Development of a bioaugmented membrane filtration unit for removal of 2,6-dichlorobenzamide (BAM) from drinking water

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Thesis to obtain the Master of Science Degree in

**Biological Engineering**

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“Obrigada!”
Abstract

The surface and groundwater reservoirs used for the production of potable water are nowadays at risk due to the increasing occurrence of micropollutants. One such micropollutant is the regularly found 2,6-dichlorobenzamide (BAM), originated from the herbicide dichlobenil. Dichlobenil was banned in the European Union since 2008 but its adsorption on soil still leads to accumulation of BAM in water bodies. Due its high chemical stability, water treatment plants based on traditional methods such as activated carbon, ozonation or chlorination, are not efficient at removing BAM from this kind of contaminated water.

The bacterial strain Aminobacter sp. MSH1 was isolated from dichlobenil contaminated soil and showed the capacity for degradation and complete mineralization of BAM. The tests already performed in sand-filters, despite promising, revealed an extensive loss of cells and could not keep the concentration of BAM at a lower value than the established norm of 0.1 µg/L.

This work intended to resolve the wash-out of MSH1 cells with the use of membrane technology, which by studying several membranes on cell and on micropollutant retention allowed to determine the best performing membranes. MSH1 bacteria efficiently degraded BAM to a certain extent being however verified the formation of a degradation product, DCBA. Membrane retention tests showed to be efficient, however not enough to discard its combination with bioaugmentation: it was demonstrated the need of MSH1 cells in combination with the membranes for an efficient removal of micropollutants, from the water.

Keywords: Aminobacter sp. MSH1, bioaugmentation, 2,6-dichlorobenzamide, membrane technology, water purification
Resumo

A situação dos reservatórios de águas subterrâneas e de superfície é atualmente fragilizada devido ao crescente risco do uso de micropoluentes como produtos farmacêuticos, produtos de higiene pessoal ou pesticidas. Um composto frequentemente encontrado é o micropoluente 2,6-dichlorobenzamida (BAM), proveniente do herbicida dichlobenil. Este herbicida está banido da União Europeia desde 2008, mas a sua elevada capacidade de adsorção pelos solos culmina na grande acumulação de BAM nos reservatórios de água. Este composto altamente hidrofílico é altamente permeável nos solos e devido à sua grande estabilidade química estações de tratamento de águas residuais não são capazes de solucionar este problema através do uso de métodos tradicionais tais como carbono ativado, ozonização e cloração.

A partir do solo contaminado por dichlobenil, a estirpe *Aminobacter* sp. MSH1 foi isolada e demonstrou grande capacidade de degradação e de completa mineralização de BAM. Já correntemente testados, os filtros de areia verificaram uma significativa perda de células, sendo que o limite máximo permitido de BAM em água potável (0.1 µg/L), nunca foi atingido, sendo sempre superior.

Neste trabalho pretendeu-se solucionar o *wash-out* verificado da bactéria MSH1, pelo uso de tecnologia membranar, cujo passou pelo estudo de diversas membranas para retenção de células e de retenção de micropoluentes. O uso de membranas mostrou ser eficiente, e concluiu-se que a existência de células em combinação com as membranas, é uma necessidade absoluta para a remoção deste micropoluente da água.

**Palavras-chave:** *Aminobacter* sp. MSH1, bio-aumentação, 2,6-diclorobenzamida, purificação de água, tecnologia-membranar
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## Nomenclature

<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGW</td>
<td>Artificial Ground Water</td>
</tr>
<tr>
<td>AOC</td>
<td>Assimilable Organic Carbon</td>
</tr>
<tr>
<td>AS</td>
<td>Amidase Signature</td>
</tr>
<tr>
<td>BAM</td>
<td>2,6-dichlorobenzamide</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DCBA</td>
<td>2,6-dichlorobenzoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DWTP</td>
<td>Drinking Water Treatment Plant</td>
</tr>
<tr>
<td>EfOM</td>
<td>Effluent organic matter</td>
</tr>
<tr>
<td>EPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FC</td>
<td>Flow Cytometer</td>
</tr>
<tr>
<td>GAC</td>
<td>Granular Activated Carbon</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>HT</td>
<td>High Throughput</td>
</tr>
<tr>
<td>MBR</td>
<td>Membrane Bioreactor</td>
</tr>
<tr>
<td>MF</td>
<td>Microfiltration</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectroscopy</td>
</tr>
<tr>
<td>MS</td>
<td>Mineral Salts</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-off</td>
</tr>
<tr>
<td>NF</td>
<td>Nanofiltration</td>
</tr>
<tr>
<td>NTU</td>
<td>Nephelometric Turbidity Unit</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OP</td>
<td>Organo-phosphates</td>
</tr>
<tr>
<td>PAC</td>
<td>Powdered Activated Carbon</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse Osmosis</td>
</tr>
<tr>
<td>THM</td>
<td>Trihalomethanes</td>
</tr>
<tr>
<td>TMP</td>
<td>Transmembrane Pressure</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltration</td>
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<td>UPRO</td>
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<tr>
<td>UPLC</td>
<td>Ultra-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater Treatment Plant</td>
</tr>
</tbody>
</table>
1. Literature Review

1.1 Micropollutants

Anthropogenic chemicals responsible for drinking water contamination are most typically polar or semi-polar organic compounds that are present in a concentration range of pg per liter to µg per liter and are commonly referred as micropollutants. All foodstuffs intended for human or animal consumption in the European Union are subject to a maximum residue level of pesticides in their composition in order to protect animal and human health. Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC, established a default limit of 0.01 mg/kg (or 0.1 μg/L) (EIONET, 2016) (Eur-Lex, 2015).

Atrazine, metolachlor ESA, lincomycin, sucralose, and nonylphenol are well known examples of micropollutants (Benner, et al., 2013). Micropollutants can enter the urban water cycle via both diffuse and point sources. Anthropogenic chemicals from domestic and industrial origin enter the urban water cycle via sewer networks, reaching wastewater treatment plants (WWTP) where they are removed or discharged into surface waters (Benner, et al., 2013) (Mihelcic, Hand, & Auer, 2010).

Pesticides applied in agriculture and urban areas to control unwanted organisms such as insects, weeds, and fungi are examples of anthropogenic chemicals that primarily enter the water cycle from diffuse sources. Most pesticides are mobile in the aqueous phase and their direct application to land enhances their mobility after rain events, resulting in significant fluxes of pesticides in groundwater even at distant locations from the application point. Additionally, processes like biotransformation or photolysis can result in the formation of persistent degradation products, often more polar and thereby more mobile than the parent compound. It is frequent to find degradation products at higher concentrations than the parent compounds (Benner, et al., 2013). A schematic representation of the micropollutant motion in the environment is shown in Figure 1.1.

![Figure 1.1 - Common pathways of micropollutants into drinking-water sources (Benner, et al., 2013).](image)
1.2 Pesticides in drinking water sources

Throughout history, the thrive to survive lead mankind to always find ways of protecting cultivation and food production from all kind of risks. However, since 1950, this has resulted in a massive utilization of synthetic compounds for pest control called pesticides. A pesticide is any substance or mixture of substances containing both “active” and “inert” ingredients. Active ingredients prevent, destroy, repel or mitigate a pest, as well as they are plant regulators, defoliants, desiccants or nitrogen stabilizers. Inert ingredients are needed for product performance and usability (EPA, 2015) (EPA, 2016) (EPA, 2016). The classification of pesticides is based on the target group or according to the chemical structure and hazard. Based on the target group the classes of pesticides include herbicides, insecticides, rodenticides, fungicides, and bactericides (Ye, et al., 2013) whilst e.g. organophosphorus, carbamates or organochlorine compounds are the common pesticide chemical structures. By hazard, the pesticides can belong to the class Ia, Ib, II, III or U going from an extremely hazardous classification to slight risk of toxicity (WHO, 2010).

Overviewing the general scenario, the introduction of synthetic insecticides – organo-phosphate (OP) insecticides in the 1960s, carbamates in 1970s and pyrethroids in 1980s and the introduction of herbicides and fungicides in the 1970s–1980s contributed greatly to pest control and agricultural output (Aktar, et al., 2009). The total pesticide consumption amount is decreasing (EEA, 2004). The main reason seems to be the application of more efficient pesticide substances which are applied in smaller quantities. But it is the toxicity of an individual pesticide, not necessarily the amount used, which determines its potential for environmental damage (Aktar, et al., 2009). Although agriculture has a considerable share in the use of pesticides, also households, industry and the authorities use significant amounts of these compounds (MIRA, 2013).

The quality of the drinking water sources is increasingly deteriorating due to anthropogenic activities. Fresh surface and groundwater resources are becoming increasingly scarce due to unsustainable practices such as food production, energy generation, industrial applications and general public use. Also increasing is the salinity in coastal and agricultural areas and continuous input of nutrients and hazardous chemicals compromises the water quality which is assessed by physical, chemical and biological characteristics relative to those required for human use (Benner, et al., 2013) (Pukkila, 2015).

Specifically, for the Flanders region in Belgium and based on the 2011 indicator report on the Flanders environment, an exceedance of the quality standard for one or more degradation products of pesticides was observed in more than half (56 %) of the measurement locations in the phreatic monitoring network. This means that the concentration for one substance was greater than 0.1 μg per liter or that the total concentration of all the substances together was higher than 0.5 μg per liter. Furthermore, pesticides or pesticide degradation products in concentrations below the legal standard were found in one-third of the measurements, meaning that only approximately 10% of the cases did not show any trace of these pollutants (Steertegem, 2011). The standard exceedances occur nearly everywhere in Flanders. This does not mean that the pesticide problem is of the same nature everywhere. The substances that are encountered differ from place to place, depending on the land use. The land use determines which
pesticides can be used in a certain area. The extent to which these substances and their degradation products get into the groundwater and spread further from there, depends in turn on the characteristics of the subsoil such as permeability, presence of organic matter or clay to which pesticides and their degradation products attach themselves and depth of the water table (Steertegem, 2011).

Concerning surface water, the physical-chemical and biological water quality in Flanders is insufficient to meet the drinking water standards and targets (MINA plan, Decree on Integral Water Policy, Water Framework Directive). In 2010 the Belgian Biotic Index was determined at 376 locations and 37 % of the monitored locations achieved a good or very good quality. Of the almost 500 measurement points that were sampled at least five times in the period 2000-2010, the Belgian Biotic Index showed a significant improvement in 17 % and a significant worsening in 0.4 %. Concerning groundwater, measurements (in percentage) of different kinds of pesticides are represented in Figure 1.2. Hazardous pesticides such as atrazine, diuron or simazine are and it is also possible to notice the presence of the compound BAM, which toxicity is much more threatening than its original compound, the pesticide dichlobenil (Steertegem, 2011).

![Figure 1.2 - Different types of pesticides present in groundwater (MIRA, 2013).](image)

### 1.3 Dichlobenil and the metabolite 2,6-dichlorobenzamide (BAM)

Dichlobenil, is a nitrile herbicide widely used for weed control, mainly in non-agricultural areas and in the aquatic environment. It has been applied for control of annual and perennial grasses and broad leaved weeds and in some cases to remove tree roots and inhibit their growth in sewers and to control submerged and floating aquatic weeds (Björklund, et al., 2011). Dichlobenil is one of the 84 substances of the third stage part B of the review program covered by Commission Regulation (EC) No. 1490/2002, as amended by Commission Regulation (EC) No. 1095/2007. After the examination of dichlobenil by the Commission of the European Communities, it was concluded that there were clear indications of harmful effects, leading to the adoption of a decision on non-inclusion in Annex I to Council Directive 91/414/EEC (Authority, 2010). This directive concerns the authorization, placing on the market, use and control within the Community of plant protection products in commercial form, and the placing on the
market and control within the Community of active substances intended for a specific use (EUR-Lex, 2014).

When the degradation of dichlobenil occurs, 2,6-dichlorobenzamide (BAM) is formed. Represented in Figure 1.3, are the chemical structures of dichlobenil and its metabolites BAM and 2,6-dichlorobenzoic acid (DCBA).

![Image](image)

Figure 1.3 - Structures of (a) 2,6-dichlorobenzonitrile (Dichlobenil); (b) 2,6-Dichlorobenzamide (BAM); (c) 2,6-dichlorobenzoic acid (DCBA) (Sigma-Aldrich, 2016).

Some of the physico-chemical properties of the dichlobenil pesticide and its metabolites are shown in Table 1.1 (Björklund, et al., 2011).

<table>
<thead>
<tr>
<th></th>
<th>Molecular Weight, MW (g/mol)</th>
<th>Vapor pressure (mPa)</th>
<th>Water solubility (mg/L) at 25ºC</th>
<th>Sorption distribution coefficient, K_d (L/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichlobenil</td>
<td>172.01</td>
<td>88 (20ºC)</td>
<td>20</td>
<td>1.5 – 17.4</td>
</tr>
<tr>
<td>BAM</td>
<td>190.03</td>
<td>0.4 – 4 (25ºC)</td>
<td>2700</td>
<td>0 – 0.93</td>
</tr>
<tr>
<td>DCBA</td>
<td>191.01</td>
<td>N.A.</td>
<td>N.A.</td>
<td>&lt; 18¹</td>
</tr>
</tbody>
</table>

Dichlobenil is relatively persistent in the environment and is primarily bound to solid matrices such as soils (Benner, et al., 2013). This is a result of the verified high K_d (Table 1.1) which makes this component very keen to be absorbed by the organic matter in the soil. Of great concern is its main degradation product, BAM, which is water soluble and therefore transported downward in aquifers, contaminating groundwater resources. BAM is a hydrophilic compound and is easily dissolved due to its high water solubility and low sorption distribution coefficient (Table 1.1). The potentially produced DCBA metabolite can also leach to the groundwater due to a rather low organic carbon sorption coefficient (Fava, et al., 2005) (Holtze, et al., 2007).

1.3.1 BAM degrading *Aminobacter* sp. MSH1: bioaugmentation studies

Among 39 locations previously treated with dichlobenil, six locations showed extensive mineralization of BAM indicating that microbial adaptation to BAM degradation (Simonsen, et al., 2006). *Aminobacter* sp. MSH1 was isolated from one of these locations (Simonsen, et al., 2006) (Simonsen, et al., 2007).

¹ Value of K_{oc} - normalized partition coefficient between soil organic carbon and water (Fava, et al., 2005).
The bacterial strain *Aminobacter* sp. MSH1 is known for the complete degradation and mineralization of dichlobenil and BAM to CO$_2$ while using them as carbon, nitrogen and energy source (Simonsen, et al., 2012). *Aminobacter* spp. are ubiquitous in natural environments and are characterized as α-proteobacteria and facultative methylotrophs, able to utilize methylamine and methyl halides for growth (Simonsen, et al., 2012). According to Sørensen et al. (2007), after analyzing the degradation and mineralization of $^{14}$C BAM to $^{14}$CO$_2$ by *Aminobacter* sp. strain MSH1 for concentration ranges from 7.9 nM to 263.1 µM, it was observed that this degradation was very efficient. Despite apparent higher concentrations resulting in higher degradation of micropollutants, high concentration of dichlobenil revealed to inhibit the degradation activity from the MSH1 bacteria (Simonsen, et al., 2007). Moreover, *Aminobacter* sp. MSH1 commonly degraded BAM into the metabolite DCBA, which was accumulated, suggesting that BAM biodegradation occurred through DCBA formation involving amide hydrolysis (Simonsen, et al., 2012). The genetic identification of an amidase, designated as BbdA, was found to initiate the degradation of BAM in *Aminobacter* sp. MSH1 (T’Syen, et al., 2015). The corresponding gene *bbdA*, appears to be indispensable for BAM degradation in strain MSH1. According to T’Syen et al. 2015, MSH1 mutants defective in BAM conversion lacked *bbdA*, indicating that the amidase can be linked to the conversion of BAM into DCBA. Moreover, the loss of BAM degradative phenotype was found to be due to loss of the plasmid pBAM1, that contains the contig carrying *bbdA*. Likewise, the *bbdB* gene was also lost, being this gene located on the pBAM2 plasmid, which is responsible for both DCBA degradation and BAM mineralization.

Hence, BbdA’s high affinity for BAM and its constitutive expression are of interest for using strain MSH1 in the treatment of groundwater containing this micropollutant for drinking water production (T’Syen, et al., 2015).

The comparison of reported $K_M$ values of enzymes in Table 1.2 shows two $K_M$ values of BbdA for BAM, which is one of the lowest $K_M$ values between AS Family Amidases (T’Syen, et al., 2015). For many enzymes, experimental evidence suggests that $K_M$ is an approximation of *in vivo* substrate concentration. Thus, BbdA’s low $K_{\text{M,BAM}}$ value translates the low BAM concentrations that are expected in soil contaminated with dichlobenil (to which MSH1 bacteria were subjected) (T’Syen, et al., 2015).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein function</th>
<th>Substrate</th>
<th>$K_M$ (µM)</th>
<th>$T_{\text{opt}}$ (ºC)</th>
<th>Host organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbdA</td>
<td>AS amidase</td>
<td>BAM</td>
<td>0.71</td>
<td>62.5</td>
<td><em>Aminobacter</em> sp. MSH1</td>
</tr>
<tr>
<td>BbdA</td>
<td>AS amidase</td>
<td>benzamide</td>
<td>2.51</td>
<td>62.5</td>
<td><em>Aminobacter</em> sp. MSH1</td>
</tr>
</tbody>
</table>

The lower the Michaelis-Menten constants, the higher the enzyme affinity to the substrate. It is then possible to verify on Table 1.2 that BAM is the substrate with higher affinity and even with residual concentrations, maximum degradation rates are possible to attain. On the other hand, T’Syen et al. (2015) observed that BbdA was better tuned to degrade benzamides, which are less chlorinated than BAM. Thus, a higher maximal rate is likely to be attained for non-chlorinated benzamides. In fact,
substantially higher rates were verified when analyzing the conversion of both substrates in Table 1.2, by BbdA (T’Syen, et al., 2015) (Fonseca & Teixeira, 2007).

The *Aminobacter* sp. MSH1, besides being the only strain seen to be capable of degrading the herbicide as well as several metabolites, also presented very high degradation capacity, making it the first known dichlobenil-mineralizing bacterium (Sørensen, et al., 2007).

As implied throughout this section, the pesticide degrading bacteria acts by the process called bioaugmentation. It consists of adding actively growing, specialized microbial strains into a microbial community in an effort to enhance the ability to degrade certain compounds (Gerardi, 2006). The occurrence of this bacteria intended to solve the detected BAM pollution in aquifers, since studies on natural attenuation showed insignificant dichlobenil degradation in the aquifers. And also demonstrated that BAM is a very mobile and recalcitrant compound. Since small or no degradation was detected for BAM, it therefore represents a threat to groundwater (Clausen, et al., 2007).

Previous studies on sand-filters bioaugmented with *Aminobacter* sp. MSH1 already aimed to solve the persistent micropollutant problem in contaminated water (Simanjuntak, 2014) (Albers, et al., 2014).

The two following cases, Albers et al. (2015) and Sekhar et al. (submitted), studied MSH1 cell characteristics such as their affinity to low BAM concentrations, the amidase role and the biofilm formation ability for BAM degradation/mineralization. In order to assess the use of BAM as carbon source by MSH1 cells, optimal BAM concentrations and the importance of assimilable organic carbon AOC were equally studied. These studies allowed to infer on different MSH1 cell aspects, being of great interest to identify in the present work.

AOC, a fraction of the total concentration of organic carbon present in water, is typically the most readily degradable fraction, which tends to be composed of small molecular weight compounds, being also regarded as a measure for the biological stability of the water (Escobar & Randall, 2001).

Recently, Albers et al. (2015) assessed several characteristics of MSH1 behavior during bioaugmentation in sand-filters. Efficient removal of a 0.2 µg/L BAM solution to a value below the legal threshold limit (0.1 µg/L) was attained and no BAM degradation products were detected. Nonetheless, the loss of the inoculated bacteria for time periods longer than a few weeks caused efficiency loss in the system. Backwashing effects were proven to be related to the large decrease of inoculated cells, being this however needed for the removal of precipitated metal oxides. Equally important was the measurement of the amount of assimilable organic carbon (AOC), being glucose admitted to be the C-source, from where the basis of the AOC calculations were done in this study (Albers, et al., 2015). MSH1 bacteria were not able to assimilate this carbon source, which pointed for the existent competition from the original indigenous microorganisms (protozoa) on the sand-filter and pointed also to MSH1 starvation due to the limitation in easily AOC (Albers, et al., 2015). This revealed that the AOC amount was very low in the groundwater media utilized for feeding the filters, which was not enough to sustain a sufficient population of MSH1-bacteria. The bioaugmented process furthermore showed not to be affected by temperature or BAM concentration, as well as genetics showed that the *bbdA* encoding gene was not lost and it was constitutively expressed (Albers, et al., 2015).
Bacterial strains used for bioaugmentation in sand filters in drinking-water treatment plants (DWTPs) are faced with three challenges: the occurrence of micropollutants at very low concentrations, adaptability in oligotrophic conditions and short hydraulic retention times. A study performed in suspended batch conditions and in flow chambers characterized cell attachment and biofilm mode of growth (Sekhar, et al., submitted).

This study allowed to infer on the colonization of a solid surface, mimicking the conditions occurring in biofilter systems for water treatment. Additionally, BAM degradation rates by Aminobacter sp. MSH1, under controlled feeding regimes in flow cells and in batch cultures, were compared (Sekhar, et al., submitted).

Since cell growth was affected by low BAM substrate concentrations and oligotrophic environment, in case of biofilm formation, this was considered to be an additional constraint to the diffusion of BAM from the bulk liquid to the biofilm surface, and from that to the cells. Short hydraulic retention times resulted in a short contact of the microbial catalyst with BAM, while at the same time high flow rates caused the loss of cells due to shear stress (Sekhar, et al., submitted). This study showed that MSH1 is capable of colonizing the flow chambers at microconcentrations of BAM. With a concentration of 1 µg/L BAM, an efficient colonization of the solid surface was observed. This was due to the surface properties (inert glass surface with silica-based materials), that for being similar to the sand-filters, proved to be an appropriate material for MSH1 cell attachment and further biofilm development (Sekhar, et al., submitted).

On the other hand, cell growth in the flow chambers fed with BAM micro concentrations was not attained, suggesting that biomass formation was mainly supported by the AOC present in the minimal medium. It was then hypothesized that not all imaged cells were metabolically active but a large amount would be in a dormant state (Sekhar, et al., submitted). Concerning BAM degradation, it was overall verified a removal efficiency above 70%. As shown by T'Syen et al. 2015, the presence of MSH1 theoretically assures BAM degradation irrespective of BAM concentration. Ultimately, one reasonable explanation for the hypothetical bacterial dormant state, is that despite the constitutive character of BbdA, there wasn’t enough enzyme production, due to the starvation conditions. Else, upper cells in the biofilm were efficiently fed with BAM while deeper cells were fed from their metabolites.

For the batch systems, degradation rates at BAM microconcentrations were seen to be higher, since the steady presence of AOC and BAM allow an efficient activity, in opposition to the flow chambers, where not enough substrate was available for the cells.

It was concluded that sand-filters used for bioaugmentation are consequently not fully predictable for the cells activity under starvation conditions in batch systems showing also the importance of AOC in their activity and number in the system (Sekhar, et al., submitted) (Benner, et al., 2013).

1.4 Drinking water production

Drinking water production is a stepped treatment process aimed at eliminating pathogens, removing particulate matter, reducing turbidity and even the adapting tastes and odors according to the
consumer’s acceptancy (Benner, et al., 2013) (Hand, Zhang, & Mihelcic, 2010). Drinking water can be produced from both surface and groundwater. However, due to the increasing risk of droughts by overexploitation in natural areas, a shift from groundwater to surface water is noted. The treatment of groundwater for drinking water production requires only a few steps, since aquifers have natural filtration capacities. On the other hand, surface water is exposed to a wide range of contaminants, thus an efficient treatment is crucial for its further use as drinking water (Van der Bruggen, et al., 2004).

1.4.1 Conventional drinking water production processes

Traditional surface water treatment involves techniques such as aeration, coagulation/flocculation, sand filtration, physico-chemical softening, activated carbon adsorption and disinfection (Figure 1.4). On a preliminary phase the water is prepared by retaining coarse solids (large objects). Subsequent aeration of the water increases the level of dissolved oxygen and oxidizes metals, causing their precipitation. Although promising, studies on the role of the aeration process on the removal of biological micropollutants showed its dependency on the metabolic activity of the microbial community and on the hydraulic residence time of the aeration basin, which combined did not favor significant micropollutant removal (Benner, et al., 2013). Coagulation by the addition of coagulants such as Fe III (usually in the form of FeCl₃) is followed by flocculation, commonly using anionic and nonionic polymers to aggregate destabilized particles, trapping unwanted matter. These are very important steps to reduce the turbidity arising from suspended organic and inorganic particles, but Stackelberg et al. 2007 showed that polar and semi-polar pesticides remained partitioned in the aqueous phase (Stackelberg, et al., 2007). In sand-filters the water is driven by gravity flow through a bed of granular material and the particles are collected within the bed. This allows the removal or transformation of inorganic species and residual particles. Sand filters are normally colonized by native microbial populations attached to the sand grain surface that grow on nutrients from the source water. The next process, disinfection, makes use of oxidizing agents, heavy metals cations and physical agents (heat or UV radiation). Common disinfectants are free chlorine, ozone, chlorine dioxide and UV light. The disinfection in drinking-water practice refers to two activities: primary disinfection where the inactivation of microorganisms in the water occurs, and secondary disinfection also designated as residual maintenance, consisting on maintaining a disinfectant in the treated-water distribution system (Hand, Zhang, & Mihelcic, 2010) (Benner, et al., 2013). Similarly to the previous treatment, also the production of drinking water from groundwater follows the same principles, though often little or no treatment is required to be suitable for drinking purposes (WHO, Groundwater, 1996).
Currently used processes and disinfection agents for the treatment of contaminated water are following described.

1.4.1.1 Activated carbon

Activated carbon filters are applied to remove residual growth substrates (AOC) and to minimize regrowth of bacterial contaminants in the distribution system. Powdered activated carbon (PAC) and granular activated carbon (GAC) are widely used for the removal of synthetic organic chemicals and odor compounds from drinking-water supplies. GAC is able to remove nonpolar and semi-polar to polar micropollutants from water. The adsorption process transfers molecules from a fluid stream onto a solid surface, concentrating them by physical forces. These are controlled by van der Waals-type forces, non-specific binding mechanisms or attractive forces between the adsorbate and the adsorbent surface. The treatment is carried out in a fixed-bed mode of operation either as gravity feed or under pressure. Operation and maintenance are costly and the filtration does not eliminate the pollutant. Moreover, despite the compounds being sequestered in the filter, desorption processes might occur, exposing the effluent to the adsorbed chemical. Likewise, highly polar pesticides and pharmaceuticals can easily breakthrough activated carbon filters, which compromises the sustainability of the method (Hand, Zhang, & Mihelcic, 2010) (Benner, et al., 2013) (Albers, et al., 2015).

1.4.1.2 Chlorination

Free chlorine is the most frequently used disinfectant, while the use of combined chlorine is often limited to secondary disinfection. The necessity of a turbidity below 20 Nephelometric Turbidity Unit (NTU) was established and the World Health Organization guideline established threshold, of 5 mg/L (WHO, Groundwater, 1996). Such amount is however above the taste threshold of 0.6 – 1 mg/L, being desirable
the attainment of a value within this range (Hand, Zhang, & Mihelcic, 2010). When gaseous chlorine (Cl\textsubscript{2}) is added to water it rapidly reacts to form hypochlorous acid (HOCl), which together with the hypochlorite ion (ClO\textsuperscript{-}) is often referred to as free chlorine. Although disinfection using chlorine is very effective and non-costly, the concern goes to by-product formation: chlorine reacts with dissolved organic matter naturally existent in waters, forming carcinogenic trihalomethanes (THM) (Hand, Zhang, & Mihelcic, 2010). Chloramine compounds are also used and are formed when both chlorine and ammonia are present in the water. Chlorine dioxide has higher oxidizing power than chlorine, however, at neutral pH, typical of most waters, it has only 70% of the oxidizing capacity of chlorine. While chlorine dioxide does not produce significant amount of TMH as by-products, it produces inorganic compounds, for instance chlorite and chlorate, which at certain levels of exposure become a concern. The risks of explosion at high temperatures, exposure to light or in presence of organic substances, causes restraints to the use of chlorine dioxide (Hand, Zhang, & Mihelcic, 2010). Oxidative chlorine species can be selective or non-selective oxidants that inactivate pathogens and also react with micropollutants to produce halogenated disinfection by-products which inconveniently may have equal or greater toxicity comparatively to the parent chemical (Benner, et al., 2013).

1.4.1.3 Ozonation

Ozonation is an advanced oxidation technique that in combination with hydrogen peroxide and/or UV radiation, becomes a favorable alternative to chlorination. Ozone, which is a highly reactive gas, decays rapidly under ambient conditions, hence it has to be generated on site by electrical discharges in the presence of O\textsubscript{2}. It is a strong oxidant that reacts with microbes by direct oxidation or through the action of generated hydroxyl radicals. A strong affinity between micropollutants containing phenol, aniline or deprotonated-amine substructures and ozone is verified, whereas hydroxyl radicals can non-selectively oxidize a variety of organic chemicals (Gunten, 2003) (Hand, Zhang, & Mihelcic, 2010) (Benner, et al., 2013).

Ozonation is a disinfection process usually occurring in reactors called contactors, where ozone is generally introduced in bubble chambers in series. Conditions such as high pH favors hydroxyl radical oxidation reactions, while low pH, high alkalinity, low concentrations of organic matter and low temperature will increase the stability of aqueous ozone residuals. The residuals originated by the use of ozone are not tolerable to continue in the water distribution system and the inevitable generation of bromate composes a great risk due to the evidence of carcinogenic effects on humans. High costs in operation and maintenance of the ozonation process labels it as difficult when assessing costs versus benefit (Gunten, 2003) (Hand, Zhang, & Mihelcic, 2010) (Benner, et al., 2013).

Though, both techniques chlorination and ozonation, have shown to produce persistent oxidation products with equal toxicity as the parent chemical (Benner, et al., 2013).

1.4.2 Membrane-based drinking water production processes

Besides the traditional strategy for water treatment, there is another option to accomplish the same purpose (drinking water production): a membrane-based water treatment system (Figure 1.5). This
system uses ultrafiltration to replace coagulation, flocculation and sand filtration. Conventional softening and activated carbon filtration are replaced by nanofiltration, where the addition of sulphuric acid prevents membrane scaling due to CaCO$_3$ precipitation. Post-treatments involve the use of crushed marble/remineralization and chemical disinfection. Remineralization is important for human health and a minimum total hardness is required to meet legal standards (60 mg Ca/L, 15ºF in Belgium). Activated carbon sorption might be used as a treatment extent to remove remaining organic compounds from the nanofilter permeate because of its run time. Run time increases in this sort of treatments due to natural organic matter and micropollutants being removed beforehand, to an already great extent, consequently diminishing operating costs (Van der Bruggen, et al., 2004).

Both traditional and membrane-based water treatment strategies present advantages and limitations. Considering economical aspects, traditional treatments have generally better performance than membrane-based alternatives, but in terms of quality, public health and society acceptance the last is highly favored. Thus, membrane technology is perceived as a promising technology, very likely to become the future trend to produce drinking water (Van der Bruggen, et al., 2004).

Recent studies reported a gravity driven ultrafiltration membrane pilot plant operating during 10 months, where it was seen that the microbiological quality of the permeate was within recommended and acceptable limits of the WHO and South African standards for drinking water quality. Despite these promising results, more research is needed to evaluate health impact, maintenance and cost-effectiveness, of the process. It is also important to assess bacterial regrowth and water quality deterioration, as well as the public awareness for the technology acceptability (Molelekwa, et al., 2014).

Similarly to the traditional systems for drinking water production, activated carbon and disinfection processes detailed in sections 1.4.1.1, 1.4.1.2 and 1.4.1.3 are also used on these membrane-based drinking water production processes.

Alternatively, to traditional and membrane-based drinking water treatments, treatments in situ are extensively used such as pump and treat, air sparging, in-situ flushing, permeable reactive barriers, monitored natural attenuation and bioremediation. This remediation technology is selected based on the specific hydrogeological and contaminant conditions, desired cleanup levels, remedial time and cost (Reddy, 2008).
1.5 Membrane technology

Over 20,000 membrane plants including reverse osmosis, were operating worldwide by the year of 2007 with perspectives of significant growth as the water consumption increases. The use of membrane technology provides a good retention system working as a selective barrier between two phases, providing a very efficient and economical way of separating components. Depending on the physical or chemical properties, specific compounds will pass through providing an immense possibility with different membrane types (Hand, Zhang, & Mihelcic, 2010) (Mulder, 1997).

1.5.1 Pressure driven membrane processes

Technically and commercially established membrane processes for water treatment, include reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF) and microfiltration (MF). Although there is no sharp distinction, these processes are defined mainly according to the pore size of the respective membranes, and to a lesser extent by the level of driving force for permeation, i.e. the pressure difference across the membrane. With decreasing porosity (i.e. from MF to UF and NF to RO), the hydrodynamic resistance of the respective membranes increases and consequently higher pressures are applied to obtain the required water fluxes. MF and UF systems generally operate at a pressure of ~1.7 to ~10.3 bar, while some operate under vacuum at less than 0.8 bar. The porous MF and UF membranes are characterized by the molecular weight cut-off (MWCO), which is expressed in Dalton indicating the molecular weight of a hypothetical non-charged solute that is 90% rejected. NF can be characterized either by MWCO or ionic retention of salts such as NaCl or CaCl₂; RO membranes being dense, are characterized by salt retention, although studies from Kimura et al. 2004 have modeled molecular retention to determine a MWCO (Karabelas & Plakas, 2011) (Mulder, 1997).

Being NF and RO specially used when low molecular weight solutes have to be separated from the solvent, it is predictable that micropollutants such as herbicides, present high retention in these types of membranes (Mulder, 1997). These membranes typical operation pressure range from 7-30 bar and from 20-100 bar, respectively. Recently, the application of membrane technology in water and wastewater...
treatment is increasing due to stringent water quality standards. Nanofiltration is one of the widely used membrane processes for water and wastewater treatment in addition to other applications such as desalination. NF is also seen as a good replacing alternative to RO membranes in many applications, due to lower energy consumption and higher flux rates (Shon, et al., 2013).

1.5.2 Filtration modes

These systems can be operated in dead-end or cross-flow mode (Figure 1.6). In the dead-end filtration, the liquid that enters the membrane surface is pressed through the membrane and some solids and components will stay on the membrane while the flow goes through. This filtration depends on the pore size of the membrane that will determine the resistance that the membrane presents. The pressure needed is called Trans Membrane Pressure (TMP). During membrane cleaning, components are removed hydraulically, chemically or physically and when this cleaning process is performed, the module is temporarily out of order, which results in a discontinuous process. On the other hand, when cross-flow (also referred as tangential flow) filtration takes place, the feed is recycled being that during recirculation the feed water flow is parallel to the membrane. Only a small part of the feed water is used for permeate production whereas the largest part will leave the module. The purpose of this type of flow is to control cake thickness and since the flowing forces are high, it enables the suspended solids to be carried away in the water flow. Stable fluxes are easily achieved in these systems and cleaning in this installations is performed by means of backward flushing or chemical addition. Consequently, cross-flow filtration has high energy costs since the entire feed needs to be brought under pressure (Azevedo, 2013).

![Figure 1.6 - Schematic representation of (a) cross-flow and (b) dead-end filtration modes (Sciences, 2016).](image)

1.5.3 Polarization phenomena and fouling
Although membrane manufacturers give very definite and straightforward information about membrane cut-off and pore size, there is no possible way of rigorously predict their behavior (Mulder, 1997).

During permeation (transport of atoms, molecules and ions in a permeable media due to the gradient of concentration/temperature/pressure) the flux through the membrane is measured as function of the applied pressure (Hinková, 2011) (Azevedo, 2013). When a certain minimum is reached, the largest pores become permeable, while the smaller pores still remain impermeable. Theoretically, the increase in flux is proportional to the increase in applied pressure; however, synthetic microfiltration and ultrafiltration membranes generally do not possess a uniform pore size and thus, this proportionality may not be observed (Hinková, 2011). The concept of “cut-off” is more frequently used in ultrafiltration membranes and is defined as the molecular weight which is 90% rejected by the membrane. Nevertheless, other parameters should be considered, such as shape and flexibility of the macromolecular solute, interaction with the membrane material and occurrence of concentration polarization phenomena. Such phenomena results from the membrane that retains the solutes to a certain extent, where there will be an accumulation of retained molecules near the membrane surface. This results in a highly concentrated layer near the membrane which exerts a resistance towards mass transfer i.e. a concentration polarization resistance. The concentration of the accumulated solute molecules may become so high that a gel layer can be formed. This exerts the gel layer resistance as seen in Figure 1.

Membranes range from porous to non-porous and depending on the type of separation involved, different characterization techniques will be required. In general, the membrane characterization becomes progressively more difficult as the pore size decreases. The pore size and pore distribution mainly determines which particles or molecules are retained and which will pass through. On the other hand, non-porous membranes are used to perform separations on a molecular level. However, rather than molecular weight or molecular size, the chemical nature and morphology of the polymeric membrane and the extent of interaction between the polymer and the permeants are the important factors to consider. The separation is achieved either by differences in solubility and/or diffusivity. So methods like permeability or surface analysis can determine the physical properties related to the chemical structure of the membrane (Mulder, 1997).

Often, a typical flux-time behavior may be observed: the flux through the membrane decreases over time and is mainly due to concentration polarization and fouling.

In microfiltration and ultrafiltration, the flux decline is very severe, with the process flux often being less than 50% of that corresponding to pure water flux. Nanofiltration and reverse osmosis are denser membranes, where a much higher pressure must be applied to force the same amount of solvent through the membrane (Mulder, 1997).

The performance of a membrane operation is diminished by polarization phenomena and fouling, being very important to distinguish between them, although both are not completely independent of each other since fouling can result from polarization phenomena. Membrane fouling occurs due to mechanisms such as pore blocking, cake formation, concentration polarization, and organic adsorption. When existent, fouling depends on physical and chemical parameters such as concentration, temperature, pH,
ionic strength and specific interactions (hydrogen bonding, dipole-dipole interactions). It can be classified as reversible fouling and irreversible fouling: distinction is in which membranes are operated and cleaned. Normally foulants can be classified into four categories such as particulates, organic, inorganic and micro-biological organisms (Mulder, 1997).

The membrane fouling mechanisms, induce additional resistances on the feed side to the transport across the membrane. The extent of these phenomenon is strongly dependent on the type of membrane process and feed solution employed. Schematic representation in Figure 1.7.

![Figure 1.7 - Overview of various types of resistance towards mass transport across a membrane in pressure driven processes (Mulder, 1997).](image)

The various resistances depicted in Figure 1.7 contribute with different extent to the total resistance, where ideally only the membrane resistance is involved. With porous membranes, it is possible for some solutes to penetrate into the membrane and block the pores, leading to the pore-blocking resistance. Finally, a resistance can arise due to adsorption phenomena i.e. $R_a$. Adsorption can take place on the membrane surface as well as within the pores themselves. Flux decline has a negative influence on the economics of a given membrane operation, and for that reason, measures must be taken to reduce its incidence (Mulder, 1997).

There are methods to reduce fouling, however, each separation problem requires its own specific treatment. Several approaches can be distinguished: pre-treatment of the feed solution, change of membrane properties, change of module and process conditions and cleaning of the membranes (Mulder, 1997).

Thus, in microfiltration and ultrafiltration the actual flux through the membrane can be only a fraction of the pure water flux. With polarization phenomena, the flux at a finite time is always less than the original value, but when steady state conditions are attained, the flux will become constant as a function of time. Despite being a reversible process, a continuous decline can often be observed. This results from
deposition of retained particles, colloids, emulsions, suspensions, macromolecules or salts, on or in the membrane (Mulder, 1997).

Membrane technology offers several possibilities. Faced with adsorption, it does not require phase change, occurs without accumulation of substances and does not need a regeneration cycle. It is also free of chemical additives, which is relevant when it comes to environmental impacts. Besides all the possibilities, there is also some drawbacks such as required costly pre-treatments and lack of robustness. However, progress has been made in developing membranes that have a significantly better overall performance and exhibit much more stability.

1.5.4 Removal of emerging trace-organic pollutants by membrane processes

A study by Xu et al. (2005) on the rejection of emerging trace organics by a variety of commercial reverse osmosis (RO), nanofiltration (NF), and ultra-low-pressure RO (ULPRO) membranes, was performed to simulate operational conditions for drinking-water treatment.

The rejection of trace organic compounds by high-pressure membranes is known to represent a complex interaction of steric hindrance, electrostatic repulsion, solution effects on the membrane, and solute and membrane properties. Some interactions considered of great importance, such as electrostatic exclusion, hydrophobic–hydrophobic interactions between solute and membrane, solution chemistry and membrane fouling, were assessed in this study (Xu, et al., 2005).

Rejection is affected by key membrane properties that include molecular weight cutoff (MWCO), pore size, surface charge, hydrophobicity–hydrophilicity and surface morphology. In addition, solution chemistry (such as feed water pH, ionic strength, hardness and the presence of organic matter), extent of membrane fouling, and hydrodynamic conditions, may also considerably influence the rejection of trace-organic pollutants (Xu, et al., 2005).

It was found that negatively charged hydrophilic solutes were further rejected by electrostatic repulsion through negatively charged membrane surfaces. The degree of electrostatic repulsion and rejection of negatively charged solutes was mainly dependent on the amount of membrane surface charge, but it also depended on the molecular weight of the compound and on the pore size of the membrane. Since membrane surface charge is dependent on the feed water’s pH and ionic strength and on the accumulation of organic foulants, the rejection of negatively charged solutes was subjected to changes during membrane operation. For nonionic hydrophobic compounds, hydrophobic–hydrophobic interactions with the membrane and steric hindrance have been reported as the driving mechanisms for rejection. The rejection of hydrophobic nonionic compounds has also been found to be dependent on operational conditions and solution chemistry. Findings from this study indicated that tight NF and ULPRO membranes, while operating at lower feed pressure, performed similar to conventional RO membranes in the removal of emerging trace-organic pollutants. Hydrophobic non-ionic compounds were only partially removed by a conventional RO membrane. Tight NF and ULPRO membranes could achieve a similar and elevated degree of rejection for hydrophobic nonionic compounds depending on
the membrane surface properties. For tight high-pressure membranes, the membrane surface charge was more important for rejection than the MWCO (Xu, et al., 2005).

The presence of effluent organic matter (EfOM) resulted in an improved removal of negatively charged compounds, as a result of increased membrane surface charge. Furthermore, the presence of EfOM seemed to completely neutralize the influence of hydrodynamic conditions, on rejection performance of high-pressure membranes (Xu, et al., 2005).

Martínez et al. 2015, tested the performance and behavior of a thin film composite polyamide RO membrane on 75 micropollutants including VOCs (Volatile organic compounds), endocrine disrupting compounds, fragrance allergens and pesticides. Consistent rejection levels above 83% were verified for fragrance allergens and EDCs, mainly attributed to their polarity and molecular weight. The RO showed an overall suitability in the rejection of the tested compounds (Martínez, et al., 2015).
2. Context and Objectives

Bioaugmentation of existing sand filters in DWTPs with *Aminobacter* sp. MSH1 cells for the removal of BAM from groundwater was tested in Denmark and Belgium (Albers et al., 2014) (Albers et al., 2015) (Sekhar, et al., submitted). However, rapid cell loss from these sand filters due to shear and grazing by protozoa led to a limited period of BAM removal under the required EU norm for drinking water. Embedding MSH1 cells in porous stones extended this period, but steady state BAM removal was insufficient to meet drinking water standards (Simanjuntak, 2014). As such, it was suggested that cells should be more efficiently retained in the system. As a solution for this problem, membrane technologies such as microfiltration and ultrafiltration, are able to efficiently retain bacterial cells. Moreover, when using e.g. nanofilter or reverse osmosis membranes, both the micropollutant BAM and the MSH1 cells are potentially able to be retained.

The main goal of this thesis was to test and develop two direct flow membrane filtration systems that (i) either retain MSH1 cells or (ii) retain the micropollutant BAM and MSH1 cells. In the first setup (Setup 1), the BAM contaminated groundwater was passed through a membrane of high permeability with a sufficiently small pore size for retaining MSH1 cells. In this case, BAM removal was based on the BAM-degrading activity of the MSH1 cells in the stirred cell, also referred in this work as membrane bioreactor (MBR). In the second setup (Setup 2), a membrane of low permeability was used which allowed the retention of BAM itself. In this case, BAM removal was a combination of BAM retention and subsequent degradation of BAM by the MSH1 cells in the MBR.

Two main specific objectives determined the course of all performed experiments and are described as follows:

1. Development of a direct flow MBR relying on MSH1 cell retention for BAM degradation
   a. MSH1 cell retention tests (viability measurements): three types of microfilters having a suitable pore size were chosen to study those with the best relation retention/permeability, concerning the cells inside the reactor. Staining procedures aimed to quantify and confirm viability of the inoculated MSH1 culture;
   b. Long-term tests evaluating BAM removal efficiency and cell viability: different BAM concentrations in contact with MSH1 cells aimed to mineralize BAM to a norm respecting value (<0.1 µg/L);
2. Development of a direct flow MBR relying on BAM retention and MSH1 cell retention for BAM degradation
   a. BAM retention tests: assessing the best relation retention/permeability of ultrafilters, nanofilters and reverse osmosis filters with BAM filtration media, to perform Setup 2 tests with the chosen membrane;
   b. Effect of MSH1 cells on membrane permeability: filtration of the cells to assess their effect on the membrane’s permeability;
   c. Long-term tests evaluating BAM retention/removal efficiency and MSH1 cell viability: tests with both retentions of BAM and MSH1 cells were performed, intending to optimize the long-term filtrations, by increasing the contact time between BAM and MSH1 cells.
For Setup 1, MSH1 cell retention efficiency by several MF membranes was tested as a function of MSH1 cell density in the MBR. More importantly, viability of MSH1 cells during the filtration period was tested as to ascertain BAM degradation activity. To quantify dead and live cell populations in effluent and reactor samples, a flow cytometric method was first developed. The viability and BAM degradation activity in MBR were monitored during long-term continuously fed MBR operation.

For Setup 2, several NF and RO membranes were tested for BAM retention efficiency and permeability when filtering groundwater contaminated with different BAM concentrations. Moreover, the same membranes were tested with different concentrations of MSH1 cell suspensions to evaluate the cell’s effect on permeability.
3. Materials and Methods

3.1 Dichlobenil metabolites

2,6-dichlorobenzamide (BAM) PESTANAL® analytical standard was purchased from Fluka Analytical (Germany). BAM has a molecular weight of 190.03 g/mol and the chemical formula is C$_7$H$_5$Cl$_2$NO. 2,6-dichlorobenzoic acid (DCBA) 98% was purchased from Aldrich Chemistry (China). DCBA has a molecular weight of 191.01 g/mol and the chemical formula is C$_7$H$_4$Cl$_2$O$_2$.

3.2 Media formulation and bacterial cultivation

R$_2$B is a rich medium used for rapid growth of bacteria and is composed of 0.5 g tryptone, 0.5 g yeast extract, 0.55 g glucose D$_+$ (monohydrate), 0.5 g (soluble) starch, 0.3 g sodium pyruvate, 0.3 g K$_2$HPO$_4$ and 0.1 g MgSO$_4$·7H$_2$O per liter of ultrapure water (Milli-Q®). R$_2$B was autoclaved at 121°C for 20 min. For the preparation of R$_2$A agar plates, 13 g of Select Agar (Invitrogen) was added prior to autoclaving.

Mineral salts (MS) medium is a minimal medium without a carbon source. To prepare one liter of MS medium, 978 mL ultrapure water (Milli-Q®) were autoclaved, to which 10 mL phosphate buffer solution were added, as well as 10 mL nutrient solution, 1 mL trace metal solution and 1 mL FeCl$_3$ solution. All solutions were added under sterile conditions. The phosphate buffer consisted of 1.36 g/L KH$_2$PO$_4$ and 1.78 g/L Na$_2$HPO$_4$·2H$_2$O. The nutrient solution was made of 0.05 g/L MgSO$_4$·7H$_2$O and 0.0132 g/L CaCl$_2$·2H$_2$O. The trace metal solution contained 2.86 mg/L H$_3$BO$_3$, 1.54 mg/L MnSO$_4$·H$_2$O, 0.039 mg/L CuSO$_4$·5H$_2$O, 0.021 mg/L ZnCl$_2$, 0.041 mg/L CoCl$_2$·6H$_2$O and 0.025 mg/L Na$_2$MoO$_4$·2H$_2$O. The FeCl$_3$ solution was made up of 5.14 mg/L FeCl$_3$·6H$_2$O (Appendix 2 - Media Preparation).

The species *Aminobacter* sp. MSH1 used in this study was isolated from a dichlobenil-treated soil in a courtyard of a former plant nursery located above a BAM-contaminated aquifer near Hvidrove (Denmark) (Sjøholm, et al., 2010). In this study, the green fluorescent protein (GFP)-producing MSH1-GFP variant was used (Sekhar, et al., submitted). MSH1-GFP was cryopreserved in -80°C until its use. MSH1-GFP was plated on R$_2$A plates amended with 200 mg/L BAM which were incubated at 25°C for 4 days. Afterwards, colonies were transferred to 25 mL R$_2$B supplemented with 200 mg/L BAM and incubated 25°C in Erlenmeyer flasks (100 mL) on a horizontal shaker (100 rpm). Depending on the experiment, when requiring a high incidence of BAM-mineralizing MSH1 cells, MSH1 colonies were also transferred to 200 mL MS medium supplemented with 200 mg/L BAM and incubated for 7-10 days at 25°C in Erlenmeyer flasks (1L) on a horizontal shaker (100 rpm).

Cells were harvested in the late exponential phase by centrifugation (6000 x g, 20°C, 10 min) and washed two times with a 10 mM MgSO$_4$ solution. Cell suspensions were adjusted to the required cell density using an OD spectrophotometer (Bausch & Lomb spectronic 21) by measuring the culture’s OD at 600 nm (OD 1 = approx. 10$^9$ cells/mL).
3.3 Artificial groundwater formulation

An artificial groundwater (AGW) medium was prepared, based on the composition of groundwater extracted in the Egenhoven DWTP (Egenhoven-West battery, De Watergroep, Belgium) with its macroelements specified in Table 3.1.

Table 3.1 - Macroelements in Egenhoven (Belgium) groundwater

<table>
<thead>
<tr>
<th>Element</th>
<th>mg/L</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄</td>
<td>0.02</td>
<td>0.001</td>
</tr>
<tr>
<td>NO₂</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>NO₃</td>
<td>47.79</td>
<td>0.771</td>
</tr>
<tr>
<td>Ca</td>
<td>154.88</td>
<td>3.872</td>
</tr>
<tr>
<td>K</td>
<td>2.19</td>
<td>0.056</td>
</tr>
<tr>
<td>Mg</td>
<td>17.03</td>
<td>0.701</td>
</tr>
<tr>
<td>Na</td>
<td>17.57</td>
<td>0.764</td>
</tr>
<tr>
<td>PO₄</td>
<td>0.11</td>
<td>0.001</td>
</tr>
<tr>
<td>SiO₂</td>
<td>27.46</td>
<td>0.458</td>
</tr>
<tr>
<td>Cl</td>
<td>50.50</td>
<td>1.423</td>
</tr>
<tr>
<td>F</td>
<td>0.20</td>
<td>0.011</td>
</tr>
<tr>
<td>SO₄</td>
<td>97.57</td>
<td>1.016</td>
</tr>
</tbody>
</table>

AGW medium was composed of solutions of 4.62 g/L NaCl, 3.37 g/L CaCl₂, 6.46 g/L Ca(NO₃)₂, 0.012 g/L NH₄Cl, 0.42 g/L KCl, 0.02 g/L CaHPO₄, 2 g/L CaSO₄, 0.07 g/L NaF and 17.3 g/L MgSO₄, using ultrapure water (Milli-Q®). All solutions were autoclaved prior to their use and 1 mL of each solution was added to 950 mL of sterile ultrapure water.

Carbon sources such as glucose, DCBA and BAM were added to the different media (bacterial cultivation media or artificial groundwater) prior to autoclaving, when appropriate. A stock solution of the antibiotic kanamycin (Km) (50 mg/mL) was filter sterilized (0.22 µm) and added to the media (bacterial cultivation media or artificial groundwater) after autoclaving when temperature was below 50°C.

3.4 MSH1 live/dead cell enumeration

3.4.1 Flow cytometry method development

R2B grown MSH1 cell suspensions were prepared as previously described. A 5 mL cell suspension in 10 mM MgSO₄ was subjected to 60°C overnight. A 0.5 mL cell suspension was suspended in 70% isopropanol and kept at 4°C overnight. A 0.5 mL cell suspension was diluted in 20 mL of 70% isopropanol and incubated at 20°C for one hour. A 0.5 mL cell suspension was diluted in ultrapure water to which 90 µL of sodium hypochloride (NaOCl) were added and incubated at 25°C for one hour on a horizontal shaker. The cell suspension in 10 mM MgSO₄ was used as a negative control. A volume of 200 µL of each treated cell suspension was transferred in duplicate to a 96-well plate and serially diluted (10-fold). The plate was incubated for 30 minutes at 37°C and subsequently 2 µL of SYBR Green I (SYBR-I) 100x diluted in DMSO and 2 µL of 0.3 mM or 1.5 mM propidium iodide (PI) were added (Appendix 3 - Extraction of genomic DNA using the CTAB-Lysozyme method). Each well was analyzed with flow cytometry (BD Accuri™ C6 Flow Cytometer). The outline of the samples in the 96 well plate is presented in Table 3.2. Evian water was used as a blank for cell enumeration by flow cytometry.
3.4.2 Flow cytometry method application

Live and dead cells of MSH1 were enumerated using the already mentioned device, BD Accuri™ C6 Flow Cytometer. Cell suspensions taken during experiments were diluted aiming at 1000 events/sec using sterile 10 mM MgSO\textsubscript{4} and analyzed in 96-well plates. Evian water was used as a blank. The 96 well plate was incubated at 37°C for 30 min. Afterwards, 2 µL of SYBR I 100x diluted in DMSO (from Thermo Scientific) and also 2 µL of 0.3 mM PI stain were added to each well having a final volume of 200 µL. The 96 well plate was again incubated at 37°C (± 2°C) for 10 min. Start up and calibration of the BD Accuri ™ C6 Flow Cytometer were performed according to manufacturer guidelines. All samples were analyzed at fast pre-set flow rate of 66 µL per min with a run limit of 10 µL collecting only events above 10000 in the forward scatter channel (FSC). SYBR I signal was detected in the FL1 channel (Excitation: 488 nm, emission: 533/30 nm) and the PI signal in the FL3 channel (excitation 488 nm, emission > 670 nm). Live (only SYBR I stained) and dead (SYBR I and PI stained) cells were gates in a scatter plot (FL3 vs. FL1).

Wash steps and agitation of the 96 well plate was performed regularly to avoid carry over from one well to the other and prevent cell precipitation.

3.5 Membranes

Several types of membranes were tested and can be roughly categorized into microfilters (MF), ultrafilters (UF), nanofilters (NF) and reverse osmosis (RO) membranes (
Table 3.3). Membranes were cut from sheets (305 x 305 mm or 1016 x 305 mm) into discs of 49 mm for the use in the stirred cell (Figure 3.1) and 18 mm for the 16-single feed spider experiments (Figure 3.3 and Figure 3.4) using the manual presser Berg&Schmidt with the adaptor size A.T20.
Table 3.3 - Tested membranes and respective specifications and properties

<table>
<thead>
<tr>
<th>Membranes</th>
<th>Pore Size</th>
<th>Polymer</th>
<th>Flux (GFD/psi)</th>
<th>Rejection</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF Supor®</td>
<td>0.2 µm</td>
<td>PES&lt;sup&gt;2&lt;/sup&gt;</td>
<td>N.A.&lt;sup&gt;3&lt;/sup&gt;</td>
<td>N.A.&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Pall Corporation</td>
</tr>
<tr>
<td>MF V0.2</td>
<td>0.22 µm</td>
<td>PVDF&lt;sup&gt;4&lt;/sup&gt;</td>
<td>245-280/20</td>
<td>N.A.&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Synder™</td>
</tr>
<tr>
<td>MF TM10</td>
<td>0.22 µm</td>
<td>PVDF&lt;sup&gt;4&lt;/sup&gt;</td>
<td>50/10</td>
<td>N.A.&lt;sup&gt;3&lt;/sup&gt;</td>
<td>TriSep™</td>
</tr>
<tr>
<td>UF XT</td>
<td>1000 Da</td>
<td>PES&lt;sup&gt;2&lt;/sup&gt;</td>
<td>75-80/50</td>
<td>N.A.&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Synder™</td>
</tr>
<tr>
<td>NF SB90</td>
<td>≈150 Da</td>
<td>Cellulose Acetate Blend</td>
<td>30/225</td>
<td>97.0% MgSO&lt;sub&gt;4&lt;/sub&gt; (85% NaCl)</td>
<td>TriSep™</td>
</tr>
<tr>
<td>NF 90</td>
<td>= 200 to 400 Da</td>
<td>Polyamide</td>
<td>46-60/130</td>
<td>99.0% MgSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Dow Filmtec™</td>
</tr>
<tr>
<td>RO SB50</td>
<td>&lt; 100 Da</td>
<td>Cellulose Acetate Blend</td>
<td>30/420</td>
<td>95.0% NaCl (99.0% MgSO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>TriSep™</td>
</tr>
<tr>
<td>RO X201</td>
<td>&lt; 100 Da</td>
<td>Polyamide-urea</td>
<td>30/225</td>
<td>99.5% NaCl</td>
<td>TriSep™</td>
</tr>
</tbody>
</table>

3.6 Membrane filtration experiments

3.6.1 MSH1 cell retention by MF membranes

MSH1 cell retention (setup 1) at different cell densities was assessed by testing MF membranes using a stirred cell device. The stirred cell HP4750 from Sterlitech is a high-pressure (max 69 bar) and chemically resistant device that performs a wide variety of membrane separations. The structure made of stainless steel (316L) with good chemical resistance, is composed of three main components: a cylindrical body with removable top and bottom, a stir bar assembly and a porous stainless steel membrane support disk on which ø 49 mm membrane discs can be used (Figure 3.1). For filtration, pressure (max 8 bar, pressurized air) was applied to a volume of max 300 mL. Operation and maintenance of the stirred cell is described in the Assembly and Operation manual (Appendix 5 - HP4750 Stirred Cell Assembly and Operation Manual).

---

<sup>2</sup> Polyethersulfone  
<sup>3</sup> N.A. – Non available  
<sup>4</sup> Polyvinylidene-difluoride
In a first experiment, the MF membrane Supor was tested for the retention of MSH1 cells. MSH1 cell suspensions were prepared as previously described and diluted in AGW medium at $10^6$, $10^7$, $10^8$ and $10^9$ cells/mL. In a second experiment, MF membranes V0.2 and TM10 were tested for the retention of $10^6$ and $10^9$ cell/mL. For each experiment, glass reaction tubes (18 mL) for permeate collection were washed and dried at 64ºC. For each filtration session, filtering around 100 mL and stirred at 600 rpm, the feed before and after filtration was sampled in duplicate (10 mL) and five effluent samples (20 mL) were taken during filtration. Sample volumes were determined by weighing tubes before and after sample collection. Permeability for each membrane was calculated as previously described. Subsamples of 200 µL were collected and transferred to a 96 well plate for cell enumeration with flow cytometric analysis. After filtration, each membrane disc was collected in a sterile tube and stored at -20°C until DNA extraction for real-time qPCR.

### 3.6.2 Continuous bioaugmented membrane reactors for BAM removal from groundwater

The previously described stirred cell (section 3.6.1) was placed on a magnetic stirrer agitating reactor volume at 250 rpm. Sterile AGW medium (10 L SCHOTT bottles) amended with the specified BAM concentration (Table 3.4) was used as influent and was pumped using a 205S peristaltic pump (Watson-Marlow) through the specified membrane disc of ø 47 mm and flow rate (Table 3.4). The setup was connected using Viton tubing as depicted in Figure 3.2. When a stable flux was achieved, MBRs were inoculated with MSH1-GFP pre-grown in MS with 200 mg/L BAM according to the specified cell concentration (Table 3.4).
Figure 3.2 - Long-term filtration setup: (1) stirred cell, (2) pump, (3) feed reservoir and (4) waste reservoir.

Table 3.4 - Overview of conditions tested in a continuous bioaugmented MBR setup

<table>
<thead>
<tr>
<th>BAM concentration (µg/L)</th>
<th>Membrane</th>
<th>Cell concentration (cells/mL)</th>
<th>Experiment duration (days)</th>
<th>Flow (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>MF Supor</td>
<td>5×10⁹</td>
<td>7</td>
<td>1.2</td>
</tr>
<tr>
<td>10</td>
<td>MF Supor</td>
<td>7×10⁷</td>
<td>11</td>
<td>1.1</td>
</tr>
<tr>
<td>10</td>
<td>MF V0.2</td>
<td>1×10⁸</td>
<td>12</td>
<td>1.3</td>
</tr>
</tbody>
</table>

The inoculum was sampled in triplicate for cell enumeration with flow cytometry. Immediately after inoculation and at regular time intervals, permeate samples were collected in triplicate during intervals of 5 minutes (5 mL). Flux and permeability was calculated as previously described. Experiments had a duration of 7-12 days as specified in Table 3.4. At the end of the experiment, effluent samples were collected in triplicate, pumping was stopped and the reactor volume in the MBR was sampled in triplicate. Subsamples of 1 mL were collected in microcentrifuge tubes and 5 µL of 37% HCl solution was added to avoid bacterial growth. Samples were centrifuged at 10 000 x g for 5 min to remove particulate matter. A volume of 900 µL was transferred to clear glass vials and stored at -20°C for UPLC or UPLC-MS/MS analysis. The MBR was disassembled and the membrane was immediately stored at -20°C for DNA extraction for real time qPCR.

In the last MBR experiment, 100 µL of the last effluent samples were collected and transferred to a 96 well plate, under sterile conditions. A dilution series was made with 10 mM MgSO₄ and 10 µL of each dilution were plated in triplicate on R₂A plates amended with 50 mg/L kanamycin to selectively grow MSH1-GFP. Plates were incubated for 4 days at 25°C and colony forming units were determined.

3.6.3 BAM and DCBA retention test by UF, NF and RO filtration
A 16-single feed and 8-single feed spider were used to test the retention of BAM and DCBA by different membranes. The 16-single feed spider is composed of 16 positions for 18 mm membrane discs supported by porous stainless steel discs and one common feed reservoir with a Teflon-coated magnetic stirrer bar (Figure 3.3 and Figure 3.4) (Vankelecom, 2010). Operation and maintenance of the high throughput module (HT-module) is described in Appendix 5 - HP4750 Stirred Cell Assembly and Operation Manual (Vandezande, et al., 2005) (Belgium Patente Nº WO2006002500 A1, 2006).

AGW medium was amended with either 1, 10 µg/L BAM or with 1000 µg/L DCBA. According to the maximum capacity of the 16-single feed spider, 600 mL of feed solution was applied each time. In case of 1 and 10 µg/L BAM, filtration was performed twice in separate experiments to correct for erroneous feed sampling in the first experiment. The membranes UF XT were tested in duplicate and the membranes NF 90, NF SB90, RO SB50 and RO X201 tested in triplicates. For 1000 µg/L BAM, the 8-single feed spider was used to which 400 mL was applied. The tested membranes were the NF 90, NF SB90, RO SB50 and RO X201 in duplicate. Pressure was applied to the feed reservoir using nitrogen at 60 bar. During filtration, on average 10 feed samples (1 to 5 mL) were collected at regular time intervals and for each filter three effluent samples (1 to 5 mL) were collected at variable time intervals when pressure was stable. Sample volume was determined by weighing vials before and after sample collection. Permeability for each membrane was calculated by the following equation (1).

\[
\text{Permeability (L/m}^2\text{h.bar}) = \frac{\text{Flux (L/m}^2\text{h)}}{\text{Pressure (bar)}}
\]  

Subsamples of 1 mL were collected in microcentrifuge tubes and 5 µL of 37% HCl solution was added to avoid bacterial growth. Samples were centrifuged at 10 000 x g for 5 minutes to remove particulate matter. A volume of 900 µL was transferred to clear glass vials and stored at -20°C, for UPLC or UPLC-MS/MS analysis.
3.6.4 Permeability of UF, NF and RO membranes in the filtration of MSH1 cell suspensions

MSH1 cell suspensions were prepared as previously described and diluted in AGW medium. The effect of MSH1 cells on the permeability of membranes was assessed for UF XT, NF SB90, NF 90 in triplicate and for the RO SB50 and RO X201 in duplicate. A volume of 600 mL of a MSH1 cell suspension of $10^6$ cells/mL in AGW was applied to the 16-single feed spider. During filtration, on average 10 feed samples (10 mL) were collected at regular time intervals and for each filter three effluent samples (1 to 5 mL) were collected at variable time intervals when pressure was stable. Sample volume was determined by weighing vials before and after sample collection. Permeability for each membrane was calculated by equation (1).

3.7 Analytical methods

3.7.1 Ultra-Performance Liquid Chromatography (UPLC)

Samples with BAM and DCBA concentration below 1 µg/L were analyzed by the UPLC-MS/MS and above 1 µg/L with UHPLC-UV/VIS. UPLC-MS/MS analysis was performed as described by Sekhar et al. (submitted) (Appendix 1 - Ultra Performance Liquid Chromatography). UHPLC-UV/VIS analysis of samples was done with a reversed phase VisionHT C18 column HighLoad (100 mm x 2 mm, 1.5 µm). Samples of 10 µL were injected and were eluted using 85% H$_3$PO$_4$ at pH 2.5 and 15% acetonitrile. BAM and DCBA were detected at 210 nm and were identified based on their respective retention time (3.5 min and 5.3 min respectively). Concentrations were determined based on standard series of BAM and DCBA correlating area under the peak (mV x min) with concentration (µg/L).

3.7.2 DNA (deoxyribonucleic acid) extraction

DNA extraction from membranes followed the CTAB-Lysozyme method (Larsen, et al., 2007). Frozen membranes were used in as a whole or cut in pieces and suspended in 450 µL GTE solution inside 2 mL cryotubes to which 100 mg of sterilized glass beads (1 mm) were added. The mixture was mechanically disrupted (3 x 5000 rpm during 45 sec and pause of 30 sec between the 3 cycles) using the ribulizer precellys 24 (Bertin technologies). Afterwards, a volume of 50 µL 10 mg/mL lysozyme solution was added and incubated overnight. A volume of 150 µL of 2:1 SDS:proteinase K (10 mg/mL) solution was added and incubated on the Bioer Mixing Block MB-102 at 55°C for 40 min. The supernatans were transferred to sterilized 2 mL nuclease free microcentrifuge tubes to which 200 µL of 5 M NaCl were added and gently mixed. A volume of 160 µL CTAB solution (preheated at 64°C) was added and a 10-minute incubation followed at 65°C. Approximately 1 mL of 24:1 chloroform/isoamyl alcohol was added and vigorously shaken and centrifuged at 13000 x g during 5 minutes. The resulting water layer at the top (900 µL) was transferred to sterilized 2 mL nuclease free microcentrifuge tube and 900 µL of 24:1 chloroform/isoamyl alcohol were added and centrifuged at 13000 x g during 5 minutes. The water layer on top was transferred to a sterilized 1.5 mL Eppendorf tube to which 560 µL of 70%
isopropanol was added and mixed by inversion until the DNA precipitated. After a 5-minute incubation at room temperature, the DNA was precipitated by centrifugation at $13000 \times g$ for 10 minutes. The supernatant was decanted leaving the DNA pellet in the tube and was subsequently washed with 1 mL of 70% ethanol and centrifuged at $13000 \times g$ during 5 minutes. The supernatant was decanted and the DNA pellet air-dried for 15 minutes. DNA was dissolved in 50 µL of a 25 mM Tris solution at pH=8,5 and stored at -20ºC (Appendix 3).

3.7.3 Real time quantitative PCR

The catabolic genes $bbdA$ and $bbdB$ in MSH1 were used to quantify the BAM-degrading and BAM-mineralizing cells in filter samples using real time qPCR. The analysis was performed in a RotorGene (Corbett research) as described by (Raes, 2015).
4. Results and Discussion

4.1 Optimization of a flow cytometric method for live-dead cell enumeration of *Aminobacter* sp. MSH1 culture

The experimental work performed in this thesis required and relied strongly on *Aminobacter* sp. MSH1 cell enumeration and viability assessment during filtration sessions. Hence, a flow cytometric approach was developed for rapid and accurate enumeration of live and dead MSH1 cells in MBR content and MBR effluent samples. Live and dead cells were separately enumerated by selective binding of two intercalating nucleic acid stains SYBR Green I and propidium iodide (PI). SYBR Green I binds to DNA of both live and dead cells, while PI binds only to DNA of dead cells since this molecule can only permeate damaged cell membranes.

In order to calibrate the flow cytometer for acquisition of live and dead cell enumeration, both cell population types needed to be generated. As such, live cell cultures were exposed to stresses and oxidizing agents to generate dead cells (McDonnell & Russell, 1999) (A.D.Russell, 2003). Since *Aminobacter* sp. is a Gram-negative genus, the outer membrane acts as a barrier limiting the entry of many compounds due to its hydrophobic cell surface. The bacterial cells were subjected to a heat shock at 60°C overnight, to 70% isopropyl alcohol for one hour and overnight, and to NaOCl.

Live/dead staining with low (0.3 mM) and high (1.5 mM) PI concentration of the cells subjected to the different treatments was analyzed by flow cytometry. Solutions with different PI concentrations were used to verify whether a higher PI concentration stained cells more efficiently, but also whether this wouldn’t influence the state of the cells itself, as PI is toxic to bacterial cells. Channel FL1 (excitation: 488 nm, emission: 533/30 nm) and FL3 (excitation: 488 nm, emission > 670 nm) were used for the detection of SYBR green I and PI, respectively.

The results on both staining concentrations SYBR I + 0.3 mM PI and SYBR I + 1.5 mM PI, for all the treatments applied to the cells diluted 1:100 are represented in Figure 4.1, and the differences are described as follows. Nomination of Figure 4.1 was given by default, for a staining with 0.3 mM PI (images on the left column: A03 to E03). Images on the right column were named from A09 to E09, for a staining with 1.5 mM PI. Thus, each row represents two different samples containing MSH1 cells, which were both subjected to the same treatment. Also noticeable in Figure 4.1 are the fluorescence channels FL1 and FL3, both used on the cells signal detection.

Starting with samples A03 and A09, these were used as a live-cell control for the rest of the treatments applied. The cells were suspended in 10 mM MgSO\(_4\), where they were growing in a stable and nutrient-abundant environment, having assured that an exponential growing culture was harvested and measured. It was then expected that the cluster resulting from this analysis corresponded to an alive bacterial cell cluster, from which the P6 gating was created.

Since the *Aminobacter* sp. MSH1 is a soil bacteria, a treatment with 60°C was admitted to be very likely harmful for live cells. When analyzing the treatment where cells were incubated overnight at 60°C, the
resulting cluster was identified as a cluster of dead but intact cells, from which the P7 gating was created. All following treatments were analyzed based on this treatment where coherent results were seen for all. Cells detected by the flow cytometer were positioned on the P7 gate area, confirming the intended aim in obtaining dead cells. When stained with 1.5 mM PI, the cell cluster was equally positioned on the P7 gate, reinforcing the assumption.

The signal's intensity of the detected dead cells by the fluorescence channels FL1 and FL3, was becoming lower, indicating a more disintegrating effect of the cells in each treatment. Based on previous reports, (Barbesti, et al., 2000) the use of higher concentrations of PI stain on cells, showed to result in a lower cell signal, which was yet admitted as these cells were still alive (metabolically active) and that such observations were due to an incipient membrane damage impeding the exclusion of the propidium dye. Such behavior was equally noticed in the current study.

This effect was more clearly noticed on the NaOCl treatment, which resulted in a lower amount of dead cells than any other treatment as well as a shifting that led to the creation of the new gating, P8. Cells submitted to this oxidizing agent did not effectively stain as a result of NaOCl destroying effect, leaving few DNA molecules to bind with the stain. Thereby, the heating treatment provided a sharp dead cell identification by its wider and well defined cluster. Followed by the isopropanol treatments, the cluster despite being thinner, also allowed a clear distinction of the dead cells cluster.

In each image in Figure 4.1, a high density of ungated black dots is noticeable in the lower left corner, being considered as noise and having no contribution on live-dead cell enumeration.
Figure 4.1 - Comparison between diluted cells 1:100 subjected to different treatments, stained with SYBR-I and PI 0.3 mM on the left column, and SYBR-I and PI 1.5 mM on the right column. (A03 and A09) cells in MgSO₄, (B03 and B09) cells in MgSO₄ incubated at 60°C overnight, (C03 and C09) cells in 70% isopropanol, (D03 and D09) cells in 70% isopropanol left overnight and (E03 and E09) cells in NaOCl. P6 gating quantifies living cells, P7 gating quantifies dead cells and P8 gating quantifies the amount of dead cells for the NaOCl treatment (percentage values in the figure). Fluorescence channels FL1 and FL3 represented, were used for signal detection. Ungated dots were considered background noise.

Based on this study, a template was created for further MSH1 cell analysis in the flow cytometer, where gating for noise and live/dead cells are represented in Figure 4.2.
Figure 4.2 - Channels FL1 and FL3 for the signal detection of live (gated in green) and dead (gated in red) cells used as a template on further experiments. Noise gating in purple.

Once the gates were determined and settled for the identification of dead and live cells, it was of great importance to assess the effects of the staining agents on cell’s viability. Aiming to determine if a higher amount of dead cells would be detected when a higher concentration of PI stain was used, two different PI concentrations, namely 0.3 mM and 1.5 mM, were applied to the different sets of cells. This results are represented in Table 4.1.

Table 4.1 - Cell viability study based on several applied cell disruption treatments. The amount of cells/µL is represented as a function of cell dilution. Graphs on the left column represent cells that were stained with a 0.3 mM PI solution and those on the right column, represent cells that were stained with a 1.5 mM PI solution.
Treatments

<table>
<thead>
<tr>
<th>0.3 mM PI</th>
<th>1.5 mM PI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Live (with MgSO₄)</strong></td>
<td><strong>Live (with MgSO₄)</strong></td>
</tr>
<tr>
<td>Dead (60°C+MgSO₄)</td>
<td>Dead (60°C+MgSO₄)</td>
</tr>
<tr>
<td>Dead (with fresh isopropanol)</td>
<td>Dead (with fresh isopropanol)</td>
</tr>
</tbody>
</table>
In each graph, the amount of cells from 1:10 to 1:10000 diluted samples were represented in stack columns where live cells were represented under the dead cells, for the same treatment applied with 0.3 mM PI on the left, or with 1.5 mM PI on the right.

Comparing the amount of dead cells for each concentration of PI stain applied, it is seen that all cell disruption treatments, except for the isopropanol applied overnight, registered higher amount of dead cells/µL, when the 1.5 mM PI stain was used.

A higher PI concentration resulted in a higher proportion of dead cells, possibly due to PI toxicity. On a 0.3 mM concentration the lower number of events suggest that despite the number of cells in the dilution series, the dye is only staining previously dead cells, and not interfering with them by causing their death. Given the fact that 1.5 mM PI would be too toxic for bacterial cells influencing the accuracy of the measurement of dead cells in the analyzed samples, a concentration of 0.3 mM PI was preferred and used on all onwards performed experiments.
4.2 Effect of cell density on the retention of *Aminobacter* sp. MSH1 by MF membranes

In the previously described setup 1 strategy for removing BAM from drinking water, *Aminobacter* sp. MSH1 cells should be efficiently retained by the used membrane without affecting its permeability. As such, MF membranes were chosen to study MSH1 cell retention. Three different microfiltration membranes were tested, namely: MF Supor, MF V0.2 and MF TM10. The MF Supor and MF V0.2 membranes are composed of PES and PVDF polymers, respectively, having similar permeability. The MF TM10 membrane has a lower permeability (125 L/hm²bar, Table 3.3) and is composed of PVDF polymer. Both the effect of MSH1 cell density on membrane permeability and on cell retention efficiency were assessed. Different cell densities $10^6$ and $10^9$ cells/mL were tested aiming to assess whether the retention capacity of the membrane was affected by these different concentrations.

Clear differences were observed in terms of the time needed to filter 100 mL of AGW with a certain MSH1 cell density. For $10^6$ cells/mL, the time needed to filter 100 mL was 46.7±6.1 sec, 42.5±3.5 sec and 5.9±7.1 min for MF Supor, MF V0.2 and MF TM10, respectively. For $10^9$ cells/mL, the time needed to filter 100 mL was 19.7±1 min, 21.1±3.3 min and 53.9±14.1 min for MF Supor, MF V0.2 and MF TM10, respectively. This led to a difference in permeability for the different tested MF membranes (Figure 4.3). For a $10^6$ cells/mL concentration, the average permeability was higher for MF Supor (3871.9±578 L/m²hbar) and MF V0.2 (4002.6±547 L/m²hbar) compared to the MF TM10 membrane (45.7 ±7.2 L/m²hbar). This observation can be compared with the average permeability for Table 3.3 in section 3.5. Filtering a $10^9$ cell/mL suspension led to a decrease in permeability, namely 72.4±4.2 L/m²hbar, 47±7.1 L/m²hbar and 10.7±2.4 L/m²hbar, for MF Supor, MF V0.2 and MF TM10, respectively (Figure 4.3).

Starting by the evaluation of the permeability, it was intended to understand how the retention of the cells would be affected by this parameter. According to the permeability on Table 3.3 membranes MF V0.2 and MF TM10 are characterized by a permeability of 349 L/m²hbar and 125 L/m²hbar, respectively. MF Supor has no permeability specification provided by the manufacturer. In the experiment using a concentration of $10^6$ cells/mL, while MF TM10 permeability was 3x lower than the specified, MF V0.2
registered the opposite behavior, since it was noticed a permeability 11x higher than the specified. However, with the concentration of $10^9$ cells/mL, a decrease of 12x was registered for MF TM10 and of 7x for MF V0.2. It is then noticeable that the cells had a significant effect on membrane’s permeability.

The amount of cells present in the permeate when filtering a volume of 100 mL with the membranes MF Supor, MF V0.2 and MF TM10 was determined and the results are shown in Figure 4.4 for a cell suspension of $10^6$ cells/mL and in Figure 4.5 for a cell suspension of $10^9$ cells/mL.

![Figure 4.4](image1.png)
**Figure 4.4** - Amount of cells lost in the permeate stream during the filtration of 100 mL of AGW with $10^6$ cells/mL using the membranes MF Supor (green triangles), MF V0.2 (blue dots) and MF TM10 (red squares).

![Figure 4.5](image2.png)
**Figure 4.5** - Amount of cells lost in the permeate stream during the filtration of 100 mL of AGW with $10^9$ cells/mL using the membranes MF Supor (green triangles), MF V0.2 (blue dots) and MF TM10 (red squares).

The amount of cell loss in the permeate stream was highest in the first 20 mL filtered, but decreased progressively to stabilize to a steady state cell loss. However, clear differences were observed between each tested MF membrane. When filtering $10^6$ cells/mL, initial cells loss differed between MF membranes ranking from high to low: MF TM10, MF Supor and MF V0.2 with an average of $120.8\pm24.9$ cells/µL, $64.7\pm14.7$ cells/µL and $47.5\pm11.0$ cells/µL, respectively, for the first 20 mL filtered. Steady state
cell loss corresponded to 7.5±3.3 cells/µL, 4.0±2.3 cells/µL and 4.0±1.7 cells/µL respectively. When filtering 10^9 cells/mL, initial cells loss differed between MF membranes ranking from high to low: MF TM10, MF V0.2 and MF Supor with an average cell loss of 5258.2±2541.5 cells/µL, 2717.4±361.6 cells/µL and 497.9±296.5 cells/µL, respectively, for the first 20 mL filtered. Steady state cell loss corresponded to 320.2±119.7 cells/µL, 281.4±441.9 cells/µL and 18.6±12.3 cells/µL for MF TM10, MF V0.2 and MF Supor respectively.

Filtrations of AGW with 10^6 cells/mL occurred at a pressure of 1 bar for the membranes MF Supor and MF V0.2, while filtration with MF TM10 membrane occurred at 5 bar. For AGW with 10^9 cells/mL a pressure of 2 bar, 3 bar and 5 bar was applied for MF Supor, MF V0.2 and MF TM10, respectively.

Based on the cells lost in the permeate, total cell loss was estimated for all membranes for the two tested cell densities. At the low cell density, MF Supor, MF V0.2 and MF TM10 achieved 98.9 ± 0.0 %, 98.9 ± 0.0 % and 99.3 ± 3.9 % retention, respectively. At high cell density, MF Supor, MF V0.2 and MF TM10 achieved a retention of 99.9 ± 0.0 %, 99.8 ± 0.0 % and 100.0 ± 0.0 %, respectively. For the estimation of the retention capacity of the membrane, for the steady state, the calculation was based on the amount of lost cells/µL for the last permeate sample. The steady state retention at the low cell density, for MF Supor, MF V0.2 and MF TM10 was 99.9 ± 0.0 %, 99.8 ± 0.0 % and 99.7 ± 0.0 %, respectively. At high cell density, a complete retention was estimated for all membranes, since it was obtained an approximate retention of 100.0 ± 0.0 %, for all membranes.

To determine whether MSH1 cells remain in the reactor volume instead of being embedded in or attached onto the MF membrane, the amount of cells in the membrane was calculated based on the cell enumeration in the permeate and MBR. The mass balance was as follows: Cells MBR Initial = Cells permeate + Cells membrane + Cells MBR Final. Mass balance calculations are shown in Table 4.2. The calculated fraction of cells in the membrane was compared to the cells in the membrane enumerated by qPCR (Table 4.2).
Table 4.2 - Mass balance of the total cell amount of the $10^6$ cells/mL and $10^9$ cells/mL cell retention experiment for the microfilters MF Supor, MF V0.2 and MF TM10

<table>
<thead>
<tr>
<th>Cells MBR initial</th>
<th>Cells permeate</th>
<th>Cells MBR final</th>
<th>Calculated cells membrane</th>
<th>Measured cell number on membrane</th>
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<tr>
<td>Low cell density test ($10^6$ cells/mL)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>$9.3 \times 10^7$</td>
<td>$1.9 \times 10^8$</td>
</tr>
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<td>$1.6 \times 10^8$</td>
<td>$1.4 \times 10^8$</td>
</tr>
<tr>
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</tr>
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<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>MF TM10</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
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<td>High cell density test ($10^9$ cells/mL)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MF Supor</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>$3.5 \times 10^{9}$</td>
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<tr>
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<tr>
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<td>$5.5 \times 10^{10}$</td>
<td>$2.3 \times 10^{10}$</td>
</tr>
<tr>
<td>MF V0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter 1</td>
<td>$7.9 \times 10^{10}$</td>
<td>$1.1 \times 10^8$</td>
<td>$8.2 \times 10^{10}$</td>
<td>$2.6 \times 10^{9}$</td>
</tr>
<tr>
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<td>$6.0 \times 10^{10}$</td>
<td>$2.8 \times 10^{9}$</td>
</tr>
<tr>
<td>Filter 3</td>
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<td>$6.1 \times 10^7$</td>
<td>$4.8 \times 10^{10}$</td>
<td>$1.6 \times 10^{10}$</td>
</tr>
<tr>
<td>MF TM10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>$6.2 \times 10^{10}$</td>
<td>$6.9 \times 10^7$</td>
<td>$5.0 \times 10^{10}$</td>
<td>$1.2 \times 10^{10}$</td>
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<tr>
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<td>$1.3 \times 10^8$</td>
<td>$5.4 \times 10^{10}$</td>
<td>$2.2 \times 10^{10}$</td>
</tr>
<tr>
<td>Filter 3</td>
<td>$8.3 \times 10^{10}$</td>
<td>$1.9 \times 10^9$</td>
<td>$4.4 \times 10^{10}$</td>
<td>$3.8 \times 10^{10}$</td>
</tr>
</tbody>
</table>
From the previous balance, it is possible to notice that for a lower cell density, a significant part of the introduced cells was essentially in suspension in the MBR when using the MF Supor, MF V0.2 and MF TM10 membranes, while the remaining amount of cells was retained by the membrane. These observations result from a comparison with the enumeration by qPCR analysis, where a difference of 10-fold or 100-fold higher amount of cells is noticed between the measured and the calculated amount expected. At a higher cell density, practically all cells were evenly distributed between the remaining concentrated volume of the feed and on the membrane, being however in some cases, in lower amount on the membrane (with 10-fold difference).

The mass balance of the cells is then indicative of the cell distribution throughout the filtration, namely if cells have a sinking behavior or remain in suspension during filtration. Negative values indicate that no cells were expected to be found in the membrane.

Also of importance was the live/dead cell enumeration, performed to determine whether permeation through the membrane was mainly done by live or dead cells. For a cell density of $10^6$ cells/mL, initial dead cell amount of 6.3±0.0%, 1.4±0.0% and 3.6±0.0% was determined for MF Supor, MF V0.2 and MF TM10 respectively, while the dead cell amount from the total lost in permeate was 22.9±20.6%, 33.3±23.6% and 14.9±11.0%, for MF Supor, MF V0.2 and MF TM10, respectively. For a cell density of $10^9$ cells/mL, initial dead cell amount of 1.1±0.0%, 1.0±0.0% and 0.1±0.0% was measured for MF Supor, MF V0.2 and MF TM10, respectively, while the amount of dead cells from the total cell loss in the permeate stream was 16.2±26.4% 4.5±2.2% and 4.3±1.7% for MF Supor, MF V0.2 and MF TM10, respectively. It is then possible to verify that a higher amount of dead cells was found in the permeate, than in the feed. No significant difference was noted when overlooking the ratio of live/dead cells, for both densities.

Figure 4.6 schematically represents the proportion of all living and dead cells/µL throughout the filtrations using the membranes MF Supor, MF V0.2 and MF TM10 for both lower and high cell densities, being noticeable a higher density of green dots in each plot, indicative of a higher amount of living cells than dead cells.

![Figure 4.6](image_url)

Figure 4.6 - On the left (a), cell densities (in cells/µL) in the permeate for filtrations of AGW with a cell density of $10^6$ cells/mL using the membranes MF Supor, MF V0.2 and MF TM10 with live cells in green dots and dead cells in red triangles. On the right (b) the same is represented but for AGW with a cell density of $10^9$ cells/mL.
Despite the favorable low proportion of dead cells detected, this short-term test does not reproduce the working time of a direct flow system combining cells and membranes, meaning that the follow-up of live/dead changes and metabolic activity should be done for a longer period of time.

It was assessed what would be the extent of cells retained, namely, if higher cell retention would correspond to a lower membrane permeability, leading to a possible correlation with fouling phenomena.

From all the microfilters tested, the MF TM10 had the most accentuate loss of cells in the permeate, despite having also the lowest permeability, which confirmed the inexistence of fouling.

These results suggest $10^6$ cells/mL as the most suitable cell concentration, for an efficient cell retention, since similar retention levels were obtained when compared to a concentration of $10^9$ cells/mL, but the higher permeability on both MF Supor and MF V0.2 membranes presents an advantage on the use of such cell concentration.

On the other hand, it was noticed a high cell loss on the first 20 mL of permeate collected, which was significantly different from the steady state loss. It was then determined the amount of volume of media needed to filtrate, in order to have the same cell loss of the first permeate. Both experiments were compared. Confirming once again that for a $10^6$ cells/mL concentration, an amount of volume 1294 mL, 950 mL, 2416 mL for MF Supor, MF V0.2 and MF TM10 is needed to equalize the amount of cells lost on the first 20 mL permeate. This, when compared for a $10^9$ cells/mL concentration resulted in a loss of 2142 mL, 773 mL, 1497 mL for the respective membranes. Analyzing both groups of values it is possible to recognize the same order of magnitude indicating that higher cell densities would need an identical amount of volume to lose the same quantity as the first permeate, during a longer period of time. This translates the advantage of using of a higher cell density, since a 1000-fold higher concentration ($10^9$ cells/mL in comparison with $10^6$ cells/mL) registered less cell loss.

The exponential behavior of the cell loss in permeate, seen in Figure 4.4 and Figure 4.5, was of great interest to understand. A simplified explanation is based on a physical phenomenon where the sudden change in pressure possibly takes the cells to immediately reach the membrane, that is dry at the start of the experiment and thus, with a higher chance of the pores being loose and open, letting the first set of cells coming through. However, a more abstract assumption can explain this evidence. Looking at the polymer constitution of the MF V0.2 and MF TM10 membranes, both are constituted by PVDF (polyvinylidene-difluoride) but exhibited complete opposite retention behaviors, excluding the hypothesis of a possible cell-polymer interaction. Despite cell concentrations, higher permeabilities led to the lowest amount of cell loss for MF V0.2 membrane. But the opposite was noticed for the MF TM10 membrane.

Intrinsic properties of MF TM10 membrane shows to be the reason for the lower retention, once pore-size characterization and membrane polymer composition were apparently not responsible for such behavior.

As indicated by the specifications, MF TM10 membrane possesses a working pressure of 0.7 bar. As informed by the manufacturer, membranes are tested against target compounds, therefore it was admitted that cells, were unlikely used as a target compound for this membrane. Therefore, such pressure specification was not admitted to accurately define a working pressure for this work. A pressure
of 5 bar was applied on this cell retention experiment, and by the results obtained (high cell loss), a possible intrinsic membrane damage might have occurred. Despite, the apparent integrity of the membrane in all tests did not raise suspicious of an excessive amount of pressure applied.

Based on the results obtained, MF V0.2 seemed a suitable membrane for a future application on the setup 2 direct flow system since time, retention, permeability, and concentration all showed to be appropriate. MF Supor is equally pointed to be suitable since overall results showed similarity with MF V0.2 for the same parameters.

4.3 Continuous bioaugmented membrane reactors for BAM removal from groundwater

To test Setup 1 for long term BAM removal, three membrane bioreactors were continuously fed with AGW amended with 0.2 µg/L and 10 µg/L BAM. Two MF membranes were tested: MF Supor and MF V0.2 based on the MSH1 cell retention tests from section 4.2.

The amount of cells needed for inoculation of the MBR was calculated using the suspended batch specific degradation rates, i.e., following Monod kinetics (Sekhar, et al., submitted).

4.3.1 1st long-term filtration

In the first long term MBR experiment, the microfilter Supor was used to retain MSH1 cells in the MBR and was fed with AGW amended with 0.2 µg/L BAM. Upon inoculation of $4.1 \times 10^9$ cells in the MBR, BAM removal was immediate under the norm of 0.1 µg/L (Figure 4.7) and remained under the norm for the whole duration of the experiment with an average removal efficiency of 74.9%. For all sampling moments BAM concentration kept under the established norm with an average of 0.03±0.02 µg/L.

Cells loss in the permeate is shown in Figure 4.8. Until around the third day of operation, a high amount of cell loss was registered with $2.2 \times 10^4 \pm 5.5 \times 10^3$ cells/mL and once the steady state was reached, the amount of cell loss dropped to $4.2 \times 10^3 \pm 1.9 \times 10^3$ cells/mL. The total cumulative amount of live and dead cell loss through time is shown in Figure 4.9. A total amount of $2.3 \times 10^8$ live cells was estimated, while the total amount of dead cells was $9.4 \times 10^7$. A total cell retention of 92.2% was achieved.
Figure 4.7 - Evolution of BAM concentration in the permeate throughout the extent of the first long term experiment using the MF Supor membrane. The dashed line corresponds to BAM concentration in the feed solution.

Figure 4.8 - Evolution of the concentration of living (blue) and dead (red) cells lost in the permeate throughout the experiment. Data points extrapolated by linear regression are marked as yellow triangles.

Figure 4.9 - Cumulative curve of the total amount of living (blue) and dead (red) cells lost in the permeate throughout the experiment. Data points extrapolated by linear regression are marked as yellow triangles.
Making a cell mass balance and tracking the evolution of BAM concentration in the permeates, allowed the study of the theoretical removal rates in comparison with the ones actually verified, i.e. the total amount of BAM that should have been degraded and the actual degraded amount at both the beginning and end of the experiment. The theoretical rate $1.3 \times 10^{-4}$ µg BAM/min was found to be 2x lower than the one measured at the beginning, of $2.5 \times 10^{-4}$ µg BAM/min. At the end of the experiment a lower degradation rate was measured of $2.14 \times 10^{-4}$ µg BAM/min although, based on the suspended cell BAM degradation kinetics and on the amount of cells in the MBR, $2.51 \times 10^{-3}$ µg BAM/min was expected (91.44% lower measured rate). The specific degradation rate, at the beginning was 1.7x lower with $6.1 \times 10^{-14}$ µg BAM/cell/min and by the end this even changed to 11.6x lower with $9.3 \times 10^{-15}$ µg BAM/cell/min compared to specific degradation rates in suspended batch ($1.1 \times 10^{-13}$ µg BAM/cell/min) (Sekhar, et al., submitted). Even though specific degradation rates for BAM were lower than expected, overall BAM removal rate was sufficient to attain effluent concentrations of BAM below the norm.

Analysis of the membrane allowed the determination of the amount of new cells generated during the 7 days of experiment by applying an overall mass balance of the cells inoculated, with the total amount lost in permeate and amount retained on the membrane.

Quantification of the total amount of cells present in the membranes, for all three long-term experiments, was done by quantification of \textit{bbdA} and \textit{bbdB}. The duplicates in qPCR were analyzed by the average of obtained \textit{bbdA} and \textit{bbdB} copies per filter. Intended to obtain the total amount of cells retained by the membrane, only \textit{bbdA} copies were accounted for mass balance purposes. Thus, the presence of \textit{bbdB} copies revealed to be of importance to conclude on the still existent BAM mineralization capacity by \textit{Aminobacter} sp. MSH1.

It was then possible to ‘keep track’ of the course of the cells since the moment they were introduced in the reactor until reaching the end of the experiment. With a total of $4.1 \times 10^9$ cells introduced, $3.2 \times 10^8$ lost and $2.3 \times 10^{10}$ accumulated, indicates a growth of $1.9 \times 10^{10}$ cells, i.e., 78.3% growth percentage.

4.3.2 2\textsuperscript{nd} long-term filtration

In the second-long term MBR experiment, the microfilter Supor was again used to retain MSH1 cells in the MBR and was fed with AGW amended with 10 µg/L BAM. Upon inoculation of $5.4 \times 10^{10}$ cells in the MBR, BAM removal was not enough to reach the norm of 0.1 µg/L (Figure 4.10) with an average removal efficiency of 99.1%. In opposition to the first long-term filtration, DCBA formation was detected on the seventh day of the experiment, with a concentration of 4.4±0.5 µg/L, remaining however BAM with a stable concentration. For all sampling moments the measured average BAM concentration was 0.09±0.07 µg/L, while the average DCBA concentration was 8.75±3.78 µg/L from the moment it was detected.

Cells loss in the permeate is shown in Figure 4.11. Up to the third day of operation, a higher amount of cell loss was registered with $6.7 \times 10^3 \pm 3.2 \times 10^3$ cells/mL and an overall steady state cell loss was not noticed.
The total cumulative amount of live and dead cell loss through time is shown in Figure 4.12. A total amount of $3.4 \times 10^8$ live cells was estimated, while of the total amount of dead cells was $1.5 \times 10^8$. A total cell retention of 99.1% was achieved.
Figure 4.10 - Evolution of BAM (blue dots) and DCBA (orange triangles) concentration in the permeate throughout the extent of the second long term experiment using the MF Supor membrane. The dashed line corresponds to BAM concentration in the feed solution.

Figure 4.11 - Evolution of the concentration of living (blue) and dead (red) cells lost in the permeate throughout the experiment. Data points extrapolated by linear regression are marked as yellow triangles.

Figure 4.12 - Cumulative curve of the total amount of living (blue) and dead (red) cells lost in the permeate throughout the experiment. Data points extrapolated by linear regression are marked as yellow triangles.
A cell mass balance and evolution on BAM concentration in the permeates allowed the study on theoretical removal rates in comparison with the ones actually verified, i.e. the total amount of BAM that should have been degraded and the actual degraded amount at both the beginning and end of the experiment. The theoretical rate $1.3 \times 10^{-2}$ µg BAM/min was found to be in accordance with the one measured at the beginning, of $1.07 \times 10^{-2}$ µg BAM/min. At the end of the experiment a lower degradation rate was measured of $1.08 \times 10^{-2}$ µg BAM/min although, based on suspended cell BAM degradation kinetics and the amount of cells in the MBR, $6.4 \times 10^{-1}$ µg BAM/min was expected (98.31% lower measured rate). The specific degradation rate, at the beginning was 25x lower with $2.0 \times 10^{-13}$ µg BAM/cell/min and by the end this even changed to 58x lower with $8.4 \times 10^{-14}$ µg BAM/cell/min compared to specific degradation rates in suspended batch ($4.9 \times 10^{-12}$ µg BAM/cell/min) (Sekhar, et al., submitted). The specific degradation rates for BAM were lower than expected and overall BAM removal rate was not sufficient to attain effluent concentrations of BAM below the norm, reason why the DCBA might have been formed.

Equally analyzed the membrane, the amount of new cells generated during the 11 days of experiment was determined by applying an overall mass balance of the cells inoculated, with the total amount lost in permeate and the amount retained on the membrane. It was then possible to ‘keep track’ of the course of the cells since the moment they were introduced on the reactor until reached the end of the experiment. With a total of $5.4 \times 10^{10}$ cells introduced, $4.9 \times 10^8$ lost and $1.3 \times 10^{11}$ accumulated, indicates a growth of $7.6 \times 10^{10}$ cells, i.e., 28.9% growth percentage.

### 4.3.3 3rd long-term filtration

In the third long term MBR experiment, the microfilter MF V0.2 was used to retain MSH1 cells in the MBR and was fed with AGW amended with 10 µg/L BAM. Upon inoculation of $6.0 \times 10^{10}$ cells in the MBR, BAM removal however never reached the norm of 0.1 µg/L (Figure 4.13) with an overall average removal efficiency of 58.7%. This removal efficiency suffered however some oscillations, specifically from the fourth day, where DCBA accumulation began. Before the seventh and eighth day, BAM removal efficiency of 94% was noticed, but after a quick stabilization on these days, BAM concentration significantly increased and the removal efficiency dropped to 71.4%.

DCBA formation was detected on the fourth day of the experiment at a concentration of $7.8 \pm 2.6$ µg/L. For all sampling moments the measured average BAM concentration was $1.7 \pm 1.5$ µg/L, while the average DCBA concentration was $7.7 \pm 1.0$ µg/L from the moment it was detected.

Cells loss in the permeate is shown in Figure 4.14. Until the first day a higher amount of cell loss was registered with $1.9 \times 10^4 \pm 8.8 \times 10^3$ cells/mL and a steady state cell loss of $5.4 \times 10^3 \pm 2.0 \times 10^3$ cells/mL. The total cumulative amount of live and dead cell loss through time is shown in Figure 4.15. A total amount of live cells was estimated of $6.1 \times 10^8$, while the total amount on dead cells was $1.9 \times 10^8$. A total cell retention of 98.5% was achieved.
Figure 4.13 - Evolution of BAM (blue dots) and DCBA (orange triangles) concentration in the permeate throughout the extent of the third long term experiment using the MF V0.2 membrane. The dashed line corresponds to BAM concentration in the feed solution.

Figure 4.14 - Evolution of the concentration of living (blue) and dead (red) cells lost in the permeate throughout the experiment. Data points extrapolated by linear regression are marked as yellow triangles.

Figure 4.15 - Cumulative curve of the total amount of living (blue) and dead (red) cells lost in the permeate throughout the experiment. Data points extrapolated by linear regression are marked as yellow triangles.
Applying again a cell mass balance and tracking the evolution of BAM concentration in the permeates, allowed the study on theoretical removal rates in comparison with the ones actually verified, i.e. the total amount of BAM that should have been degraded and the actual degraded amount at both the beginning and end of the experiment. The theoretical rate $1.29 \times 10^{-2} \, \mu g \, BAM/min$ was found to be in accordance with the one measured at the beginning, of $1.26 \times 10^{-2} \, \mu g \, BAM/min$. At the end of the experiment an equal degradation rate was measured of $1.27 \times 10^{-2} \, \mu g \, BAM/min$ although, based on suspended cell BAM degradation kinetics and the amount of cells in the MBR, $2.46 \times 10^{-2} \, \mu g \, BAM/min$ was expected ($48.28\%$ lower measured rate). The specific degradation rate, at the beginning was $21x$ lower with $2.35 \times 10^{-13} \, \mu g \, BAM/cell/min$ and by the end this even changed to $50x$ lower with $9.87 \times 10^{-14} \, \mu g \, BAM/cell/min$ compared to specific degradation rates in suspended batch ($4.93 \times 10^{-12} \, \mu g \, BAM/cell/min$) (Sekhar, et al., submitted). The specific degradation rates for BAM was lower than expected and overall BAM removal rate was not sufficient to attain effluent concentrations of BAM below the norm, reason why the DCBA might have been formed.

Equally analyzed the membrane, the amount of new cells generated during the 12 days of experiment was determined by applying an overall mass balance of the cells inoculated, with the total amount lost in permeate and amount retained on the membrane. It was then possible to ‘keep track’ of the course of the cells since the moment they were introduced on the reactor until reached the end of the experiment. With a total of $6.0 \times 10^{10}$ cells introduced, $8.1 \times 10^{8}$ lost and $5.0 \times 10^{9}$ accumulated, indicates an amount of $5.4 \times 10^{10}$ missing cells, which was admitted to possibly be in the stirred cell remaining volume by the end of the long-term filtration, leading to analysis of the cell amount in this sample. It was found a total of $9.5 \times 10^{3}$ cells in suspension, from which it was concluded that the DNA extraction procedure on the membrane sample might have had an experimental problem.

In order to assure that the bacteria used in the filtration experiments corresponded to *Aminobacter* sp. MSH1 strain, confirmation tests were carried out by using a permeate sample collected at the last day of this third long-term filtration. The sample was streaked in three petri dishes containing a growth media of R2A with kanamycin, where after two days the formation of yellow round-shaped colonies was possible to observe. The results confirmed the identity of the species as being Aminobacter-gfp labeled, due to its kanamycin resistance that is not possessed by other possible contaminant microorganism.

Once all long-term filtrations executed, it was mainly concluded that retention of BAM and DCBA were of big priority for an efficient drinking water production system. Its persistency throughout the second and third long-term filtrations were approached in two ways, where at first the amount of cells was interrogated as being the cause of this issue. However, high cell retention from the MF Supor and MF V0.2 were in accordance with the previous results on cell retention, with a higher than 90% range of retentions achieved. Since cell retention was effective, intrinsic aspects of the cells were seen as the most likely cause for the BAM and DCBA accumulation, which led to the verification of the degradation capacity of the cells and a significant lower value was detected. An apparent dormancy state was admitted and thought to be an outcome of a not sufficient retention time of the micropollutant, leading them to interrupt their mineralization capacity or even losing it. This assumption was taken after the analysis of the volume remaining in the bioreactor, that confirmed respectively 86.8% and 67.1% of live
cells on the second and third long-term filtrations. The first long-term filtration was not analyzed, due the BAM degradation efficacy.

4.4 Retention of BAM and DCBA by UF, NF and RO membranes

Once performed the previous two main experiments: direct flow filtration to test the suitability of membranes for cell retention and micropollutant degradation by retaining MSH1 cells, the need for a BAM and DCBA retention, led to the third phase of the experiments.

To meet our first objective i.e. finding a suitable membrane for the retention of BAM and its metabolite DCBA, several membranes were tested with different micropollutant concentrations. The ultrafiltration membrane UF XT, the nanofiltration membranes NF SB90 and NF90, and the reverse osmosis membranes RO SB50 and RO X201 were chosen based on pore size, intrinsic properties and suitability for drinking water production. BAM and DCBA possess a similar molecular weight, respectively, 190.03 g/mol and 191.01 g/mol. Given their MWCO, UF membranes are unlikely to retain BAM and DCBA. Although NF and RO membranes (MWCO < 400 Da) are likely to retain these micropollutants, the unpredictable predisposition to fouling-phenomena or concentration-polarization factors needed to be studied for its suitability to use in further applications, i.e. drinking water treatment.

Alongside with its retention, for all five membranes, the study on the impact of BAM and DCBA on membrane permeability was also of great importance. The permeability of a membrane allows to evaluate how easily a filtration occurs time wise. It is also particularly important for possible applications at an industrial level where if a certain flux is required, the membrane area can be correctly adjusted to have an effective retention as a function of its permeability.

The retention efficiency of the filtration of AGW with 1 µg/L, 10 µg/L, 1 mg/L BAM and 1 mg/L DCBA are shown in Table 4.3 and Figure 4.16. Filtration experiments were performed twice for BAM (Exp 1 and Exp 2). It was observed that for 1 µg/L BAM (Exp 1) for all membranes except for the RO X201, the permeate concentrations were higher than the feed concentrations. As such, it was hypothesized that sampling of the feed was done incorrectly and corrected in Exp 2, therefore only data of Exp 2 will be further described and discussed.

Only one of the tested membranes showed significant BAM retention at all tested concentrations. At 1 and 10 µg/L BAM, approx. 30% of BAM was retained by the RO X201 and increased to 60% at 1 mg/L BAM. None the other NF membranes or RO SB50 showed BAM retention.

Regarding the UF XT, some indication of BAM retention was observed for 1 and 10 µg/L BAM. However, likely BAM absorption was the main mechanism explaining retention. For each UF XT membrane tested, three sample vials were consecutively collected. The first sample collected showed a lower BAM concentration than the following two ones. Specifying, for 1 µg/L BAM the first permeate registered 37±24% retention while the next two 11±7% and 5±10%, also for 10 µg/L BAM retentions 49±26%, 33±17%, 13±12% and for 1 mg/L BAM 15±3%, 7±1% and 5±3% showing a pattern that was verified throughout the filtrations tests with UF XT. BAM is likely absorbed until a saturation point is reached.
The UF XT is hydrophilic and composed of PES. Also hydrophilic is the micropollutant BAM, making it prone to interact with the PES polymer.

The NF90 was included in the experiment, as BAM retention was previously observed Madsen & Sogaard (2014) with an overall retention of >90% achieved for a BAM solution at a concentration of 1 mg/L. However, in this study BAM retention was not observed.

In order to understand the disparity of results, two differences were pointed out. The experiment by Madsen & Sogaard (2014) subjected the membrane NF90 to a previous stabilization of one hour to accommodate for compression of the membrane and complete homogenization of the solution, while both permeate and concentrate streams were returned to the feed bank during that time. In the current work, the membrane was directly used without any kind of previous treatment. Also explained by Madsen & Sogaard (2014) as known to reduce membrane capacity of rejection, is the existence of an interaction between the membrane and the dipole moment of the pesticides. Facing the disparities of about 90% rejection on the previous study vs 2% rejection on the present study, tests mimicking membrane preparation described by Madsen & Sogaard (2014) should be executed as well as applying the same pressure, identical filtration media, equipment and other relevant parameters (Madsen & Sogaard, 2014).

For the metabolite DCBA, in all membranes except for the UF XT, retention above 50% was observed (Table 4.3 and Figure 4.16). DCBA retention was ranked from low to high: NF90 < NF SB90< RO X201< RO SB50. As such, DCBA was much better retained by the NF and RO membranes compared to BAM. BAM and DCBA have the same molecular weight, which doesn’t explain the difference in retention.

Since DCBA possesses a carboxyl group and admitting that the solution is at a neutral pH, this group was admitted to be negatively charged. This retention by the NF90 and RO X201 membranes, is seen as a cause from an interaction with this membrane constitution polymer, polyamide which possesses the strongest polar functional group, amide, resulting in a high retention by the membrane. DCBA was also retained by cellulose acetate blend membranes, though cellulose is an uncharged compound, not polar due to its functional groups.
Figure 4.16 - BAM retention (orange bars) and permeability (blue dots) for different BAM and DCBA concentrations solutions, filtered through the indicated membranes.
Table 4.3 - Retention efficiency at different concentrations of BAM and DCBA for different membranes tested

<table>
<thead>
<tr>
<th>RETENTION (%)</th>
<th>1 µg/L BAM Exp 1</th>
<th>1 µg/L BAM Exp 2</th>
<th>10 µg/L BAM Exp 1</th>
<th>10 µg/L BAM Exp 2</th>
<th>1 mg/L BAM Exp 1</th>
<th>1 mg/L BAM Exp 2</th>
<th>1 mg/L DCBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>UF XT</td>
<td>-30±35</td>
<td>18±20</td>
<td>-27±14</td>
<td>32±23</td>
<td>-</td>
<td>9±5</td>
<td>6±3</td>
</tr>
<tr>
<td>NF SB90</td>
<td>-9±3</td>
<td>-1±11</td>
<td>-6±4</td>
<td>-20±12</td>
<td>-4±7</td>
<td>5±4</td>
<td>77±18</td>
</tr>
<tr>
<td>NF 90</td>
<td>-13±28</td>
<td>14±14</td>
<td>13±6</td>
<td>-21±23</td>
<td>16±24</td>
<td>2±2</td>
<td>54±21</td>
</tr>
<tr>
<td>RO SB50</td>
<td>-8±5</td>
<td>9±18</td>
<td>1±4</td>
<td>-1±6</td>
<td>10±12</td>
<td>2±2</td>
<td>91±9</td>
</tr>
<tr>
<td>RO X201</td>
<td>90±1</td>
<td>38±21</td>
<td>94±1</td>
<td>25±11</td>
<td>73±17</td>
<td>63±10</td>
<td>89±1</td>
</tr>
</tbody>
</table>

Table 4.4 - Permeability at different concentrations of BAM and DCBA for different membranes tested

<table>
<thead>
<tr>
<th>PERMEABILITY (L/m²hbar)</th>
<th>1 µg/L BAM Exp 1</th>
<th>1 µg/L BAM Exp 2</th>
<th>10 µg/L BAM Exp 1</th>
<th>10 µg/L BAM Exp 2</th>
<th>1 mg/L BAM Exp 1</th>
<th>1 mg/L BAM Exp 2</th>
<th>1 mg/L DCBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>UF XT</td>
<td>13.3±1.4</td>
<td>11.7±2.0</td>
<td>18.5±0.4</td>
<td>9.6±2.2</td>
<td>-</td>
<td>17.0±1.9</td>
<td>5.4±1.4</td>
</tr>
<tr>
<td>NF SB90</td>
<td>1.9±0.1</td>
<td>1.9±0.3</td>
<td>2.7±0.3</td>
<td>1.7±0.2</td>
<td>1.3±0.1</td>
<td>0.5±0.1</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>NF 90</td>
<td>17.2±11.0</td>
<td>4.3±0.7</td>
<td>4.2±02</td>
<td>3.8±0.1</td>
<td>2.6±0.4</td>
<td>3.6±0.2</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>RO SB50</td>
<td>1.6±0.3</td>
<td>1.5±0.8</td>
<td>2.8±1.1</td>
<td>1.4±0.4</td>
<td>1.1±0.1</td>
<td>2.8±0.5</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>RO X201</td>
<td>1.1±0.1</td>
<td>2.7±1.5</td>
<td>1.6±0.2</td>
<td>1.3±0.2</td>
<td>0.7±0.1</td>
<td>1.5±0.2</td>
<td>1.1±0.1</td>
</tr>
</tbody>
</table>
Permeability was compared between the tested membranes when filtering AGW amended with BAM or DCBA (Figure 4.17). The UF membrane showed the highest permeability which was 6-fold higher compared to the NF membranes and RO membranes, irrespective of the applied BAM concentrations. However, permeability was 5-fold lower when filtering DCBA using the UF XT.

Both 1 and 10 µg/L BAM were filtered with permeabilities of 11.7±2.0 L/m² h bar and 9.6±2.2 L/m² h bar, respectively (Table 4.4 and Figure 4.17). However, at 1 mg/L BAM permeability was 17.0±1.9 L/m² hbar and at 1 mg/L DCBA 5.4±1.4 L/m² hbar. The higher permeability at 1 mg/L BAM is explained by the apparent correlation with the UF phenomena of absorption, that leads to micropollutant accumulation until a saturation point is reached. This test registered the lower value in retention, 9±5% that is possibly explained by a quicker reach of saturation at higher concentrations, leading to the higher permeability of the membrane. The same apparent absorption phenomenon was noticed for DCBA, but a lower permeability was obtained at 1mg/L DCBA. This might be explained by the formation of hydrogen-hydrogen bonds between DCBA molecules and since polyethersulfone is a non-polar molecule, hydrophobic interactions are established, and saturation occurs. Such behavior has been reported in previous studies and it is related with fouling, which was seen as a possible cause for the lower permeability detected (Zhao, et al., 2013).

The permeability of both tested nanofilters (NF90 and NF SB90) for the different BAM concentrations and DCBA are shown in Table 4.4 and Figure 4.17. When comparing NF90 with NF SB90, the permeability of the NF SB90 is generally lower, varying between 0.4±0.2 L/m² hbar and 1.9±0.3 L/m² hbar, while the NF 90 varied between 3.6±0.2 L/m² hbar and 4.3±0.7 L/m² hbar. This is due to possible fouling on the NF SB90 membrane. The permeability of the NF SB90 membrane was lowest for 1 mg/L BAM and 1 mg/L DCBA, compared to the 1 and 10 µg/L BAM, while for the NF 90 permeability remained practically unchanged for all concentrations.

Permeability of the tested RO membranes (RO X201 and RO SB50) for the different BAM and DCBA concentrations are shown in Table 4.4 and Figure 4.17. No apparent difference in permeability was noted between the two RO membranes. The permeability of RO SB50 ranged from 0.2±0.1 L/m² hbar to 2.8±0.5 L/m² hbar and of that of RO X201 ranged from 1.1±0.1 L/m² hbar to 2.7±1.5 L/m² hbar. For the RO X201 membrane, permeability was highest for 1 µg/L BAM, and remained stable for 10 µg/L and 1 mg/L BAM and 1 mg/L DCBA. For the RO SB50 membrane, permeability was highest for 1 mg/L BAM and lowest for 1 mg/L DCBA.

All results in BAM and DCBA retention, as well as permeability, provided a general panorama on all particularities from each of the tested membranes. Throughout the results analysis, it is seen that no perfect solution can stand out, but relating retention and permeability, some membranes are more likely suitable for BAM and DCBA retention. A membrane satisfying this criterion would be the RO X201. When tested, all BAM solutions with different concentrations indicated highest retention for this membrane that largely stood out from the other membranes. Thus, filtration of DCBA achieved an overall good retention, including for the RO X201. Moreover, the permeability of this membrane kept its consistency throughout all tests, which is trustworthy when future applications require predicting the
membrane's behavior. Variations in concentration did not seem to have a significant effect on permeability, as this was observed for all NF and RO tested (Figure 4.17).

Testing retention with the aim of seeing at what extent BAM could be retained, the goal was a future application on direct flow systems such as the previously mentioned, setup 2.

Retentions for all membranes, especially RO X201 as seen to be promising, did never obey the maximum allowed 0.1 µg/L from the European Union norm.

Despite not being proportional, the microgram range concentration of BAM registered lower retention than the 1 mg/L, indicating the chance that at lower concentrations the membranes will not be able to retain at a great extent, and the norm will continue not being satisfied. Exemplifying for a 1 µg/L BAM and 0.2 µg/L BAM with a 40% retention, the attainment of 1.3±0.5 µg/L consequently, leads to the drinking water norm still not being respected, reaffirming the need of combining the membrane technology with bioaugmentation.

Concerning DCBA, its formation during the second and third long-term filtrations determined the performed tests for a 1 mg/L concentration.

Since no membrane retained DCBA until a value below the norm, bioaugmentation was necessary for the achievement of a drinkable water. Once known that RO X201 retains BAM, and registering 89% retention for DCBA, this membrane would have the capacity to retain both.
Figure 4.17 - Obtained permeability for DCBA and three different BAM concentrations for the ultrafilter XT on the left figure, for the nanofilters NF SB90 (blue) and NF90 (orange) at the central figure, and for the reverse osmosis membranes RO SB50 (orange) and RO X201 (blue) on the right figure.
4.5 Effect of MSH1 Cells on the permeability of UF, NF and RO membranes

It was proposed that one of the setups to remove BAM from drinking water would combine BAM retained by a UF, NF or RO membrane with MSH1 cells degrading BAM simultaneously. However, as UF, NF and RO are prone to fouling by e.g. bacterial growth, effects of MSH1 cells on membrane permeability had to be tested. This complementary test used a MSH1 cell suspension of $10^6$ cells/mL, that was filtered using the UF XT, NF SB90, NF90, RO SB50 and RO X201. Permeability is represented in Figure 4.18.

Analyzed all membranes, the highest permeability was obtained by the NF SB90 with 10.5±0.2 L/hm² bar, as the lowest was obtained for RO X201 with 1.8±0.1 L/hm² bar. As observed by these results, the most selective membrane range, reverse osmosis, obtained lower permeabilities, fact that does not favor the efficacy of the membrane since only from the cell size, all membranes represented in Figure 4.18, are prone to retain cells.

When thinking about the second direct flow system, the use of reverse osmosis membranes was pointed as the solution for BAM retention. Thus, seen the cells permeability of the RO membranes, as well as the NF90, it should be taken into account the possible combination of another kind of membrane as a first selective barrier to avoid fouling phenomena, prone to happen due to the low cell permeability on these membranes.

Since the RO X201 had the best retention performance on the previous tests and its permeability values when filtering MSH1 cell suspensions matched the range of permeabilities obtained in the BAM and DCBA filtration tests, it can be concluded that apparently the presence of the cells does not present a negative effect on the filtration, making this membrane, making it suitable to be used for BAM, DCBA and cell retention.
5. Conclusions and Future Work

Our objective was to develop an efficient biological treatment for the removal of the micropollutant BAM from groundwater as an alternative to the currently used physico-chemical treatments in drinking water production. Previously, the BAM-degrading *Aminobacter* sp. MSH1 was applied to existing sand filters in drinking water treatment plants for BAM removal at concentrations as low as 0.2 µg/L. Initially, adequate BAM removal was observed producing drinking water under the EU norm of 0.1 µg/L BAM. However, substantial loss of MSH1 cells from the sand filter led to a decreasing BAM removal rate and hence led to BAM concentrations above the EU norm. In this thesis, we sought to solve the observed problems by the use of membranes to prevent the wash-out of the cells possibly in combination with the retention of BAM or formed metabolites.

In a first setup (Setup 1), membranes were selected to retain MSH1 cells efficiently and BAM removal relayed on the BAM-degrading activity of MSH1 cells. Stepwise tests were performed starting by assessing cell retention at different cell densities using MF membranes. Cell retention efficiency was tested for three different microfilters where, MF Supor and MF V0.2 showed efficient retention of MSH1 while no effect on permeability was observed. Lower cell densities registered a higher loss of cells once the system reached a steady state (< 0.5% loss), while higher cell densities showed a lesser amount of cell loss (< 0.05%). Cell viability was also monitored and it was verified that a major part of the cells remained alive for both concentrations tested. In fact, for $10^6$ cells/mL, approximately 76% showed to be live cells, while 92% living cells were accounted for $10^9$ cells/mL.

The membranes MF Supor and MF V0.2 were both used in continuously fed MBRs to see MBR efficiency during long term operation. This was mainly to assess viability and BAM degradation activity of MSH1 cells in the MBR operated under Setup 1. BAM degradation was immediate upon starting the MBR at both 0.2 and 10 µg/L BAM and produced water under the EU norm of 0.1 µg/L. However, in the case of 10 µg/L BAM, BAM concentrations increased in the permeate stream and accumulation of the metabolite DCBA (in the permeate stream) was observed after 5 days of operation. Likely, part of the MSH1 cells are no longer viable or are in a dormant state and, as such, are no longer degrading BAM, leading to the increasing of BAM concentration. However, in case of DCBA accumulation, other explanation has to be found since the genes e.g. *bbdB* needed for BAM mineralization were still present. As such, the genotype for BAM mineralization and DCBA degradation should still be present in the MBR.

At this point, it is believed that the enzymes responsible for BAM mineralization, which are plenty in the MSH1 cells upon inoculation, were not replaced under the tested oligotrophic conditions and a net loss of these enzymes occurred.

As such, the so far conducted experiment to test and develop a direct flow MBR as proposed for Setup 1, has great potential to remove BAM from groundwater. However, great care should be taken to assure complete BAM removal without the formation of any metabolites. It is therefore of extreme importance to assess the state of MSH1 in the MBR and investigate which parameters control its state.

In a second case (Setup 2), it was proposed the execution of a filtration test with both micropollutant and cell retention. UF, NF and RO membranes were tested for the retention of BAM and DCBA at
different concentrations. The RO membrane X201 showed a BAM retention of 38±21%, 25±11%, and 63±10% for 1 µg/L, 10 µg/L and 1 mg/L. All other membranes couldn’t retain BAM at any of the tested concentrations. Likely, being BAM a low molecular component, RO membranes are the ones expected to effectively retain this micropollutant. Besides, the high selectivity of the membrane material towards the filtration media seems to also be a reason for the observed behavior.

Permeability was also measured to demonstrate the ‘ease’ of filtration, serving as a comparison term between membranes. The RO X201 possessed a stable permeability throughout the entire range of concentrations tested and is, then, a behavior-predictable membrane offering stable filtration conditions.

The RO X201 membrane retained DCBA, but all the other tested RO and NF membranes also retained DCBA at an average of 74±19%. DCBA, having a similar molecular weight to BAM, is retained by all these membranes with another mechanism than size exclusion: membrane material selectivity. This retention of DCBA by a multitude of different membranes offers some possibilities in solving the problem with DCBA accumulation seen for Setup 1.

When MSH1 cells are combined with BAM or DCBA retention, it is important to see how the permeability of the membranes is affected by the presence of MSH1 cells in the MBR. Consequently, for each membrane type, the permeability in the presence of cells was tested and it was not significantly affected, as when compared to the permeabilities achieved for BAM and DCBA. As such, fouling effects due to bacteria were not an immediate problem. However, great care should be taken when cells start to grow or when bacterial cells other than MSH1 are continuously supplied with the groundwater.

The reverse osmosis membrane RO X201 showed to be effective for both cell and micropollutant retention and its use for long-term filtrations should take into account different possibilities. Since cells are constantly growing throughout time, the continuous feed of micropollutant with cells multiplying at a certain speed may cause fouling effects in the long term. In order to avoid such situation, the microfilters shown to effectively hold cells could be used as a first barrier followed by the RO membrane.

The direct flow system as proposed in this thesis has some disadvantages. These systems are known to easily foul and require higher pressure for performance. A possible solution is the use of a cross-flow system, where the feed flows tangentially across the surface of the membrane, rather than straight through the filter.

Having in mind the potentialities of this work, all mentioned situations must be simulated in realistic conditions of their intended use. Thus, long-term tests shall take place starting by the fundamental viability and functionality of the cells. As determined for lower scales, a cell break-through reaches a steady state from which possible re-inoculations might become necessary. Membranes being known by predisposition to fouling or concentration-polarization effects, must be assessed to estimate their durability, when subjected to realistic conditions of continuous flow systems.

As a next step, a pilot-scale tangential flow system is going to be tested to treat actual groundwater or tap water contaminated with BAM. Important aspects here are the availability of other organic carbon sources for bacterial growth, as well as the presence of other microorganisms such as bacteria and
protozoa. Other bacteria can compete for nutrients with MSH1 and protozoa graze bacterial cells. Both mechanisms can lead to a decreasing BAM removal in the MBR over time and should be addressed.
6. References


Kimura, K., Toshima, S., Amy, G., & Watanabe, Y. (2004). Rejection of neutral endocrine disrupting compounds (EDCs) and pharmaceutical active compounds (PhACs) by RO membranes. *Journal of Membrane Science 245*, 71-78.


Appendix 1 - Ultra Performance Liquid Chromatography

Hundred µL sample was injected on a Waters Acquity UPLC HSS T3 (1.8 µm 2.1 x 5 mm) column and elution was performed using a mobile phase consisting of acetonitrile and ultrapure water (Milli-Q®) (volume ratio 90/10) at a flow rate of 0.3 mL min⁻¹. The MS was equipped with a heated electrospray ionization (HESI II) source in positive ion mode, operated at a spray voltage at 4 kV; vaporizer temperature of 350°C and capillary temperature of 315°C; sheath and auxiliary gas of 45 and 5 arbitrary units, respectively. S-lens RF amplitude was 63 V. Collision energy (CE) in Q2 was respectively 35eV for transition m/z 249.04>124.99; 33eV for transition m/z 249.04>132.95; 17eV for transition m/z 249.04>159.98 and 16eV for transition m/z 249.04>182.04 of BAM. Quantification data were acquired based on these four transitions in the selected reaction monitoring (SRM) mode. Instrument control and data processing were carried out by means of Xcalibur Software 2.0.7 SP1 (Thermo Scientific, San José, USA).
Appendix 2 - Media Preparation

Protocol for
MEDIA PREPARATION

In this document you will find the composition and preparation procedures for different bacterial growth media, and one algal growth medium.

Index
A.4. RA .................................................................................................................. 2
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A.4. R₂A

Complex medium that is less rich than LB but has more constituents. Excellent for isolations.

- Protease peptone: 0.5 g (use tryptone)
- Yeast Extract: 0.5 g
- Casein hydrolysate: 0.5 g
- Glucose D+ (monohydrate): 0.55 g
- Starch (soluble): 0.5 g
- Sodium pyruvate: 0.3 g
- K₂HPO₄: 0.3 g
- MgSO₄: 0.05 g (MgSO₄·7H₂O: 0.1 g)
- Distilled H₂O: 1000 mL

pH should be between 7.0 and 7.6. If not, adjust before autoclaving.

B.7. MS

B.7.1. Stock solutions

All stock solutions should be autoclaved except for the trace metal and FeCl₃-solution which should be be filter sterilized (0.22 μm).

Buffer

- KH₂PO₄: 136 g
- Na₂HPO₄·2H₂O: 178 g
- mQ·H₂O: 1000 mL

Nutrient

- MgSO₄·7H₂O: 5 g
- CaCl₂·2H₂O: 1.32 g
- mQ·H₂O: 1000 mL

Trace metal

- H₂BO₃: 2.86 g
- MnSO₄·H₂O: 1.54 g
- CuSO₄·5H₂O: 0.039 g
- ZnCl₂: 0.021 g
- CoCl₂·6H₂O: 0.041 g
- Na₂MoO₄·2H₂O: 0.025 g
- mQ·H₂O: 1000 mL

FeCl₃-solution

- FeCl₃·6H₂O: 0.514 g
- mQ·H₂O: 100 mL

B.7.2. Preparation of medium

Fill a bottle with about 978 mL mQ·H₂O
Add 10 mL Buffer solution
Autoclave and cool down
Add 10 mL nutrient solution
Add 1 mL of filter sterilized trace metal solution
Add 1 mL of filter sterilized FeCl₃-solution
Appendix 3 - Extraction of genomic DNA using the CTAB-Lysozyme method

Protocol
1. Add 450 μl GTE solution to the membranes inside 2 mL cryotubes and homogenize.
2. Transfer the supernatant to a 2-ml microcentrifuge tube containing 50 μl of a 10 mg/ml lysozyme solution.
3. Mix gently and incubate overnight at 37°C.
4. Make a 2:1 solution of 10% SDS solution and 10 mg/ml proteinase K. Add 150 μl of this solution to the cells and mix gently. Incubate 20 to 40 min at 55°C.
5. Add 200 μl of 5 M NaCl and mix gently.
6. Preheat CTAB solution to 65°C, add 160 μl, and mix gently. Incubate 10 min at 65°C.
7. Add an equal volume (~1 ml) 24:1 (v/v) chloroform/isoamyl alcohol, shake vigorously to mix, and microcentrifuge for 5 min.
8. Transfer 900 μl aqueous layer to a fresh 2-ml microcentrifuge tube.
9. Repeat extraction with 24:1 (v/v) chloroform/isoamyl alcohol, shake vigorously to mix, and spin in microcentrifuge for 5 min.
10. Transfer 800 μl to fresh 2-ml microcentrifuge tube.
11. To 800 μl aqueous layer, add 560 μl (0.7 vol) isopropanol, mix gently by inversion until the DNA has precipitated out of solution.
12. Incubate 5 min at room temperature. Microcentrifuge for 10 min, room temperature.
13. Aspirate supernatant and add 1 ml of 70% ethanol to wash DNA pellet. Mix gently by inversion and microcentrifuge 10 min at room temperature.
14. Carefully aspirate supernatant, avoiding the pellet, and air-dry DNA pellet for 15 min. Do not overdry.
15. Add 50 μl Tris 25 mM buffer to dried DNA pellet and store overnight at room temperature or 4°C to allow pellet to dissolve. Store up to 1 year at ~20°C.

References
Current Protocols in Microbiology 10A.2.1-10A.2.21, August 2007
Appendix 4 - Eawag Protocol

366.1 Determining the total cell count and ratios of high and low nucleic acid content cells in freshwater using flow cytometry

6 Method execution

6.1 Preparation of the fluorescent stain
- The fluorescent stain SYBR Green I (10'000x) is diluted 1:100 in 0.2 μm filtered DMSO.
Note: The prepared stain aliquots can be stored at -20 °C in glass vials over a long time period (also see 5.4). Protective gloves should be worn while handling the stain.

6.2 Staining the samples
- The stain is added in a ratio of 1:100 to the sample (for example: 10 μl SYBR Green I 100x in DMSO (6.1) in 1000 μl sample).
- The sample is mixed on the vortex for 5 seconds.
- The stained sample is incubated in a heating block at 37°C (±2°C) for a minimum of 13 minutes in the dark.
- In this manner, many samples can be prepared simultaneously and stored in the dark until needed.
Note: In laboratories, samples are usually stored at 4 °C. With a sample volume of 1 ml, three minutes are necessary to increase the core temperature of the sample from 4 °C to 37 °C. Based on this the 13-minute incubation time was calculated - 3 minutes to reach core temperature and 10 minutes incubation time.

6.3 Sample dilution and measurement range
- During the measurement, an upper limit of approximately 1’000 counts/second must not be exceeded (depending on specific FC instrument specifications). This represents a cell count in the sample of approximately 1.0 × 10^3 – 2.0 × 10^4 cells/ml (depending on the sample background signals). Otherwise, the sample should be diluted with the dilution media listed under 4.3 until a value below the upper detection limit is reached.
1. Wet the O-rings with a small amount of water or the fluid to be processed.

   **Note:** HP4750 Stirred Cells are shipped with Buna-N O-rings and gaskets by default; other materials such as Viton and PTFE are available as options. Table 2 on page 10 outlines O-ring and gasket material compatibility.

2. Insert the O-rings in the bottom of the cell body. (Photo 2.1 & 2). Check to be certain that the O-rings fit properly in the grooves.

3. Place a piece of precut membrane over the center O-ring. The membrane should be installed with the active side toward the cell reservoir. In general, membranes coated on substrate have a shiny, active side and a dull substrate side. (Photo 2.3)

4. Place the stainless steel porous membrane support disk on top of the membrane to hold the membrane in place. (Photo 2.4)

   **Note:** If you cut your own membranes, the stainless steel porous disk can be used as a template. See Appendix 1 on page 14 for more details.

5. Fit the cell bottom onto the cell body, aligning the circular groove with the circular ridge on the bottom of the cell body. (Photo 2.5)

6. Use the 3-inch high pressure coupling to clamp the cell bottom to the cell body. Tighten the high pressure coupling with 16 foot pounds of torque for 1000 psi (69 bar) operation. (Photo 2.6)

7. Insert Permeate Tube into side of cell body, tighten using a wrench. (Photo 2.7)

   **Note:** The HP4750 uses Swagelok connector fittings. More detailed instructions, information, and images of the fittings can be found in Appendix 1 on page 13.

8. Insert the Stir bar assembly by dropping it into the Cell Body through the 2-inch-diameter opening at the top. (Photo 2.8a). Alternatively, the stir bar assembly can be lowered into the cell with the stir bar retriever. (Photo 2.8b). Figure 2.8c shows the correct position of the Stir Bar Assembly. After the assembly is in place, pour the solution to be filtered into the Cell Body.

9. Insert the gasket on top of the Cell Body, making sure it fits properly in the grooves. (Photo 2.9)

10. Use the 2 inch high pressure coupling to clamp the cell top to the cell body. Tighten the high pressure coupling with 16 foot pounds of torque for 1000 psi (69 bar) operation. (Photo 2.11) After both clamps are properly attached, the HP4750 Stirred Cell should be centered on top of a magnetic stirrer.

Figure 2.8c: Proper Stir Bar Position