Preface

This thesis is the result of the research work I carried out in the period of 2009 -2010 at the Department of Biotechnology and the Department of Hydraulic and Environmental Engineering, Norwegian University of Science and Technology (NTNU). Over these years, many people have accompanied and supported me. I would like to express my sincere gratitude to everyone who has made this work possible.

Particularly, I would like to thank my supervisors Professor Liv Fiksdal, Professor Kjetill Østgaard and Professor TorOve Leiknes for good advice, support, feedback, help and encouragement. A special thanks to Professor Live Fiksdal for giving me the opportunity to present my work on the Water Research Conference in Lisbon, Portugal.

I am grateful to PhD Cheng Sun for good supervising with the experimental set up, planning the experiments, and operating the experiments when I was busy with lab work. I will also thank Professor Olav Vadstein and Ingrid Hjort for good support, help and encouragement.

Several people have given me assistance with the work in this thesis. I thank PhD Ingrid Bakke for teaching me DGGE, and for good support and help. I thank Gøril Thorvaldsen and Trine Håberg for assistance in the laboratory, and Arne Grostad for assistanc with the experimental set up.

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I will also like to thank the PhD students whom I sheared office with. Thanks for help, support and discussions both professionally and socially.

This thesis would never have been complete without the support from Torstein Tønnessen. Thanks for your support and for always being there. Finally, I will give a special thanks to my mother and my father for your kind support and confidence to my future.

Abstract

NF membrane processes have the ability to produce drinking water of high quality, and may be an important tool for solving the increasing demand for drinking water in the world. However, membrane biofouling is one of the major drawbacks, resulting in higher operating cost and lower filtration efficiency. Therefore controlling the biofouling is of high interest.

The aim of this work was to investigate the feasibility of molecular methods applied to identify and quantify biofouling of NF membranes, and to compare different microbial communities exposed to different operating conditions. The conditions to be explored include different operating modes, *e.g.* crossflow and dead-end, feed water qualities, *e.g.* tap water and surface water, and operating time. The experiments were preformed on a lab-scale NF-membrane module. The molecular techniques, DGGE and FISH, were tested and optimized.

In this thesis, the molecular techniques were targeting the 16s ribosome RNA to analyze whole microbial communities. With DGGE all the bacterial species from each sample were separated on a gel, due to small differences in their target gene. With FISH the chosen bacteria species was quantified with CLSM and ImageJ software, to investigate the community dynamic.

Using molecular methods for characterizing the biofouling of NF membranes was shown to be feasible. DGGE and FISH managed to identify and quantify biofouling, and the results was used to compare different microbial communities exposed to different operating conditions.

The DGGE results showed that the type of feed water had the largest effect on the communities, even though the measured water qualities and the flux decline (fouling rate) was similar. The communities on the membrane operated for the same time, with different feed waters (tap, surface and pre-treated surface water) showed large differences in the DGGE pattern. The choice of pre-treatment of the water is therefore an important parameter that is crucial to investigate, and may be used for optimizing the full scale NF plants in the future.

The effect of operating mode (dead-end *vs.* crossflow) had larger effect for the experiments operated with tap water, than the experiments operated with surface water. The operating time

was not found to have a large effect on the bacteria structure. There were no large resemblance between the bacteria in the water, and the bacteria in the early biofilm formation.

Combination of FISH, CLSM and the software ImageJ, a quantification of the relative proteobacteria was obtained. FISH were suitable for detection of changes for the chosen bacteria under different operating conditions.

The fouling on the NF system was rapidly formed, and the flux decline was the highest during the first two days of operation, and was not affected by the feed water quality. By use of a cleaning solution a higher flux was maintained, but despite of less fouling the bacteria structure seemed to have relatively high species richness and diversity.

The molecular methods were investigated and showed that

- The choice of extraction kit could affect the community DNA results with different community structures.
- Large PCR products (>500 bp) do not manage to penetrate the gel in the INGENY phorU DGGE system.
- The optimal denaturing gradient in this theses were between 35 - 55 %.

The lab-scale NF-membrane module has shown to be a reproducible system, after comparison of two microbial community patterns from two membranes operated under the same conditions.

The results of the thesis has been represented as a poster on the Water Research conference (from 12-14 may) in Lisbon, as well as a oral presentation on the workshop to the Safe water conference (14 may) in Sintra, Portugal.

Abbreviation

CLSM – Confocal laser scanning microscope

DGGE – Denaturing gradient gel electrophoresis

EPS – Extracellular polymeric substances

FISH – Fluorescent *in situ* hybridization

HF – Hollow-fiber

HS – Humic substances

IBET– Instituto de Biologia Experimental e Tecnológica, Lisboa Portugal

MBR – Membrane bioreactor

MF – Microfiltration

NDP – Normalized pressure drop

NF – Nanofiltration

NOM – Natural organic matter

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PFA – Paraformaldehyde

RO – Reverse osmosis

rRNA – Ribosomal ribonucleic acid

SW – Spiral-wound

THM – Trihalomethane

TMP – Transmembrane pressure

TOC – Total organic carbon

UF – Ultrafiltration

VIVA – Vikelvdalen vannbehandlingsanlegg

Contents

1. Introduction

1.1 Membrane filtration

1.1.1 Membrane technologies

Membrane treatment of water has existed since the 1960s, and there has been a tremendous development until now (Hendricks, 2006, Horsch *et al.*, 2005, Delong *et al.*, 1989). Today membrane technologies are used in a wide area, from basic removal of particles from wastewater, to desalination of seawater. The relatively low cost compared to other technologies is the main reason for the great development and wide use of membrane technology (Hendricks, 2006).

The membranes can be divided into four groups due to the pore size; microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) (Hendricks, 2006). Figure 1.1 gives an illustration of the differences in pore sizes. Most of the membrane technologies have been developed for wastewater treatment, but the technology has been adapted to treatment of drinking water.

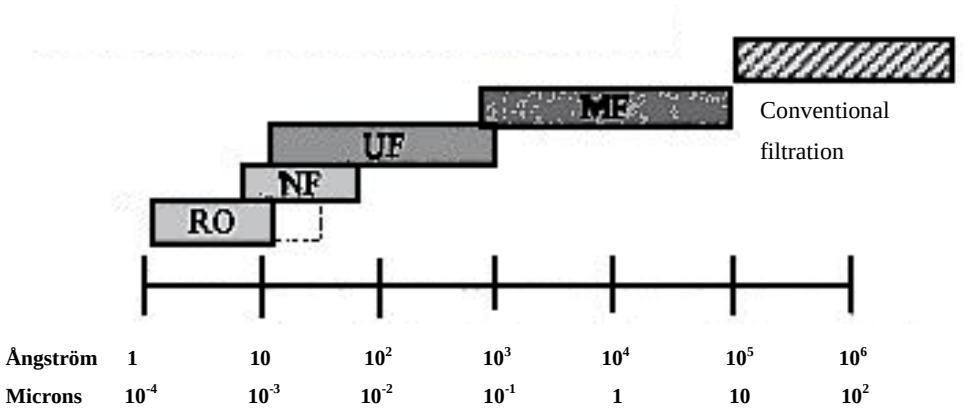


Figure 1.1: Membrane definitions (Leiknes, 2008)

The development has created a large variety of modules available on the market. The spiral-wound (SW) and the hollow-fiber (HF) modules shown in Figures 1.2 and 1.3 are the most commonly used. The SW module is most popular when using RO, NF and UF membranes, whereas the HF module is used in UF and MF applications (Sing, 2006).

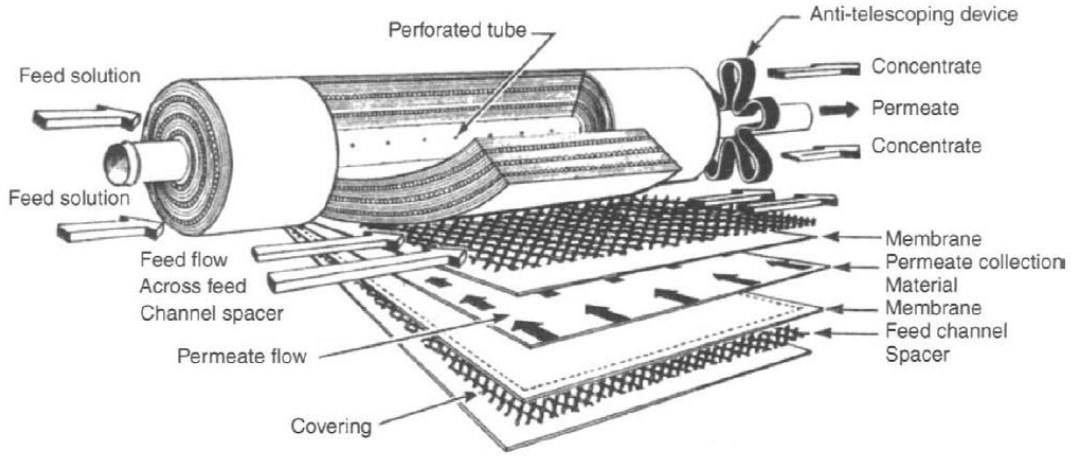


Figure 1.2: Spiral-wound membrane module (Sing, 2006)

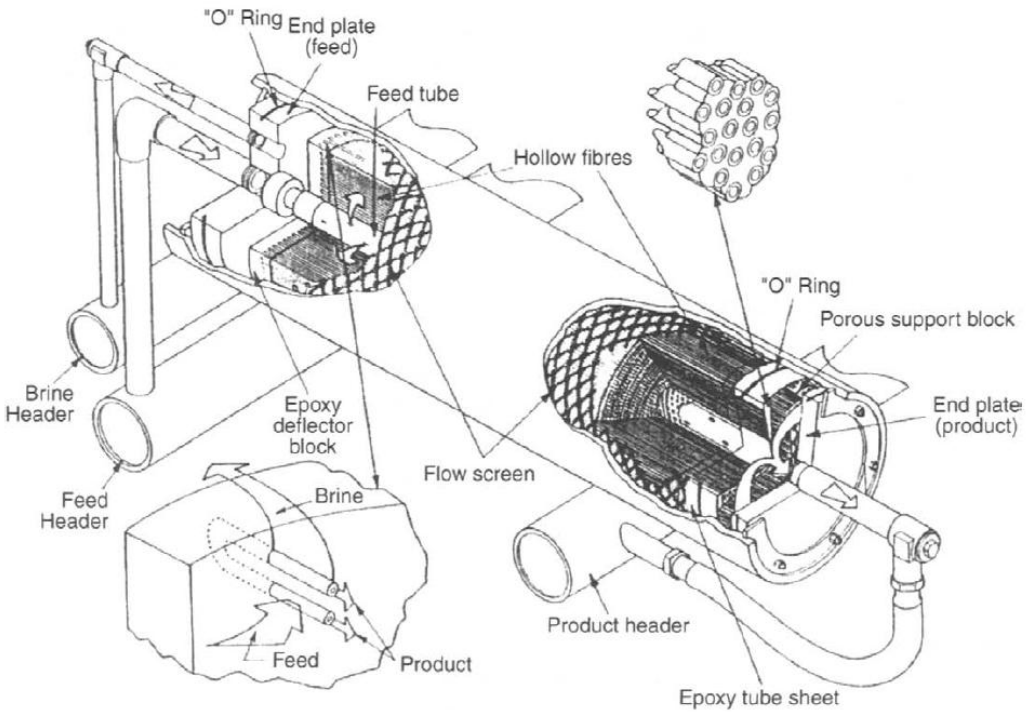


Figure 1.3: Hollow fiber RO membrane module (Sing, 2006)

Membrane processes, such as NF have the ability of producing drinking water of high quality. The demand for drinking water in the world is increasing, and regulations of drinking water quality become stricter (Shannon *et al.*, 2008). Not only the demand of water, but the need for clean water becomes crucial. The removal of pathogenic microorganisms and organic and inorganic pollutants is not only important for the human health, it is also important to do this in a way which will be gentle to the environment, *e.g.* membrane filtration, where no chemicals are included in the treatment.

1.1.2 Membrane fouling

Membrane fouling is used to describe loss of membrane function. This could be due to pore plugging or external pore blocking caused by deposition of colloids and particles on the surface as well as in the membrane pores, leading to a decline in flux and increased pressure drop across the membrane (Ramesh et al., 2006).

Almost all substances in the water can cause or contribute to fouling. Factors to be considered are particles, dissolved organic and inorganic compounds, as well as biofilm growing on the membrane surface. The particles and dissolved compounds in the water will cause fouling when it accumulates on the membrane surface, when it is embedded within the membrane, and when it causes changes in the chemical character of the membrane (Hendricks, 2006).

In a fouling cycle, the flux decreases with time due to fouling, followed by partly restoration by cleaning. There are three different fouling concepts to consider in a fouling cycle; a) total fouling, which is the overall loss of flux, b) reversible fouling which is the part that may be restored by cleaning, and c) irreversible fouling, which cannot be restored. For SW membranes, often used in NF treatment of drinking water, the optimal time between cleaning cycles depends on the raw water quality, which in turn depends on pretreatment such as use of cartridge filters (Hendricks, 2006).

Natural Organic Matter (NOM) may be a significant cause of fouling. The main NOM component in Norwegian water is humic substances (HS). Because of the large size of humic substance molecules (MW 1000-100 000), it is possible to separate HS directly from water by molecular sieving through a sufficiently tight membrane such as NF(Ødegaard *et al.*, 2009).

1.1.3 Nanofiltration

Nanofiltration (NF) of drinking water was introduced in the late 1980s, mainly aiming at combining softening and organics removal (Eriksson, 1988). Since then, new applications have been developed for drinking water production, such as arsenic removal (Figoli et al., Waypa et al., 1997, Kosutic et al., 2005), removal of pesticides (Kosutic et al., 2005), and partial desalination (Diawara, 2008). During the last decade, the use of NF has expanded both in wastewater and drinking water treatment, due to factors such as the growing demand of water with high quality, the growing pressure to reuse wastewater, better reliability and integrity of the membranes, lower price and more stringent standards in the drinking water industry (Van Der Bruggen *et al.*, 2008).

Different membrane processes such as RO and NF can produce water of high quality. NF is closely related to RO, and was developed because there was a need for filtration of high quality, but not as high as for RO, and with lower cost. NF membranes have a surface charge due to ionisable groups, *e.g.* carboxylic or sulphonic acid groups. Ions larger than the pore size are rejected because of Donnan exclusion, that is the electrical potential between the charged membrane and the bulk solution (Sing, 2006).

The traditional material used for NF membranes are organic polymers. Most NF membranes are packed into spiral-wound elements, but tubular, hollow fiber and flat-sheet modules are also in use.

1.1.4 NF plants in Norway

The first full-scale Norwegian NF plant was put into operation in 1990 (Ødegaard et al., 2009). Today, more than 100 NF plants are in operation, and the technology has become commonplace. All the plants in Norway are based on spiral wound modules, and the majority with cellulose acetate membranes. The pore size of the membrane is typically 1-5nm, and is operated with a pressure between 4-8 bar (Ødegaard et al., 2009). Typical flow diagram of a NF plant is showed in Figure 1.4.

The raw water passes through a pre-filter with a sieve opening of 50 µm, to remove the largest particles before the NF. The membranes are run with a crossflow, which is a flow passing over the membrane, to remove the loose particles and maintain the pressure. The water flow pressed through the membrane is denoted the permeate, and will be free of all particular material that are larger than the pores. An alkaline calcium carbonate filter is often included in order to increase the level of calcium and bicarbonate in typically soft and corrosive Norwegian waters, before the water is transported to the distribution network (Ødegaard et al., 2009).

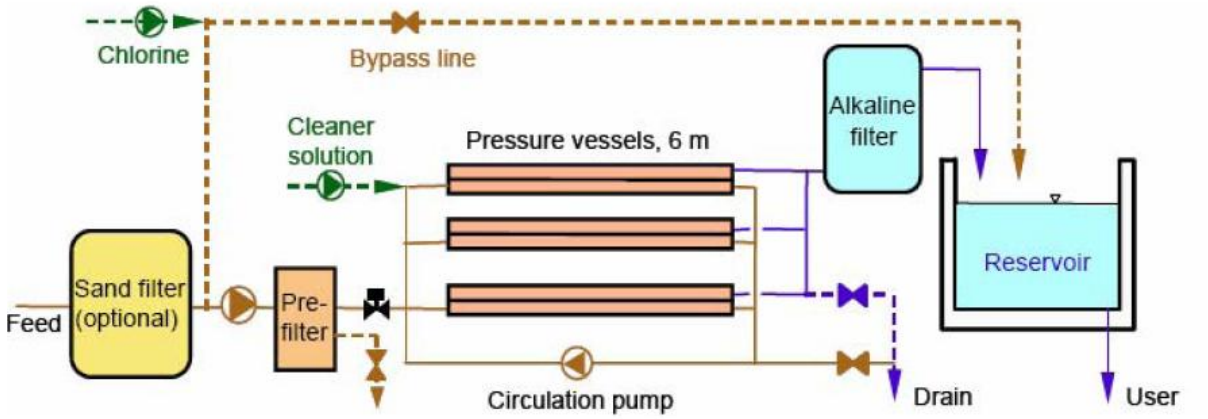


Figure 1.4: Flow diagram of a NF plant (Ødegaard et al., 2009).

The common practice of membrane cleaning in Norway today is one daily chemical cleaning and one major cleaning once or twice a year, to avoid capacity reduction over time as a result of membrane fouling. The daily cleaning is preformed with a chemical solution usually recommended by the manufacturer of the membrane, and chosen on the basis of the quality of the water. It usually contains an oxidant such as chlorine for disinfection. The cleaning routine is performed during the night for 20-40 minutes (Ødegaard et al., 2009).

The NF process is often selected for NOM removal from drinking water when the NOM content/color is high (>30 mg pt/l), and the turbidity is low (< 1 NTU) (Ødegaard et al., 2009).

1.2 Biofouling

1.2.1 General aspects

The fouling caused by biota growing on the membrane surface is called biofouling. Unlike other types of fouling, good pretreatment of the water have little effect, and over time microbes will always invade and colonize the system, even if the nutrient concentration is low. By cell adhesion they may develop and form biofilms capable of scavenging nutrients. Effective prevention of microbial growth can be achieved if a continuous and sufficiently high chlorine concentration is maintained. This is not however an ultimate solution due to growing environmental concerns (Flemming and Schaule, 1992). Biofouling is considered to be the major fouling type in NF, and the fouling seems mainly to be caused by biofilm formation in the feed channel spacer (se figure 1.2) (Vrouwenvelder *et al*., 2009).

1.2.2 Biofilm

A biofilm forms when bacteria adhere to a surface and begin to reproduce. A mature biofilm composed of living, growing and reproducing microorganisms, as well as high molecular-weight extracellular polymeric substances (EPS), multivalent cations, biogenic, colloidal and inorganic particles, and dissolved compounds. It has a complex structure which protects and allows the microorganisms to grow. The microbial cells in the biofilm are held together by EPS, which mainly consist of polysaccharides and proteins, but other macromolecules such as DNA, lipids, and humic substances may also contribute. The EPS provide three-dimensional gel-like networks which protect the microorganisms from adverse environmental conditions, often enforced by divalent cations, among which Ca^{2+} plays an important role. It is estimated that more than 99 % of all microorganisms live in aggregates such as in biofilm and flocs (Wingender and Flemming, 1999).

The life cycle of a biofilm, as shown in Figure 1.5, may be divided into 3 phases; 1) attachment, 2) growth and 3) dispersal. In the attachment phase free-floating, or planktonic, bacteria encounter a submerged surface and become attached. The cells begin to divide, and increase the EPS amount by production and dead-cell debris. In the growth phase, the biofilm develop to a complex three dimensional structure, due to EPS, and bacterial growth. The structure is influenced by different environmental factors. In the dispersal phase the biofilm is

able to propagate through detachment of clumps of cells, or a type of seeding dispersal, which allows individual cells to detach. This enables the bacteria to reattach to a surface downstream of the original community (Cunningham et al., 2008).

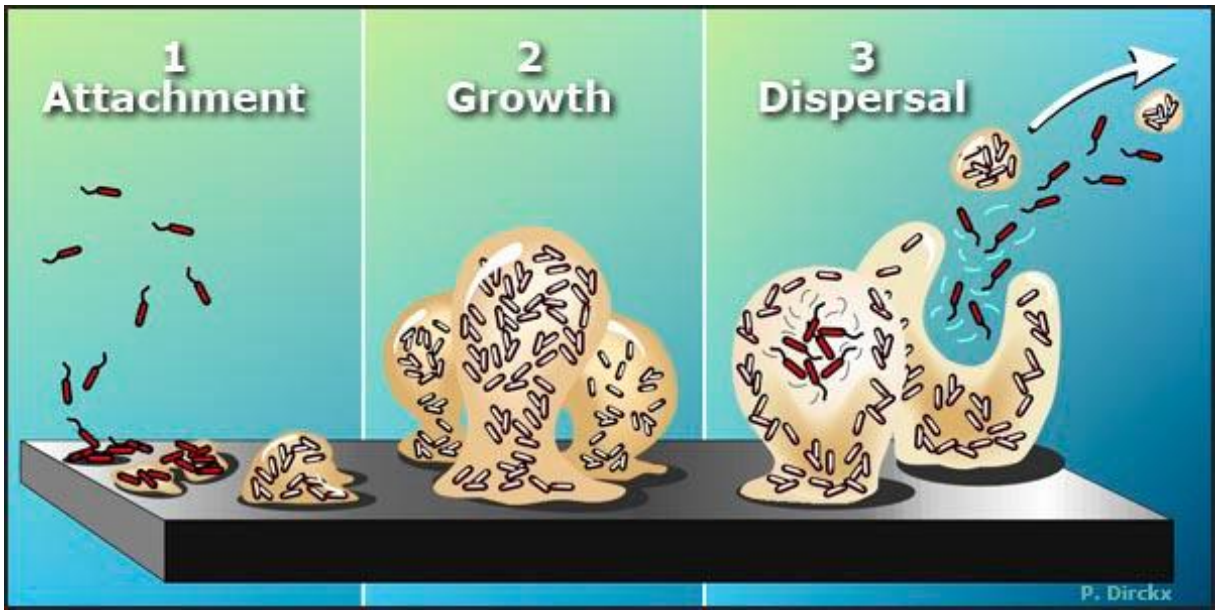


Figure 1.5: The biofilm life cycle; 1) Attachment, 2) Growth, 3) Dispersal (Cunningham et al., 2008)

The biofilm is a complex heterogenic structure. Once the nascent microcolony is established, cells of other species are recruited to the biofilm from the passing bulk fluid. This recruitment can be random or specific, some biofilm formations such as oral biofilm cells accumulating in dental plaque, develop in a very specific sequence. In addition to cell division and aggregation, the bulk of the biofilm is increased by the accumulation of nonliving materials, sludge, sand, and organics of many sorts. This will increase the mass, as well as the complexity of the biofilm, and may serve as structural elements as well as nutrient substrate (Cunningham et al., 2008).

According to Wingender and Flemming (1999) there is no such thing as a general biofilm model, as biofilms formed by various organisms under a wide range of conditions. A number of conceptual models exist for the structures of microbial biofilms (see Figure 1.6), reflecting the various biofilm phenomenon observed.

Among this models are the heterogeneous mosaic biofilm model, the water-channel mushroom-like model, and the dens confluent biofilm model. Examples of all these models can be found in nature, and the structure is largely determined by the prevailing substrate concentration and shear forces. Other factors that also contribute to the development, composition, and structure are summarized in table 1.1(Wingender and Flemming, 1999).

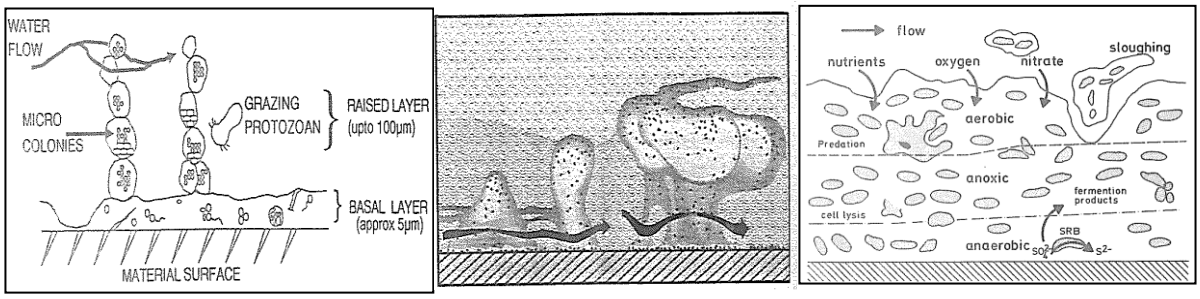


Figure 1.6: Conceptual models for biofilm structure; heterogeneous mosaic model (left), water-channel mushroom-like model (in the middle), and dens confluent biofilm model (right) (Wingender and Flemming, 1999)

Biofilm formation decreases the efficiency of the water treatment industry. The demand for controlling biofilm formation is substantial. Biofilm are remarkably resistant to antimicrobial reagents and several possible mechanisms have been proposed. Lack of penetration is not considered as a significant contributor to antimicrobial resistance. Scientists believe the resistance may be due to 1) degradation of the antimicrobial agent, 2) due to high heterogeneity and complexity of metabolically quiescent cells which repopulate the membrane when the microbial agents have killed the other species, 3) production of new proteins by cells in the biofilm, which help the cells to pump out the antibiotics to keep the concentration below a lethal level (Cunningham et al., 2008).

Table 1.1: Major factors that control biofilm development, composition and structure (Wingender and Flemming, 1999)

Surface properties of the substratum	*e.g.* roughness, hydrophobicity
Surface properties of the microorganisms	
Physicochemical conditions of the bulk liquid phase	*e.g.* pH, temperature, salinity
Concentration of available organic substrate	
Morphology of microorganisms	*e.g.* filaments
Physiological activity of microorganisms	
Lysis of biofilm organisms	
Grazing protozoa	
Activity of invertebrates	
Formation of gas bubbles	*e.g.* N_2, CH_4
Continuous detachment of small particles	(erosion)
Sporadic detachment of large fragments of biofilm	(sloughing)
Age of biofilm	
Adsorption of exogenous material from the bulk phase	
Hydraulic conditions	*e.g.* flow rate, shear stress
Presence of antimicrobial agents	

1.3 Molecular methods for biofilm detection

Vrouwenvelder *et al.* (2009) claims that the membrane fouling in drinking water systems with good pre-treatment is mainly caused by the biofouling. Studies of the microorganisms are therefore crucial for a better understanding of the fouling events in drinking water. Still, studies of biofilm growth on NF membranes in drinking water systems have been less explored than biofilms in membrane bioreactors (MBR) systems in wastewater treatment (Vrouwenveder et al., 2009). No studies, including denaturing gradient gel electrophoresis (DGGE), on biofouling characterization in NF drinking water systems have been founde. Only a few articles about fluorescent i*n situ* hybridization (FISH) have been found.

1.3.1 rRNA - based methods

The ribosome is a part of the protein synthesis system, and is therefore universal (Madigan and Martinko, 2006). They consist of two subunits, one small and one large, which is build up from ribosomal ribonucleic acid (rRNA), and proteins (se Figure 1.7).

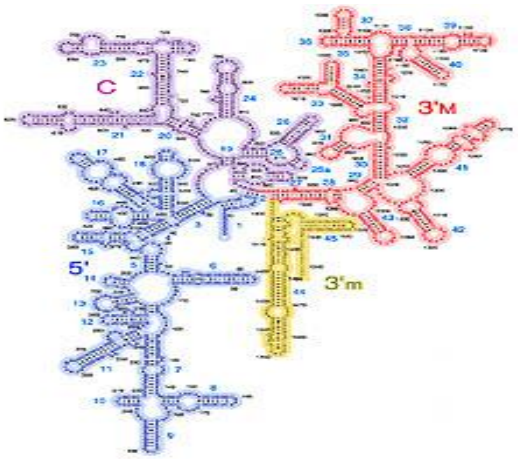


Figure 1.7: 16S rRNA of *Escherichia coli*.*(Madigan and Martinko, 2006)*

The rRNAs have signature sequences, which are short oligonucleotides unique to certain groups of organisms, *e.g.* signature sequences specific for each of the domains of cellular life are known (Archea, Bacteria and Eukarya). It also contains signature sequences defining specific groups within a domain or groups of organisms with the same functions, *e.g.* proteobacteria or ammonium oxidizing organisms. If the specific gene sequence is known, one may choose which hierarchy level to work with and then design or buy a commercial probe or primer, which fit the desired hierarchy. Ribosomes are naturally multiple in all cells, unlike DNA in chromosomes, which may give a amplified signal when using *in situ* methods (Madigan and Martinko, 2006).

1.3.2 PCR-DGGE

Denaturing gradient gel electrophoresis (DGGE) is a method for separating DNA molecules that may be copies of rRNA sequences. The technique combines gel electrophoresis with DNA denaturation. The samples are always products of the polymerase chain reaction (PCR) (Clark, 2005).

Extraction

When analyzing a microbial community, the total community DNA is extracted from samples with commercialized DNA extraction kits. The DNA obtained is a mixture of genomic DNA from all of the microorganisms originally presented in the habitat.

PCR

PCR is a method for amplifying DNA sequences. It is a sensitive method, which may start with DNA from only one cell, and amplify it into an amount which is sufficient for cloning or sequencing (Clark, 2005).

The DNA from the sample, specific primers, the enzyme DNA polymerase, and a supply of nucleotides are mixed together and placed on a PCR machine. The chosen primers; two nucleic acids, hybridizes to complementary sequences in the target nucleic acid, before the DNA polymerase copies the target gene, by adding new complementary bases (Figure 1.8). An increase in temperature denaturizes the new double-stranded nucleic acid, and the polymerase can once more copy the target gene. The amplification takes action through a series of identical temperature cycles. After each cycle, the DNA is doubled. It is therefore ideally an exponential amplification (Clark, 2005).

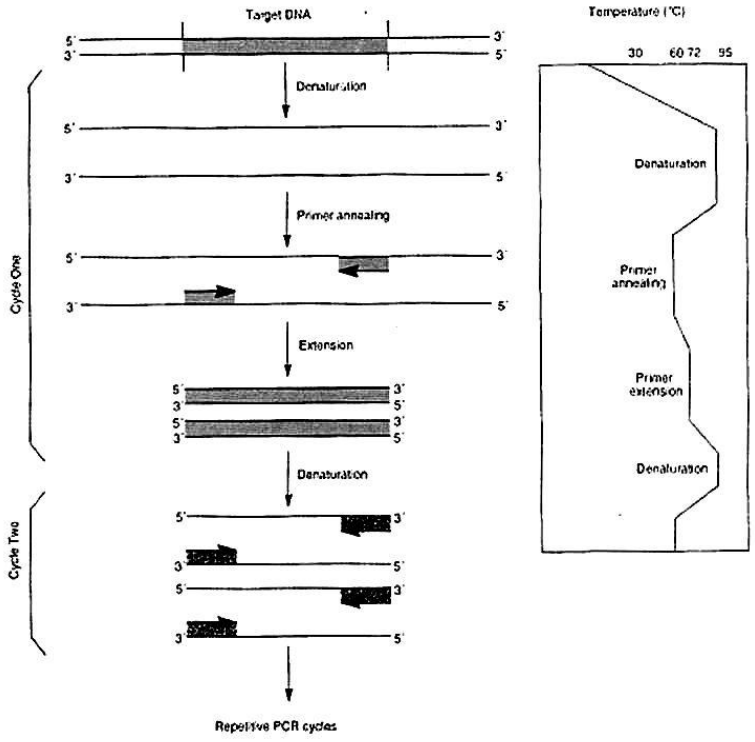


Figure 1.8: PCR for the amplification of a target gene sequence (Atlas and Bartha, 1986)

As mentioned earlier, when choosing primer the desired hierarchy level has to be chosen, and when analyzing a whole community, a commonly amplified gene is the one encoding the 16S ribosomal RNA. When analyzing a community, it is important to chosen primers that target all the different 16sRNAs in the sample, to include all the bacteria (Madigan and Martinko, 2006). In DGGE there is one forward and one backward primer, where one of the primers has a GC-rich sequence added, a so called GC-clamp on the 5´-end. The GC-clamp acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands when run on the DGGE gel (Muyzer and Smalla, 1998).

DGGE

Using a single set of primers on a microbial community in PCR, leads to a single gel band in a pure agarose gel containing amplified DNA fragment of the same size (Figure 1.9). This band contains many highly related but not identical sequences. The nucleotide sequence between the priming sites can vary as a result of evolutionary divergence, and an additional step is therefore required to separate the different species DNA (Madigan and Martinko, 2006).

DGGE is a gel electrophoresis method where separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (Muyzer and Smalla, 1998). The sequences of the same size differ in their melting or denaturing profile because of differences in the base sequences. The DNA denaturant, will denaturate the double stranded DNA when it reaches the region which contains sufficient concentration, which will make the migration to stop (Madigan and Martinko, 2006). Each band in the gel will therefore represent one version of the target gene from one bacteria species (se Figure 1.9).

Once DGGE has been preformed one may compare the band patterns from different samples, calculate diversity indexes, make clusters which will give the relatedness, or cut out individual bands from the gel, and send them in for sequencing.

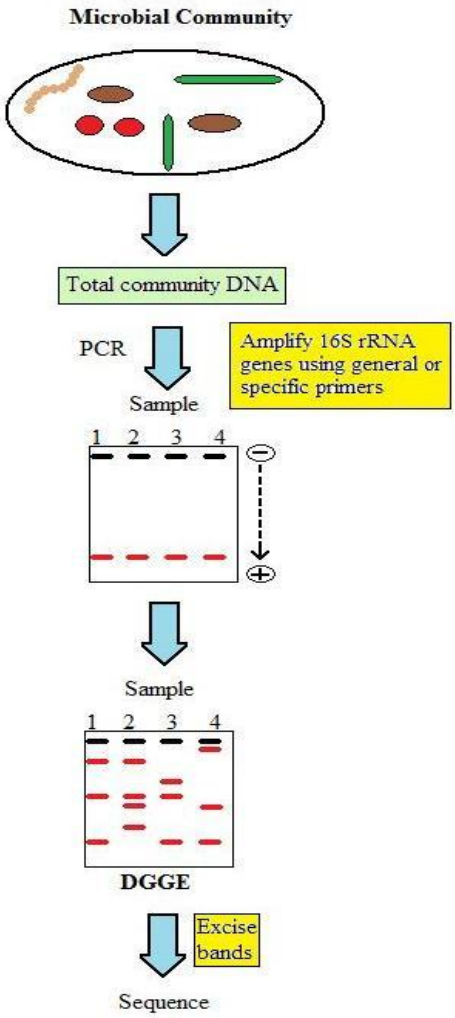


Figure 1.9: Procedure for analyzing community DNA, with PCR and DGGE

1.3.3 Analyzing the DGGE gel

Fingerprinting

Each band in the DGGE gel represents one variant of the chosen gene amplified by the PCR. If the 16sRNA were amplified, each band will represent single bacterial species. When comparing two samples, the differences in bacterial species may be observed, due to different band patterns as well as different intensity on the bands.

Shannon-Weaver diversity index

It may be easier to analyze the DGGE patterns of microbial communities, and to compare the result with research performed elsewhere, when calculating diversity indexes. The Shannon-Weaver diversity index is a widely used index when calculating the diversity. The index is

simply the information entropy of the distribution, treating species, in this case each band, as symbols and their relative population sizes, the band intensity, as the probability. The advantage of this index is that it takes into account both number of species and the evenness of the species. The index is increased either by having additional unique species, or by having a greater specie evenness (Krebs, 1989). Equation 1 shows the equation for calculating the Shannon-Weaver index for discrete distribution.

$$\mathbf{H` = -\Sigma\ P_i log[P_i]} \qquad \mathbf{(1)}$$

Where P_i is the abundance of a given subspecies in a lane divided by the total number of bands (k`), *e.g.* species, observed in that lane.
Using species richness (k`) and the Shannon-Wiener index (H`), one may also calculate a measure of evenness (shown in equation 2).

$$\mathbf{J` = H`/ln(k`)} \qquad \mathbf{(2)}$$

Evenness (J`) is a measure of how similar the abundances of different species are. When there are similar proportions of all species then evenness is one, but when the abundances are very dissimilar, some rare and some dominant species, then the value decrease (Rewhc, 2000).

Jaccard's Cluster analysis

When using resemblance coefficients, the matrix in Figure 1.10 is used for comparing two samples X and Y.

		Sample Y	
		1	0
Sample X	1	a	c
	0	b	d

Figure 1.10: Matrix for comparing two samples X and Y (Romesburg, 1984).

In the matrix, a is all the bands they have in common, b represent the bands Y but not X have, c is the bands X but not Y have, and d is the bands they do not have at all, in the total set.

Equation 3 shows the Jaccard similarity coefficient calculations.

$$J = a / (a+b+c) \quad (3)$$

The Jaccard coefficient indicates maximum similarity when the two samples have identical values at J = 1, while indicates maximum dissimilarity when there are no matches at J = 0 (Romesburg, 1984).

Sequencing

The bands may also be marked out, and sequenced. The sequences revile the species that is responsible for that band. Marking out bands may be difficult when whole communities is analyzed, the bands may be so dense that many of the bands composing of more than one versions of the gene (Madigan and Martinko, 2006).

1.3.4 FISH

Fluorescent *in situ* hybridization (FISH) is a molecular method for identifying the presence or absence of specific nucleic acids *in situ*. The method was first used in clinical studies, where probes were used to target chromosomes. In 1986 DeLong *et al* reported the use of fluorescently labeled rRNA-targeted oligonucleotide probes applied as phylogenetic stains for cultivation-independent identification of microorganisms for the first time. Since then, the method has been developing rapidly, and is now the method of choice for rapid identification of microorganisms in environmental and medical samples (Wagner *et al.,* 2003*)*. Since the method is *in situ* it will not change the sample composition, like the PCR where the DNA is copied after extraction. Instead, FISH will give a quantitative or qualitative picture of the specific bacteria present at a specific moment.

Principle

Bacteria are stained by using fluorescent dyes labeled probes. A probe is a strand of nucleic acid which is used to hybridize to a complementary nucleic acid, which can target general- or specific bacteria (Madigan and Martinko, 2006). The nucleic acid target is usual 16S rRNA or the 23S rRNA (Figure 1.11). The fluorescent dyes, also called fluorochromes, will excite in a specific light wave, and emit a specific color (Madigan and Martinko, 2006).

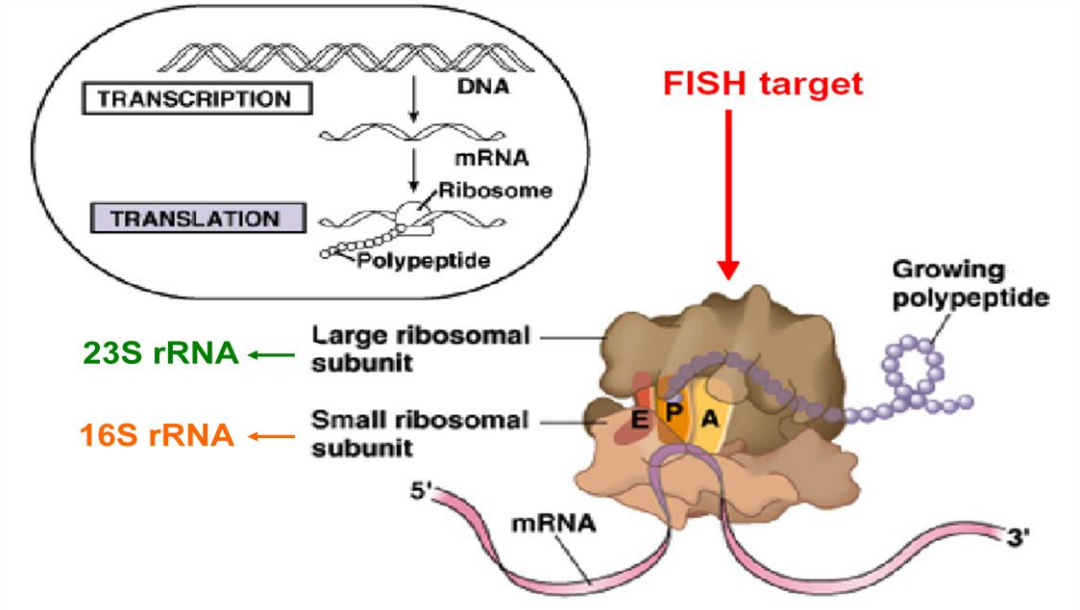


Figure 1.11: Probe-target the ribosome (Carvalhio and Silvia, 2009)

After hybridization, the cell can be observed under a fluorescent microscope. Specific organisms can therefore be discovered in a sample, and the relative abundance of an organism can be estimated (Madigan and Martinko, 2006).

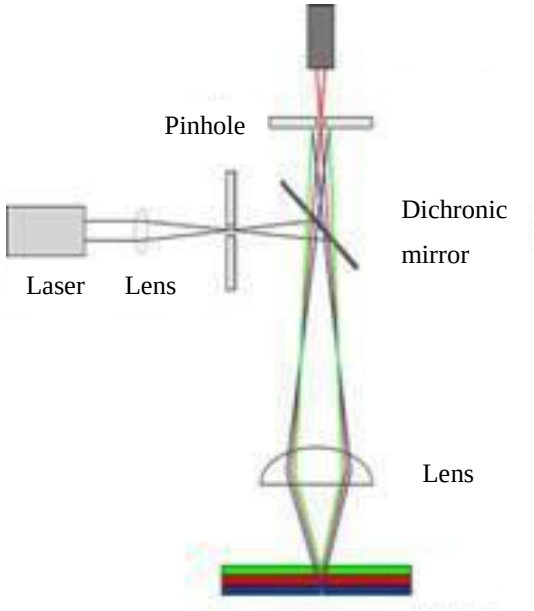
Area of application

FISH using rRNA-targeted oligonucleotide probes has become a widely used approach when studying microorganisms directly in complex systems. There is no need for cultivation or isolation, which makes the method quick and easy. When working with FISH, one has to know what to look for, since the number of bacterial species that can be analyzed at the same time is limited (Nielsen et al., 2009).

FISH can also be used for *in situ* quantification of uncultured microorganisms. The abundances and population dynamics of selected microbes can be monitored in their *in situ* environment. In a quantification experiment two different labeled probes have to be used; one universal which marks all the bacteria in the sample, and one specific, so all bacteria will get one fluorescent color, and the target bacteria will get another fluorescent color.

The simplest way of quantification is to use a confocal laser scanning microscope (CLSM), which shoots two pictures simultaneously; one of each fluorochrom, which may be used to calculate the relative biomass, by counting the colored pixels (Nielsen et al., 2009). In CLSM,

the illuminating radiation is laser light, which passes through a pinhole before striking a half silvered or dichroic mirror (Figure 1.12). The light passes down through the objective and illuminates the fluorescent stained object. The object will emit light of a longer wavelength, which then passes back into the objective lens, and through the dichroic mirror (Cunningham *et al.*, 2008).



Figur 1.12: The light path in confocal microscopy(Cunningham *et al.*, 2008)

The published work on biofilm characterization in drinking water systems is almost absent, but Hörsch *et al*. (2005) used FISH on a flat-channel test unit with NF and UF membranes, to analyze the proteobacteria structure. They showed that the bacteria composition of the primary fouling layer after 5 days was dominated by the gamma-subclass of the proteobacteria, and a significant amount of the beta proteobacteria. The mature fouling layer after 33 days was dominated by bacteria of the alpha- and beta- proteobacteria, and they found that the mature biofilm was similar to the bacteria community in the feed water. These results tell that there is a dynamic in the biofilm development, and that the gamma proteobacteria has the ability to attach to a clean surface.

1.4 Objectives

One objective of this work was to investigate the feasibility of molecular methods applied to identify and quantify biofouling of NF membranes. A second objective was to compare different microbial communities on membranes exposed to different operating conditions, *i.e.* to investigate if changes in the operating conditions may be detected by analyzing the microbial communities. The conditions included different modes, *e.g.* crossflow and dead-end, different feed water types, *e.g.* surface water and tap water, and different filtration times.

One sub goal was to test and optimize the molecular techniques with regard to choice of PCR primers, PCR reagents and denaturing gradients for separation. Another sub goal was to examine the reproducibility of molecular techniques and the reactor system, *i.e.* lab scale membrane filtration (NF) units monitored by measuring the water quality of the influent and the effluent water.

2. Material and Methods

2.1 Experimental set-up

2.1.1 NF system

The NF modules used in this study was built by Arne Grostad, Staff engineer at department of hydraulic and environmental engineering, based on a similar lab-scale reactor used by the Instituto de Biologia Experimental e Tecnológica (IBET), Portugal. The two modules were run in parallel, one module had a crossflow configuration, and the other was a dead-end mode (without crossflow). The experimental system and the NF test units are shown in Figure 2.1a and 2.1b respectively.

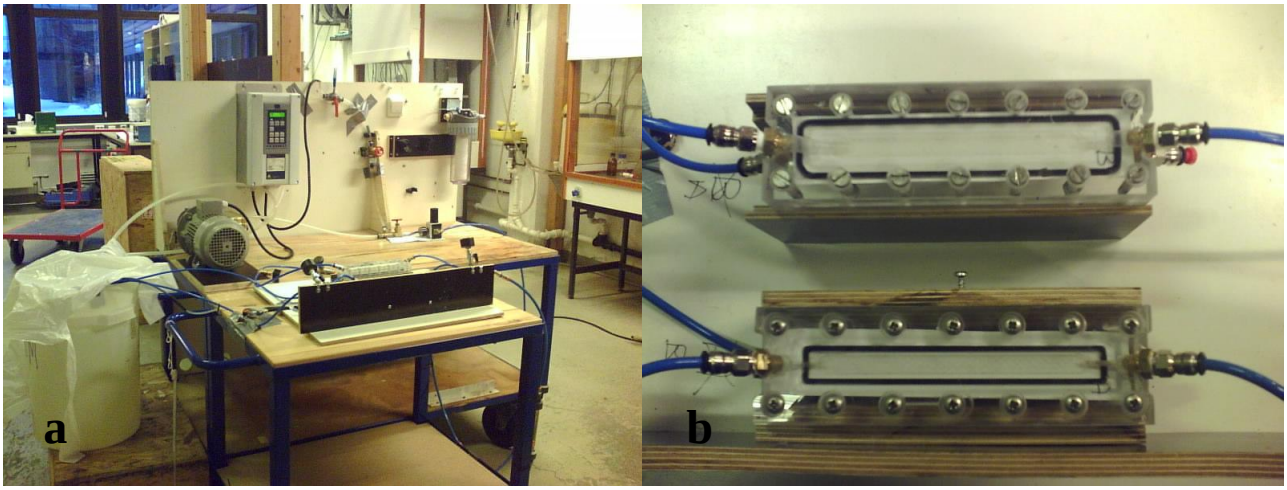


Figure 2.1: Experimental system (a) and NF test units (b) that were used in the experiment

When tap water was used as feed-water, the pressure from the tap was used to maintain a constant pressure. In the experiments using surface water, a high pressure pump (Hydra-Cell^{TM} pump, produced by Lönne) was used to maintain a constant pressure.

Figure 2.2 shows the flow chart of the system. Permeate produced in the experiment with surface water, was recycled back to the feed water tank, to avoid the increasing concentration of the feed water.

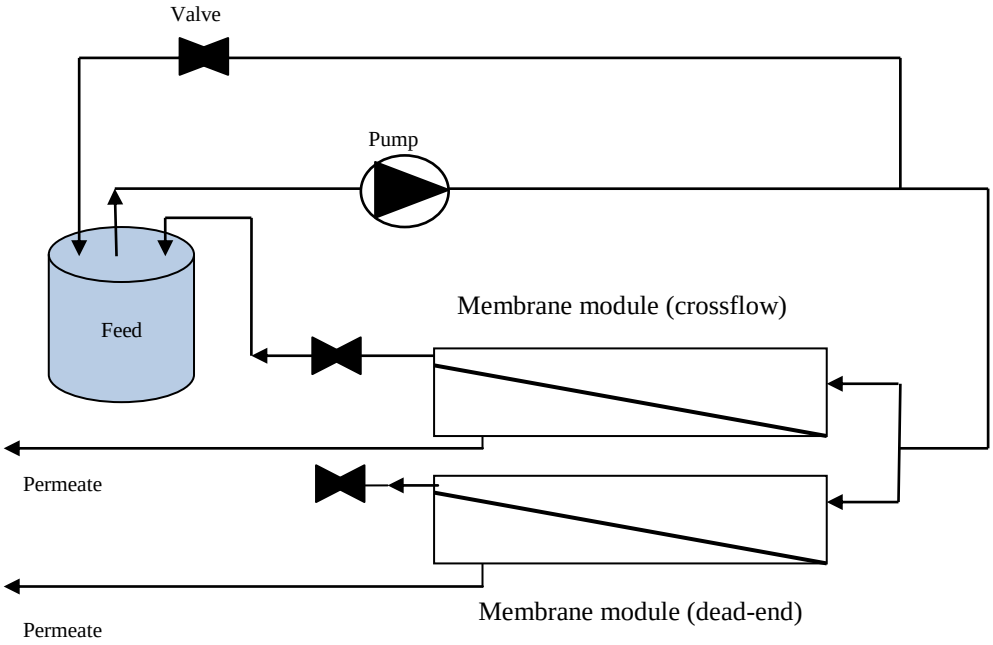


Figure 2.2: Flow chart of the pilot

2.1.2 Membrane

A NF membrane (FILMTEC™ NF270) was used in the experiments. Table 2.1 shows the characterizations of the membrane. The membrane is designed to remove high percentage of total organic carbon (TOC) and trihalomethanes (THM), while having a medium to high salt passage (Dow, 2010).

Table 2.1: Characterization of the NF membrane

	NF
Type	FILMTEC™ NF270
Manufacture	FilmTec/Dow
Material	Polyamide thin-film composite
pH range	2-11
Salt passage	Medium/high
T_{max}, °C	45°C

2.1.3 Chemical cleaning

After each experiment, the whole system was cleaned and disinfected with sodium hypochlorite NaClO, 0.01 % w/V, for 10 minutes.

Into experiment 9, 50 ml cleaning solution, containing 0,1 % wt NaOH pH13 (Dow, 2010) was used on the membranes for 20 minutes every day to remove biofouling. The system was stopped so the cleaning solution could soak the membrane. After the soaking, the membranes were flushed with 200 ml of surface water. The permeate flow was measured before and after each cleaning.

2.1.4 Feed water source

Jonsvatnet

Lake Jonsvatnet is the main drinking water source in Trondheim. The water was collected from the influent of the Jonsvatnet drinking water treatment plant, Vikelvdalen vannbehandlingsanlegg (VIVA), and was stored in refrigerators at 5°C. VIVA collects the water on 50 meters depth, 7 meter from the bottom. Under the experiments with Jonsvatnet feed water, a high pressured pump was used to keep the pressure constant. 40 liters of water were used for each experiment. After each experiment, the water in the test unit was replaced with fresh water.

Tap water

For the tap water experiment, the influent tube was connected to the water tap in the lab. The pressure was contained at 5 bars due to the constant pressure in the tap water system.

2.1.5 Operating conditions

The membrane filtration experiments were carried out under different conditions such as various filtration time and water quality, but with the same transmembrane pressure (TMP), *i.e.* 5 bar. Membrane module A had a crossflow of 12.5 l/h trough the entire experiment. The different operating conditions are shown in table 2.2.

Table 2.2: Test name with feed water type and days of operation

Experiment number	Test A crossflow	Test B dead-end	Feed water type	Days of operation
1	A1a A1b	B1	Tap water	17
2	A2	B2	Tap water	8
3	A3	B3	Jonsvatnet (surface water)	7
4	A4	B4	Jonsvatnet (surface water)	12
5	A5	B5	Jonsvatnet (surface water)	16
6	A6	B6	Jonsvatnet (surface water)	2
7	A7	B7	Jonsvatnet (surface water)	8
8	A8	B8	Jonsvatnet (surface water)*	8
9	A9	B9	Jonsvatnet (surface water)**	8

*Pretreated with microfiltration, 20µm
**Pretreated with microfiltration (20µm), and run with disinfection solution for 15 minutes every day

2.1.6 Comments to the experiments

Test A1a (table 2.2) was just a trial test to estimate how the experimental pilot system worked.

Experiment 3 was designed to test if the two pilots were comparable, *i.e.* the crossflow and dead-end configurations was shifted, test A3 was operated as a dead-end and test B3 as a crossflow. These two tests results will not be exposed to further analyses on the biofilm.

The first experiment with surface water (experiment 5, Table 2.2) had some problems with unstable pressure. The plan was to operate the membrane for eight days, but because it had to be stopped and restart several times, the membrane was operated for a few more days than the initial plan. Because it was of interesting to see whether such types of pulses may affect the biofilm, the membranes were analyzed further.

The influent feed water of experiment 8 and 9 were pretreated with a wound polypropylene cartridge filter, whit pore size of 20 µm.

2.1.7 Monitoring fouling

The fouling was monitored by measuring the decrease in flow from the effluent to the two modules. This was done by holding a beaker under the effluent for two minutes, and then measuring the amount by weighing. Since the membrane area for the two modules, A and B, were different, 21.7 and 32 cm^2 respectively, the flux (l/m^2h) was calculated for comparison.

2.2 Water quality analysis

Feed water quality and membrane filtration was monitored by measuring the water quality of the influent and effluent under different operating conditions. The methods used in this study have been developed by the Norwegian Standardization Association (NSF), which is a member of the International Organization for Standardization (ISO) and the European Committee for Standardization (CEN).

2.2.1 TOC (NS ISO - 8245)

Total organic carbon (TOC) is the organic carbon content of a water sample. TOC analyzator Tekmar Dohrmann Apollo 9000 was applied, using a catalyst and heat while supplying oxygen to convert organic C into CO_2 (Droste, 1997). The TOC analyzes was performed by Trine Margrete Hårberg Ness, staff engineer at the department of hydraulic and environmental engineering, NTNU.

2.2.2 Turbidity (NS EN ISO - 7027)

Fluids can contain suspended solid matter consisting of particles of many different sizes. The turbidity of a fluid is a result of the scattering and absorption of light by suspended solids in the liquid. Natural water or wastewaters will contain many different sized particles at different concentrations (Droste, 1997). To monitor changes in the turbidity, a HACH 2100N turbidimeter was used.

2.2.3 Color analysis (NS - 4787)

For color analysis, HITACHI U-300 spectrophotometer was used to beam with light on a sample (Droste, 1997).The absorbed light with wave length 410 nm was measured.

2.2.4 UV-absorbance (NS – 9462)

The same instrument was used for measuring the UV absorbance at 254 nm.Unsaturated double bounds will absorb strongly in this region.

2.3 PCR-DGGE

The protocol applied in this experiment has been developed by Ole-Kristian Hess-Erga at the department of Biotechnology, NTNU, for marine bacteria, and is a modification of the DNeasy protocol from QIAGEN. There have been done some modifications in order to fit the protocol to this assignment. In the DNA extraction, DNeasy Blood & Tissue Kit from QIAGEN was used on all the samples, and to compare it with another kit, some of the samples were extracted with the MoBIO UltraClean^{TM} Soil DNA Isolation kit by Ana Filipa Silva at IBET, Portugal. In the PCR reaction Taq PCR core kit from QIAGEN was used.

2.3.1 DNA extraction for Bacteria on the NF membrane

A scalpel was used to remove some biofilm from the membranes into an Eppendorf tube. Then 180 µl enzymatic lyses buffer (see Appendix 1, table A1.1) was added, and the mix was incubated at 37°C for 30 minutes. After incubation, 40 µl of protein kinase K and 180 µl ATL buffer was added and the mixture was incubated at 55°C for 30 minutes.
After incubation 200 µl AL buffer was added, and the sample was vortexed and incubated at 70°C for 10 minutes. Then 300 µl of 96% ethanol was added, and the sample was mixed. Then the solvent was transferred to a DNeasy column, and centrifuged at 8000 rpm for one minute, before the filtrate was removed. 500 µl of AW 1 buffer was added to the column and centrifuged at 8000 rpm for one minute. The filtrate was removed. Then 500 µl AW 2 buffer was added, and the column was centrifuged at full speed (1500 rpm) for three minutes. The

column was then transferred to a sterile Eppendorf tube. 50 µl AE buffer was added directly on the membrane, and incubated for a minute at room temperature, before centrifuged at 800 rpm for one minute. The AE buffer step was repeated.

2.3.2 DNA extraction for Bacteria in a water sample

60 ml of surface water was filtrated trough a 0.2 µm Dyngard filter. Then the filter was placed into a sterile Eppendorf tube, and centrifuged for a short time. The excess water was discarded. A PCR tube was used as a plug on the end of the filter, and the filter with the plug was placed into a new Eppendorf tube. A volume of 90 µl of enzymatic lyses buffer was added on the top of the filter before incubated at 37°C for 30 minutes. After incubation, 20 µl of protein kinase K and 90 µl ATL buffer was added and incubated at 55°C for 30 minutes. After the incubation, the Dyngard filter was put upside down in a new Eppendorf tube, and centrifuged at 8000 rpm for two minutes. The extract was put on ice, while the two different incubation procedures were repeated. The extracts were combined, and 200 µl AL buffer was added. The mixture was vortexed and incubated for 10 minutes at 70°C. After this step, the protocol was the same as the one for the bacteria on the NF membrane.

2.3.3 Measuring DNA concentration

The extract was brought to a NanoDrop ND-1000 spectrophotometer from Thermo Scientific. The DNA contents were measured twice for each sample. The measurements were used to calculate the amount of template used in the PCR reaction described below.

2.3.4 PCR

The PCR were carried out on VWR^{TM} unocycler from VWR. Two different PCR reactions were tried out, a simple PCR with one round and a nested PCR with two rounds, where the product from the first round is used as a template in the second round. First a master mix was made for all the PCR samples (for recipes for the different master mixes, see Appendix 1, table A1.3, A1.5). In the simple PCR reaction, two primers were used, *e.g.* 338f_GC and 518r, while in the nested PCR reaction three different primers were used, *e.g.* 27F and 1492R in the first round, and 968F_GC and 1492R in the second round, for more detailed information about the primers see Appendix 1, table A1.2. The PCR reaction regime was

decided, and all the samples were copied. For PCR regimes for the simple and for the two nested PCR rounds see Appendix 1, table A1.4, A.1 6 and A1.7 respectively.

After the PCR reaction a 0.8 % agarose gel was made. After the gel was set, 1xTAE buffer (Appendix 1, table A1.8) was added. Then a mixture of 1 µl 6x loading dye and 5 µl sample was added to the wells in the gel, and operated with 140 V for approximately 60 minuttes, before the gel was photographed on a UV table, to see if there was any PCR product or contaminations.

2.3.5 DGGE

The INGENY phorU system from Ingeny, Netherland, was used in the DGGE experiment. All the DGGE gels were made by 8 % acrylamide. After installing the system, a gradient had to be chosen. A wide denaturing gradient (25 % -60 %) was first tried out, then narrowed down to 35 % -55 % for a better separation. For mixing of the gradients see Appendix 1, table A1.9.

Two Falcon tubes were labeled with the chosen high and low gradient. The high and the low gradient, each whit 24 ml acrylamid, were mixed from 80 % and 0 % acrylamide standard solutions, se Appendix 1, table A1.10 and A1.11. The acrylamid were filtrated trough a 0.2 µm filter, before mixing it with tetramethylethylenediamine (TEMED) and ammonium persulfate (APS), see Appendix 1, table A1. 9. APS was the last reagent in the solution, and was added right before the solutions were poured into the gradient maker. A pump applied the solutions from the gradient maker trough a syringe needle in to the INGENY phorU system. After the gradient maker were empty, 10 µl TEMED and 40 µl APS were added to 8 ml 0 % acrylamid solution, which were poured into the gradient maker, and pumped on top of the gel. The gel was left for polymerization for 2 hours.

The buffer tank was filled with 0.5xTAE, and heated to 60°C. After the heating and the polymerization the gel chamber were placed in the tank. 4 µl loading dye were added to a 15 µl sample, and then loaded in to the wells in the gel. The gel was run with 100 V for 17 hours.

After the 17 hours, the voltage was turned off, and the gel chamber was lifted out from the buffer tank. Then the gel was withdrawal from the gel chamber and placed in a box. A 30 ml

SYBR Gold (Invitrogen) solution (3 µl SYBR Gold + 600 µl 50xTAE + 30 ml MilliQ water) were poured on the gel, so each lane were covered, and then stained for 60 minutes in the dark. After the staining, the gel were washed with MilliQ water, and then photographed on a UV-table (G-BOX from Syngene).

2.3.6 Analysis of the DGGE gel

The gel was analyzed with the free software Gel2k (Norland, 2004), which count the bands and measure the band intensity. From this data the Shannon –Weaver diversity indexes and evenness for all samples were calculated according to equations 1 and 2, given in section 1.3.3.

A similarity cluster was made with the Gel2k software, based on the Jaccard similarity coefficient, according to equation 3 in section 1.3.3.

2.4 FISH

The protocol used in this FISH experiment, was developed by IBET, Lisbon in Portugal. The recipe of the reagents, the probes and the fluorochrom which were used are described in Appendix 2.

2.4.1 Sample fixation

Gram positive bacteria

0.5 ml of ethanol (98 %) and 0.5 ml of sample were mixed together, and stored at 4°C for 4 - 16 hours. After the incubation, the sample was centrifuged at 10 000 rpm for three minutes. The liquid was removed, and the pellet was washed with 1 ml of phosphate buffered saline (PBS) 1 x, and centrifuged. The washing step was repeated. After the last centrifuge, the pellet was resuspended with 0.5 ml PBS x1, and 0.5 ml of 98 % ethanol (-20°C) was added to the sample. The samples were stored at -20°C.

Gram Negative Bacteria

1.5 ml of PFA and 0.5 ml of sample were mixed together, and stored at 4°C for 1 - 3 hours. After the incubation, the sample was centrifuged at 10 000 rpm for three minutes. The liquid was removed, and the pellet was washed with 1 ml of PBS 1x, and centrifuged. The washing step was repeated. After the last centrifuge, the pellet was resuspended with 0.5 ml PBS x1, and 0.5 ml of 98% ethanol (-20°C) was added to the sample. The sample was stored at -20°C.

2.4.2 Sample application and dehydration

Black teflon coated slides (from Thermo Scientific) with ten wells were used (Figure 2.3). After labeling the wells, 3 to 30 µl of the sample was applied to each well, and the sample was then air dried or dried in an oven at 46°C. After the sample was dried, the slide was dehydrated in a series of Falcon tubes, filled with respectively 50 %, 80 % and 98 % ethanol, for three minutes in each tube. After the dehydration, the slides were dried under compressed air.

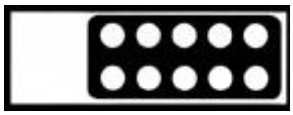


Figure 2.3: Teflon coated slides with eight wells

2.4.3 Probe hybridization

A bed with a tissue paper was made inside a Falcon tube. 8 µl hybridization buffer (Appendix 2) was applied in each well, while the rest of the buffer was used to moisturize the tissue in the Falcon tube. 1 µl of each fluorochrome marked probe (Appendix 2, table A2.3), cytochrome 3 for EUB bacteria and cytochrome 5 for the target bacteria, was applied to each well, and mixed gently without touching the slide. The slide was then placed into the Falcon tube, and the tube placed in the oven at 46°C for 1 to 2 hour.

2.4.4 Washing

A washing buffer (Appendix 2) was prepared before the hybridization was over, and heated in a bath to 48°C. Then each slide was washed with a Pasteur pipette before the slides were placed in the washing buffer tube, and put into the 48°C bath for 10-15 minutes. After the

bath, each slide was washed with MilliQ water at 4°C, and the excess water was removed with compressed air.

2.4.5 Mounting slides

After drying the slides, a few drops of Vectashield were applied, and a cover slip was put gently over the slide, to force Vectashield solution to cover all wells. Nail polish was applied to the edge of the cover slip to prevent it from moving away from the slide, and prevent immersion oil to combine with Vectachield. After the nail polish was dried, the slides were stored at -20°C.

2.4.6 FISH quantification

A Zeiss confocal LSM 510 Meta with HeNe 633 and 543 lasers, was used to excite fluorochrom Cy5 and Cy3 respectively. For every sample 30 pictures was taken. The software ImageJ (Rasband, 2006) was used with a macro for calculating the pixel in the pictures for the two lasers. The threshold was decided for each sample.

3. Results and discussion

3.1 Membrane operation

A cake layer of fouling developed on the membrane, and the membrane was turning browner and darker as a function of days of operation. Differences in fouling on the two different modules were also observable (Figure 3.1).

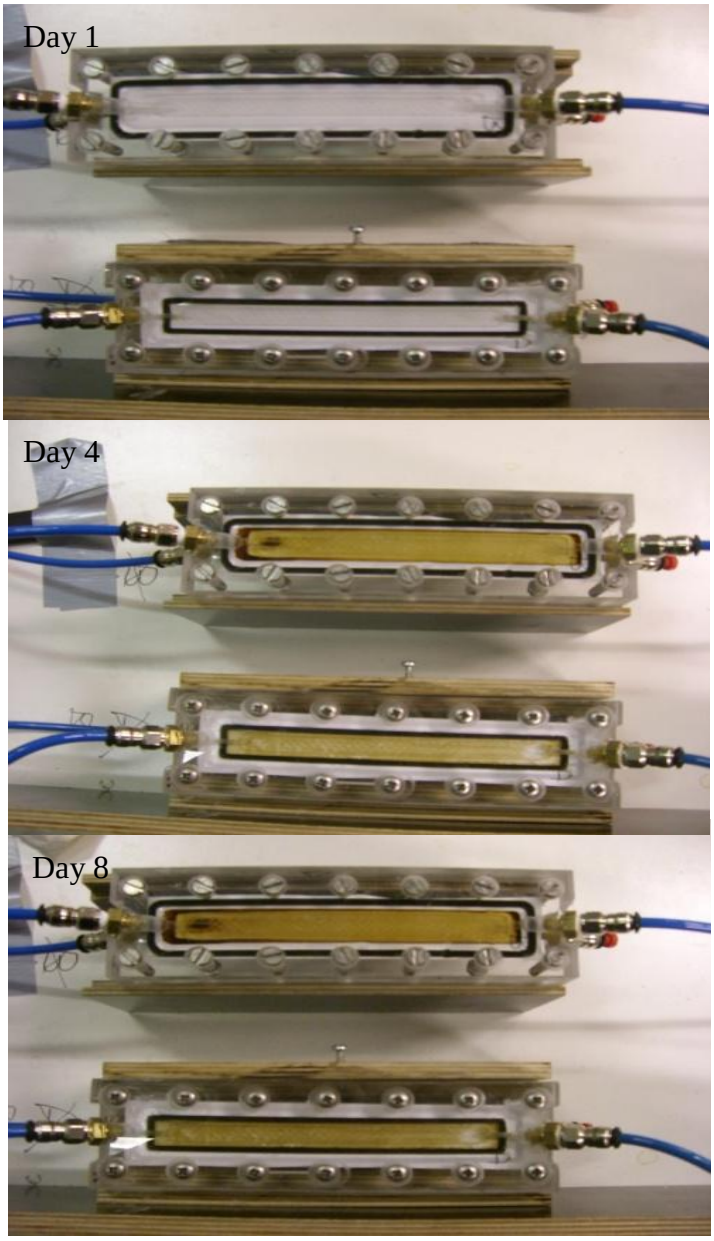


Figure 3.1: Pictures of the membranes, operated for 1, 4 and 8 days. The upper membrane in each picture represents the dead-end mode, and bottom the crossflow mode.

The membrane operated as a dead-end module became darker after a few hours, and the cake layer was thicker compared to the crossflow system (Figure 3.1). This was expected, since the crossflow removes some deposits that are unable to stick to the membrane, while nothing is removed in the dead-end module. The cake layer formed on the dead-end was observed to be less dense than the cake layer on the crossflow, under the removing of the biofilm from the membrane. This corresponds to the observation of Cunningham *et al.* (2008), that the biofilm will be denser under conditions with low nutrient and high crossflow.

In this project, the flux decline (l/m^2h) was used as an indicator for fouling, instead of measuring increase in pressure. The following results show the effect of the operating parameters on the flux.

3.1.1 Effect of feed water quality

Figure 3.2 and 3.3 show the flux decline, dead-end and crossflow module respectively, operated with different feed water qualities (experiment 2, 7 and 8, Table 2.2).

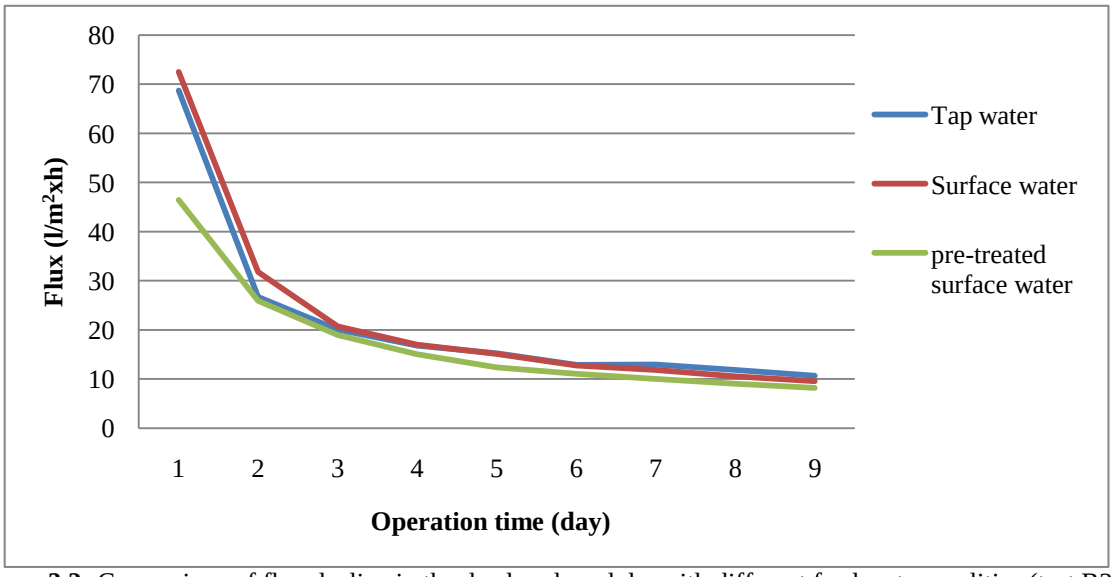


Figure 3.2: Comparison of flux decline in the dead-end module, with different feed water qualities (test B2, B7 and B8)

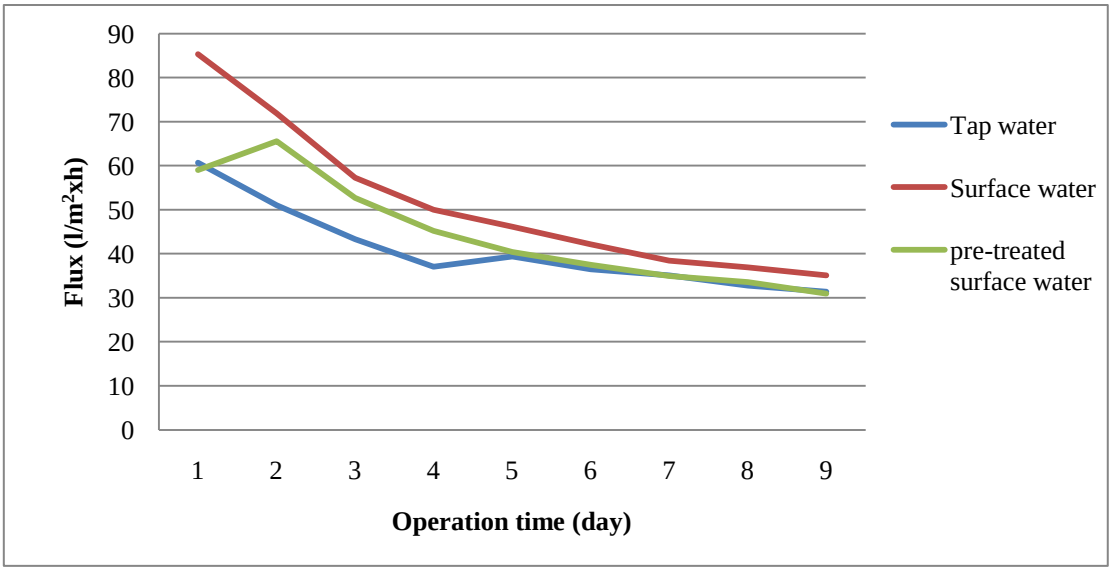


Figure 3.3: Comparison of flux decline in the crossflow module, with different feed water qualities (test A2, A7 and A8)

The flux decline was not affected by the feed water quality (Figure 3.2 and 3.3), *i.e.* the rate of fouling was unaffected by the feed water type. This corresponds to the measured water qualities discussed later. The measurements are similar for the different water qualities, and the effect on the flux is therefore minimal.

Some differences could be detected at the start of the experiments, but this was probably due to air in the system or unstable flow at the start up, which may have affected the measurements. The flux decline was rappid, and waiting for an hour before measuring, may have affected the results initial.

3.1.2 Effect of operating mode

Figure 3.4 shows the flux decline of experiment 1 and 5 (Table 2.2), and Figure 3.5 shows the flux decline for experiment 2 and 8 (Table 2.2). The Figures compare the two modules, crossflow and dead-end.

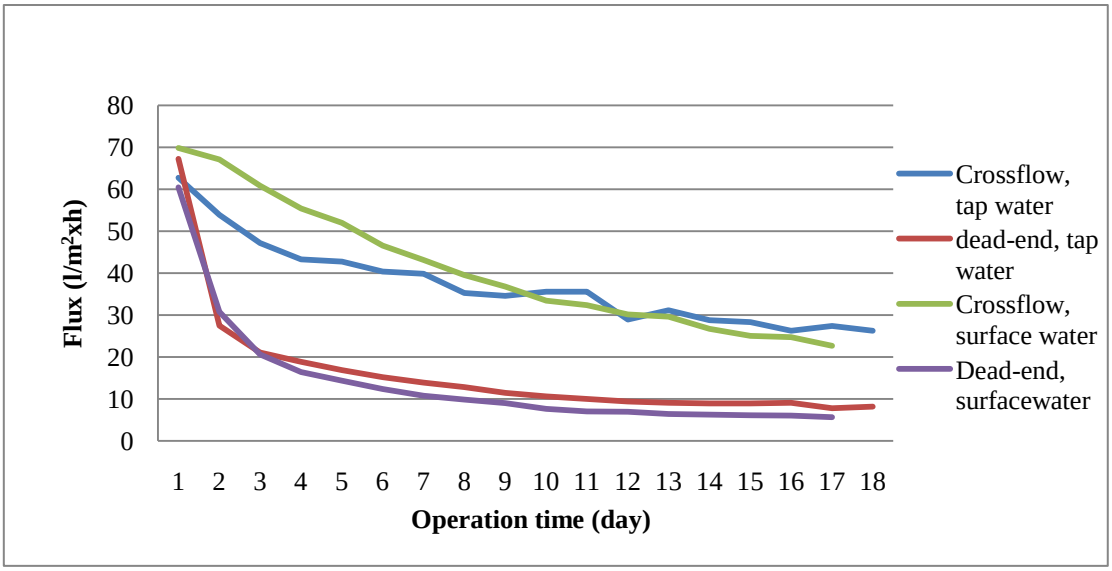


Figure 3.4: Crossflow *vs*. dead-end module, 18 days

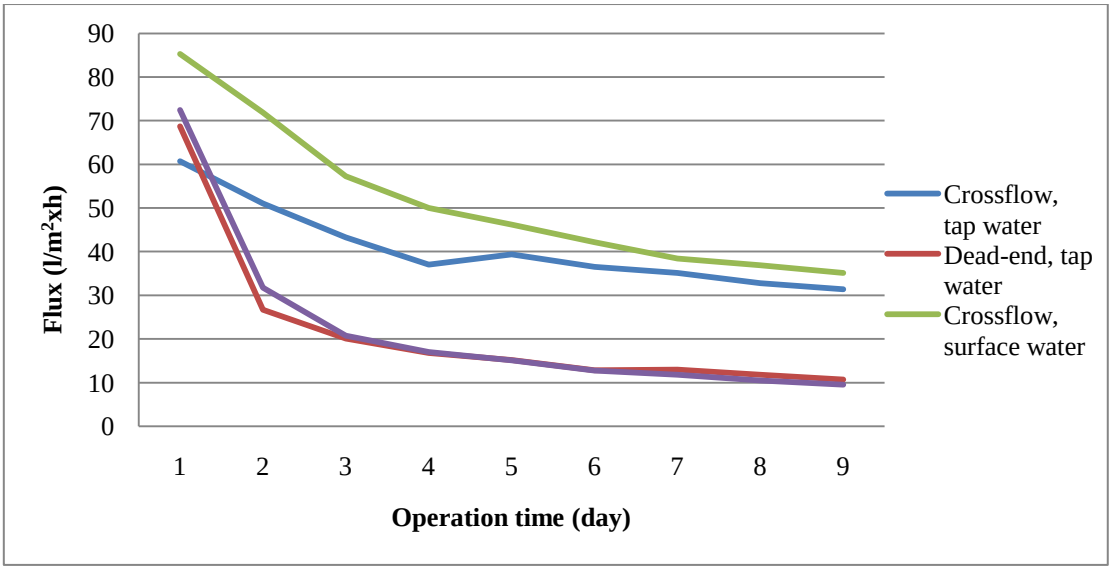


Figure 3.5: Crossflow *vs*. dead-end module, 9 days

Figure 3.4 and 3.5 demonstrate that the flux decline was larger for the dead-end module compared to the crossflow, and that the crossflow module maintains a higher flux trough the whole experiment time, compared to the dead-end module. This was expected since no fouling components were carried away by a crossflow in the dead-end module. The flux decrease was highest during the first 3 days, and then reduced.

3.1.3 Effect of membrane area

Reactor A and B had different membrane surface areas, so in order to see if this had some effect on the system the modes were switched. Figure 3.6 shows experiment 3 (Table 2.2), where the reactors (A and B) exchange operating mode, compared with experiment 8 (Table 2.2).

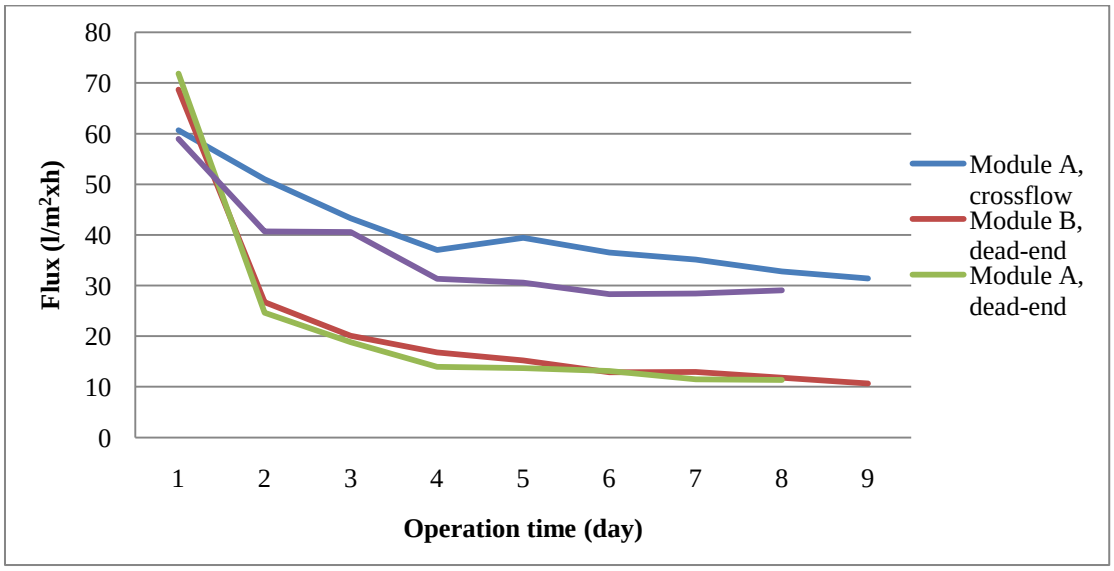


Figure 3.6: Comparison of the two parallel pilots, A and B

The results from the two experiments are similar. This means that the differences in area do not affect the flux results.

3.1.4 Cleaning cycle

In experiment 9 (Table 2.2), a cleaning procedure were done (Figure 3.7). The membranes in this experiment were cleaned with a solution of NaOH for 15 minutes each day. The flux was measured immediately before and after each cleaning to detect the recovery.

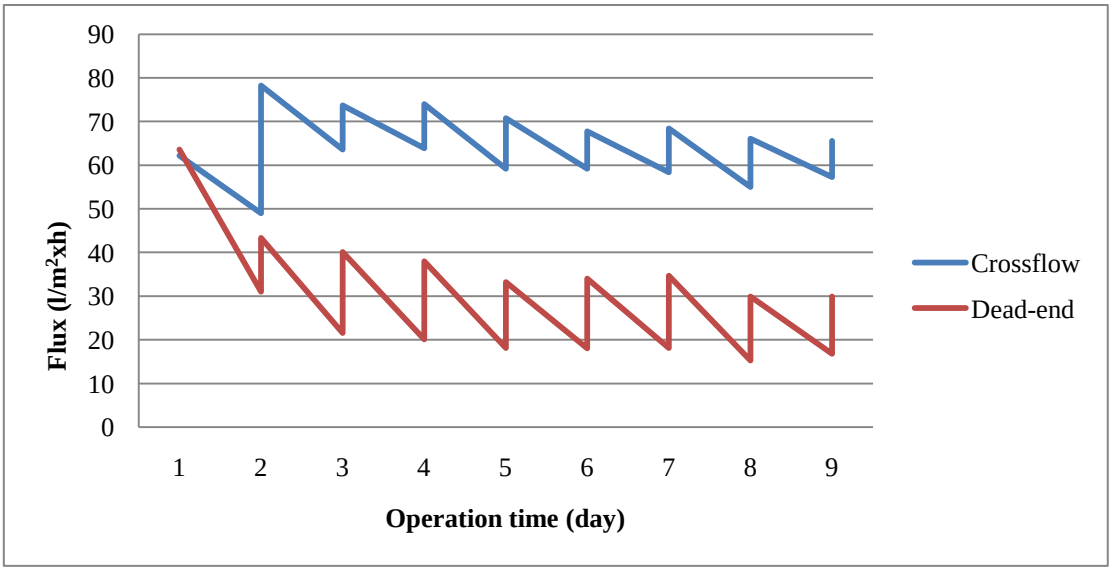


Figure 3.7: Cleaning cycles for dead-end and crossflow module.

The flux was relatively higher compared to the membranes operated without cleaning. After the first cleaning, on day 2, the recovery of flux was much higher for crossflow (test A9) than for dead-end (test B9) filtration. It looks like it was more irreversible fouling on the dead-end than on the crossflow system after the first day, which cannot be removed by the cleaning solution. This gave the crossflow a lower flux in general. The decline was more similar for the two testes after day 3, *e.g.* accumulation of irreversible fouling had almost the same rate. After the cleaning on day 2 the flux for crossflow and dead-end was 78 l/m^2 h and 43 l/m^2 h respectively. At day 9 the flux had decreased to 65 l/m^2 h for crossflow module and 29 l/m^2 h for dead-end module, which was a capacity decrease of 16 % and 31 %. Since the recovery after cleaning was decreasing with time, there may be a limit for how long one may continue the procedure, before the membrane is totally clogged. The experiment should have been tried out for a longer period of time, to get a better understanding of the long-term effect, but due to time limits, the work was not continued.

3.2 Water quality

Three types of feed water were used to investigate the effect on NF process and the biofilm characteristics. The qualities of the feed waters are represented in table 3.1.

Table 3.1: Feed water quality for tap water, surface water and pre-treated surface water

Feed water	TOC (mg/L)	$Color_{410}$ (Pt)	UV_{254} (Absorb)	Turbidity (NTU)
Tap water	3.156±0.233	14.31±1.01	0.090±0.003	0.115±0.017
Surface water	4.938±1.643	15.68±1.60	0.110±0.012	0.176±0.021
Pre-treated surface water	4.521±0.548	15.61±0.32	0.106±0.002	0.158±0.007

The differences between the measured feed waters qualities are not large, they are almost the same, but this does not mean that there is no difference. The TOC does not distinguish between biological degradable and no degradable substances, and the turbidity recordings do not distinguish between the size of the particles, this may have a effect on the microbial community (Droste, 1997).

The pre-treatment of the surface water with a 20µm filter gave no significant changes in the measurements. Permeate from the membranes operated with surface water was recycled back to the feed water tank to avoid up-concentration of the feed water. This may have affected the water quality over time, since the biofilm that were growing on the membranes may have used some of the nutrient sources in the water. This was not detectable by methods used in this experiment.

The effluent water qualities from test A and B were measured frequently, which are summarized in table 3.2 and 3.3 respectively.

Table 3.2: The effluent water quality of test A (crossflow)

	TOC (mg/L)	$Color_{410}$ (Pt)	UV_{254} (absorb)	Turbidity (NTU)
Tap water	0,323±0,128	0,04±0,07	0,002±0,003	0,067±0,011
Surface water	1,541±1,020	0,04±0,10	0,007±0,004	0,102±0,032
Pre-treated surface water	1,180±0,394	0,00±0,00	0,007±0,002	0,057±0,001

Table 3.3: The effluent water quality of test B (dead-end)

	TOC (mg/L)	$Color_{410}$ (Pt)	UV_{254} (absorb)	Turbidity (NTU)
Tap water	2,314±0,333	4,52±1,59	0,101±0,149	0,105±0,032
Surface water	3,894±1,437	5,00±1,05	0,067±0,010	0,110±0,040
Pre-treated surface water	3,509±0,387	4,56±1,41	0,058±0,005	0,058±0,005

The filtration efficiency was also calculated and is showed in table 3.3 and 3.4 respectively.

Table 3.4: Removal efficiency, test A (Crossflow)

	TOC	$Color_{410}$	UV_{254}	Turbidity
Tap water	90 %	100 %	98 %	41 %
Surface water	69 %	100 %	94 %	42 %
Pre treated surface water	74 %	100 %	93 %	64 %

Table 3.5: Removal efficiency, test B (dead-end)

	TOC	$Color_{410}$	UV_{254}	Turbidity
Tap water	27 %	68 %	12 %	8 %
Surface water	21 %	68 %	39 %	38 %
Pre treated surface water	22 %	71 %	46 %	63 %

The removal efficiency of TOC was significantly larger for the crossflow than for the dead-end module. This shows that when the test A operating with a crossflow, particles causing the TOC accumulation tends to be washed away, rather than pressed through the membrane. The same was observed for the color and the UV_{254} absorbance, the removal was 100% for color and over 90% for UV_{254} absorbance, when using a crossflow test, independent on the feed water, while it was down to 70% for color and under 50% for UV, when using dead-end. The difference in removal efficiency was not that big when it comes to turbidity. This may be due to the low turbidity (< 1NTU) in the influent as well as permeate.

3.3 PCR-DGGE

3.3.1 Method improvement

Extraction

Ana Filipa Silva from IBET preformed a DNA extraction with MoBIO UltraClean™ Soil DNA Isolation kit on test A1, A4 and A5. The extraction products were brought back to Norway, and handled with the same PCR reaction and run on a DGGE gel with the other samples extracted with the DNeasy Blood & Tissue Kit. Figure 3.8 shows the DGGE patterns

from some of the samples extracted with the MoBIO UltraClean™ Soil DNA Isolation kit, compared to the same samples extracted with the DNeasy Blood & Tissue Kit.

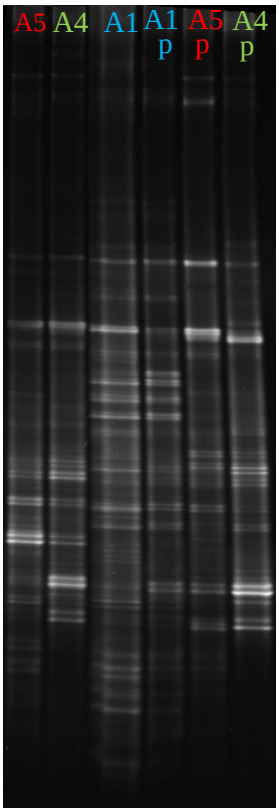


Figure3.8: Comparison of DGGE patterns, when using different extraction protocols. The tests marked with p have been extracted in Portugal.

The two extraction kits have produced resembling DGGE patterns, but there are some differences. A4 and A4p resemble the most, only two bands separating them. For the A5 and the A5p the difference was larger, *e.g.* there is one band which is dominating on the A5 lane, that is almost absent on the A5p. There is also some smaller band separating them. A1 and A1p do also show some differences, but it is more of the dominating part, *e.g.* some bands more dominating in one sample but still present in the other. The differences in the DGGE patterns can also be due to the PCR reaction. There were some problems to amplify the DNA from A5 and A4, probably because of contaminations which inhibited the PCR reaction. This has an effect on the amount of DNA after the PCR, and therefore has an effect on the intensity of the bands. This comparison shows that the choice of extraction kit may affect the results, and it is therefore important to choose the right extraction kit for the actual purpose.

PCR

The oligonucleotid products from the nested PCR were larger (500 bp) than the oligonucleotid products from the single PCR reaction (200 bp). This could be an advantage, since the probability for one bacterium to be represented more than one time on the gel decrease. And if further analyzing is required, the 200 bp oligonucleotids is so small that the probability for finding more than one species which mach the sequenced oligonucleotid is high, while with the bigger oligonucleotids in the nested PCR the probability is decreasing.

Figure 3.9 shows the DGGE patterns after the products from the nested PCR, compared to one sample from a simple PCR.

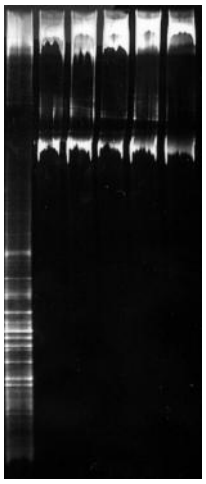


Figure 3.9: The lane to the left shows the DGGE fingerprint from a sample with simple PCR, rest of the lanes is DGGE patterns with nested PCR products

Figure 3.9 shows that the nested PCR products have not managed to migrate in the gel, and all the products are stuck on top of the gel. The 500 bp oligonucleotids were unsuccessful to entering the gel. There were also done an experiment with nested PCR products from IBET, for excluding the PCR reaction as a source of the migration failure, but the results remained the same. This could be related to the problems of the INGENY phorU system. It is not designed for oligonucleotids that big. At IBET they use a BioRad system, and they had no problems with the same PCR product. There were also other experiments, apart from this, that had the same problem when using large oligonucleotids (>500 bp). One possible solution could have been to lower the acrylamid percentage, but the BioRad system had the same percentage as in this experiment, so this was not tried out, since it was excluded as a possibility. Since the nested PCR did not work, the rest of the experiments were preformed with the simple PCR.

DGGE

To get a god suitable separation, a wide denaturing gradient (25-60%) for the acrylamide gel were first tried out. Figure 3.10 shows a comparison between the wide gradient, and a more narrowed gradient (35-55%).

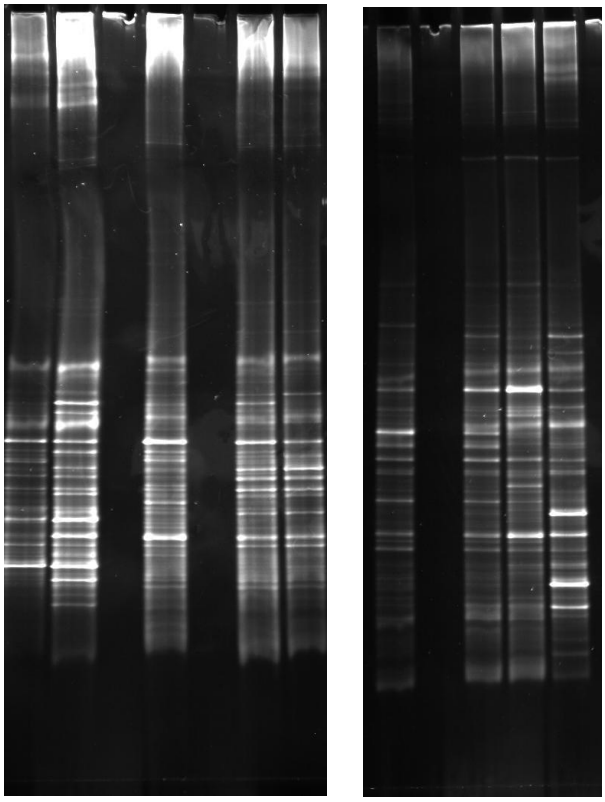


Figure 3.10: Comparison between wide denaturing gradient from 25 - 60 % (to the left), and low gradient from 35 - 55 % (to the right)

Figure 3.10 shows that the band that are separated on the gel with a wide denaturing percentage are compact and spreads over a smaller area. This is not optimal separation, therefore the gradient was narrowed down to 35-55% (Figure 3.10 to the right) which obtained better separation of the bands on the DGGE gel.

3.3.2 Reproducibility

It is important that reproducible results can be obtained for the same operating conditions. Figure 3.11 shows the DGGE fingerprint of the community for two replicates, test A1a and test A1b, both operated for 16 days, with tap water as the feed water, in a crossflow module.

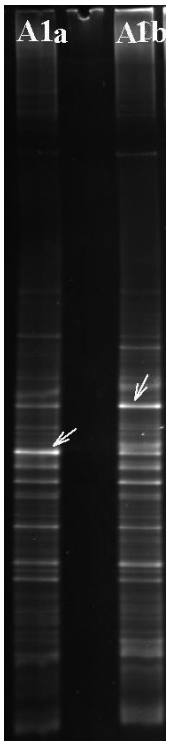


Figure 3.11: The DGGE-fingerprint from two tests A1a and A1b operated under the same conditions.

The two DGGE fingerprints are similar, with just some small differences. The biggest differences, labeled with arrows, are two bands, which are more intense than the corresponding bands on the other lane, but they are not absent. So the picture illustrate that the system is reproducible.

The two membranes, compared in Figure 3.11, were operated when the system was new. When such experimental systems have been used for a period of time, biofilm formation may occur in the tubing and other areas of the system where the chemical cleaning do not reach. The reproducibility of biofouling DGGE fingerprints observed initially in the testing period may have been different later in the period. To verify that the system really was reproducible, one should have performed a new experiment at the end of the period, but due to time limits this was not preformed.

3.3.3 DGGE gel

All the PCR products from the different experiments that were analyzed, was run together on one DGGE-gel. Figure 3.12 shows a picture of the gel. The samples may be identified from table 2.2. The lanes named L and C, is a ladder and a control respectively.

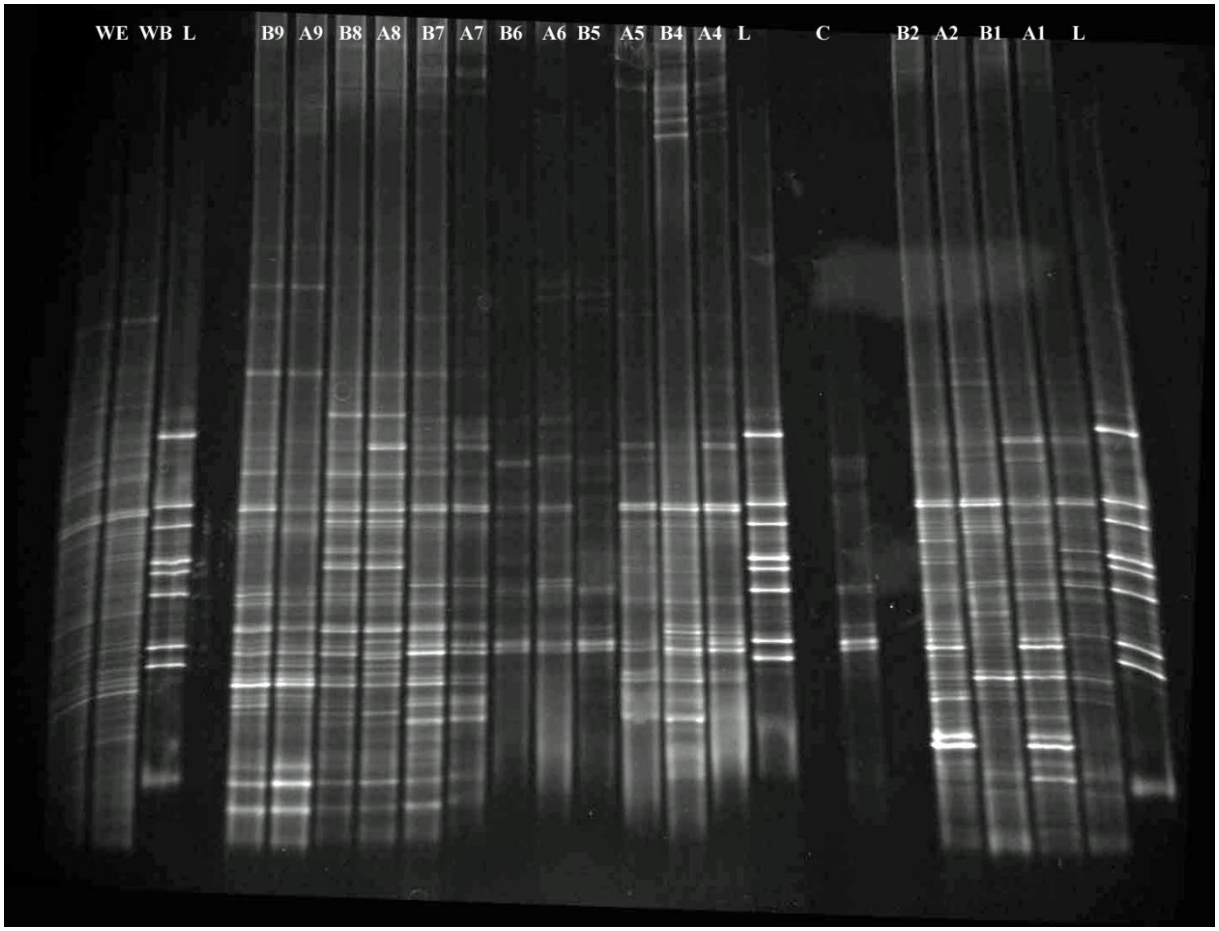


Figure 3.12: DGGE patterns of the NF membranes, which may be identified in table xxx, as well as the DGGE patters for the surface water quality (WB and WE) at the beginning and the end of the experiment.

The following results discuss the effect of the operating parameters on the microbial community.

Effect of the feed water type

Experiments 2, 7 and 8 (Figure 3.12) represent the membranes operated for 8 days, with tap water, surface water and pre-treated surface water respectively. The figure shows that the bacteria communities have differences, *i.e.* the feed water type has a large effect on the

bacteria structure. The dominating bands in the different samples do not correspond, which mean that the feed water affects the competitions for the bacteria (Figure 3.12).

It was demonstrated in section 3.1.1 that the feed water type had no effect on the flux decline, and therefore no effect on the overall fouling. This means that the bacteria composition do not have a big effect on the initial fouling, but a large effect on the biofouling. One interesting aspect of this result is the long termed effect. How the community structure effects the operation over time, *e.g.* maybe some structures cause more irreversible fouling than other.

Pre–treatment of the surface water before filtrating it with a NF membrane is common practice in Norway, and studies of the effect if the filtration is negative or positive is absent. In this experiment, the filter pre-treating the water was 20 micron due to available supply, common practice is 50 micron. An interesting aspect is if the pre-filtration pore size affect on the community. By analyzing this effect, maybe it would be possible to optimize the operation of a NF treatment plant by choice of pre-filtration.

It was also demonstrated in Table 3.1 that the differences between the measured feed water qualities were similar, this does not mean that they are the same. The TOC does not distinguish between biological degradable and no degradable substances, and the turbidity recordings do not distinguish between the size of particles. This may have a big influence on the competition between the bacteria.

Effect of operating mode

The different operation modes (A was operated as a crossflow and B as a dead-end) had effect on the bacteria structure (Figure 3.12). The mode of operation had a larger effect on the bacteria community in experiments where tap water was used as feed water (experiment 1 and 2), compared with the experiments with surface water. A1 and B1 have a lot of bands that not corresponding, the same with A2 and B2 (figure 3.12). While, *e.g.* A7 with B7, which was operated with surface water have more resemblance. The reason for the modes different effects after the different feed water qualities may be due to the bacteria in the water. When the tap water leaves the treatment plant, it should not contain microorganisms, while the surface water has not been trough any disinfection, and therefore contains natural amount of microorganisms. When filtrated trough the membrane, the natural contents of microorganisms

in the surface water is higher, and it will therefore affect the recruitment to the membrane surface. However, in drinking water the biofilm will mainly be due to growth rather than large recruitment of bacteria from the water. The community is therefore more affected by the conditions on the membrane, while the bacteria communities in the surface water membranes are more affected by the recruitment from the water. The effect of operation modes is therfor depending on the feed water quality.

Effect of operating time

The effect of operating time is difficult to evaluate when comparing the two surface water experiments (7 and 5, Figure 3.12) operated for 8 and 16 days, since the PCR reaction was less successful, and the pattern less bright, especially for the dead-end module (B5). The membrane operated for 2 day (experiment 6), is similar to the one operated for 8 days. The bands are not that intense, but the bands are corresponding with the bands in experiment 7. This means that the initial bacteria structure is similar, but with fewer species, than after 8 days.

The tap water experiment with 8 and 17 days shows that the bacteria structure is similar. The operating time do not have a large effect on the system. Nanofiltration of tap water for two days was not done. So comparing the different results from tap water and surface water is not possible.

Due to time limits, the long lasting experiments were only operated for 17 days. The membrane in a full scale NF plant is operated for a longer period of time, before changed. One interesting aspect would have been to investigate the community for a longer period of time, *e.g.* study of a mature biofilm.

Bacteria in the feed water

The lanes named WB and WE (Figure 3.12), shows the DGGE-pattern for the surface water right after it had been collected (WB), and the end of the experiment (WE). The patterns are similar, and have only small differences, telling that the handling and storage of the water have been successful. The bacteria community has not changed dramatically over time and the storage did not affect the experiments.

By comparing the membranes operated for 2 days (experiment 6) (Figure 3.12) and the surface water (WB and WE), one may see that the bacteria structure do not resembles. The surface water has more species, and the dominating bands in the water are not corresponding to the dominating bands on the membrane. The bacteria dominating in the biofilm after two days are different from those of the water phase.

Cleaning cycles

The membranes that had been treated with a cleaning solution every day (experiment 9, Figure 3.12), showed more resemblance to the membranes operated with the untreated surface water (experiment 7). This is not an expected result, since the membranes operated with cleaning was feed with the pre-treated surface water, and therefore should have a bigger resemblance to the membranes operated with the same pre-treated water (experiment 8).

The results show that the community has the same amount of species after cleaning the membrane for 8 day. This may be because the community is adapting, and after some time biofilm is resisting the cleaning solution.

Unstable system

The membranes from the unstable system (experiment 4) were similar to the membrane operating for 8 days (experiment 7), but there are some corresponding bands that are more intense and test B4 have some band on the top of the gel, that is unique for this sample.

3.3.4 Analyzing the DGGE fingerprints

The DGGE-gel in Figure 3.12 was analyzed by the software Gel2k which made a cluster shown in Figure 3.13, from the Jaccard equation (se equation 3). The closer the samples are to each other, the more relatedness.

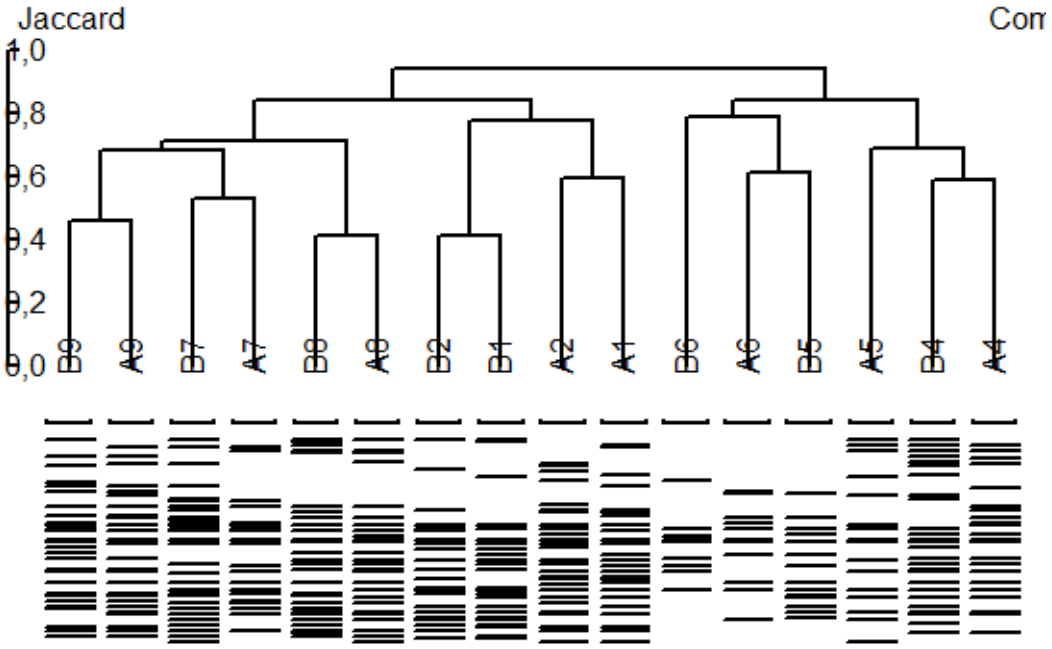


Figure 3.13: Cluster made from the Jaccard equation

The cluster illustrated in Figure 3.13 shows that the biofouling of all the membranes operated with tap water (experiment 1 and 2), have a close resemblance in the DGGE pattern, and that the operating time has less effect on the community than the mode of operation. The cluster shows that the dead-end reactors (A2 and A1) have closer resemblance to each other than the crossflow reactors (B1 and B2). This corresponds to the observation on the gel above (Figure 3.12).

The membranes operated with not pre-surface water for 8 and 16 days (experiment 7 and 8) show a different pattern. Unlike the tap water membranes, the two surface water modules operated for 8 days a more similar to each other than to the membranes with longer operating time. As mentioned earlier, there were some problems with the PCR reaction for experiment 5. The PCR products were small compare to the other, and therefore it gave DGGE patterns which were less bright (se Figure 3.12). This have an effect on the cluster, since some of the bands may not be registered as bands. The Jaccard cluster does not separate bands due to their intensity.

The two modules operated for 2 days (experiment 6), have also weak DGGE patterns, but this may be due to the short operation time. They are close on the cluster, but not as close as for the corresponding modules operated for 8 days.

The two membranes operated for 12 days, with unstable conditions (experiment 4) have a high relatedness toward each other, but they are not close related to the surface water modules operated for 8 days. As discussed earlier, experiments (4 and 7) look similar, but the individual bands that experiment 4 have, give a large outcome on the Jaccard cluster. The different community is also observed in the FISH analysis (Figure 3.21 and 3.22) discussed later.

Differences in feed waters affected how the other parameters affect the membranes. When using surface water, the modes of operation had not a big effect, while using tap water, the mode had a larger effect on the community structure. This is shown in the DGGE patterns and on the cluster analysis (Figure 3.12 and 3.13). As discussed earlier the reason for this may be due to the bacteria in the feed water.

3.3.5 Shannon-Weaver diversity index

Data obtained from Gel2k when analyzing the gel (figure 3.12) were used to calculate the Shannon–Weaver diversity indexes for the different samples, as well as the band richness and the evenness (Table 3.6).

Table 3.6: Band richness (k), Shannon-Weaver diversity index (H´) and the evenness (J´)

	k	H'	J'
A1	24	2,748	0,865
B1	24	2,580	0,812
A2	27	2,741	0,832
B2	22	2,307	0,746
A4	22	2,487	0,805
B4	26	2,798	0,859
A5	16	2,413	0,870
B5	14	2,213	0,839
A6	11	2,132	0,889
B6	9	1,842	0,838
A7	19	2,607	0,885
B7	30	2,840	0,835
A8	26	2,918	0,895
B8	28	2,891	0,868
A9	26	2,737	0,840
B9	28	2,842	0,853

The diversity indexes tell that the diversities in the different membranes are similar (Table 3.6). The Shannon index tells that the diversity in the samples is almost the same for all the samples. Also the evenness is nearer 1 than 0 for all the samples. This tells that the distribution of all the species is even.

There were no significant different ($P<0.05$) for the experiments, except experiment 6, which has low band richness and a relatively low Shannon–Weaver index (Table 3.6). Experiment 6 were the membranes operated for 2 days. This shows that the diversity of the community and the species richness in the beginning of a biofilm formation is low, and is increasing when the biofilm is more established. This may be due to pioneer bacteria that are better to attach to a clean surface and grow relatively fast in the beginning.

Experiment 9 (Table 3.6), which was cleaned with a cleaning solution have a relative high Shannon-Weaver index and species richness. As discussed earlier, most of the fouling was removed, when cleaning the membrane (Figure 3.7), but as shown in Table 3.6 and in Figure 3.12, the communities have high species richness. This means that the cleaning solution remove the overall fouling, but do not remove or affect the membrane community in biofilm. Further investigation would have been interesting to see if the biofilm formation will increase. As shown in Figure 3.7, the cleaning efficiency is decreasing trough time, and this may because the biofilm is adapting.

3.4 FISH

More than 1000 pictures was photographed with the CLSM, and used in the analyzing of the biofilm structure. The relative alpha, beta, and gamma proteobacteria biomass were calculated shown with their respective standard deviation of the mean (SDOM).

Surface water

Figure 3.14 shows an example of two pictures of the biofilm on the membrane operated for 16 days, in a dead-end mode. The left picture shows the alpha proteobacteria (yellow) relative to the total bacteria (red) in the sample, while the right shows the beta.

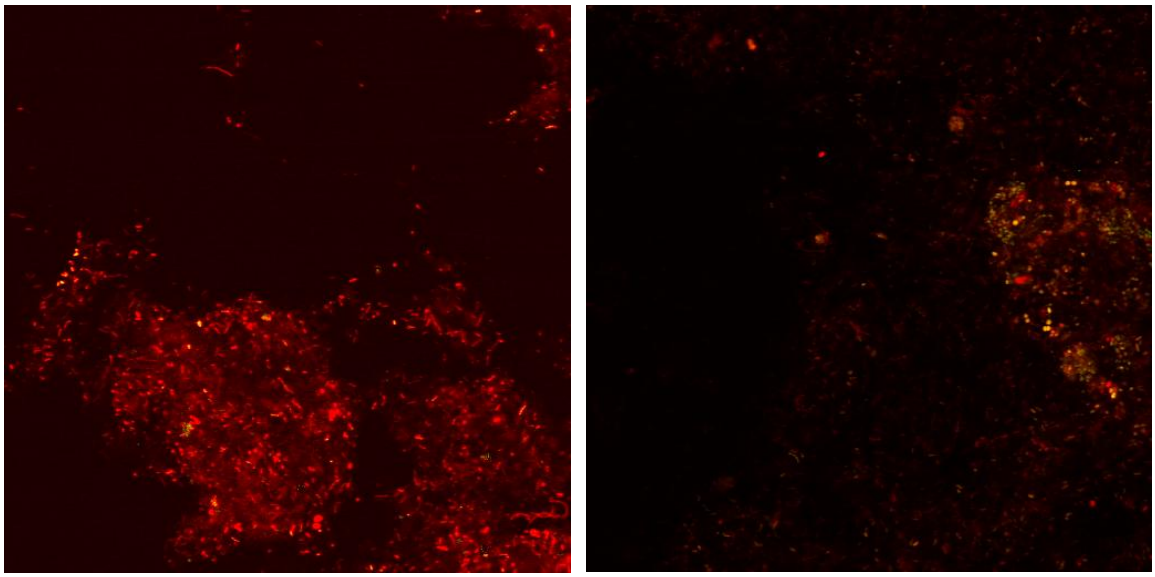


Figure 3.14: Left picture shows the alpha proteobacteria (yellow) relative to the total bacteria in the sample (red), while the right shows the beta. The membrane operated for 16 days, in a dead-end mode.

As illustrated in Figure 3.14, the pictures by themselves do not give any information, apart from showing that the chosen bacteria are absent. And that the beta proteobacteria is growing in more dens clusters (right picture), while the alpha is more evenly distributed, and are not giving the bright yellow colors as beta do. Therefore it is crucial to quantify with software that count pixels, to be able to compare two different samples.

Figure 3.15 and 3.16 shows the relative biomass of the proteobacteria as a function of time, for crossflow and dead-end module (experiment 5, 6 and 7, Table 2.2).

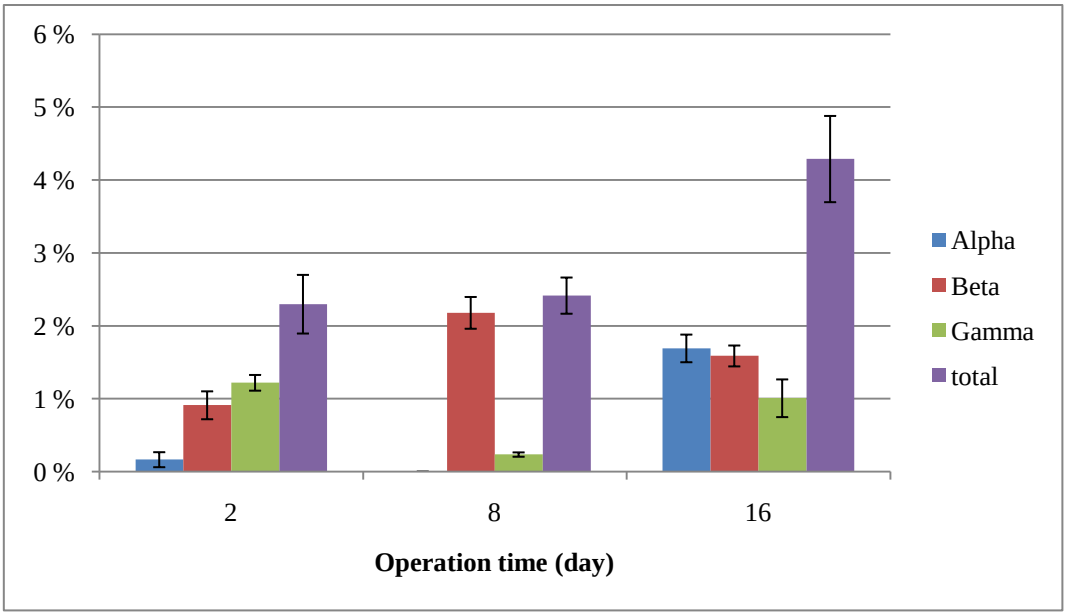


Figure 3.15: Relative biomass of the proteobacteria, with surface water as feed water, in the crossflow module, test A5, A6 and A7

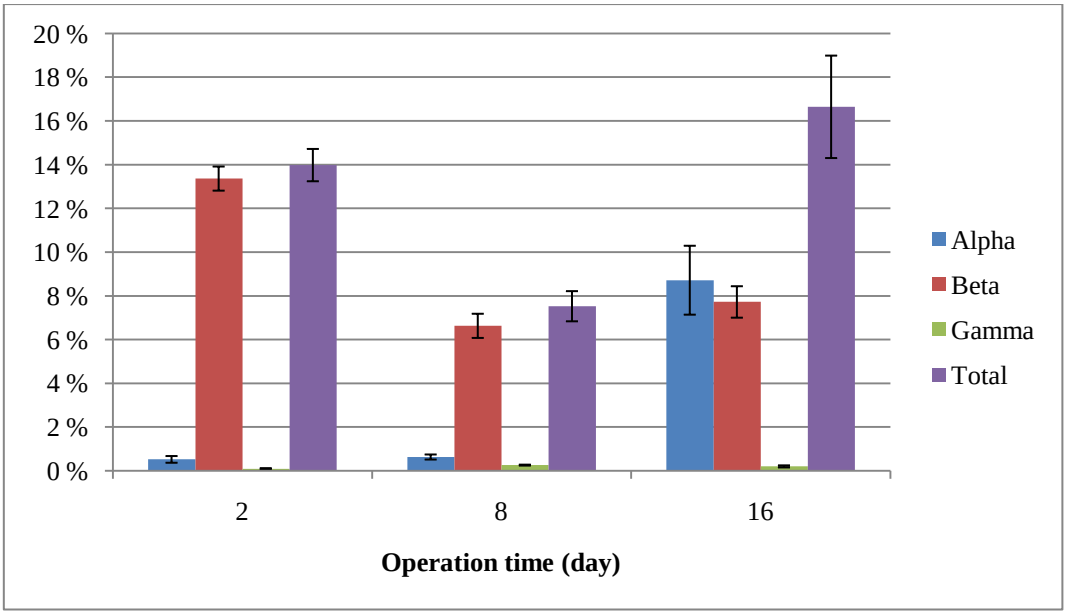


Figure 3.16: Relative biomass of the proteobacteria, with surface water as feed water, in the dead-end module, test B5, B6 and B7

Figure 3.15 and 3.16 show the relative proteobacteria as a function of time, when using surface water as feed. In Figure 3.15 the amount of total proteobacteria was increasing with operating time from 2.3 - 4.3 %. It seems that the gamma and beta proteobacteria were dominated in the beginning of the biofilm formation, and then the alpha and beta

proteobacteria gradually taking over. Hörsch *et al.* (2004) also found out that the gamma proteobacteria was the dominating proteobacteria in the beginning of biofilm formation in a drinking water system. This may be due to the fact that the gamma is better in attaching to a clean surface, while the alpha group grow faster, and dominating after some time.

However, this is not the case in the dead-end module, as Figure 3.16 illustrates that the total proteobacteria was much higher than in the crossflow (14 % vs. 2.3 %), and the total proteobacteria increased after 16 days. Also the dominating bacteria in the beginning were beta proteobacteria (13.4 %). In the end at day 16, the alpha subgroup increased to 8.7 %, while the beta have decreased a little (7 %).The gamma proteobacteria fraction was lower than 1% during the whole experiment. In a dead-end module, the bacteria do not need to attach and stick to the surface, they may be directly deposited on the membrane, and this will give different initial conditions for the biofilm formation. If the hypothesis of Hörsch *et al.* (2004) is correct, that the gamma have a property of attaching to a surface, this may become a disadvantage in the dead-end module. Since all the bacteria stays, the selection will be mainly determined by substrate competition. In these two modules, dead-end and crossflow, the alpha seems to increase with time. An explanation for this is that the alpha needs some time to establish.

As discussed, the module configuration did not have big effects on the communities on the DGGE gel (Figure 3.12). There were some corresponding bands with different intensity, but the patterns were similar. DGGE is not a true quantitative method, since the PCR reaction is not always reliable. In Figure 3.15 and 3.16 the differences is clear, the proteobacteria biomass is higher for the dead-end than for the crossflow. DGGE is therefore not sufficient to decide the quantity, but give a good illustration of the quality, which species which is absent.

3.4.1 Tap water

Figure 3.17 and 3.18 show the relative proteobacteria as a function of filtration time, for the crossflow module and the dead-end module, when using tap water as feed (experiment 1 and 2).

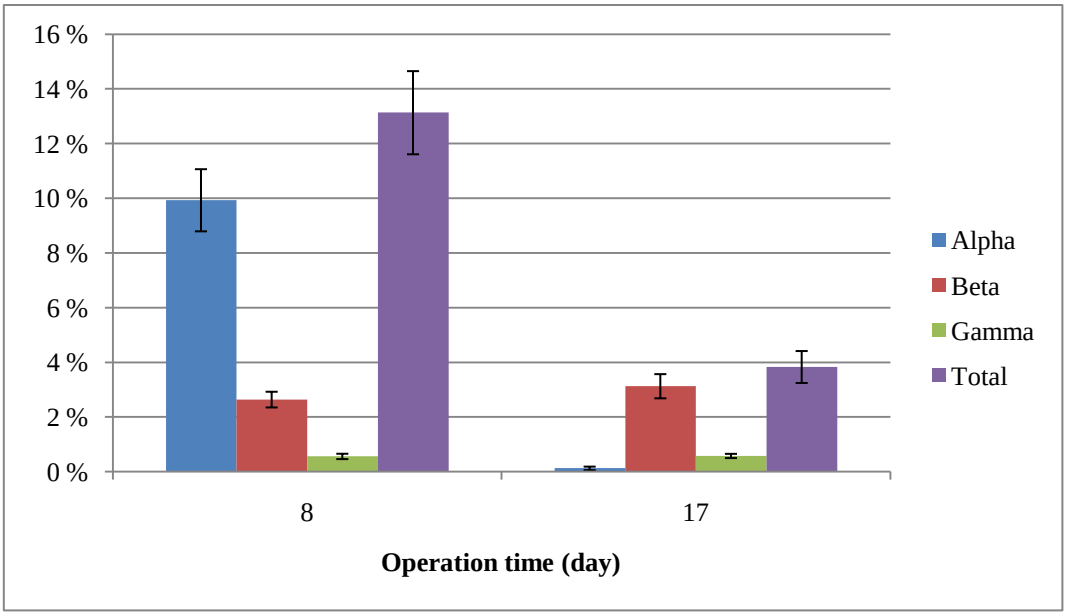


Figure 3.17: Relative biomass of the proteobacteria, with tap water as feed water, in the crossflow module, test A1 and A2

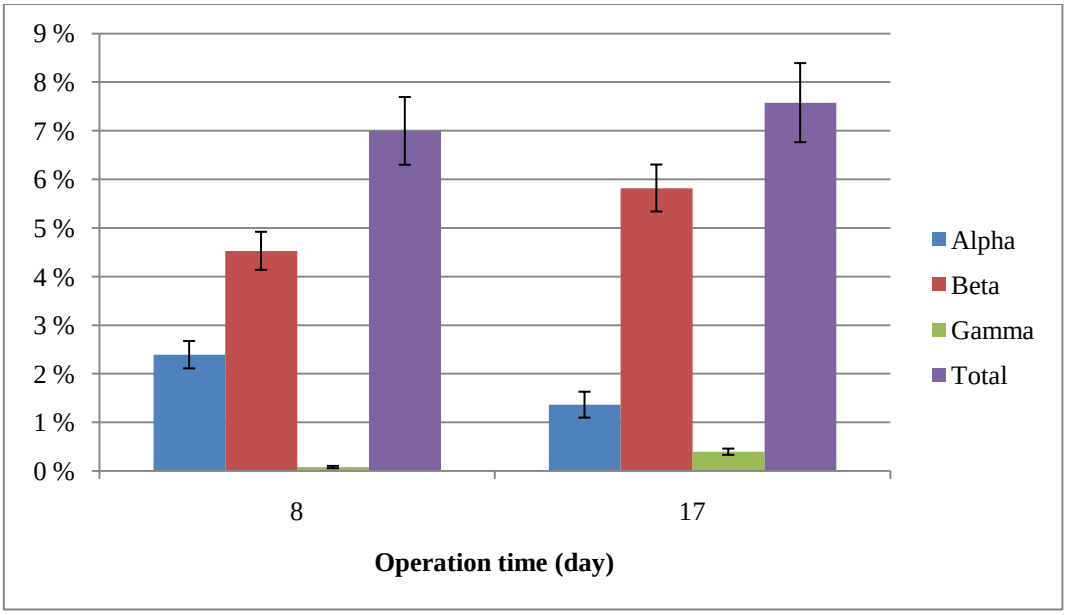


Figure 3.18: Relative biomass of the proteobacteria, with tap water as feed water, in the dead-end module, test B1 and B2

The crossflow module (Figure 3.17) had a decrease in the total proteobacteria biomass, from 13.1 % at 8 days to 3.8 % after 17 days. This was due to the alpha proteobacteria, which dominated at first, and then decreased. The beta and the gamma proteobacteria seem to be insignificantly affected over time.

In the dead-end module (Figure 3.18), the total relative proteobacteria seemed more stable, but the dynamic was changing. The beta proteobacteria increased from 4.53 % to 5.82 %, while the alpha proteobacteria decreased from 2.39 % to 1.36 %. This may be due to faster growth of the beta proteobacteria, thereby dominating the system.

However, in the surface water experiment the case was different as mentioned above. This may be due to the feed water quality, *e.g.* the particles in the water. Even if the turbidity was similar, there could be differences in the size of the particles, the origin, or its availability as nutrient for the bacteria. As discussed in the results from DGGE in section 3.3, the community is highly affected by the feed water quality.

Different operating conditions

Different operating conditions were also compared. Figure 3.19 and 3.20 shows the relative proteobacteria biomass after 8 days, with different feed water qualities (tap water, surface water and pre-treated surface water), as well as a membrane washed with NaOH, for the two different modes, crossflow and dead-end.

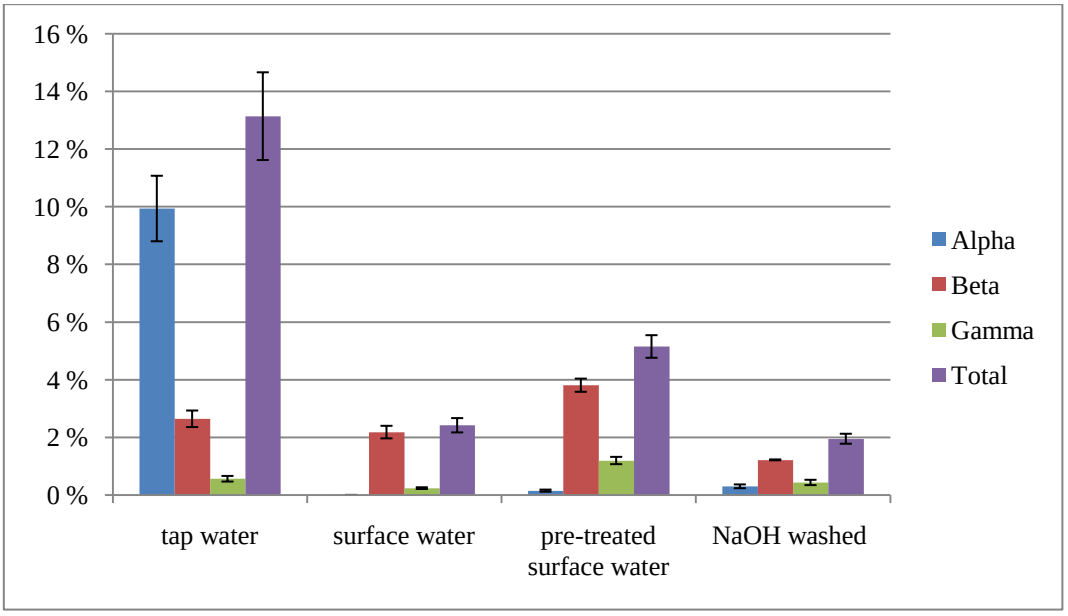


Figure 3.19: Relative biomass of the proteobacteria, after 8 days, with different feed water qualities, crossflow mode

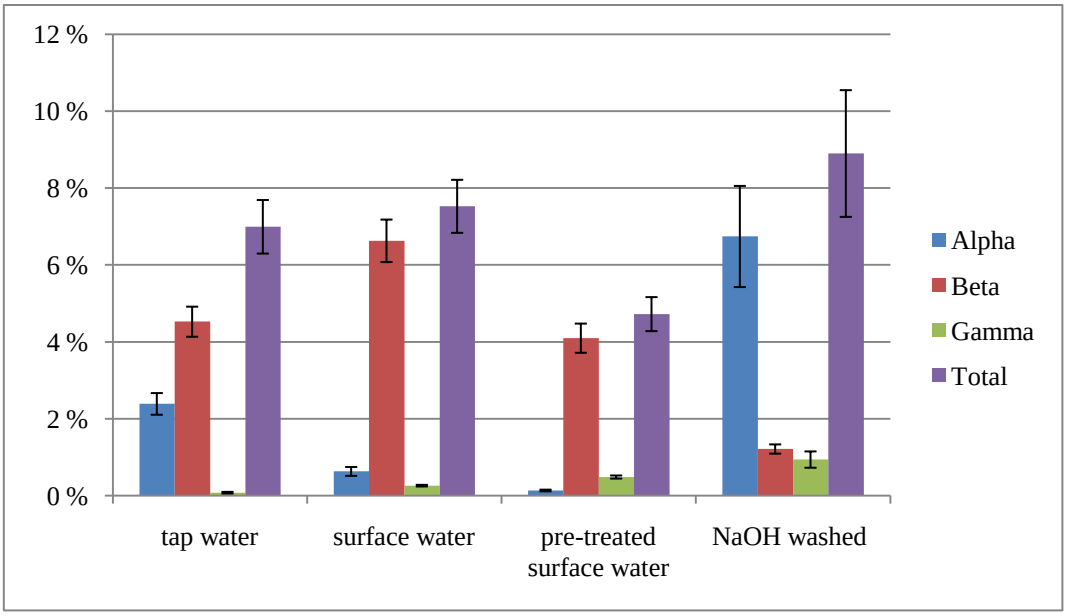


Figure 3.20: Relative biomass of the proteobacteria, after 8 days, with different feed water qualities, dead-end mode

In Figure 3.19 it was observed that the feed water quality had a significant effect on the crossflow module. The membrane operated with tap water, had a high presence of proteobacteria(13.1 %) with the alpha group dominating, while the membrane operated with surface water and pre-treated surface water, had a lower percentage of proteobacteria (2.4 % and 5.3 %). These results are the same as the results from the DGGE gel discussed earlier (section 3.3.3), the feed water quality have a large effect on the community.

The NaOH washed membrane had a lower number of proteobacteria. This may be due to lover tolerance towards the cleaning solution, therefore other, more resistant bacteria dominated during NaOH washed test.

In Figure 3.20, the feed water did not seem to have such large effect as in the crossflow module. However, the community structures changed. In the membranes operated with tap water, surface water and pre-treated surface water, the beta proteobacteria seemed to dominat in the biofilm. In the tap water test, there was also a significant amount of alpha proteobacteria. Apparently the alpha proteobacteria grew better when using tap water as a feed water source. This may be due to the water quality, including the available nutrients.

As shown in Figures 3.19 and 3.20 the NaOH washed membranes were clearly different in the two different operating modes. The relative proteobacteria was much higher in the dead-end than in the crossflow, and dominated by the alpha proteobacteria. The reason for this is that there is more accumulation of particles in the dead-end module, which may enhance quick biofilm formation, in contrast to the crossflow module, where all the extra particles are washed away by the crossflow. When the cleaning solution was used, the biofilm was thicker and the amount of bacteria larger, and this may have been an advantage for the alpha proteobacteria group.

An unstable operation

As mentioned earlier, experiment 4 was stopped and started many times under the experiment, since the system were difficult to keep stable. Still the proteobacteria analysis was performed on this membrane as well.

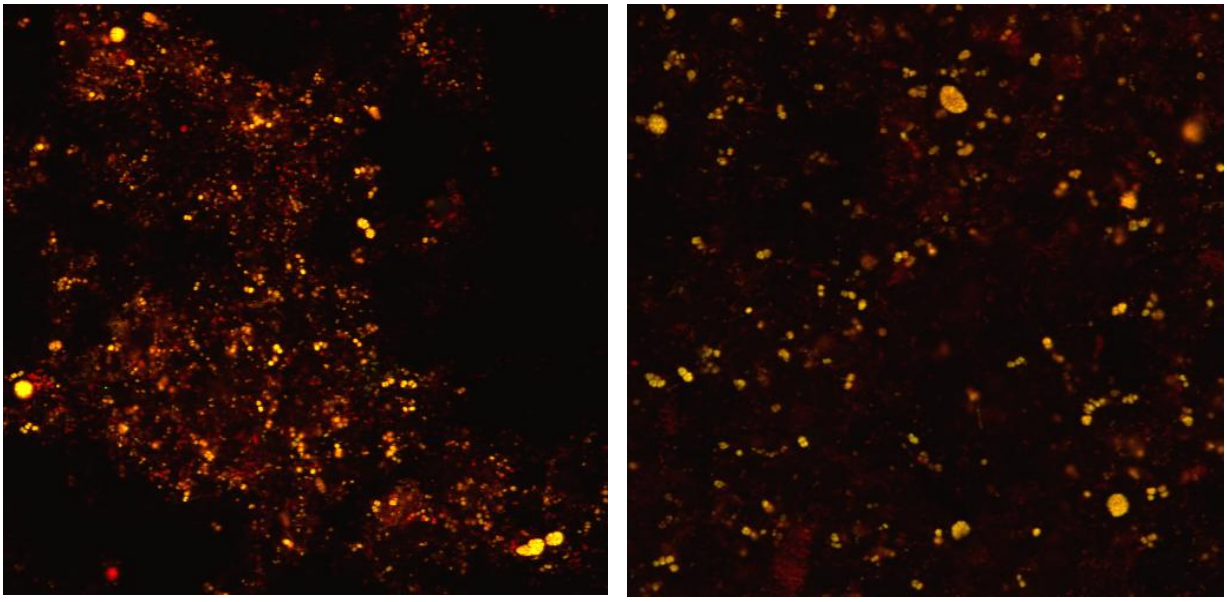


Figure 3.21: The beta-proteobacteria mass (yellow), relative to the total bacteria mass (red). Left: Crossflow mode, right: dead-end mode

Figure 3.21 shows pictures of the beta proteobacteria. As you can see in both of the modules, the beta proteobacteria (yellow) was growing in big clusters. This behavior was not observed for any of the other experiments.

Figure 3.22 shows the calculations of the relative proteobacteria for the unstable system.

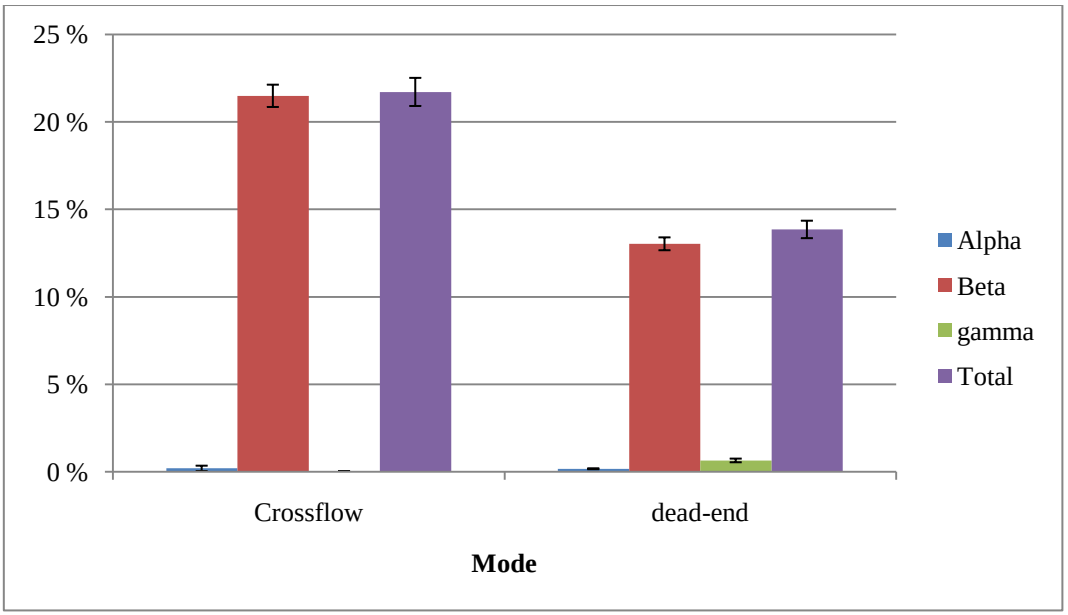


Figure 3.22: The FISH results from the unstable experiment.

The relative proteobacteria biomass is higher than all the other experiments. This membrane was not supposed to be a part of the experiment, but the results are really interesting, and illustrate that a small variation in the system may have a big effect on the community structure. One interesting aspect of these results is that the treatment plants have to shut down part the system or part of the system, due to maintenance. How these irregularities affect the biofilm in the long run may therefore be interesting since it have this large effect on the community.

3.5 DGGE and FISH

The DGGE-patterns showed that changes in the operating conditions affect the community structure of the biofilm. This may also be easily detected with FISH. The aim for the future would be to get a better understanding of the changes, and in that way gain a better control over the system. A big challenge in water- and wastewater treatment is the biofouling problem. As shown in this assignment, it is possible to use DGGE to detect small changes in the community, probably also discovering some bacteria that are responsible for the fouling, or may be good indicators for the membrane operation or forecasting irreversible fouling.

In this thesis the proteobacteria were quantified by FISH, but as the results showed, they are in small number in the NF membrane filtration system used in this thesis. Other bacteria may have been more suitable for monitoring the system with FISH, but when using FISH the desired bacteria has to be known, since analyzing is limited to only a few bacteria in the same time.

A future work may be to solve this problem by sequenced the DGGE bands and therefore identify the problem bacteria. This makes it possible to make new FISH probes, to be custom designed for the bacteria causing the problem, or indicating that something soon will happen if the parameters are not changed. FISH is an easy and quick method, which could be standard method on a treatment plant in the future, but more research on the subject is required to get a better understanding of the complicated community structure.

4. Conclusion

Using molecular methods on the NF membranes has shown to be feasible. DGGE and FISH managed to identify and quantify biofouling, and the results was used to compare different microbial communities exposed to different operating conditions.

The DGGE results showed that the type of feed water had the largest effect on the communities, even though the measured water qualities and the flux decline (fouling rate) was the same. The communities on the membrane operated for the same time, with different feed waters (tap, surface and pre-treated surface water) showed large differences in the DGGE patterns. The choice of pre-treatment of the water is therefore an important parameter that is crucial to investigate, and may be used for optimizing the full scale NF plants.

The effect of operating mode (dead-end *vs.* crossflow) had larger effect for the experiments operated with tap water, than the experiments operated with surface water. The operating time was not found to have a large effect on the bacteria structure. There were no large resemblance between the bacteria in the water, and the bacteria in the early biofilm formation.

Combination of FISH, CLSM and the software ImageJ, a quantification of the relative proteobacteria was obtained. FISH were suitable for detection of changes for the chosen bacteria under different operating conditions.

The fouling on the NF system was quickly formed, and the flux decline was the highest after the first two days of operation, and was not affected by the feed water quality. Use of a cleaning solution kept the flux level significantly higher, but despite of less fouling the bacteria structure seemed to have relatively high species richness and diversity.

The molecular methods were investigated and showed that

- The choice of extraction kit could affect the community DNA results with different community structures.
- Large PCR products (>500 bp) do not manage to penetrate the gel in the INGENY phorU DGGE system.
- The optimal denaturing gradient in this theses were between 35 - 55 %.

The lab-scale NF-membrane module has shown to be a reproducible system, after comparison of two microbial community patterns from two membranes operated under the same conditions.

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Appendix list

Appendix 1 – Media and solutions used in DGGE

Enzymatic lyses buffer

Table A1.1: Recipe for enzymatic lyses buffer

Reagent and its concentration
20mM Tris-Cl, pH 8
2mM EDTA
1.2 % Triton® X-100
29mg/ml lysozyme

Primers

In the simple PCR reaction, two primers were used, *e.g.* 338f_GC and 518r, while in the nested PCR reaction four different primers were used, *e.g.* 27f and 1492r in first round, 968F_GC and 1492r for the second round.

Table A1.2: Primers with their respective nucleotide sequence, and references

Primer	Nucleotide sequence	References
338f_GC	cgc ccg ccg cgc gcg gcg ggc ggg gcg gGG GCA CGG GGG GAC TCC	(Amann *et al.*, 1990b) (Muyzer *et al.*, 1993)
518r	ATT ACC GCG GCT GCT GG	(Amann *et al.*, 1990b)
27f	AGA GTT TGA TYM TGG CTC AG	(Lane, 1991)
1492r	GGY TAC CTT GTT ACG ACT T	(Lane, 1991)
968f_GC	cgc ccg ccg cgc ccc gcg ccc ggc ccg ccg ccc ccg ccc cAAC GCG AAG AAC CCT AC	(Muyzer *et al.*, 1995)

PCR master mix

When analyzing more than one sample, a master mix was used, as shown in table A1.3.

Table A1.3: The reagents and the amount used in the simple PCR reaction

Reagent and its concentration	Amount (µl)
Template	2
10 x reaction buffer	5
dNTP (10mM)	1
$MgCl_2$ (25mM)	1
BSA (10mg/ml)	1.5
Forward primer (3µm)	1.5
Reverse primer (3µm)	1.5
Taq polymerase	0.25
H_2O (sterile, ion-free)	36.25

PCR cycles

In the simple, one round PCR reaction, 35 repetitions were used. Table A1.4 gives an illustration on the temperature and time for each step in the PCR reaction.

Table A1.4: PCR reaction regime

Temperature (°C)	Time	Repetition
95	3 min	1
95	30 sec	35
55	30 sec	35
72	60 sec	35
72	30 min	1
4	∞	

In the nested PCR, two different rounds with PCR reaction, was preformed. Table A1.5 shows the master mixes used in 1^{st} and 2^{st} round of the nested PCR.

Table A1.5: The reagents and the amount, for both rounds, used in the nested PCR reaction

Reagent and its concentration	Amount (µl) 1st	Amount (µl) 2st
Template	2	2 (from 1st Round)
10 x reaction buffer	2.5	5
dNTP (10mM)	0.5	1
$MgCl_2$ (25mM)	1	1.5
BSA (10mg/ml)	1.25	0
Forward primer (3µm)	1.5	1.5
Reverse primer (3µm)	1.5	1.5
Taq polymerase	0.2	0.2
H_2O (sterile, ion-free)	39	41.3

The regimes for the two rounds are showed in table A1.6 and A1.7 respectively.

Table A1.6: The regime of the 1st round in the nested PCR reaction

Temperature °C	Time	Repetitions
94	5 min	1
94	30 sec	20
50	30 sec	20
72	60 sec	20
72	5 min	1
4	∞	

Table A1.7: The regime of the 2st round in the nested PCR reaction

Temperature °C	Time	Repetitions
94	5 min	1
94	30 sec	30
50	30 sec	30
72	60 sec	30
72	5 min	1
4	∞	

TAE buffer

Table A1.8: recipe for 50x TAE buffer

Reagent	Amount	Final concentration
Tris base	242.0g	2M
Acetic acid glacial	57.1ml	1M
0.5 M EDTA, pH 8	100ml	50mM
dH_2O	to 1000ml	

Acrylamid solutions

Table A1.9: recipes for different denaturing gradients, by mixing 0% and 80% denaturing solutions.

Denaturing percent	0%	80%	TEMED + 10% APS	Total volume
25%	16.5ml	7.5ml	16µl +87µl	24ml
35%	13.5ml	10.5ml	16µl +87µl	24ml
55%	7.5ml	16.5ml	16µl +87µl	24ml
60%	6ml	18ml	16µl +87µl	24ml

Table A1.10: 0% denaturing solution

Reagent	8% gel
40% acrylamid (bioRasLab Inc.)	100ml
50 x TAE	5 ml
dH_2O	To 500ml

Table A1.11: 80% denaturing solution

Reagent	8% gel
40% acrylamid (bioRasLab Inc.)	50ml
50 x TAE	2.5ml
Urea	84g
Formamide (deionized)	80ml
dH_2O	To 250ml

Appendix 2 - Media and solutions used in FISH

Hybridization buffer

360 µl NaCl 5M

40 µl TRIS-HCl 1M

MilliQ Water (see table A2.1)

Formamide (see table A2.1)

2 µl SDS 10 %

Washing buffer

NaCl 5M (see table A2.2 for amount)

1 ml of TRIS-HCL 1M

EDTA 0.5M (see table A2.2 for amount)

Raise the volume with MilliQ water up to 50 ml

50 µl SDS 10 %

Table A2.1: Amount of formamide and milliQ water to prerare the hybridization buffer

Formamide amounts (µl)	% Formamide	H_2o MilliQ amount (µl)
0	0	1598
100	5	1498
200	10	1398
300	15	1298
400	20	1198
500	25	1098
600	30	998
700	35	898
800	40	798
900	45	698
1000	50	598

Table A2.2: Amount of NaCl and EDTA to prepare washing buffer

% Formamide	NaCL amount (µl)	EDTA amount (µl)
0	9000	-
5	6300	-
10	4500	-
15	3180	-
20	2150	500
25	1490	500
30	1020	500
35	700	500
40	460	500
45	300	500
50	180	500

Phosphate Buffered Saline, ph7.2

30x PBS: add 38.7g $Na_2HPO_4.12H_2O$, 6.6g $NaH_2PO_4.2H_2O$ and 113,1g NaCl to 500ml of MilliQ water. Autoclave and store as stock.

Dilute 1:10 for 3x PBS (for PFA solution) and 1:30 for 1x PBS (for direct application).

% Paraformaldehyde

Set up the balance and a heated stirrer in the fume hood. Warm 65ml of purified water to 60°C. Weight out 4g of PFA powder. Add PFA to the water to obtain a cloudy solution. Add 2 drops of 2M NaOH and the PFA should be dissolved in 1-2min. Cool to room temperature and add 33ml of 3x PBS. Adjust the pH to 7.2 with 1M HCl. Filter through 0.2µm filter to remove any undissolved crystals. Aliquot applicable volumes and freeze.

5M NaCl

Add 58g of NaCl to 200ml of MilliQ water. After diluting, autoclave.

1M Tris-HCl

Add 31.5g of Tris-HCl to 150ml of MilliQ water, dissolve and adjust pH to 7.2 with 2M NaOH. Make up to 200ml with MilliQ water and autoclave.

0.5M EDTA

Add 18.6g of EDTA disodium hydrat to 75ml of MilliQ water. Adjust pH to 7.2 with 2M NaOH pellets. Make up to 100ml with MilliQ water and autoclave.

10% SDS

Dissolce 10g of SDS in 100ml of MilliQ water.

Table A2.3: The probes target bacteria, fluorochrom and there sequences

Target	Probe	Fluorochrom	Sequence 5`-3`	References
Most Bacteria	EUB338	Cy 5	GCT GCC TCC CGT AGG AGT	(Amann *et al.*, 1990a)
	EUB338 II	Cy 5	GCA GCC ACC CGT AGG TGT	(Daims *et al.*, 1999)
	EUB338 III	Cy 5	GCT GCC ACC CGT AGG TGT	
Alpha proteobacteria	ALF969	Cy 3	TGG TAA GGT TCT GCG CGT	(Oehmen *et al.*, 2006)
	cALF969a	-	AGG TAA GGT TCT GCG CGT	
	cALF969b	-	GGG TAA GGT TCT GCG CGT	
Beta proteobacteria	BET42a	Cy 3	GCC TTC CCA CTT CGT TT	(Manz *et al.*, 1992)
	cBET42a	-	GCC TTC CCA CAT CGT TT	
	c1033	-	GCC TTC CCA CCT CGT TT	(Yeates *et al.*, 2003)
Gamma proteobacteria	GAM42a	Cy 3	GCC TTC CCA CAT CGT TT	(Manz *et al.*, 1992)
	cGAM42a	-	GCC TTC CCA CTT CGT TT	
	c1033	-	GCC TTC CCA CCT CGT TT	(Yeates *et al.*, 2003)