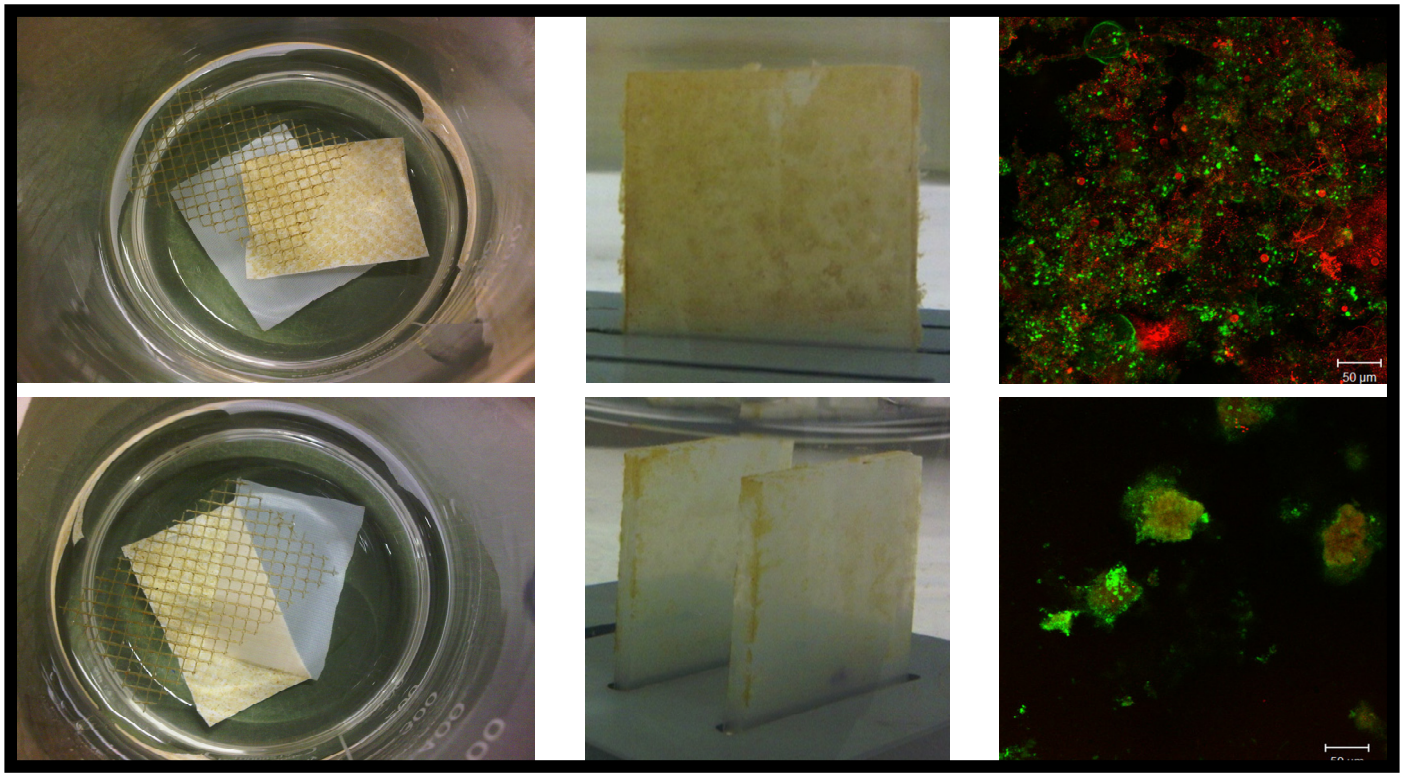

Assessment of the biofilm removal efficiency of cleaning agents and procedures for RO/NF membranes

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KWR Watercycle Research Institute

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Postbus 1072 3430 BB Nieuwegein T 030 606 95 11 F 030 606 11 65 E info@kwrwater.nl I www.kwrwater.nl

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Dick van der Kooij

Authors
Wim Hijnen[a], Castillo Cristina[b], Anke Brouwer-Hanzens[a], Emile Cornelissen[a] and D. van der Kooij[a]

[a] KWR, Nieuwegein
[b] Technical University München

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Preface and acknowledgement

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Samenvatting/summary

Biofouling van hoge druk RO/NF membranen is een van de belangrijkste operationele problemen bij de toepassing van dit proces. Dit probleem wordt beheerst door voorbehandeling, membraan ontwerp en membraan reiniging. Deze laatste beheersmaatregel is meestal ontworpen lokaal specifiek door trial en error en gebaseerd op de verbetering van operationele condities. Kwantitatieve informatie over biofilm verwijdering is geen selectie criterium. De wetenschappelijke literatuur over de doeltreffendheid van reinigingsmiddelen en procedures om biofilm te verwijderen van oppervlakken is beperkt en meestal kwalitatief. Een kwantitatieve laboratorium test was ontwikkeld om een breed range van producten en procedures te screenen op hun reinigingsefficiëntie op een kosten effectieve, snelle en simpele wijze.

In dit rapport de productie van uniforme biofilm monsters was verder geoptimaliseerd en deze monsters zijn toegepast in een breed screenende test met verschillende chemische producten en reinigings protocols die bij membraan reinigingen worden toegepast. De studie bevatte basische en zure reinigers, detergenten, compexeerders, biocyden en enzymen (analytisch gedefinieerde middelen en commerciële mengsels) als ook specifieke reinigings protocollen toegepast onder door de Nederlandse waterbedrijven. Aanvullend onderzoek was uitgevoerd om het effect van de biofilm karakteristieken te bepalen en om biofilm monsters van full-scale RO membranen te testen. De parameters die zijn gebruikt om de reiniginseffecten te kwantificeren zijn de biochemische parameters ATP voor het effect op levende bacteriële biomassa en koolhydraten (CH) als belangrijkste component van de bacteriële exopolymere substanties (EPS). Om de biofilms voor en na reiniging te visualiseren is gebruik gemaak van confocal laser scanning microscopie (CLSM).

De globale waarneming in de test resultaten was dat ATP verwijdering meer duidelijke verschillen vertoonde tussen de geteste chemicaliën en protocollen dan de verwijdering van CH. ATP verwijdering varieerde van 0 - > 99%. Het toont het effect van de behandeling op de levende bacteriële biomassa maar dit kan niet direct worden gerelateerd aan biomassa verwijdering. CH verwijdering als een indicatie voor de verwijdering van bacteriële EPS was meestal minder dan 50%. Het gebruik van het oxidatieve middel als NaOCl als een positieve controle voor biomassa verwijdering toonde dat een 70% verwijdering van EPS haalbaar is. Dit middel kan echter niet worden gebruikt in de praktijk door de schade die het aanricht aan het membraan.
Een mengsel van NaOH en Natrium Dodecyl Sulfaat (SDS; 1%) is gebruikt in de testen als een referentie behandeling. De resultaten met dit mengsel toonden dat de ATP removal van de PVC-P biofilms varieerde van 50 - >99% en de CH verwijdering van 20 – 80%. Een hoge verwijderingefficiëntie werd waargenomen voor de biofilms op PVC-P biofilms slang met een lage verhouding tussen CH en ATP (µg/ng) van <0,8. Bij waarden van >0,8 die werden waargenomen bij de biofilms op PVC-P plaatjes nam het effect van de CH/ATP verhouding op de verwijderingefficiëntie af. Dit toont aan dat de biofilm condities de reinigingsefficiëntie beïnvloeden. De breed screenende test is uitgevoerd met de PVC-P plaatjes met een CH/ATP ratio van > 0,8. De NaOH/SDS efficiëntie voor CH varieerde van 21 – 36%. De gegevens toonde dat alleen NaOCl en de kation detergent CTAB meer effectief waren in de verwijdering van biofilm dan NaOH/SDS en al de andere individuele chemicaliën als ook de commerciële mengsels presteerden minder dan NaOH/SDS. Bovendien, ook alle Multi-stap behandelingen met analytische gedefinieerde producten en commerciële mengsels bij 35°C lieten geen duidelijke toename in biofilm verwijdering zien. De combinatie van Divos 2 en Divas 116 (zuur+base) was de enige Multi-stap behandeling met een verhoogde CH verwijdering van 47%.

Voorlopige resultaten met de membraan biofilms van een ontzoutinginstallatie toonden dat biofilm verwijdering bij deze monsters in dezelfde orde van grootte of hoger waren dan waargenomen voor de PVC-P biofilms ondanks de veel hogere CH/ATP verhoudingen. Dit laat zien dat deze eigenschap van de biofilm niet universeel kan worden gebruikt om de weerstand van biofilm tegen reiniging te karakteriseren. Het onbekende effect van de vergelijking tussen een zeewater en zoetwater biofilm en het beperkte aantal gegevens over membraan biofilms benadrukt de noodzaak voor aanvullende vergelijkende testen om de voorspellende waarde van de laboratorium reinigingstest om optimale chemicaliën en protocollen te selecteren te verifiëren.

De aanvullende waarde van de CLSM analyse is aangetoond in deze studie. De beelden toonden duidelijk de verschillende effecten op de nucleotiden (ATP) en EPS (CH) concentraties en ook op de ruimtelijke structuur van de biofilm laag die duidelijk 'implodeert'. Kwantitatieve CLSM gegevens over de reductie van de biofilm dikte door een aantal chemische behandelingen toonde een vergelijkbare trend als berekend uit de ATP en CH verwijderingen.

Eindconclusie van de studie is dat de laboratorium test met uniforme biofilm monsters en ATP en CH analyses een betrouwbare test is om de biofilm verwijderingefficiëntie van chemicaliën en procedures vast te stellen. De resultaten van de biofilm productie en testen verhogen het inzicht van de effecten van reinigingsmiddelen en procedures op biofilms. De resultaten aangevuld met CLSM analyses vergroot daarmee het algemene beeld van biofilm groei op oppervlakken en de weerstand tegen chemische stress factoren.

Deze studie was echter niet uitputtend en sommige aspecten moeten verder worden onderzocht om te komen tot een algemeen toepasbare methode:

- Meer gegevens zijn nodig over de invloed van de biofilm eigenschappen op de verwijderingefficiëntie;
- Aanvullende laboratoriumtesten zijn nodig om het kwantitatieve effect van de volgende belangrijke variabelen te bepalen: diverse combinaties van middelen, pH, temperatuur, reinigingsduur en mechanische krachten;
- Aanvullende onderzoek naar het gebruik van de test om de verwijdering van anorganische vervuilende als componenten (scalants) en organische colloïdale verbindingen (biocolloïdale stoffen als TEP);
- Meer onderzoek naar innovatieve reinigingsstrategieën zoals de toepassing van de fenton reactie geïnduceerd door de aanwezigheid van Fe en het gebruik van peroxide of andere potentiële combinaties met de milde oxidator peroxide (detergenten);
- NF/RO membraan experimenten om
    - te verifiëren of de best presterende reinigingsoptie inde laboratorium test ook het best presteert bij membraanreiniging;
    - te verifiëren of deze beste optie ook goed is voor de algemene prestatie karakteristieken van de membranen (drukval, water flux en zout passage) die door biofouling kunnen worden beïnvloed.

Summary

Biofouling of high pressure membranes is one of the major operational problems in the applications of this process. This problem is controlled by pre-treatment, membrane design and membrane cleaning. The latter control measure is usually designed locally specific by trial and error and focussed on the effects on improvement of operational conditions. Quantitative information on biofilm removal is no selection criterion. The scientific literature on the efficacy of cleaning agents and procedures to remove biofilms from surfaces is limited and mostly qualitative. A quantitative laboratory test was developed to screen a broad range of product and procedures for their cleaning efficiency in a cost effective, rapid and simple manner.

In this report the production of uniform biofilm samples was optimized further and these samples were applied in a broad screening test with different cleaning agents and protocols applied in membrane cleaning. The survey consisted of alkaline and acidic cleaners, detergents, chelates, biocides and enzymes (analytical grade and commercial blends) as well as the specific cleaning protocols applied under Dutch Water Companies. Additional research was done to investigate the effect of the biofilm characteristics and test also biofilm samples from full-scale RO membranes. The parameters used to quantify the cleaning effect were the biochemical parameters ATP for the effect on the living bacterial biomass and carbohydrates (CH) as the major component of the bacterial exopolymeric substances (EPS). To visualize the biofilms before and after cleaning confocal laser scanning microscopy (CLSM) was applied.

The overall observation in the test results was that ATP removal showed more distinct differences between the tested agents and protocols than the removal of CH. ATP removal ranged from 0 - >99%. It reflects the effect of the treatment on the living bacterial biomass but this is not necessarily related to biomass removal. CH removal as an indication of the removal of bacterial EPS was usually less than 50%. The use of the oxidant NaOCl as a positive control showed that 70% of EPS removal is achievable. But this agent can not be used in practice due to the damage of the membranes.
A mix of NaOH and Sodium Dodecyl Sulphate (SDS; 1%) was used in the tests as a reference treatment. The results with this mixture showed that the ATP removal from the PVC-P biofilms ranged from 50 - >99% and the CH removal from 20 – 80%. The high removal efficiencies were observed for biofilms on PVC-P tubing with a low CH/ATP ratio (µg/ng) of <0.8. At values >0.8 observed for the PVC-P plates the effect of this ratio on the removal efficiency diminishes. This demonstrates the effect of the biofilm conditions on the cleaning efficiency. The broad screening test was performed with biofilms on PVC-P plates with CH/ATP ratio >0.8 where the NaOH/SDS efficiency to remove CH ranged from 21 – 36%. The results showed that only NaOCl and the cationic detergent CTAB were more effective in removing biofilm and all other individual agents as well as the commercial blends performed less than the mixture NaOH/SDS. Furthermore, also all of the multi-step treatments with analytical grade chemicals and commercial blends tested at 35°C did not show a significant increase in biofilm removal. The combination of Divos 2 and Divos 116 (acid+alkaline) was the only multi-step treatment with an elevated CH removal of 47%.

Preliminary tests with membrane biofilms from a desalination plant showed that biofilm removal efficiencies from these samples were in the same order of magnitude or higher as observed for the PVC-P biofilms despite much higher CH/ATP ratio's, indicating that this characteristic can not be used universally to characterize the resistance of biofilms against cleaning. The unknown effect of comparing seawater and fresh water biofilms and the limited amount of data on membrane biofilms, however, emphasize the need for additional comparative tests to verify the predictive value of the laboratory cleaning test for selecting the most optimal cleaning agent or protocol.

The additional value of CLSM analysis was demonstrated in this study. The images clearly showed the different effects of cleaning on the nucleotide (ATP) and EPS (CH) concentrations and also on the spatial structure of the biofilm layer which collapsed. Quantitative CLSM data on reduction of the biofilm thickness by a number of chemical treatments showed similar trends as calculated for the removal of ATP and CH.

Overall conclusion of the study is that the laboratory test using uniform biofilm samples and ATP and CH analysis is a reliable test to assess the biofilm removal efficiency of agents and procedures. The results of biofilm production and testing increase the insights on the effects of cleaning agents and procedures on biofilms. The results, supplemented with CLSM analysis, additionally increase the general views on biofilm growth on surfaces and its resistance against chemical stress.

This study, however, was not an exhausted study and some aspects need further research to obtain a general applicable method:

- More data are needed on the influence of the biofilm characteristics on the cleaning efficiency;
- Additional laboratory tests are required to assess the quantitative effect of the following major cleaning variables on the clening efficiency: combined chemical treatments, pH, temperature, cleaning duration and shear forces during cleaning;
- Additional studies after the application of the laboratory test to assess the efficacy of agents and procedures to remove inorganic fouling deposits (scalants) as well as biocolloidal deposits (TEP);
- More research is needed to explore innovative cleaning strategies such as the fenton process induced by the presence of Fe and the use of H_2O_2 or other potentially interesting combinations such as peroxide with a detergent.
- SWM cleaning experiments to
    - verify whether the 'best' selected cleaning agent/protocol in the laboratory test is also the 'best' cleaning agent/protocol for SWM elements;

- verify what the effect is of chemical cleaning on the general performance characteristics of membrane processes (NPD control, water flux and salt passage) impaired by biofouling.

Inhoud

1 Introduction

Currently the global drinking water demand is increasing, together with more and stricter regulations on its quality. Due to the decrease in costs of membrane applications, an increasingly important role for membrane filtration is expected in the future (Mallevialle, 1996). A downside of both nanofiltration (NF) and reverse osmosis (RO) technologies is the tendency to membrane fouling, especially by biofouling, which causes an increase of the normalized pressure drop and/or decrease of normalized flux. These types of operational problems will lead to higher plant operation costs, and they may limit the application of membrane filtration in (drinking) water treatment (Vrouwenvelder, 2008).
The problem of membrane fouling is controlled by balancing pretreatment of the feed water and membrane cleaning practices. The latter control measure causes however costs due to loss of production time and costs on chemicals. In the current literature, most of the research on operational problems and efficacy of cleaning agents and protocols for membranes is based on the effect on restoration of the operational conditions such as, pressure drop, salt passage and flux decrease. Therefore, there is a lack of information on the quantitative relationship between water quality and biomass parameters related to biomass accumulation causing these operational problems in NF/RO membranes. This information is needed for diagnosis purposes, improvement of pretreatment and, in the case of the presented research, for assessing the efficacy of cleaning procedures to control biofouling. Recent studies have shown that bioassays and specific biomass parameters can quantitatively be related to biofouling of spiral wound membranes (Hijnen et al., 2009; 2011; Van der Kooij, 2010).
Chemical cleaning is an important part of the membrane process operation and it has a deep influence on the performance and economics of membrane processes (Liu et al, 2001). Cleaning in Place (CIP) protocols in RO/NF full-scale installations are usually tailor made and specific for the plant operational conditions and feed water without performing an exhausting and costly comparative study. There are only a few studies available presenting some generalized results on chemical cleaning. Therefore it is necessary to carry out an evaluation of the major used chemicals and procedures in membrane practices in order to have a better understanding of the biofilm removal dynamics. This assessment is done in this research by means of a laboratory-scale test which mimics the mechanical cleaning step and rinsing step of the CIP procedures.
In the research done at KWR a laboratory cleaning test is being developed to find a cost effective way of screening and developing cleaning strategies with an optimized biofilm removal efficacy. In earlier studies (Lahondes, 2010; Van der Kooij et al., 2011) the principle components of such a test were developed. The aim of the current research is to evaluate the efficay of chemical agents as individual cleaning agent or in a sequence of cleaning steps (sequences of Dutch Drinking Water Companies) and the influence of cleaning conditions (temperature and pH) on the biofilm removal from surfaces. To achieve this, one of the most important aspects is to ensure the production of homogeneous and reproducible biofilm samples, in order to guarantee a proper evaluation of the cleaning efficiency of each cleaning chemical. For this, different biofilm production units working under various controlled conditions (feed water quality and flow, nutrients dosing, etc.) were employed. This enabled to verify the influence of the biofilm characteristics of the cleaning efficiency. To validate and complement the data obtained from these laboratory measurements, CLSM imaging of cleaned biofilm samples were carried out to assess the value of CLSM as an additional tool.

2 Membrane cleaning

The purpose of chemical cleaning is to break down the structure of the fouling layer to allow the removal of the foulants by hydraulic rinsing (Li and Elimelech, 2004). This requires a synergy between the chemical and physical conditions of the cleaning process since the chemical reaction between the cleaning agent and the foulant layer is related to the mass transfer of cleaning agent from the bulk solution to the fouling layer and the foulants from the fouling layer back to the bulk solution (Figure 2.1). Therefore, a highly effective cleaning process will be accomplished when both chemical and physical interactions are favorable (Ang, 2006).
Assuming that the cleaning agent has a favorable chemical reactivity with the foulant, their chemical reaction will weaken the structure of the foulant layer. After this step the physical (hydrodynamic) conditions will play an important part in removing the foulants from the fouling layer by means of the mass transfer of the reaction products. Nevertheless, it should be stressed that if the cleaning agent does not have a favorable chemical reaction with the foulant, the improvement of the physical conditions will not increase the cleaning efficiency (Ang, 2006).

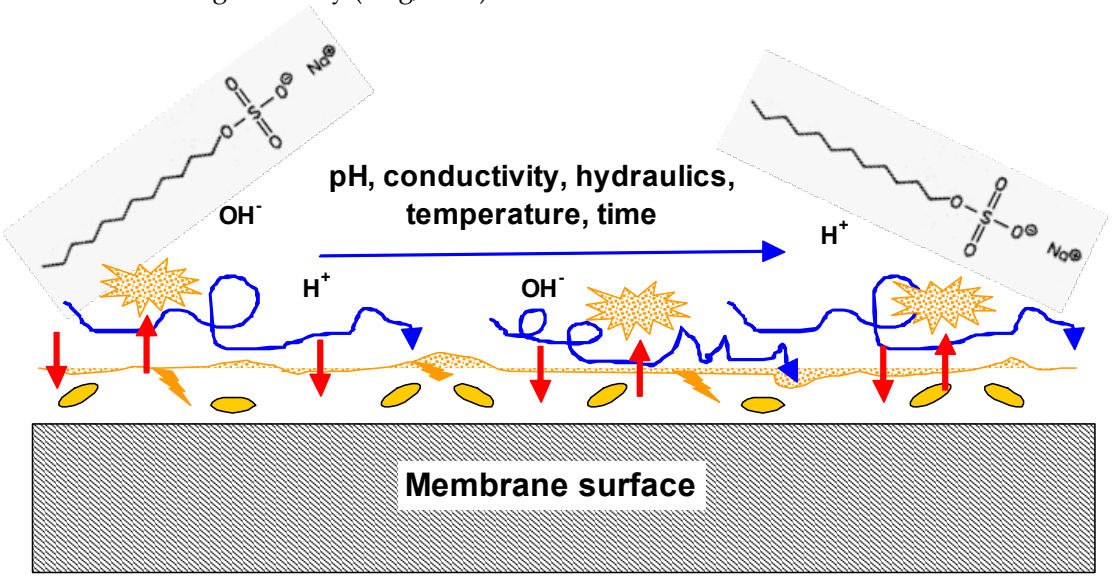


Figure 2.1. Conceptual model of the cleaning process.

These interactions are represented in Figure 2.1, where a scheme of the hypothesized mechanism of the chemical cleaning is shown. Considering this, different factors affect cleaning efficiency such as: chemical agent concentration, pH, cleaning time, temperature and hydrodynamic conditions during the cleaning process, as mentioned by different authors (Ang et al, 2006; Liu et al, 2001; Zondervan and Roffel, 2007; Liikanen et al, 2002). Hydrodynamic conditions are one of the most important factors since mass transfer within the fouling layer is supposed to be the rate-limiting factor. Concentration of chemicals, cleaning time and temperature affect the mass transfer and consequently the chemical reaction. Temperature is presumed to change the reaction equilibrium by enhancing reaction kinetics and by increasing the solubility of solutes, causing an impact on the efficiency and rate of membrane cleaning (Liu et al, 2001).

2.1 Cleaning Agents

There are different types of cleaning agents available in the market, and their effectiveness or reaction with the foulant layer will depend on its chemical characteristics and on the characteristics of the membrane foulant layer. Other important aspects to take into consideration are the compatibility of the chemical with the membrane material, the safety, stability, price and waste treatment of the cleaning agent (Zondervan and Roffel, 2007).

Cleaning agents can be divided into the following categories (Ang, 2006; van der Kooij et al., 2010; Liu et al., 2001):

1. Alkaline: their cleaning mechanism is through hydrolysis and solubilization. Alkaline chemicals are commonly used for dissolving organic deposits and removing biological material from the membrane surface. Organic foulants are normally negatively charged in aqueous solution (Liikanen et al., 2002); this negative charge is enhanced due to the high pH (pH 10-12) of the alkaline chemicals which increases the electrostatic repulsion within the biofilm and as a result the penetration of the cleaning agent is enhanced. Additionally, a high negative charge results in an increase in the solubility of the organic foulant. Alkaline conditions will also dissolve bacterial cells causing increased cell lysis and death (inactivation) which affects enzymatic reactions.
2. Acid: because of their low pH (pH 2-4) they are able to dissolve inorganic precipitates (scaling) and the inorganic matrix in a biofilm, e.g., iron, manganese and calcium, formed on the surface of the membrane and the pores. Possible mechanisms employed by acids are hydrolysis/saponification and solubilization/chelation (Madaeni, 2010).
3. Detergents: they are a type of surfactants which have both a hydrophobic and a hydrophilic group. They work by decreasing the surface tension of the water, which results in a better hydration and solubility of the fouling layer. Detergents solubilize macromolecules by forming micelles around them which helps to remove the foulant from the membrane surface. They are used to remove mainly organic and biological material from membrane surfaces.
4. Biocides: they can be divided in oxidizing and non-oxidizing. Oxidizing biocides act by oxidizing the cell membrane of microorganisms, causing a loss of structure and leading to cell lysis and death. Non-oxidizing biocides employ different mechanisms to attack microbiological species, such as: disruption of enzymes, interference with their metabolism and acting on proteins (Selby, 2011).
5. Chelating agents: remove metals and other precipitating ions (Ca+2, Mg+2) from the foulant layer on the membrane by complex formation. They are usually not applied as the sole agent but in combination with other cleaning chemicals.
6. Enzymes: they hydrolyze the polymers in the EPS of the biofilm, and thus destroying the internal structure and ultimately may break down the attachment of the biofilm to the membrane surface.

2.2 Literature review

A literature review was carried out to have an overview of the different cleaning studies which assessed the cleaning efficacy on different biofilms or fouled surfaces with different detection methods. The researches presented in Table 2.1 were performed under a wide range of experimental conditions, such as: types of feed water and membranes used (spiral-wound and hollow fiber membranes for RO, NF and UF) and chemical (type and concentration of cleaning agent, pH) and physical (time, temperature, flow) conditions. Also, the scale of the research included laboratory-scale as well as full-scale installations. Most studies assessed the cleaning performances with the improvement of the membrane characteristics.

2.3 Cleaning practices in the Netherlands

A survey among different water companies in the Netherlands with reverse osmosis and nanofiltration plants was carried out to have an overview of the cleaning conditions used in these full-scale installations. Due to the wide range of feed water quality, quality control and cleaning criteria, different CIP procedures are used and are specific for each installation (tailor-made solutions). NaOH and HCl are mainly used as cleaning agents together with commercial blends in cleaning procedures of two or three steps at a high temperature (35 ^{o}C). The cleaning frecuency for the different locations varied from every 4 weeks to once a year (see Table 2.2). The information from the literature study data (Table 2.1) and the full-scale protocols (Table 2.2) were used as the selection criterion for the experimental work in the current study.

Table 2.1. **Literature review of the best membrane cleaning agents, according to each author**

Author	*Cleaning Agents*	*Biofilm and evaluation Parameters*	*Experimental Conditions cleaning*	*Results*
Corpe, W. 1974	HCl, NaOH, Phosphate buffer, Tris-Glycerine buffer, EDTA, H-xyquinoline, Na-desoxycholate, periodote, perchlorate, Mercaptoethanol, urea, Triton-X100, enzymes, Na laurylsulphate	Pseudomonas M8 on glass slides, Microscopic cell count	Incubation for 5-30 min. at 25oC	loss of cells from surface for NaOH, anionic/nonanionic detergents, chelating/oxidizing agents, protein denaturants Most effective: NaOH, Na-laurylsulphate, urea,
Whittaker (1984)	Esterase, EDTA, Trypsin, Triton, Urea, SDS, Papain, Protease, Biz, Pancreatin	Biofilm on SWM membrane material; Visual inspection by Scanning Electron Microscopy TDC ATP Protein analysis	Water with low (3-5 mg) and high (15-20 mg) chlorine concentration Feed water: secondary (activated sludge) treated municipal wastewater RO spiral wound membranes T= 35 °C	Cleaning treatments in the category of enzyme, antiprecipitants, bactericides and denaturing agents were the most successful Bacterial counts did not correlate well with the visual observations via SEM
Madaeni, Mohamamdi (2001)	EDTA+NaOH EDTA+SDS+NaOH EDTA+SDS+KOH	Pretreated water fouling a RO membrane Resistance removal (RR) Flux recovery (FR)	FilmTec polyamide FT30 RO membrane Temp. 25°C Feed water: mainly calcium sulphate+ calcium phosphate	Acids were the weakest cleaning agents for the experimental conditions The combination of chelating agent, surfactant with alkali provide the best cleaning efficiency Operating conditions have dominant effects on cleaning efficiency
Liikanen (2002)	0.1% Na5P3O10+ 0.2% Na4EDTA 0.8% Citric acid+ 0.1% Oxalic acid+ 0.2% Na4EDTA	Fouled NF membranes Flux recovery Foulant removal	NF spiral wound membrane Feed water: conductivity (14.8 mS/m), organic content (2.7 mg/l) Cleaning solutions were circulated for 10min at low flow rate (800-1000 l/h) and for 15min at a high flow rate (1800-2000 l/h) Temp 25°C	Alkaline chelating agents were the most efficient Alkaline cleaning improved the membrane flux, but resulted in a decrease in ion retention Acidic cleanings may be used for membrane ion retention recovery

Author	*Cleaning Agents*	*Biofilm and evaluation Parameters*	*Experimental Conditions cleaning*	*Results*
Li (2004)	1 mM EDTA (pH 11) 1% SDS (pH 11)	NF membrane fould with humic river water TOC Permeate flux Recovered water flux	Thin-film composite NF membranes; Feed water (pH 8.1) + humic acid (20 mg/l) Mixing of the cleaning solution at 470 rpm for 10 min + rinse Evaluation with Ca+2 and Mg+2	Membranes fouled in absence of divalent cations were easily cleaned by all chemicals. Efficiency of EDTA is highly sensitive to pH Efficiency of SDS was found to be dependent on the SDS concentration
Ang et. Al (2006)	EDTA 2 mM pH 11 SDS 10 mM pH 11	Alginate, river natural organic matter on RO membrane; Pure water flux before fouling and after cleaning	Feed water: organic foulants (EfOM) RO membrane: thin-film composite LFC-1 Laboratory-scale crossflow test unit (membrane cell)	EDTA and SDS were quite effective in reacting with organic foulants Cleaning efficiency with SDS and EDTA was improved by optimizing cleaning agent dose and solution pH
Madaeni, Samieirad (2010)	0.1%w/v (SDS+NaOH)+HCl 0.6%w/v HCl	Waste water fouled membrane Resistance removal (RR) Flux recovery (FR)	FilmTec polyamide FT30 RO membrane Feed water: wastewater	An appropriate combination fo cleaning agents can highly remove the foulants from the membrane surface An increase in temperature, cross-flow velocity and time, the cleaning efficiency was improved
Creber and Vrouwenvelder (2010)	NaOH, pH 12 NaOH+10mM SDS	MFS flow chamber acetate feeding; Magnetic Resonance Imaging (MRI) % Effective surface area Accesible biofilm surface (ABS)	Dechlorinated tap water + nutrient solution of sodium acetate, sodium nitrate and sodium phospate (1000 µg/l, 200 µg/l and 100 µg/l). Temp. 45 °C. Time: 90min Flow: 100 ml/min. RO spiral wound membranes (MFS)	None of the cleaning strategies employed were able to remove completely the biofilm present MRI was employed successfully to visualize and quantify the effectiveness of various membrane cleaning operations

Author	Cleaning Agents	Biofilm amd evaluation Parameters	Experimental Conditions cleaning	Results
Balthazard (2009)	NaOH, SDS, Citric acid, Urea	PVC and SWM biofilms, ATP, carbohydrates, TDC	Shaker test, CIP in biofilm monitor and MFS, temp. 20oC	Biofilm growth 60 days for acceptable CH conc., efficiency calculated from reference and cleaned sample: closely related (location), test protocol with shaker most simple and flexible test but optimization required, some observations on cleaningefficiency: - multiple step treatment more effective than single step - first acid than alkaline better than alkaline – acid - alkaline/SDS > citric acid and SDS > NaOH - NaOH 1% and 0.05% similar
Lahondes (2010)	NaOH, SDS, Oxalic acid, HCl, Citric, lactic acid (OA)	PVC and SWM biofilms, ATP, carbohydrates, TDC	Shaker test, CIP in SWM, temp. 20oC	Optimalisation of the biofilm production and analysis: higher temperature, no growth limitations, better homogenized biofilm samples, reference sample with shaker treatment in Milliqu, growth period biofilm >4 weeks, PVC biofilm problems (skin like removal), SWM samples: removal shaker test and CIP similar, NaOH/SDS as reference treatment (biofilm effects), Single NaOH < NaOH/SDS but > SDS, TOC as biofilm parameter more unexplained variabilities

Table 2.2. **Survey of the cleaning protocols at different RO full-scale plants in the Netherlands**

Location	Source water	Pretreatment	Frequency	Step 1	Step 2	Temp.	Cleaning Criteria
1	Permeate MBR sewage plant	MBR	-	Floclean MC11	-	-	-
2	Seawater	UF	-	NaOH/SDS	HCl	-	Mass Transfer Coef. (MTC)
3	Effluent sewage plant	Fe-inline UF	6 weeks	NaOH pH 12	HCl pH 2	35 °C	NPD
4	Permeate MBR sewage plant	Microstrainer 50 µm	4 weeks	NaOH pH 12	HCl pH 2	35 °C	NPD
5	Biesbosch water	Fe-inline UF	1- 8 weeks	NaOH pH 12	Divos 2 pH 2	35 °C	NPD
6	Drinking water/Surface water from reservoir	Cation-softener	yearly preventive	NaOH pH 12	HCl pH 2	-	NPD/MTC
7	Anaerobic groundwater	-	1 - 0.5 per year	Citric Acid pH 2	NaOH pH 12	35-40 °C	Time/Feed pressure
8	Pretreated groundwater (aerobic)	Sand filtration	1 per year	Citric Acid pH 2	Novoclean 135 pH 12	35-40 °C	NPD/MTC
9	Anaerobic groundwater	-	2 - 3 per year	Permaclean 33	Permaclean 77	-	NPD/MTC
10	Surface water	Fe-inline UF	< 3 per year	Na-Bisulfate+NaOH pH 10-11.5	Divos 2 pH 2-2.5	30 °C	Slope increase
11	Surface water	UF	6 weeks	H_2SO_4 pH 2	NaOH pH 12	-	Time/Feed pressure
12	River Meuse water	Sand filtration, UF	3 weeks – 3 months	Divos2 pH 2.2-2.5	Divos 116 pH 11	30 °C	NPD

3 Materials & Methods

3.1 Laboratory cleaning test: a test with produced biofilm samples

One of the most important aspects of the current research was to ensure the production of homogeneous and reproducible biofilm samples, in order to guarantee a proper evaluation of the cleaning efficiency of each cleaning chemical. For this, different biofilm production units working under various controlled conditions (feed water quality and flow, nutrients dosing, etc.) were employed. This variety of conditions also allowed observing their influence in the growth of biofilm on PVC surfaces. PVC was used as a substratum in all lab-scale monitors due to its high biofilm formation potential (BFP) and biomass production potential (BPP) as shown by van der Kooij and Veenendaal (2001). This is an ideal characteristic that stimulates the production of the biofilm samples.

3.1.1 Biofilm Plate Unit (BPU)

This monitor uses 2.5×2.5 cm plasticized PVC pieces (0.3 cm thickness) as substratum, placed vertically. The flow chamber (width 4.2 cm and height 2.5 cm) was filled with five rows of these plates which created six flow channels of 0.45 cm in width (W) and 2.5 cm in height (H). The device is fed with drinking water, which is pre-filtered with a 10 µm cartridge filter to ensure the removal of iron and other larger particles. The equipment consists of a reservoir tank of 2 l with a constant water temperature of 25 °C provided by a submersible glass heater (Speed, 200 W), a centrifugal recirculation pump (Verder International B.V., the Netherlands) and a container of non-plasticized PVC for the PVC pieces which has a transparent Perspex cover lid to allow the observation of the biofilm development, as shown in Figure 3.1.

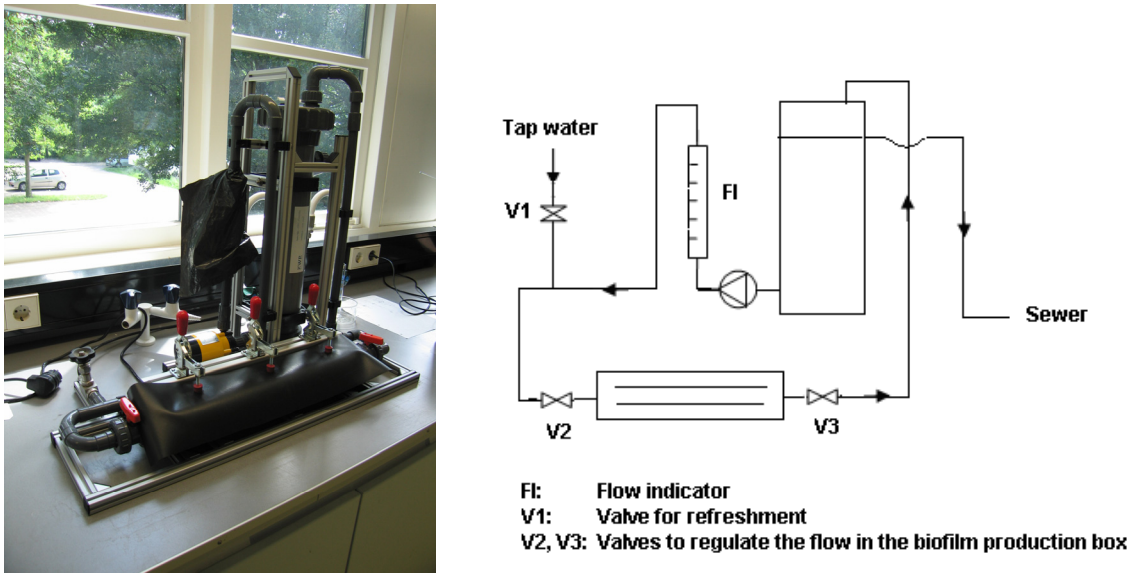


Figure 3.1. **Biofilm production unit (BPU) picture and scheme**

The system works with a recirculation flow of 350 l/h and a water refreshment flow of 20 l/h, which ensures a complete refreshment of the system every 12 min. To ensure a wide variety of microorganisms in the system, the monitor was inoculated with pre-filtered (1.2 µm) surface water from the Lek canal (located in Nieuwegein, the Netherlands) for 24h. With the purpose of promoting a proper and faster growth of the biofilm, two nutrients solutions were dosed to the refreshment flow of the system: a solution of KNO_3 and K_2HPO_4 as a source of nitrogen (1 mg N/l feed water) and phosphate (0.31 mg P/l feed water) and a solution containing plasticized PVC tubes to supply

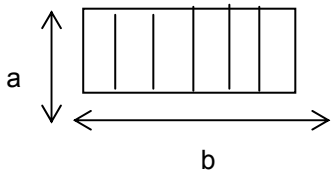
external carbon source (1 µg C/l feed water). The dosing rate for each solution was of 0.4 ml/min with a pulse pump (Masterflex, Cole-Parmer). To prepare the solutions, sterile 5 l glass flasks filled with Milli-Q water (18.2 mΩ/cm^2) were used. The equipment is located in a dark room, to avoid the growth of phototrophic bacteria.

The Reynolds number is defined as follow (Schock and Miquel, 1987):

$$\mathrm{Re} = \frac{\rho \cdot \upsilon \cdot L}{\mu}$$

where,
ρ: water density at 25 °C (997.0479 kg/m^3)
υ: velocity based on the actual cross section area of the duct or pipe (m/s)
μ: water viscosity at 25 °C (9.00x10^{-4} N.s/m^2)
L: hydraulic diameter (m)
The hydraulic diameter was determined using the formula for rectangular ducts with W<<H,

$$d_h = \frac{4ab}{2(a+b)} = \frac{4x(0.042 - 6*0.003)x0.025}{2(0.042+0.025)} = 0.0076$$



where,
a= width of the duct (m)

b= height of the duct (m)

In order to calculate the flow velocity in the plate monitor, the cross-section area (m^2) of the empty flow channel was determined:

$$Area_{BPU} = Width\ x\ Depth = 0.042x0.025 = 0.00105$$

Considering that the PVC plates occupy a volume in the flow channel, this area was subtracted from the total cross-section area to obtain the actual area where the water it's flowing.

$$Area_{PVC-plate} = Thickness\ x\ Heigth = 0.003x0.025 = 7.5xE-5$$

The flow velocity in the filled flow chamber in the chamber is calculated from the flow of 350 l/h assuming a proportional distribution,

$$Flow\ velocity = Qx\frac{1}{3600}x\frac{1}{Flow\ Channel} = 0.35x\frac{1}{3600}x\frac{1}{0.000675} = 0.14$$

Finally, the Reynolds number for the BPU with no plates is:

$$\mathrm{Re} = \frac{997.0479kg/m^3 \cdot 0.144m/s \cdot 0.0076m}{9.00x10^{-4}N.s/m^2} = 1217 \text{ (Laminar)}$$

In a final test the flow was reduced to 200 l/h which resulted in a flow of 0.81 m/s and Re number of 695.

3.1.2 Biofilm Formation Setup (BFS)

In this monitor the biofilm grows inside of 8 parallel plasticized PVC tubes (ØID 1.2 cm, 55 cm), using drinking water at 12-16 °C as feed water (Figure 3.2). The system works with no water recirculation and no nutrients dosing. Each tube has a feed flow of 125 l/h. The equipment is covered with a black plastic bag, to avoid the growth of phototrophic bacteria.

The Reynolds number was calculated following the same procedure as for the BPU, but including the calculations for a tube:

$$Area_{BFS} = \frac{\pi D(mm)^2}{4x1000^2} = \frac{\pi(12mm)^2}{4x1000^2} = 0.0001m^2$$

$$Flow\ velocity = Qx\frac{1}{3600}x\frac{1}{Flow\ Channel} = 125\frac{l}{h}x\frac{1m^3}{1000l}x\frac{1h}{3600s}x\frac{1}{0.0001m^2} = 0.369m/s$$

$$d_h = \frac{ID(mm)}{1000} = \frac{12mm}{1000} = 0.012m$$

The Reynolds number for the BFS is:

$$\mathrm{Re} = \frac{997.0479kg/m^3 \cdot 0.173m/s \cdot 0.0120m}{9.00x10^{-4}\,N.s/m^2} = 4900 \text{ (Turbulent)}$$

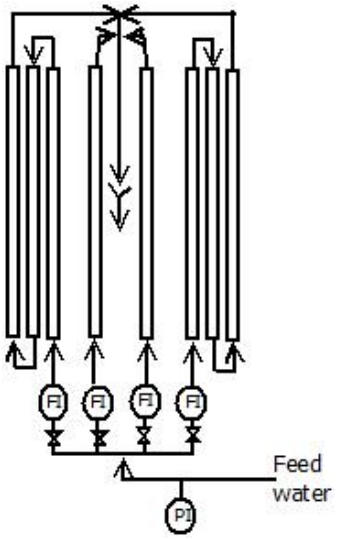


Figure 3.2. Biofilm formation setup picture and scheme

3.1.3 Biofilm Formation Monitor (BFM)

The monitor consists of two parallel glass columns packed with PVC plates (1.5x3 cm) working in an open cycle (Figure 3.3), which means that there is no recirculation. The monitor is connected directly into a low treated surface water supply, at a temperature of 18 °C and with a feed flow of 100 l/h. No nutrients are dosed in this system.

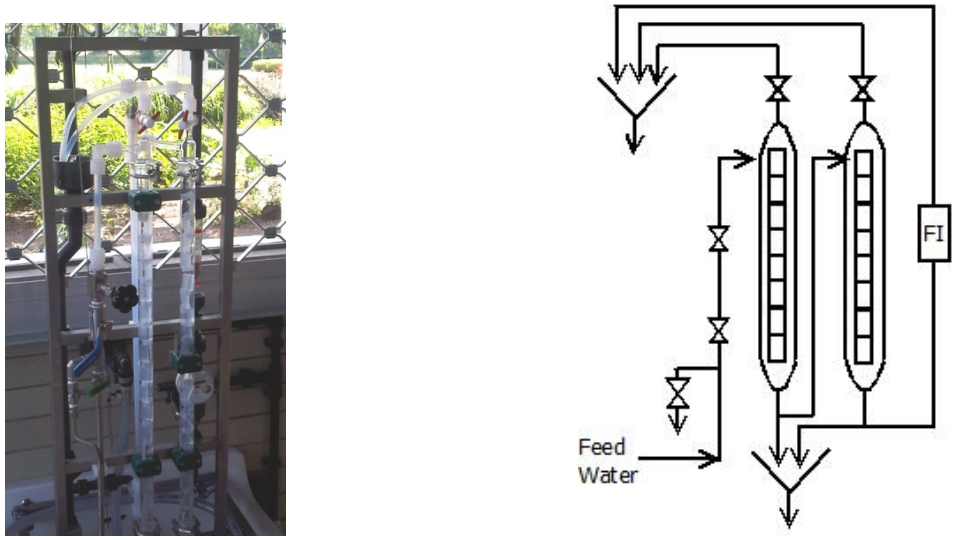


Figure 3.3. Biofilm formation monitor picture and scheme

The Reynolds number was calculated following the same procedure as for the BFS:

$$Area_{BFS} = \frac{\pi D(mm)^2}{4x1000^2} = \frac{\pi(18mm)^2}{4x1000^2} = 0.000254m^2$$

$$Flow\ velocity = Qx\frac{1}{3600}x\frac{1}{Flow\ Channel} = 120\frac{l}{h}x\frac{1m^3}{1000l}x\frac{1h}{3600s}x\frac{1}{0.0002m^2} = 0.131m/s$$

$$d_h = \frac{ID(mm)}{1000} = \frac{18mm}{1000} = 0.018m$$

The Reynolds number for the BFM is:

$$\mathrm{Re} = \frac{997.0479kg/m^3 \cdot 0.131m/s \cdot 0.0180m}{9.00x10^{-4}N.s/m^2} = 2612 \text{ (Transient)}$$

3.1.4 Spiral-Wound Membrane System

Three parallel spiral-wound membranes (DOW™ Filmtec™ TM30-2540) were installed vertically in a pilot plant scale setup and fouled for 10 weeks (Figure 3.4). The system was feed with pre-filtered tap water using a 1 µm cartridge filter (Meltblown, Van Borselen) to avoid particulate fouling. The pre-filtered water was then stored in the feed tank, from which it was fed into the system by means of a vertical pump (DP Pumps, the Netherlands).

Figure 3.4. Spiral-wound membrane system setup.

To ensure a rapid biofilm growth on the membrane, all three elements were dosed with a nutrient solution of 10 µg C/l. Element 1 was dosed with a solution of sodium acetate, and Element 2 and 3 with a mixture of carbon compounds (sodium acetate, glucose, glutamate and sodium benzoate; each 2.5 µg C/l). The nutrient solution was dosed using a peristaltic pump (ProMinent Verder B.V., the Netherlands) set at a flow of 0.5 l/h per membrane. To prepare the solutions, 80 l dark plastic containers filled with demineralized water were used and placed on a scale to record the dosing to the system.
All three elements were equipped with feed and permeate flow meters (Tecfluid) and a pressure gauge (Yokogawa Electric Corporation) to measure the pressure difference between the feed and concentrate side of the membrane.
Parallel to the biofilm formation on each membrane, the effect of air/water cleaning (AWC) on the membranes was also evaluated. Each element worked under different AWC conditions: element 1 and element 2 were used as a reference, therefore no mechanical cleaning was performed; element 3 had an AWC every 24h. The daily air/water cleaning was carried out automatically for 5 min. at an air/water ratio of 4:1 (air flow: 1400 l/h, water flow: 350 l/h). The air inlet of each element is controlled by an air valve which is connected to an air flowmeter (Festo B.V., the Netherlands). The air pressure used was between 2-3 bars, as recorded by a pressure gauge (Festo B.V., the Netherlands). The detailed scheme of the setup is presented in Figure 3.5.

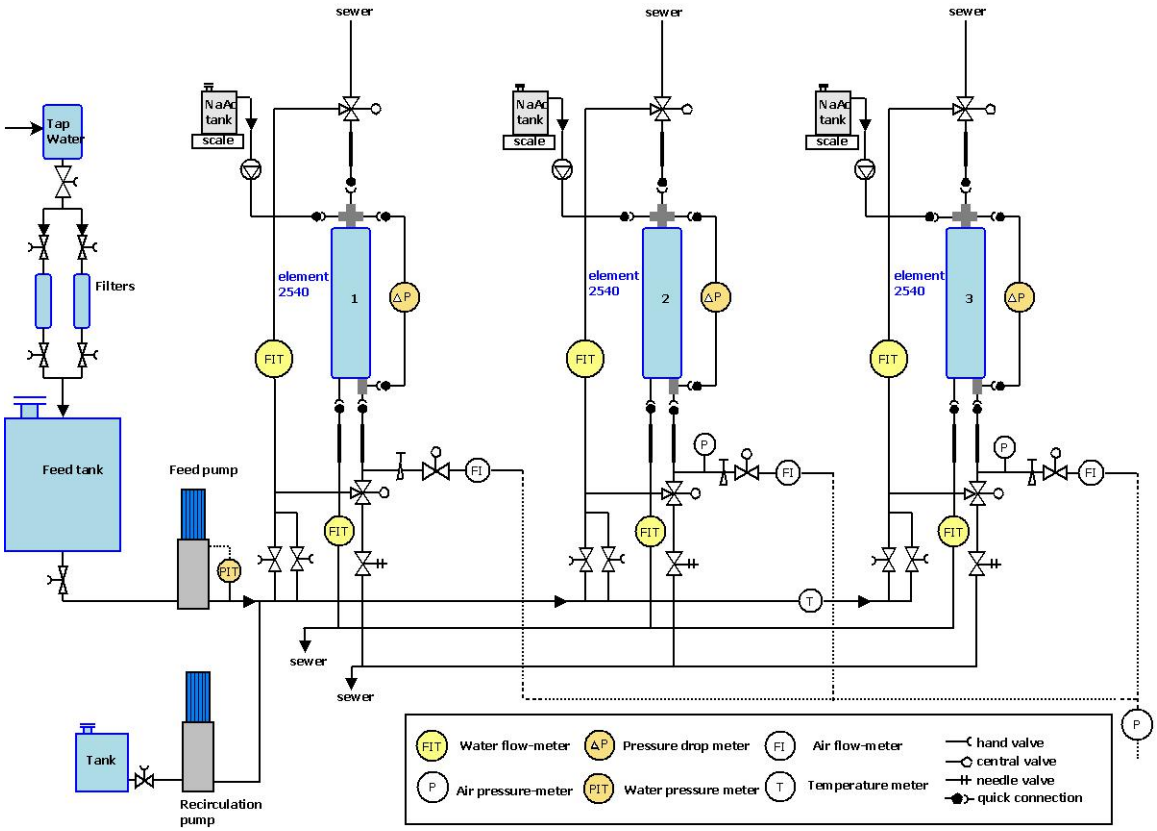


Figure 3.5. Spiral-wound membrane scheme

The production parameters (feed and permeate flow, feed pressure, pressure difference of each element and the feed water temperature) were recorded by a data-logger (Phoenix Contact B.V.) every minute. These parameter were also checked on-site daily, together with pH and conductivity measurements of the feed and concentrate streams of each membrane.
The system worked at a constant flow to ensure the same water production throughout the run; this, evidently, produced an increase in the pressure difference of the system (NPD). The feed flow was set at 350 l/h and the permeate flow at 50 l/h for each membrane. When the pressure difference reached a certain pressure drop, the setup was stopped and the membranes taken out for autopsies. An overview of the operational conditions of each monitor is presented in Table 3.1.

Table 3.1. Biofilm production conditions in the different units

	BPU[a]	***BFS***	***BFM***	***SWM***
Flow (l/h)	350	125	100	350
Surface biofilm (cm^2)	70x12.5	28x7.5	20x12.5	$4x10^4$
Flow surface channel (m^2)	0.0008[a]	0.0002	0.00025	0.0008[c]
Water type	Prefiltered Tap water	Tap water	Pretreated surface water	Tap water
Recycling Water refreshment flow (l/h)	20	0	0	0
Nutrient supply	P,N (C[b])	no	no	Organic-C
V (m/s)	0.120	0.369	0.12	0.12
Temperature (^oC)	25	15	18	15

[a] corrected for PVC surface in the flow direction (5 lines of plates); [b] very low C-supply of 1 µg C/L from a plasticized PVC tube suspension; [c] flow channel of 1 m and height 0.0008 m

Table 3.2. Chemical quality of the feed water of the different set up

		BFM	BPU[a]	BFS/SWM[b]
Parameter	**Unit**	**Average value ±SD**	**Average value ±SD**	**Average value ±SD**
TOC	mg C/L	2.2 ±0.4	2.0±0.1	1.9
Orthophosphate	mg P/L	<0.02	0.02	0.034
NO_3	mg N/L	2.1 ± 0.7	0.12 ±0.04	<1
Fe	mg/L	0.01± 0.02	0.06	0.34
Ca	mg/l	-	<18	71.9
Turbidity	Fte	0.09±0.02	nd	-
pH		7.9 ± 0.2	7.9	7.9
Conductivity	mS/cm	65.6	43.1	37.9

[a] Hijnen et al., 2011; [b] Le Pemp, 2011

3.2 Analytical Procedures

In the current literature, most of the research on cleaning agents and protocols is based on the control of operational conditions such as pressure drop and flux decrease. In this study, the assessment of the biofilm growth and the cleaning efficiency of each cleaning chemical were based on the removal of biomass assessed by two biochemical parameters: adenosine triphosphate (ATP) which represents the active bacterial cell biomass, and the carbohydrates, which relate to the extracellular polymeric substances (EPS) of the biofilm. ATP and carbohydrates have been proved to be quantitatively related with the pressure drop development in spiral-wound membranes and thus with the thickness of biofilm blocking the feed channel (Hijnen et al., 2011).

3.2.1 Sampling of the biofilm production units

According to the biofilm production unit chosen for the cleaning test, different sampling methods were applied:

- For the BPU, plates from the same row were selected using one plate as reference and the two plates adjacent to the reference plate as the samples exposed to cleaning. After sampling, the free spots in the monitor were supplemented with clean plasticized PVC plates. In an overview the age of the plates in the monitor were carefully registered.
- For the BFS, pieces of PVC tubing were cut from the selected tube and then divided longitudinally, one half as a reference and the other half as the cleaned sample. Two pieces of tube were used in each beaker.
- For the BFM, the plates were taken out of the columns, one plate as a reference and the plate below as the cleaned sample.
- In the case of the spiral-wound membranes, one membrane sheet was selected and pieces from the feed side were cut, one as a reference and the adjacent one as the cleaned sample. In this case, two samples were used for each beaker.

3.2.2 Preparation of the biofilm samples

Each biofilm sample to be analyzed was placed in a sterile cup with 40 ml Milli-Q water and brushed with an autoclaved and carefully cleaned electric toothbrush (Braun) to remove the biofilm from the surface. The solution was then sonified with High Energy Sonication (HES) using a Branson W-250 equipment with a sonotrode tip (Ø 6.5 mm) for 5 min at 45% amplitude. This step is performed to release the residual biofilm from the toothbrush and substratum surface and to homogenize the biofilm suspension. It is considered that these two steps, brushing and sonication, remove completely the biofilm from the surface.

3.2.3 ATP Analysis

ATP is a compound that can be found in every living cell, where it is used as energy provider. The ATP analysis is based on the extraction of the compounds from biomass using a nucleotide-releasing

agent, followed by a light-generating luciferine-luciferase reaction. The light signal generated from this reaction is measured as relative light units (RLU) with a luminometer (van der Kooij and Veenendaal, 2001).
To obtain the ATP concentration of the samples, 100 µl from the biofilm suspension were placed in a 500 µl vial in duplicate to be analyzed with the luminometer. The obtained reading is in RLU/ml which has to be converted to pg of ATP per cm2 by using the coefficient from the calibration curve and the surface area of the sample. 2 pg ATP/ml and 100 pg ATP/ml standards are used for the calibration of the equipment.

3.2.4 Carbohydrates Analysis

To determine the carbohydrates present in the biofilm samples a procedure based on the Dubois method is employed, using glucose as the reference carbohydrate. In this method the poly-saccharides in the biofilm matrix are hydrolyzed by sulphuric acid and complexated with phenol. The absorbance of the samples is then measured directly using a spectrophotometer.
The procedure starts by preparing several glucose solutions to calibrate the spectrophotometer. Four millilitres of each standard are placed in a vial where 100 µl of phenol and 10 ml of pure sulfuric acid (96 %w/v) were added and mixed using a Vortex. The samples are left stand for 15 minutes, for the orange-yellow coloured complex to form. During the assessment of the calibration curve the same procedure is applied.
The absorbance of the standards was measured by using a spectrophotometer at a wavelength of 490 nm. With these values the calibration curve was obtained. Afterwards the samples were measured and the µg/ml concentrations obtained were converted to µg/cm2 by using the surface area of the substratum.
A dilution of the biofilm suspension samples was done using Milli-Q water at a factor of 1:4 to secure that the absorbance measured was within the range of the calibration curve, since the average carbohydrate concentration of the reference biofilm samples used in this research was of 50 mg/l and the maximum standard concentration is 30 mg/l.

3.2.5 Laboratory Scale Cleaning Protocol

Periodic cleaning in place (CIP) is commonly applied for controlling biofouling of spiral-wound membranes used for desalination and in water treatment to ensure water production. The collection of detailed quantitative information about effects of cleaning procedures at full-scale plants is an option to obtain the optimal cleaning conditions. A precondition is that the information is complete and collected systematically during daily practice, but inquiries show that this is not easy to establish and cleaning conditions are tailored to the local situation. Therefore, objective comparison of the efficacy of cleaning agents and procedures with field data is difficult.
An additional method to obtain the required information is by using a laboratory test under standardized conditions. Such a test is developed in previous studies (van der Kooij et al., 2010). The major requirements for a robust and cost effective laboratory test which gives reproducible results on distinctive features are:
(i) standardized production of biofilm samples as surrogate for membrane biofilms,
(ii) distinctive parameters to quantify the biofilm concentration;
(iii) a simple test which simulates soaking and rinsing of a CIP procedure;
(iv) the cleaning efficiencies calculated from this test using certain (combinations of) chemicals should give the same ranking as for the cleaning efficiencies of the CIP procedures of spiral-wound membranes using the similar (combinations of) chemicals.

The applied laboratory test was carried out on a shaker where 600 ml beakers were placed (Figure 3.6). One beaker was filled with 200 ml of the cleaning solution, and a reference beaker was filled with 200 ml of Milli-Q water. The biofilm samples were placed inside the beakers, and agitated for 1 h at 100 rpm to simulate the mechanical cleaning. The temperature was fixed according to the cleaning procedure.

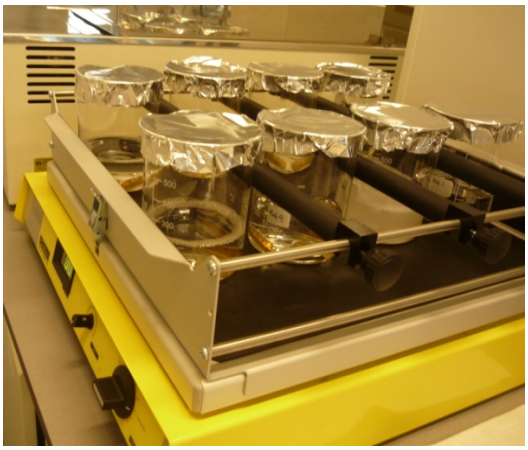


Figure 3.6. Picture of the orbital shaker with the cleaning test beakers

After this step, the samples in the beakers filled with the cleaning solution were placed in beakers filled with 200 ml of Milli-Q water to simulate the rinsing step of the CIP. The beakers were agitated for 15 min maintaining the 100 rpm. Afterwards, the samples were taken out to carry out ATP and CH measurements to determine the biofilm removal of each chemical.
The objective of the cleaning protocol is to mimic the cleaning procedures (CIP) carried out in full-scale installations under a controlled environment. One of the main differences of the lab-scale protocol with CIP procedures is the exposure of the biofilm to the cleaning agents. Under the test this is optimal in the suspension but under practical conditions this can be limited by blocking of the feed channel. To validate the lab test method, the efficacy of the cleaning agents and procedures must be compared with the efficacy assessed in a CIP. Additionally, membrane samples can be treated in the laboratory test to verify the effect of exposure differences between a real CIP and the laboratory test.

3.2.6 Single step treatments

The labscale cleaning test was divided in cleaning protocols with single and multiple steps. The selected conditions were based on the information found in literature and also supplied by manufacturers. In the single step protocols, the cleaning chemicals were evaluated individually under the same conditions of temperature (20±1 ^{o}C) and time (60 min.). The chemicals to evaluate were selected according to the categories used: alkaline, acid, detergents, biocide, chelating agents and enzymes, as shown in Table 3.3.

3.2.7 Multiple steps

In full-scale installations, the CIP procedure is usually carried out in combined steps to target the different types of fouling affecting the membrane. For this reason, multiple step protocols were performed using different cleaning chemicals in several steps. The evaluated cleaning sequences were: acid/alkaline, alkaline/acid, alkaline/detergent, alkaline/biocide and detergent/biocide following the CIP protocols used in several reverse osmosis full-scale installations in the Netherlands (see Table 2.3 and 3.4).
The compatibility of the cleaning agents with the reverse osmosis membranes was taken into account when choosing the cleaning conditions for the laboratory scale cleaning test. As pointed out by van der Kooij et al. (2010), critical properties of the cleaning chemicals are: pH, oxidizing properties and adsorption into the membrane. Also, temperature should not exceed a critical value. Considering this, the pH range evaluated in this research was between 2-12 and the temperature did not exceed the 35 ^{o}C.

Table 3.3. Cleaning chemicals used according to their categories

Category	*Agent*	*Concentration, pH*	*Category*	*Agent*	*Concentration, pH*
Alkaline	NaOH	0.03-1%; pH 12, 12.7	Acid	Citric acid	1%, pH 2
	Floclean MC11	pH 11		HCl	0.01M, pH 2
	Permaclean PC-33	pH 12		Divos 2	0.4%, pH 1.6
	Novoclean 135	pH 11.6		PermacleanPC-77	4%, pH 4
	Divos 116	pH 12		P3-ultrasil 73	1%, pH 2.5
	P3-ultrasil 141	0.1%, pH 11			
Detergent	Na Dodecyl Sulphate[a]	1%, pH 8	Biocide	DBNPA	50 ppm, pH 6
	Na Triphosphate	2%, pH 9		Na Bisulfite	500 ppm, pH 4
	Trisodium Phosphate	1%, pH 12		NaOCl	0.5%, pH 11.4
	CTAB	1%, pH 5		P3-oxyzan ZS	0.1%, pH 3
	NaDDBS	0.025%, pH 7		H2O2[b]	0.5%
Enzyme	P3-ultrasil 53	1%, pH 9	Chelating agent	EDTA	10 mM, pH 11
	BAN 480	pH 5			
	Dextrozyme	pH 5			
	Savinase	pH 7			
	Everlase	pH 7			

[a] Combined with NaOH pH 12; [b] combined with SDS

Table 3.4. Multiple steps protocols evaluated

Step 1	***Step 2***
NaOH/SDS 0.02%	HCl pH 2
NaOH pH 12	HCl pH 2
NaOH pH 12	Divos 2 pH 2
Citric Acid pH 2	NaOH pH 12
Divos 2 pH 2	Divos 116 pH 12
Citric Acid pH 2	Novoclean 135 pH 12
Permaclean 33	Permaclean 77
Na-Bisulfate+NaOH pH 12	Divos 2 pH 2

3.2.8 Confocal Laser Scanning Microscopy (CLSM)

In order to analyze and observe the structure and distribution of the main components of the biofilm (microorganisms and extracellular polymeric substances (EPS)) grown on PVC plates and to study the influence of the chemical treatment of the biofilm structure, Confocal Laser Scanning Microscopy analyses were carried out. CLSM is a one-of-a-kind type of microscopy since it allows the optical sectioning of thin layers (slices) by doing two things: controlling the depth of field and rejecting the out-of-focus image-degrading objects in the field of view (Lewandowski, 2007). This is achieved by illuminating a single point with a focused laser beam and by using a pinhole in front of the detector (PMT) which blocks the light emitted above and below the focal plane (Neu, 2011). Confocal Laser Scanning Microscopy has proven to be an indispensable tool for studying *in situ* biofilm structure and composition (Lawrence et al., 1998) since it provides sharp images of the spaces occupied by completely hydrated biofilms (Lewandowski, 2007). The CLSM used was an upright laser scanning microscope (Carl Zeiss AG, LSM510 META) controlled by the AIM software version 3.2 (Carl Zeiss AG, Jena, Germany) and equipped with four different lasers (Enterprise, Ar, HeNe1, HeNe2).

Preparatory method for analysis of the biofilm samples

The biofilm sample on the PVC plate is placed in a Plexiglass box and filled with tap water. In order to observe with the CLSM the microorganism and polymeric components (EPS) of the biofilm, the sample must be stained. The stains used were: *Aleuria aurantia* lectin (LINARIS Biologische Produkte GmbH, Wertheim-Bettingen, Germany) labeled with Alexa Fluor® 633 (Invitrogen/Molecular Probes, Eugene, USA) for the EPS glycoconjugates and SYBRgreen (Invitrogen/Molecular Probes, Eugene,

USA) for the nucleic acids (see Table 3.5). The staining procedure was carried out as indicated by Staudt et al. (2004).

Table 3.5. Stains used to identify the different components of the biofilm

	Nucleic Acids	EPS Glycoconjugates
Stain	SYBRgreen	AAL633
Excitation λ (nm)	488	633
Emission λ (nm)	495-545	650-780
Color allocation	Red	Green

Digital image analyses
For each biofilm sample, four to five spots were chosen randomly and scanned to determine the distribution of EPS and nucleic acids. These samples were scanned in the xy direction with a water inmersible 20× (Achroplan, N.A.=0.5) lens with a field of view of 460×420 µm. The captured intensity images had a frame size of 512×512 pixels and were acquired with a data depth of 8-bit. For the image analyze of the pictures, the software JImage Analyzer (version 1.2) together with ImageJ (version 1.44p, http://imagej.nih.gov/ij) were used to obtain the area fraction coverage of bacteria and EPS glycoconjugates and the maximum and mean thickness of the biofilm. These images were converted to gray-scale images than were then converted to binary (threshold) images in order to compute their areal parameters (Lewandowski, 2007). The threshold was chosen manually for each image stack.

For each stack, the foreground pixels were counted and the coverage *C* of every single image of the image stack was quantified. Then the average coverage C_{stack} was calculated for each image stack, as shown by Wagner et al. (2009):

$$C_{stack} = \frac{1}{n_{\max}} \sum_{n=1}^{n=n_{\max}} C$$

with *n*= number of slices

$$C = \frac{1}{n_{\max}} \sum_{n=1}^{n=n_{\max}} C_{stack}$$

with *n*= number of image stacks

Additionally, all values of average coverage C_{stack} of all analyzed image stack on one plate for the samples treated with each of the selected chemicals, were averaged once more to obtain one single value C_{total}, representing the averaged amount of scanned EPS glycoconjugates and nucleic acid on one sample.

3.2.9 Statiscal analysis

This study aimed at a broad screening of different cleaning agents and protocols. Due to the longevity and laborious character of both the biofilm production and biomass analysis the tests were performed in duplicate. This hampers extended statistical analysis of the data. To present the variability of the assessed cleaning efficiency both values are presented in the tables.

4 Results and discussion

4.1 Assessment of the cleaning efficiency: quantification parameters

The laboratory-scale cleaning test employed in this research consisted of three important elements: (i) the production of stable and homogenous biofilm samples, (ii) an optimal method to isolate the biofilm from these samples and (iii) a reliable method to determine the concentration of biomass. ATP (Magic-Knezev and van der Kooij, 2004) and carbohydrate analysis (Dubois, 1956) were used as the biomass parameters. These methods have been evaluated as proper to determine the biomass concentrations in biofouled spiral-wound membranes (Hijnen et al., 2011). In this section some important analytical aspects of these methods, such as recovery, precision and reliability, were evaluated.

4.1.1 Efficiency of the biofilm isolation method

As mentioned in the Materials and Methods chapter, the biofilm was removed mechanically from the PVC plate using an electric toothbrush. To evaluate if this isolation method was effective, three reference biofilm samples were chosen to determine the ATP and CH concentrations after mechanical brushing. To do this, the biofilm on the PVC plate was brushed in a cup with 40 ml Milli-Q water, taken out from the biofilm suspension and placed in another cup filled also with fresh Milli-Q water and brushed again. This was done a third time in a new cup filled with fresh Milli-Q water. The ATP and CH concentration of these three suspensions were analyzed to determine the remaining biofilm in each of the suspensions.

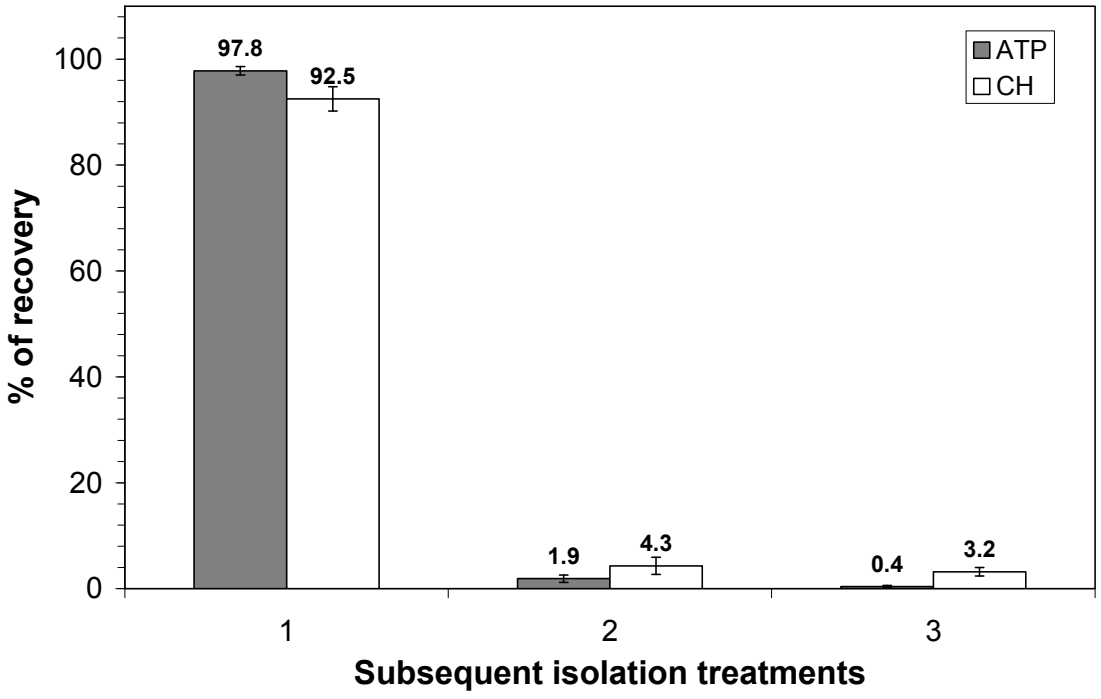


Figure 4.1. Biofilm in suspension after mechanical isolation from the PVC plate. 1, 2, 3, respectively first, second and third isolation treatment

The results of the biofilm isolation method (n=6) evaluation showed that on the first brushing, 98% and 92% of the ATP and CH are removed respectively (see Figure 4.1; data in Appendix I), demonstrating that the brushing of the PVC plate efficiently removes almost all the biofilm from the plate surface.

This high removal efficiency was validated by means of CLSM imaging. In Figure 4.2 an image of a reference PVC biofilm is presented and compared with the clean surface of a plate after mechanical isolation. The image after the brushing shows only one floc of biofilm (16 µm thickness) on the plate surface, whereas the reference sample was completely covered with a mean biofilm layer of 70 µm (image analysis data). This confirms the suitability of this method for the removal of biofilm. Image analyze of this picture (Figure 4.2, right picture) using the software JImage (JImage Analyzer, v1.2) showed that the volume fraction of the EPS glycoconjugates (1.3%) is quite similar than of the nucleic acids (1.4%), which correlates with the values obtained in the laboratory measurements. The volume fraction was calculated dividing the pixel volume of each channel (bacteria, EPS) obtained by the software JImage, by the total volume of the image (512 µm×512 µm×step size µm).

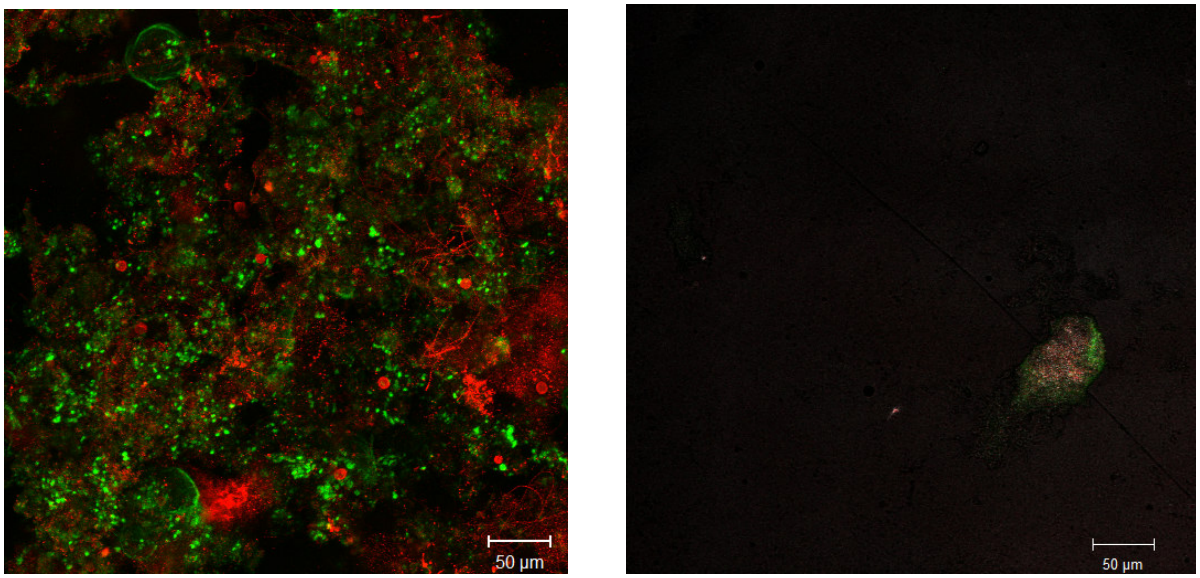


Figure 4.2. Maximum Intensity Projection of the biofilm on a reference PVC plate (left) and the remaining biofilm on a PVC plate surface after mechanical brushing (right).

In a separate test, it was demonstrated that the biomass homogenization method with High Energy Sonication (HES) applied for 5 minutes, which was optimized in a former study (Lahondes, 2010), did not reduce the ATP recovery as previously shown by Lahondes (data in Appendix II). Therefore, HES treatment during 5 min. is a proper method to homogenize the biofilm suspension without ATP degradation, as shown in Figure 4.3.

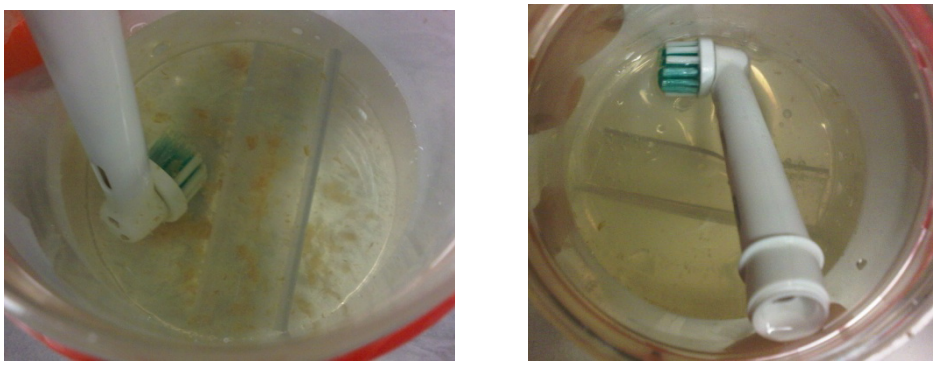


Figure4.3. Biofilm suspension before (left) and after (right) 5 minutes of HES treatment

4.1.2 Analytical characteristics of the carbohydrate method

To measure the CH concentrations in biomass samples, standard solutions of Glucose were prepared to determine the calibration curve. A glucose solution with a concentration of 25 mg/l (referred as

control sample) was prepared to evaluate the consistency of the glucose solution used to prepare the standards.

Additionally, a standard addition sample was applied to assess the possible disturbing influence of the biofilm sample matrix to the recovery of the standard, glucose. This was done by mixing 2 ml of a chosen calibration standard (different concentrations employed) with 2 ml of a selected biofilm sample. The sum of the total absorbance of this mixture was compared with the sum of half of the absorbance of the two solutions chosen.

For ten calibration curves, control samples and standard addition samples, the statistics are presented in Table 4.1. For the full data, see Appendix III. Considering that the standard addition sample had a different selected concentration on each test, the average of this measurement is not appropriate to show. These data show the high accuracy of the carbohydrate analytical, since the control samples and standard addition samples have a low standard deviation.

Table 4.1. Statistics of the parameters of the calibration curve, control and standard addition samples of the carbohydrates analysis

	n	***Average***	***Standard Deviation***
Calibration curve: slope[a]	10	0.0890	0.0056
Control (mg/l)	10	25.936	1.5%[b]
Standard addition (mg/l)	10	-[c]	2.6%[b]

[a] r^2 = 0.99 for all curves; [b] Relative standard deviation; [c] Not representative, since samples within a wide range of concentrations were chosen to prepare the standard addition sample.

The biofilm samples were analyzed in duplicate for both ATP and CH measurements, obtaining an average relative standard deviation of 2% for all measurements (min: 0%, max.: 5%), demonstrating the precision and reproducibility of the analysis performed.

4.2 Biofilm samples

The production of standard biofilm samples for the laboratory test was one of the major challenges in this research. In former studies, the biofilm growth on PVC concept was developed (van der Kooij et al, 2011; Balthazard, 2009; Lahondes, 2010) but still unexplained problems with the biofilm were encountered during the presented research. Especially a 'skin-like' biofilm with easy detachment from the PVC plate surface was the remaining problem observed. This condition was also observed in PVC biofilm samples prepared in continuous flow systems with PVC tubing and tap water recirculation (Lahondes, 2010). Although not proven, it was hypothesized that nutrient limitation and high iron concentrations could be two possible causes of this skin-like biofilm. The current study evaluated several biofilm monitors working in a continuous flow system with tap water or pretreated surface water. As detailed in the Materials and Methods chapter, the following aspects were adapted for each monitor:

- the Biofilm Formation Setup (BFS; PVC tubing) supplied continuously with cold tap water (15°C) and no recirculation;
- the Biofilm Plate unit (BPU) with pre-filtered tap water (10 μm), N/P and external substrate addition, increased tap water refreshment regime and temperature (25 °C);
- the Biofilm Formation Monitor (BFM) loaded with PVC plates and continuously supplied with pretreated surface water at ambient temperature (18 °C);
- Membrane system setup with spiral-wound membranes supplied continuously with cold tap water (15 °C) and external substrate dosage.

Drinking (tap) water is usually considered to have low concentration of carbon, nitrogen and phosphorous (Manuel, 2007). From the four monitors used, the biofilm plate unit (BPU) and the spiral-wound membrane setup were the only systems with external nutrient dosage (nitrogen and phosphorous). Each of the four systems was operated under different hydraulic flow conditions. The BFS and biofilm monitor showed the highest dynamic flow conditions with a Reynolds number of

4900 and 2612, respectively, followed by the BPU filled with five rows of plates with the lowest hydrodynamic condition (Re=1217).

4.2.1 Biofilm growth in the Biofilm Production Unit (BPU)

Figure 4.4 shows the flow cell of the BPU loaded with new PVC plates and also with plates showing some biofilm development after 12 days of cultivation. Visually, this biofilm is characterized for being homogenously distributed over the plate surface in a thin layer and with a slight orange color, due to iron deposits (see Figure 4.4). The iron content of the feed water after filtration (10 µm) is 0.06 mg/l. With a feed flow of 20 l/h, the iron dosage in the BPU monitor is 1.2 mg/h. Iron measurements in the biofilm resulted in an average iron accumulation of 2 µg/cm².

Figure 4.4. BPU loaded with new PVC plates and biofouled PVC plates after 12 days of cultivation and a biofilm sample after 29 days of incubation

4.2.2 Biofilm formation set up (BFS)

The biofilm formed on the PVC tubes from the BFS monitor is a thick and dense biofilm, as observed in Figure 4.5. It is also noticeable its stronger brown color (compared with the BPU biofilm), consequence of a higher iron input in the system. Visually, the biofilm coverage was not uniform through the whole length of the tube. The iron content of the feed water of this setup (no pre-filtration) is 0.34 mg/l due the high Fe concentration in the supplied tap water. With a feed flow of 125 l/h, the iron content in the BPU monitor is 42.5 mg/h, obtaining an iron accumulation in the biofilm of around 100 µg Fe/cm².

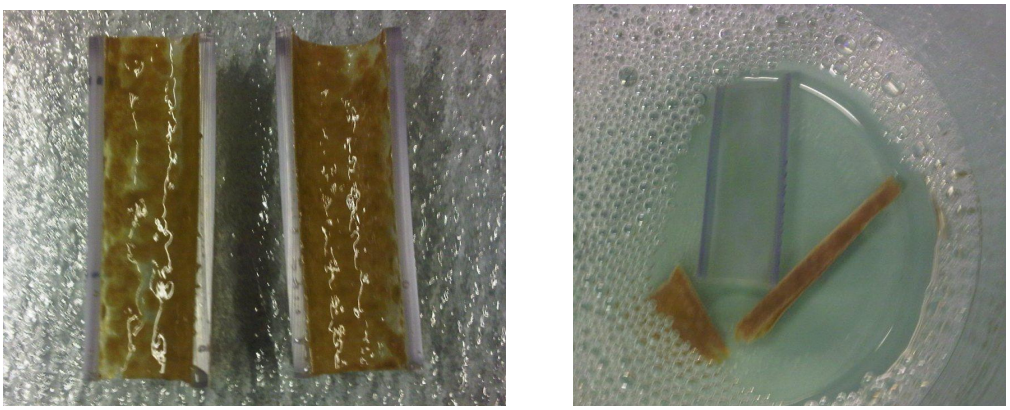


Figure 4.5. Biofilm development on PVC tubes after 75 days of cultivation and biofilm detachment after treatment with SDS

This biofilm was observed to be weakly attached to the tube surface, in addition to its non-uniform distribution on the surface. This was specially noticed after treatment with a solution of 1% SDS (see Figure 4.5, where the biofilm layer on the PVC tube was completely detached. Although not proven, it was hypothesized that the considerably higher iron concentration in the biofilm produced this sort of instability in the biofilm structure.

4.2.3 The Biofilm Monitor

This biofilm monitor supplied with prefiltered surface water (coagulation, sedimentation and rapid sand filtration) produced a low biofilm concentration, as observed in Figure 4.6. It can be seen that the biofilm layer on the PVC plate is very thin and with a light color after 50 days of cultivation. One explanation for this slow biofilm growth could be the low phosphate concentration found in the feed water (<0.02 mg/l), which could have limited the biofilm development on this monitor due to the insufficient nutrients concentration. Additionally, the iron concentration in the feed water (no filtration) was also low, 0.01 mg/l. With a feed flow of 100 l/h, the iron content in the monitor is 1 mg/h, which is considerably lower than in the previously described monitors.

Figure 4.6. Biofilm growth on the PVC plates in the BFM after 50 days of cultivation

4.2.4 Biofilm density on the different biofilm samples

As visually shown before, the different conditions of temperature, flow, feed water and substratum under which each monitor was operated, produced a different (concentration of) biofilm (see Figure 4.7). The biofilm growth can be demonstrated by the increase of the ATP and CH concentration in time. The results showed that each monitor had a different biofilm production. In the HP and BFS tube monitors (data from Lahondes, 2010), the ATP concentration kept increasing after 60 days of cultivation, having a maximum ATP concentration three times higher than observed on the plates in the BPU and BFM. After a fast growth during the first 30 days, the ATP concentration in the BPU and the BFM plate maintained a relatively constant value for the rest of the operational period. Considering that the BPU monitor was the only with nutrients dosage and warmer feed water temperature, this was an unexpected result. Possible explanation for the difference in biofilm production between the plate monitors and the tube monitors (HP; BFS) is a lower nutrient concentration in the PVC plate compared to the PVC tubing. First of all due differences in the material composition and secondly due to the lower nutrient availability caused by the difference in flow conditions: in tubing growth at one side and in BPU at two sides of the PVC plates (>50% reduction).
On the other hand, the CH results for this monitor did have a similar increase tendency as the three other monitors evaluated. Taking into account that the BPU had a lower concentration of ATP, this means that this biofilm had a higher CH/ATP ratio which might indicate more EPS due to higher attachment to the plates than in the tube monitors (HP, BFM). The CH/ATP ratio (μg/ng) for the BPU biofilm was 1.0±0.4 and for the BFS samples 0.5±0.2.

A possible explanation for the high carbohydrate concentration found on the PVC plates (BPU) could be the hydraulic conditions expressed in the Reynolds number, which is a condition that has proven to be of importance on biofilm growth (Manuel, 2007; Wäsche et al., 2002). The hydraulic conditions in both systems were different: the flow conditions in the BPU channels were laminar (Re = 1217) and in the tubes of the BFS more turbulent (Re of 4900). Besides the difference in material composition the other major difference between the BFS tubes and the plates in the BPU is the water flow: in the tube

with a inner diameter of 1.2 cm there is one flow channel and in the BPU there are six flow channels of 0.45 cm thickness between the PVC plate surfaces. Although calculations reveal laminar conditions in the BPU channels the flow may be more turbulent due to the fact that above the plates there is an open space of a few mm's and each row consists of 14 separate plates with small open spaces between each plate. Furthermore, 100% outlining of the plates is not possible and therefore drag forces may occur at the edges of the plates. These conditions which are hard to quantify in hydraulic forces may have caused higher turbulence flows and shear forces in the BPU monitor which would lead to more dense biofilms. As observed by Wäsche et al. (2002), the biofilm grown under turbulent flow conditions has a tendency to be more dense and with a higher attachment to the substratum in order to protect the bacteria from the shear forces. An explanation given is that high flow velocities increase cells hydrophobicity, favoring cell aggregation and, as a consequence, biofilm accumulation (Manuel, 2007).

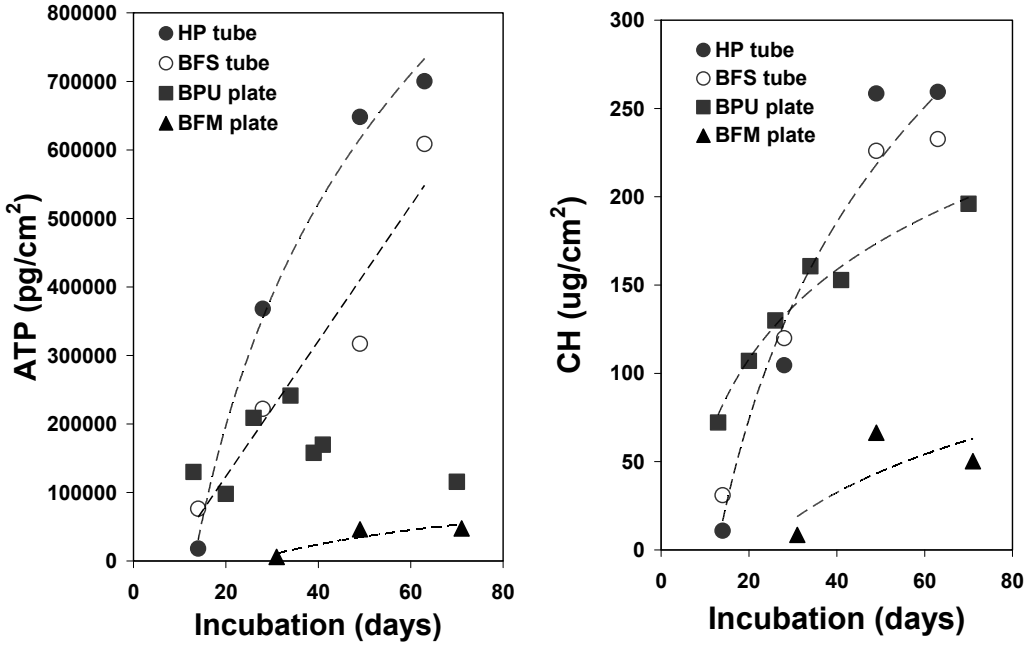


Figure 4.7. Comparison of the biofilm growth assessed with ATP and CH concentrations in the different biofilm monitors. HP: hexa-penta monitor, BFS: biofilm formation setup, BPU: biofilm formation unit, BFM: biofilm formation monitor. (Lahondes, 2010; Castillo, 2011)

It is important to notice that both HP and BFS tube monitors had the same tendency of biofilm growth and their biofilm concentration parameters were on the same range. This indicates the influence of the material used to grow the biofilm, since both monitors used PVC tubes as substratum. Although not verified, the concentration biodegradable plasticizers in tube might be higher than in the plate PVC pieces. The BFM device with PVC plates presented the lowest biofilm growth rate, also lower than in the BPU. A possible explanation could be the low phosphate (≤ 14 µg PO4-P/l) and iron concentration (≤ 6 µg/l) present in the feed water (Table 3.2). Especially the Fe content was very low compared to the BPU system with prefiltration (0.06 mg/l). Furthermore, the monitor worked under a laminar flow regime (Re=2612) which can limit the mass transport of the nutrients (Manuel, 2007).

In this research the BPU was the common used biofilm set up. The biofilm concentration on the plates of the BPU used for the successive experiments presented variations as shown by the ATP and CH measurements on the plates used as reference in the different cleaning tests (see Table 4.2). These plates only had the effect of the mechanical step of agitation with Milli-Q water. Although there was a clear growth on the plates, as shown by the ATP and CH parameters measured in Figure 4.7, a clear correlation with the age of the biofilm plates with an age in the range of 26-41 days could not be found (Table 4.2). The standard deviation of the measured parameters represents the heterogeneity of the

biofilm, which is expected in this kind of systems. This variation observed from plate to plate can also be caused by the location of the plate in the monitor, whether it is closer to the inlet, outlet, or to the walls of the device. Another possibility is the different flow conditions in the channel along the plates which were placed in the monitor. Despite this variation, again a clear relationship between the parameters measured and the location of the plate could not be established. This is subject for further studies.

4.2.5 Reproducibility of the cleaning test

In every separate test a standard treatment with NaOH/SDS was conducted. In Table 4.2 the results of ATP and CH removal assessed for this single test for every produced batch of biofilm plates was presented. The biomass concentrations and the CH/ATP ratios in this table were calculated from the reference samples (n=4-5).

Table 4.2. Biofilm concentration parameters of separate batches of plates produced in the biofilm plate unit (BPU) and two other units (HP and BFS; Lahondes, 2010)

Biofilm age (days)	ATP (pg/cm2)	CH (µg/cm2)	CH/ATP	NaOH/SDS pH12 or 12.7 removal (%) ATP – CH
26 BPU	$2.1x10^5 \pm 4x10^4$	130 ± 10	0.63 ± 0.07	69 – 36
30 BPU	$6.0x10^4 \pm 2x10^4$	96 ± 9	1.43 ± 0.38	99 – 45 (54-29) [a]
33 BPU	$2.0x10^5 \pm 1x10^4$	131 ± 15	0.78 ± 0.12	96 – 36
34 BPU	$2.4x10^5 \pm 3x10^4$	161 ± 10	0.67 ± 0.09	66 – 36
36 BPU	$1.0x10^5 \pm 2x10^4$	119 ± 11	1.13 ± 0.21	90 – 25 [b]
36 BPU	$1.0x10^5 \pm 2x10^4$	100 ± 13	0.93 ± 0.11	80 – 21 [b]
37 BPU	$1.3x10^5 \pm 2x10^4$	141 ± 18	1.15 ± 0.12	75 – 23
39 BPU	$1.6x10^5 \pm 3x10^3$	119 ± 5	0.75 ± 0.03	58 – 28
41 BPU	$1.7x10^5 \pm 9x10^3$	153 ± 10	0.90 ± 0.03	60 – 34
41 BPU	$1.7x10^5 \pm 9x10^3$	153 ± 10	0.90 ± 0.03	81 – 35 [a]
28 HP	$4.3x10^5 \pm 8x10^4$	106±19.6	0.25±0.02	≥99 – 78 [a]
28 BFS	$4.7x10^5 \pm 2x10^4$	174.3±25.3	0.37±0.05	≥99 – 61 [a]
49 HP	$6.6x10^5 \pm 6x10^3$	275.0±10.3	0.42±0.02	≥99 – 62 [a]
49 BFS	$3.1x10^5 \pm 7x10^4$	216.7±24.4	0.71±0.10	≥99 - 44 [a]
63 HP	$6.8x10^5 \pm 1x10^5$	268±32.6	0.40±0.03	≥99 – 60 [a]
63 BFS	$6.0x10^5 \pm 2x10^4$	246.1±9.3	0.41±0.02	≥99 – 54 [a]

[a] pH 12.7; [b] Test performed at 35 °C

These results showed that the biofilm characteristics as well as the cleaning efficiencies showed variations, though the standard treatment was not always performed identical. Two treatments were performed at a higher pH of 12.7 and two at a higher temperature of 35°C. Based on ATP removal the elevated pH and temperature showed a tendency of increase but based on the removal of CH the

effect of both conditions on the removal was not significant. The lowest CH removal rate was observed at the elevated temperatures. Other variables which may have an impact on the cleaning efficiency are the age of the biofilm and the ratio of CH/ATP as a parameter of the fraction EPS in the biofilm, a protective matrix for the bacteria in the biofilm. The ATP removal ranged from 66 – 99% and the CH removal from 21 – 45% for the NaOH/SDS treatment on BPU biofilm.

The effect of the pH was tested in the current study to compare the results performed under feasible environmental conditions (pH not higher than 12) with the results of former experiments with biofilms produced in plasticized PVC tubes (HP and BFS; Lahondes, 2010). The effect of a mixture of NaOH/SDS at a pH of 12.7 showed a higher cleaning efficiency on the HP/BFS biofilms (Table 4.2). Besides the higher pH in the cleaning these biofilms had a lower CH/ATP ratio indicating different biofilm characteristics. When all the NaOH/SDS tests are accumulated, assuming a minor effect of the pH, and are correlated with the CH/ATP ratio of the reference biofilm sample of the same test there was a clear logarithmic trend observed of a decreasing cleaning efficiency with an increasing CH/ATP ratio (see Figure 4.8).

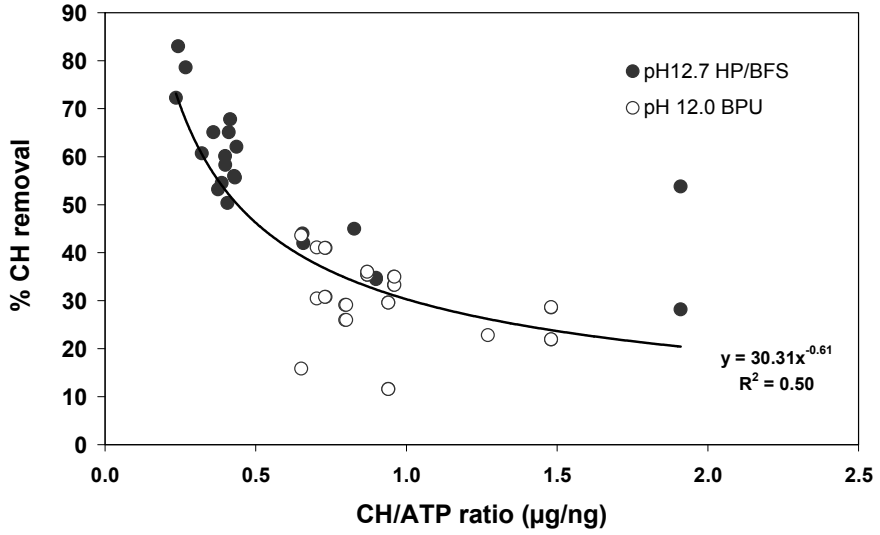


Figure 4.8. Influence of CH/ATP ratio and pH on the cleaning efficiency of the NaOH/SDS standard treatment for different biofilms

The results of NaOH/SDS demonstrate that the lab-scale test is to some extent reproducible and meets the requirement of having an objective test to evaluate agents and procedures, but the effect of the biofilm characteristics on the cleaning efficiency should be included in the test. Consequently for a proper and objective comparison of the cleaning efficiency of agents and protocols the standard NaOH/SDS test should be the reference cleaning as a parameter to account for differences in biofilm characteristics.

4.3 Cleaning efficiency assessment of agents and procedures with BFS biofilms

The biofilm samples produced with the BFS system in the current study (no recirculation and low temperature) was found to be not suitable for further studies. An initial test with NaOH pH12, SDS, EDTA, citric acid and NaOH/SDS showed complete removal for SDS (Images see 4.2.2) but unreliablie data for the removal of biofilms for the other treatments (Table 4.3). From the comparison of the biofilm and Fe data it became clear that the removal of Fe is similar to the removal of biofilm for NaOH/SDS (separate or combined) but not for citric acid and EDTA. This demonstrates the effect of the latter two agents usually applied to remove scalants from the surface of the membranes.

Table 4.3. ATP and CH removal (%) of the chemicals evaluated on the initial screening (duplicate values presented). Substratum: PVC tubes

	Biofilm age (d)	*NaOH/SDS (pH 12)*	*SDS 1% (pH 8)*	*NaOH (pH 12)*	*Citric Acid (pH 2)*	*EDTA (pH 11)*
ATP (%)	75	35; -52	99.8; 99.9	-471; -171	30; -14	-198; -216
CH (%)	75	57; -5	≥98	-75; 19	35; 18	5; -2
Fe (%)	75	35; -1	99.3; 99.6	-45; 22	94; 94	81; 76

[a] nd = not determined

4.4 Cleaning efficiency assessment of agents and procedures with BPU biofilms

The evaluated cleaning chemicals were divided into the following categories according to their cleaning mechanisms (number of chemicals): Alkalines (6), Detergents (5), Acids (5), Biocides (5), Chelating (1), and Enzymes (5) (see section 2.4.1 for more detail). Each of these categories included both analytical grade chemicals (referring to NaOH, HCl, EDTA, etc.) and commercial blends. Commercial blends are constituted by a mixture of different analytical grade chemicals and are produced by different manufacturers (Diversey, Ecolab®, Nalco, etc.). The commercial blends evaluated are the ones used by reverse osmosis full-scale installations in the Netherlands. All chemicals were evaluated in single steps cleaning protocols at room temperature (20 °C) to assess the individual cleaning effect of each chemical. Considering that in full-scale reverse osmosis installations cleaning-in-place procedures are carried out with more than one step, a cleaning protocol using multiples steps and a higher temperature (35 °C) were also evaluated, following the CIP protocols used in several reverse osmosis full-scale installations in the Netherlands (see section 2.5.2).

4.4.1 Initial screening: biomass and iron removal

One cleaning agent from each category (see section 2.4.1) was selected to make an initial screening using PVC plates from the BPU monitor as substratum. Analytical grade chemicals were used according to their pH: acid chemicals at pH 2 and alkaline chemicals at pH 12, which are the lowest/highest pH allowed by membrane manufacturers respectively (Hydranautics, Filmtec™). Detergents were used at the concentration recommended by literature or manufacturer.

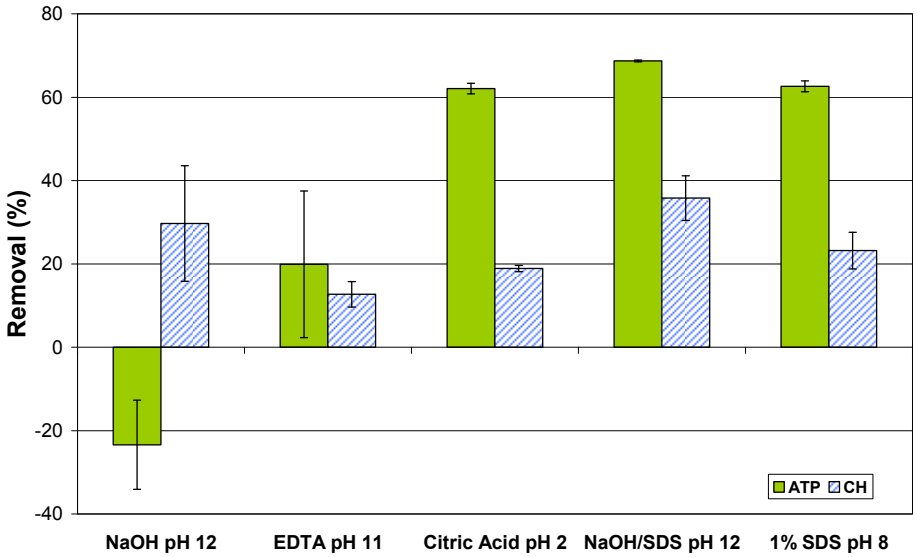


Figure 4.9. ATP and CH removal (%) of each chemical evaluated on the initial screening. Substratum: PVC plates (age 26 days)

Table 4.4. ATP and CH removal (%) of the chemicals evaluated on the initial screening. (duplicate values presented). Substratum: PVC plates

	Batch of BPU (CH/ATP)	*Biofilm age (d)*	*NaOH/SDS (pH 12)*	*SDS 1% (pH 8)*	*NaOH (pH 12)*	*Citric Acid (pH 2)*	*EDTA (pH 11)*
ATP (%)	1 (0.63)	26	68.5; 69	61.3; 64	no removal	63.3; 60.8	37.6; 2.3
	2 (1.10)	33	nd[a]	67.9; 64.3	47.3; 48.7	nd	61.7; 32.6
CH (%)	1 (0.63)	26	30.4; 41.1	18.8; 27.6	15.8; 23.6	18.2; 19.7	15.7; 9.6
	2 (1.10)	33	nd	21.6; 18.6	22.1; 35.2	nd	30.5; 4.5
Fe (%)	1 (0.63)	26	39.6	35.9	nd	65.6; 65.1	73.4; 42.0
	2 (1.10)	33	nd	20.8	9.7	nd	58.6

[a] nd = not determined

The highest ATP and CH removal was obtained in the standard treatment NaOH/SDS (Figure 4.9). Citric acid, SDS and EDTA had an ATP removal close to the standard treatment, but the CH removal in those treatments was 10 – 20% lower. Remarkable was the observation of the ATP data for the single treatment with NaOH where the ATP concentrations increased in stead of decreased. The CH removal, however, was slightly lower than the CH removal of the standard test. The results showed variation as presented in Table 4.4. This test was repeated for SDS, NaOH and EDTA with a second batch of biofilm samples. The cleaning efficiencies of SDS and NaOH for batch 1 and 2 were within limits reproduced but for EDTA the data were too variable for such a conclusion (Table 4.4).

EDTA was employed at a high pH (pH 11) since it has been proved that at an alkaline pH the chelating ability of EDTA to remove calcium ions increases since more carboxylic groups of EDTA are deprotonated at a high pH (Ang, 2006). In these biofilms also the Fe content was measured before and after cleaning to investigate to possibility to assess the effects on adsorbed inorganics in the biofilm. In batch one the Fe content in the three reference samples were 2.2; 2.3; 2.7 $\mu g/cm^2$ and in batch two this concentration was 12.2 and 11.5 $\mu g/cm^2$. The high effect of EDTA as well as citric acid to remove inorganic ions from the surface was demonstrated by the high efficacy to remove Fe (Table 4.4). The Fe concentrations were reduced by citric acid and EDTA for 42 – 74% in test 1 and 2 whereas NaOH/SDS, NaOH and SDS reduced these concentrations with lower efficacy <35%. Despite of this, EDTA and citric were slightly less effective to remove biofilm than the alkaline treatments (Table 4.4). Considering that these ions, especially divalent Ca acts through a chelating mechanism, this result indicates that both ions in the structure of the BPU biofilm do not play a significant role. Moreover, the results also indicate that biofilm and Fe removal are not directly correlated as also presented for the BFS biofilms before (see §4.3).

The standard cleaning method NaOH/SDS works by a synergy between the alkaline NaOH and the detergent SDS. The high pH of the NaOH increases the negative charge of the biofilm, intensifying the repulsive forces and thus destabilizing the biofilm structure (Liikanen et al., 2002). This allows for a better penetration of the SDS, an anionic detergent with the ability to disrupt cellular structures and denature them. The detergent molecules attach firmly to the protein molecules, masking their native charge and giving the protein the negative charge of the SDS (G-Biosciences). Additionally, it has been proved that a greater cleaning efficiency is obtained when combining NaOH with SDS than with a single cleaning with the individual chemicals (Madaeni and Samieirad, 2010; Li et al., 2005).

SDS was used at a concentration higher than its critical micelle concentration (CMC) reported to be 7-10 mM. It is important to work with a detergent concentration higher than the micelle concentration in order to ensure the spontaneous formation of micelles (G-Biosciences). During the cleaning test it was noticed the high foaming potential of the SDS. Its influence on the cleaning efficiency on spiral-wound membranes should be further investigated.

Other studies found in literature (Ang et al, 2006; Li and Elimelech, 2004; Zondervan and Roffel, 2007; van der Kooij, 2011; Arnal et al., 2008) show a low to moderate efficiency of citric acid and NaOH when used individually. Even though these studies evaluated their cleaning efficiency by means of operational parameters such as the membrane flux, the results can relate to the ones found in this

research. In this case, citric acid proved to be a strong chelating agent (high Fe removal), with a higher ATP removal than EDTA. This was also noticed visually, as observed in Figure 4.10, where after treatment with citric acid the brownish color of the biofilm had decreased considerably, probably due to the removal of iron deposits by the acid solution. This indicates that citric in general is a better agent when the inorganic fouling is combined with biofilms.

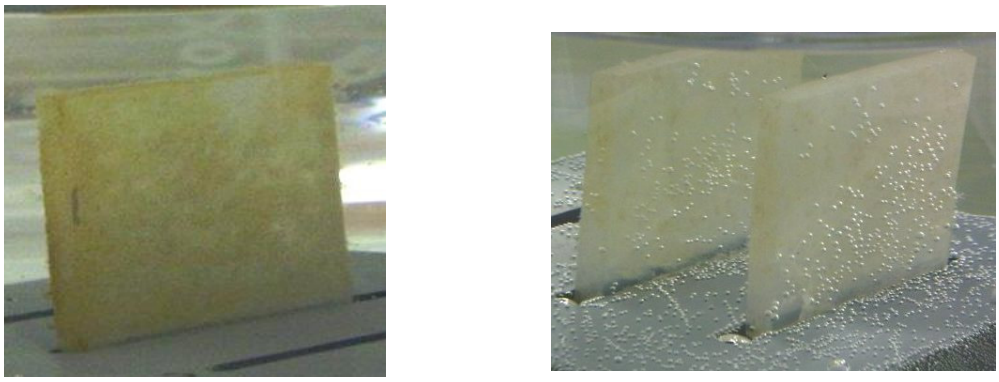


Figure 4.10. Biofilm on a PVC plate before (left) and after (right) treatment with Citric Acid (pH 2) during 60 minutes

4.4.2 Detergents

A selection of several detergents was evaluated. The cleaning chemicals were all analytical grade and were evaluated at the concentration recommended in the literature. The detergents evaluated included CTAB (Decyltrimethylammonium bromide) a cationic surfactant, STP (Sodium triphosphate) a chelating agent used in detergents, TSP (Trisodium phosphate) a phosphate based detergent and NaDDBS (sodium dodecylbenzenesulfonate) an anionic surfactant.

CTAB had a complete ATP removal whereas the carbohydrate removal was comparable with the removal of the standard test (Figure 4.11, Table 4.5). The other detergents had a very poor biofilm removal. The biofilm layer is considered to have a negative charge (Liikanen et al., 2002); this can explain the high interaction between the biofilm layer and CTAB which is a cationic detergent, and therefore can be easily bonded with the biofilm compounds destabilizing its structure and denaturing the bacteria.

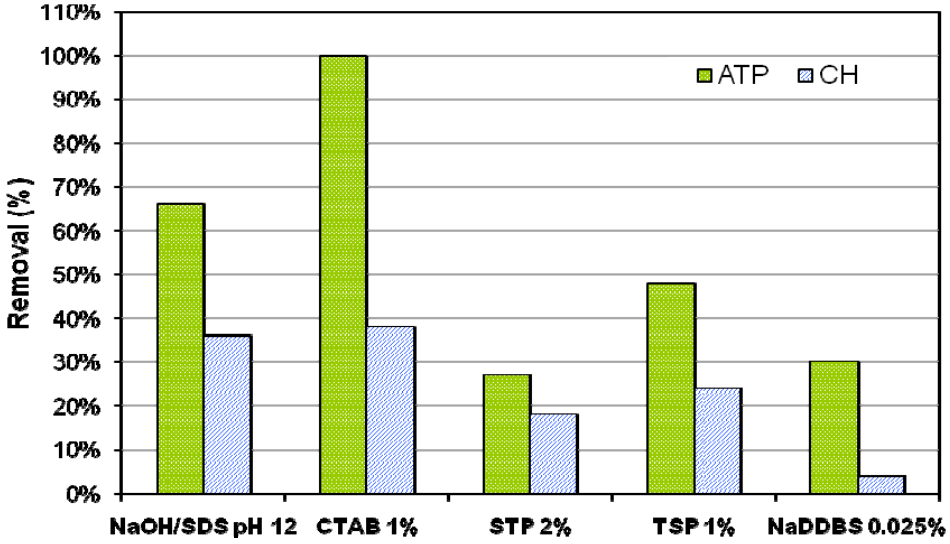


Figure 4.11. ATP and CH removal (%) of the detergents evaluated. Substratum: PVC plates (age 34 days

Table 4.5. ATP and CH removal (%) of the detergents evaluated (duplicate values presented). Substratum: PVC plates (age 34 days)

	NaOH/SDS (pH 12)	*CTAB 1% (pH 5)*	*STP 2% (pH 8.75)*	*TSP 1% (pH 12)*	*NaDDBS 0.025% (pH 7)*
ATP (%removal)	59; 72	100;100	24; 31	43; 53	28; 33
CH (%removal)	31; 41	36; 41	17; 19	19; 28	7; 2

In results presented by Whittaker et al. (1984) using secondary treated municipal wastewater as feed water of spiral-wound RO membrane units, CTAB showed a low removal of the biofilm layer (<50%, determined by observation with scanning electron microscopy SEM). The high effect on ATP means that this detergent is effective in inactivation of the active biofilm on RO systems fed with water with low organic compounds concentration as used in the presented research.
The charge theory explained for the combination NaOH/SDS may also explain the low removal with NaDDBS at neutral pH, since it is an anionic detergent and it's more difficult to penetrate the biofilm structure. STP and TSP are components mainly used in commercial detergents. STP is a highly charged chelating agent therefore it is employed as a water softener (buider) (Schrödter, 2008). Both chemicals can present a higher efficacy when combined with surfactants, as shown by van der Kooij et al. (2011), where a solution of STP and NaDDBS presented an increase in the membrane permeability of 60%. The lack of a surfactant in the current study might explain the low biofilm removal obtained for these chemicals when evaluated individually. A visual inspection of the plates after chemical treatment confirmed the low removal results obtained in the laboratory measurements, since no considerable difference was observed between the reference samples and the cleaned samples.

4.4.3 Biocides

The biocides evaluated were DBNPA (2,2-Dibromo-3-nitrilopropionamide), sodium bi-sulphite (both non-oxidizing biocides) and sodium hypochlorite (NaOCl), a strong oxidizer. The biocides were used at the concentration recommended by literature or manufacturer. The high biofilm removal of the NaOCl, with a complete ATP removal and a considerably high CH reduction of 71% are shown in Figure 4.12 and Table 4.6.

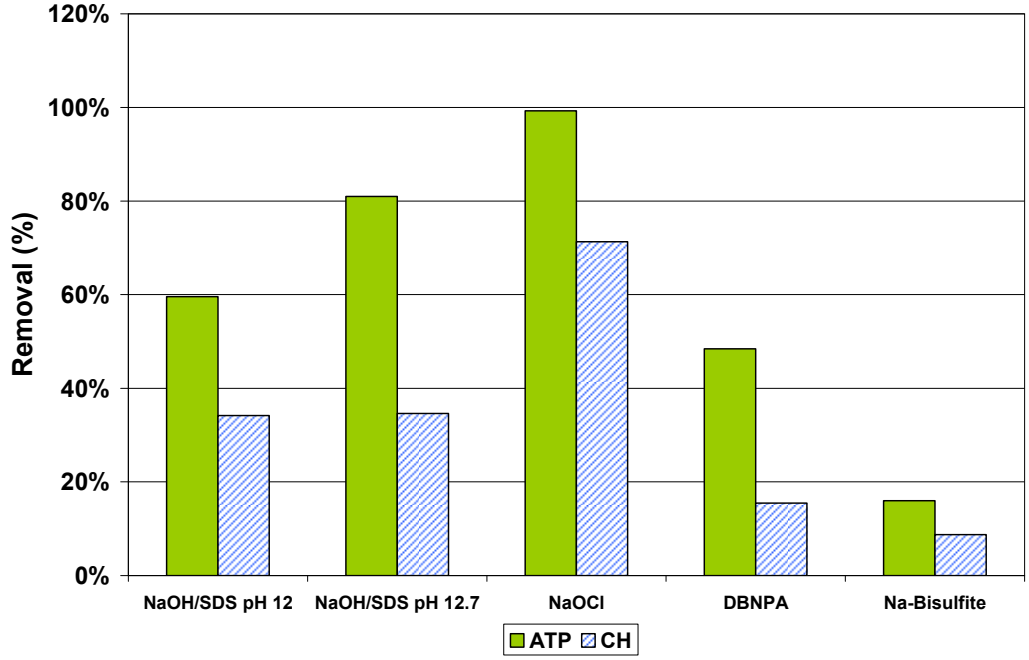


Figure 4.12. ATP and CH removal (%) of the biocides evaluated. Substratum: PVC plates (age 41 days).

This behavior is explained by the dissociation mechanism of sodium hypochlorate at high pH. NaOCl dissociates in hypochlorous acid and sodium hydroxide when added to water (see Eq. 1):

$$NaOCl + H_2O \rightarrow HOCl + NaOH \quad (1)$$

In this research, the solution of sodium hypochlorite was evaluated at a pH of 11.4, which means that the hypochlorous acid (HOCl) is mainly dissociated in hypochlorite ion (OCl-) (Selby, 2011). It is believed that hypochlorous acid (found mainly at a pH of 6) is a more effective biocide than hypochlorite ion, but according to a research carried out by Charaklis (1990) the biofilm destruction is improved when using sodium hypochlorite at high pH, meaning that the hypochlorite ion is more effective at removing biofilm. This statement is confirmed also visually in Figure 4.13, where a considerable decrease of the biofilm layer after chemical treatment with NaOCl. The high biofilm removal rate presented by the NaOCl coincides also with the findings of Zondervan and Roffel (2007) and Arnal et al (2008), both using surface water as feed water in UF systems. And also the study of Chen and Stewart (2000) showed a considerably higher biofilm removal for hypochlorite at higher pH.

Table 4.6 ATP and CH removal (%) of the biocides evaluated (duplicate values presented). Substratum: PVC plates (age 41 days)

	NaOH/SDS (pH 12)	***NaOH/SDS (pH 12.7)***	***NaOCl 0.5% (pH 11.4)***	***DNBPA 50 ppm (pH 6)***	***Na Bisulfite 500ppm (pH 4)***
ATP (%removal)	61; 58	76; 85	99; 99	53; 44	22; 11
CH (%removal)	33; 35	35; 34	68; 75	16; 15	14; 4

Non-oxidizer biocides proved to be not effective for the type of biofilm used, obtaining very low %removal for both ATP and carbohydrates.

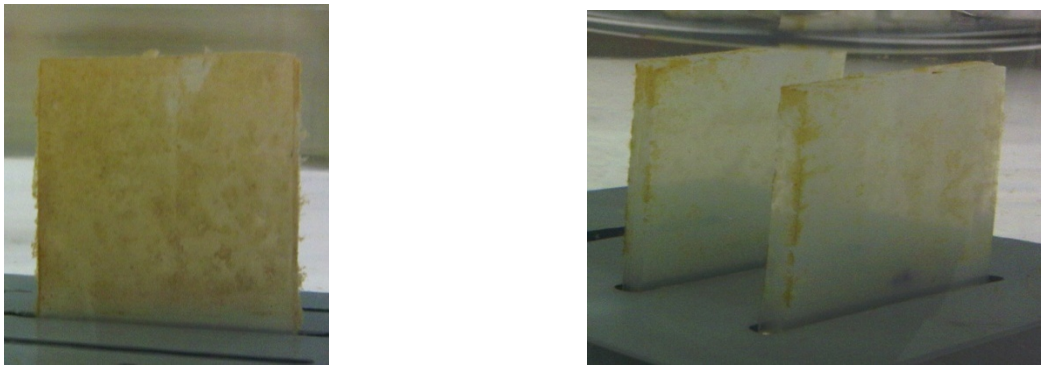


Figure 4.13. Biofilm on a PVC plate before (left) and after (right) treatment with NaOCl (0.5%, pH 11.4) during 60 minutes

4.4.4 Acid solutions

The cleaning efficacy of two strong (HCl and Divos 2, both at a pH of 2) and a mild (PermaClean 77 at a pH of 4) acid solutions was evaluated. Analytical grade chemicals were used according to their pH (acid at the lowest pH allowed by membrane manufacturers). Commercial cleaners were used at the concentration recommended by literature or manufacturer.

PermaClean 77 and Divos 2 are acid commercial blends used at reverse osmosis full-scale installations. Both solutions showed very low biofilm removal efficiency (≤15%) together with HCl, an analytical grade chemical commonly used as cleaning agent (see Figure 4.14, Table 4.7). Of the three solutions evaluated, Divos 2 (a mixture of nitric and phosphoric acid at a pH of 1.6) had the highest ATP removal, demonstrating the influence of a strong pH on the deactivation of bacteria. Considering that the fouling layer on the plates was mainly composed by biofilm rather than by salts and minerals (no data available), the low efficacy of the acid cleaners was expected, since they are more applied for the removal of scaling.

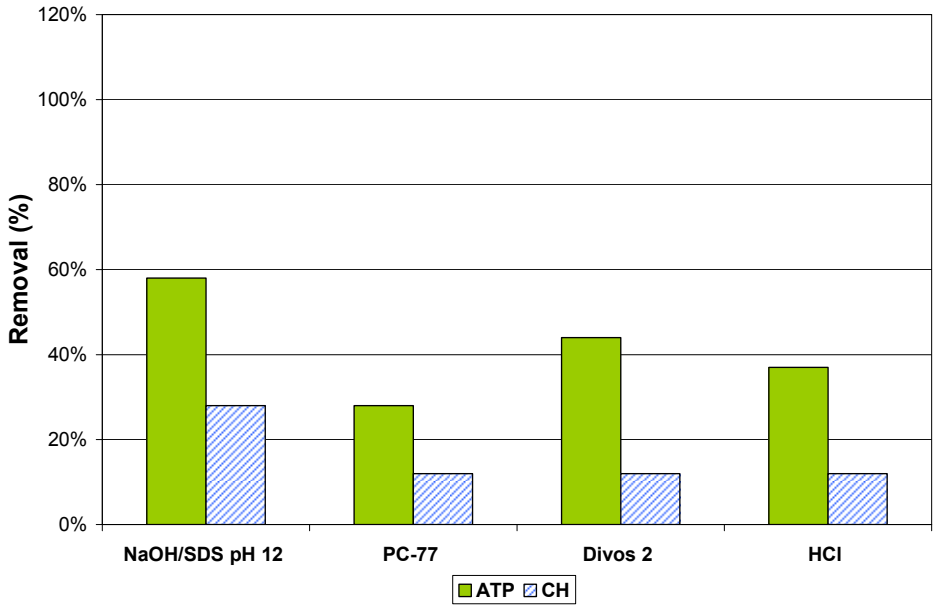


Figure 4.14. ATP and CH removal (%) of the acid solutions evaluated. Substratum: PVC plates (age 39 days)

Table 4.7 ATP and CH removal (%) of the acid solutions evaluated (duplicate values presented). Substratum: PVC plates (age 39 days)

	NaOH/SDS (pH 12)	***PermaClean 77 4% (pH 4)***	***Divos 2 0.4% (pH 1.6)***	***HCl (pH 2)***
ATP (%removal)	61; 56	26; 30	44; 45	38; 36
CH (%removal)	26; 29	10; 14	15; 8	21; 3

Permaclean 77 is a membrane cleaner designed especially for the removal of iron fouling. After a direct observation of the plate samples after chemical treatment (Figure 4.15) a considerable reduction on the color of the biofilm was observed, which demonstrates the high efficiency of the cleaner in the removal of iron deposits (no data collected).

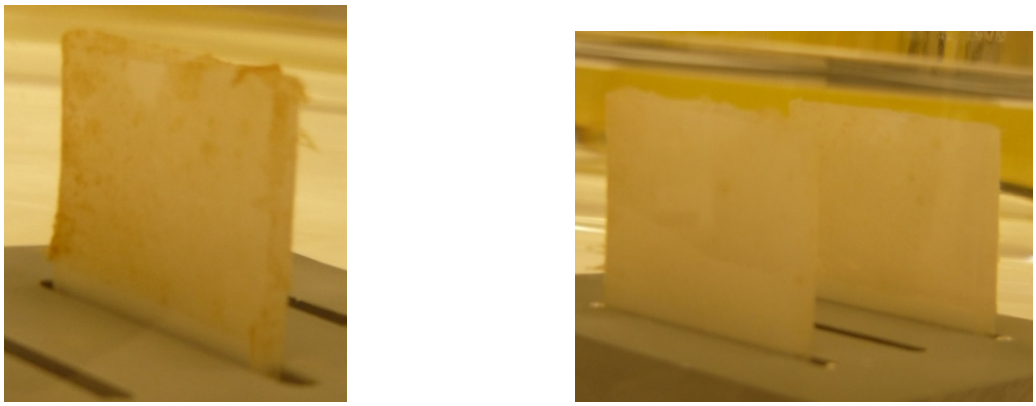


Figure 4.15. Biofilm on a PVC plate before (left) and after (right) treatment with Permaclean 77 (4%, pH 4) during 60 minutes

4.4.5 Alkalines

Several alkaline commercial blends used at full-scale reverse osmosis installations were evaluated. The commercial cleaners were used at the concentration recommended by the manufacturer. From the

results presented at Figure 4.16 and Table 4.8, the cleaner with a highest removal efficacy was Divos 116, which used a lower concentration than the other cleaning solutions showing the high efficiency of this cleaner for the removal of ATP. For the carbohydrates, however, the %removal for all cleaner evaluated was quite low (<30%). Floclean MC11, despite being a mixture of detergent builders (STP, TSP), chelating agents (EDTA, citric acid) and pH buffer (Floclean® MC11, BioLab Water Additives, UK) showed to have the lowest cleaning efficiency for the biofilm used.

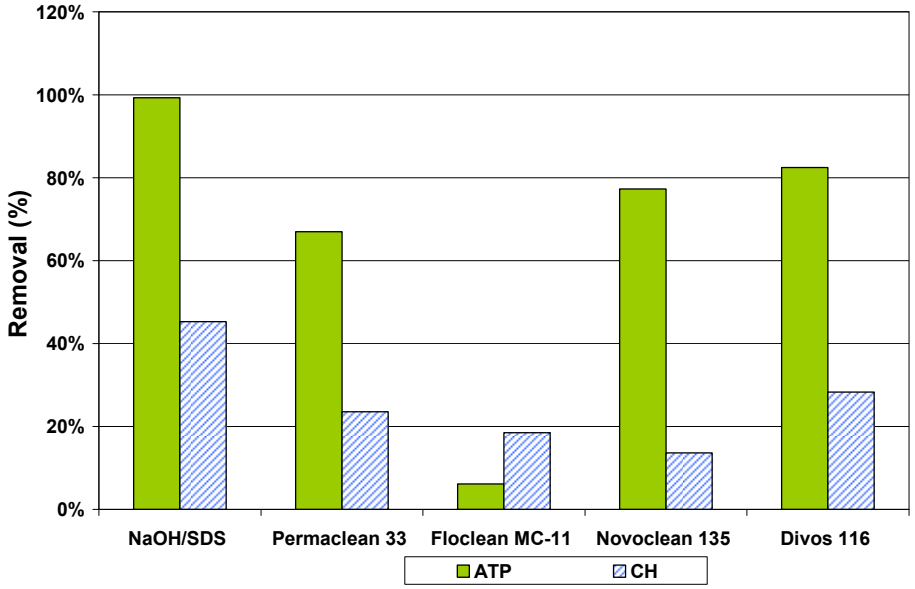


Figure 4.16. ATP and CH removal (%) of the alkaline solutions evaluated. Substratum: PVC plates (age 30 days).

Table 4.8. ATP and CH removal (%) of the alkaline solutions evaluated (duplicate values presented). Substratum: PVC plates (age 30 days).

	NaOH/SDS (pH 12.7)	***PermaClean 33 2% (pH 12)***	***Floclean MC11 2% (pH 11)***	***Novoclean 135 0.4% (pH 12)***	***Divos 116 0.8% (pH 12)***
ATP (%removal)	99; 99	77; 74	2; 10	78; 77	85; 80
CH (%removal)	29; 54	27; 20	18; 19	21; 6	34; 22

Despite the low carbohydrate removal obtained by the alkaline cleaners, the ATP removal was considerably higher than for the acid cleaners, confirming that alkaline chemicals are more recommended for reducing fouling caused by biofilm and organic substances. The low carbohydrate removal showed by the alkaline cleaners was also observed after a visual inspection of the biofilm samples, observing no considerable differences between the reference and the cleaned samples.

4.4.6 Ecolab products

Several commercial cleaning products by the manufacturer Ecolab® recommended for membrane cleaning were evaluated. The used products are: P3-Ultrasil 73, an acid cleaner containing citric and lactic acid among others; P3-Ultrasil 141, a mild alkaline cleaner with KOH and phosphoric acid tripotassium salt; P3-Ultrasil 53, a enzymatic cleaner containing mainly EDTA and phosphates; and P3-Oxyzan ZS, a biocide with acetic acid as main component and peracetic acid and hydrogen peroxide at low concentrations (information obtained from the MSDS of each product). The same trend observed for the commercial cleaners previously presented, was shown by the Ultrasil products evaluated (see Figure 4.17, Table 4.9). P3-Oxyzan ZS and P3-Ultrasil 53 had the highest ATP removal, but were not able to reduce the carbohydrates concentration of the biofilm, which means that these cleaners are effective at deactivating bacteria but not at removing the biofilm EPS. For the others Ultrasil products, their biofilm removal efficiency was quite low.

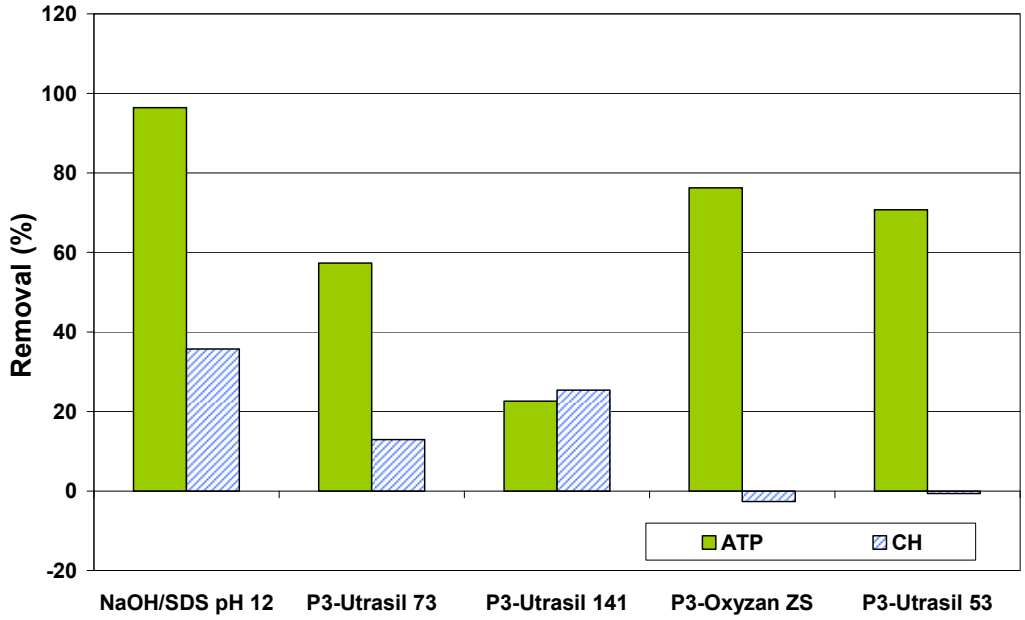


Figure 4.17. ATP and CH removal (%) of the Ecolab® solutions evaluated. Substratum: PVC plates (age 33 days)

Table 4.9. ATP and CH removal (%) of the Ecolab® solutions evaluated (duplicate values presented). Substratum: PVC plates (age 33 days)

	NaOH/SDS (pH 12.7)	***P3-Ultrasil 73 1% (pH 2.5)***	***P3-Ultrasil 141 0.1% (pH 11)***	***P3-Oxyzan ZS 0.1% (pH 3)***	***P3-Ultrasil 53 1% (pH 9)***
ATP (%removal)	95; 98	55; 60	18; 27	77; 76	72; 70
CH (%removal)	35; 36	9; 17	30; 21	0; 5	10; 0

4.4.7 Enzymes

The following enzyme categories were evaluated: alpha-amylase (BAN 480), lucoamylase/pullulanase (Dextrozyme) and protease (Savinase, Everlase), all from Novozymes (Novozymes A/S, Denmark). Some researchers have shown the efficacy of enzymes for the degradation of the biofilm EPS (Augustin et al., 2004; Johansen et al., 1997; Melo et al. 1997; Lequette et al., 2010). Molobela et al. (2010) studied the removal efficacy of Savinase, Everlase and BAN, obtaining an 80% reduction of *P. fluorescens* biofilms and on the degradation of EPS for the protease enzymes. In the research presented, the ATP and CH removal achieved by the enzymes evaluated on this test was quite low (see Figure 4.18, Table 4.10), with some enzymes showing even an increase on the ATP and CH concentration of the biofilm after the treatment. This behavior observed could mean that the biofilm used the enzymes as substrate, enhancing the biofilm growth.

Table 4.10. ATP and CH removal (%) of the enzyme solutions evaluated (duplicate values presented). Substratum: PVC plates (age 37 days)

	NaOH/SDS (pH 12)	***NaOH/SDS + BAN 480***	***NaOH/SDS + Savinase***	***BAN 480 (pH 5)***	***Dextrozyme (pH 5)***	***Savinase (pH 7)***	***Everlase (pH 7)***
ATP (%)	73; 77	88; 86	85; 79	-22; -10	-11; -22	-3; 17	-21; -22
CH (%)	18; 23	25; 16	32; 21	-7; -7	-6; -11	-20; -27	-26; -22

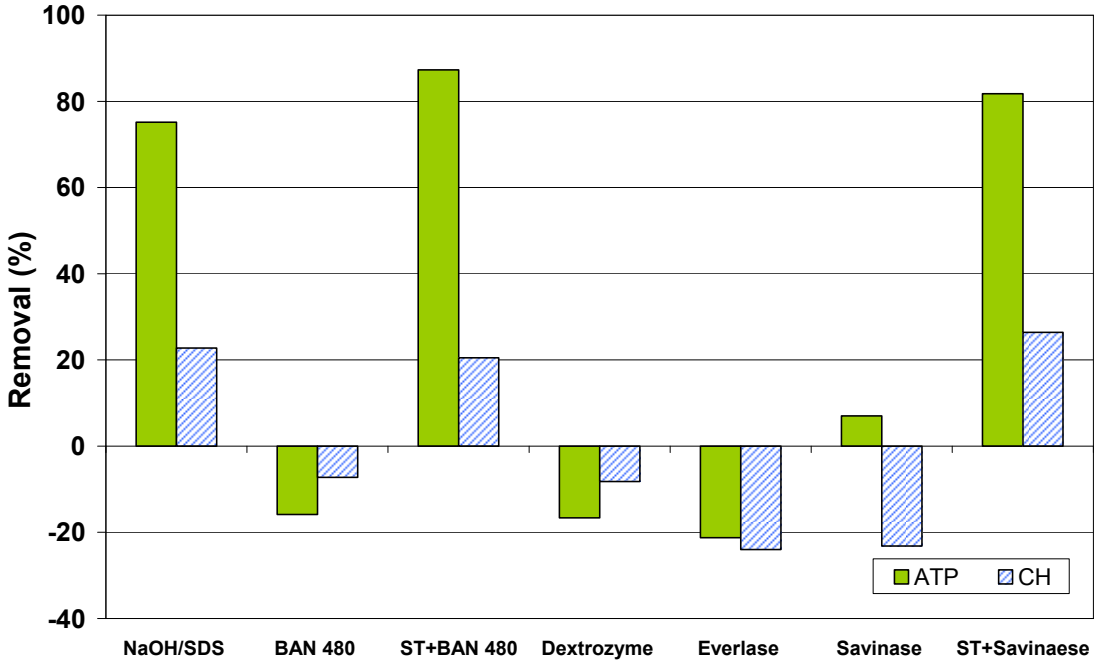


Figure 4.18. ATP and CH removal (%) of the enzyme solutions evaluated separately or with a preceending NaOH/SDS treatment (ST) for two of the enzymes. Substratum: PVC plates (age 37 days)

To avoid this, a multiple step protocol consisting of a first step with NaOH/SDS followed by a step with an enzyme (BAN480 and Savinaese) was evaluated. The hypothesized mechanism is that the solution of NaOH/SDS will remove the main layers of the biofilm and deactivating most of the bacteria present in it, leaving the base biofilm layer exposed for the enzyme action. The %removal obtained for this two step protocol was similar to the single standard treatment indicating no additional removal during the second enzyme treatment step (Figure 4.18). One of the reasons for the low efficacy obtained in the tests is that enzymes are recommended to be used in a mixture due to the heterogeneity of the extracellular polymeric substances of the biofilm. When combining different enzymatic activities a considerable degradation of the bacterial biofilm could be obtained (Augustin et al., 2004).

4.4.8 Multiple steps

Cleaning protocols consist usually of several cleaning steps. In this study the protocols of the Dutch water companies were evaluated used at full-scale installations (Table 3.4). The multiple steps protocols were carried out at a temperature of 35 ºC. In Test 1, three alkaline + acid steps and one acid + alkaline step were evaluated. The results presented on Figure 4.19 and Table 4.11 show no real increase of biomass removal compared to the standard test in one step with NaOH/SDS. Thus, no additive effect of the second cleaning step was observed. The protocol with an alkaline treatment as first step had twice the ATP removal obtained with the protocol starting with an acid step (Citric acid+NaOH). This is in agreement with previous results (see 4.3.4 and 4.3.5) and expected since alkaline conditions make biofilms more susceptible for penetration due to its electrostatic interaction. The treatment with NaOH + HCl and NaOH + Divos 2 had similar results, meaning that there is not a considerable difference between HCl and the commercial cleaner Divos 2. The detergent in the double treatment NaOH/SDS + HCl caused a slightly higher removal compared to NaOH + HCl. From the results it became clear that the cleaning efficiency of these multiple steps protocols at higher temperatures was hardly better than observed for the individual chemicals (Table 4.11).

Table 4.11. Comparison of the cleaning efficiencies of the chemicals used in multiple step Test 1 and the cleaning efficiencies of the individual chemicals

	Cleaning Protocol	ATP (%removal)	CH (%removal)
Multiple steps	NaOH/SDS pH12	90 – 91	22 – 29
	NaOH/SDS pH12 + HCl pH2	54 – 63	34 – 25
	NaOH pH12 + HCl pH2	47 – 47	21- 22
	NaOH pH12 + Divos 2 pH1.6	41 – 56	29 – 23
	Citric A. pH2 + NaOH pH12	27 – 23	26 – 25
Individual steps	NaOH pH12	47 – 49	18 – 35
	Citric Acid pH2	63 – 61	18 - 20
	SDS 1% pH8	61 – 64	19 – 28
	Divos 2 0.4% pH1.6	44 – 45	15 – 8
	HCl pH2	38 – 36	21 – 3

In Figure 4.20 and Table 4.12, the results of a second test with multiple steps protocols are presented. In this test, two acid + alkaline steps and two alkaline + acid steps protocols were evaluated. The results show also a quite low removal for both protocols, again demonstrating that the sequence of the pH step does not have a large impact on this biofilm. The highest CH removal was observed for the combination of Divos 2 + Divos 16 (acid + alkaline) showing a cumulative effect, while this was not observed for the other two step protocols.
A visual inspection of the plates after chemical treatment from Test 1 and 2 confirmed the low removal results obtained in the laboratory measurements, since no considerable difference was observed between the reference samples and the cleaned samples.

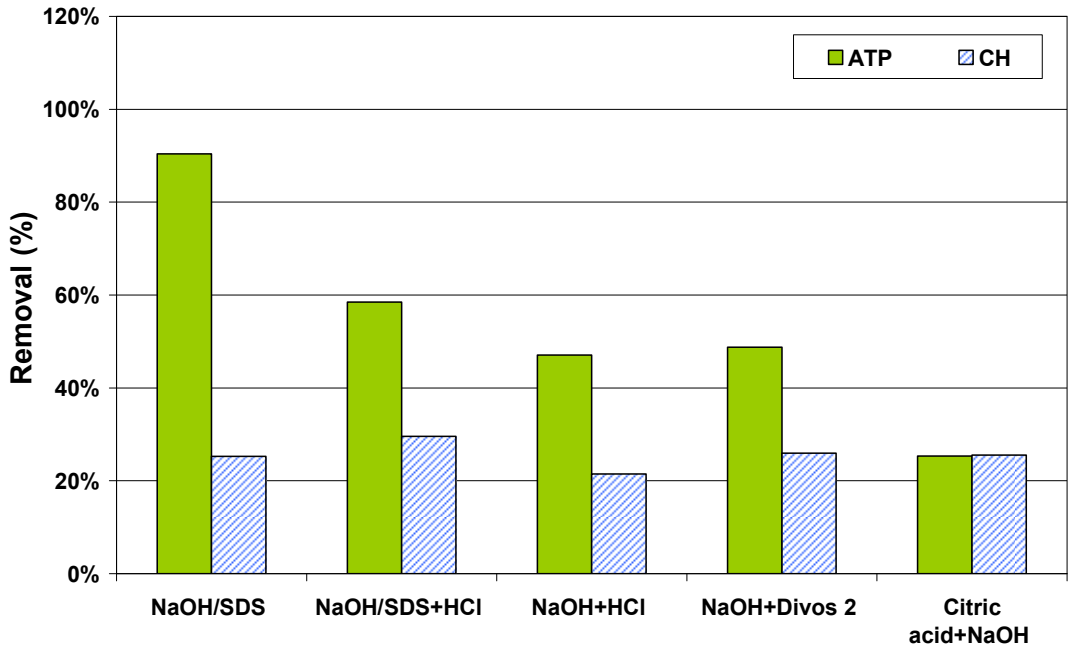


Figure 4.19. ATP and CH removal (%) of the multiple steps protocols evaluated on Test 1. Substratum: PVC plates (age 36 days)

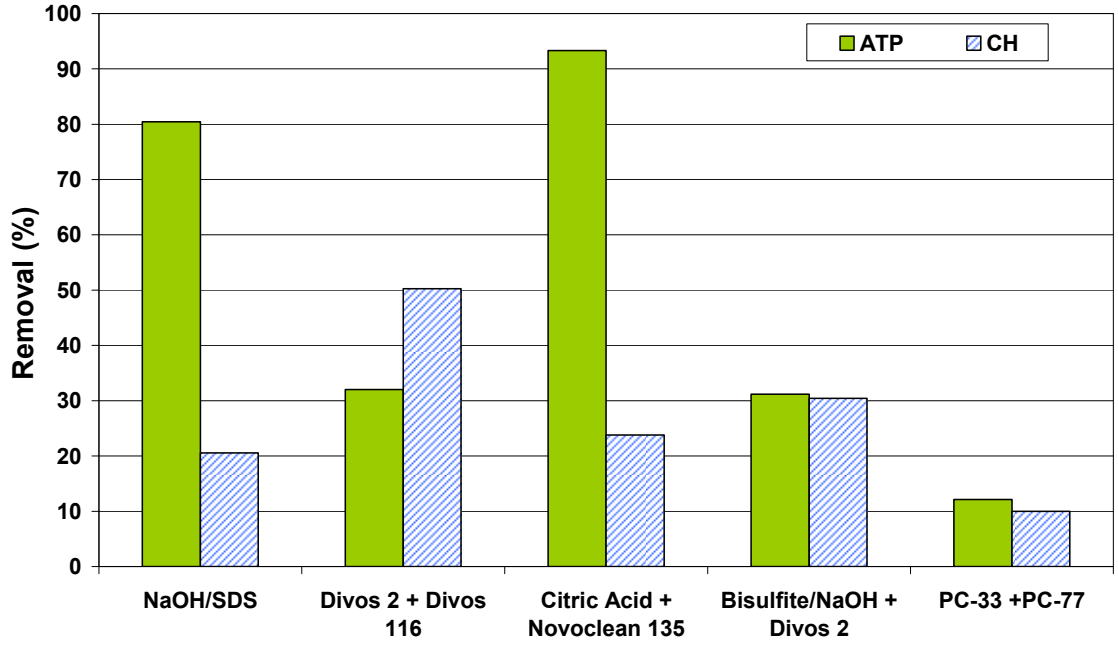


Figure 4.20. ATP and CH removal (%) of the multiple steps protocols evaluated on Test 2. Substratum: PVC plates (age 36 days)

Table 4.12. Comparison of the cleaning efficiencies of the chemicals used in multiple step Test 1 and the cleaning efficiencies of the individual chemicals

	Cleaning Protocol	ATP (%removal)	CH (%removal)
Multiple steps	NaOH/SDS pH12	73 – 86	27 – 23
	Divos 2 pH1.8 + Divos 116 pH12	33 – 24	49 – 46
	Citric A. pH2 + N135 pH12	94 – 92	27 – 30
	Bisulfite/NaOH pH12 + Divos2 pH1.8	20 – 39	27 – 32
	PC-33 pH12 + PC-77 pH4	13 – 12	14 – 6
Individual steps	NaOH pH12	47 – 49	18 – 35
	SDS 1% pH8	61 – 64	19 – 28
	Divos 2 pH1.6	44 – 45	15 – 8
	Divos 116 pH12	80 – 85	22 – 34
	Citric Acid pH2	63 – 61	18 – 20
	Novoclean 135	78 – 77	21 – 6
	PC-33 pH12	77 – 74	27 – 20
	PC-77 pH4	26 – 30	10 – 14

4.5 Cleaning efficiency for other biofilms

In paragraph 4.2.5 it was concluded that the biofilm characteristics have an impact on the cleaning efficiency assessed for NaOH/SDS. This effect of the biofilm characteristics was also investigated for other biofilms.

4.5.1 Biofilm samples from the BFM

The biofilm samples grown in the biofilm monitor supplied with pretreated surface water were evaluated with the same analytical grade chemicals of the initial screening test with the BPU samples. The biofilm concentration on the plates was lower than in observed in the BPU monitor and a variable CH/ATP ratio (Table 4.13).

Table 4.13. The biomass concentration on the plates in the BFM (age 99 days) supplied with pretreated surface water and the cleaning efficiency of some analytical grade chemicals in comparison with the efficiency assessed on BPU biofilms

References	*ATP; CH (pg or μg/cm²)*	*CH/ATP (μg/ng)*	*Cleaning agents*	*ATP (% removal) (BFM ; BPU)*	*CH (% removal) (BFM ; BPU)*
1	5.8x10⁴ ;49.4	0.86	NaOH/SDS pH12	59–71 ; 68–69	35–41 ; 30–41
2	4.9x10⁴; 32.4	0.66	NaOH pH12	48–66 ; 47–49	54–64 ; 18–35
3	2.9x10⁴; 43.5	1.51	Citric A. pH2	-3–5 ; 63-61	34–18 ; 18–20
4	6.6x10⁴; 40.9	0.62	SDS pH8	78–56 ; 61–64	86–40 ; 19–28
5	3.6x10⁴; 47.3	1.31	EDTA pH11	-7–25 ; 62–33	62–86 ; 31–5

Visual inspection of these biofilms showed that the Fe content of this biofilm sample was most likely low (no brown colour see Figure 4.8; no data presented). The results showed higher ATP removal for NaOH and SDS compared to citric and EDTA. The CH removal from the BFM biofilm samples was variable and for Citric and EDTA higher than the ATP removal. The removal by NaOH/SDS from BFM and BPU biofilm samples was similar but for the other agents the removal from the BFM biofilm samples was higher. Due to exhaustion of samples no further tests were preformed on the BFM biofilm samples. These results were more variable than observed in former tests, but clearly show the effect of the biofilm characteristics on the cleaning efficiency assessment.

4.5.2 Spiral-wound membrane biofilms

There were two biofilms on spiral-wound membranes tested in the current study. The first was derived from a full-scale desalination plant using *seawater* as the feed water. Approximately 24 hours after the sampling of an element from the plant the module was opened for autopsy.

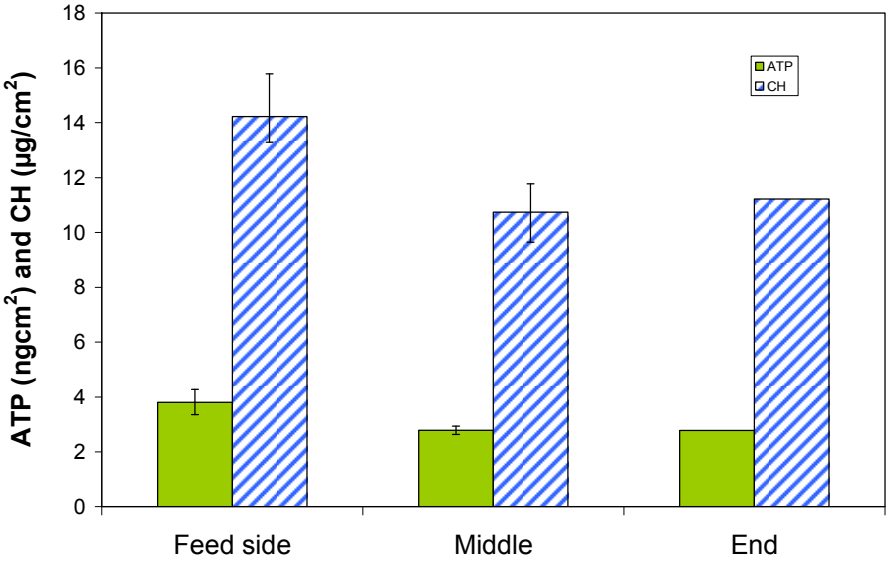


Figure 4.21. The biofilm concentrations (ATP and CH) on a RO membrane element from a full-scale desalination plant fed with seawater (Evides Water company)

The highest biomass concentration was observed at the feed side of the element but the spatial distribution of the biomass showed no large differences in concentrations on the membrane (Figure 4.21). Based on these concentrations of ATP and CH and the threshold values assessed for pressure

drop increase in spiral-wound membranes for these parameters of respectively 3.7 ng ATP and 8.1 µg CH per cm² for 100% pressure drop increase (Hijnen et al., 2011) it is concluded that the RO membrane suffers from biofouling. This membrane material (Fig. 4.22) was used in a cleaning test with selected agents and the cleaning protocol applied at the site.

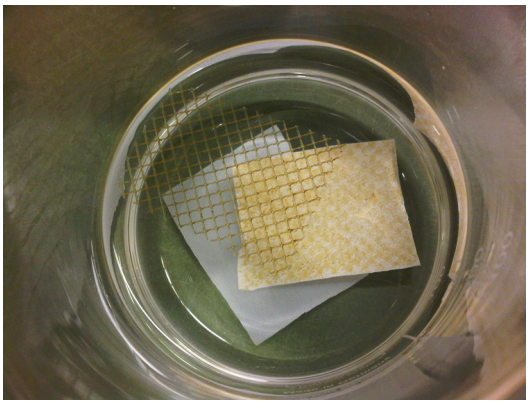


Figure 4.22. RO membrane sample in the laboratory cleaning test

The results presented in Table 4.14 showed that treatments with high pH caused the highest ATP removal. The CH removal ranged from -21% for Divos 2 to 56% for Citric acid. The applied protocol from the site of the RO installation (NaOH/SDS + HCl) was less efficient than the same protocol in a different sequence (acid – alkaline) or the other individual agents. In the laboratory test using BPU biofilms the cleaning efficiencies were lower for most agents and protocols, despite the lower CH/ATP ratio. As to the relative comparison between the agents and protocols these results showed the same conclusion for Divos 2 as the least effective agent, but for the other agents and protocols the sequence in efficacy derived from these results was different from the sequence assessed on the RO membrane material. Though the results should be regarded as indicative (RO membrane test done with one sample) it again shows the effect of the biofilm characteristics on the cleaning efficacy. Here we compare the effect on biofilms grown in seawater and fresh water environment with quite different characteristics. The data demonstrate that the CH/ATP ratio can not be used as a general biofilm characteristic for the cleaning efficacy as concluded from Figure 4.8. For an objective and decisive conclusion on the use of the laboratory cleaning test for assessment of the biofilm removal efficiency of cleaning agents and protocols the BPU bioflm on plasticized PVC should be produced in seawater.

Table 4.14. The cleaning efficiency assessed for a RO membrane in the laboratory test compared to the test results of the same agents and protocols determined for BPU biofilms in separate experiments

	RO membrane (CH/ATP 5.3-16.0)		*BPU biofilm (CH/ATP 0.6-1.2)*	
	ATP removal (%)	CH removal (%)	ATP removal (%)	CH removal (%)
NaOH/SDS(1.0%) pH12	99	41	58-96	23-36
EDTA pH11	57	43	2-62	5-31
Citric A. pH2	45	56	61-63	18-20
Divos 2 pH1.64	63	-21	44-45	8-15
NaOH/SDS(0.02%) + HClpH2[a]	99	29	54-63	25-34
HClpH2 + NaOH/SDS(0.02%)	100	45	47-47	21-22

[a] Cleaning protocol applied at the RO membrane site

The second membrane biofilm was produced in a *fresh water* environment. As presented in the Materials and Methods chapter, three spiral-wound membrane elements were employed in a pilot-plant setup. This experiment had three major objectives:

- to evaluate the influence of the nutrients solution composition on the fouling rate in the membranes;
- to assess the efficiency of a mechanical cleaning by means of an air/water cleaning;
- and to assess the cleaning efficiency on the membrane biofilm samples.

The setup was fed with drinking water plus an external dosing of easily biodegradable compounds in a concentration of 10 µg C/l. The conditions under which each element was operated are presented in Table 4.15.

Table 4.15. Operation conditions for the spiral-wound elements used in the pilot-plant setup

	***Nutrients supply** (10 µg C/l)*	***Air/Water Cleaning***
Element 1	Na-Acetate	No
Element 2	Mixed nutrients [a]	No
Element 3	Mixed nutrients [a]	Yes (daily)[b]

[a] Sodium acetate, glucose, glutamate and sodium benzoate; [b] With a duration of 5 minutes and an air/water ratio of 4:1

In Figure 4.23 it can be seen that after 75 days the pressure drop in all elements increased quite fast due to biofouling of the membrane elements, even with feed water with a low carbon content of 10 µg C/l. The duration of the lag phase of the increase in pressure drop in this experiment was long compared to former experiments because in the first stage a nutrient solution with a carbon content of 15 ng C/l was dosed. When the nutrient dosing was set at the level presented above (10 µg C/l) the pressure drop increase started after approximately 10 days. Element 1 presented a slightly higher pressure drop compared to Element 2. Considering that both elements had no cleaning during the run, this increase might be due to the different composition of the nutrient solutions used for each element. With only acetate as the solely nutrient, the biofilm composition will be composed of the bacterium with the highest affinity for this substrate. With the mixed nutrient one might expect a lower biofilm formation rate because of the more complex microbial population.

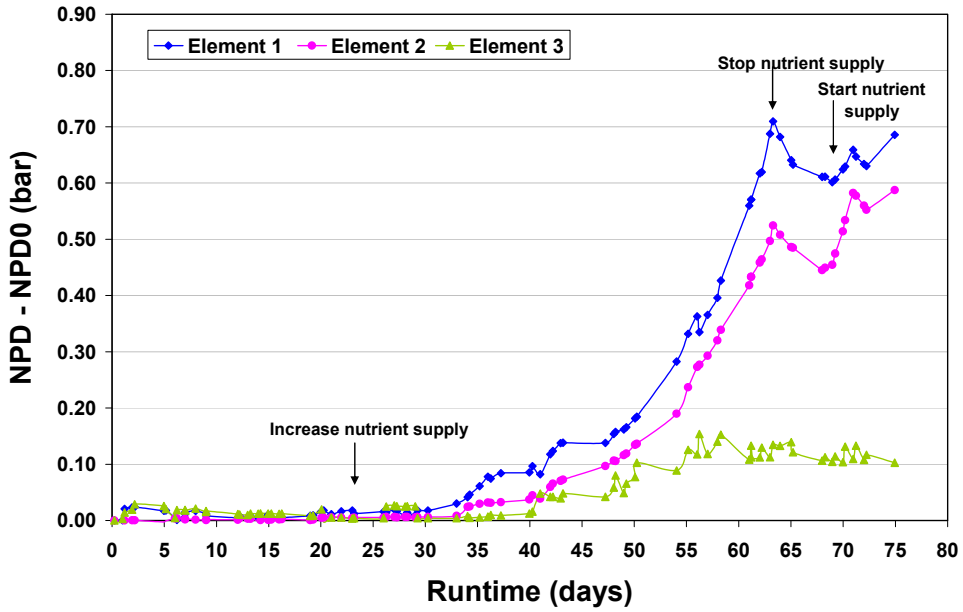


Figure 4.23. Normalized pressure drop difference for each of the elements evaluated on the spiral-wound membrane setup

It is also noticeable that the daily Air/Water Cleaning performed in Element 3 was able to stabilize the pressure difference in the element after an initial increase of 0.15 bars, showing that mechanical cleaning is effective in controlling the pressure drop increase in spiral-wound membranes caused by biofilm formation. The pressure drop showed to be the only variable affected by the biofilm growth on the membrane, since the other operational parameters of the elements evaluated (see table 4.16) had very similar values. Unclear is whether the observed difference in ΔMTC decrease is significant and can be explained by the different operational conditions.

Table 4.16. Operational variables of the spiral-wound elements setup at the time of the autopsy

Variable	*Element 1 SWM 1*	*Element 2 SWM 2*	*Element 3 SWM 3*
Flux decrease	0.989	1.002	0.955
Recovery (%)	12.276	14.279	13.483
Mass Transfer Coefficient (MTC)	2.278	2.222	2.225
ΔMTC ($MTC_0 - MTC_t$)	0.278	0.206	0.384
Max. ΔP (bar)	0.709	0.582	0.154

The elements were opened afterwards for autopsy and cleaning test. The ATP and CH concentration obtained in the autopsy (see Table 4.17) were higher in the feed side of the membrane and decreased towards the end of the element. This biofilm distribution behavior on the membrane surface was expected (Vrouwenvelder et al, 2008; Hijnen et al., 2011) considering that the feed side receives the higher load of nutrients which stimulates a higher biofilm production in this side of the membrane.

The results showed relatively low ATP concentrations in the membrane, much lower than observed in the PVC biofilm monitors (see section 4.2.5) and also lower than observed by Hijnen et al. (2011) using the Membrane Fouling Simulator at the same nutrient concentration of 10 µg of acetate-C/l. In this research the ATP concentrations were $>10^4$ pg/cm^2. The CH results were more in line with the published data. Thus the high CH/ATP ratios obtained suggest a low active biomass on the membrane surface. A possible reason for this low ATP concentration and high CH/ATP ratio is the period of no dosing prior to the autopsy which reduced the pressure drop (see Figure 4.23) due to a loss of active biofilm.

Table 4.17. Autopsy results of the spiral-wound membrane (SWM) elements used in the pilot-plant setup

SWM 1	*Total ATP*	*CH*	*Total*	*CH/ATP*
Sample	*(pg/cm2)*	*(µg/cm2)*	*Surface (cm2)*	
Feed	$1.9x10^3$; $1.6x10^3$	27.6; 19.9	41.9; 42.3	14.5; 12.4
Middle	$7.8x10^2$; $1.1x10^3$	10.5; 9.0	42.0; 42.5	13.5; 8.6
End	$8.5x10^2$	12.0	48.2	14.0
Avg. (SD)	$1.2x10^3$ (495)	15.8 (7.9)		12.4 (2.6)
SWM 2				
Feed	$2.7x10^3$; $2.4x10^3$	17.6; 17.1	37.4; 38.8	6.6; 7.2
Middle	$1.1x10^3$; $1.1x10^3$	11.0; 11.1	40.9; 42.1	9.9; 9.9
End	$6.5x10^2$	14.5	460	22.3
Avg. (SD)	$1.6x10^3$ (882)	14.3 (3.2)		11.2 (6.4)
SWM 3				
Feed	$2.0x10^3$; $2.0x10^3$	13.2; 13.3	46.3; 45.8	6.8; 6.6
Middle	$8.1x10^2$; $8.8x10^2$	8.6; 8.1	43.1; 42.9	10.7; 9.2
End	$8.7x10^2$	6.3	50.5	7.1
Avg. (SD)	$1.3x10^3$ (620)	9.2 (2.3)		8.1 (1.8)

Despite the differences in pressure drop and visual conditions, the autopsy results showed no significant differences in the ATP concentration in the elements. The ATP and CH concentrations in

elements 1 and 2 dosed with a different nutrients solution composition were similar. The CH concentration in element 3 was lower than in element 1 and 2 which correlates with the lower pressure drop increase in this element. Due to this result the CH-ATP ratio was lower, but in general this ratio in the elements is much higher than for the different PVC biofilm samples presented before. This is an indication of a relative low active biofilm due to the nutrient depletion period prior to the autopsy.

The behaviour observed for the pressure drop difference of each element was demonstrated visually in Figure 4.24. These images show a higher biofilm accumulation on SWM 1 and 2 than on the sheets of SWM 3. Especially on SWM 1 it is noticeable the high biofilm accumulation at the feed side, whereas for SWM 3 the biofilm has a lighter color and a more uniform distribution on the membrane surface. On SWM 3 it was observed a horizontal pattern on the membrane surface in the shape of white stripes (see Figure 4.26), probably due to the shear effect of the air bubbles during air/water cleaning.

After performing the autopsy of the three spiral-wound membrane elements from the pilot-plant setup (see section 4.2.4), pieces from the feed side were taken to perform a cleaning test with two selected multiple steps protocols using commercial cleaners, together with the standard NaOH/SDS treatment. The data presented Table 4.18 show very interesting results. First of all, despite the different operational conditions of each element (nutrient solution dosed and cleaning frequency) the general trend in biofilm removal efficiency obtained for each protocol was in the same order of magnitude, except for the CH removal by the standard treatment NaOH/SDS. This was low in SWM 1 compared to SWM 2 and 3.

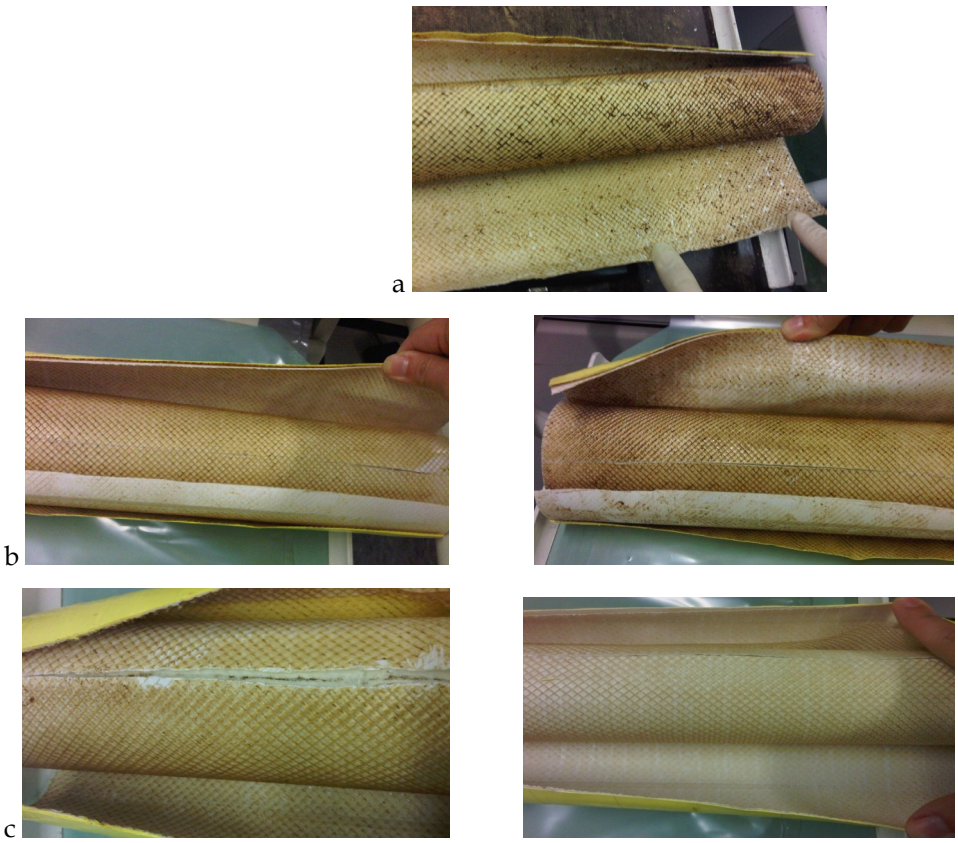


Figure 4.24. Autopsy images of the biofilm formation on a) SWM 1 (feed side), b) SWM 2 (left, feed side; right, end side) and c) SWM 3 (left, feed side; right, end side

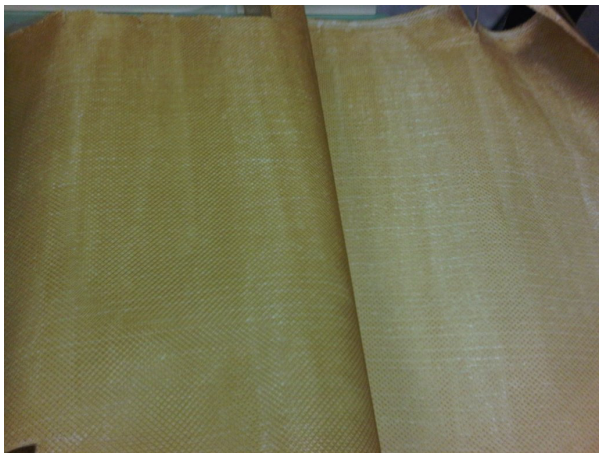


Figure 4.25 Pattern observed on SWM 3 during the autopsy

The comparison of the effects on the SWM and BPU biofilms showed differences in ATP removal, which was lower for the BPU biofilms and in CH removal which was similar or somewhat higher in the BPU biofilms for NaOH/SDS and the Divos protocol but significantly lower for the permaclean protocols. Thus, these data presents a preliminary conclusion on the predictive value of the cleaning test using artificial biofilms: the results did not fully represent the cleaning efficiency assessed for the SWM biofilms. Additionally the conclusion presented on the CH/ATP ratio before is confirmed by these data: the much higher CH/ATP ratio for the SWM biofims did not result in lower cleaning efficiencies.

Possible explanation for these observations is the difference in the nature of the biofilms. The SWM biofilms used in this cleaning test were observed to be a more "fluffy" biofilm after mechanical cleaning in Milliqu water (reference samples) which was easily detached from the membrane surface by the orbital shaker; this observation could explain the higher removal efficiency for CH by the two step commercial brand agents obtained for the permaclean chemicals. Further validation experiments are required for a more definitive conclusion, with either cleaning experiments on SWM biofilms performed as Cleaning In Place (CIP) and in the cleaning test with optimal exposure to the chemicals.

Table 4.18. ATP and CH removal (%) of the multiple steps protocols evaluated. Substratum: Spiral-wound membranes (SWM age 75 days) and BPU plates

Biofilm	*Biomass*	*NaOH/SDS (pH 12)*	*Divos 2 (pH 1.8) +Divos 116 (pH 12)*	*PC-33(pH 12) +PC-77 (pH 4)*
SWM 1	ATP (%)	96 – 96	87 – 87	68 – 74
SWM 2	ATP (%)	98 – 98	91 – 90	81 – 81
SWM 3	ATP (%)	97 – 97	96 – 96	93 – 95
BPU	ATP (%)	58 – 96	33 – 24	13 -12
SWM 1	CH (%)	-2 – nd	19 – 37	53 – 60
SWM 2	CH (%)	21 – 19	29 – 41	46 – 49
SWM 3	CH (%)	19 – 0	37 – 56	37 – 43
BPU	CH (%)	21 – 36	49 – 46	14 – 6

4.6 Confocal Laser Scanning Microscopy analysis

In order to visualize the cleaning process of cleaning BPU biofilm samples were used to analyze with CLSM. Untreated biofilm samples were analyzed to evaluate the structure and components distribution on the initial biofilm. Also samples treated with some of the chemicals evaluated in the laboratory test were analyzed to observe the effect of the chemicals on the biofilm structure.

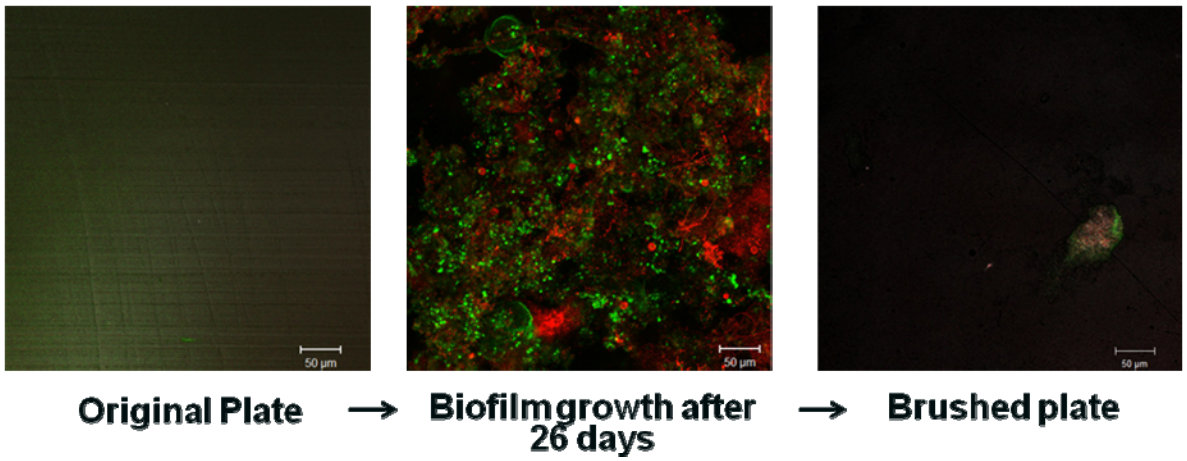


Figure 4.26. Comparison of Maximum Intensity Projection images of an original PVC plate (left), a plate with biofilm growth after 26 days of cultivation in the biofilm production unit and mechanical treatment with Milli-Q water (middle), and a plate after being brushed with an electrical toothbrush (right). Green represents EPS glycoconjugates and red nucleic acids (e.g. bacteria)

From each sample analyzed, four to five images were taken. The most representative pictures are shown in this section (for more images see Appendix V). In Figure 4.26 it can be seen the initial state of a PVC plate that was conserved in Milli-Q water. As expected there is no biofilm present, only the stain absorbed by the plate material can be observed. After 26 days of cultivation, it can be seen a full biofilm coverage in the image (middle picture), and the presence of bacteria surrounded by EPS glycoconjugates. As explained in the Materials and Methods chapter, the PVC piece is brushed with an electric toothbrush to isolate the biofilm from the PVC plate for the biomass quantification. In the right image it is shown the high efficacy of this mechanical treatment to remove almost all the biofilm present on the plate, which relates to the analytical assessment of this method performed at the laboratory (see section 4.1.1).

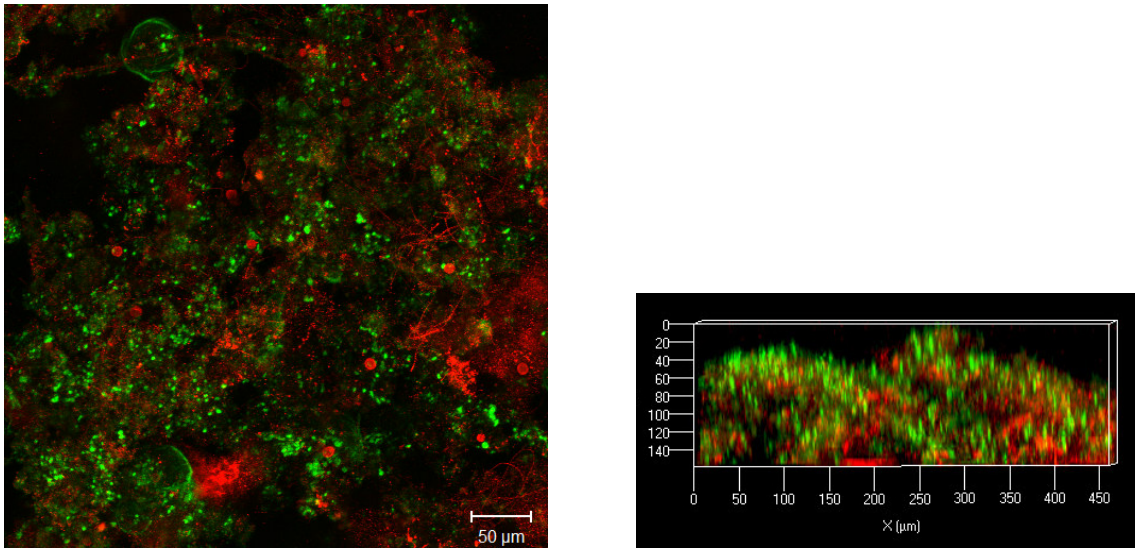


Figure 4.27. Maximum Intensity Projection (left) and cross-section view (right) of a CLSM image of a reference sample. Green represents EPS glycoconjugates and red nucleic acids (e.g. bacteria)

The representative image of a reference sample grown on a PVC plate together with its cross section image is shown in Figure 4.27. The presence of filamentous bacteria can be observed in this image. In the cross-section view (right), it can be seen a relatively flatten layer of biofilm, although with this view only one side of the biofilm can be observed. Image analyze of this picture (Figure 4.28) shows the distribution of the bacteria and EPS area fraction throughout the biofilm thickness (180 µm represents the surface of the PVC plate, 0 µm represents the top of the bulk phase).

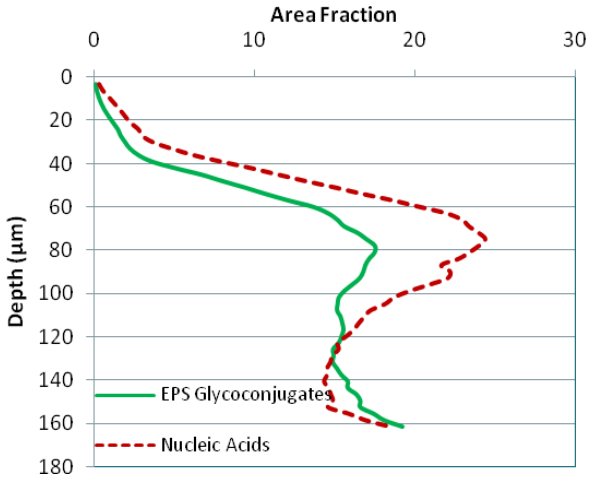


Figure 4.28. Development of bacteria and EPS area coverage according to the biofilm depth for the CLSM image of a reference plate shown in Figure 4.26

For the reference image selected, the trend of nucleic acids and glycoconjugates over the biofilm is similar with a higher presence of bacteria than EPS, with the larger biomass concentration located in the middle of the biofilm (60-80 µm depth). In most of the references images analyzed the highest coverage was found close to the PVC surface, which shows that the biofilm characteristics were varying. Since the PVC plate acts as attachment and nutrient substratum (plasticizers present in the material), the latter biomass distribution in the samples was expected.

Six biofilm samples treated with selected cleaning chemicals were evaluated. For all the cleaned samples two different phenomena were observed, as shown in Figure 4.29 for the sample cleaned with the alkaline detergent Divos 116.

- In the pictures it can be seen that the cleaning mechanism is not a peeling off but a more heterogeneous effect which decreases the biofilm coverage of the surface compared to the reference image (Figure 4.26);
- Another phenomenon is depicted in the picture on the right showing large agglomerates of remaining biofilm which are most likely less susceptible for cleaning.

This difference on the structure of the remaining biofilm after chemical cleaning can be explained by considering the inherent heterogeneity of the biofilm layer. Since the biofilm layer is not equally distributed on the plate surface and, additionally presents a hill-like structure, this causes a non-uniform penetration of the cleaning agent. As a consequence, some areas of the biofilm are more exposed to the detergent action than others and for this reason some areas of the biofilm layer are easier to be removed. The image analysis of the pictures for the sample treated with Divos 116 (alkaline detergent) calculated a reduction of the average mean thickness of the biofilm from 70 to 20 µm, which represents a removal of 70% of the biofilm layer. This is in the same order of magnitude as calculated for the ATP removal (80-85%) but higher than for the CH removal (22-34%). This indicates that the quantitative data from this CLMS image does not correspond with the quantitative data assessed with the chemical analysis. There are examples though which show that the chemical and CLSM data were more in agreement. This was the case for the NaOCL treatment.

In Figure 4.30 it is shown that CLSM imaging allows differentiating the cleaning effect of each of the chemicals evaluated. Sodium hypochlorite was the cleaning agent with the highest removal efficiency as shown by the results from the laboratory measurements (ATP removal: 99%, CH removal 71%). This trend was confirmed by CLSM imaging (left picture) since a very low biofilm coverage with only a few flocs is observed and the average mean biofilm thickness was reduced from 70 µm to 9 µm (86% removal). NaOCl is a strong oxidizer, and this effect can be observed on the cross-section of Figure 4.30. As mentioned in section 4.3.3, the hypochlorous acid has a higher effect on biofilm at alkaline pH and this strong penetration is observed in the large voids found in the biofilm structure.

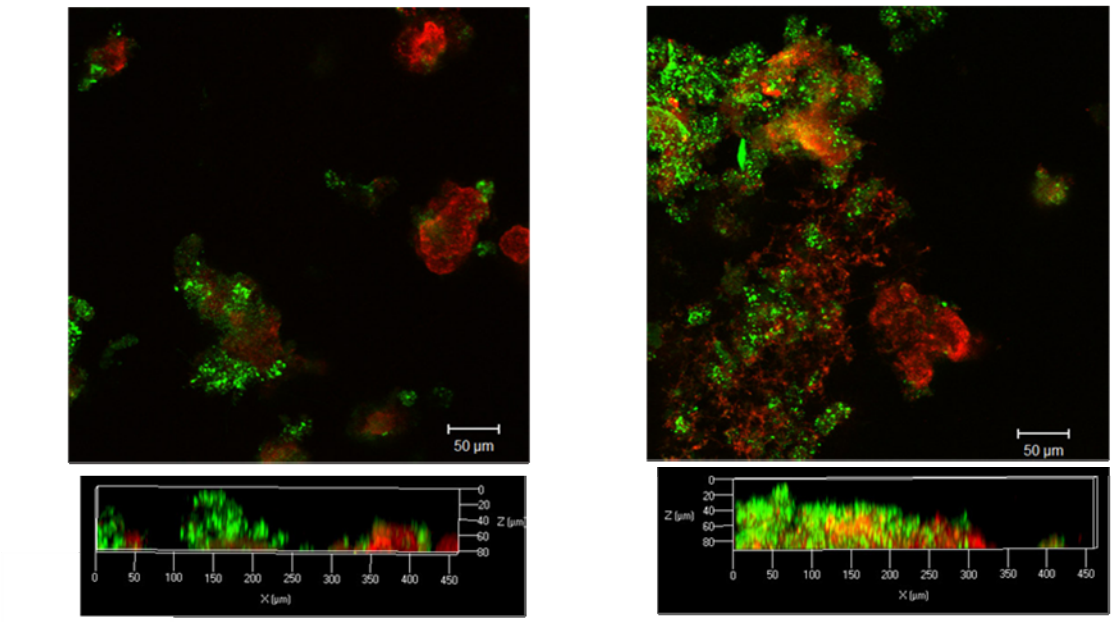


Figure 4.29. Maximum Intensity Projection (top) and cross-section view (bottom) of a CLSM image of a biofilm sample treated with Divos 116. ATP removal: 80 -85%, CH removal 22-34% (according to laboratory analysis). Green represents EPS glycoconjugates and red nucleic acids (e.g. bacteria)

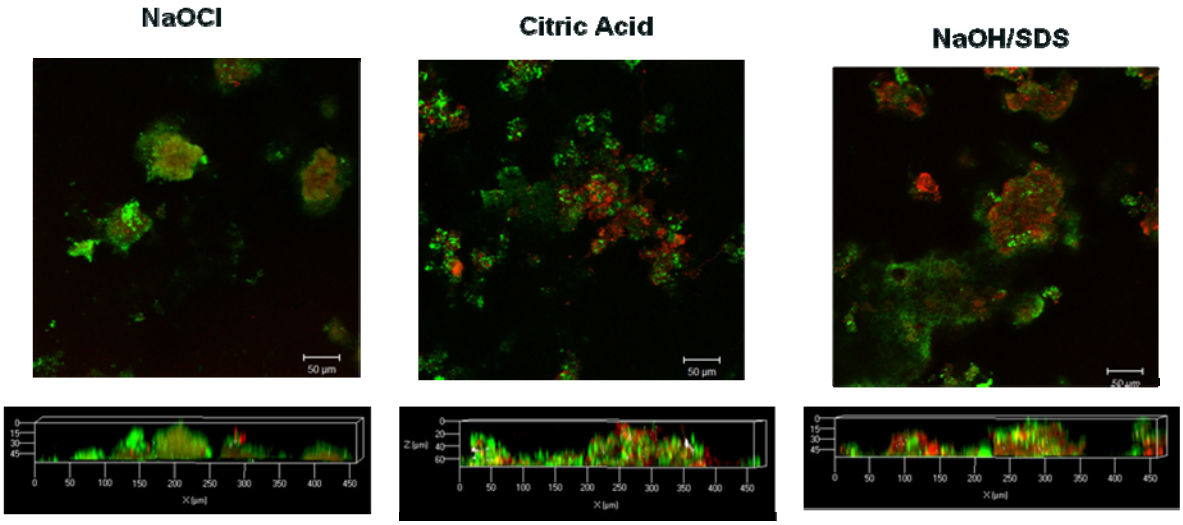


Figure 4.30. Maximum Intensity Projection (top) and cross-section view (bottom) of CLSM images of biofilm samples treated with NaOCl (left), citric acid (middle) and the standard treatment solution of NaOH/SDS (right). Green represents EPS glycoconjugates and red nucleic acids (e.g. bacteria)

For citric acid and the standard treatment of NaOH/SDS, a larger residual biofilm coverage is presented in the CLSM images (Figure 4.30). This relates to the efficacy obtained in the laboratory measurements for each chemical (ATP: 62% and CH: 19% for citric acid and ATP: 63%, CH: 33% for NaOH/SDS), which is a moderate biofilm removal. In the image for citric acid, it seems that the chemical removed the larger agglomerations of the biofilm layer, and only a thin layer with small flocs remained. The NaOH/SDS solution increases the negative charges of the biofilm and the surface tension of the water, destabilizing the biofilm structure. This mechanism seems not to be strong enough for an effective removal of the biofilm, since large residual agglomerates of biofilm can be observed for this cleaning solution. A reduction of the average mean thickness of 70 to 31 µm for citric acid (56% removal) and to 52 µm for NaOH/SDS (26%) after image analyzes of both pictures. For citric acid higher than chemically assessed and for NaOH/SDS the efficiencies estimated from the chemical analysis and CLSM images were in the same order of magnitude.

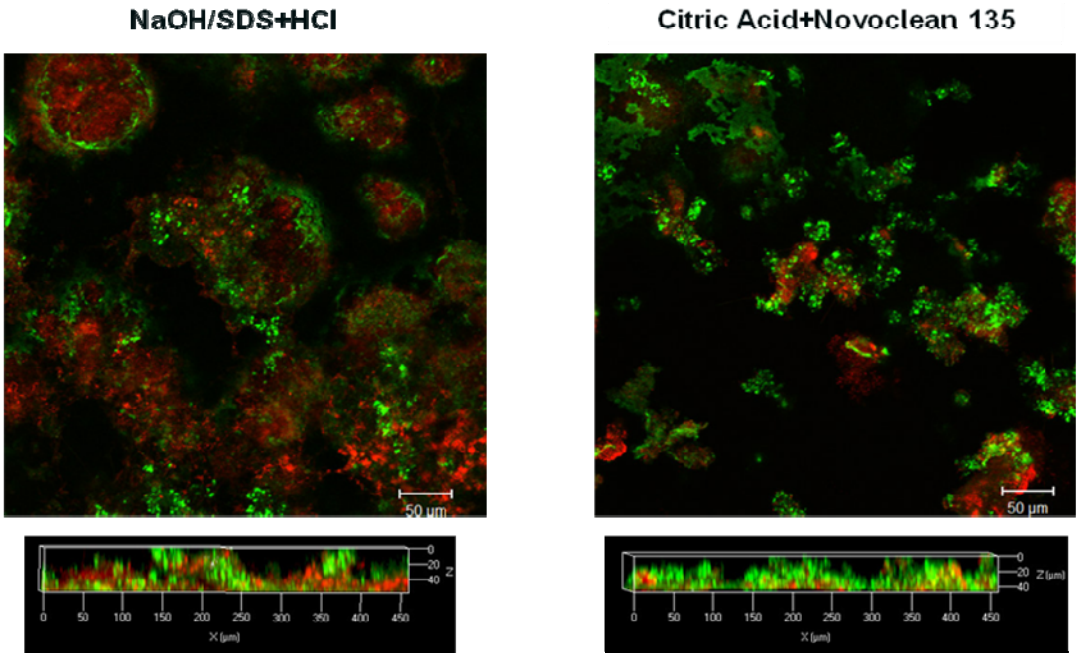


Figure 4.31 Maximum Intensity Projection (top) and cross-section view (bottom) of CLSM images of biofilm samples treated with NaOH/SDS+HCl (left) and citric acid +Novoclean 135 (right). Green represents EPS glycoconjugates and red nucleic acids (e.g. bacteria)

In the case of multiple steps treatments, the protocol with citric acid and the alkaline detergent Novoclean 135 (Figure 4.31, right) presented the same phenomenon as for the individual treatment with citric acid, although in the multiple step treatment the biofilm coverage is more uniform (as seen in the cross-section image) and the mean thickness reduction was higher (from 70 to 13 µm; 81% removal) probably due to the combined action with the alkaline detergent. For the treatment with NaOH/SDS and HCl the same large biofilm coverage from the individual treatment is observed (Figure 4.31 left), but for the multiple step treatment a higher reduction of the biofilm thickness was obtained (from 70 to 14 µm).

In Figure 4.32 an overview of the results of the image analyze of the CLSM pictures is presented. The values shown in this graph are an average of the values obtained for each of the five pictures taken for each sample (as explained in section 3.4.1). CLSM imaging allows us to study in detail the performance of the cleaning chemicals in removing biofilm from a surface. In general, a clear decrease of the mean biofilm thickness is observed for all chemicals, being NaOCl the cleaning agent with the highest removal. But the main difference between the chemicals is in the area coverage; this is noticeable in treatments 5-7 which presented a lower reduction of the bacteria and EPS glycoconjugates area fraction, compared with treatments 3 and 4. This trend is also observable on the CLSM images for the respective chemicals.

From the results shown in this graph, a relationship between biofilm thickness removal and biofilm components (bacteria, EPS glycoconjugates) removal of the cleaning chemicals cannot be established. In the same manner, ATP and carbohydrate results cannot be related with the results obtained in the CLSM image. The biofilm removal observed in the images seems to be higher than the one obtained with the laboratory measurements. Despite of this, the same removal trends were also observed.

This same discrepancy between visual techniques and analytical techniques were obtained by Whittaker et al. (1984). In this case, the decrease in biofilm was observed by means of Scanning Electron Microscopy (SEM) and the analytical measurement used was the count of bacteria.

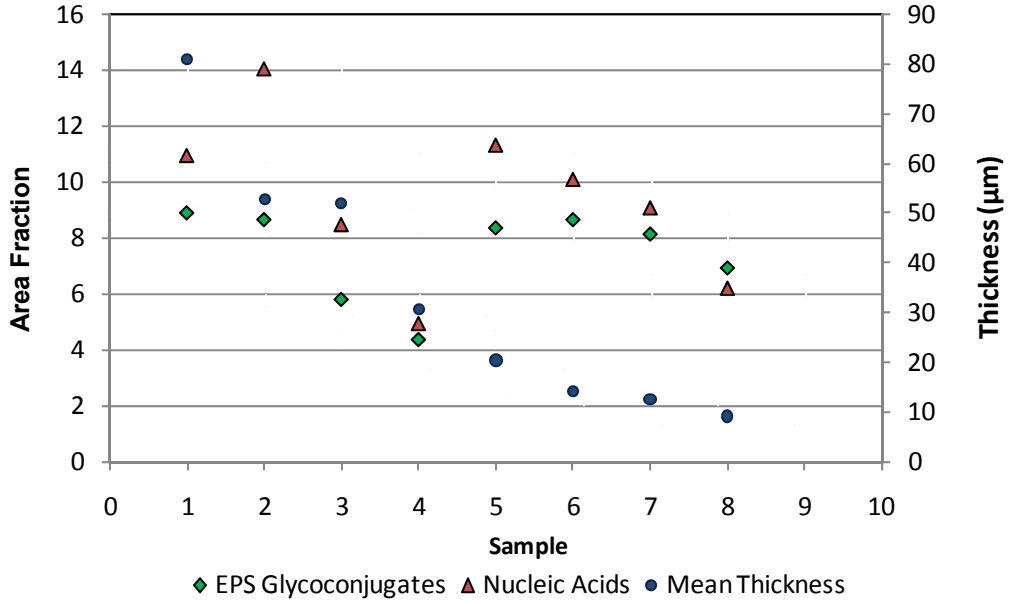


Figure 4.32 Image analysis parameters evaluated for each of the samples analyzed with the CLSM, where: 1- Reference 1, 2- Reference 2, 3- NaOH/SDS, 4- Citric Acid, 5- Divos 116, 6- NaOH/SDS+HCl, 7- Citric Acid+N135, 8- NaOCl

From the data in Figure 4.32 the biomass removal percentages of the different cleaning steps were estimated from the mean thickness of the biofilm and presented in Figure 4.33 together with the removal rates assessed in a separate laboratory test using ATP and CH data.

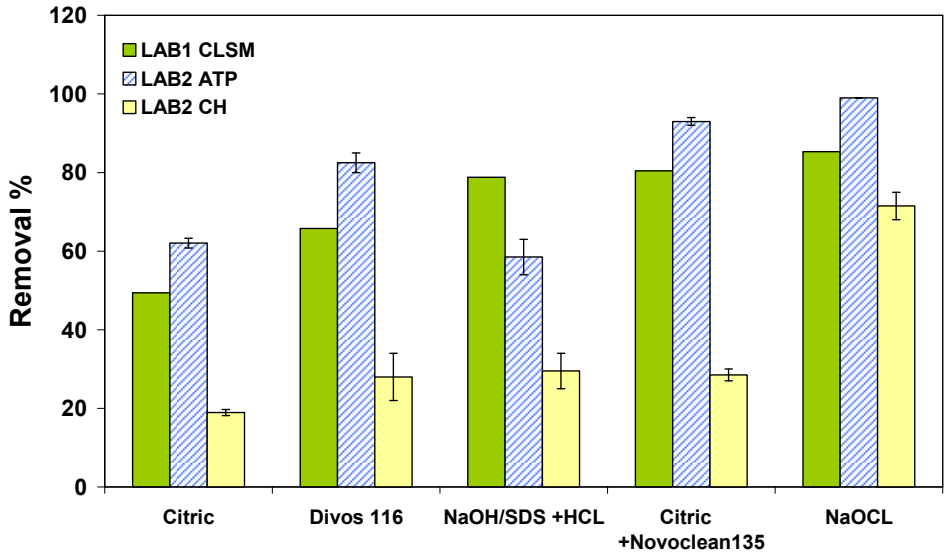


Figure 4.33. The cleaning efficiency (%) estimated from the mean thickness of the biofilm determined with CLSM for a selected cleaning agents and protocols tested with the laboratory test using BPU biofilms and the cleaning efficiencies (%) calculated from the ATP and CH concentrations determined under the same conditions in a separate laboratory test (error bars duplicates)

It was remarkable to notice that the sequence observed for the different agents and protocols assessed in the laboratory test using CLSM analysis was almost similar to the sequence observed for the same agents and protocols assessed in a separate laboratory test using ATP and CH analysis. This

demonstrates that both analytical methods lead to similar conclusion on the distinct effects of agents and protocols on biofilms and that the laboratory test produces reproducible results.

It is important to note that the results obtained with the CLSM images and image analyzes of the pictures refer only to the stained biofilm material. Some of the known downsides of this technique are the limitation of the staining procedure and laser penetration in the sample (Wagner, 2009; Walters, 2008). Despite of this, considering that these limitations affect equally all the samples analyzed, CLSM evaluation demonstrates to be a good method for assessing the efficacy of the chemical cleaning. Further interpretation of the fraction coverage as presented in Fig. 4.26 and 4.28 is not presented in this report. As shown in Figure 4.26 it depicts the distribution of the stained material in the biofilm. The data also demonstrate that the staining presents only part of the biofilm volume and it remains to be clarified of which material the residual biovolume is composed; H_2O is most likely an important part of this.

5 Increased views on biofilm removal by chemicals

The results of this study have increased the views on the quantitative effects of chemicals on the removal of attached biofilms defined as the cleaning efficiency. The laboratory cleaning test was optimized to a robust test which generates reproducible results which made it possible to compare the cleaning efficiency of chemicals and cleaning protocols. In the test standard biofilms grown on plasticized PVC are used. To compare also biofilms on RO/NF membrane materials are used in the test.

5.1 Parameters to quantify biomass removal

General conclusion was that the PVC biofilms produced in the Biofilm Pate Unit (BPU) was not easily removed by the chemicals used in the practices of RO/NF membrane cleaning. The cleaning efficiency was assessed with ATP as the parameter for the inactivation and/or removal of active bacterial cells and with carbohydrates as the parameter for EPS in the biofilm matrix. In the study the cleaning efficiency for both parameters were calculated. The average ATP removal of all tests on BPU biofilms (n=49) was 50% and ranged from -23.4–99.6% (Table 5.1). The efficiency to remove carbohydrates was 20.1% on an average with a range of -24-71.3%. These results demonstrate that even though it is possible to deactivate the bacteria present in the biofilm, it is rather difficult to remove the biomass where they are embedded.
Both parameters represent different parts of the biofilm as shown in the CLSM images. Therefore the removal of the biofilm is the cumulative effect of the chemicals on both parameters. This is clearly demonstrated in Figure 4.33 where the estimated biofilm reduction by the CLSM analysis followed the same trend for both parameters and was in order of magnitude more in agreement with the ATP reduction than with the CH removal. The CLSM images also showed that the fraction of both biofilm components in the biofilm matrix was similar. The removal of the total biomass was estimated from the ATP and CH removal by

- estimating the active bacterial biomass (µg of wet weight/cm^2) from the ATP concentrations (multiplication the ATP results (pg/cm$^{2)}$ with 0.001 based on characteristics of 1 cell of 3.6x10^{-7} ng/cell (Magic-Knezev and Van der Kooij, 2004) and 0.2 pg wet weight/cell, Wickipedia);
- summation of the active bacterial biomass with the CH concentration and calculating the removal rates.

This revealed that the average removal rate of total biomass is 36% which is approximately the average of the ATP and CH removal (Table 5.1).

Tabel 5.1 The average ATP, CH and total biomass removal for the BPU biofilms

	ATP	*CH*	*Total biomass*
Average (±SD)	50.3±33.2	20.1±16.7	36.4±23.0
Min.	-23.4	-24.0	-22.8
Max.	99.6	71.3	86.0

Further data analysis revealed that the ATP and CH removal was linearly related (Figure 5.1). The same Figure also showed that in the removal based on the total biomass the ATP removal had a higher impact on the assessed removal than the CH removal. These data must be regarded however as indicative because the nature of biofilms is variable and rather complex. In these simplified calculations a constant ratios of ATP/cell and dry weight per cell was assumed. These ratios are variable as shown for ATP/cell ratio before (i.e. Magic-Knezev and van der Kooij, 2004). Some general characteristics of biofilms which are of importance to interpret the observed data have been described recently by Flemming and Wingender, 2010:

- In most biofilms micro-organisms itself account for less than 10% of the dry matter. This is also estimated from the biovolume in the current study: at a level of 2x10^5 pg of ATP/cm^2 the

number of cells is $5.6x10^8$ per cm^2 which represents a biovolume of approximately $3x10^{-4}$ cm^3 which is 4% the estimated volume of the biofilm ($7x10^{-3}$ cm^3 estimated from the CLSM analysis with a mean thickness of 70 μm);

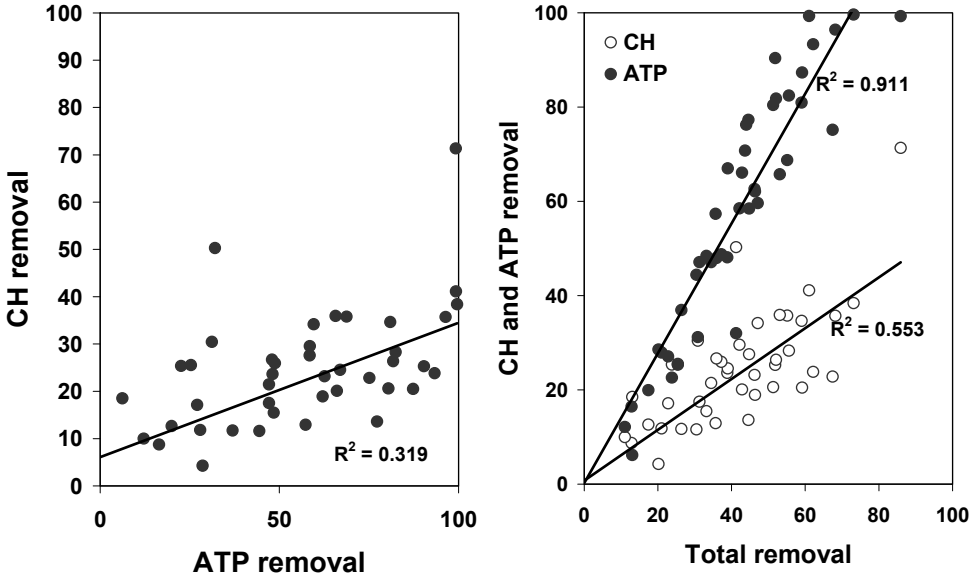


Figure 5.1. The relationship between the ATP and CH removal (left) and (right) between the estimated Total biomass removal calculated from ATP and CH concentration

- Polysaccharides are the main component of biofilms. The proportion of excreted polysaccharides does not necessarily reflect the proportion of the cells present. Furthermore, the composition of the polysaccharides is variable which will lead to different results in the CH method where glucose was used as the calibration carbohydrate. That implicates that conversion of CH data to biovolume is not straightforward;
- Biofilms also contain proteins or enzymes and external DNA. Some non enzymatic proteins are part of the mechanical structure of a biofilm (lectins) and also DNA molecules play a role in the stability of biofilms;
- Extracellular polysaccharides are highly hydrated and water is the major component of biofilms. Removal of ATP and/or polysaccharides quantified with the CH method will also result in a non quantified volume of hydrated water.

Assuming that the CLSM estimation of biofilm removal is correct, based on the above mentioned characteristics of biofilms an accurate and quantitative agreement of these data with the biochemical data of biofilm removal as applied in the laboratory test is not to be expected. The agreement of the trends shown by both techniques as shown in Figure 4.33 is probably the best result one could imagine.

5.2 Effects of the different agents and protocols on biofilm

The results of all laboratory tests performed with PVC biofilms produced in the BPU are presented for the different categories of single agents and for the different multiple step treatments categorised in the sequence of the steps in Appendix 2. In every separate test NaOH/SDS was used as the reference treatment. To correct for the variation in cleaning effect observed for this standard treatment (which is most likely caused by the natural differences in biofilm characteristics as presented in paragraph 4.2.5, Table 4.3) the cleaning efficiencies of the different agents were normalized by calculating the ratio with the NaOH/SDS efficiency. Larger than 1.0 means more removal and < than 1.0 means lower removal. For ATP and CH removal the normalized cleaning efficiencies caused by individual agents are presented in Figure 5.2 and for the double step treatments in Figure 5.3.

The agents permaclean 33, Novoclean 135, Divos 116, CTAB, NaOH/SDS pH12.7 and NaOCl had normalized cleaning efficiencies of > 1.0 for ATP removal. For CH removal however, only NaOCL had a normalized cleaning efficiency which was significantly higher than 1.0 and NaOH and the standard treatment at a higher pH of 12.7 were slightly more efficient. The other agents were less efficient compared to the standard treatment with NaOH/SDS at a pH of 12.0. Thus, sodium hypochlorite was the most effective cleaning agent. This strong oxidizer, however, is used as an extreme treatment that cannot be used in all RO/NF membranes which showed that it is possible to obtain a high biofilm removal.

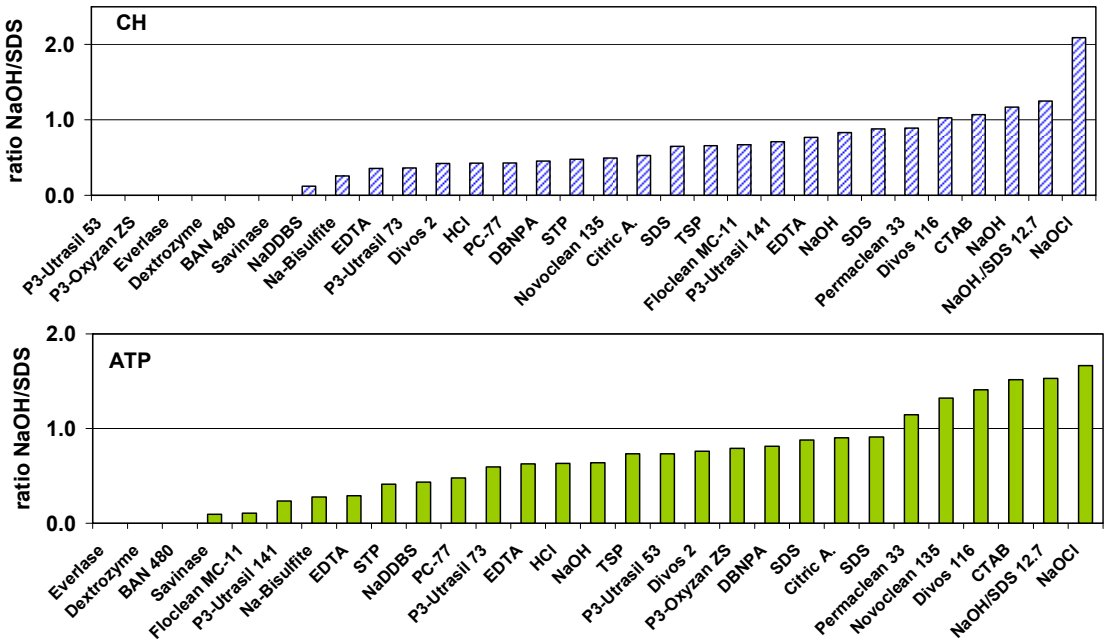


Figure 5.2 The normalized cleaning efficiencies of the single step treatments (ratio with NaOH/SDS pH 12) for the different agents arranged in increasing order

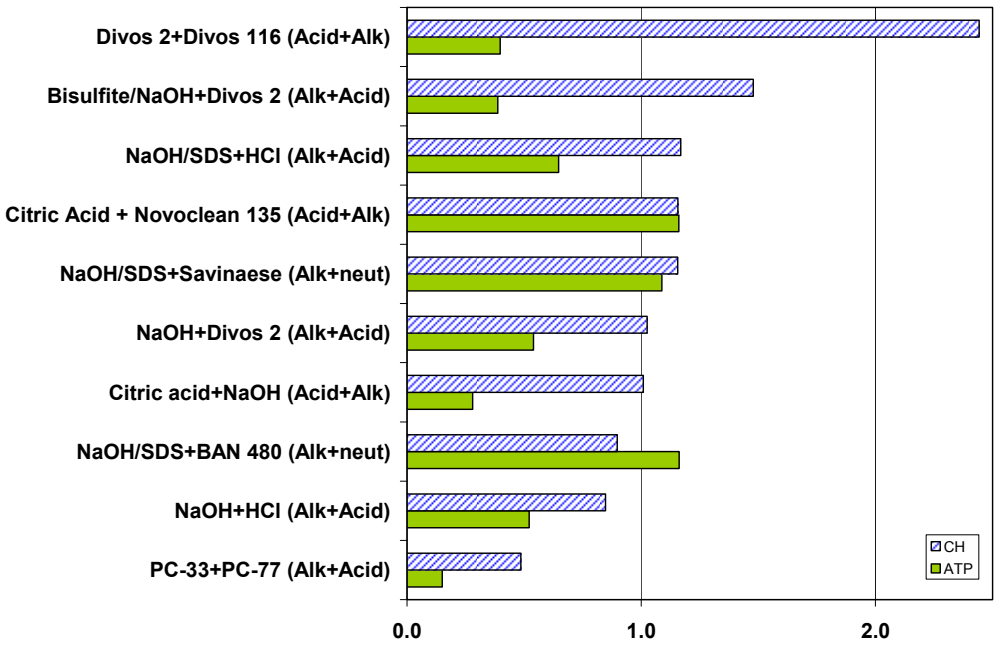


Figure 5.3. The normalized cleaning efficiencies of the double step treatments (ratio with NaOH/SDS pH 12) arranged in increasing order of CH removal

The same normalized cleaning efficiencies were calculated for the double step treatments and presented in Figure 5.3. Most double treatments had a normalized cleaning efficiency of ≤1.0 for ATP and of ≥ 1.0 for CH removal. The double Divos treatment showed a significant higher normalized cleaning efficiency (Figure 5.3).

Subsequently, the different categories of agents were compared in Figure 5.4 for both the single and double step treatments based on the average cleaning efficiencies and the range (maximum and minimum values, error bars). This is a rough comparison rather than an accurate comparison. The range of values within one category is sometimes high and the number of tested agents for every category was not equal. The results showed that treatments (single or double) with an alkaline agent at increased pH values and detergents were the most effective ones. Cleaning protocols using alkaline cleaning agents are recommended due to their ability to dissolve organic deposits and remove biological material from the membrane surface. The results for CH removal confirm this statement. As stated before the alkaline effect is caused by the increased repulsive forces in the biofilm. In a recent presented study the effect of the pH on the structure of EPS excreted from *Bacillus megaterium* was clearly demonstrated (Wang et al., 2011). The EPS matrix they studied had an iso-electric point at pH 4.8. Below this value the matrix is positively charged and more dense and compact. At high pH values the EPS structure is more negatively charged and repulsive which results in weaker intra- and inter-colloid interactions and release of chains, swelling and lower density structures. The highest effectiveness of the detergents was caused by the positively charged cationic CTAB (Decyltrimethylammonium bromide at pH of 5.4, an agent which is used in DNA extraction methods). The other tested detergents SDS, TSP and STP were anionic detergents (negatively charged) or a non-ionic detergent such as NaDDBS. This illustrates the importance of the surface properties of the chemicals used for biofilm removal.

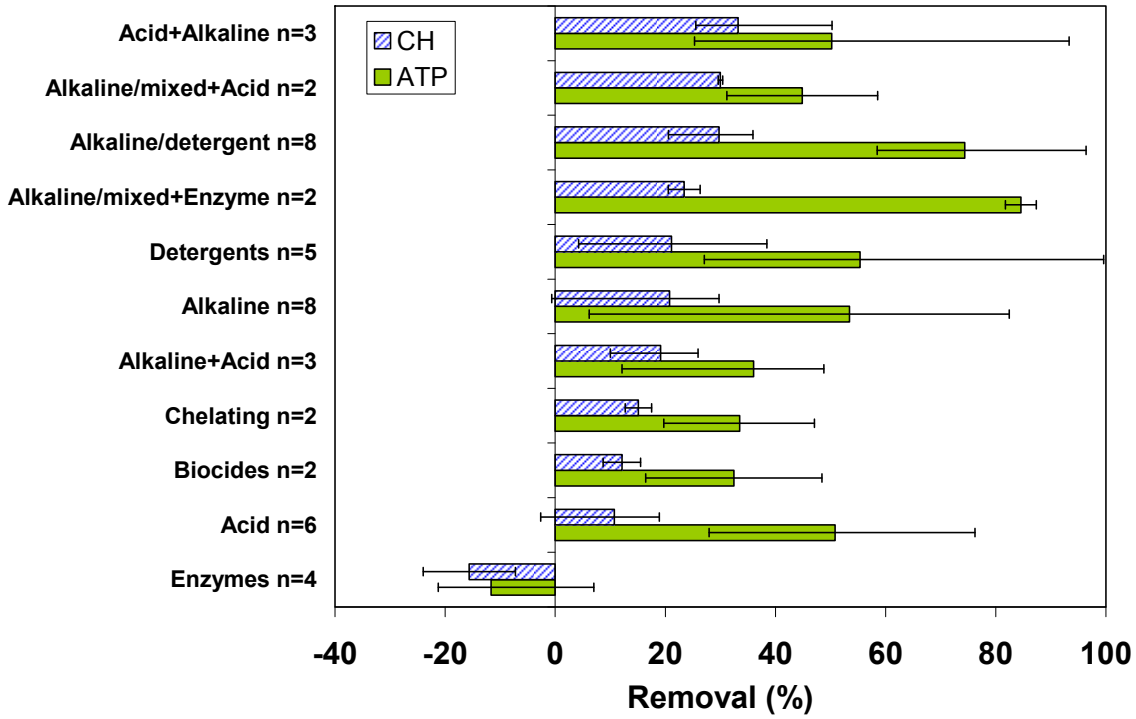


Figure 5.4 Average ATP and CH removal percentages observed for the different categories of agents (n = number of values or agents included; error bars depicts the range of percentages; NaOCL was not included in the biocides) arranged in increasing CH removal

The ATP removal rates by acids, alkaline and detergents agents were similar, but the CH removal by the acids was clearly lower compared to both other agents and in the same order of magnitude as observed for the chelating agent EDTA and both biocides DBNPA and Na-bisulfite. The enzymes showed a negative removal (increase of ATP and CH concentrations) indicating that at these

conditions and contact times these agents cause increased microbial activity in the biofilms. A low effect of enzymes on the EPS matrix was also described by Flemming and Wingender (2010).

There is a limited number of studies reporting the effects of cleaning agents on biofilm concentrations. The study of Whittaker et al. (1984) measured the cleaning efficiency by scanning electronic microscopy for a number of individual and combined agents. They observed removal rates ranged from 50 – 100%. Corpe (1974) presented a study in which he measured the cell density decrease after exposure to chemicals. For HCl and NaOH (0.1 N) the reduction was 7 and 85%. The reduction was higher for EDTA and SDS (0.1%; respectively 95 and 99%). More in line with the current study was the study of Chen and Stewart (2000). For a number of chemicals the removal of biofilm could be compared with the results of the current study (Table 5.2). These data showed similar conclusion with respect to the removal of bacterial cells and biofilm components such as proteins or carbohydrates: the removal rates of cells and EOPS components are not always correlated demonstrating that both biofilm components are removed by different mechanisms and/or are not equally distributed in the biofilm matrix. Furthermore, removal of bacterial cells (cells or ATP) is usually more effective than removal of EPS components such as proteins or carbohydrates. The sequence in removal efficiency presented in Table 5.2 based on the EPS components was almost similar for both studies.

Table 5.2. Biofilm removal reported by Chen and Stewart (2000) in comparison to the biofilm removal assessed in the current study for similar chemical treatment

	Chen and Stewart, 2000	*Protein*[a]	*Cells*[b]	*Current study*	*CH*	*ATP*
Acid	Acid pH2.9	16	66	Acid pH1.6-3.9	11	51
Chelating	EDTA 0.01M	26	-49	EDTA 0.01M	15	34
Alkaline	Alkaline 11.2	47	99.8	Alkaline 9.2-12.2	21	54
Biocide	NaOCL 15 mg/l pH 10.9	65	99.6	NaOCL 5000 mg/l 11.4	71	99.3
Detergent	SDS 0.1%	71	33	SDS 1%	22	64

[a] Lowry method; [b] culture R2A

An overall observation in literature is that the use of mixtures of compounds and/or series of treatments showed enhanced effects. The multi-step treatments were categorized in the sequence of the pH step (low-high or high-low). From the data presented in Figure 5.4 a multistep treatment hardly resulted in increased biofilm removal and the sequence of pH treatment (low-high or high-low pH) was not a selecting criterion for the choice of the most effective protocol. Based on the CH removal the protocol of Divos 2 + Divos 116 was the most effective protocol (acid + alkaline; Fig. 5.3).

5.3 Removal of biofilm linked scaling (Iron and Calcium)

In membrane cleaning acids and chelating agents such as EDTA are used to remove scalants such as Fe and Calcium. These cations are associated with the anionic carboxylic groups in the EPS. Multivalent cations such as Ca^{2+} are related to the formation of thick and compact biofilms with increased mechanical stability (Flemming and Wingender, 2010). There was limited attention in the current study for the removal of cations from the biofilm. Preliminary measurements showed that Ca removal rates could not be measured. The Fe removal efficiency was measured in a test with BFS and BPU biofilms. The PVC tube from the BFS system contained higher CH and Fe concentrations (Fig. 5.5). In the tests the removal of biofilm (CH) and Fe was determined for EDTA and citric acid and compared with the CH and Fe removal by alkaline and detergents (NaOH/SDS). The Fe and CH removal rates for the alkaline and detergent treatments showed similar values and trends for the BFS and BPU biofilms. For EDTA and citric acid however it was clearly shown that the Fe removal rate was significantly higher than the CH removal rate. The results clearly show that these agents cause different reactions in the biofilm. They cause an effective removal of mono-valent anions such as Fe. On the other hand, assuming that multi-valent anions such as Ca were also present in the current biofilms and explain partly the persistent character of the PVC biofilms, under the applied conditions as single agents EDTA and citric acid were not effective enough to break Ca-bridges in the biofilm structure and cause a significant biofilm removal.

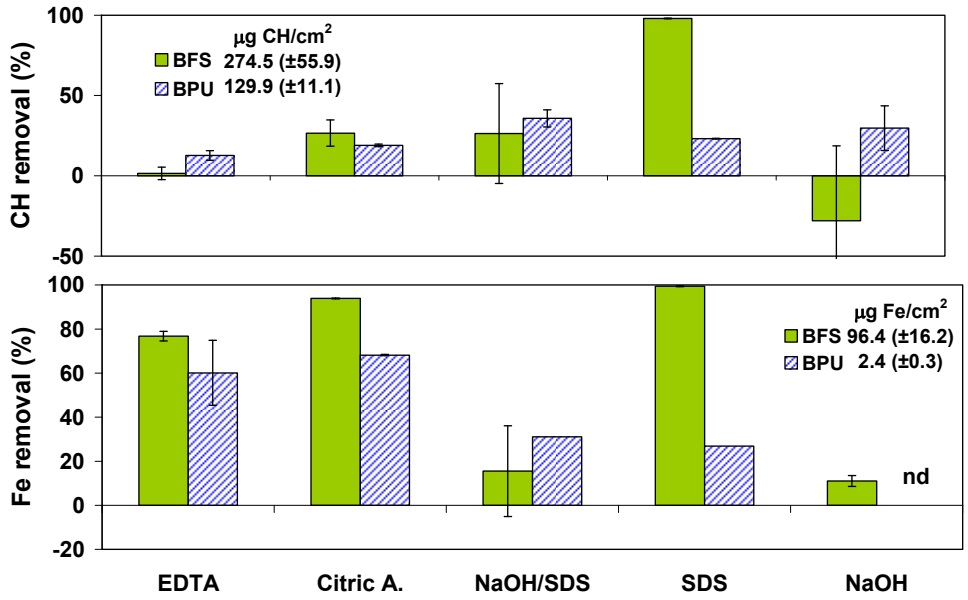


Figure 5.5 Fe and CH removal percentages (error bars = duplicates; nd = not determined) asssed for different agents in a laboratory test using BFS and BPU biofilms

5.4 Translation to spiral-wound membrane cleaning

5.4.1 Comparison with biofilm removal from membranes

One of the major objectives of the laboratory test on PVC biofilms was to select agents and protocols for the removal of biofilms from high pressure membranes. Therefore it is of importance to verify whether the selection based on the laboratory test can be translated to the removal of biofilms from membranes. In a former study preliminary comparative results have been presented for biofilm removal from PVC and spiral-wound membrane (SWM) surfaces (Van der Kooij et al., 2011). The ATP and CH removal was measured for PVC tubes (BFS produced), SWM membrane in the labtest and in a CIP procedure using the same cleaning agents and protocols in a pilot plant with SWM elements. The data showed a high variation due to the use of different agents and protocols (Figure 5.6). Therefore only some preliminary conclusions can be presented.

A higher average CH removal from the PVC biofilm was observed compared to the average CH removal from the SWM samples in the labtest and the CIP. The other average removal rates for the different materials and cleaning conditions were in the same order of magnitude.

In the current study the comparison with SWM biofilms was also preliminary addressed (Fig. 5.6). No CIP procedures was tested, only the biofilm removal from PVC and SWM membrane material could be compared for the same cleaning procedures in the laboratory test. The results showed the opposite compared to the former results: a lower biofilm removal (ATP/CH) for the PVC compared to the biofilm removal assessed for SWM membrane material. This discrepancy in comparative results between PVC and SWM biofilms of the former and current study is caused by the use of different PVC biofilms with different resistance to biofilm removal explained by the increased CH/ATP ratio. Since this ratio for the SWM biofilms was much higher (±10; Table 4.15, 4.18), this ratio cannot be regarded as a reliable parameter to predict biofilm resistance to cleaning.

These preliminary results demonstrate that removal of biofilm from PVC and SWM material lie in the same order of magnitude but shows differences probably caused by unqualified differences in biofilm characteristics. Major question in these comparative studies is, however, whether the selected cleaning agents and protocols as the 'best' based on the relative differences assessed with the laboratory test are also the 'best' for biofilm removal from full-scale membranes. More comparative and validations experiments using 'real world' membrane elements are required for definitive conclusions on the predictive value of the laboratory test results for membrane cleaning in practice.

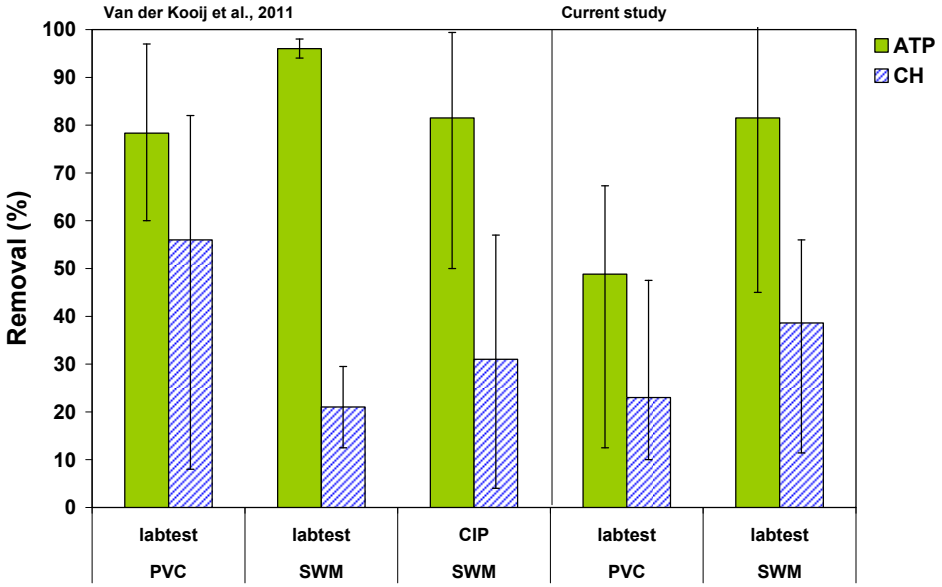


Figure 5.6. The biofilm removal rates (ATP and CH) assessed for PVC biofilms in the labtest and for SWM biofilms in the labtest and a CIP procedure in the current study and a previous published study (Van der Kooij et al., 2011)

5.4.2 Cleaning efficienies of the full-scale protocols of the Dutch water companies

In the current study the cleaning protocols applied at Dutch full-scale NF/RO installations were tested in the laboratory test. These membranes are cleaned with different frequencies based on different criteria (Table 5.3). In this Table the absolute and normalized cleaning efficiencies are presented for ATP and CH and arranged in normalized cleaning efficiency for CH (EPS). The protocol with the highest efficiency at location 12 is cleaned based on NPD and has a relatively high cleaning frequency. At location 5 the cleaning frequency was even higher and also based on NPD. The cleaning efficiency assessed with the laboratory test however was 25% less compared to the protocol at location 12. .

Table 5.3. The cleaning efficiencies assessed with the laboratory test for the full-scale cleaning protocols used by the Dutch drinking water companies

Loc.	*Cleaning*	*pH1*	*pH2*	*ATP %*	*CH %*	*ATP ratio*	*CH ratio*	*Freq*	*Crit.*
9	PC-33+PC-77	11.7	3.9	12.1	10.0	0.15	0.49	2-3 year	NPD/MTC
3/4	NaOH+HCl	12	2	47.1	21.5	0.52	0.85	4-6 weeks	NPD
7	Citric acid+NaOH	2	12	25.3	25.5	0.28	1.01	1-2 year	Time/feed P
5	NaOH+Divos 2	12	1.6	48.8	25.9	0.54	1.03	1-8 weeks	NPD
8	Citric A. + Novocl 135	2	11.6	93.3	23.8	1.16	1.16	1 year	NPD/MTC
2	NaOH/SDS+HCl	12	2	58.5	29.6	0.65	1.17	?	MTC
10	Bisulf./NaOH+Divos 2	12	1.6	31.2	30.4	0.39	1.48	>16 weeks	
12	Divos 2+Divos 116	1.6	12.2	32.0	50.3	0.40	2.44	3-12 weeks	NPD

5.4.3 Biofilm removal and operational conditions

Membrane cleaning has been reviewed by Cornelissen (2005). Some general conclusions of this report were:

- Cleaning conditions of NF/RO membranes such as frequency or criteria and mode of cleaning (chemicals/protocols) depends largely on the local experiences;
- Pressure drop increase of 50% or flux decline of 10% are common criteria for cleaning;

- Low amount of scientific studies available on cleaning directly related to biofouling;
- Most effective cleaning agents described were chelating agents (EDTA), surfactants en denaturising agents (Ureum).

Biofouling is described as a common type of fouling in spiral-wound membranes (Khedr, 2000; Schippers et., 2004) resulting in operational problems. Therefore the assumption is that an effective biofilm removal will benefit the overall process performance related to water flux, salt passage and pressure drop increase. The role in biofilm accumulation in pressure drop increase problems has been described before ((Flemming, 1997; Flemming et al., 1993a; Ridgway and Flemming, 1996) and recently quantified for ATP and carbohydrates as major components of biofilms (Hijnen et al., 2011). Thus, optimized biofilm removal is of importance to minimize energy consumption caused by pressure drop in NF/RO membranes. Undecided and subject for further studies is the effect of optimized biofilm removal during cleaning on the flux decline and salt passage in these membranes.

The preliminary membrane experiment presented in the current study showed that a periodic air/water cleaning had a positive effect on the pressure drop development, though unclear yet is the long term effect on the flux decline and salt passage. This positive effect was also confirmed by lower CH concentrations in the membrane. No significant differences, however, were observed in the laboratory cleaning test with respect to differences in cleaning efficiency. This might indicate no real impact on the resistance of the biofilm to cleaning caused by the increased shear stress during air/water cleaning. This conclusion however is possibly compromised by the test conditions at the end (biofilm stress due to substrate limitation) and should be confirmed by additional experimental work with prolonged operational periods.

5.4.4 Environmental aspects of cleaning

One aspect that also needs to be considered when evaluating the usage of a cleaning chemical is its effect on the environment. Sometimes a chemical presents a high cleaning efficiency but it use is restricted by its damaging effects on the membrane and the negative impact on the environment (van der Kooij et al., 2011). The environmental aspects and disposal of the cleaning chemicals evaluated were not investigated but these aspects definitely affect the applicability of a cleaning agent. The German EPA provides a good indication of the risks involved in the use of different cleaning chemicals (Lattemann, 2010) by establishing three water hazard classes (VwVwS): low, considerable and severe hazard to waters. Most of the chemicals tested in this research are classified as low hazard to waters, including acids and phosphates, with the exception of EDTA, NaDDBS, detergents, and NaOCl which are classified as hazardous to waters.

6 General conclusions

To summarize the results of the current study the following main objectives have to be evaluated:

- optimization of the production of reproducible and homogenous biofilm samples;
- screening cleaning agents and protocols with varying pH and temperature;
- investigate the effect of the biofilm on the cleaning efficacy;
- to explore the use of CLSM analysis in the test as additional tool.

6.1 Optimized laboratory test

The study started with three biofilm production set ups using plasticized PVC as substratum and nutrient supply: the Biofilm plate unit (BPU), the Biofilm formation set up (BFS) and Biofilm formation monitor (BFM). The BPU in which the growth conditions (nutrient limitation, iron removal and temperature) and test conditions were optimized (simplified operational, sampling and testing conditions) produced PVC biofilm samples suited for the cleaning tests. The biofilm concentrations were used after 4-6 weeks of incubation and showed some variation between the different batches in biofilm density and ratio between carbohydrates (CH) and ATP. The laboratory scale cleaning test evaluated showed to be an appropriate protocol for the assessment of the cleaning efficiency of chemicals, proved by the good reproducibility of the standard NaOH/SDS treatment results. Using this NaOH/SDS as a standard cleaning test it was demonstrated that the cleaning efficacy depended on the CH/ATP ratio as a characteristic of the biofilm. The effect was strong for ratios ranging from 0.25 - 1.0, but since the effect showed a logarithmic trend, the effect was negligible at higher ratios. Therefore it is recommended to include the standard cleaning with NaOH/SDS as a reference to account for the biofilm characteristics in the test.

6.2 Broad screening survey of membrane cleaning agents and procedures

The screening tests of agents and protocols used in membrane cleaning were conducted in a sequence of the different categories of products, starting with the individual agents followed by the multiple step treatments.

- In general, a moderate removal (>60 %) of ATP was obtained for several chemicals at conditions of both low and high pH. Carbohydrate removal, which is regarded to be more representative for the removal of the EPS structure, however, was low for all chemicals applied in membrane cleaning (<45%). Sodium hypochlorite was the cleaning chemical with the highest biofilm removal (ATP: 99%, CH: 71%), even though this chemical cannot be used for RO/NF membranes. These results demonstrate that even though is possible to deactivate the bacteria present in the biofilm, it is rather difficult to remove the EPS matrix where they are embedded. Furthermore, it demonstrates that oxidation is an effective way to destabilize and break down the biofilms EPS matrix and subsequently remove it from the surface. Based on this idea in combination with the possible incorporation of Fe in the biofilm it would be interested to investigate the potential use of an oxidative process such as the fenton process induced by the addition of a more mild oxidant H_2O_2.
- Alkaline and detergents were more effective in removing carbohydrates or EPS than acids, EDTA, biocides and enzymes. Enzymes as individual agents did not remove the biofilm. The increase in ATP and CH values after exposure to the enzymes indicates that they enhance metabolic activity in the biofilm. A preceding cleaning with NaOH/SDS did not enhance cleaning activity by enzymes;
- Contrary to expectations, the cleaning efficiency of commercial blends as individual agents was observed to be lower than the analytical grade chemicals, for the biofilm employed.
- And also no increase of the cleaning efficiency was obtained when using combined cleaning steps (multiple steps) and an elevated temperature of 35^0C.
- In the multi step treatments a clear preference for acid – alkaline or alkaline – acid was not observed. One test with the former sequence Divos 2 and Divos 116 (acid + alkaline) showed a

synergistic effect on the biofilm resulting in one of the highest CH removal percentages of 47%.

6.3 Influence of the biofilm

In the study different biofilms were tested. PVC biofilms produced under different conditions and biofilms on SWM material. The results clearly showed that the biofilm characteristics had a significant effect on the cleaning efficacy. For the PVC biofilms the CH/ATP ratio was identified as a characteristic which impacted the biomass removal at values <1.0 µg CH/ng of ATP where the removal was higher than at CH/ATP rtios of >1.0. The results on SWM biofilms, however, showed the opposite conclusion for this ratio. At high ratios of ±10, more biofilm removal for SWM biofilms was observed compared to the PVC biofilms under the same cleaning conditions. Thus, this ratio cannot be regarded as a reliable general predictor for the resistance of biofilms to membrane cleaning. The major conclusion deduced from the comparative results of PVC and SWM biofilms in the discussion was that further verification tests are required for a definitive conclusion on the predictive value of the laboratory test for the cleaning efficiency in CIP procedures at full-scale membrane plants. The focus in these studies should be on the question whether the 'best' selected cleaning agents and protocols in the laboratory test are also the 'best' agents and protocols for biofouled SWM elements.

6.4 Confocal Laser Scanning Microscopy as an analytical tool

CLSM imaging proved to be a useful tool to evaluate the biofilm structure and the influence of the cleaning protocol on its components. It also allowed a deeper insight on the cleaning mechanisms of the chemicals evaluated. From the CLSM images of the biofilm samples after treatment with selected cleaning chemicals, it was observed how the chemicals used had different penetration mechanisms on the biofilm surface. Sodium hypochlorite showed to have a very strong penetration producing a considerable reduction of the biofilm thickness, whereas for NaOH/SDS the removal seemed to be more uniform. The trends observed in the chemical quantification were to some extent confirmed with CLSM imaging but there were also clear differences. Despite the limitations of this technique for a quantitative study on the effects of chemical cleaning on biofilms, CLSM evaluation is still a good method for assessing the efficacy of the chemical cleaning.

6.5 Recommendations for future studies

The results of the current study showed that the removal of biofilms can be studied under well defined conditions which enable the assessment of the conditions which influence the process. The study was set up as a broad screening study for cleaning agents and procedures used in NF/RO membrane cleaning practices.

This study, however, was not an exhausted study and some aspects need further research to obtain a general applicable method:

- More data are needed on the influence of the biofilm characteristics on the cleaning efficiency;
- Additional laboratory tests are required to assess the quantitative effect of the following major cleaning variables on the clening efficiency: combined chemical treatments, pH, temperature, cleaning duration and shear forces during cleaning;
- Additional studies after the application of the laboratory test to assess the efficacy of agents and procedures to remove inorganic fouling deposits (scalants) as well as biocolloidal deposits (TEP);
- More research is needed to explore innovative cleaning strategies such as the fenton process induced by the presence of Fe and the use of H_2O_2 or other potentially interesting combinations such as peroxide with a detergent.
- SWM cleaning experiments to
    - verify whether the 'best' selected cleaning agent/protocol in the laboratory test is also the 'best' cleaning agent/protocol for SWM elements;

- verify what the effect is of chemical cleaning on the general performance characteristics of membrane processes (NPD control, water flux and salt passage) impaired by biofouling.

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Appendix 1: Biofilm isolation method

TableA.I.0.1. Remaining biofilm concentration (ATP and CH) on a PVC plate after brushing. Test 1

Sample	Milli-Q vol. (ml)	Blank (RLU/ml)	Total Surface (cm2)	Total ATP (pg/cm2)	CH average	Remaining ATP	Remaining CH
Ref 1	40	17	16.051	8.30E+04	120.50	98	88
Ref 1 A	40	17	16.051	1.39E+03	9.59	2	7
Ref 1 B	40	17	16.467	3.02E+02	6.29	0.4	5
Ref 2	40	17	16.467	9.92E+04	128.49	98	94
Ref 2 A	40	17	16.732	1.91E+03	5.11	2	4
Ref 2 B	40	17	16.231	2.15E+02	3.78	0.2	3
Ref 3	40	17	15.000	1.07E+05	104.24	97	92
Ref 3 A	40	17	14.856	3.07E+03	5.54	3	5
Ref 3 B	40	17	17.246	5.21E+02	3.79	0.5	3

Ref 1: first brushing. Ref 1A: second brushing. Ref 1B: third brushing

TableA.I.0.2. Remaining biofilm concentration (ATP and CH) on a PVC plate after brushing. Test 2

Sample	Milli-Q vol. (ml)	Blank (RLU/ml)	Total Surface (cm2)	Total ATP (pg/cm2)	CH (µg/cm2)	Remaining ATP	Remaining CH
Ref 1	40	17	16.051	8.30E+04	88.98	97	93
Ref 1 A	40	17	16.051	2.14E+03	3.93	2	4
Ref 1 B	40	17	16.467	5.26E+02	3.28	1	3
Ref 2	40	17	16.467	9.92E+04	85.60	98	94
Ref 2 A	40	17	16.732	1.22E+03	2.59	1	3
Ref 2 B	40	17	16.231	4.39E+02	2.55	0.4	3
Ref 3	40	17	15.000	1.07E+05	94.47	99	94
Ref 3 A	40	17	14.856	1.25E+03	3.29	1	3
Ref 3 B	40	17	17.246	2.40E+02	2.37	0.2	2

Ref 1: first brushing. Ref 1A: second brushing. Ref 1B: third brushing

Appendix 2: Cleaning test data

Overview of the biomass removal of all laboratory tests with BPU biofilms: single step treatments

	pH	*ATP*	*CH*	*Total Biomass*	*Ratio ATP*	*Ratio CH*	*Ratio total*
NaOH	12.0	-23.4	29.7	-2.5	-0.34	0.83	-0.04
Floclean MC-11	11.4	6.2	18.5	13.0	0.11	0.67	0.29
P3-Utrasil 141	10.9	22.6	25.4	23.8	0.23	0.71	0.35
NaOH	12.0	48.0	26.7	35.9	0.64	1.17	0.53
P3-Utrasil 53	9.2	70.7	-0.6	43.7	0.73	-0.02	0.64
Permaclean 33	11.7	67.0	24.5	39.0	1.15	0.89	0.87
Novoclean 135	11.6	77.3	13.6	44.6	1.32	0.49	1.00
Divos 116	12.2	82.4	28.3	55.6	1.41	1.03	1.24
Alkaline		*(53.5)[a]*	*20.8*	*31.6*			
NaOH/SDS	12.0	68.7	35.8	55.1	1.00	1.00	1.00
NaOH/SDS	12.7	81.0	34.6	59.0	1.36	1.01	1.25
NaOH/SDS	12.7	99.3	41.1	61.1	1.70	1.49	1.36
Alkaline/detergents		*90.1*	*37.9*	*60.0*			
STP	8.8	27.1	17.1	22.8	0.41	0.48	0.43
NaDDBS	7.1	28.6	4.3	20.2	0.43	0.12	0.38
TSP	12.0	48.1	23.6	38.9	0.73	0.66	0.73
SDS	8.0	66.1	20.1	42.9	0.88	0.88	0.64
SDS	8.0	62.6	23.2	46.2	0.91	0.65	0.84
CTAB	5.4	99.6	38.4	73.2	1.52	1.07	1.38
Detergents		*55.3*	*21.1*	*40.7*			
PC-77	3.9	27.9	11.8	21.0	0.48	0.43	0.47
P3-Utrasil 73	2.5	57.3	12.9	35.7	0.59	0.36	0.52
HCl	2.0	36.9	11.7	26.4	0.63	0.43	0.59
Divos 2	1.6	44.4	11.6	30.5	0.76	0.42	0.68
P3-Oxyzan ZS	3.4	76.2	-2.6	44.0	0.79	-0.07	0.65
Citric A.	2.0	62.1	18.9	46.4	0.90	0.53	0.84
Acids		*50.8*	*10.7*	*34.0*			
Na-Bisulfite	4.0	16.5	8.7	12.9	0.28	0.26	0.27
DBNPA	6.0	48.4	15.5	33.2	0.81	0.45	0.70
NaOCl B	11.4	99.3	71.3	86.0	1.67	2.09	1.82
Biocides		*32.4[b] (54.7)*	*12.1[b] (31.9)*	*23.0[b] (44.0)*			
EDTA	11.0	19.9	12.7	17.4	0.29	0.35	0.32
EDTA	11.0	47.1	17.5	31.3	0.63	0.77	0.46
Chelating		*33.5*	*15.1*	*24.4*			
Everlase	7.0	-21.2	-24.0	-22.8	-0.28	-1.05	-0.34
Dextrozyme	5.1	-16.6	-8.2	-12.3	-0.22	-0.36	-0.18
BAN 480	5.1	-15.8	-7.2	-11.3	-0.21	-0.32	-0.17
Savinase	7.1	7.0	-23.2	-8.1	0.09	-1.02	-0.12
Enzymes		*-11.7*	*-15.6*	*-13.6*			

[a] negative value for NaOH not included; [b] efficiency without NaOCl

The removal of biomass assessed in the laboratory test using BPU biofilms: double step treatments

	pH1	*pH2*	*ATP*	*CH*	*Total Biomass*	*Ratio ATP*	*Ratio CH*	*Ratio total*
PC-33+PC-77	11.7	3.9	12.1	10.0	11.0	0.15	0.49	0.22
NaOH+HCl	12	2	47.1	21.5	34.5	0.52	0.85	0.66
NaOH+Divos 2	12	1.6	48.8	25.9	37.2	0.54	1.03	0.72
Alkaline+Acid			36.0	19.1	27.6			
Citric acid+NaOH	2	12	25.3	25.5	25.4	0.28	1.01	0.49
Divos 2+Divos 116	1.6	12.2	32.0	50.3	41.2	0.40	2.44	0.80
Citric Acid + Novoclean 135	2	11.6	93.3	23.8	62.2	1.16	1.16	1.21
Acid+Alkaline			50.2	33.2	42.9			
Bisulfite/NaOH+Divos 2	12	1.6	31.2	30.4	30.9	0.39	1.48	0.60
NaOH/SDS+HCl	12	2	58.5	29.6	42.2	0.65	1.17	0.81
Alkaline/mix+Acid			44.9	30.0	36.5			
NaOH/SDS+Savinaese	12	7	81.8	26.4	52.1	1.09	1.16	0.77
NaOH/SDS+BAN 480	12	5.1	87.3	20.5	59.2	1.16	0.90	0.88
Alkaline/DET+Enzyme			84.5	23.4	55.6			

Postbus 1072 3430 BB Nieuwegein T 030 606 95 11 F 030 606 11 65 E info@kwrwater.nl I www.kwrwater.nl