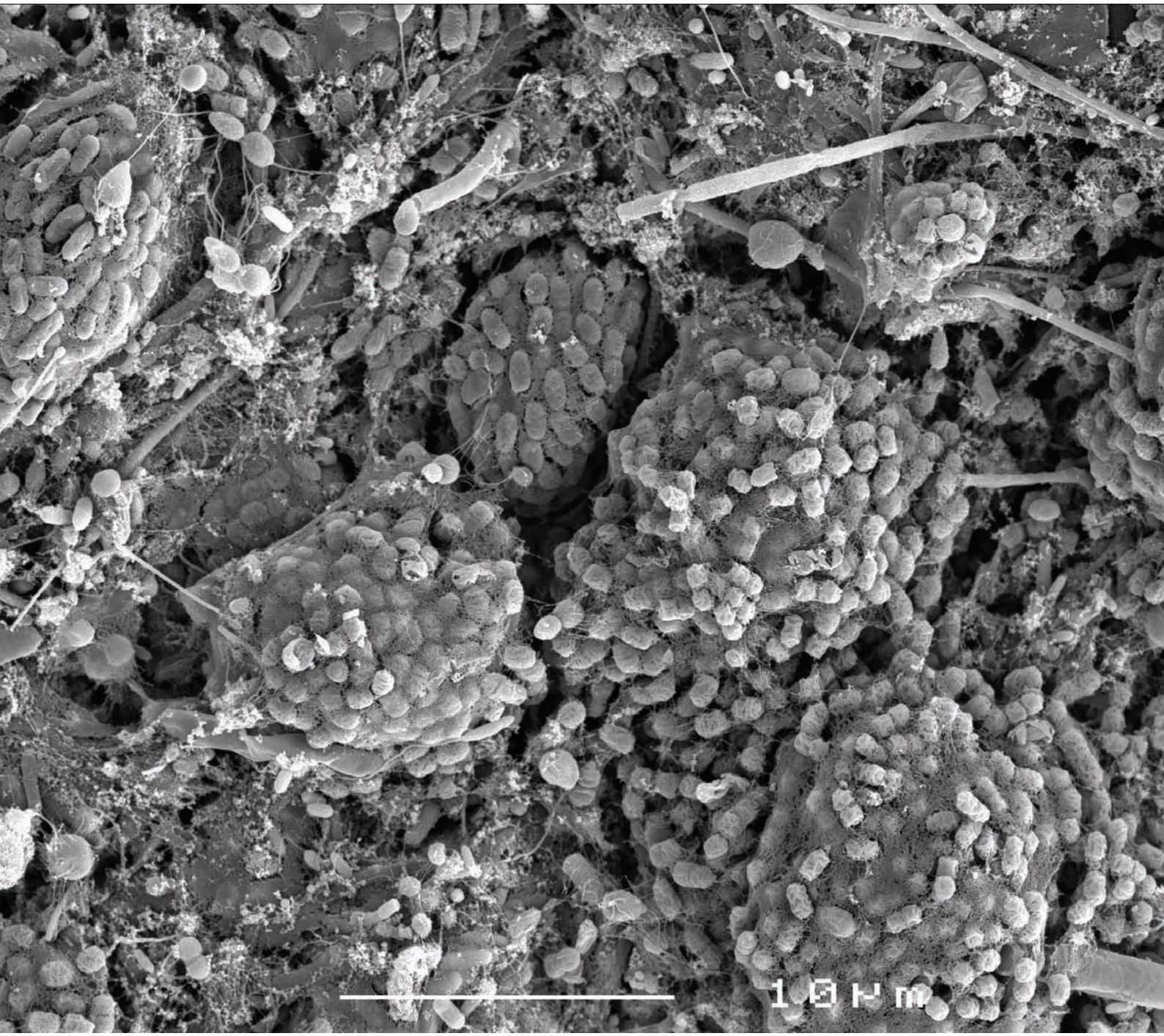
Summary & Samenvatting



SUMMARY

Reverse osmosis systems play an important role in removal of a wide variety of contaminants from water streams of different quality. Maintenance and operational costs of such systems are affected by biofouling, a process in which bacteria attach to membranes and support structures and develop into a thick film that can choke the entire RO system. Management of this problem requires basic understanding of the mechanism of this biological phenomenon. Essential questions that need to be answered are: which organisms are involved, how do they interact to form the films, can anything be done to avoid attachment of such organisms and establishment of the films. If biofouling cannot be prevented we should try to manage the system with a minimal impact? The basic questions of this PhD research project therefore addressed the origin, succession and spatiotemporal development of microbial biofilms in full-scale RO systems, in particular in relation to operational aspects of the system itself, that is, when biofouling affects the engineering aspect of the system by influencing performance parameters (chapter 1). To find the answers, a multifaceted research strategy involving the acquisitions of representative samples and the use of many molecular and microscopic analysis techniques in parallel was employed (chapter 6). The sampling procedure and sampling analysis are crucial to understand the biofouling phenomenon within a full-scale water treatment plant. An inappropriate sampling approach provides only a snapshot of free-floating population or surface-associated microbial consortia and a limited gain in knowledge of their actual structural complexity (chapter 6). The bacteria that form the biofilms in a full-scale RO system originated from the water fed to the system (chapters 2, 3 and 5). In the water phase of different stages of a full-scale RO plant, they occur as clumps or single cells (chapters 4 and 5). A large fraction of these cells appear to be dormant and are just transported by the flowing water (chapter 4). The biofilms grow on any surface in an RO water treatment facility (chapter 3). This gives local niches for growth and detachment of biomass either as single cells or cell clumps, spreading bacteria to the further stages of the RO plant. In the membrane modules of RO system (chapters 2 and 3), the enriched bacteria might more easily colonise the surfaces since they will be better adapted to growth in the system than bacteria present in the feed water. The colonisation pattern is rather distinct (chapters 4 and 5). Initially, a layer formation by the single cell colonizers (sphingomonads) and accumulation of flocculated material (mainly *Beta*- and *Gammaproteobacteria*) on the RO membrane and feed-side spacer surfaces occurs. The sphingomonads quickly spread over the membrane and feed-side spacer surfaces. Within 4 days of system operation, they mainly

develop in a flat monolayer of the abundantly EPS-embedded cells. This mode of growth makes them rapid and efficient colonisers of available surfaces. In contrast, the clumps-associated pioneering organisms appear to be trapped mainly in the first part of the RO membrane module, most likely due to a filtering action of the spacer. In time, these microorganisms develop in a certain pillar-like structures and slowly spread throughout the whole membrane module on top of the established sphingomonads biofilm. During this process it was observed that the secondary colonisers (bacteria and eukaryotes) occur in the resulting biofilm structures. Although the composition of the biofilm microbial community undergoes a succession in time, the architecture of an established mature biofouling layer appears to be rather stable. Cleaning of the RO membrane modules with chemicals did not lead to large changes in the biofilm morphology; a thin sphingomonads layer remained, which form the basis for other bacteria to attach and grow (chapter 5). The conventional cleaning agents appeared to mainly affect the bacteria in the top layers of the biofouling layer. The top layer disappeared from the film and only the deeper laying sphingomonads cells can be detected under the collapsed but obviously not removed biofilm EPS matrix. Due to the killing of cells in the biofilm matrix and re-growth of a new microbial layer on its top, the basal EPS layer even increases somewhat in time. The killing of cells during cleaning leads to the availability of extra substrates in the system and, consequently, the growth of a wider diversity of bacteria. Especially, the *Actinobacteria* seemed to profit from such additional substrates. After cleaning the biofouling layer seemed to grow faster than a fresh biofilm. Without cleaning it takes around 16 days before biofilm becomes spread over the entire membrane surface (chapter 4), whereas after cleaning this happens already within 6 days of operation (chapter 5). Likely, this is related to the extra substrate supply derived from the dead cells. To conclude, the RO biofouling is a complex phenomenon with two appearances: a fouling layer on the membrane limiting the water flux through the membrane and a fouling layer on the spacer limiting the water flow through the spacer channel and giving an increased pressure drop over the membrane module (chapter 6). The fouling layer on the spacer might also lead to a non-uniform flow through the module resulting in parts of the membrane being a dead zone (i.e., no flow), which also may result in the decreased water fluxes. It became clear that cleaning strategies should focus more on the removal of the accumulated biomass and not only on killing of cells. Moreover, the basal *Sphingomonas* layer requires further research to appropriately control biofouling in RO systems. It might also be possible to design the RO - membrane module in a different manner, leading to a different biofilm morphology which gives less rise to operational problems.

Samenvatting

Omgekeerde osmose (RO) systemen spelen een belangrijke rol bij het verwijderen van vele verontreinigingen uit verschillende typen waterstromen. Operationele kosten en onderhoudskosten van deze systemen worden in belangrijke mate beïnvloed door biofouling, een proces waarin bacteriën zich aan membranen hechten en ontwikkelen tot een dikke laag die het hele RO systeem kan blokkeren. Beheersing van dit probleem vereist fundamentele kennis van het biofoulingsproces. Cruciale vragen die beantwoord moeten worden zijn: welke organismen zijn betrokken, welke interactie treedt op bij het vormen van de biofilm, is er een mogelijkheid om hechting van deze organismen en de vorming van de biofilm te voorkomen. Wanneer biofouling niet voorkomen kan worden, dan is het zaak het probleem zo efficiënt mogelijk te beheersen. De fundamentele vragen van dit onderzoek waren daarom gericht op de vorming en ontwikkeling (in ruimte en tijd) van microbiële biofilms in full scale RO systemen. Het ging met name om de operationele aspecten van het systeem zelf, dat wil zeggen wanneer biofouling, door het beïnvloeden van de bedrijfsvoering, gevolgen heeft voor het functioneren van een installatie (hoofdstuk 1). Om antwoorden te vinden werd een uitgebreide onderzoekstrategie toegepast voor het verkrijgen van representatieve monsters en voor het gebruik van een aantal complementaire moleculaire en microscopische analysetechnieken (hoofdstuk 6). De procedure van bemonstering en analyse van het monster zijn cruciaal om inzicht van het biofouling fenomeen van waterzuiveringsinstallatie te verkrijgen. Een benadering waarbij een momentopname van de vrij-zwemmende of van de aan het oppervlakte gehechte microbiële populatie bestudeerd wordt geeft een beperkt beeld van de structurele complexiteit van biofouling (hoofdstuk 6). De bacteriën die de biofilm vormen in full scale RO systemen, waren afkomstig uit het voedingwater van het systeem (hoofdstukken 2, 3 en 5). In de water fases van de verschillende compartimenten van de RO installatie, kwamen ze voor als vlokjes of als enkele cellen (hoofdstukken 4 en 5). Een groot deel van deze cellen bleken dormant te zijn en werden enkel vervoerd met het stromende water (hoofdstuk 4). De biofilms groeien op elk oppervlak in de RO installatie (hoofdstuk 3). Dit geeft de lokale niches voor de hechting, groei en loslaten van biomassa, hetzij als losse cellen of cellen in vlokjes, zodat de bacteriën doorstromen naar verdere compartimenten van de RO installatie. In de membraanelementen van een RO systeem (hoofdstukken 2 en 3), kunnen de opgehoopte bacteriën zich gemakkelijker gaan koloniseren aan oppervlakken, omdat zij zich beter aanpassen aan de groei in het systeem dan de bacteriën die aanwezig zijn in het voedingswater. Het patroon van de vorming van kolonies is nogal divers (hoofdstukken

4 en 5). Eerst treedt de vorming van een laag van de eencellige bacteriën (sphingomonaden) op en daarna de accumulatie van ***geflocculeerde*** materiaal (vooral *Beta-* en *Gammaproteobacteriën*) op het RO membraan en het voedingsspaceroppervlak. De sphingomonaden verspreiden zich snel over het membraan en spaceroppervlak. Binnen 4 dagen na de opstart van een systeem, ontwikkelen ze zich tot een vlakke monolaag met EPS (extracellulaire polymere substanties) tussen de cellen. Deze groeiwijze maakt ze snelle en efficiënte kolonisatoren van de beschikbare oppervlakken. De pioniers die vlokvormig groeien lijken daarentegen met name in het eerste deel van het RO membraanelement te worden afgevangen, waarschijnlijk als gevolg van de filtrerende werking van de spacer. In de loop van tijd ontwikkelen deze micro-organismen zich tot kolomvorige structuren, die zich langzaam over het gehele membraan element verspreiden bovenop de al aanwezige sphingomonas biofilm. Tijdens dit proces is ook waargenomen dat zich secundaire kolonievormers (bacteriën en eukaryoten) ontwikkelen in de biofilm structuren. Hoewel de samenstelling van de microbiële populatie in de biofilm een successie ondergaat, blijkt de architectuur van een volgroeide biofouling laag tamelijk stabiel te zijn. Reiniging van de RO membraanelementen met chemicaliën leidt niet tot grote veranderingen in de biofilm morfologie; een dunne sphingomonads-laag blijft namelijk achter, die weer de basis vormt voor andere bacteriën om te hechten en te groeien (hoofdstuk 5). De conventionele reinigingsmiddelen blijken vooral de bacteriën in de bovenste lagen van de biofilm aan te pakken. De bovenste laag van de film is verdwenen en alleen de dieper liggende *Sphingomonas* cellen kunnen nog gedetecteerd worden onder de ingeklapte maar niet verwijderde EPS structuur in de biofilm. Door het afdoden van cellen in de biofilm matrix en groei van een nieuwe microbiële laag daar bovenop, neemt de dikte van primaire EPS-laag nog enigszins toe in de tijd. Als gevolg van het afdoden van cellen tijdens het reinigen komt extra substraat beschikbaar in het systeem, hetgeen resulteert in groei van een grotere diversiteit aan bacteriën. Vooral de *Actinobacter**iën*** lijken te profiteren van die extra substraten. Na het reinigen lijkt de biofouling laag sneller te groeien dan een initiële biofilm. Zonder reinigen duurt het ongeveer 16 dagen voordat de biofilm zich verspreid heeft over het gehele membraanoppervlak (hoofdstuk 4), terwijl dat na een reiniging al binnen 6 dagen geschiedt (hoofdstuk 5). Waarschijnlijk houdt dit verband met het extra substraat dat beschikbaar is gekomen uit de afgedode cellen. Geconcludeerd kan worden dat RO biofouling een complex fenomeen is met twee verschijningsvormen: een fouling laag op het membraan oppervlak die de flux van het water door het membraan beperkt en een fouling laag op de spacer die weerstand biedt aan de waterstroom door het voedingskanaal en daardoor een

verhoogde drukval over het membraan element veroorzaakt (hoofdstuk 6). De fouling laag op de spacer kan ook leiden tot een ongelijkmatige stroming en een verlaagde waterflux. Het is duidelijk geworden dat de reiniging strategieën zich dienen te richten op het verwijderen van de opgehoopte biomassa en niet alleen op het afdoden ervan. Bovendien, vereist de vorming van de sphingomonas-laag verder onderzoek om te komen tot een adequate controle van biofouling in RO systemen. Het is misschien ook mogelijk om de RO membraanmodule op een andere manier te ontwerpen, met als doel een andere biofilm morfologie te bereiken, hetgeen minder aanleiding zal geven tot operationele problemen.

About the author

Ludmila Bereschenko was born on 2th Agustus, 1963 in Cherson, Ukraine. Her curiosity for the amazing world of A. Van Leeuwenhoek's minuscule “dierkens” was initiated by her father Anatolij P. Bereschenko. During her study and graduation in Microbiology at the Kyiv University, Ukraine (1981-1986) this curiosity was transformed in a profound admiration for the barely visible dimension and its scientific investigation. This admiration emerged once again as she “submerged” into the world of DNA technology as a researcher at Microscreen, a molecular microbiology laboratory in Groningen, The Netherlands. Three years later she dove deeper into the watery world of microbiological research as Head of laboratory at Paques Bio Systems, a water purification laboratory in Balk, The Netherlands. At the age of 41, she took the opportunity to realize a long-held dream of reaching the honored academic degree of *Philosophiæ Doctor*. She accepted an assignment on a PhD project entitled “Biofilm development on new and cleaned membrane surfaces”, which project was a joint venture between the Technological Top Institute for Water Technology “Wetsus” in Leeuwarden, the Microbiology Department of the Wageningen University, the Environmental Biotechnology Department of the Delft University of Technology, and a number of Dutch private companies in the field of water treatment. This project resulted in a number of peer-reviewed scientific publications and, ultimately, this thesis.

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Oral Presentations

- Biofilm Systems VI, 24-27 September 2006, Amsterdam, The Netherlands
- Symposium WRCD: theme Asia, 16 January 2007, Delft, The Netherlands
- Sensible Water Technology (SENSE Symposium), 12-13 April 2007, Leeuwarden, The Netherlands
- IWA Biofilm Technologies Conference, 8-10 January 2008, Singapore, Singapore

SENSE Coordinator PhD Education and Research

Mr. Johan Feenstra

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