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Abstract

The main metabolite of the herbicide dichlobenil, 2,6-dichlorobenzamide (BAM), is frequently detected in drinking water at concentrations above the European threshold limit of 0.1 µg/L. *Aminobacter* sp. MSH1 is capable of mineralizing BAM at ecologically relevant concentrations till below the limit of 0.1 µg/L, making MSH1 a promising candidate for bioaugmentation in sand filters of drinking water treatment plants as a cost-effective and sustainable alternative for granular activated carbon (GAC) for BAM removal. However, the success of this bioaugmentation depends on the capability of MSH1 to invade the indigenous bacterial community effectively and to survive in oligotrophic sand filter conditions. The main goal of this thesis is to gain insight in key processes occurring during the survival of MSH1 when invading the sand filter environment and to identify 'invasion' genes that encode for these processes, since the expression of these genes can be linked to the success of bioaugmentation.

In the first part of this thesis, different continuously fed flow cell experiments were used to examine what governs the incidence of pBAM2, the plasmid responsible for BAM mineralization, in the MSH1 population when growing as biofilms in oligotrophic conditions. Loss of pBAM2 occurred when low BAM-concentrations and additional AOC were present, while the incidence of pBAM2 increased with increasing BAM-concentrations. No horizontal gene transfer occurred but the MSH1 population used for bioaugmentation is composed of two subpopulations: one pBAM2-carrying MSH1 subpopulation capable of BAM-mineralization and one pBAM2-deficient MSH1 subpopulation which simply converts BAM to DCBA. In oligotrophic conditions, the presence of residual AOC and the concentration of BAM largely determines the abundance of each subpopulation. For example, when BAM concentrations increased, BAM mineralizing cells increased due to the selective advantage of this subpopulation when growing on BAM. Next, the effect of an indigenous sand filter community on BAM removal efficiency of MSH1 was examined in two possible bioaugmentation strategies: invasion and co-colonization. In case of co-colonization, MSH1 was negatively affected by the co-colonizing sand filter community resulting in a lower survival and, hence a lower BAM removal efficiency. When the surface was colonized by a sand filter community and MSH1 had to invade this existing biofilm a positive effect was observed.

In the second part, a continuously fed flow chamber and a lab scale sand filter experiment were used to identify 'invasion' genes using three different strategies, since gene identification is challenging as only little bacterial biomass is available in oligotrophic conditions for e.g. RNA extraction for RNA-Seq. We were able to extract sufficient total RNA (for comparative transcriptomic analysis), insert fragments (for differential fluorescence induction) and gDNA (for transposon mutagenesis). However, it is possible that the sequences will be insufficient to identify 'invasion' genes, since we are reaching the lower limits of the approaches.

Samenvatting

De belangrijkste metaboliet van het herbicide dichlobenil, 2,6-dichloorbenzamide (BAM), wordt vaak aangetroffen in het drinkwater met concentraties boven de limiet van 0.1 µg/L, opgelegd door de EU. *Aminobacter* sp. MSH1 is in staat om BAM te mineraliseren bij ecologisch relevante concentraties tot onder de grens van 0.1 µg/L, waardoor MSH1 een hoopvolle kandidaat is voor bioaugmentatie van zandfilters van drinkwater zuiveringsinstallaties als een kosteneffectief en duurzaam alternatief voor granulaire actieve kool. Maar het succes van deze bioaugmentatie hangt af van het vermogen van MSH1 om de inheemse bacteriële gemeenschap effectief binnen te dringen en om te overleven in de voedselarme condities van de zandfilter. Het belangrijkste doel van deze thesis is om inzicht te krijgen in de belangrijkste processen tijdens de overleving van MSH1 wanneer deze de zandfilter omgeving binnen dringt en om de 'invasie' genen die coderen voor deze belangrijke processen te identificeren aangezien de expressie van deze genen gekoppeld kan worden aan het succes van de bioaugmentatie.

In het eerste deel van deze thesis werden verschillende experimenten gebruikt om te onderzoeken wat de incidentie van pBAM2, het plasmide verantwoordelijk voor BAM mineralisatie, in de MSH1 populatie beïnvloedt bij het groeien als biofilm in voedselarme condities. Verlies van pBAM2 werd vastgesteld wanneer lage BAM-concentraties en additionele AOC aanwezig waren, terwijl de incidentie van pBAM2 toenam met toenemende BAM-concentraties. Er vond geen horizontale gen overdracht plaats maar de MSH1 populatie gebruikt voor bioaugmentatie bestaat uit twee subpopulaties: een pBAM2-dragende MSH1 subpopulatie die BAM kan mineraliseren en een pBAM2-deficiënte MSH1 subpopulatie die BAM omzet tot DCBA. In oligotrofe condities, zal de aanwezigheid van rest AOC en de concentratie van BAM grotendeels de abundantie van elke subpopulatie bepalen. Bijvoorbeeld, wanneer BAM concentraties stegen, nam het aantal BAM mineraliserende cellen toe door het selectief voordeel van deze subpopulatie door groei op BAM. Vervolgens werd het effect van een inheemse zandfilter gemeenschap op de efficiëntie waarmee MSH1 BAM degradeert, onderzocht in twee mogelijke bioaugmentatie strategieën: invasie en co-kolonisatie. In het geval van co-kolonisatie, werd MSH1 negatief beïnvloed door de zandfilter gemeenschap resulterend in een lagere overleving en dus een lagere BAM afscheidingsefficiëntie. In het geval van invasie werd een positief effect door de zandfilter gemeenschap waargenomen.

In het tweede deel, werd een continu stroom cel experiment en een laboratoriumschaal zandfilter experiment gebruikt om 'invasie' genen met behulp van drie verschillende strategieën te bepalen, aangezien gen identificatie uitdagend is bij oligotrofe condities doordat maar zeer weinig bacteriële biomassa aanwezig is voor bv. RNA-extractie van RNA-Seq. We waren in staat om voldoende totaal RNA (voor een vergelijkende transcriptoom analyse), insert fragmenten (voor differentiële fluorescentie

inductie) en gDNA (voor transposon mutagenese) te extraheren. Het is echter mogelijk dat de sequenties van onvoldoende kwaliteit zijn om ‘invasie' genen te identificeren, aangezien we aan de ondergrenzen van de benaderingen zitten.

List of Abbreviations

AFPs	autofluorescent proteins
AGE	Agarose gel electrophoresis
AOC	Assimilable organic carbon
ASFC	Artificial sand filter community
BAM	2,6-dichlorobenzamide
BSA	Bovine serum albumin
bp	Base pairs
CLSM	Confocal laser scanning microscopy
DCBA	2,6-dichlorobenzoic acid
DFI	Differential fluorescence induction
DWTP	Drink water treatment plant
EPS	Extracellular polymeric substances
FACS	Fluorescence-activated cell sorting
FC	Flow cell
GAC	Granular activated carbon
GFP	Green fluorescent protein
Gm	Gentamycin
kb	kilobase
Km	Kanamycin
M lib.	Transposon mutant library
MS	Mineral salts
MW	Molecular weight
OBAM	*ortho*-chlorobenzamide
OD	Optical density
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PP	Promoter-probe
qPCR	Quantitative polymerase chain reaction
RNA-Seq	RNA sequencing
UPLC	Ultra-performance liquid chromatography
wt	Wild type
WWTP	Waste water treatment plant

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1. Context and objectives

Pesticides and its metabolites are frequently detected in drinking water at concentrations above the European threshold limit of 0.1 µg/L. As a result, these micropollutants are of increasing concern for drinking water treatment plants (DWTPs), since these pollutants need to be removed using physico-chemical treatment techniques such as granular activated carbon (GAC) filtration. However, some disadvantages are associated with this technique. For instance, the technique has high operational and maintenance costs and in addition the micropollutant is merely moved from one medium to another (from water to activated carbon). A possible alternative for this expensive technique is the bioaugmentation of sand filters of DWTPS with a micropollutant degrading bacterium. The main metabolite of the broad-spectrum nitrile herbicide dichlobenil, 2,6-dichlorobenzamide (BAM), is an important contaminant of groundwater which is used for drinking water production. *Aminobacter* sp. MSH1 is capable of mineralizing BAM at ecologically relevant concentrations till below the limit of 0.1 µg/L, making this bacterial strain a promising candidate for bioaugmentation in sand filters of DWTPs. The success of this bioaugmentation depends on the capability of the strain to invade the indigenous bacterial community effectively and to survive in oligotrophic sand filter conditions where social interactions such as cooperation and/or competition occur. These invasion processes such as metabolism in oligotrophic conditions, biofilm formation and interactions with other bacteria in sand filters lie encoded in 'invasion' genes of strain MSH1. The expression of these genes can be used as an indicator for the activity of MSH1 in each process and can be linked to the success of introduction in sand filters and hence the success of bioaugmentation.

The main goal of this thesis was to gain insight in key processes occurring during the survival of MSH1 when invading the sand filter environment and identify genes that encode for these processes. Several key processes were previously identified such as the loss of BAM mineralization, dormancy and lower cell viability of MSH1 in biofilms grown in oligotrophic conditions and are further looked into in a first part of this thesis. How are these processes furthermore impacted by the presence of an indigenous bacterial sand filter community and how is this influenced by the presence of assimilable organic carbon? In a second part, genes encoding for these processes are identified as these 'invasion' genes are responsible for the success of the bacterium to invade the sand filter community by interacting with the sand filter environment.

In a first part of this thesis, a first important aspect for the survival of MSH1 in oligotrophic conditions is investigated, more specifically the mineralization stability of *Aminobacter* sp. MSH1 in oligotrophic conditions. The BAM-degradation is, according to T'Syen (unpublished), encoded on two plasmids of strain MSH1, pBAM1 and pBAM2. The amidase BbdA degrades BAM to DCBA and is encoded by the

constitutively expressed *bbdA* gene, located on plasmid pBAM1. The genes responsible for further degradation of DCBA, such as *bbdB* and *bbdC*, are located on plasmid pBAM2 and its loss is linked with the loss of BAM mineralization. As a result, a single cell is capable of mineralizing BAM only when both pBAM1 and pBAM2 are present. The natural abundance of MSH1 cells that possesses pBAM1 is estimated at 99-99.9%. However, only 0.1-1% of MSH1-cells has both plasmids. A **first objective**, related to mineralization stability, was to investigate **what governs the incidence of pBAM2 in the MSH1 population when growing as biofilms in oligotrophic conditions**. Can the pBAM2 incidence increase with increasing BAM-concentrations? Does assimilable organic carbon play a role in the stability of pBAM2 in the MSH1 population? Especially, the impact of a high or low incidence of pBAM2 in the MSH1 population might be of great importance for the full mineralization of micropollutant BAM-concentrations in the sand filter environment. A **second objective**, related to mineralization stability, was to determine whether **horizontal gene transfer of pBAM2 occurred amongst the MSH1 cells in biofilms grown under oligotrophic conditions** or whether pBAM2 is only passed on vertically during mitosis.

A third objective, was to study whether ***Aminobacter* sp. MSH1 is able to colonize surfaces as a pioneer (co-colonization) or a successor (invasion)** in the presence of an indigenous sand filter community. MSH1 probably needs to compete for nutrients and space in both conditions, but is invasion more difficult compared to co-colonization since a well-adapted sand filter community already colonized the sand filter environment? Also, the extent of available AOC should be considered as a variable determining the survival of MSH1 in biofilms developed under both scenarios.

A second part of this thesis, focuses on the objective to identify 'invasion' genes specifically expressed in the presence of the sand filter community under oligotrophic conditions using three independent high-throughput approaches. The gene identification using these approaches is based on the differential expression of genes in MSH1 in the presence and absence of a sand filter community. In a first approach, called comparative transcriptomics, mRNA transcripts in MSH1 are compared between both conditions. For the second approach, called differential fluorescence induction, a MSH1 promoter probe library was previously constructed by introducing random fragments (1-2 kb) of gDNA of MSH1 into a promoter vector in front of a promoterless *gfp* gene. Next, this various vectors were introduced in MSH1 cells making them reporters of the expression of its own genes. When a promoter on this fragment is activated, the cell will be GFP fluorescent as the promoterless *gfp* gene is transcribed. The promoter probe library will be grown in the absence and presence of the sand filter community. By separating the GFP and non-GFP MSH1 reporters by FACS and sequencing the inserted DNA fragments, the activated promoters can be identified. By comparing active promoters in the absence and presence of the artificial community, 'invasion' genes specifically expressed as a response to the artificial community can be

identified. A last approach used, is transposon mutagenesis. A MSH1 transposon mutant library will be used, created by a mini-Tn5 transposon system with a promoterless *gfp* reporter gene that knocks out the genes of *Aminobacter* sp. MSH1 by randomly introducing a transposon in the genomic DNA. After growing the mutant library both in the absence and presence of the sand filter community, mutants with a disrupted gene essential for 'invasion' will be lost in the mixed-species condition but will be present in mono-species conditions. Consequently, these genes will be considered as essential 'invasion' genes. In addition, this technique also provides information on non-essential 'invasion' genes. When the promoter of the knocked out gene is activated, the cell will be GFP fluorescent as the promoterless *gfp* gene is transcribed. By separating the GFP and non-GFP MSH1 mutants by FACS and sequencing the DNA fragments located next to the transposon, the activated promoters can be identified. By comparing active promoters in the absence and presence of the artificial community, non-essential 'invasion' genes can be identified. These genes are non-essential since these MSH1 mutants survived in the multispecies condition, but are 'invasion' genes because they are specifically expressed as a response to the artificial community.

Gene identification will be mainly based on a comparative analysis of MSH1 gene expression when growing in continuously fed flow chambers. Although, these chambers mimic the sand filter environment, e.g. oligotrophy, shear stress, biofilm formation and species interactions, some important mechanisms might be missed. Therefore, a last objective of this thesis, was the identification of 'invasion' genes in MSH1 using comparative transcriptomics when MSH1 invades lab scale sand filters in the absence and presence of the sand filter community.

2. Literature study

2.1. The problem of micro-pollutants and in particular pesticides

Nowadays, clean drinking water is so widely available in the western world. However, this accessibility of clean drinking water was only possible due to the efforts to make water treatment more successful. In the 19th century, water became more and more polluted as a result of the Industrial Revolution. Consequently there was a need for improved water treatment systems. By these developments, it became clear that water quality has a significant impact on health since the outbreak of waterborne diseases as cholera decreased due to the improved water treatments (1)(2). In addition to these diseases, there are still other threats to drinking water. For instance, during the last years, there is more attention given to the occurrence of micropollutants, such as pesticides and pharmaceuticals, in water.

2.1.1. Definition micropollutant

Micropollutants are organic pollutants that occur in contaminated water at extremely low concentrations ranging from 0,1 to 10 µg/l (3). The very low concentrations make it difficult to detect and analyse these micropollutants in the environment and, in addition, complicates the removal of these components out of water and waste water (4). Micropollutants have various kinds of origin such as urban settlements (domestic wastewater), agriculture (runoff), industry (industrial waste water), hospital effluents and traffic (5). Pharmaceuticals, endocrine disruptors, metals and metalloids, industrial chemicals, pesticides, surfactants, personal care products and hormones are some examples of products that occur as micropollutants (4). The increased interest in micropollutants during the last years can be explained by the more frequent detection of micropollutants in waters bodies used as drinking water sources. A reason for the more frequent detection is the increased production and consumption of these mass-produced materials and commodities, although this is not the only explanation. Also the improvement in analytical capabilities contributes to micropollutants being more frequently detected because of lower detection limits (6). For many techniques, these detection limits are up to parts per trillion levels for some substances. These improvements in analytical techniques have resulted in identification of hundreds of natural and synthetic organic chemicals in the aquatic environment. A lot of these contaminants are likely to exist for some time, although they are only now being measured (7).

2.1.2. Occurrence of micropollutants in the aquatic environment

The occurrence of micropollutants in the aquatic environment can be studied according to the type of water.

Initially a lot of micropollutants end up in waste water. The concentrations of these micropollutants in the influent and effluent of waste water treatment plants know fluctuations with location and time (4). A number of factors may explain these variations, such as the production amounts and usage/consumption patterns of products containing micropollutants, consumption of some pharmaceuticals coinciding with current diseases and climatic conditions (rain, temperature and level of sunlight) (4,8). As example, the use of some pesticides can be seasonal, because many pests only occur in certain conditions (4).

The current waste water treatment plants (WWTPs) are not designed to remove micropollutants efficiently out of water. In this way, they end up in the surface water by the effluent of the WWTP's. The influent concentrations are usually one to two orders of magnitude higher than effluent concentrations (0,001-1 µg/L) (4). When micropollutants end up in surface water, they will be exposed to natural attenuation. Dilution in surface water, sorption onto sediments or suspended solids, biodegradation and direct or indirect photolysis are examples of causes responsible for natural attenuation. Wang (2011) demonstrated that also climatic conditions, such as rainfall, temperature, etc. have their impact on the concentrations of micropollutants in surface water. When there is more rainfall, more materials can dissolve which results in higher pollution of these emerging components (9). In addition, the population density as well, has an impact on the occurring concentration of micropollutants. In more densely populated regions, more products containing micropollutants are used, which results in higher concentrations of these micropollutants in surface water of the populated regions (4).

Micropollutants can also end up in groundwater, but this pollution was found to be less in comparison to surface water (10,11). A number of mechanisms can result in this groundwater contamination. Landfill leachate, interaction between groundwater and surface water, infiltration of contaminated water from agricultural land or seepage of septic tanks and sewer systems are the main mechanisms (4). Additionally, also bank filtration or artificial recharge using recycled water can introduce micropollutants into groundwater. The lower concentration in groundwater is a result of dilution, adsorption, degradation and travel time, which ensure decreasing concentration from the sources to groundwater. The physicochemical properties of the micropollutants are consequently very important for determining the amount of what finally is found in groundwater (4).

Drinking water is either produced from groundwater, surface water or a mixture of both. Therefore, the occurrence of organic micropollutants in ground- and surface waters has become a concern for the drinking water industry. Surely because there are possible health effects that may be related to these micropollutants (4). Organic micropollutants in drinking water are possible contributors to cancer risks. This is immediately the major public concern of contamination in drinking water. People want to avoid these concerns and risks by protecting the quality of water provisions, despite the point that actual risks

are probably small in most cases. Nevertheless, there are examples of incorrect waste disposal practices which has led to severe water contamination (7). Carcinogenic substances in animals have been treated as non-threshold mechanisms. As a consequence, each dose above zero will theoretically pose a risk of cancer (7). But because the concentrations of these carcinogenic micropollutants in drinking water are so low, the risks associated with it will be minimal or even unmeasurable. Account where the risks anyway exist, they are small in magnitude compared to overall cancer risk rates (7). Besides, a vast majority of micropollutants have not been tested yet for carcinogenicity (12). Standards imposed by the EU do not only look at pesticides separately but also look at the sum of all pesticides in drinking water, because toxicity of mixtures are different than toxicity of individual substances. Individual pesticide residues may not exceed the limit of 0,1 µg/litre for each active ingredient, while the sum of all pesticides must remain below 0,5 µg/litre (13), (14). Cancer, reproductive and teratogenic risks are chronic diseases caused by long-term exposure to some pesticides. In addition, also acute toxicity occurs, mostly through functional and biochemical action in the central and peripheral nervous system (14).

The occurrence levels of micropollutants in drinking water is determined by several factors. First, the water source is an important element, because groundwater was found to be less contaminated with micropollutants in comparison to surface water (10). As a result groundwater requires less intensive treatment. But groundwater is not inexhaustible since replenishment of groundwater is slower than the rate at which the water is pumped up for use in industry, agriculture and drinking water. So there is a lot of pressure on the availability of groundwater in order to meet the demand for potable water. Another possibility is the use of surface water for the production of drinking water (15). Furthermore, seasons also have an effect. According to Y. Luo et al. winter water samples are showing higher concentrations in comparison to summer water samples. In addition, the concentration of micropollutants in drinking water is also determined by the drinking water treatment, because it eliminates micropollutants from drinking water (4). In addition, disinfection of drinking water, used to reduce water-borne diseases, is also an origin for micropollutants. The disinfectants react with organic matter resulting in the formation of disinfection by-products (DBPs) (16–18). These treatment generated products are known to be produced by all chemically active treatment chemicals, such as ozone, chlorine dioxide, iodine and chloramines but the most studied DBPs are derived from chlorination (7), (12).

2.2. The drinking water production process

To assure the safety of the finished drinking water, water of different sources always needs to be treated. These treatment processes must be tailored to the potential contaminants in the source (7).

Drinking water is either produced from groundwater, surface water or a mixture of both. The drinking water production process differs for these different sources. Suspended solids and pathogenic bacteria must be removed in order to be suitable for drinking water. But also the removal of micropollutants has become necessary over the last years (19–21).

2.2.1. The drinking water production process: groundwater

Groundwater flows through an aquifer and is hereby naturally filtered. In combination with a long residence time underground, this filtering ensures that groundwater is generally free from disease-causing micro-organisms. Groundwater also contains less suspended material and undissolved solids compared to surface water due to this natural filtering. In addition, the exchange of ions and molecules with the aquifer material usually takes place over an extended period when the retention time is longer, leading to water quality improvement. One can regard this as a 'natural water treatment' (22,23).

Groundwater can be either aerobic or anaerobic. Aerobic groundwater has a high oxidation potential allowing bacteria to degrade organic matter and start to grow. After a while, organic matter starts to deplete, with the consequence that bacteria will stop growing and eventually die. The drinking water production process from aerobic groundwater is thus simple, incorporating only disinfection and possibly softening of the water. The drinking water production process from anaerobic groundwater includes a lot more steps, necessary to deal with some additional problems. First, micro-organisms are forced to use other electron acceptors, like sulphate and nitrate, due to oxygen limitation. Sulphate and nitrate are hence reduced respectively to sulphides and ammonia. They want to avoid these reduced compounds in drinking water since they are toxic for humans and produce unpleasant odours. Additionally, anaerobic water contains iron and manganese in the soluble and reduced form, i.e. Fe^{2+} and Mn^{2+}. But when this water comes in contact with oxygen, iron and manganese will be oxidized (Fe^{3+} and Mn^{4+}) which results in insoluble oxides and consequently in turbidity. These oxides need to be removed from the water before distribution takes place. The treatment of groundwater to produce drinking water consists of the following steps: aeration, decantation, filtration and disinfection (20).

2.2.2. Drinking water production process: surface water

In contrast to groundwater, which is often pumped up from very great depths, surface water has an advantage in its accessibility. However, surface water is more easily exposed to pollution than groundwater and it lacks the profit of 'natural water treatment' since it doesn't percolate in soils. As a consequence, surface water has to be treated with extra intensive water purification techniques (22). First will the untreated water enter a basin, where self-purification can take place, e.g., sedimentation of suspended solids and ammonium oxidation. Next, Fe^{3+}-salts are added in order to destabilize the colloidal particles, which will coagulate and form flocs. Mechanical stirrers can be used to cause

turbulence in the water to facilitate the coagulation and flocculation (growth of the flocs) (19,21). In this process, metal hydroxides are precipitated and turbidity caused by suspended inorganic and organic particles is reduced (20). Flocs and remaining suspended particles are subsequently removed by sand filtration. This can be preceded by sedimentation because the flocs are heavier than water. After sand filtration, water softening is done with sodium hydroxide and disinfection and oxidation takes place to stabilize the biological quality of the finished water, remove colour and odour or to inactivate pathogens (20). Due to oxidation, organic compounds will degrade into CO_2 and H_2O. Chemical oxidants are added such as hydrogen peroxide or ozone for oxidation and free chlorine or chloramines for disinfection (20). These processes with oxidative chlorine species generate unwanted halogenated by-products (7). Activated carbon filtration is used to remove those by-products. As presented in Figure 1, disinfection takes place only after the activated carbon filtration. Since chlorination also generates micropollutants, only a limited dose of Cl_2 is added (22).

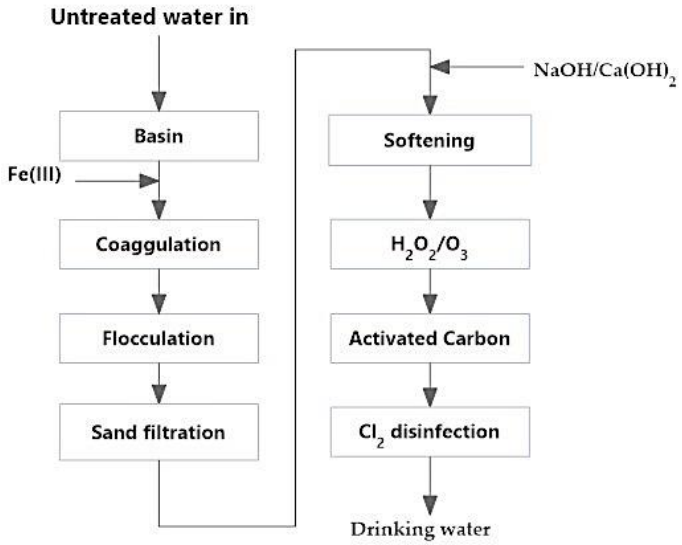

Figure 1: An illustration of a traditional drinking water treatment process (22).

2.2.3. Current techniques for the removal of micropollutants from drinking water

Bank filtration is one of the most basic methods of water purification and for some micropollutants it has been demonstrated to be efficient for removal (20,24). But, bank filtration is just an effective pre-treatment preceding the intake at a drinking water treatment plant (20). Aeration of anoxic groundwater creates an oxic environment in which a wide range of pharmaceuticals were preferentially and at least partially removed. This proves that aeration is not only used to physically strip gases, but that it also plays a role in the removal of micropollutants during drinking water treatment. However, the removal is often not significant because it depends on the metabolic activity of the microbial community and the hydraulic residence time of the aeration basin (20). Also, in the step of coagulation, flocculation and sedimentation, removal of micropollutants can take place, depending on the physicochemical

properties of the chemicals. Hydrophobic micropollutants with relatively high log K_{ow} (>6) values and low water solubility can be removed. Polar and semi-polar pesticides and pharmaceuticals on the other hand will remain partitioned in the aqueous phase and consequently insufficient removal of micropollutants takes place (20). After sedimentation, inorganic species and remaining particles are removed or transformed in rapid and slow sand filters. Next are the advanced oxidation and disinfection practices, designed to control biological stability of drinking water. The removal of micropollutants from drinking water can be a side-effect of these processes. But persistent and potentially toxic by-products can be formed during oxidation or disinfection. This together with high operational and maintenance costs creates uncertainty in the advantage of these processes in comparison with the cost (20,25). At last, activated carbon filters, particularly granular activated carbon (GAC), are used to remove residual assimilable organic carbon (AOC) and other nonpolar and semi-polar to polar micropollutants from water. The operational and maintenance costs are also high for this process and as a result is not considered to be a sustainable technology (20,26). In a study, implemented by Ormad (2007), it was demonstrated that adsorption onto activated carbon is very efficient for removing several studied pesticides from water, but, the pesticides pass only from one medium to another and are not degraded (26).

2.3. Case specific: Dichlobenil and BAM

2.3.1. Dichlobenil and BAM contamination of groundwater resources

Dichlobenil is a worldwide used broad-spectrum nitrile herbicide (Figure 2) and is mostly used in non-agriculture areas, such as private gardens, in plant nurseries, railway lines, motorways, fruit orchards etc. (27)(28). It has been applied for control of annual and perennial grasses and broad leaved weeds but also to remove tree roots and prevent their growth in sewers (28). In aquatic habitats dichlobenil has also been implemented for control of submerged and floating aquatic weeds, particularly in association with aquaculture (27). The intensive use of this pesticide and other pesticides in agriculture, on public land and in private gardens induces a potential risk of contamination of groundwater resources. This threat is especially relevant for persistent and mobile compounds because it entails a greater risk of leaching from topsoil to underlying aquifers (29). Dichlobenil is relatively persistent with high sorption to soil and sediment (Table 1) and therefore it is not as mobile (27). As a result of the low mobility, there is little or no downward transport of dichlobenil with water movement. Groundwater contamination of this pesticide is very low or even negligible due to this immobilization and is thus not a great problem (28)(30). However, when added directly to a waterbody, dichlobenil can still be problematic (28). Besides its high sorption to particles, dichlobenil is also relative volatile compared to

other herbicides. So transportation to the ocean takes place not only through rivers but also through the atmosphere (27,28).

Not only pesticides, but also their persistent metabolites, which are produced from partial degradation, are of increasing concern (31). The stable metabolites are more frequently detected than the pesticide itself (32). Dichlobenil has some possible metabolites like 2,6-dichlorobenzoic acid (DCBA), *ortho*-chlorobenzamide (OBAM) and 2,6-dichlorobenzamide (BAM) (Figure 2). The latter one is the main metabolite of dichlobenil in environmental samples and pure bacterial cultures (29).

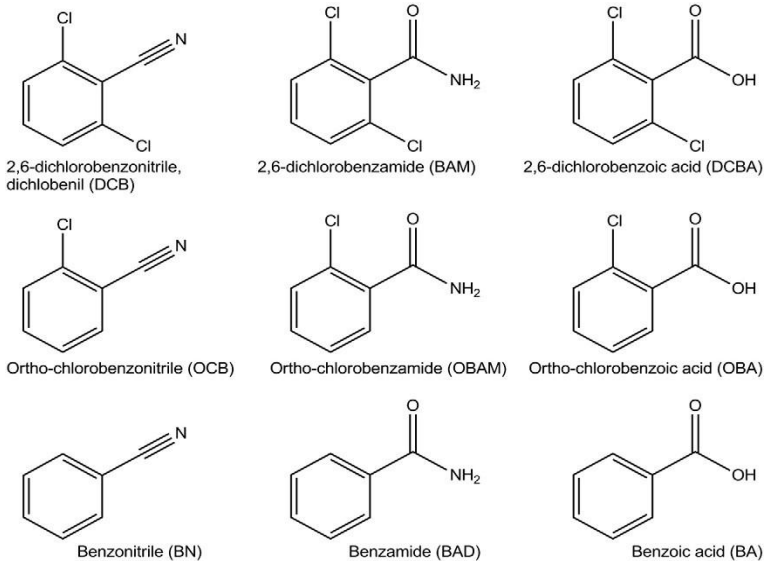

Figure 2: Dichlobenil, 2,6-dichlorobenzamide and degradation products thereof as suggested by Holtze et al. (2007). E. Björklund et al. / Science of the Total Environment 409 (2011) 2343–2356 (31)

Compared to dichlobenil, the metabolite BAM is more water soluble (see Table 1). Because BAM is less volatile, resulting in a higher Henry constant (K_H), it is highly unlikely that BAM is transported from soil and water bodies to the air. But BAM can be present in the air since dichlobenil is relative volatile and thus transported through air, where it can be degraded to BAM (28). In addition, sorption of BAM to soil and surface sediments is 10-100 weaker compared to sorption of dichlobenil. As a result, BAM is much more mobile than dichlobenil where it will contaminate groundwater more readily (28,29,31). As such BAM is detected in an increasing number of European aquifers, while detection of dichlobenil in groundwater is relatively rare (31,33). Furthermore, BAM has been demonstrated to have low degradation rates in aquifers and as a result it remains in the water body over time. Since drinking water production in Flanders and many other countries is based for more than 50% on groundwater, BAM may pose a risk to human health (27,34).

Table 1: Physical and chemical parameters of dichlobenil and BAM [15].

IUPAC name	2,6-dichlorobenzonitrile	2,6-dichlorobenzamide
Abbreviation	DCB	BAM
CAS RN	[1194-65-6]	[2008-58-4]
Molecular weight (g mol^{-1})	172.0	190.0
Melting point (C)	139; 145	196; 199
Vapour pressure (mPa)	88 (20 C)	E
Henry's law constant, K_H (atm m^3 mol^{-1})	1.01 x 10^5	1.22 x 10^9
K_{oc} (l kg^{-1})	500; 896	33; 35
Solubility in H_2O (mg l^{-1})	18 (20 C)	2730 (23 C)
LD50 (mg kg^{-1})	1014; 4460	1144; 2330

In 1997, dichlobenil was banned in Denmark because during 1992 until 2002 BAM was detected in 21.4% of the investigated groundwater wells. When looking at the concentrations allowed by the European Union drinking water guideline, 6.6% of the wells in Denmark exceeds this limit. As a result numerous groundwater abstraction wells had to be closed, which was very costly because 99% of drinking water in Denmark originates from groundwater (32,35). It can be inferred that in other countries, where dichlobenil was still intensively used, groundwater would also be found contaminated with BAM, if, of course, this metabolite was included in their monitoring programs (36). In 2008, herbicides containing dichlobenil formulations were banned in EU. Despite this, concentrations of BAM in groundwater is still growing since BAM has a high half-life (29,30,37). In Flanders, groundwater analyses are carried out twice a year by the Flanders Environment Agency. In 2013, BAM was detected in 10-20% of the measurement points. In Figure 3 the measured concentrations of BAM in 2010 are mapped. So we can deduce that there are three problem areas in Flanders (Southwest of West-Flanders, southeast of Ghent and around Sint-Truiden). As seen in Figure 4, BAM has the highest percentage of water abstraction for which the concentration is above the European threshold of 0,1 µg/L (13) compared to other pesticides. In addition, BAM was also detected in drinking water but exceedance of the norm is less common compared to groundwater samples (37).

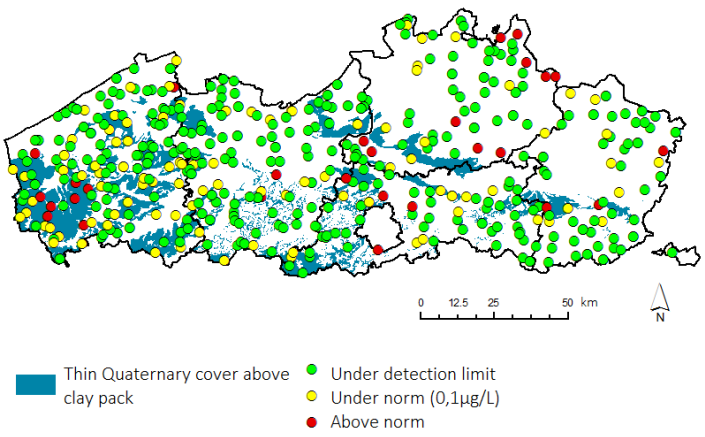

Figure 3: BAM detected in concentrations under detection limit, under norm or above norm (measured in 2010). Blue areas are zones with a thin Quaternary cover above clay pack (37).

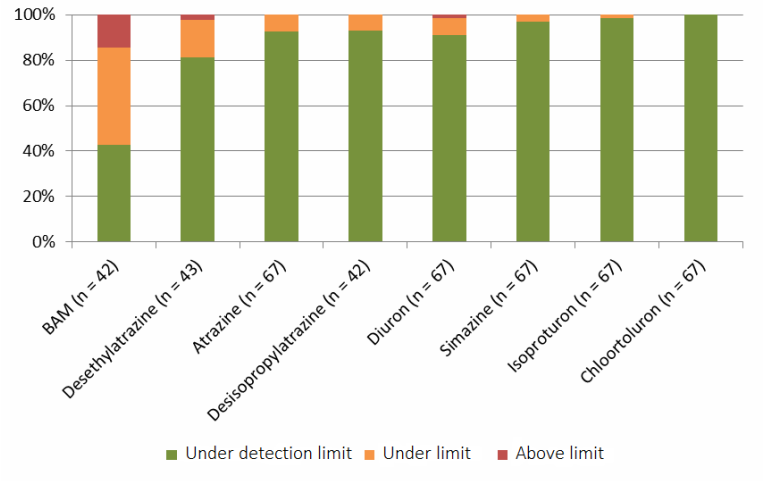

Figure 4: Percentage of abstracted waters for which the pesticide concentration in 2009 remains below detection limit, norm (0,1 µg/L) or exceeds the norm (37)

2.3.2. Risks

Dichlobenil and BAM have two kinds of potential risks, one to biota and one to humans. Looking at the potential risks to biota, most studies indicate LC_{50} values for dichlobenil and BAM respectively in the low mg/L range and high mg/L range. Compared to the maximum reported concentrations of dichlobenil and BAM in river or surface waters in different countries, the LC_{50} values are 10000 times higher. Risk quotients (RQ) can be calculated for dichlobenil using the highest concentration of dichlobenil found in the environment, the most sensitive end point found and a safety factor, defined by the Uniform Principles of the EU Directive 91/414/EEC Annex VI. When RQ is lower than one, there is an acceptable risk. If on the other hand RQ is above 1, measures to reduce the exposure should be taken since the risk is unacceptable. The RQ for photosynthesis inhibition in natural planktons is equal to 0,023 according

to E. Björklund (27). Even lower RQ's are found for BAM (2.4 x 10^{-4}). From this it shows that any immediate toxic threat is unlikely because the RQ's are much lower than 1.

Next, there are the potential risks to humans by exposure via food or drinking water. Despite the fact that studies on dichlobenil and BAM in food are few, it is most unlikely that they should cause any risk to human health via food (27), (38). In addition, humans are mainly exposed to dichlobenil and BAM via drinking water, obtained from surface water and mostly groundwater. BAM and dichlobenil show toxicity in the same range of several hundred mg/kg, but BAM has been observed in groundwater at higher concentrations compared to dichlobenil (27). A margin of safety (MOS) can be calculated when using the highest concentration in groundwater and the no observed adverse effect level (NOAEL) from chronic experiments on dogs, respectively equal to 2.8 x 10^{-3} mg/L and 1.25 mg/kg/day. Assuming a daily drinking water intake of two litres and a body weight of 70 kilo, the MOS for dichlobenil is above 15000 which is demonstrating that there is no significant risk (27). BAM has higher concentrations in groundwater than in surface water. Although these concentrations are higher than those of dichlobenil, they are still much lower than the concentrations needed for acute toxicity. Thus risks to humans due to dichlobenil and BAM via drinking water are extremely low and even lower in Europe, as dichlobenil was banned since 2008 (27,31). Despite this ban, BAM will still be present in groundwater for a long time because this metabolite has a very long half-life (29,37). According to Björklund *et al.* (27), the low toxicity of BAM can be explained by its high water solubility which ensures rapid filtration in the kidneys and excretion in the urine. Also one should bear in mind that carcinogenic and endocrine disrupting effects of long-time exposure to low concentrations of BAM in drinking water are unknown (31). Both dichlobenil and BAM are divided into the group of substances that have a proven, probable or possible carcinogenicity, but only BAM was detected in drinking water (39). Dichlobenil is considered possible human carcinogen according to the US EPA and the Pesticide Action Network, since data show limited evidence of carcinogenicity in the absence of human data. BAM is suggested to be equal or even less carcinogenic compared to dichlobenil. US EPA has placed BAM in toxicity category III. Last but not least, it is important to consider the fact that BAM which has reached groundwater is able to create transformation products that may be potentially toxic (27).

2.3.3. Degradation and mineralization of dichlobenil

A lot of studies about the dissipation of dichlobenil are available, but the underlying process or its metabolites were still barely explored. These studies show that biodegradation of dichlobenil is widespread since it occurs both in dichlobenil pre-exposed soils and not pre-exposed soils (31). The effect of dichlobenil pre-treatment of soils on the degradation of dichlobenil was accessed in a study by Maria S. Holtze et al. In this study, dissipation of dichlobenil was seen in all soils (pre-exposed, not pre-exposed and sterile controls). This dissipation in sterile soils can be explained by evaporation and

sorption to soil particles. In order to determine whether the dissipation in non-sterile soils is due to degradation by microorganisms, the concentration of dichlobenil over time in each of the non-sterile soils is compared to the concentration of their sterile control. This study shows that significant degradation of dichlobenil occurred in both pre-exposed and not pre-exposed soils (29,33). This indicates that numerous native microorganisms are capable of degrading dichlobenil to BAM, through nitrile hydratases, see Figure 5 (30,33). It was demonstrated, by Sørensen (32) that nitrile hydratases have a low specificity. Strains, capable of hydrolysing dichlobenil to BAM were isolated from not dichlobenil pre-exposed soils and they were all enriched using different aliphatic or aromatic nitriles as a single source for nitrogen and carbon (31). Degradation of dichlobenil to its main metabolite BAM can be described by first-order rate law equations and as such kinetics indicate no proliferation of the degrader population. Other explanations could be that the degradation is co-metabolic or that sorption of dichlobenil has an influence on the degradation (31,33).

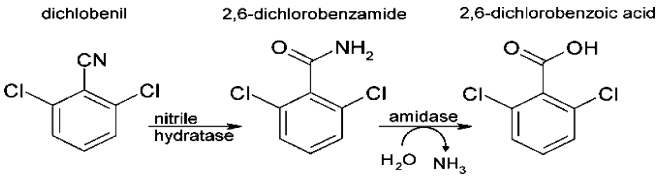

Figure 5: Hydrolysis of dichlobenil to 2,6-Dichlorobenzamide (BAM) catalysed by nitrile hydratase and further degradation of BAM to 2,6-dichlorobenzoic acid catalysed by amidase (30).

2.3.4. Degradation and mineralization of BAM by *Aminobacter* sp. MSH1

Dichlobenil is biodegradable in soils but total mineralisation is rare. A bacterial adaptation, caused by prior exposure to dichlobenil, is required for this total mineralisation (28,31). This adaptation, obtained by different events such as horizontal gene transfer or the modification of the specificity of existing enzymes, allows particular bacteria to grow on the persistent metabolite BAM derived from dichlobenil (31). In the study by Maria S. Holtze et al., the effect of prior exposure of soils to dichlobenil on the degradation of BAM was also accessed. Dissipation should relate to biotic degradation because no decrease in BAM-concentration was detected in the sterile soils (29,33,35). This study also showed conversion of BAM to 2,6-DCBA but no significant mineralisation of BAM occurred in soils not previously exposed to dichlobenil, resulting in accumulation of 2,6-DCBA. In pre-exposed soils, however, BAM was mineralised following a sigmoid curve. It was concluded that the degradation of BAM resulted in growth of indigenous microorganisms. As conclusion, dichlobenil is slowly degraded into BAM by indigenous soil microorganisms independent of exposure history. BAM, on the other hand, is only mineralized to CO_2 by adapted microorganisms from dichlobenil pre-exposed soils (29).

For isolation of BAM-mineralizing bacteria, a large-scale screening of areas treated with dichlobenil was completed to find soils capable of mineralizing BAM fast. Only two BAM-mineralizing bacteria were

isolated (30). A first was enriched and isolated from a soil with BAM mineralization but no obvious dichlobenil mineralization. This bacterium was eventually identified and named *Aminobacter* sp. strain ASI1 using 16S rRNA gene sequencing and fatty acid analysis. From another soil a BAM- and dichlobenil-mineralizing bacterium was isolated, identified as *Aminobacter* sp. strain MSH1 (32,35). This bacterium uses BAM as well as dichlobenil as energy, carbon and nitrogen source for growth. According to a study performed by Sørensen, *Aminobacter* sp. strain ASI1 showed lower cell densities compared to strain MSH1 after growth on different BAM-concentrations (32). Both strain ASI1 and MSH1 are capable of mineralizing the ring structure. In addition, ecologically relevant concentrations of BAM can be degraded efficiently by both strains, resulting in the capacity to mineralize BAM below the limit of 0.1 µg/L. Thus *Aminobacter* sp. strain MSH1 was suggested as a promising candidate for use in bioremediation processes of BAM-contaminated drinking water resources with very low concentrations of BAM or dichlobenil (32,35).

Accumulation of 2,6-DCBA was detected during growth on BAM, which indicated that the first step in the BAM-degradation pathway of *Aminobacter* sp. MSH1 and ASI1 was catalysed by BbdA, an constitutively expressed amidase (35,40). This amidase was encoded on a 40.6 kb IncP-1β plasmid (pBAM1). This constitutive expression of the amidase, derived from the fact that BAM-degradation rate was equal whether the cells were pre-grown in the presence or absence of BAM, indicates that its expression does not depend on BAM-mediated induction. This is important when the strain is used to remove trace levels of BAM from drinking water (40). 2,6-DCBA is a potential toxic groundwater contaminant due to its high leaching potential. Additional research showed that DCBA catabolic pathway in *Aminobacter* sp. MSH1 is initiated by the oxygenase BbdD, encoded on the 57.8 kb plasmid pBAM2 (*repABC*-type plasmid). Other genes that are possibly involved in the DCBA catabolic pathway; are also encoded on this plasmid (T'Syen, unpublished). According to T'Syen, the loss of the competence to mineralize DCBA is linked to the loss of the entire plasmid pBAM2. In order to mineralize BAM, it is demonstrated that an *Aminobacter* sp. MSH1 cells must possess both pBAM1 and pBAM2 (T'Syen, unpublished). Further research is need to identify different metabolites in the degradation pathway and to eventually known the entire degradation pathway (32,35).

2.4. Bioaugmentation in sand filters

2.4.1. Bioremediation: natural attenuation, biostimulation and bioaugmentation

Since contamination is most of the time already present, remediation is used to reduce the pollutant level. Microorganisms can have the capability to degrade or transform xenobiotic compounds to non-toxic compounds or to mineralize the pollutants to energy, carbon (CO_2) and nutrients (N, P, H_2O) for their growth. These processes are typically brought together under the term "biodegradation". When

organisms, mostly microorganisms, are used to clean up the contamination, this is called bioremediation. Bioremediation was studied mostly in the soil because of the natural occurrence of microorganisms and since soils are often very strongly polluted (41).

A first class of bioremediation technology is the monitoring of natural attenuation. Natural attenuation refers to a passive remediation process which requires the presence of intrinsic microorganisms in the environment that are able to degrade or transform a contaminant within a reasonable time (42). This natural attenuation should be monitored to confirm that degradation takes place rapidly enough and without the production of unwanted metabolites. When natural systems are not adapted for rapid degradation of the anthropogenic compound of interest, biostimulation could be a good bioremediation technology. In this technique, the physical and/or chemical characteristics of the soil are altered in order to stimulate natural biodegradation. An example is the use of biosparging whereby air is injected under the water table to increase the oxygen concentration in the groundwater to maintain aerobic processes (42,43). Since the indigenous bacteria are already accustomed to the conditions of the site (temperature, pH and nutrient availability), they are better suited for use at that site compared to foreign bacteria (43). However, when the organisms responsible for the degradation of the target pollutant do not exist at the site or are not present in sufficient numbers, bioaugmentation is used. This is a third class of bioremediation technology (41,44).

Bioaugmentation is the addition of a preadapted bacterial strain or consortium, isolated or enriched from a contaminated site where rapid degradation of the pollutant was detected (41,44). To monitor this process, biomarkers based on g*fp* or *luc* were used to track the efficiency of the inoculated bacterium or consortium (42). This foreign bacterium or consortium is responsible for the partial degradation or total mineralization of the xenobiotic compound (20). When partial degradation takes place, oxidized metabolites are formed, which can then be used as primary substrates for heterotrophic members of the population. This is called co-metabolic bioaugmentation, wherein the degrading bacteria will grow on nutrients, other than the pollutant (20,45). On the other hand, when total mineralization takes place, the specific catabolic strains will grow on the nutrients obtained by mineralization of the pollutant (see Figure 6) (20).

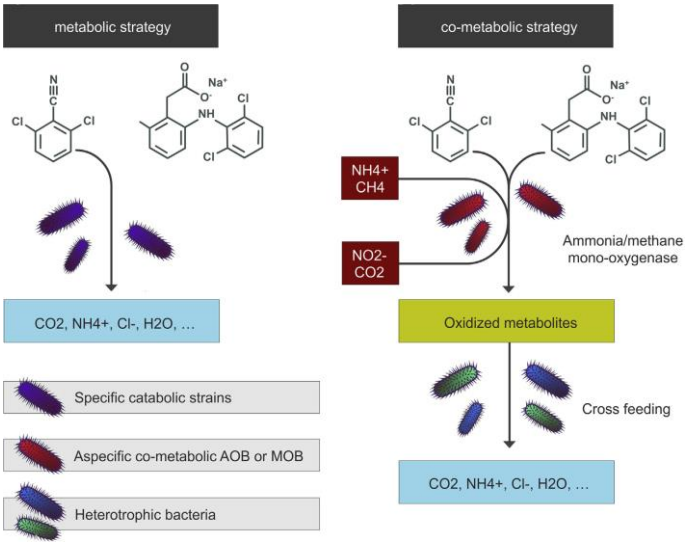

Figure 6: Schematic of the metabolic and co-metabolic bioaugmentation strategies; AOB: ammonia oxidizing bacteria; MOB: methane oxidizing bacteria (20).

A bioaugmentation study, which was successful in the laboratory, does not necessarily lead to a success for decontamination *in situ* (44). This is because a number of challenges need to be faced. A first challenge is that the foreign bacteria are outcompeted by endogenous microorganisms, resulting in insufficient population levels for biodegradation. Thus integration of the strain in the existing microbial community would determine partly the success of bioaugmentation together with the level of activity of the strain used for bioaugmentation (20,43,44,46). A second problem can occur when partial degradation is responsible for the production of potentially volatile compounds or metabolites that are more toxic compared to the parent pollutant (20,42,43). Co-contaminated sites (e.g. contaminated with heavy metals and pesticides) can also ensure a challenge, since high concentrations of co-contaminants (e.g. heavy metals) may inhibit the organic compound degradation capacity of the microbial population (44). The use of a consortium is preferred since the pollutant degradation is supported through a synergy between the bacteria and a community of other bacteria. In addition, a consortium has a higher robustness and metabolic diversity (41,43,44). A fourth challenge arises from the actual inoculation since the inoculum for bioaugmentation is grown under ideal conditions in bioreactors. When this inoculum is then inoculated, the microbial inoculants come in contact with complex natural conditions so that they certainly will experience stress. As a result, a decrease of the introduced population will occur (44,45). Other factors of concern (listed in Table 2) are extremes in pH, temperature or moisture content, nutrient availability, predation, etc. which are all factors that influence microbial growth in general (44,45).

Table 2: Factors affecting microbial growth in general and the bioaugmentation potential (44).

Factors	Description
pH	Biodegradation is inhibited by extreme pH conditions
Temperature	Microbial growth and degradation are both influenced by temperature
Moisture	High moisture reduces aeration in soils; low moisture inhibits microbial growth and
Competition	Inoculated microorganisms undergo competition for nutrients with the autochthonous
Predation	Decrease of inoculated microorganisms due to overgrowth of protozoa
Loss of microbial viability during inoculation	Stress as a result of environmental changes during inoculation
Cell death after inoculation	Death because of toxicity of contaminants or depletion of nutrients

Apart from the challenges pointed out above, bioaugmentation also has some advantages. Bioremediation strategies are natural processes that use resources available in nature to clean up the contamination. Therefore they are accepted by the public as a waste treatment process. Furthermore, it is a more eco-friendly technique when total mineralization takes place since the pollutant is no longer transferred from one medium to another but is completely destructed. Besides, harsh chemicals are no longer used in bioremediation techniques resulting in a reduction of damaging effects on the environment (42). In addition, this technique is also more cost-effective and efficient compared to chemical treatment or physical removal as it can be carried out on site and thus eliminates the need to transport quantities of waste. So this also removes the potential threats to human health and the environment associated with this transport (43,44).

2.4.2. Interactions between bacteria

2.4.2.1. Interaction mechanisms of bacteria

To understand the interactions in natural microbial communities, the mechanisms used by microorganisms to interact are first discussed. According to Little et al., two main types of interactions can be distinguished, symbiotic and antagonistic interactions (47). The symbiotic interactions are divided into three overlapping categories. A first category of symbiosis is a mutualistic association in

which both organisms derive profit from one another. Parasitism is the other extreme of symbiosis. One speaks about parasitism when an organism lives on or in another organism and is completely or partly dependent on its host, while the host will consequently experience negative effects. At last, commensalism is a final type of symbiosis which fits in between parasitism and mutualism. One organism still derives benefit from another organism but in this case the latest organism experiences neither harm nor benefit (47). Finally, it should also be mentioned that commensalism and mutualism can be either obligated or facultative. When the symbiosis is facultative, the population can survive in the absence of the other partner, while this is not the case with obligate associations (47). In addition to symbiotic interactions, microorganisms can also interact antagonistically with others via competition. Two kinds of competition can be separated, exploitative and interference competition. Exploitation competition is indirect and can also be described as competition for nutrient and space. On the other hand, interference competition occurs directly between individuals via antagonism. As an example, organisms can produce compounds that will directly inhibit growth of potential competitors or will kill them off (47).

2.4.2.2. Interactions between bacteria in biofilms

It is generally known that biofilms are the dominant lifestyle of bacteria both in natural environments, except for deep ground- and seawaters, and on manmade settings (48,49). A biofilm can be defined as a highly organized, cohesive community of either a single, but mostly of multiple microbial species attached to a biological or inert surface and embedded in a self-synthesized extracellular polymeric substances (EPS) matrix (42,48–51). As it appeared from looking at intact biofilms under microscope, biofilms are will-organized communities with specialized configurations instead of bacteria who randomly stick together (48). According to Flemming, the EPS matrix can account for over 90% of the dry mass, although the creation of this matrix is a very energy-consuming process (51). The composition of EPS can vary greatly between biofilms, depending on the microorganisms present, the experienced shear forces, the temperature and the nutrients available (52). But nevertheless the main components remain polysaccharides, proteins, nucleic acids and lipids in all EPS matrices and they provide a number of advantages for bacteria in biofilms. The main function of the EPS matrix is to protect biofilm bacteria against different stress factors, such as UV radiation, extreme pH values, etc. (50). Other functions of extracellular polymeric substances in bacterial biofilms are shown in Table 3.

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Table 3: Functions of extracellular polymeric substances in biofilms (52)

Function	Relevance for biofilms
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of desiccation in water-deficient environments
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protozoa
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms

Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community
Exchange of genetic information	Facilitates horizontal gene transfer between biofilm cells
Electron donor or acceptor	Permits redox activity in the biofilm matrix
Export of cell components	Releases cellular material as a result of metabolic turnover
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides

Due to the high cell density and the coexistence of diverse microbial species very close together, complex social interactions occur within and between species. These interactions can be ether competitive or cooperative (48,52). Examples of competitive interactions in biofilms are production of toxins, preventing adhesion of other microorganisms, acidifying surrounding, predation, etc. (49). They all lead to separate growth. In contrast, species are located very close to each other when cooperative interactions such as division of labour, collective actions, etc. take place. An improved overall fitness of the biofilm consortium is the result of cooperative interactions (49).

2.4.2.3. Role of biofilms in bioremediation

Relative to free-floating planktonic cells, bacteria in biofilms have a greater tolerance to pollutants, protective immune factors, unfavourable environmental factors etc. due to cell-to-cell interactions (42,49,50). These cell-to-cell interactions between bacteria in biofilms control the expression of numerous genes at different locations in the biofilms and at different stages of their development (48,50,51). Consequently, biofilms are very dynamic allowing them to survive environment fluctuations (49). In addition, degradation of different pollutants by numerous metabolic pathway can be promoted by such variable gene expressions (42). Lappin et al. (1985) observed that a synergistic community was able to degrade the herbicide Mecoprop. However, none of the individual strains could degrade the herbicide (49). This demonstrates that cooperation between bacteria in biofilms stimulates the degradation of otherwise non-degradable complex compounds. A biofilm should therefore not be viewed as just the sum of all individuals present in the biofilm (49). Furthermore, biofilms absorb immobilize and degrade various environmental pollutants making biofilms interesting to use for bioremediation (42).

2.4.3. Bioaugmentation in DWTPs (sand filters)

As mentioned above in the section of 'current techniques for the removal of micropollutants from drinking water', sand filters used for drinking water production contain native bacterial populations. But microorganisms that possess the required catabolic genes to degrade or mineralize micropollutants, are unfortunately rarely dominating in these autochthonous bacterial communities (20,53). Consequently, bioaugmentation of sand filters, used for drinking water treatment processes, with microbial strains that are able to degrade pesticides and micropollutants might be a promising strategy for improving the degradation and mineralization of pesticides. A lot of examples of successful bioaugmentation of soils are available. In contrast, research on the use of bioaugmentation for drinking water applications was still limited but the results were quite promising (20). For example, it was shown by McDowall *et al.* (54) that bioaugmentation of laboratory-scale sand filters with a geosmin-degrading bacterial consortium resulted in improved removal of geosmin and 2-methylisoborneol (MIB), both taste and odour compounds. This bacterial consortium was isolated from the sand filter at a full-scale DWTP that showed geosmin and MIB degradation (54). Another example showing the potential for bioaugmentation of sand filter materials is given by Krüger *et al.* (55). In this study, mineralization of 4-chloro-3methylphenoxyacetic acid (MCPA) was promoted by inoculating the laboratory-scale sand filters with MCPA-degrading bacteria (55). Nevertheless, first a successful isolation and identification of a micropollutant degrading or mineralizing strain must take place. As mentioned above in Section 2.4.1, two main catalytic processes employed by the microorganisms were observed, growth-linked processes and co-metabolic reactions (20). Before bioaugmentation can take place, a pre-culturing of the isolated strain is required to obtain the right cell density. Next, the success of the actual inoculation in a sand filter depends on the capability of the strain to invade successfully in the autochthonous bacterial community. In addition, the microorganisms need to survive in oligotrophic conditions, since groundwater contains only 100 µg AOC/L (53,56). Until now, most observations were made at higher concentrations of the pollutant, so research must still determine whether mineralization will also occur at lower, environmental micropollutant concentrations (20).

Before implementation of the bioaugmentation into sand filters, one must also take into account the additional technical limitations. First, the hydrodynamics of the process can affect the efficiency of micropollutant removal. However, further studies are needed to determine the actual influence of hydraulic retention time and hydraulic loading rates on pollutant degradation (20). A second limitation occurs when the process is diffusion limited since at that point only little removal will be realised, even when the individual strain is physiologically capable of degrading minor concentrations of a pollutant (20,53). Third, limited attachment can occur as a result of hydraulic or physical conditions. The retention of biomass in the system, which is of great importance for the success of bioaugmentation, can be

enhanced using carrier materials (20,53). As a final point, the primary function of the sand filter must not be forgotten. As a consequence, strains or consortia that were added to the sand filter must be able to bear the routine and maintenance operations (e.g. backwashing) while still providing the intended additional benefits (20).

2.4.4. Bioaugmentation to remove BAM in pilot scale sand filters

As mentioned above, *Aminobacter* sp. MSH1 strains are able to degrade BAM to DCBA or to mineralise BAM totally. In a previous laboratory sand filter study of Albers *et al.*, it was shown that MSH1 stick well to sand particles and degrades BAM-concentrations of 3 µg/L well to below to European threshold limit of 0.1 µg/L (57). Next, two pilot scale studies were executed in Denmark and Belgium.

A first experiment was set up by Albers *et al.* to examine the potential of bioaugmentation with *Aminobacter* sp. MSH1 into pilot waterworks sand filters to remediate groundwater containing 0,2 µg/L 2,6-dichlorobenzamide (BAM) (53). The second study compares both the use of suspended MSH1 cells and the use of MSH1 cells embedded in porous carriers for bioaugmentation of sand filters (Horemans, unpublished). Both studies finally showed that BAM-concentration decreased immediately after inoculation with *Aminobacter* sp. MSH1 to below 0.1 µg/L. In addition, no BAM-degradation products or adverse effects on other filter processes, such as oxidation of iron and ammonium, were detected in the first experiment. However, this efficiency of BAM-degradation is hard to maintain as seen in both studies, which showed that this efficiency decreased gradually to less than 20% resulting in BAM-concentrations finally exceeding the norm. This can be explained by loss of the inoculated bacteria, since the ratio of the *bbdA* mRNA transcripts per *bbdA* gene, responsible for the first step in the BAM-degradation, was stable (53). The period of BAM removal under 0.1 µg/L was two to three times longer when embedded cells were used compared to the strategy with suspended MSH1 cells (Horemans, unpublished). In the first study, Albers *et al.* gave four reasons for this loss of cells. The first explanation are losses through backwashing as many bacteria were mainly associated with iron oxide precipitate which were backwashed from the sand filter. Two other phenomena which contributed to the loss of bacteria are detachment with washout and predation by protozoa. Also starvation contributes to the decrease in BAM-degradation efficiency. Starvation could occur because groundwater used for feeding the sand filter contained only a small amount of AOC of which only a part could be utilized by *Aminobacter* sp. MSH1. In addition, the small amount of BAM (0,2 µg/L) in the influent is also inadequate to contribute as an energy source for *Aminobacter* sp. MSH1 (53). In the second study it was shown that cells embedded in porous carriers have higher biomass in the column compared to suspended cells. However, the total loss of cells is calculated to be equal for both strategies at steady state conditions. The embedded cells are lost due to grazing by protozoa or cell death by decreasing availability of AOC and nutrients, but not due to shear loss from the column. In addition, also an equal BAM removal (20%)

at steady state was obtained compared to suspended cells. Two different explanations are possible; a three times lower removal rate for embedded cells or that only one third of this embedded cells had access to BAM. It is demonstrated that MSH1 grows on residual AOC in oligotrophic conditions while degrading BAM, which influence BAM-mineralization since growth on AOC results in a decrease of BAM-mineralizing MSH1 cells. As such the amount of AOC available to MSH1 determines MSH1's growth and consequently steady state cell numbers and BAM removal. As a result, it makes no difference which strategy is used since the outcome is the same. Only the time needed to reach steady state is different. More specifically, steady state was reached faster with suspended cells compared to embedded cells. (Horemans, unpublished)

2.5. Methods for identification of invasion genes

As mentioned above in 2.4.3 is the success of bioaugmentation in a sand filter mainly determined by the capability of the foreign strain to successfully invade the autochthonous bacterial community and to survive the oligotrophic sand filter conditions. In this sand filter environment, social interactions such as cooperation and/or competition occur (53). Consequently, insight on how the invading bacterial strain interacts with the autochthonous bacterial community is needed to predict the success of bioaugmentation. During interspecific bacterial interactions, responsive gene functions might be activated to produce antibiotics or resistance against toxins in order to compete or even cooperate with the autochthonous bacterial community (49). Hence, when *Aminobacter* sp. MSH1 is used for bioaugmentation of sand filters used in drinking water production, the success of the invasion depends on specific gene functions allowing MSH1 to interact with the sand filter community. Those genes responsible for a successful invasion are called social 'invasion' genes. Below, three techniques used to identify these 'invasion' genes are discussed, but in Figure 7 the advantages and disadvantages of each technique are already given.

Transcriptomics	Differential fluorescence induction	Transposon mutagenesis
✚ high reproducibility	✚ enrichement of cultures possible	✚ enrichement of cultures possible
✚ simple: no library construction	✚ semi-automated screening of large population	✚ semi-automated screening of large population
▬ bias due to cDNA synthesis and amplification	✚ detection of changes in expression level	✚ Distinction between essential and non-essential genes
▬ sufficient mRNA needed	✚ genes essential for growth also identified	▬ genes essential for growth NOT identified
	▬ Never complete genome coverage by Promoter probe library	▬ Never complete genome coverage by Transposon mutant library
	▬ post-transcriptionally regulated genes not detected	▬ FACS: •cell aggregation hinders FACS •bacterial cells at low end of detection capabilities •calibration needed before each experiment
	▬ FACS: •cell aggregation hinders FACS •bacterial cells at low end of detection capabilities •calibration needed before each experiment	

Figure 7: Advantages and disadvantages of three 'invasion' genes identification techniques; FACS: fluorescence-activated cell sorting; RNA-Seq: RNA sequencing.

2.5.1. Transcriptomics

The complete set of transcripts in a cell (messenger RNA) for a specific developmental stage or physiological condition is called the transcriptome (58,59). It reflects the genes that are actively expressed and is the template for protein synthesis in the translation process. Transcriptomics refers to the study of the mRNA within a cell or organism (58). Using transcriptomics, all species of transcripts can be catalogued, including mRNAs, non-coding RNAs and small RNAs. In addition, also the transcriptional structure of genes can be determined. A key aim of transcriptomics is to quantify the differential expression of each transcript during development and under various conditions (59).

Hybridization- and sequence-based technologies have already been developed to deduce and quantify the transcriptome. For the first type of approaches custom-made or commercial high-density oligo microarrays are used to incubate fluorescently labelled cDNA. Transcriptomic analysis using microarrays have already been conducted for various bacteria. Some examples are *Drosophila melanogaster* (60), *Porphyromonas gingivalis* (61), *Burkholderia cepacia* (62), *Escherichia coli* (63), *Zymomonas mobilis* (64).

The benefits are that these hybridization-based approaches are high throughput and relatively inexpensive. However, these techniques can only be used for model organisms since knowledge about the genome sequence is required. Further limitations are the high background levels due to cross-hybridization and the saturation of signals. These two limitations together create a limited detection range. At last, complicated normalization methods can be required so it becomes possible to compare expression levels across different experiments (59). In the second type of approaches, the cDNA sequence was directly determined. But the use of traditional sequencing is limited because it is rather low throughput, expensive and not quantitative. Moreover, only a part of the transcript is analysed and isoforms are in general not easy distinguished from each other (59).

Recently, RNA sequencing (RNA-Seq) was developed for both mapping and quantifying transcriptomes and uses deep-sequencing technologies. First, RNA is converted to cDNA fragments with adaptors at one or both ends. Then these fragments may or may not be amplified after which they are sequenced using single-end or pair-end sequencing. 454 pyrosequencing system is often used (59,65–67). RNA-Seq has some advantages compared to the existing technologies, including no limitations for non-model organisms, the ability to study complex transcriptomes and to quantify expression levels very accurately, and high levels of reproductivity. In addition, no or very low background signals and no upper limit for quantification result in a large dynamic range of expression level detection. At last, less RNA samples are required for RNA-Seq (59,68). On the other hand, there are still some challenges that must be taken into account. A first challenge is the production of cDNA libraries since several manipulations should be performed which can complicate the use of the libraries or the analysis of RNA-Seq results. Secondly, there are also bioinformatics challenges that should be addressed whereas large amounts of data has to be stored, retrieved and processed. This requires the development of efficient informatics methods. A last challenge refers to the relation between sequence coverage and cost. Sequence coverage is the percentage of transcripts that are measured. The cost increases when a greater coverage is preferred since this requires more sequencing depth. According to Z. Wang et al., there can generally be stated that the larger the genome, the more complex the transcriptome and the more sequencing depth is required for adequate coverage. As a result, a consideration must be made between the preferred coverage and the associated cost (59).

Differential transcriptomic is a first technique used to identify 'invasion' genes of *Aminobacter* sp. MSH1. The benefits and disadvantages of this technique are given in Figure 7. MSH1 is grown in biofilms with and without the sand filter community, whereupon mRNA is extracted and sequenced. The sequences with elevated abundance in the multispecies biofilms will be considered as possible 'invasion' genes.

2.5.2. Differential fluorescence induction

Differential fluorescence induction (DFI) is a technique used to monitor promoters that are preferentially active under given growth conditions (69–73). For this technique, a plasmid-based promoter probe library needs to be constructed (70). Previously, chromosomal DNA of the bacterium of interest was fragmented randomly. Hereafter, the random fragments are fused upstream to a promoterless reporter gene in a plasmid vector, which is next harboured in the bacterium of interest. This constitutes the promoter probe library (70). Recently, autofluorescent proteins (AFPs) are used widely as reporter gene such as green fluorescent protein (GFP) or its derivatives. GFP has a great advantage as reporter protein because it does not involve any cofactors for expression, it is not toxic in the target bacterium and it can be detected in many conditions such as in agar plates or fluorescence-activated cell sorting (FACS), etc. This results in the possibility to analyse promoter activity on the single-cell level (70,72,74). However, the use of GFP also brings disadvantages including restrictions to the pH range, oxygen requirement for cyclization of the chromophore and the absence of signal amplification (71). After construction, the promoter probe library can be cultured in different conditions. GFP is expressed in a library cell when the promotor is active under a specific condition and those green fluorescent cells can be isolated and analysed by FACS (70,71). The sequence of the genomic promoter-containing inserts of the isolated fluorescent cells can be determined and compared to the entire genome sequence of the target organism, resulting in the identification of DNA sequences that caused increased expression of the promoterless *gfp* gene in a certain condition. The products of those genes may be associated with the response of the organism to a given environmental signal (70,75). By comparing these DNA sequences between different conditions, it is possible to identify differential gene expression in various conditions (69,70). By sequencing promoter-containing library fragments, in addition, new genes can be identified. The function of these unknown induced genes may be determined by means of DFI, based on their induction profiles. The sequences of those genes could next be mapped on the genome sequence of the bacterium of interest. But further research is absolutely required before a function can be allocated to a gene (70,75).

This technique can be used to identify 'invasion' genes of *Aminobacter* sp. MSH1 by using a continuous flow cell system. After construction of a MSH1 promoter probe library, this library is grown in multispecies and monospecies biofilms, with and without an ASFC respectively. For both conditions, the fluorescent and non-fluorescent MSH1 cells are separated using FACS and regrown as monospecies biofilms. Using Illumina sequencing, the sequences of the DNA sequences in front of GFP are determined for both GFP and non-GFP population and compared with genes in the MSH1 draft genome sequence. By comparing the sequences of the GFP population with the non-GFP population, differentially expressed genes, which are possible 'invasion' genes are identified.

DFI is an attractive technique because it provides a semi-automated screening of large populations with high reproducibility (71,75). Secondly, the fluorescence window can be altered to detect changes in level of expression (71,72). Next, the chromosomal copy of specific genes is not affected and genes essential for growth in culture are also identified by DFI. Furthermore, DFI allows identification of differentially regulated genes regardless of the basal expression level (70,75). Lastly, the isolation of *in vivo*-induced promoters is facilitated by using plasmids (71). However, it is difficult to construct a promoter probe library that completely covers the genome of the bacterium of interest. Another disadvantage includes the inability to detect genes that are regulated post-transcriptionally as DFI is based on promoter trapping. Next, aggregation of bacteria or macrophages can cause problems during the flow cytometric analysis and sorting (71). In addition, the bacteria are at the low end of the detection capabilities of flow cytometers which complicates the sorting of the bacteria (72). Since the fluorescent signals of GFP are nonlinear, the linear range of the signal needs to be calibrated and determined for each experiment to allow quantification of gene expression (71). The benefits and disadvantages are also given in Figure 7.

2.5.3. Transposon mutagenesis

To identify genes that are responsible for survival in specific conditions, a large transposon mutant library is often constructed of chromosomal DNA (76–78). Two techniques of mutagenesis are known, chemical and transposon mutagenesis. In chemical mutagenesis, DNA is exposed to a mutagen to alter one locus in the chromosome per cell. For transposon mutagenesis on the other hand, a piece of foreign, heterologous DNA is inserted into the chromosome to disrupt the expression of a certain gene (76). Transposon mutagenesis has some benefits compared to chemical mutagenesis such as the presence of antibacterial resistance determinants on the transposons that facilitate selection of mutants. In addition, the site of the mutation can be mapped in the transposon mutant by using semi-random arbitrary PCR sequencing methods. For chemical mutagenesis, such a method is not available. Third, the delivery of transposons to the receiver is relatively easy due to conjugation, transduction or transformation (76). A last benefit, described by H.D. Kulasekara, is that no additional transposition events will occur within the same chromosome since the suicide vector carrying the transposase is located outside the transposable element (76). Consequently, transposon mutagenesis is preferred relative to chemical mutagenesis. S. Lewenza *et al.* demonstrated that a transposon mutant library can be used to identify differentially regulated genes from bacteria under different conditions (77). This was as well confirmed by a study of G.C. Langridge *et al.*, who also claim that this technique should work for any bacterium, as long as a sufficiently large mutant library can be obtained. This is determined by the presence of a suitable transposon and depends on the transformation rates (78).

Tn*5* and Tn*10* platforms are most frequently used as base for transposons in bacterial genetics (76). An example of the use of random mini-Tn*5* transposon mutagenesis was given by S. Lewenza et al. involving the generation of mutants in *Pseudomonas aeruginosa* PAO1 (77). Insertion of this transposon into a gene results in a random knockout mutation. The transposon can also encode a promoterless reporter gene (*phoA, lacZ, gfp, lux*), which may serve as a real-time reporter of gene expression of the inactivated gene (77). The pRL27 plasposon (given in Figure 8) is a transposable element from *Escherichia coli* that carries a modified Tn*5* transposon. One of the processes used by bacteria as a mode of genetic transfer is conjugation, which is used to deliver the transposon from *E. coli* into the bacterium of interest. The plasmid carries a segment for kanamycin resistance, allowing the mutants to grow on this antibiotic (79,80).

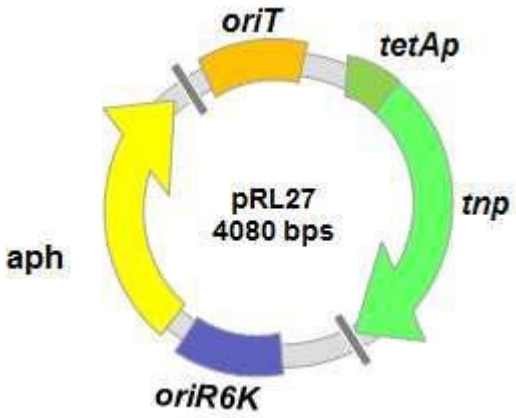

Figure 8: "pRL27 is a suicide plasmid that contains a transposon. The plasmid also carries kanamycin resistance (aph) and an origin of replication (oriR6K) to allow later cloning of transposon insertion sites. The oriT origin allows for transfer from host to donor. TetAp is a promoter that is functional in a wide array of organisms. Tnp codes for transposase, the protein responsible for the transposition event." (79).

Transposon mutagenesis is a third technique used to identify 'invasion' genes of *Aminobacter* sp. MSH1 by using a continuous flow cell system. The advantages and disadvantages of this technique are given in Figure 7. After construction of the transposon mutant library, pools of the mutants can be grown in different conditions, such as monospecies and multispecies conditions. When a gene essential for the growth in the multispecies condition is knocked out, this mutant will be lost in this condition and will diminish in number compared to the starting mutant library and the monospecies condition. After fragmentation of DNA from different conditions and the starting mutant library, an Illumina library preparation is used with a transposon specific forward primer and a universal custom Illumina reverse primer (78). Next, the amplified samples are sequenced using Illumina platform and a transposon based primer to sequence across the junction of the transposon (76,77). Genes present in the starting library but absent in a specific condition will be considered as essential for survival in this condition. Thus by recovering all non-essential genes and comparing them with the starting library, an estimate for the

number of essential genes can be obtained (77,78). When comparing the essential genes between different conditions, it is possible to identify condition-specific essential genes, such as the 'invasion' genes. This are the genes that are essential in the multispecies condition but not in the monospecies one. In addition, the promoterless reporter gene on the transposon can give information about non-essential genes that are still specific for the multispecies condition, since these mutants are fluorescent in this condition but not in the monospecies condition (78).

3. Materials & methods

3.1. Used chemicals, bacteria and culture condition

2,6-dichlorobenzamide (BAM) PESTANAL® analytical standard was purchased from Sigma-Aldrich (CAS number: 2008-58-4). The linear formula of BAM is $Cl2C6H_3CONH_2$ with a molecular weight of 190.03 g/mol.

3.2. Media preparation

R_2A and R_2B were used for rapid growth of bacterial cultures to obtain high density cell suspensions to be used in experiments. R_2A was composed of 0,5 g/L tryptone, 0,5 g/L yeast extract, 0,5 g/L casein hydrolysate and 0,5 g/L soluble starch, 0,55 g/L glucose D+, 0,3 g/L sodium pyruvate, 0,3 g/L K_2HPO_4, 0,05 g/L $MgSO_4$ and 13 g/L Select Agar (Invitrogen) in ultrapure water (MilliQ®) and was autoclaved at 121°C for 20 minutes. A broth variant (R2B) was made by leaving out the Select Agar.

Mineral salts (MS) medium was a carbon deficient mineral medium used in experiments. Different sterile stock solutions were prepared. A phosphate buffer stock solution (136 g/L KH_2PO_4 and 178 g/L $Na_2HPO_4.2H_2O$) and a nutrient solution (5g/L $MgSO_4.7H_2O$ and 1.32 g/L $CaCl_2.2H_2O$) was made in ultrapure water (MilliQ®) and autoclaved (121°C, 20 min). A trace metal solution (2.86 g/L H_3BO_3, 1.54 g/L $MnSO_4.H_2O$, 0.039 g/L $CuSO_4.5H_2O$, 0.021 g/L $ZnCl_2$, 0.041 g/L $CoCl_2.6H_2O$ and 0.025 g/L $Na_2MoO_4.2H_20$) and a $FeCl_3$ solution (5.14 g/L $FeCl_3.6H_2O$) was filter sterilized using a sterile 0.22 µm filter. To prepare 1L of MS medium, 987 mL MilliQ water and 10 mL of phosphate buffer were autoclaved (121°C, 20 min). After cooling down to 50°C, 10 mL nutrient solution, 1 mL trace metal solution and 1 mL $FeCl_3$ solution were added under sterile conditions.

When appropriate, a volume of a 1 g/L BAM solution was added to the medium to achieve a certain concentration of BAM prior to autoclaving. When needed, 50 mg/L kanamycin and 20 mg/L gentamycin were added from a 1000x concentrated sterile stock solution after autoclaving and cooling down to 50°C

3.3. Bacterial cultivation

All cultures and libraries used in this study were stored at -80°C in glycerol stock solution prior to use. *Aminobacter sp.* MSH1 and *Aminobacter sp*. MSH1-GFP (32,81), a green fluorescent protein (GFP) labelled variant of MSH1 carrying Gfp2X-miniTn5Km (82), was grown on R_2A plates containing 200 mg/L BAM for 4 days at 25°C . Several colonies were transferred to R_2B medium amended with 200 mg/L BAM or to MS medium with 200 mg/L BAM allowing growth for 2 days and 7-10 days respectively, at 25°C on a horizontal shaker (100 rpm). The MSH1 promotor probe sublibraries made by using the pRU1097

promoter vector containing 1-2 kb MSH1 genome fragments (83) and the MSH1 transposon mutant sublibraries created by using pRL27-Gfp containing a mini-Tn5 transposon system with a promoterless *gfp* gene (84) were grown directly in R_2B medium amended with 20 mg/L gentamycin and 50 mg/L kanamycin, respectively. Sand filter bacterial isolates were grown in R_2B medium according to the scheme in Table 4 to obtain cell suspensions of each isolate at the same time.

Table 4: Time to inoculate different sand filter bacteria before the start of the experiment.

Time of inoculation	Sand filter bacteria			
4 days before start	K 129			
3 days before start	K 169			
2 days before start	S 9	S 22	S 51	S 158
2 days before start	K 27	K 52	K 89	K 112
1 day before start	S 164,2	K 62	K 67,1	

Cultures and sublibraries were harvested at exponential growth around OD at 600nm of 0.4-0.9 by centrifugation (6000 x g, 15 min, 15°C) and washing with 10 mM $MgSO_4$ thrice.

3.4. Continuous flow cell setup for bacterial biofilm development

A schematic overview of the flow cell setup for the growth of bacterial biofilms in continuously fed conditions is shown (Figure 9). Bacteria are allowed to grow in flow chambers (Figure 10) on a cover glass. Assembly, sterilization and inoculation of the setup was previously described by Tolker-Nielsen *et al.* (85) and a detailed overview is given in Appendix 1. Bacterial cell suspensions inoculated in the flow cells were brought to the same OD at 600nm of 0.2 (OD_{600nm} of 1 = 10^9 cells/mL) with 10 mM $MgSO_4$ solution of which 300 mL was inoculated per flow chamber using an insulin needle (Terumo). Cells were allowed to attach during one hour before pumping was resumed (3.5 mL/h).

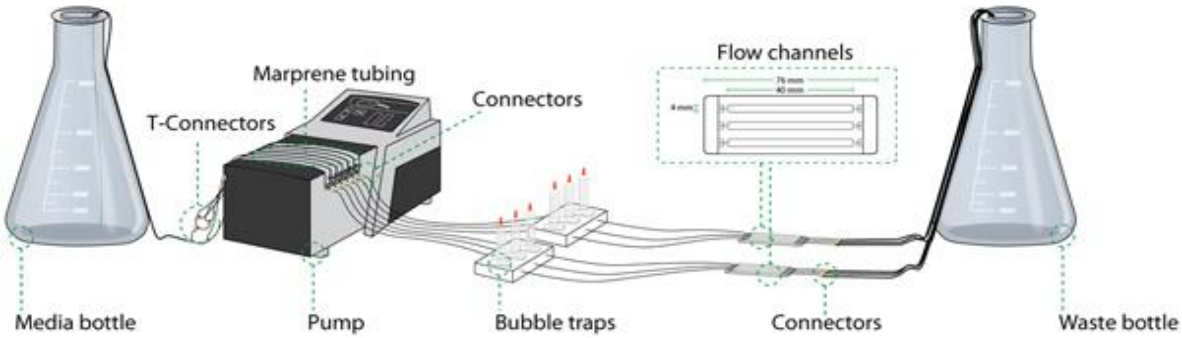

Figure 9: General set-up of a flow cell system (86).

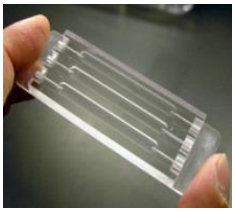

Figure 10: A flow cell with three flow chambers. The length of a flow chamber is 40 mm, the width is 4 mm and the height is 1 mm.

In general, during experiments, effluent samples were collected regularly in microcentrifuge tubes (1.5 mL) to which 5 µL of 37% HCl was added to kill bacterial cells. Samples of 1 mL were centrifuged (10000 rpm, 5 min) and 700 µL was transferred to clear glass vials (1.5 mL) and stored at -20°C until UPLC-UV/VIS analysis to determine BAM and DCBA concentrations. In general, at the end of the experiments, flow cells were disconnected and each chamber was studied using epifluorescence microscopy and confocal laser scanning microscopy (CLSM) to visualize the developed bacterial biofilm. Also, after CLSM analysis, bacterial cells were flushed from the flow chamber using 1 mL sterile 10 mM $MgSO_4$ solution and were collected in sterile Eppendorf tubes. Cell suspensions were centrifuged at 6000 *x g* for 15 min of which the pellet was stored at -20°C until DNA extraction.

3.5. Biofilm experiment for studying the BAM mineralization stability of MSH1 in oligotrophic conditions

Two separate biofilm experiments were executed to determine the stability of pBAM2 carrying the genes for BAM mineralization. For both biofilm experiments, *Aminobacter sp*. MSH1-GFP was grown in R_2B amended with 200 mg/L BAM and MS with 200 mg/L BAM to generate cultures with a respectively low (not enriched) and high (enriched) incidence of MSH1-GFP cells carrying pBAM2. In the second experiment, MSH1-GFP was grown in R2B amended with 200 mg/L BAM (not enriched) and was also repeatedly grown in MS with 200 mg/L BAM (enriched) for three consecutive times. In both experiments, a continuous flow cell system was fed with MS medium containing different nominal concentrations of BAM, i.e., 1 mg/l, 100 µg/l, 10 µg/l, 1 µg/l or no BAM. In experiment 1, MS medium contains AOC (± 100 µg/L), while in experiment 2, AOC was removed by acid washing influent bottles overnight with 2 N HCl solution and afterwards rinsing with ultrapure water (MilliQ) three times. Three flow chambers for each condition were inoculated with the not enriched and enriched MSH1-GFP cell suspension prepared as described previously. Experiments were running for four weeks. Effluent samples were taken regularly for UPLC-UV/VIS analysis for determining BAM and DCBA concentrations. 3D-images of MSH1-GFP biofilms were taken using CLSM at the end of the experiment and biofilm cells were collected for DNA extraction. DNA extracts were analysed with qPCR for the determination of *bbdA* and *bbdB* gene copy numbers.

3.6. Biofilm experiment for the study of horizontal gene transfer in MSH1 under oligotrophic conditions

Aminobacter sp. MSH1-GFP was grown in R2B medium amended with 200 mg/L BAM and a volume was plated on R2A plates. Several colonies were picked and MSH1-GFP colonies which showed no BAM-mineralization and the presence of pBAM1 and absence of pBAM2 was confirmed with PCR targeting the *bbdA* and *bbdB* gene (Raes, Horemans, unpublished). A MSH1-GFP colony lacking BAM mineralization and *bbdB* gene was retained and subsequently grown in R2B medium. This isolate is designated MSH1-$GFP_{B+/D-}$. Wild type *Aminobacter sp*. MSH1 was grown in MS with 200 mg/L BAM and a volume was plated on R2A plates. Several colonies were picked and MSH1 colonies which showed BAM-mineralization and the presence of pBAM1 and pBAM2 was confirmed with PCR targeting the *bbdA* and *bbdB* gene (Raes, Horemans, unpublished). A MSH1 colony showing BAM mineralization and the *bbdB* gene was retained and subsequently grown in MS medium amended with 200 mg/L BAM. This isolate is designated MSH1-$wt_{B+/D+}$. For the inoculation of the flow chambers, four mixtures of MSH1-$GFP_{B+/D-}$ and MSH1-$wt_{B+/D+}$ were made (Table 5). A continuous flow cell system was fed with MS medium containing different nominal concentrations of BAM, i.e., 1 mg/l and 10 µg/l and 100 µg/L AOC. At the start of the experiment, the different inocula were injected as illustrated in Table 6. This experiment was running for 4 weeks. Effluent samples were taken regularly for UPLC-UV/VIS analysis for determining BAM and DCBA concentrations. At the end of the experiment, an effluent sample of 1000 µL was collected in a sterile Eppendorf tube (1.5 mL). Two volumes of 20 µL were diluted in 10 mM MgSO4 and kept aside for flow cytometry to determine live and dead cell numbers and single cell and aggregate numbers. A volume of 100 µL was retained for MSH1-GFP colony screening. The remaining volume (+/- 850 µL) was centrifuged (6000 *x g*, 15 min) and the pellet was kept at -20°C for DNA extraction. DNA extracts were analysed with qPCR for the determination of *bbdA* and *bbdB* gene copy numbers.

Flow cytometric cell enumeration

Cell enumeration was performed using the BD Accuri™ C6 Flow Cytometer. Cell suspensions were analysed in duplicate in 96-well plates and Evian water was used as a blank. The 96 well plate containing the samples was first incubated at 37ºC for 30 min. A first series of samples was stained for the live/dead cell enumeration by adding 2 µL of SYBR® I Green 100x diluted solution and 2 µL of 0.3 mM PI stain to each well having a final volume of 200 µL. A second series of samples was stained with SYTO® 62 nucleic acid stain solution (500 x diluted). 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 was performed according to manufacturer guidelines. All samples were analysed at 66 sec per µL 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 LP). Live (only SYBR I stained) and dead (SYBR I and PI stained) cells were gated in a scatter plot showing FL3 vs. FL1. Syto® 62 signal was detected in the FL4 channel (Excitation: 640 nm, emission: 675/25 nm). Single cells (low FSC) and aggregate numbers (high FSC) were determined based on gated events in the FL4 vs FSC scatter plot. 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.

MSH1-GFP *colony PCR*

For MSH1-GFP colony screening was used to determine the presence of the *bbdB* gene. A volume of 10 µL of the 10x and 100 x dilution of the cell suspension harvested from each flow chamber was plated on R2A plates amended with 50 mg/L kanamycin. Plates were incubated at 25°C for 4-7 days and from each chamber 32 colonies were picked and resuspended in 10 mM $MgSO_4$ in separate wells in 96 well plates. All cell suspensions were heated to 95°C for 30 minutes to lyse the cells. A PCR was performed on each cell suspension using a master mix (MM), that consists of 0.025 units *Taq* DNA polymerase per µL (DreamTaq DNA polymerase, Thermo Scientific), 0.5 µM forward primer C11 ringdiox F RT (see Table 11), 0.5 µM reverse primer C11 ringdiox R RT (see Table 11), 0.2 mM of each dNTP (Invitrogen), PCR buffer 1x (10x DreamTaq Green Buffer, Thermo Scientific), 0.1% BSA (bovine serum albumin) and PCR-H_2O. Finally, 1 µL of the extracted DNA sample was added to obtain the PCR reaction mixture. The used PCR program was 15 min at 95°C followed by 40 cycles of 20 sec at 95°C, 20 sec at 60°C and 20 sec at 72°C. The program was ended with a final increase of temperature from 72°C up to 95°C. Amplification was analysed by running 3 µl on AGE (1.5% agarose gel, 90V, 30 min, 1 kb plus ladder).

*Table 5: Composition of four inocula. MSH1-wt is a wild type Aminobacter sp. MSH1. MSH1-*GFP *is a* GFP *labelled variant of MSH1 carrying* Gfp*2X-miniTn5Km. B+: pBAM 1 is present in the bacterial cell; D+: pBAM 2 is present in the bacterial cell; D-: pBAM 2 is not present in the bacterial cell.*

	Inoculum 1	Inoculum 2	Inoculum 3	Inoculum 4
MSH1-$wt_{B+/D+}$	0%	100%	50%	1%
MSH1-$GFP_{B+/D-}$	100%	0%	50%	99%

Table 6: Conditions in flow cells. Each flow cell consists of three flow channels. The nominal concentration of BAM in the MS medium used for each flow cell is given together with the inoculated culture.

Flow cell	BAM conc.	Inoculum	Flow cell	BAM conc.	Inoculum
FC 1	1 mg/L	Inoculum 1	FC 5	10 µg/L	Inoculum 1
FC 2	1 mg/L	Inoculum 2	FC 6	10 µg/L	Inoculum 2
FC 3	1 mg/L	Inoculum 3	FC 7	10 µg/L	Inoculum 3
FC4	1 mg/L	Inoculum 4	FC 8	10 µg/L	Inoculum 4

3.7. Biofilm experiments for the study of invasion and co-colonization of MSH1 in sand filter biofilms in oligotrophic systems

For the biofilm experiment studying the invasion and co-colonization of MSH1-GFP into or with a residing artificial sand filter community, a continuous flow cell system was fed with MS medium containing 1 µg/L BAM with or without R_2A (0,1 v%). An overview of the flow cell experiment is given in Table 7. At the start of the experiment, sand filter isolates were combined in equal amount to form the artificial sand filter community (ASFC), two-times diluted with 10 mM $MgSO_4$ and were inoculated in the flow chambers when appropriate (Table 7). For the co-colonization scenario, ASFC and MSH1-GFP were combined 1:1 and injected into the flow cells when appropriate. As a control, two-times diluted MSH1-GFP was inoculated when appropriate. Both inoculated and non-inoculated flow cells were continuously fed with the aforementioned medium. After 14 days, for the invasion scenario MSH1-GFP was injected in three of flow chambers previously inoculated with ASFC and in three non-inoculated ones. The experiment was continued for four more weeks.

Table 7: Conditions in flow cells. Each flow cell consists of three flow channels. MSH1: GFP *labelled MSH1; ASFC: Artificial sand filter community; MSH1*:* GFP *labelled MSH1 injected after 14 days (invasion).*

Flow cell	Medium	Inoculum	Flow cell	Medium	Inoculum
FC 1	MS + R_2A	ASFC	FC 6	MS	ASFC
FC 2	MS + R_2A	ASFC + MSH1	FC 7	MS	ASFC + MSH1
FC 3	MS + R_2A	ASFC + MSH1*	FC 8	MS	ASFC + MSH1*
FC4	MS + R_2A	MSH1	FC 9	MS	MSH1
FC 5	MS + R_2A	MSH1*	FC 10	MS	MSH1*

Effluent samples were taken regularly for UPLC-UV/VIS analysis for determining BAM and DCBA concentrations. 3D-images of MSH1-GFP biofilms were taken using CLSM at the end of the experiment

and biofilm cells were collected for DNA extraction afterwards as described above. DNA extracts were analysed with qPCR for the determination of *bbdA* and *bbdB* gene copy numbers.

3.8. Biofilm experiment for social gene identification in MSH1 invading sand filter biofilms

The biofilm experiment for gene identification consisted of a continuous flow cell system fed with MS medium containing 1 µg BAM/L and 100 µg/L AOC. Gene identification is based on the comparison between monospecies (only MSH1) and multispecies (MSH1 with ASFC) conditions. An overview of the experiment is provided in Table 8. For the multispecies condition, the aforementioned ASFC was used and was inoculated at the start of the experiment in the appropriate flow chambers. After 14 days, MSH1 wild type (wt) was inoculated in six channels containing the ASFC and six channels previously non-inoculated. The promoter probe and transposon mutant sublibraries were combined in equal amount and inoculated in three flow chambers containing the ASFC and three previously non-inoculated ones. Two flow chambers with ASFC and two previously non-inoculated ones were inoculated with MSH1-GFP as a control for biofilm formation of MSH1-GFP and the successful invasion of sand filter biofilms. The non-inoculated control served as negative control for BAM-degradation. Effluent samples were taken regularly for UPLC-UV/VIS analysis for determining BAM and DCBA concentrations. 3D-images of MSH1-GFP biofilms were taken using CLSM at the end of the experiment for the MSH1-GFP with ASFC and MSH1-GFP control (n° 13 – 16). Also for these flow chambers, cells were harvested from the flow chambers and DNA was extracted for 16S rRNA amplicon sequencing with an operational taxonomic unit (OTU) analysis.

*Table 8: Conditions in flow cells. Each flow cell consists of three flow channels. PP lib.: MSH1 promoter-probe library; M lib.: MSH1 transposon mutant library; MSH1-wt: MSH1 wild type; MSH1-GFP: GFP labelled MSH1; ASFC: Artificial sand filter community; NI: Not inoculate; - : not used; *: after 14 days inoculated.*

Flow cell	Nr.	Inoculum	Flow cell	Nr.	Inoculum
FC 1	1	PP lib.*	FC 7	17	MSH1-wt*
	2	PP lib.*		18	MSH1-wt*
	3	PP lib.*		19	MSH1-wt*
FC 2	4	M lib.*	FC 8	20	MSH1-wt*
	5	M lib.*		21	MSH1-wt*
	6	M lib.*		22	MSH1-wt*
FC 3	7	ASFC + PP lib*.	FC 9	23	ASFC + MSH1-wt*
	8	ASFC + PP lib*.		24	ASFC + MSH1-wt*
	9	ASFC + PP lib*.		25	ASFC + MSH1-wt*
FC4	10	ASFC + M lib.*	FC 10	26	ASFC + MSH1-wt*
	11	ASFC + M lib.*		27	ASFC + MSH1-wt*
	12	ASFC + M lib.*		28	ASFC + MSH1-wt*
FC 5	13	ASFC	FC 11	29	MSH1-GFP*
	14	ASFC		30	MSH1-GFP*
	-	-		-	-
FC 6	15	ASFC + MSH1-GFP*	FC 12	31	NI
	16	ASFC + MSH1-GFP*		32	NI
	-	-		-	-

3.8.1. Differential fluorescence induction and transposon mutagenesis

Cells from the flow chambers containing the promoter-probe library and the mutant library were harvested and collected in separate sterile Eppendorf tubes (1.5 mL) and suspended in 10 mM $MgSO_4$. Green-fluorescent and non-green fluorescent MSH1 cells of flow chambers with the promoter probe library with ASFC (n° 7 – 9) and without ASFC (n° 1 – 3) and the transposon mutant library with ASFC (n° 10 – 12) and without ASFC (n° 4 – 6) were analysed and sorted using FACS. Next, all sorted cultures (GFP and non-GFP) of the promoter probe library and transposon mutant library were grown in 10 mL R_2B

medium with 10 mg/L gentamycin and 50 mg/L kanamycin, respectively, on a shaker at 25°C. In addition, the transposon mutant library samples before sorting were also grown the same conditions. Cultures were harvested at exponential growth around OD at 600nm of 0.2 by centrifugation (6000 x g, 15 min, 15°C). The pellet was resuspended in 2 mL 10 mM $MgSO_4$. 1 mL was used to make a cryostock by adding it to a cryotube with 0.5 mL glycerol (50%). The remaining 1 mL was stored at -20°C until use for DNA extraction. DNA concentration was measured using the Qubit 3.0 Fluorometer and DNA integrity was evaluated by running 2 µL of DNA samples on AGE (1% gel, 90V, 1h15, 1 kb plus).

Preparation for shotgun amplicon sequencing in DFI

Three different PCR reactions amplifying the gDNA fragments inserted in pRU1097 vector carried by cells in the promoter-probe library were executed for the gDNA extracts obtained from the enrichments after FACS analysis for the promoter-probe library. The master mix (MM) used to amplify the gDNA fragments inserted in pRU1097 vector, consists of 0.025 units *Taq* DNA polymerase per µL (DreamTaq DNA polymerase, Thermo Scientific), 0.5 µM forward primer pRU3 (see Table 11), 0.5 µM reverse primer pRU2 (see Table 11), 0.2 mM of each dNTP (Invitrogen), PCR buffer 1x (10x DreamTaq Green Buffer, Thermo Scientific), 0.1% BSA (bovine serum albumin) and PCR-H_2O. Finally, 1 µL of the extracted DNA sample was added to obtain the PCR reaction mixture. In all three PCR reactions, the polymerase was initially activated by heating the samples to a temperature of 94°C for 5 minutes. Next, the program comprises 29 cycles of 30 sec at 94°C (denaturation), 30 sec at 50°C (annealing) and 1 min 30 sec at 72°C (elongation). For the second and third PCR reactions the duration of the elongation step was extended to 3 à 4 min and a higher annealing temperature (60°C) was used. The final elongation was again the same for all three PCR reactions (10 min at 72°C). The first two PCR reactions had a final reaction volume of 12.5 µL per sample and third PCR reaction had a reaction volume of 100 µL per sample. Amplicons were analysed using AGE (2 µL sample, 1.5% agarose gel, 90V, 1h, 1 kb plus ladder) to evaluate PCR reaction and amplicon yield.

Inserts were amplified again using a PCR with elongation time of 4 minutes and an annealing temperature of 60°C. PCR reactions were carried out 4 times in 25 µL for each sample resulting in an end volume of 100 µL. A volume of 100 µL was loaded and analysed on a 1.5% low melting point gel (90V, 1h, 1 kb plus ladder). Gel samples containing amplicons of interest with a fragment size of 1000-2000 bp was excised, placed in PB buffer (100 µL buffer per 100 mg gel) from the Qiaquick PCR purification kit (Qiagen) and heated to 60°C to dissolve the gel. The protocol of the spin column was followed from step 2 using a vacuum manifold, see Appendix 6. Extracted DNA was analysed by running 2 µL of the samples an AGE (1.5%, 90V, 1h, 1 kb plus ladder) and DNA concentrations were measured using Qubit 3.0 with broad range DNA arrays.

Preparation of genomic DNA for Miseq sequencing in transposon mutagenesis

To verify whether the transposon is present a PCR analysis was performed to amplify the promoterless GFP-gene. The master mix used is the same, except for the primers, as the MM described above. In this case, GfpF' and GfpR (see Table 11) were used as primers. The PCR program for the amplification of the promoterless GFP-gene was 5 min at 94°C followed by 29 cycles of 30 sec at 94°C, 1 min at 60°C and 1 min at 72°C. The program was ended with a final 5 min at 72°C. With the intention of shearing DNA of all mutant cultures, 50 µL of each sample was required with 10 µg of total DNA. 10 µL of the cryostocks of the cultures, were inoculated in 4 mL R_2A with kanamycin (50 mg/L) to grow a denser culture from which sufficient DNA could be extracted. After DNA extraction, samples were adjusted in order to ultimately obtain 50 µL for each sample with the appropriate DNA concentration of 200 ng/µL. To shear the DNA samples into fragments of approximately 350 bp, Covaris AFA was used. Next, the samples were loaded on a 1.5% low melting point gel (90V, 1h, 1/3 100 bp plus ladder). Gel samples containing DNA fragments of interest with a fragment size of 250-350 bp was excised, placed in binding buffer (7 volumes) from the DNA Clean & Concentrator™-25 kit (ZYMO) and heated to 60 ° C to dissolve the gel. Next, the protocol of the spin column was followed from step 2, see Appendix 7. Extracted DNA was analysed by running 2 µL of the samples an AGE (1.5%, 90V, 1h, 1 kb plus ladder) and DNA concentrations were measured using Qubit 3.0 with broad range DNA arrays.

3.8.2. RNA-extraction for transcriptomics

The flow chambers with MSH1-wt (n° 17 - 22) and MSH1-wt with the ASFC (n° 23 - 28) were rinsed with 10 mM $MgSO_4$ and collected in sterile tubes, which were placed in liquid nitrogen to freeze very quickly (called snap freeze). These samples were preserved at -80 °C until used for RNA extraction. The protocol of the adjusted Guanidinium thiocyanate RNA extraction method is implemented in Appendix 5. After RNA extraction, two DNase treatments were performed using the TURBO DNA-free kit. Only the first two steps of the protocol were executed because immediately afterwards a clean-up was performed. This was carried out in the UV-cabinet. The volume of the RNA sample was set equal to 1 volume. In step 1, 0.1 volume 10 x turbo DNase buffer and 1 µL Turbo DNase was added to this RNA sample. The sample was incubated at 37°C for 20-30 minutes in step 2. Next, the RNeasy MinElute Cleanup kit from QIAGEN was completed. The protocol is included in Appendix 3. Which of the two protocols of clean-up (< 100 µL or 100 – 200 µL) should be used depends on the volume after the DNase treatment In this case, the volume after two DNase treatments was equal to 62 µL, whereby the protocol of less than 100 µL may be followed. In step 5, 80% ethanol was used. This was made by diluting 100% ethanol with nuclease free water. After the clean-up, the final volume is equal to 14 µL. Subsequently, reverse transcription qPCR for the determination of 16S rRNA gene copy numbers of *Aminobacter* sp. MSH1 was performed.

3.9. Comparative transcriptome analysis from lab scale sand filter microcosm bioaugmented with MSH1

Six microcosms with sand filter material were fed with MS medium containing a BAM-concentration of 1 µg/L and an AOC concentration of 100 µg/L at 6 mL/h. For the multispecies condition, the aforementioned ASFC was used and was inoculated at the start of the experiment when appropriate. After 30 days, MSH1 wild type (wt) was inoculated in three columns containing the ASFC and three columns previously non-inoculated (Table 9

Table 9). This invasion process was run for 7 more days.

*Table 9: Conditions in microcosms; MSH1-wt: MSH1 wild type; ASFC: Artificial sand filter community; *: after 30 days inoculated.*

Microcosm	Inoculum	Flow cell	Inoculum
1	MSH1-wt	4	ASFC + MSH1-wt*
2	MSH1-wt	5	ASFC + MSH1-wt*
3	MSH1-wt	6	ASFC + MSH1-wt*

When the experiment was terminated, all microcosms were divided in 3 samples (top, middle and bottom samples) which were snap-freezed. These samples were stored at -80°C until RNA-extraction took place.

To extract RNA from microcosms samples, consisting of sand filter material, the Powersoil Total RNA Isolation Kit was used. The protocol is included in Appendix 2. For step 20, not 100 µL of Solution SR7 but 100 µL of the Ambion RNA storage solution was used to resuspend the RNA pellet. To verify the effect of the separation column included in this kit, the bottom sample of column 5 was divided in 2 samples. For sample 1 the entire protocol was performed. In contrast, the protocol was carried out up to the resuspension of the nucleic acids pellet for sample 2. For this second sample the resuspension was the final step and was performed by using 1mL of the Ambion RNA storage solution. After RNA extraction, a DNase treatment was done using the TURBO DNA-free kit, as mentioned above. Following, the RNeasy MinElute Cleanup kit from QIAGEN was used. The protocol is included in Appendix 3. Sample 1 had an end volume of 111 µL after the DNase treatment. For sample 2, the end volume was equal to 1,101 mL. Hence for sample 1 and also for sample 2, once divided into five samples of 200 µL and one of 101 µL, the 100-200 µL protocol was used. At the end of the procedure of RNA purification, all six samples of sample 2 were pooled again, resulting in a final volume of 84 µL. Sample 1 on the other hand had a final volume of 14 µL. For sample 2, a second DNase treatment was executed using 50 µL of the 84 µL. The end volume after the clean-up was equal to 14 µL. RNA was quantified with the Qubit 3.0

Fluorometer using the high sensitivity RNA assay. Next, a first strand cDNA synthesis was performed, using 11 µL and 6 µL of RNA sample 1 and 2 respectively. The reverse transcriptase minus (RT-) negative control of the second sample was obtained similarly to the procedure used for sample 2 (6 µL sample in an end volume of 20 µL). However, for the RT- negative control of sample 1, only 2 µL was used resulting in a higher dilution factor. cDNA was analysed with qPCR for the determination of *bbdA* and 16S rRNA gene copy numbers of *Aminobacter* sp. MSH1.

To extract RNA from all remaining sand filter samples, the Powersoil Total RNA Isolation Kit was used. The protocol is included in Appendix 2. Top, middle and bottom samples were all divided in two samples of approximately 5 g (6 samples per column). In step 14 both samples of top/middle/bottom were added on the same separation column resulting in only 3 samples per microcosm. For step 20, not 100 µL of Solution SR7 was used to resuspend the RNA pellet, but 50 µL of the Ambion RNA storage solution was added. After RNA extraction, a DNase treatment was done using the TURBO DNA-free kit, as mentioned above. Next, the RNeasy MinElute Cleanup kit from QIAGEN was used. The protocol is included in Appendix 3. The end volume after the DNase treatment was equal to 56 µL, whereby the protocol of less than 100 µL may be followed. In step 3, all samples of one microcosm were added on the same clean-up column. After the clean-up, the final volume for each microcosm sample is equal to 14 µL. Next, the template RNA was diluted by adding 1 µL to 5 µL nuclease free water. 1 µL of this diluted template RNA was used for the first strand cDNA synthesis. cDNA was analysed with qPCR for the determination of 16S rRNA gene copy numbers of *Aminobacter* sp. MSH1.

3.10. Analytical techniques

3.10.1. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) analysis was done on biofilms grown in flow chambers to visualize and study biofilm architecture and composition. Prior to analysis, bacterial biofilms were counter stained using the SYTO® 62 Red Fluorescent Nucleic Acid Stain by injecting 300 µL of a 500x diluted SYTO® 62 solution in 10 mM $MgSO_4$. 3D-images of bacterial biofilms were generated using an IX81 inverted microscope (Olympus) with a Fluoview FV1000 confocal scanning unit. To detect the emission of GFP in MSH1-GFP cells, the laser of 488 nm and the band-pass filter of 505-535 nm was used. The 635 nm laser, in combination with the band-pass filter of 650-750 nm, was used to detect SYTO® 62 stained bacterial cells. For each flow cell chamber, a 3D-image was captured at three different positions at the front of the chamber. These pictures (512:512 pixel frame, 0,414 µm pixel size) were taken with a UPL SAPO x60/1, 35 objective (Olympus). Imaris 7.2. Software (Bitplane, USA) generated visualisations of the multicellular structures of the biofilms.

3.10.2. Fluorescence-activated cell sorting (FACS)

Fluorescence MSH1 cells grown in mono- and multi-species biofilms can be isolated using FACS. First, the BD Influx™ (BD Bioscince) cell sorter was calibrated with Sphero™ Rainbow Calibration particles (Sperotech) each time a new analysis was performed. After growth in R_2A medium and a ten-fold dilution with 10 mM $MgSO_4$, MSH1 wild type was used as a negative control and promoter probe sublibrary 1 in MSH1 carrying insert::pRU1097 as a positive control. By setting pulse width, forward scatter and fluorescence as a primary, secondary and third threshold, respectively, bacteria were distinguished from other particles (cell aggregates, fragments and debris). Before sorting, the samples were diluted 10 times using 10 mM $MgSO_4$. The samples were sorted until a pre-set amount of events were detected for the non-fluorescence and fluorescence cells, from which fluorescence, side scatter, forward scatter and pulse width data were collected. The sorted cells were collected in tubes with 1 mL of 10 mM $MgSO_4$ to avoid the small droplets to dry out.

3.10.3. gDNA-extraction using the CTAB-lysozyme method

To extract genomic DNA, the CTAB-lysozyme method was used. The protocol of this method is included in Appendix 4. The first step of this protocol was not performed. After step 3, the samples may optionally be stored at -20°C. After the lysozyme solution was added, the entire protocol had to be followed without a break. Both the lysozyme solution as the proteinase K solution must be freshly made each time. In step 5, 1 µL of RNase was added as well. In the last step, 50 µL Tris pH 8.5 instead of TE buffer was added.

3.10.4. Real-time quantitative PCR and reverse transcription qPCR

First strand cDNA synthesis for reverse transcription qPCR

For the first strand cDNA synthesis, the components of the kit were thawed, mixed, briefly centrifuged and next stored on ice. cDNA synthesis was done in the RNA-cabinet. Using UV, the tubes were sterilized. First an amount of template RNA was added into a sterile nuclease-free tube followed by 1 µL random hexamer primer. Thereafter the volume was further supplemented with nuclease-free water up to 12 µL. The tubes were incubated at 65°C for 5 minutes and afterwards chilled on ice, spun down and placed back on ice. Next the reagents, shown in Table 10, were added in the indicated order and the tubes were mixed gently and spun down.

Table 10: Components to add in indicated order for first strand cDNA synthesis. RiboLock RNase Inhibitor and RevertAid H Minus M-MuLV Reverse Transriptase should not thaw whether they lose their function.

Reagent	Amount
5 x Reaction Buffer	4 µL
RiboLock RNase Inhibitor (20 U/µL)	1 µL
10 mM dNTP Mix	2 µL
RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µL)	1 µL
Total Volume	20 µL

The tubes were incubated for 5 minutes at 25°C in the ThermoCell Mixing Block (BIOER Technology) followed by 60 minutes at 45°C in a water bath. To terminate the reaction, the tubes were a last time incubated for 5 minutes at 70°C in the heating block. The reverse transcription reaction product was finally stored at -80°C.

In addition, positive and negative control reactions were executed to verify the results of the first strand cDNA synthesis. First, a reverse transcriptase minus (RT-) negative control was made for each sample. These controls went through the same procedure and contain every reagent for the reverse transcription reaction except for the RT enzyme which was replaced by nuclease-free water. Secondly, a no template negative control was produced using the same procedure as above but the RNA template was replaced by nuclease-free water. In vitro produced human GAPDH control RNA and his specific PCR primers were used in the following procedure to create the positive control. All reagents were thawed, mixed and briefly centrifuged, followed by storing them on ice. Next, these reagents (see Table 12) were added into a sterile, nuclease-free tube in the indicated order.

Table 11: All primers used in this thesis. R: reverse primer; F: forward primer

Primer name	Gene	Sequence
pRU2 (R)	Insert region	TCCTCCACTAGTCTCTCTCTCTC
pRU3 (F)		TGATCCGGTGGATGACCTTTTG"
MSH1 F RT	16 S rRNA Aminobacter sp.	CAACCTTCGCCCTTAGTTGC
MSH1 R RT		TCATCTTCACCTTCCTCGCG
AmiF RT	*bbdA*	ATATCACGGCCGGTACTATGCCAA
AmiR RT		TCTTCCAAGATCGAACAACCCGGA
C11 ringdiox F RT	*bbdB*	CCGAGCTGTTGGTTTCATCG
C11 ringdiox R RT		GATCTTCAATACGCCGCTGG
GfpF'	*gfp*	TGTTCCATGGCCAACACTTG
GfpR		CCATGTGTAATCCCAGCAGC

Table 12: Components to add in indicated order for positive control first strand cDNA synthesis reaction. RiboLock RNase Inhibitor and RevertAid H Minus M-MuLV Reverse Transriptase should not thaw whether they lose their function.

Reagent	Amount
Control GAPDH RNA (50 ng/µL)	2 µL
Reverse GAPDH Primer	1 µL
5 x Reaction Buffer	4 µL
RiboLock RNase Inhibitor (20 U/µL)	1 µL
10 mM dNTP Mix	2 µL
RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µL)	1 µL
Water, nuclease free	9 µL
Total volume	20 µL

The tube had been mixed gently and centrifuged. Thereafter it was incubated for 60 minutes at 45°C followed by 5 minutes at 70°C to terminate the reaction. This positive control was stored in the -80°C just like the other controls and samples. Next, a real-time quantitative PCR (qPCR) was performed on all samples, positive and negative controls as a method to detect and quantify cDNA.

Real-time qPCR

Using real-time qPCR, a target DNA fragment is amplified, detected and quantified. For quantification, standards were measured in addition to the samples. Standards of 10^8, 10^6, 10^4, 10^3, 10^2, 10^1, 10^0 and a

blanc were used to construct a standard curve. Detection was based on SYBR Green. Therefore a mastermix was made of 7,5 µL 2x ABsolute qPCR SYBR Green Mix (Thermo Scientific), 3,9 µL nuclease-free water, 0,3 µL forward primer and 0,3 µL reverse primer per reaction. All primers used in this thesis are given in Table 11. 3 µL of a standard or a sample was added to 12 µL of this mastermix. qPCR was performed with a Rotor Gene Real-Time Centrifugal DNA Amplification Apparatus, using the program shown in Table 13.

Table 13: The program settings of quantitative PCR.

Step	Temperature (°C)	Duration (min:sec)	Repeats
Initial polymerase activation (Hold)	95	15:00	1
Denaturation	95	00:20	
Annealing	60	00:20	40
Elongation	72	00:20	
Melt	72 → 95		1

3.10.5. OTU generation and analysis

An operational taxonomic unit (OTU) generation and analysis was done on biofilms grown in flow chambers to determine which species or groups of species were dominantly present in the biofilms. First a sequencing was performed by IIT GmbH (Bielefeld, Germany), done with the Illumina MiSeq using the MiSeq v3, 2x300nt paired-end kit and the Nextera XT index kit. Only until the first PCR was performed using universal 16S rRNA primers from Klindworth (87). Thereafter, the amplicon quality was checked with the bioanalyser. A custom made 16S rRNA database was used as reference with 1170 different 16S rRNA gene sequences corresponding to the order "Burkholderiales", "Actinomycetales", "Rhizobiales" and "Anaeromonadales". The OTU generation and analysis was done following the mothur MiSeq SOP, which is based on a paper of Kozich (88), just until "Preparing for analysis – OTU's" section.

3.10.6. Ultra-Performance Liquid Chromatography (UPLC) and UPLC tandem mass-spectrometry (UPLC-MS/MS)

BAM and DCBA concentrations above 1 µg/L were determined with a UPLC-UV/VIS system (Nexera UFPLC, Shimadzu) using a VisionHT C18 Highload column (100x2mm, 1.5 µm) kept at 40°C. A volume of 10 µL was eluted with 85% H_3PO_4 pH 2.5 and acetonitrile 15% at 0.2 mL min^{-1}. BAM and DCBA was detected using UV absorption at 210 nm. Retention time for BAM and DCBA was 3.5 min and 5 min, respectively. BAM-concentrations lower than 1 µg/L were determined using a UPLC-MS/MS system (81).

3.11. Statistical analyses

A two-sided Student's t-test was performed for data with unequal variances to test the statistical significance of a difference between two obtained data sets. The hypothesis is that there is no difference in data. This hypothesis is rejected when the t-value is lower than 0.05, resulting in a significant difference between the data sets with a certainty of 95%.

4. Results

4.1. BAM mineralization stability of MSH1 in biofilms grown under oligotrophic conditions

Amidase converts BAM to 2,6-dichlorobenzoic acid (DCBA) and is encoded by the *bbdA* gene which is located on pBAM1 and is constitutively expressed (40). Further mineralization of DCBA is controlled by pBAM2 (*bbdB* and *bbdC*), which has a low incidence in MSH1 colonies from cultures with impaired BAM mineralization according to T'Syen (unpublished). The purpose of these mineralization stability experiments is to verify whether pBAM2 is stable in MSH1 biofilms grown in continuously fed flow chambers under oligotrophic conditions assuring BAM mineralization capacity at micropollutant concentrations. Moreover, it was tested whether BAM-concentration and the presence of AOC influenced this stability.

For both experiments, *Aminobacter sp*. MSH1-GFP cultures with a respectively low (not enriched) and high (enriched) incidence of MSH1-GFP cells carrying pBAM2 were generated. The *bbdA* and *bbdB* gene copy numbers were determined to calculate the percentage of cells carrying pBAM2 (Table 14). The not enriched inocula used in the experiment with AOC and the AOC depleted experiment had a *bbdB*/*bbdA* ratio of 3.16 ± 0.76% and 13.90 ± 5.42% respectively, whereas for the enriched cultures this ratios were equal to 22.11 ± 2.50% and 135.47 ± 62.76%, respectively.

Table 14: Composition of two enriched and two not enriched inocula. MSH1-GFP is a GFP labelled variant of MSH1 carrying Gfp2X-miniTn5Km. B+: pBAM 1 is present in the bacterial cell; D+: pBAM 2 is present in the bacterial cell; D-: pBAM 2 is not present in the bacterial cell.

	Inoculum 1 Not enriched	Inoculum 2 Enriched	Inoculum 3 Not enriched	Inoculum 4 Enriched
MSH1-GFP$_{B+/D+}$	3%	20%	0.1%	100%
MSH1-GFP$_{B+/D-}$	97%	80%	99.9%	0%

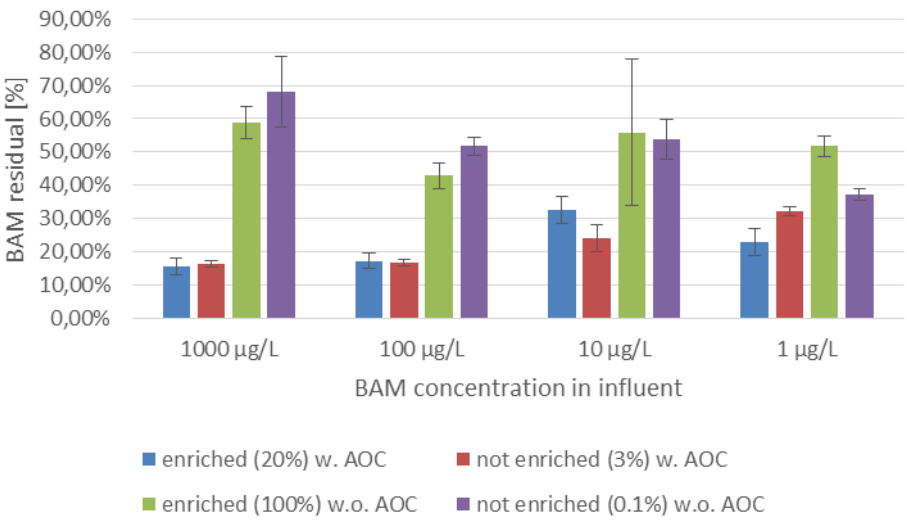

Figure 11: Residual BAM-concentrations in effluent after 32 days in percentage relative to the concentration of BAM added to the system; BAM: 2,6-dichlorobenzamide; enriched: 20% or 100% MSH1-GFP$_{B+/D+}$ with B+: pBAM 1 is present in the bacterial cell and D+: pBAM 2 is present in the bacterial cell.

The steady state BAM-concentrations in the effluent at day 32 is shown for the flow chambers continuously fed with 1000, 100, 10 and 1 µg/L BAM inoculated with the different inocula with and without AOC (Figure 11). A significant difference in BAM-degradation occurred between flow chambers with AOC and without AOC. When AOC was present, in general, a lower amount of residual BAM was detected in the effluent compared to no AOC. This difference in BAM residual in the effluent increased with increasing BAM-concentrations in the feed. However, no significant difference (95% CI) was detected between enriched and not enriched cultures except at BAM-concentrations of 1 µg/L. At the latter concentration, the not enriched culture achieved a 1.4-fold higher and 1.4-fold lower residual BAM-concentration in the effluent compared to enriched when AOC was absent or present, respectively.

Visualization of biofilms using CLSM analysis shows the spatial distribution of MSH1 in biofilms and its biomass production (Figure 12 and Figure 13). In general, it was observed that the higher the BAM-concentration in the medium, used to feed the biofilms, the higher the amount of biomass formed in the biofilm (Figure 12 and Figure 13). Biofilms developed on 100 µg/L AOC (Figure 12) showed a substantial higher amount of biomass compared to biofilms fed with no AOC (Figure 13) independent of the BAM-concentration used to fed the flow chamber. Biofilms of enriched cultures fed with BAM-concentrations of 1 µg/L or 1000 µg/L and 100 µg/L AOC (Figure 12) or no AOC (Figure 13), have a higher amount of biomass compared to the not enriched cultures under the same conditions. This difference is not apparent at other BAM-concentrations (0 µg/L, 10 µg/L and 100 µg/L).

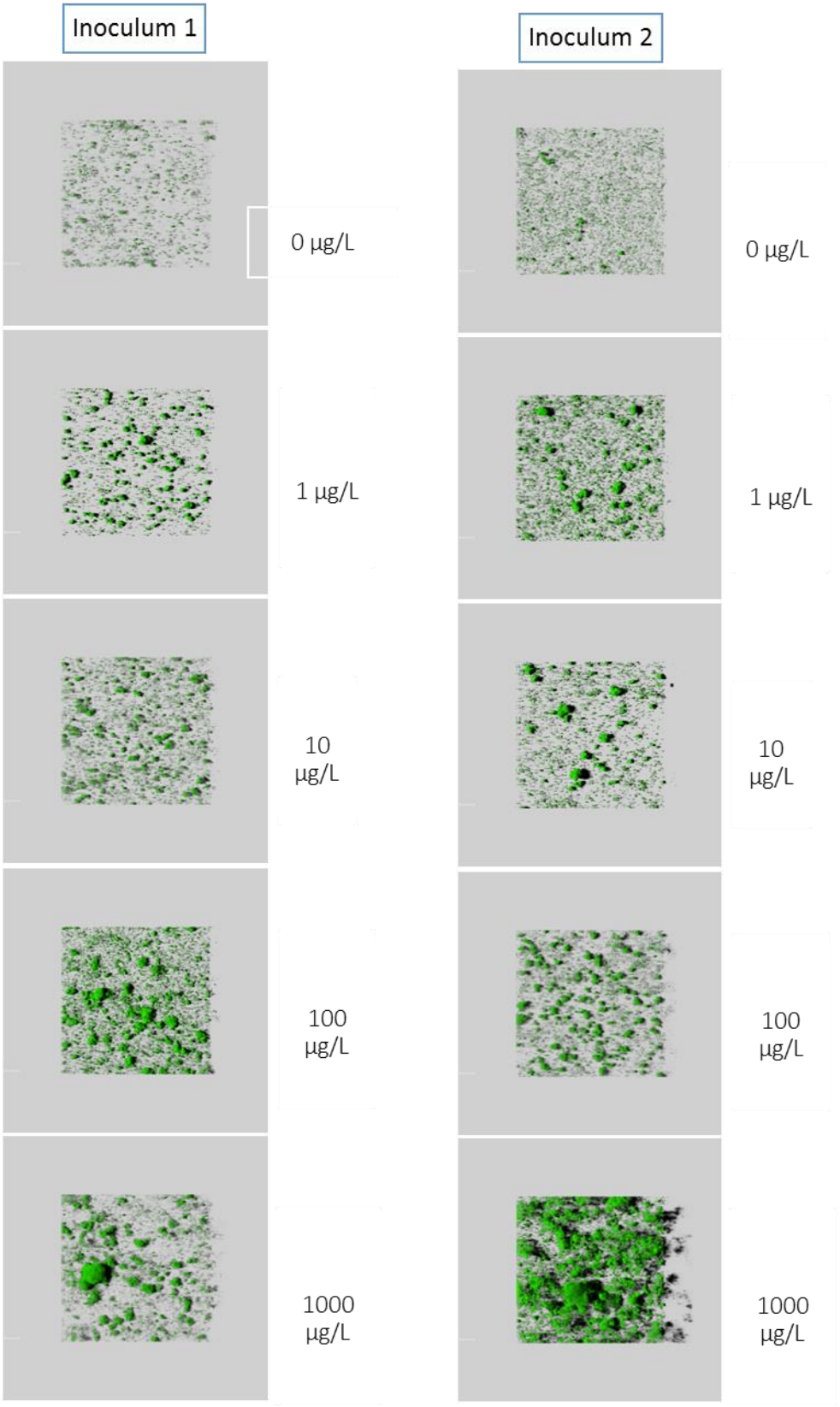

Figure 12: Top view of 3D projection of CLSM images of biofilms of inoculum 1 (not enriched) and inoculum 2 (enriched) in flow cells fed with MS medium with 100 µg/L AOC and different BAM-concentrations; MSH1-GFP: Aminobacter sp. MSH1-GFP (green).

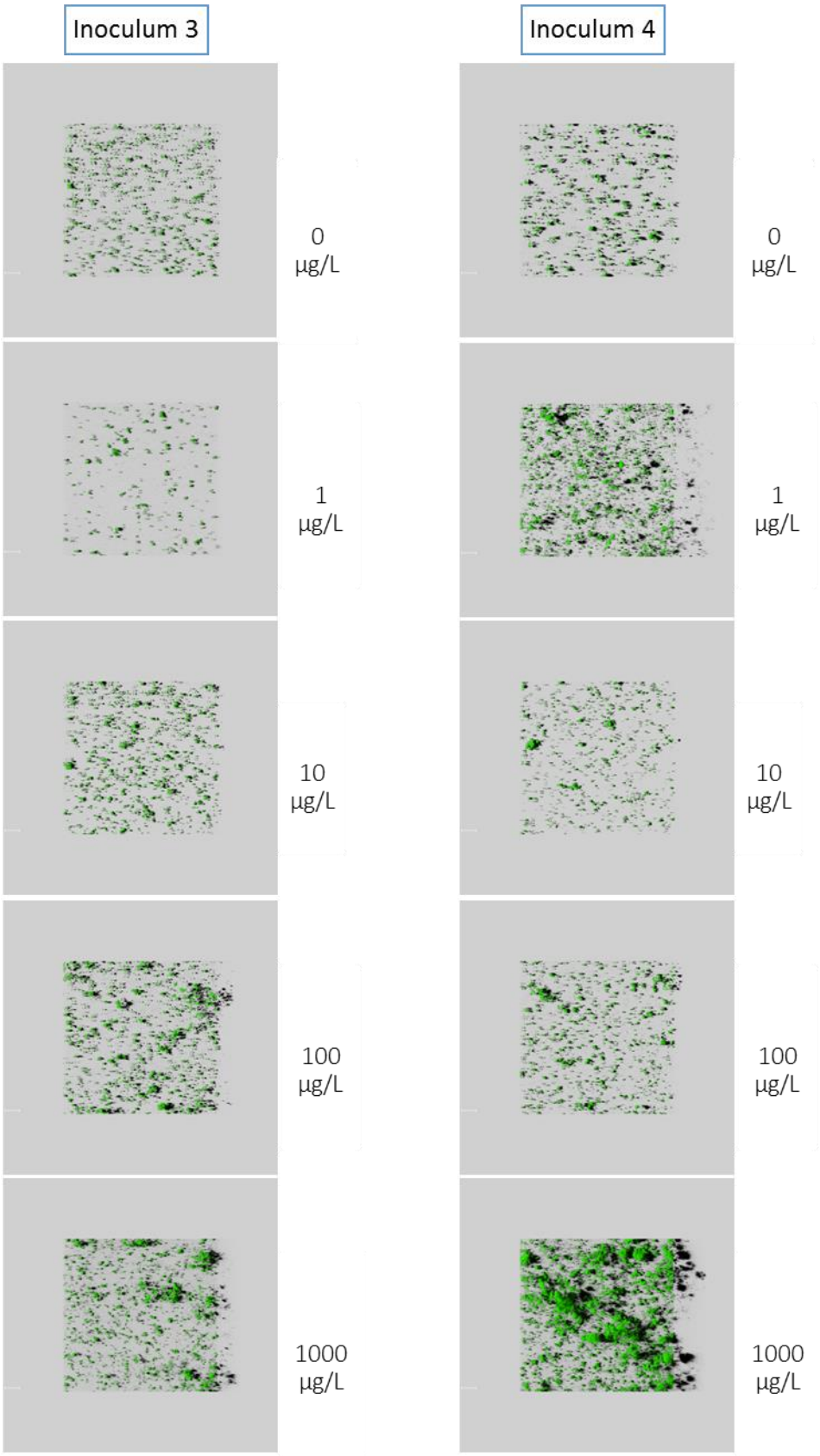

*Figure 13: Top view of 3D projection of CLSM images of biofilms of inoculum 3 (not enriched) and inoculum 4 (enriched) in flow cells fed with MS medium with no AOC and different BAM-concentrations; MSH1-*GFP*: Aminobacter sp. MSH1-*GFP *(green).*

The ratio *bbdB*/*bbdA* of the biofilms grown under the different conditions are shown (Figure 14). Standard deviation is also presented in Figure 14 but since the data are presented in log-scale, no negative values could be presented. For not enriched (3% MSH1-GFP$_{B+/D+}$) or enriched (20% MSH1-GFP$_{B+/D+}$) cultures, *bbdB* numbers decreased after being grown as a biofilm fed with MS medium containing AOC (± 100 µg/L) and BAM. For the not enriched culture this decrease was observed for BAM-concentrations below 10 µg/L, while for the enriched culture this was for BAM below 100 µg/L. However, no loss of *bbdB* was detected for the not enriched and enriched inoculum when biofilms were grown fed with MS medium without AOC with or without BAM.

bbdB numbers increased at a BAM-concentration of 100 µg/L or higher for the not enriched (3% MSH1-GFP$_{B+/D+}$) culture with AOC present in the MS medium. For the enriched (20% MSH1-GFP$_{B+/D+}$) culture with AOC present in the MS medium, *bbdB* numbers increased at a BAM-concentration of 1000 µg/L. For the not enriched (0.1% MSH1-GFP$_{B+/D+}$) culture with AOC depleted MS medium, *bbdB* numbers already increased at BAM-concentrations of 10 µg/L or higher.

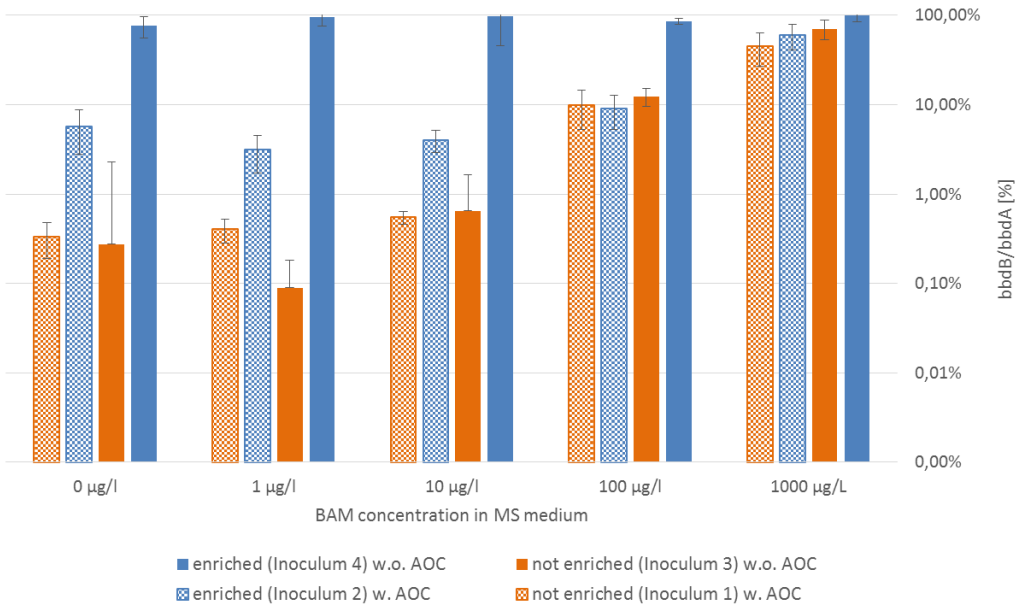

Figure 14: bbdB/bbdA [%] ratio for 4 different inocula at different BAM-concentrations in the MS medium. AOC: Assimilable Organic Carbon (± 100 µg/L); Inoculum 1: 3% MSH1-GFP$_{B+/D+}$; Inoculum 2: 20% MSH1-GFP$_{B+/D+}$; Inoculum 3: 0.1% MSH1-GFP$_{B+/D+}$; Inoculum 4: 100% MSH1-GFP$_{B+/D+}$; MSH1-wt$_{B+/D+}$: Aminobacter sp. MSH1 wild type with pBAM1 and pBAM2.

4.2. Cell viability, aggregation and horizontal gene transfer in MSH1 biofilms grown under oligotrophic conditions

A biofilm experiment with assemblies of various inocula was performed in continuously fed flow chambers mimicking the oligotrophic sand filter environment to examine whether horizontal gene

transfer of pBAM2 occurred amongst the MSH1 cells in those biofilms. In addition, cell viability and aggregation in MSH1 biofilms grown under the oligotrophic condition of the sand filter environment was investigated.

Concentrations of BAM and DCBA in the effluent of the flow cells were measured to determine BAM and DCBA degradation activity. The residual concentrations of BAM and DCBA after 30 days are shown as percentage relative to the concentration of BAM fed to the flow chambers in Figure 15. The fraction presented as removed is the percentage of BAM that is supposedly mineralized to CO_2 and H_2O.

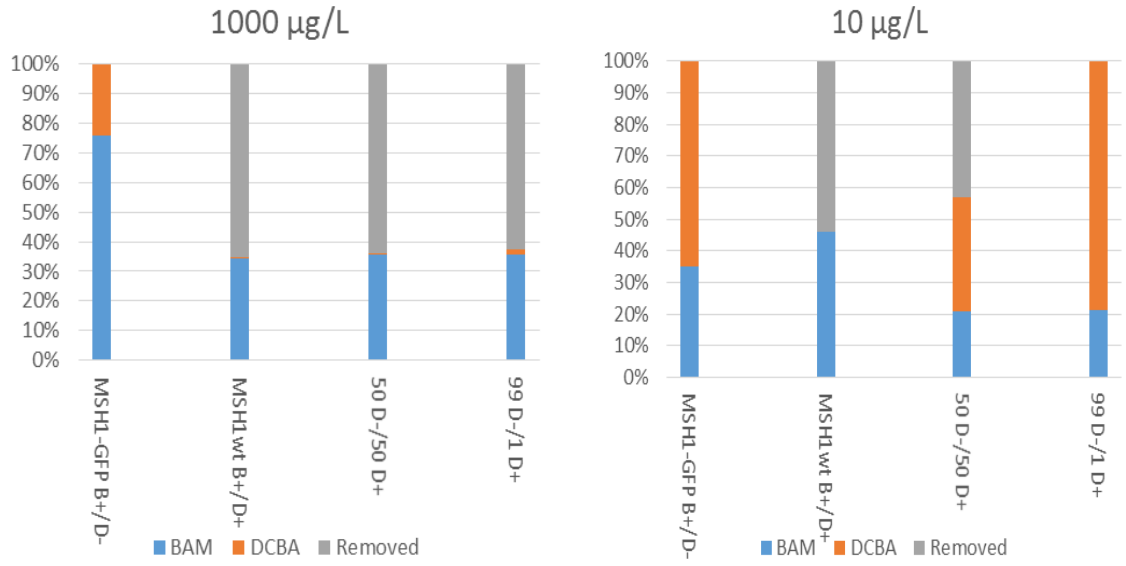

Figure 15: Residual BAM and DCBA concentrations in effluent after 30 days as percentage relative to the concentration of BAM fed to the flow chambers. Left: 1000 µg BAM/L; right: 10 µg BAM/L. BAM: 2,6-dichlorobenzamide; DCBA: dichlorobenzoic acid; MSH1-wt is a wild type Aminobacter sp. *MSH1; MSH1-*GFP *is a* GFP *labelled variant of MSH1 carrying* Gfp*2X-miniTn5Km; B+: pBAM 1 is present in the bacterial cell; D+: pBAM 2 is present in the bacterial cell; D-: pBAM 2 is not present in the bacterial cell; 50 D-/50 D+: 50% MSH1-*GFP*(B+/D-) + 50% MSH1-wt(B+/D+); 99 D-/1 D+: 99% MSH1-*GFP*(B+/D-) + 1% MSH1-wt(B+/D+).*

An *Aminobacter* sp. MSH1-GFP$_{B+/D-}$ culture, lacking the pBAM2 plasmid, converted BAM to DCBA. When BAM-concentrations of MS medium were 10 µg/L, a significantly higher percentage of BAM (65 ± 8%) was converted by this culture to DCBA compared to the BAM conversion in flow cells fed with MS medium with 1000 µg/L BAM (24 ± 2%). In addition, at 1000 µg/L BAM, 64 ± 7% of the applied BAM is degraded beyond DCBA by the other biofilms where the BAM-mineralizing MSH1-wt$_{B+/D+}$ culture was present (100% MSH1-wt$_{B+/D+}$, 50/50 MSH1-GFP$_{B+/D-}$/MSH1-wt$_{B+/D+}$ and 99/1 MSH1-GFP$_{B+/D-}$/MSH1-wt$_{B+/D+}$). At 10 µg/L BAM, only the 100% *Aminobacter* sp. MSH1-wt$_{B+/D+}$ culture was able to degrade all removed BAM (54 ± 5%) beyond DCBA as no DCBA was detected (Figure 15). The residual amount of BAM was not significantly higher for the 100% MSH1-wt$_{B+/D+}$ culture at an initial BAM-concentration of 10 µg/L (46 ± 5%) compared to 1000 µg/L as initial BAM-concentration (34 ± 7%). Then again, residual amounts of BAM were lower for the 50/50 and 99/1 MSH1-GFP$_{B+/D-}$/MSH1-wt$_{B+/D+}$ cultures at initial

BAM-concentrations of 10 µg/L (21 ± 2% and 23 ± 1% respectively) compared to 1000 µg/L as initial BAM-concentration (35 ± 4% and 36 ± 11% respectively). This difference was only significant in the case of 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$. In contrast to 1000 µg/L BAM, DCBA accumulation was detected for the 50/50 and 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ cultures at BAM-concentrations of 10 µg/L and was 36 ± 15% and 84 ± 6% respectively, of the applied BAM in the feed. As such in case of 10 µg/L BAM, the total BAM removal (i.e. beyond DCBA) was 54 ± 5% and 43 ± 13% for 100% and 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$, respectively, while no removal beyond DCBA was noted for 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$.

Table 15: Flow cytometry results: GFP *fraction given in % (standard deviation %) of biofilms after 30 days for two different conditions; MS medium (100 µg/L AOC) with BAM-concentration of 10 µg/L and 1000 µg/L; 50D-/50D+: 50% MSH1-*$GFP_{B+/D-}$ *and 50% MSH1-*$wt_{B+/D+}$*; 99D-/1D+: 99% MSH1-*$GFP_{B+/D-}$ *and 1% MSH1-*$wt_{B+/D+}$*. MSH1-*$GFP_{B+/D-}$*: Aminobacter sp. MSH1-*GFP *with pBAM1 and without pBAM2; MSH1-*$wt_{B+/D+}$*: Aminobacter sp. MSH1 wild type with pBAM1 and pBAM2;*

Condition	50 D- / 50 D+	99 D- / 1 D+
10 µg/L	43.8% (4.3%)	78.3% (2.4%)
1000 µg/L	47.4% (6.1%)	65.8% (12.8%)

At the end of the experiment, the percentage of MSH1-$GFP_{B+/D-}$ cells was determined with flow cytometry and significant differences between the biofilms in flow chambers inoculated with the different cultures were detected (Table 15). The ratio between MSH1-$wt_{B+/D+}$ and MSH1-$GFP_{B+/D-}$ remained unchanged for the 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ culture at 10 µg/L and 1000 µg/L BAM. Instead, a decrease in GFP-fraction of ± 21% in the biofilm is observed for the 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ culture at 10 µg/L BAM. This decrease is more pronounced at BAM-concentrations of 1000 µg/L (± 33% decrease).

The ratio *bbdB*/*bbdA* of both the inocula and the biofilms grown under two different conditions (1000 µg/L and 10 µg/L BAM) are given in Table 16. In general, no change in ratio *bbdB/bbdA* was found for MSH1-$GFP_{B+/D-}$. When biofilms were fed with 1000 µg/L BAM, an 1.5-fold, 5-fold and 400-fold increase of ratio *bbdB/bbdA* was detected for MSH1-$wt_{B+/D+}$, 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ and 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ cultures respectively. For MSH1-$wt_{B+/D+}$, the ratio *bbdB/bbdA* at 10 µg/L BAM was equal to the ratio of the used inoculum and an 3-fold and 7-fold increase was detected for 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ and 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ cultures respectively.

Table 16: The ratio of bbdB/bbdA [%] (standard deviation [%]) for the biofilms (fed with 100 µg/L AOC) containing 50D-/50D+: 50% MSH1-$GFP_{B+/D-}$ and 50% MSH1-$wt_{B+/D+}$; 99D-/1D+: 99% MSH1-$GFP_{B+/D-}$ and 1% MSH1-$wt_{B+/D+}$. MSH1-$GFP_{B+/D-}$: Aminobacter sp. MSH1-GFP with pBAM1 and without pBAM2; MSH1-$wt_{B+/D+}$: Aminobacter sp. MSH1 wild type with pBAM1 and pBAM2.

Culture \ Condition	Inoculum	1000 µg/L BAM	10 µg/L BAM
MSH1-$wt_{B+/D+}$	33.4% (0.8%)	51.2% (3.5%)	42.9% (28.2%)
MSH1-$GFP_{B+/D-}$	0.0% (0.0%)	0.1% (0.1%)	0.5% (0.9%)
50 D- / 50 D+	9.6% (3.1%)	46.2% (8.8%)	27.8% (5.6%)
99 D- / 1 D+	0.1% (0.0%)	38.3% (3.5%)	0.7% (0.3%)

To verify whether pBAM2-carrying MSH1-$GFP_{B+/D+}$ occurred as a result of HGT between the MSH1-$wt_{B+/D+}$ and MSH1-$GFP_{B+/D-}$, harvested biofilm cell suspensions were plated and colonies were selectively grown in the presence of kanamycin. MSH1-GFP colonies were screened for the presence of the *bbdB* gene. However, in none of the tested conditions MSH1-$GFP_{B+/D+}$ occurred (data not shown).

In addition, to the assessment of BAM and DCBA degradation activity, the occurrence of the *bbdB* gene in the total MSH1-population and the MSH-GFP cells, also viability and aggregation was assessed for biofilms and effluent samples.

For the cell viability and the ratio of free cells relative to aggregates, no significant differences were detected between the various inocula mutually. However, differences were identified between the flow chambers fed with 10 µg BAM/L and these fed with 1 mg BAM/L. Therefore, an average was taken for all flow chambers under the same conditions in order to compare these two different conditions (Table 17).

Table 17: Flow cytometry results of effluent and biofilms after 30 days for two different conditions; MS medium (100 µg/L AOC) with BAM-concentration of 10 µg/L and 1000 µg/L; Cells/(Cells + aggregates): averages given in % (standard deviation(%)); Live/(Live + Dead): averages given in % (standard deviation(%)).

Condition	Cells/(Cells + Aggregates) (%)	Live/(Live + Dead) (%)
Effluent		
10 µg/L	24.0% (12.0%)	48.1% (16.1%)
1000 µg/L	63.1% (13.2%)	80.4% (10.1%)
Biofilms		
10 µg/L	86.0% (3.8%)	10.3% (7.3%)
1000 µg/L	84.4% (5.3%)	24.7% (11.0%)

It was observed that the percentage of living cells in the effluent is 48 ± 16% for flow cells fed with BAM-concentrations of 10 µg/L and even higher (80 ± 10%) for flow cells fed with 1000 µg BAM/L (Table 17). When looking at the fraction of living cells in the biofilms, it is clear that the largest fraction of the cells are dead, since only 10 ± 7% and 25 ± 11% are alive (Table 17).

The ratio between the amount of living cells in a biofilm fed with 10 µg/L BAM and the amount of living cells in a biofilm fed with 1000 µg/L BAM is presented in Table 18Table 18 for all four inocula. These results indicate that at 10 µg/L BAM a lower amount of living cells of the 100% MSH1-$wt_{B+/D+}$, 100% MSH1-$GFP_{B+/D-}$ and 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ cultures was present in the biofilms compared to the amount of living cells of these cultures in biofilms fed with 1000 µg/L BAM. Only for the 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ culture a ratio that exceeds 100% was observed.

Table 18: Flow cytometry results after 30 days ratio of living cells in biofilms between two different conditions given in % (standard deviation %); MS medium (100 µg/L AOC) with BAM-concentration of 10 µg/L and 1000 µg/L; 50D-/50D+: 50% MSH1-$GFP_{B+/D-}$ *and 50% MSH1-*$wt_{B+/D+}$*; 99D-/1D+: 99% MSH1-*$GFP_{B+/D-}$ *and 1% MSH1-*$wt_{B+/D+}$*. MSH1-*$GFP_{B+/D-}$*: Aminobacter sp. MSH1-GFP with pBAM1 and without pBAM2; MSH1-*$wt_{B+/D+}$*: Aminobacter sp. MSH1 wild type with pBAM1 and pBAM2;*

	$\frac{\#\ living\ cells\ at\ 10\ \mu g/L}{\#\ living\ cells\ at\ 1000\ \mu g/L}$ [%]
MSH1-$wt_{B+/D+}$	25.4 ± 6.9
MSH1-$GFP_{B+/D-}$	15.6 ± 7.5
50 D- / 50 D+	49.1 ± 29.8
99 D- / 1 D+	120.1 ± 35.5

The ratio of free cells relative to aggregates is also presented in Table 17. Around 85% of the cells are not aggregated in the biofilms, independent of the BAM-concentration in the MS medium. However, looking at the cells found in effluent samples, a significant difference in single cell fraction is observed between the effluent originating from flow cells fed with 10 µg BAM/L or with 1000 µg BAM/L (24 ± 12% and 63 ± 13%, respectively).

Finally, also the ratio of the total suspended and attached amount of MSH1 cells in the flow chamber was determined for both 10 µg/L BAM and 1000 µg/L BAM (Table 19). These results show a lot of variation. Only at 10 µg/L BAM, a significant difference between 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ and 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ was detected as well as between 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ and MSH1-$GFP_{B+/D-}$. Next, it was observed that only for 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ a significant difference occurred between these ratios for 1000 µg/L BAM and 10 µg/L BAM.

Table 19: Flow cytometry results: ratio of total amount of events between effluent and biofilm after 30 days given in % (standard deviation %) for two different conditions; MS medium (100 µg/L AOC) with BAM-concentration of 10 µg/L and 1000 µg/L; 50D-/50D+: 50% MSH1-GFP$_{B+/D-}$ and 50% MSH1-wt$_{B+/D+}$; 99D-/1D+: 99% MSH1-GFP$_{B+/D-}$ and 1% MSH1-wt$_{B+/D+}$. MSH1-GFP$_{B+/D-}$: Aminobacter sp. MSH1-GFP with pBAM1 and without pBAM2; MSH1-wt$_{B+/D+}$: Aminobacter sp. MSH1 wild type with pBAM1 and pBAM2;

	MSH1-wt$_{B+/D+}$	MSH1-GFP$_{B+/D-}$	50 D- / 50 D+	99 D- / 1 D+
10 µg/L	2.33% (0.75%)	1.59% (0.76%)	3.00% (0.55%)	0.88% (0.56%)
1000 µg/L	1.05% (0.85%)	2.12% (1.28%)	1.62% (0.51%)	1.60% (0.77%)

4.3. Invasion and co-colonization of MSH1 in oligotrophic conditions in the presence of an artificial sand filter community

In order to verify whether *Aminobacter* sp. MSH1 is able to colonize surfaces as a pioneer (co-colonization) or a successor (invasion) in the presence of an indigenous sand filter community, a biofilm experiment was performed in continuously fed flow chambers mimicking the oligotrophic sand filter environment. In addition, the difference in BAM-degradation, biofilm formation and mineralizing capacity was compared between the invading or co-colonizing biofilms and whether additional AOC alters these variables.

BAM residuals, given in percentage, are shown for co-colonization (Figure 16) and for invasion (Figure 17). In case of co-colonization, a significant decrease in BAM residual was only observed two weeks after inoculation of MSH1 with or without ASFC. No BAM removal was observed when only ASFC was present. Steady-state residual BAM was probably not yet reached for 100 µg/L AOC. The residual amount of BAM was 68 ± 9% and 54 ± 2% for 100 µg/L AOC at the end of the experiment. However, steady-state residual BAM was reached after 11 days for 300 µg/L AOC with and without ASFC and the residual amount of BAM was 14 ± 5% and 23 ± 1% for 300 µg/L AOC with and without ASFC, respectively.

In case of invasion (Figure 17), no BAM removal was observed in the first 14 days of operation both in the flow chambers with and without the ASFC. When *Aminobacter* sp. MSH1-GFP was added, the residual BAM-concentration decreased instantly, except for the flow cells with an ASFC fed with 100 µg/L AOC. Steady-state residual BAM was reached after 42 days for 100 µg/L AOC and 25 days for 300 µg/L AOC with and without ASFC. The residual amount of BAM was 31 ± 2% and 28 ± 5% for 100 µg/L AOC and 27 ± 11% and 13 ± 4% for 300 µg/L AOC with and without ASFC, respectively.

As such, at the end of the experiment significantly more BAM was degraded in flow cells fed with MS medium containing 1 µg/L BAM and 300 µg/L AOC compared to flow cells fed with 1 µg/L BAM and 100 µg/L AOC. This difference is more substantial in the case of co-colonization (15 ± 6% vs 61 ± 9%) in comparison with invasion (13 ± 4% vs 31 ± 5%). In general, no significant difference in BAM-degradation was observed between biofilms with MSH1 and biofilms with MSH1 in the presence of ASFC. In addition, a significant difference in residual amount of BAM was observed between co-colonization (68 ± 9% and 54 ± 2%) and invasion (34 ± 1% and 28 ± 5%) at 100 µg/L AOC for flow chambers with and without ASFC respectively. As for flow cells without ASFC and fed with MS medium containing 300 µg/L AOC also a significant difference in residual amount of BAM was observed between co-colonization (20 ± 1%) and (11 ± 2%) invasion. Only for flow cells with ASFC and fed with 300 µg/L AOC, no significant difference in BAM-degradation was detected between co-colonization and invasion.

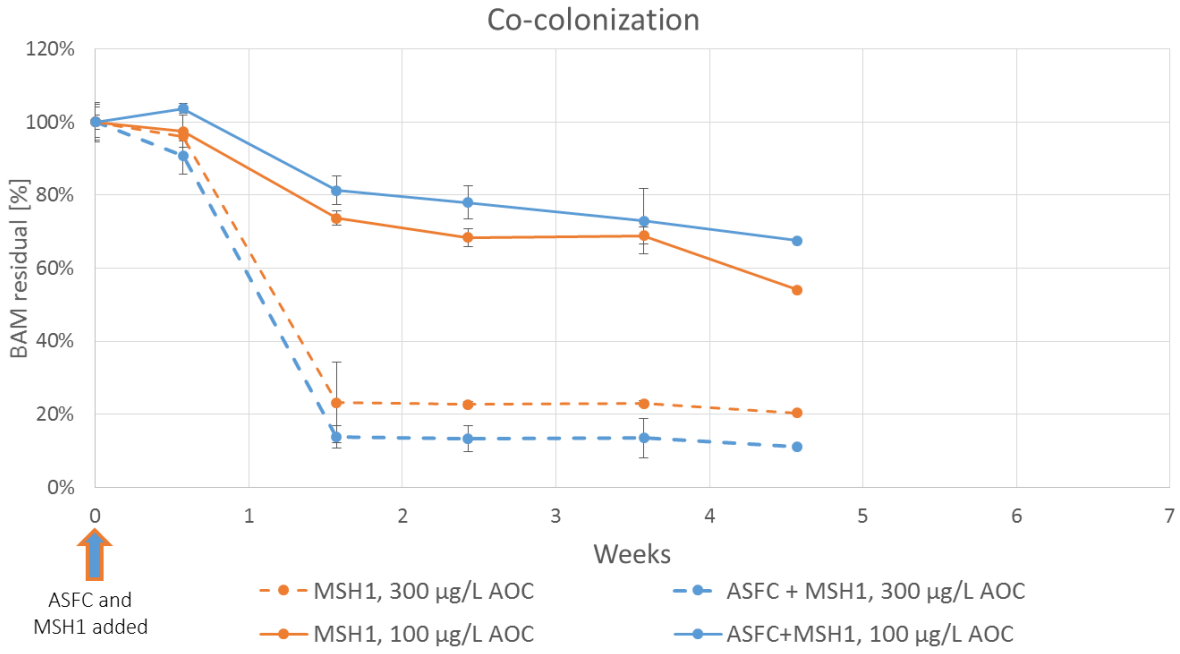

Figure 16: Co-colonization: BAM residual in effluent in function of time for four different conditions; 300 µg/L AOC: MS medium with 0.1 v% R_2A and 1 µg BAM/L; 100 µg/L AOC: MS medium with 1 µg BAM/L and no additional AOC; ASFC: Artificial sand filter community; MSH1: Aminobacter sp. MSH1-GFP.

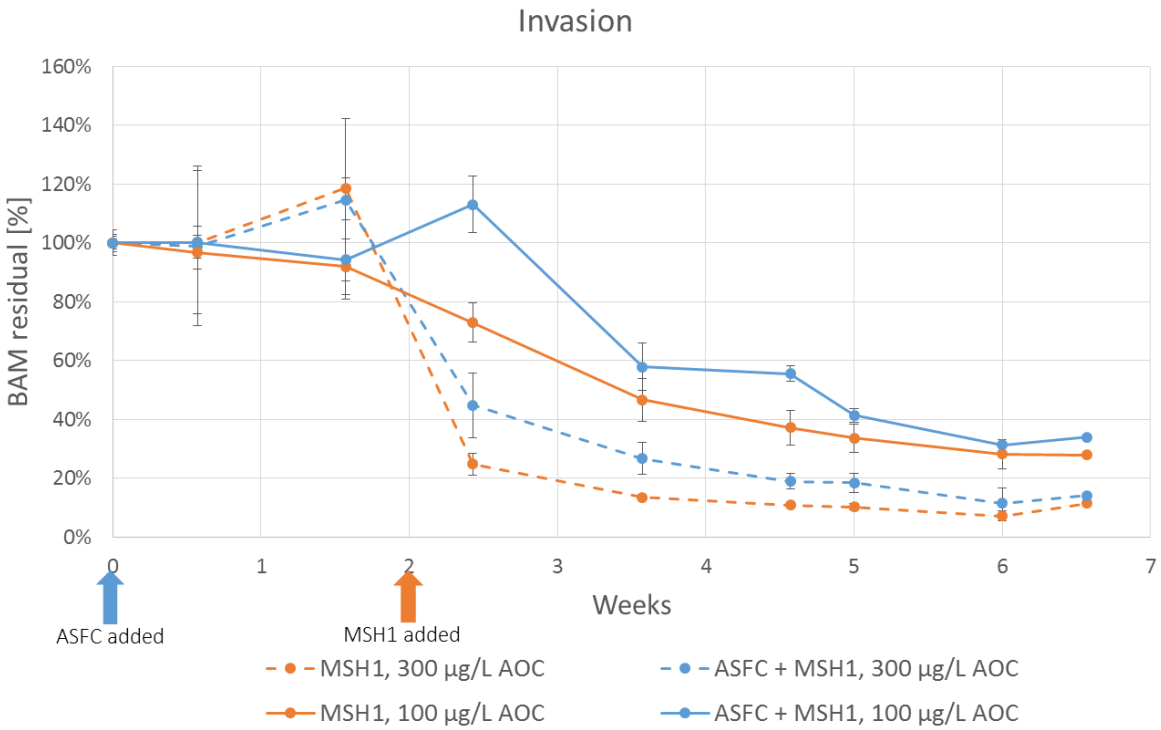

Figure 17: Invasion: BAM residual in effluent in function of time for four different conditions; 300 µg/L AOC: MS medium with 0.1 v% R_2A and 1 µg BAM/L; 100 µg/L AOC: MS medium with 1 µg BAM/L and no additional AOC; ASFC: Artificial sand filter community; MSH1: Aminobacter sp. MSH1-GFP injected 14 days after injection ASFC.

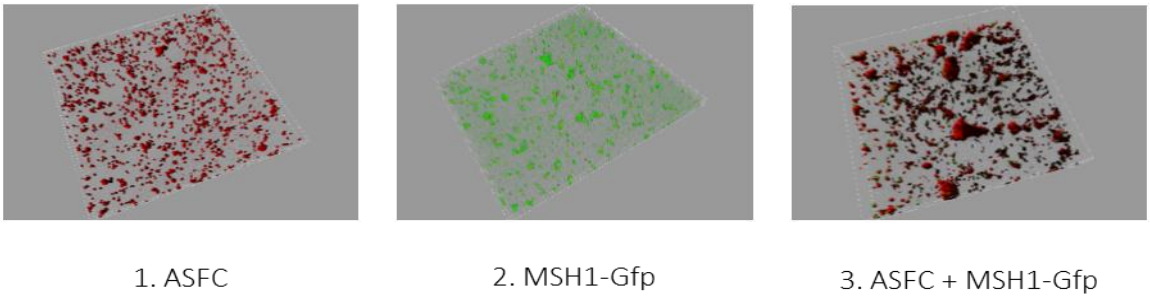

Figure 18: Top view of 3D projection of CLSM images of biofilms formed under co-colonization of MSH1-GFP with ASFC and as a control inoculated with ASFC or with MSH1-GFP in flow cells fed with MS medium with 100 µg/L AOC and 1 µg/L BAM. ASFC: Artificial sand filter community (red); MSH1-GFP: Aminobacter sp. MSH1-GFP (green).

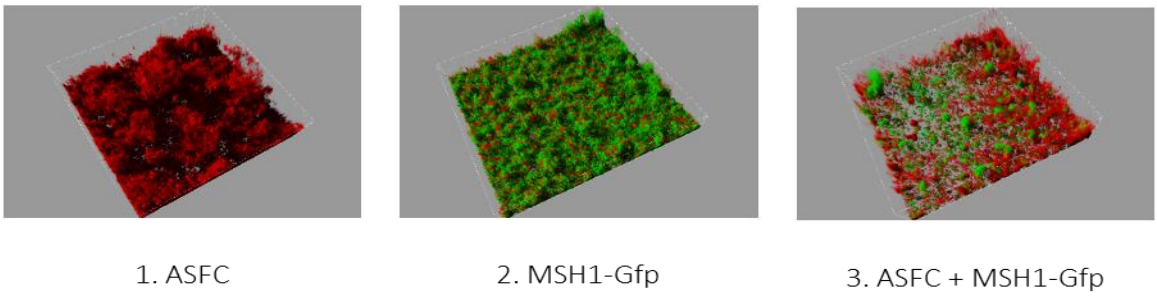

Figure 19: Top view of 3D projection of CLSM images of biofilms formed under co-colonization of MSH1-GFP with ASFC and as a control inoculated with ASFC or with MSH1-GFP in flow cells fed with MS medium with 300 µg/L AOC and 1 µg/L BAM. ASFC: Artificial sand filter community (red); MSH1-GFP: Aminobacter sp. MSH1-GFP (green).

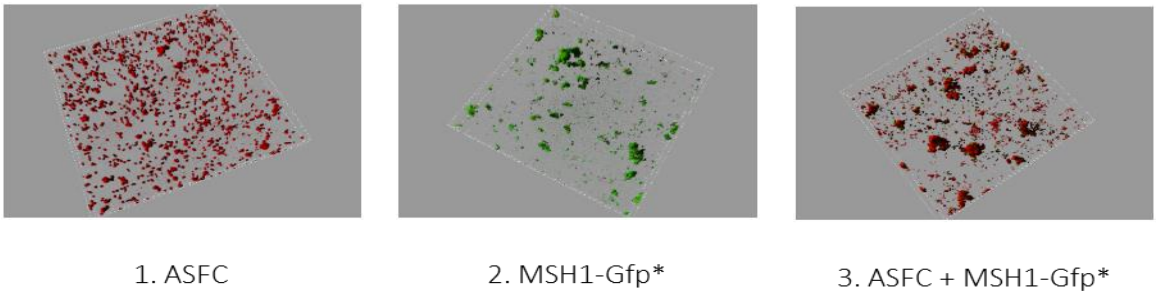

Figure 20: Top view of 3D projection of CLSM images of biofilms formed under invasion of MSH1-GFP with ASFC and as a control inoculated with ASFC or with MSH1-GFP in flow cells fed with MS medium with 100 µg/L AOC and 1 µg/L BAM. ASFC: Artificial sand filter community (red); MSH1-GFP: Aminobacter sp. MSH1-GFP (green).

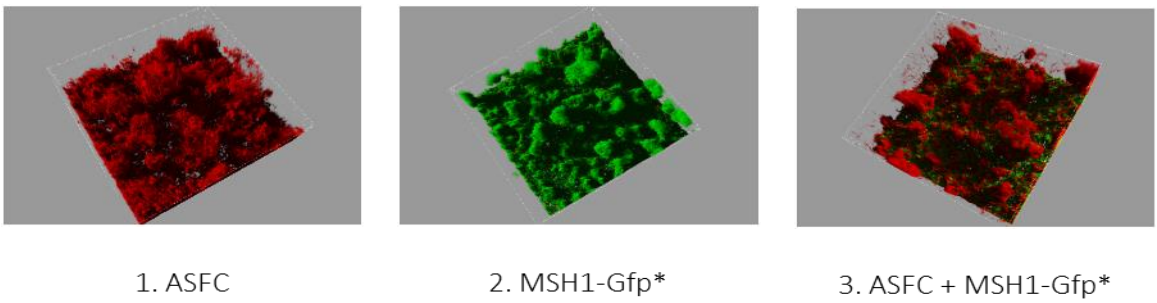

Figure 21: Top view of 3D projection of CLSM images of biofilms formed under invasion of MSH1-GFP with ASFC and as a control inoculated with ASFC or with MSH1-GFP in flow cells fed with MS medium with 300 µg/L AOC and 1 µg/L BAM. ASFC: Artificial sand filter community (red); MSH1-GFP: Aminobacter sp. MSH1-GFP (green).

Next, biofilms which developed in the flow chambers were visualized to observe the spatial distribution and microcolony formation of MSH1 in relation to the ASFC in biofilms. For biofilms formed under co-colonization CLSM analysis was performed after 35 days (Figure 18 and Figure 19). For the invasion flow cells at 46 days, CLSM images were acquired (Figure 20 and Figure 21).

Biofilms developed on 300 µg/L AOC and 1 µg/L BAM (Figure 19 and Figure 21)) showed a substantial higher amount of biomass of both ASFC and MSH1-GFP compared to biofilms fed with 100 µg/L AOC and 1 µg/L BAM (Figure 18 and Figure 20). However, less *Aminobacter* sp. MSH1-GFP biomass is detected in the presence of ASFC under all conditions. When comparing between co-colonization and invasion, differences are mainly detected for biofilms developed on 300 µg/L AOC and 1 µg/L BAM. When comparing MSH1-GFP biofilms in the latter condition, between co-colonization (Figure 19) and invasion (Figure 21), a greater amount of biomass was detected in case of invasion, while MSH1-GFP cells were allowed to grow in both cases for 4 weeks. In the same condition, for the biofilm of ASFC and MSH1-GFP combined (Figure 19), separate colonies of *Aminobacter* sp. MSH1-GFP were detected for co-colonization, while in case of invasion (Figure 21) small colonies of *Aminobacter* sp. MSH1-GFP were surrounded and overgrown by sand filter bacteria (Figure 22)

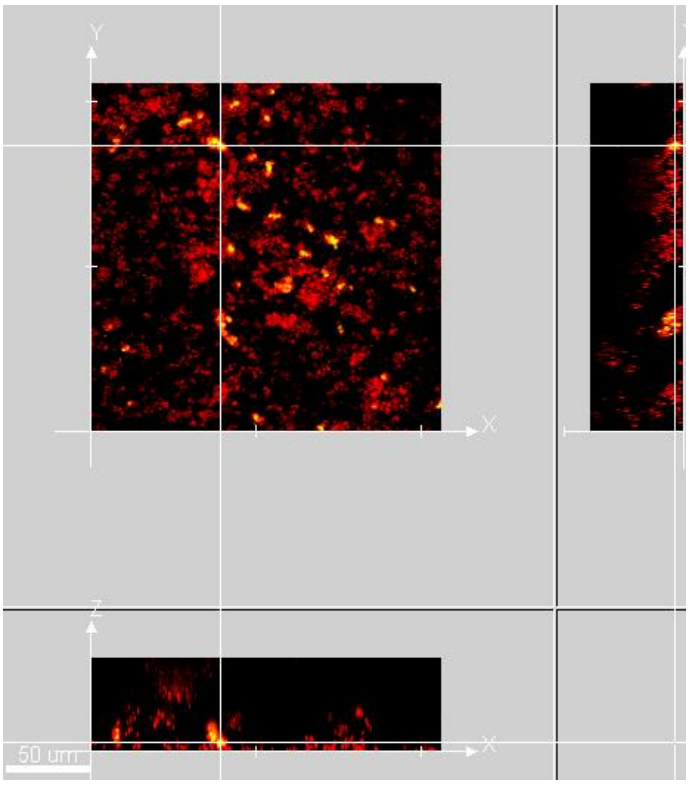

*Figure 22: a cross-section of CLSM image of biofilm formed under invasion of MSH1-*GFP *with ASFC and as a control inoculated with ASFC or with MSH1-*GFP *in flow cells fed with MS medium with 300 µg/L AOC and 1 µg/L BAM. ASFC: Artificial sand filter community (red); MSH1-*GFP*: Aminobacter sp. MSH1-*GFP *(yellow).*

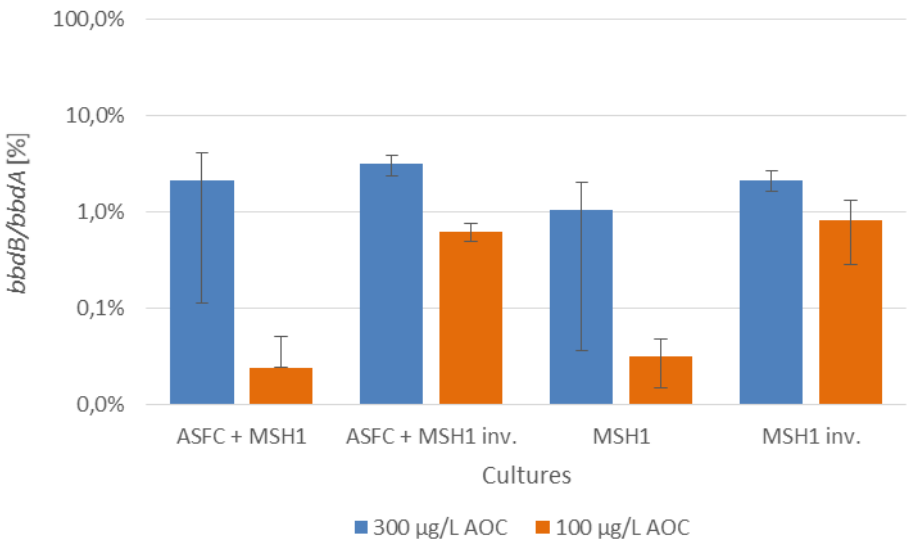

*Figure 23: The ratio of bbdB/bbdA [%] for the biofilms containing MSH1-*GFP *with or without the ASFC under the co-colonization and invasion procedure in the presence of 300 µg/L AOC or 100 µg/L AOC (Assimilable Organic Carbon); ASFC: Artificial sand filter community; MSH1: Aminobacter sp. MSH1-*GFP*; inv.: after 14 days inoculated (invasion).*

The ratio of pBAM2 to pBAM1 (*bbdB/bbdA*) was determined for the biofilms containing MSH1-GFP with or without ASFC grown under the different conditions (Figure 23). Standard deviation is also presented in Figure 23 but since the data are presented in log-scale, no negative values could be presented. For both invasion and co-colonization, no significant difference in *bbdB*/*bbdA* ratio were observed between MSH1 in the presence or absence of the ASFC. In addition, for 300 µg/L AOC, the ratio of *bbdB* to *bbdA* was higher compared to 100 µg/L AOC. From Figure 23 it appeared that this difference was much more pronounced in case of co-colonization, but based on Student's t-tests it can be concluded that this increase of the ratio *bbdB* to *bbdA* by the addition of R_2A (300 µg/L AOC instead of 100 µg/L AOC) is only significant in the case of invasion. More importantly, a significant difference in the ratio of *bbdB* to *bbdA* is observed between co-colonization (0.03 ± 0.02%) and invasion (0.72 ± 0.38%), but only at 100 µg/L AOC in the medium.

4.4. 'Social' gene identification in MSH1 during invasion of a sand filter community in oligotrophic conditions

Social interactions such as cooperation and competition between members in bacterial biofilms exist and these interactions are hypothesized to be key actors in the success of invasion of sand filter biofilms during bioaugmentation. Biological and physiological processes during invasion are encoded by genes and its identification was intended for MSH1 when forming biofilms in oligotrophic conditions on sterile surfaces (mono-species) and surfaces previously colonized by ASFC (multi-species). By comparing the genes expressed in the monospecies and multispecies condition, genes expressed as a specific response to other sand filter bacteria could be identified as social genes. For gene identification, three different

approaches were deployed. In a first approach, called comparative transcriptomics where mRNA transcripts in MSH1 are compared between both conditions. For the second approach, called differential fluorescence induction, a promoter probe library will be grown in the absence and presence of the sand filter community. By separating the GFP and non-GFP MSH1 reporters by FACS and sequencing the inserted DNA fragments, the activated promoters can be identified. By comparing active promoters in the absence and presence of the artificial community, 'invasion' genes specifically expressed as a response to the artificial community can be identified. A last approach used, is transposon mutagenesis. After growing the mutant library both in the absence and presence of the sand filter community, mutants with a disrupted gene essential for 'invasion' will be lost in the mixed-species condition but will be present in mono-species conditions. Consequently, these genes will be considered as essential 'invasion' genes. In addition, this technique also provides information on non-essential 'invasion' genes. When the promoter of the knocked out gene is activated, the cell will be GFP fluorescent as the promoterless *gfp* gene is transcribed. By separating the GFP and non-GFP MSH1 mutants by FACS and sequencing the DNA fragments located next to the transposon, the activated promoters can be identified. By comparing active promoters in the absence and presence of the artificial community, non-essential 'invasion' genes can be identified.

4.4.1. BAM removal, MSH1 survival and community composition during invasion

The residual BAM-concentration in the effluent at steady state (35 days) was determined as a percentage of the feed concentration (1 µg/L BAM) (Figure 24).

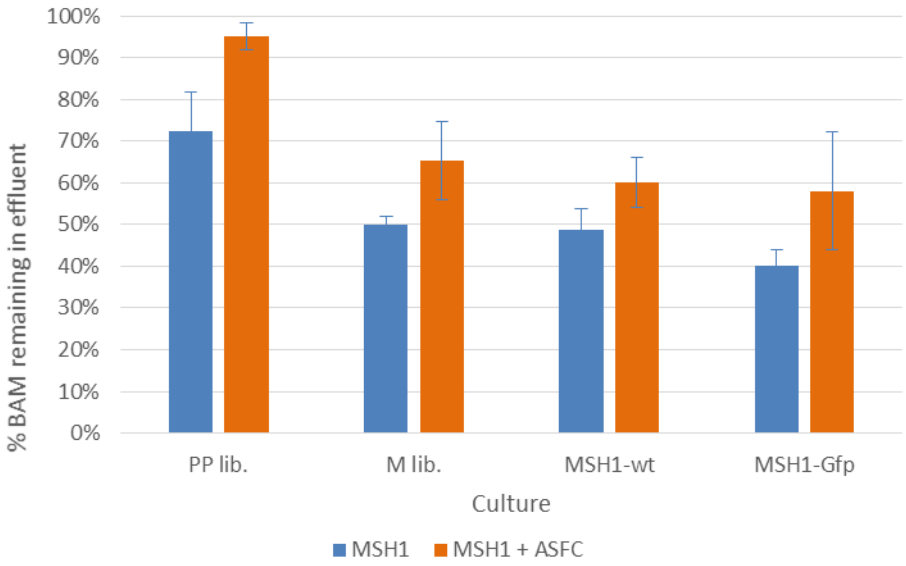

Figure 24: BAM remaining (%) in effluent after 35 days (100 µg/L AOC); PP lib.: MSH1 promoter probe library; M lib.: MSH1 transposon mutant library; MSH1-wt: MSH1 wild type; MSH1-GFP: GFP labelled MSH1; ASFC: Artificial sand filter community.

In general, a higher residual amount of BAM remained in the effluent from flow cells with the artificial sand filter community. But this difference was only significant for the promoter probe library (95 ± 3% and 72 ± 10% for with and without ASFC respectively) and MSH1 wild type (60 ± 6% and 49 ± 5% for with and without ASFC respectively). Secondly, effluent from flow cells inoculated with the MSH1 promotor probe library had a higher percentage of BAM remaining after 35 days compared to the other cultures.

3D-images of the control biofilms (MSH1-GFP, ASFC, MSH1-GFP and ASFC) grown after 35 days revealed MSH1-GFP infiltration in ASFC biofilms and surface colonization when no ASFC was present (Figure 25). Moreover, the MSH1-Gfp was able to successfully colonize the glass surface in both the condition with and without the ASFC (Figure 25). By looking at a cross-sectional view (data not shown) of the CLSM image of control biofilm with both ASFC and MSH1-GFP, it is observed that MSH1-GFP is surrounded by ASFC.

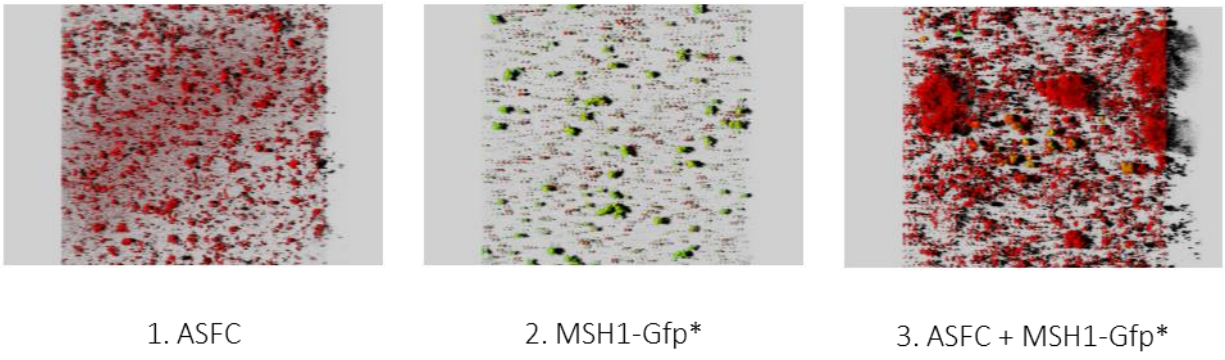

*Figure 25: Top view of 3D projection of CLSM images of control biofilms fed with MS medium with 1 µg/L BAM and 100 µg/L AOC. ASFC: Artificial sand filter community (red); MSH1-*GFP*: Aminobacter sp. MSH1-*GFP *(green).*

Also, for the control biofilms containing the ASFC with or without MSH1-GFP, bacterial species of ASFC were determined using OTU analysis. The results from this analysis demonstrated that three bacterial families were clearly dominant in the biofilms only consisting of ASFC without MSH1-GFP. The family "Comamonadaceae" is most abundant (78%) but the genus was unclassified and the ASFC consists of 8 different genera of this family (Table 4). The second most abundant OUT (16%) was "*Rhodococcus*" of the family "Nocardiaceae", followed by "Phyllobacteriaceae *Mesorhizobium*" (6%). Finally, there is even a fourth genus that is less abundant compared to the previous three but more present than all other genera detected, "Comamonadaceae *Roseateles*" (0.05%). When comparing these results with the ones of the biofilms containing MSH1-GFP when added after 14 days of ASFC biofilm development, the unclassified "Comamonadaceae" (78%) and "Nocardiaceae *Rhodococcus*" (9%) were most abundant. However, "Phyllobacteriaceae *Mesorhizobium*" was no longer detected but instead "Comamonadaceae *Xenophilus*" (1.4%) was more abundant together with a family which could not be classified (0.92%).

Finally, “Aeromonadaceae *Aeromonas*” (0.04%) was also more present compared to other genera, but to a much lesser extent than the five most prominent ones.

4.4.2. Preparation for gene identification based on DFI

The promoter probe library of MSH1 of approx. 30 000 reporters containing the promoter probe vector pRU1097 with randomly generated inserts were inoculated after 14 days in sterile flow chambers (monospecies) and flow chambers colonized by an ASFC biofilm (multispecies) in triplicate. After an additional incubation of 28 days, biofilms were harvested from flow chambers and harvested cells were sorted by FACS into a GFP-labelled and non-GFP-labelled pool, until a pre-set amount of events was sorted. For monospecies flow chambers (only promoter probe library) 10^4 and 10^5 events were sorted for the GFP-labelled and non-GFP-labelled pool respectively. For multispecies flow chambers (colonized by an ASFC biofilm) 10^3 and 10^5 events were sorted for the GFP-labelled and non-GFP-labelled pool respectively.

All sorted cell populations of the promoter probe library were selectively grown based on gentamycin resistance of the reporter cells reaching an OD of approximately 0.2. After gDNA extraction from each culture, inserts (1-2 kb) from the cell cultures were amplified using PCR. Three PCR reactions were executed to optimize the yield of amplicons for further analysis. The results of those three PCR reactions showed that an extended elongation step (4 min) and a higher annealing temperature (60°C) resulted in improved amplification of the gDNA fragments inserted in the pRU1097 vector (data not shown). In addition, a reaction volume of 100 µL was not suitable. As a result four 25 µL reactions were performed to generate the needed amount of amplicons derived from the inserts.

The amplicons of the inserts for each sorted pool were analysed on AGE (1.5%, 90V, 1h, 1 kb plus ladder) (Appendix 8) and fragments between 1-2 kb were extracted and purified. The size of the purified amplicons was verified on AGE (1.5%, 90V, 1h, 1 kb plus ladder) (Figure 26) and were of the proper size (± 1500 bp). DNA concentrations of the amplicons were measured using Qubit 3.0 (Table 20).

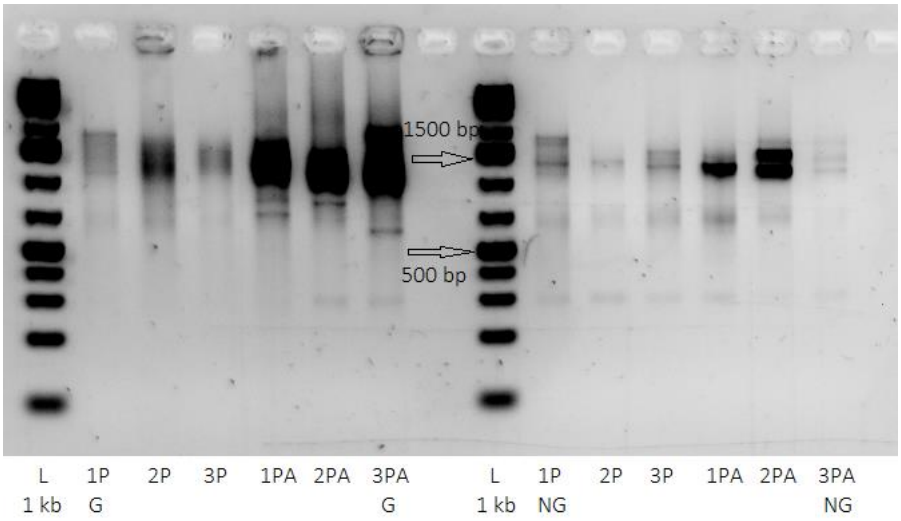

Figure 26: Verification of amplified inserts after gel extraction by AGE (1.5% gel, 90V, 1h); 1P-3P: three replicas of the MSH1 promoter probe library in monospecies condition; 1PA-3PA: three replicas of the MSH1 promoter probe library in the multispecies condition with ASFC; L: ladder, 1 kb: Generuler 1 kb plus ladder; G: cells sorted by FACS as green fluorescent; NG: cells sorted by FACS as not green fluorescent.

Table 20: DNA concentrations of sorted samples after gel extraction; PP lib.: MSH1 promoter probe library; ASFC: Artificial sand filter community; GFP*: cells sorted by FACS as green fluorescent; Non-*GFP*: cells sorted by FACS as not green fluorescent.*

	DNA concentration (ng/µL)		DNA concentration (ng/µL)
PP lib. GFP	15.2	ASFC PP lib. GFP	42.9
	33.2		47.5
	14.3		47.4
PP lib. Non-GFP	17.3	ASFC PP lib. Non-GFP	24.8
	4.61		36.5
	12.3		3.14

4.4.3. Preparation of gene identification based on transposon mutagenesis

The transposon mutant library of MSH1 of approx. 15 000 mutants obtained by conjugation with *Escherichia* coli BW29427 carrying pRL27-Gfp were inoculated after 14 days in sterile flow chambers (monospecies) and flow chambers colonized by an ASFC biofilm (multispecies) in triplicate. After an additional incubation of 28 days, biofilms were harvested from flow chambers and harvested cells were sorted by FACS into a GFP-labelled and non-GFP-labelled pool, until a pre-set amount of events was sorted. For both monospecies flow chambers (only transposon mutant library) and multispecies flow chambers (colonized by an ASFC biofilm) 10^4 and 10^5 events were sorted for the GFP-labelled and non-GFP-labelled pool respectively.

To verify whether the transposon is present a PCR analysis was performed to amplify the promoterless *gfp* gene from the mutant pools from before and after sorting in a GFP and non-GFP-labelled fraction. AGE analysis image of this PCR analysis is shown in Figure 27. This image shows that almost in all samples the promoterless *gfp* gene was amplified.

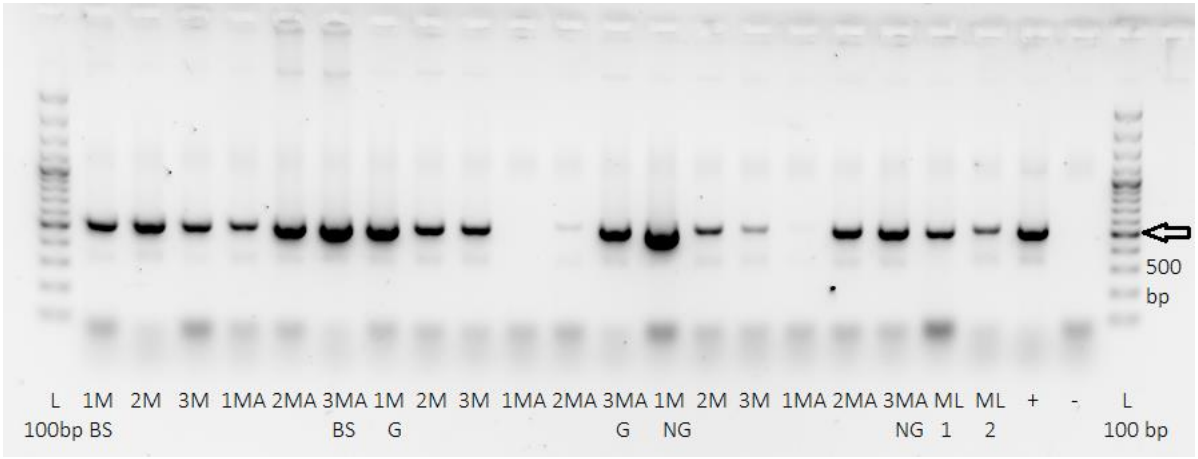

Figure 27: Verification with AGE of the presence of the transposon of the mutants by targeting the promoterless gfp gene with PCR and analysis of amplicons on AGE (1.5% gel, 90V, 75 min). 1M-3M: three replicas of the MSH1 transposon mutant library in monospecies condition; 1MA-3MA: three replicas of the MSH1 transposon mutant library in multispecies condition with ASFC; ML: MSH1 transposon mutant library inoculum; BS: before sorting; G: cells sorted by FACS as green fluorescent; NG: cells sorted by FACS as not green fluorescent; L: 100 bp plus ladder.

All separated mutant pools were cultivated under selective conditions based on kanamycin resistance. gDNA was extracted from all 12 sorted mutant pools and six mutant pools from before cell sorting and DNA concentrations were measured using Qubit 3.0 (Table 21). Within brackets is shown how much volume of this sample is required to obtain a DNA concentration of 10 µg in total in 50 µL, which was needed to shear. The MSH1 transposon mutant library of the inoculum had too low DNA concentrations in order to obtain 10 µg in total in 50 µL.

Table 21: DNA concentrations of sorted samples after growth; M lib.: MSH1 transposon mutant library; ASFC: Artificial sand filter community; GFP*: cells sorted by FACS as green fluorescent; Non-*GFP*: cells sorted by FACS as not green fluorescent; BS: all cells before sorting; TL: concentrations are too low to measure; TH: concentrations are too high to measure.*

	DNA concentration (ng/µL)		DNA concentration (ng/µL)
M lib. BS.	1980 (5 µL)	ASFC M lib. BS.	678 (15 µL)
	1920 (5 µL)		TH (5 µL)
	1560 (7 µL)		1480 (7 µL)
M lib. GFP	494 (20 µL)	ASFC M lib. GFP	112.6 (50 µL)
	1180 (9 µL)		782 (13 µL)
	1380 (8 µL)		TH (5 µL)
M lib. Non-GFP	768 (13 µL)	ASFC M lib. Non-GFP	133 (50 µL)
	1520 (7 µL)		148.6 (50 µL)
	842 (12 µL)		1320 (8 µL)
M lib. Inoculum	TL		
	TL		

gDNA was sheared to an average of 350 bp fragments and were analysed on AGE. Sheared fragments of the appropriate size were extracted and purified. Extracted DNA fragments were checked for size on AGE (Figure 28). The proper size of DNA fragments was extracted (100-600 bp). Next, the DNA concentrations were measured using Qubit 3.0 (Table 22).

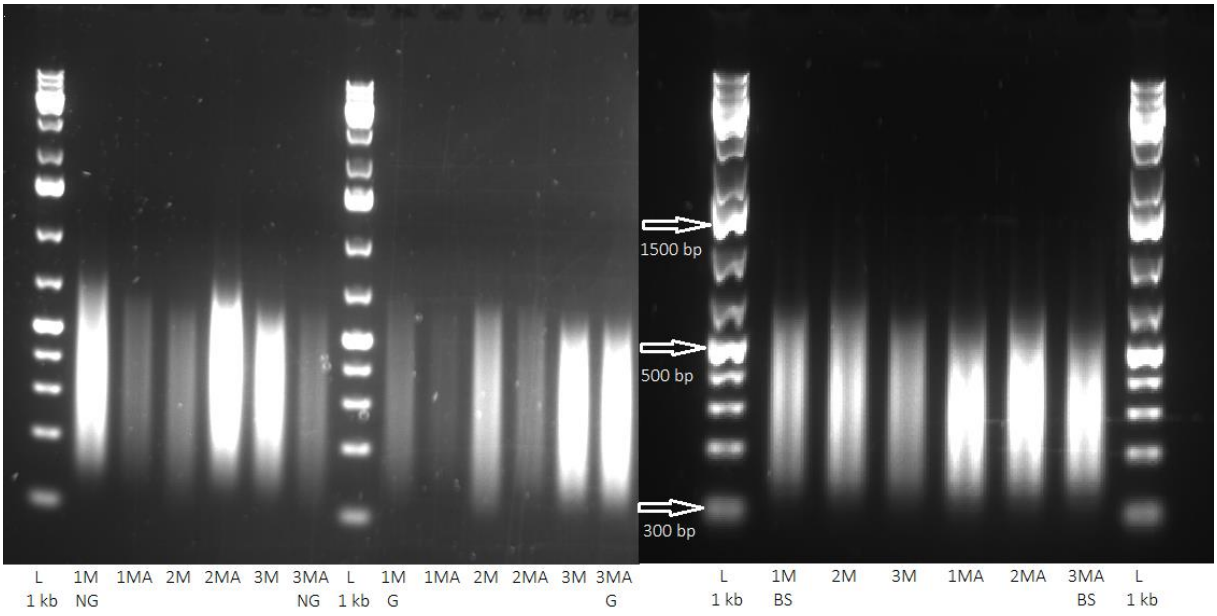

Figure 28: Verification of DNA fragments after gel extraction by AGE (1.5% gel, 90V, 1h); 1M-3M: three replicas of the MSH1 transposon mutant library; 1MA-3MA: three replicas of the MSH1 transposon mutant library with presence of Artificial sand filter community; L: ladder, 1 kb: Generuler 1 kb plus ladder; G: cells sorted by FACS as green fluorescent; NG: cells sorted by FACS as not green fluorescent; BS: before sorting.

Table 22: DNA concentrations of sorted samples after gel-extraction; M lib.: MSH1 transposon mutant library; ASFC: Artificial sand filter community; GFP*: cells sorted by FACS as green fluorescent; Non-*GFP*: cells sorted by FACS as not green fluorescent; BS: all cells before sorting.*

	DNA concentration (ng/µL)		DNA concentration (ng/µL)
M lib. BS.	55.0	ASFC M lib. BS.	79.8
	73.2		90.2
	43.0		70.6
M lib. GFP	27.0	ASFC M lib. GFP	7.6
	79.2		41.8
	152.0		196.0
M lib. Non-GFP	137.0	ASFC M lib. Non-GFP	29.6
	37.8		268.0
	119.2		36.4

4.4.4. Preparation of gene identification based on comparative transcriptomics

MSH1 wild type was inoculated after 14 days in six sterile flow chambers (monospecies) and six flow chambers colonized by an ASFC biofilm (multispecies). After an additional incubation of 28 days, biofilms were harvested from flow chambers and harvested cells were snap freezed and stored at -80°C until RNA-extraction was performed. After RNA-extraction from the harvested biofilms containing MSH1-wt with and without ASFC, DNA was removed by DNase treatment and RNA was further purified. Using reverse transcription qPCR, the MSH1 specific 16S rRNA copies were quantified (Table 23). The reverse transcriptase minus (RT-) negative control shows how much genomic DNA contamination was present in the different RNA samples. Consequently, the amount of copies per µL biofilm are reduced by the amount of copies in the reverse transcriptase minus (RT-) negative control since one is only interested in the number of rRNA copies (Table 23).

Table 23: Results of reverse transcription qPCR for cDNA samples: amount of copies 16S rRNA Aminobacter sp.; 17-19: MSH1-wt: MSH1 wild type; 23-25: ASFC + MSH1-wt: MSH1 wild type with artificial sand filter community; RT-: reverse transcriptase minus negative control.

Sample	# copies/µL (diluted)	# copies/biofilm sample (14µL)	cDNA samples - RT- (# copies/biofilm sample (14 µL))
17	1.21×10^5	3.40×10^7	$3,39 \times 10^7$
RT- 17	3.69×10^2	1.03×10^5	
18	1.28×10^5	3.59×10^7	$3,59 \times 10^7$
RT- 18	2.12×10^2	5.95×10^4	
19	9.94×10^4	2.78×10^7	$2,78 \times 10^7$
RT- 19	8.51×10^1	2.38×10^4	
23	9.72×10^3	2.72×10^6	$2,70 \times 10^6$
RT- 23	8.50×10^1	2.38×10^4	
24	1.30×10^5	3.63×10^7	$3,61 \times 10^7$
RT- 24	7.06×10^2	1.98×10^5	
25	9.60×10^4	2.69×10^7	$2,67 \times 10^7$
RT- 25	5.36×10^2	1.50×10^5	

The total amount of 16S rRNA copies per biofilm sample (volume of 14 µL) was determined (Table 23) and since 16S rRNA and 23S rRNA are always present in equal quantities also the amount of 23S rRNA

copies/µL is known. Using Avogadro's number the amount of copies per µL can be converted to an amount expressed in mol/µL. By using the molecular weight (1017720 pg/pmol and 1916640 pg/pmol for 16S rRNA and 23S rRNA respectively), the weight (pg) per µL can be determined for both 16S rRNA and 23S rRNA. By summing these weights, a good estimate for the total amount of RNA is obtained since 95% of the RNA comprises rRNA. The total amount of RNA that was extracted from a monospecies biofilm was 167.0 ± 20.5 pg. From a multispecies biofilm 176.1 ± 32.3 pg of total RNA was extracted.

4.5. Comparative transcriptome analysis from lab scale sand filter microcosm bioaugmented with MSH1

MSH1 wild type was inoculated after 30 days in columns with sterile sand filter material (monospecies) and columns with sand filter material colonized by an ASFC biofilm (multispecies), in triplicate. After an additional incubation of 7 days, biofilms were harvested from flow chambers and harvested cells were snap freezed until RNA-extraction was performed. First, the effect of using or omitting the separation column of the Powersoil Total RNA Isolation Kit was tested. Next, RNA was extracted from all remaining sand filter samples for comparative transcriptome analysis.

First, the effect of using or omitting the separation column of the Powersoil Total RNA Isolation Kit was tested for two sand filter samples from columns of the lab scale setup. Sample 1 and 2 had a weight of 4.24 g and 5.13 g, respectively. After RNA-extraction, DNase treatment, RNA clean-up and cDNA-synthesis, 16S rRNA copies were quantified with reverse transcription qPCR using the 16S rRNA *Aminobacter* sp. primers (Table 24). The amount of copies in the reverse transcriptase minus (RT-) negative control shows how much genomic DNA contamination was present in the different RNA samples. Consequently, the amount of copies per gram sand filter material are reduced by the amount of copies in the reverse transcriptase minus (RT-) negative control since one is only interested in the number of rRNA copies (Table 24).

Table 24: Results of reverse transcription qPCR for 16S rRNA: amount of copies 16S rRNA Aminobacter sp.; RT-: reverse transcriptase minus negative control.

Sample	# copies/µL	# copies/sand filter material sample	# copies/g sand filter material	samples - RT- (# copies /g sand filter material)
1	2.11×10^8	5.90×10^9	1.39×10^9	1.39×10^9
RT- 1	1.58×10^2	2.22×10^4	5.23×10^3	
2	8.31×10^6	6.52×10^8	1.27×10^8	1.27×10^8
RT- 2	6.77×10^2	5.31×10^4	1.04×10^4	

The total amount of RNA (per gram sand filter material) was estimated, as previously shown for RNA from biofilms grown in flow chambers as mentioned above. In this case, the total amount of RNA that can be extracted from one lab scale sand filter column using one of both techniques is calculated (Table 25). By using the separation column more total RNA was extracted.

Table 25: Total RNA (pg) possible to extract form one column with sand filter material using both techniques; 1: with Powersoil Total RNA Isolation separation column; 2: without this separation column.

Sample	16S pg/g sand filter material	23S pg/g sand filter material	Total RNA pg/g sand filter material	Total RNA pg per column
1	$2{,}35 \times 10^3$	$4{,}43 \times 10^3$	$6{,}78 \times 10^3$	$1{,}72 \times 10^5$
2	$2{,}15 \times 10^2$	$4{,}04 \times 10^2$	$6{,}19 \times 10^2$	$1{,}91 \times 10^4$

In addition, a second reverse transcription qPCR was conducted for the determination of *bbdA* mRNA transcripts to detect the presence of mRNA (Table 26). Also in this determination are the dilution factors and the RT- controls taken into account.

Table 26: Results of qPCR for cDNA samples: amount of bbdA gene copies; RT-: reverse transcriptase minus negative control.

Sample	# copies/µL	# copies/sand filter material sample	# copies/g sand filter material	samples - RT- (# copies /g sand filter material)
1	9.77×10^1	2.74×10^3	6.45×10^2	2.94×10^2
RT- 1	1.065×10^1	1.49×10^3	3.52×10^2	
2	6.09×10^3	4.77×10^5	9.30×10^4	9.25×10^4
RT- 2	3.76×10^1	2.94×10^3	5.74×10^2	

For all remaining sand filter samples, RNA was extracted from the lab scale sand filter columns inoculated with ASFC and with ASFC and MSH1. Each sand filter column (approx. 30 g) was divided in six samples for extraction and afterwards combined and purified. Per condition, the results of all six samples were summarized (Table 27). Reverse transcription qPCR was performed to quantify 16S rRNA copy numbers. The total amount of copies of 16S rRNA that was extracted from a lab scale column and dissolved in 14 µL (after purification) was calculated (Table 28).

Table 27: The amount of sand filter material used for RNA extraction given in gram for 6 different microcosms columns.

Condition	Amount of sand filter material (gram)	Condition	Amount of sand filter material (gram)
1 MSH1-wt	29,342	4 ASFC + MSH1-wt*	32,968
2 MSH1-wt	28,184	5 ASFC + MSH1-wt*	26,525
3 MSH1-wt	33,761	6 ASFC + MSH1-wt*	36,094

Table 28: Results of qPCR for cDNA samples: amount of copies 16S rRNA Aminobacter sp.; RT-: reverse transcriptase minus negative control.

Sample	# copies/µL (diluted)	# copies/sand filter material sample	samples - RT- (# copies /sand filter material sample)
1 MSH1-wt	1.22×10^6	2.06×10^9	$2,06 \times 10^9$
RT- 1	3.32×10^1	5.57×10^4	
2 MSH1-wt	7.77×10^5	1.31×10^9	$1,31 \times 10^9$
RT- 2	4.39×10^1	7.37×10^4	
3 MSH1-wt	1.87×10^6	3.15×10^9	$3,15 \times 10^9$
RT- 3	1.06×10^2	1.78×10^5	
4 ASFC + MSH1-wt*	1.46×10^6	2.45×10^9	$2,45 \times 10^9$
RT- 4	3.26×10^1	5.48×10^4	
5 ASFC + MSH1-wt*	2.96×10^6	4.98×10^9	$4,98 \times 10^9$
RT- 5	3.31×10^1	5.57×10^4	
6 ASFC + MSH1-wt*	5.76×10^5	9.67×10^8	$9,67 \times 10^8$
RT- 6	1.94×10^1	3.26×10^4	

The total amount of RNA (in 14 µL) was estimated, as previously shown for RNA from biofilms grown in flow chambers as mentioned above. The total amount of RNA that was extracted from a monospecies lab scale sand filter column was $1.06 \times 10^4 \pm 4512$ pg. From a multispecies lab scale sand filter column $1.36 \times 10^4 \pm 9874$ pg of total rRNA was extracted.

5. Discussion

The main metabolite of the broad-spectrum nitrile herbicide dichlobenil, 2,6-dichlorobenzamide (BAM), is an important contaminant of groundwater which is used for drinking water production. *Aminobacter* sp. MSH1 is capable of mineralizing BAM at ecologically relevant concentrations till below the limit of 0.1 µg/L, making this bacterial strain a promising candidate for bioaugmentation in sand filters of DWTPs as a sustainable alternative for the expensive GAC filtration. The success of this bioaugmentation depends on the capability of the strain to invade the indigenous bacterial community effectively and to survive in oligotrophic sand filter conditions where social interactions such as cooperation and/or competition occur. The main goal of this thesis was to gain insight in key processes occurring during the survival of MSH1 when invading the sand filter environment and identify genes that encode for these processes. Different lab scale experiments were performed to examine those key processes, such as the loss of BAM mineralization, dormancy and lower cell viability of MSH1 in biofilms grown in oligotrophic conditions.

Mineralization stability of *Aminobacter* sp. MSH1 in biofilms grown in oligotrophic conditions

A first key process is the BAM degrading and mineralization activity and stability of MSH1 in oligotrophic conditions when residing in the sand filter environment. BAM mineralization by MSH1 is encoded on two plasmids, pBAM1 and pBAM2. The amidase BbdA converts BAM to DCBA and is encoded by the *bbdA* gene which is located on pBAM1. The genes responsible for further degradation of DCBA, and hence responsible for BAM mineralization, are located on plasmid pBAM2. According to T'Syen *et al.* (unpublished), the loss of BAM mineralizing capability is linked to the loss of the entire plasmid pBAM2. In a previous study (89), it was shown that loss of pBAM2 occurred when MSH1 was grown in R_2B medium amended with 200 mg/L BAM. However, when MSH1 was cultivated in MS medium with 200 mg/L BAM, pBAM2 numbers increased and when next transferred to R_2B, no loss of pBAM2 occurred. In a **first experiment related to the mineralization stability in MSH1, the incidence of pBAM2 in the MSH1 cell population was determined when MSH1 grows as biofilms in oligotrophic conditions.** First, cultures with a low (not enriched) incidence and with a high (enriched) incidence of MSH1-GFP cells carrying pBAM2 were generated. Two continuous flow cell experiments were performed, one with 100 µg/L AOC and another without additional AOC **to examine how loss or gain of pBAM2 is impacted in the presence of AOC**. Different nominal concentrations of BAM were used **to verify whether the pBAM2 incidence increased with increasing BAM-concentrations.** From the results of this experiment, no significant difference in BAM-degradation was detected between enriched and not enriched cultures. Except at BAM-concentrations of 1 µg/L, where when 100 µg/L AOC was present the lowest residual BAM percentage was obtained (23 ± 4%) by the enriched culture while without AOC the not enriched culture

degraded BAM more efficiently (37 ± 2% BAM residual). Further research is needed to explain this difference in BAM-degradation between enriched and not enriched cultures at 1 µg/L BAM. In addition, it is observed that when 100 µg/L AOC was present, in general, a lower amount of residual BAM was detected in the effluent compared to no AOC. This can be explained by a higher amount of biomass in the presence of AOC, as was confirmed by CLSM analysis. Since the amidase is constantly produced in MSH1 cells as the *bbdA* gene is constitutively expressed, the number of MSH1 cells in the biofilm determines the overall BAM-degradation rate. This difference in the amount of residual BAM in the effluent between 100 µg/L AOC and without AOC in MS medium used to feed the flow chambers increased with increasing BAM-concentrations in the feed. This increasing difference can be explained because when the feed had a higher BAM-concentration, more BAM had to be degraded to obtain the same percentage in BAM-degradation for when the feed contained only 1 µg/L BAM. When no AOC was present in MS medium, insufficient biomass was present to achieve a high percentage of BAM-degradation resulting in higher amounts of residual BAM, even at high initial BAM-concentrations. This observation demonstrated that 1000 µg/L BAM was an insufficient source of carbon compared to 100 µg/L AOC and as a result no significant higher amount of biomass was detected. Further research should be conducted to verify what percentage of BAM is effectively assimilable. Another observation made in the presence of 100 µg/L AOC and at low concentrations of BAM (< 10 µg/L and < 100 µg/L for not enriched and enriched culture, respectively) is a decrease of the ratio *bbdB/bbdA*. However, without additional AOC and at low BAM-concentrations this ratio does not change between the time of inoculation and the harvest of the biofilms. Besides, this ratio *bbdB/bbdA* increased at a BAM-concentration of 100 µg/L and 1000 µg/L or higher for the not enriched (3% MSH1-$GFP_{B+/D+}$) culture and the enriched (20% MSH1-$GFP_{B+/D+}$) culture respectively with AOC present in the MS medium. For the not enriched (0.1% MSH1-$GFP_{B+/D+}$) culture with AOC depleted MS medium, *bbdB* numbers already increased at BAM-concentrations of 10 µg/L or higher.

Two possible explanations were formulated for the increase of pBAM2 within the MSH1 population. First, plasmids can be transferred from cell to cell by HGT such as conjugation. Also here, pBAM2 might be transferred in that manner within the MSH1 population when BAM becomes an interesting C-source at the higher concentrations. A second explanation, might be that the pBAM2 carrying MSH1 cell population has, thanks to its ability to grow on BAM, a selective advantage in conditions with high BAM-concentrations to pBAM2-deficient cells which are limited to growth on AOC.

The decrease in pBAM2 can also be explained in two ways. First, during cell division pBAM2 copies are not always equally distributed amongst the daughter cells when growing. A second explanation, is that the pBAM2-carrying MSH1 population has a lower growth rate due to the burden of carrying an extra plasmid compared to the pBAM2-deficient MSH1 population. As such when growing in conditions of

low selectivity i.e. low BAM-concentrations, the pBAM2-carrying MSH1 populations lags behind and results in a decreasing incidence of this population after several generations.

It was tested whether i) pBAM2 is gained by HGT or lost during cell division of MSH1, ii) pBAM2 dynamics can be explained by the occurrence of a slow growing pBAM2-carrying and a rapid growing pBAM2-deficient MSH1 population, where the first population has a selective advantage at higher BAM-concentrations. As such, we determined whether **horizontal gene transfer of pBAM2 occurred** amongst the MSH1 cells in biofilms grown under oligotrophic conditions or whether **pBAM2 is only passed on vertically during mitosis**. This was investigated in another continuous flow cell experiment by inoculating flow chambers with a 100% BAM-carrying (MSH1-$wt_{B+/D+}$) cell population, and a mixture of pBAM2-carrying and pBAM2-deficient (MSH1-$GFP_{B+/D-}$) population at a ratio of 50/50 and 1/99 and growing them at 10 and 1000 µg/L BAM. As a control a 100% BAM-deficient cell population was grown in the same conditions. Using a kanamycin resistant and GFP labelled MSH1-GFP deficient in pBAM2 enabled us to determine whether these cells received pBAM2 by HGT with the pBAM2-carrying MSH1-wildtype. When looking at the results for BAM and DCBA residuals in the effluent, no DCBA accumulation is observed for flow chambers inoculated with 100% MSH1-$wt_{B+/D+}$, 50/50 or 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ at initial BAM-concentrations of 1000 µg/L. However, for 10 µg/L BAM, DCBA accumulation was detected for the 50/50 and 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ cultures. However, it is possible that other unknown metabolites accumulate due to DCBA degradation. The genes for DCBA degradation need to be induced as such the concentration of DCBA can influence the expression of these genes and as such 10 µg/L might lead to a too low expression level and production of the necessary enzymes for DCBA degradation. However, the 100% pBAM2-carrying population showed full BAM-degradation as no DCBA was accumulated. So only in combination with a lower incidence of the pBAM2-carrying population DCBA was accumulated for 50% and 100% of the removed BAM in case of 50/50 and 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$, respectively. In other words, it seems that when 50% of the population had the capability to mineralize BAM (pBAM2-carrying MSH1-$wt_{B+/D+}$ cells), 50% of the removed BAM was mineralized resulting in no accumulation. The other 50% of the population (MSH1-$GFP_{B+/D-}$ cells), not able to mineralize BAM, degraded BAM to DCBA, which was released. Since DCBA accumulation was observed, it seems that this DCBA, released by MSH1-$GFP_{B+/D-}$ cells, could not be degraded by the pBAM2-carrying MSH1-$wt_{B+/D+}$ cells. Possible explanations are mass transfer limitations of DCBA from the deficient population to pBAM2-carrying MSH1-$wt_{B+/D+}$ cells or the inability to take up DCBA by the pBAM2-carrying MSH1-$wt_{B+/D+}$ cells. Mass transfer limitation seems very unlikely since no mass transfer limitations were detected for BAM. The inability to take up DCBA should be further investigated but so far this was never observed for MSH1 in suspended batch experiments.

The fraction of MSH1-wt and MSH1-GFP in the biofilms was determined by using flow cytometry. But this technique was not accurate since GFP needs to be activated with O_2. As a result, a MSH1-GFP cell that was freshly formed, would give a very weak signal causing an overlap in the results of the flow cytometer between MSH1-GFP and MSH1-wt. A correction factor could be calculated by means of control MSH1-GFP but even with this factor, the results remain inaccurate. An alternative technique to quantify both fractions is a real time qPCR that quantifies the amount of Km^R gene copies by using primer *Aph F and Aph R*. This Km^R gene encodes for kanamycin resistance and is located on the Gfp2X-miniTn5Km carried by MSH1-GFP cells. To verify whether pBAM2-carrying MSH1-$GFP_{B+/D+}$ occurred as a result of HGT between the MSH1-wtB+/D+ and MSH1-GFP B+/D+, harvested biofilm cell suspensions were plated and colonies were selectively grown in the presence of kanamycin. MSH1-GFP colonies were screened for the presence of the *bbdB* gene. However, in none of the tested conditions MSH1-GFPB+/D+ occurred and as such HGT did not occur or at a frequency that wasn't detected. Because of this determination, we can conclude that a selective advantage of the pBAM2 carrying MSH1 cell population would be the most likely explanation for the increase of pBAM2 within the entire MSH1 population used in the stability experiments above, since these cells have the ability to grow on BAM, in conditions with high BAM-concentrations.

qPCR results for the 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ culture showed a 3-fold increase of the ratio *bbdB/bbdA* at initial BAM-concentration of 10 µg/L and an 5-fold increase of this ratio at 1000 µg/L BAM. This corresponds partially with the qPCR results of previous stability experiment. No decrease of the ratio *bbdB/bbdA* was observed in this experiment given that 10 µg/L BAM probably is still a sufficient abundant carbon source. As a result, no decision can be made about which explanation appears to be the most probable one for the decrease in pBAM2 seen in the stability experiments. Both explanations should be examined more thoroughly by growing biofilms with the same mixtures of MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ in flow chambers fed with MS medium without BAM and residual AOC.

As such the decrease in the ratio *bbdB/bbdA* can still be explained in two ways. A first one is a not always equally distribution of pBAM2 amongst the daughter cells when growing. The rate of pBAM2 loss due to additional AOC, since the ratio *bbdB/bbdA* decreased only in the presence of AOC in the stability experiment, can be calculated.

The second explanation is a difference in growth rate between pBAM2-carrying and pBAM2-deficient MSH1 cells. The growth rate of both cultures depends on the concentrations of BAM and AOC. When BAM is an abundant carbon source, it is probable that MSH1-$wt_{B+/D+}$ (carrying two plasmids) still grows slower in comparison with MSH1-$GFP_{B+/D-}$ but since a larger amount of carbon source (BAM) is available for MSH1-$wt_{B+/D+}$ cells, these cells will be able to grow longer relative to MSH1-$GFP_{B+/D-}$. When BAM is insufficient as a source of carbon and as a result both MSH1-$GFP_{B+/D-}$ and MSH1-$wt_{B+/D+}$ should mainly

grow on the available AOC, MSH1-wt $_{B+/D+}$ will fall behind relative to MSH1-GFP $_{B+/D-}$because MSH1-wt $_{B+/D+}$ will grow more slowly compared to MSH1-GFP $_{B+/D-}$ since these cells carry two plasmids. This last explanation is likely to be the most probable one since in the stability experiment, a decrease in pBAM2 was observed at initial BAM-concentrations of 10 µg/L in the MS medium. While in this experiment with 10 µg/L BAM in MS medium used to feed the flow chambers, the ratio *bbdB/bbdA* remained the same as the inoculum. As a result, it is likely that no loss of the *bbdB* gene occurred. In the environment, the ratio *bbdB/bbdA* will probably decrease or stay low as seen for labscale experiments at BAM-concentrations of 10 µg/L or lower. This raises questions about the full mineralization of BAM in the environment and more importantly in sand filters bioaugmented with MSH1 for the removal of BAM at micropollutant concentrations.

In addition to HGT also other aspects important for a successful bioaugmentation of MSH1 to degrade BAM such as **the cell viability and aggregation** in MSH1 biofilms grown under oligotrophic conditions are also examined in this experiment. The cell viability in both biofilms and effluent is 2-fold higher at a BAM-concentration of 1000 µg/L compared to a BAM-concentration of 10 µg/L. This confirms that when BAM is present at higher concentrations as an additional carbon source more living cells will be present. Cells in biofilms will grow, however, the effluent consist of mainly living cells which causes a loss of live potential and active biomass. This loss of live potential and active biomass can be put into perspective as only a small percentage (1-3%) of the biofilm will rinse. The ratio of the amount of living cells at 10 µg/L BAM to the amount of living cells at 1000 µg/L BAM is very different for all four cultures. MSH1-$wt_{B+/D+}$ is able to grow on BAM resulting in a 4-fold higher amount of living cells at 1000 µg/L BAM compared to 10 µg/L BAM. For the 50/50 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ culture a 2-fold higher amount of living cells at 1000 µg/L BAM compared to 10 µg/L BAM was found. For the 99/1 MSH1-$GFP_{B+/D-}$/MSH1-$wt_{B+/D+}$ culture, the amount of living cells at both BAM-concentrations are approximately equal, because only 1% of the culture can use BAM as a carbon source. This ensures no significant influence by the presence or absence of BAM on the amount of living cells.

Another important factor for a successful bioaugmentation that is investigated in this thesis, is the **formation of aggregates** of cells in biofilms. The results showed that approximately 85% of the cells in biofilms were single cells independent of the BAM-concentration in MS. However, a significant difference in single cell fraction of the effluent was observed between BAM-concentrations of 10 µg/L and 1000 µg/L. The effluent of flow chambers fed with 1000 µg/L BAM consist mostly of single cells, while for effluent from flow chambers fed with 10 µg/L BAM mainly of aggregates were detected. A possible explanation for this difference is that at BAM-concentrations of 10 µg/L starvation takes place which can result in aggregates that break apart from the biofilm due to shear stress and are flushed out

of the flow chamber. By contrast, at BAM-concentrations of 1000 µg/L it may be that active dispersion occurs in which individual cells will colonize various and potentially more suitable micro-environments.

Invasion of the sand filter environment in oligotrophic conditions: the importance of the indigenous sand filter community

The capability of *Aminobacter* sp. MSH1 to colonize surfaces, such as sand filter material, is an important factor for the success of bioaugmentation. According to a study of Aswini *et al.* (not published), sand and the glass surface in flow cells have similar surface properties since both are silica-based materials. MSH1 has the ability to attach to sand and to develop a biofilm under shear conditions. In addition, this study of Aswini *et al.* is the first study that shows that a xenobiotic degrading microorganism is able to colonize a solid surface under conditions mimicking the conditions in biofilter systems for water treatment at environmental relevant concentrations of the micropollutant. In a study of Yanti *et al.* (89), it was observed that MSH1 was able to colonize surfaces in the presence of an indigenous sand filter community. In this thesis, it was investigated whether *Aminobacter* sp. MSH1 was able to colonize surfaces as a pioneer (co-colonization) or a successor (invasion) in the presence of an indigenous sand filter community. In both conditions, MSH1 probably had to compete for nutrients and space. Difference in BAM-degradation, biofilm formation and mineralizing capacity between co-colonization and invasion were investigated **to verify whether invasion is more difficult since a well-adapted biofilm of a sand filter community was already present**. These difference may be of importance for practical implications when in a drinking water treatment plant. For instance, the bioaugmentation of a pristine sand filter right before operation or that of a sand filter already operational for some time (and colonized by other bacteria) might lead to a different outcome for the survival of MSH1 in the sand filter and influence BAM removal as such.

Two continuous flow cell experiments were performed, one with 100 µg/L AOC and another with 300 µg/L AOC **to examine how the extent of available AOC determined the survival of MSH1 in biofilms developed under both scenarios.** From the BAM-degradation results, a first difference between co-colonization and invasion was observed. In case of co-colonization, a significant decrease in residual BAM was only observed two weeks after inoculation of MSH1 with or without ASFC. Whereas in case of invasion, no BAM removal was observed in the first 14 days of operation both in the flow chambers with and without the ASFC. But when MSH1 was added, the residual BAM-concentration decreased instantly. In addition, it appears from these BAM-degradation results that significantly more BAM was degraded at 300 µg/L AOC compared to 100 µg/L AOC. The greater extent of BAM-degradation due to a higher concentration of available AOC can be explained by a higher amount of biomass, as was confirmed by CLSM analysis. But, this difference is more substantial in the case of co-colonization (15 ± 6% vs 61 ± 9%) in comparison with invasion (13 ± 4% vs 31 ± 5%). It can therefore be concluded that the absence

of a high extent (300 µg/L) of available AOC especially has a negative influence on the degradation of BAM in case of co-colonization. A possible explanation for this negative influence of the absence of a high extent (300 µg/L) of available AOC is that when a low extent of AOC is available the competition effects between ASFC and MSH1 will increase. However, when a high extent of AOC is available, sufficient AOC is present for both cultures to grow on. Since DCBA cannot be measured below 1 µg/L, nothing could be said about mineralization from the UPLC-MS/MS results. But we can expect DCBA to accumulate probably in most cases of bioaugmentation since DCBA accumulation was already detected in the experiment investigating HGT at initial BAM-concentrations of 10 µg/L, while in this experiment only 1 µg/L BAM was present in MS medium. This low BAM-concentration is insufficient as a source of carbon to provide a selective advantage to BAM mineralizing cells. From the CLSM images of MSH1-GFP biofilms developed on 300 µg/L AOC and 1 µg/L BAM, a difference between co-colonization and invasion was observed. More specifically, a greater amount of biomass was detected in case of invasion, while MSH1-GFP cells were allowed to grow in both cases for 4 weeks. A possible explanation for this greater amount of biomass in case of invasion could be that MSH1-GFP was only inoculated after 14 days for invasion, whilst for co-colonization MSH1 was injected in the beginning of the experiment. In the case of invasion, the glass surfaces could be 'primed' with a conditioning film, formed from organic and inorganic molecules for the bulk liquid phase, which could facilitate the attachment (90). Another difference between co-colonization and invasion biofilms was observed for the biofilms of ASFC and MSH1-GFP combined. For co-colonization separate colonies of MSH1-GFP were detected, while for invasion small colonies of MSH1-GFP were surrounded and overgrown by sand filter bacteria. This difference indicates that for co-colonization most likely competition occurs between ASFC and MSH1-GFP, while in the case of invasion rather cooperation occurs. Another difference between co-colonization and invasion is the ratio of *bbdB* to *bbdA*. The ratio *bbdB/bbdA* in case of invasion is significantly higher compared to the ratio for co-colonization for both MSH1 with and without ASFC, but only at 100 µg/L AOC in the medium. In addition, it would be interesting to perform an OTU analysis for this experiment in order to determine whether co-colonization and invasion produced led to a difference in the final species abundance of the ASFC community.

We can conclude from this experiment that invasion as bioaugmentation strategy is more beneficial since lower amounts of residual BAM was detected in the effluent using this technique compared to co-colonization. In addition, the ratio *bbdB/bbdA* is higher in case of invasion which will probably result in less accumulation of DCBA but this could not be verified since DCBA cannot be measured below 1 µg/L. But since we can expect DCBA to accumulate probably in most cases of bioaugmentation, it could be interesting to use a stable DCBA-degrading bacterial strain or community simultaneously added with MSH1 during bioaugmentation. However, further research is definitely needed. Another conclusion that

could be made from this experiment is that the use of surface water would encourage BAM-degradation but the conditions of surface water knows a lot more fluctuations compared to groundwater conditions. In addition, BAM is mainly a groundwater contaminant. A number of other aspects and their influence on the success of bioaugmentation that should be investigated in further research are the change of sand filter material surface during time and differences in indigenous sand filter communities in the sand filters of different DWTPs.

'Social' gene identification in MSH1 when invading a sand filter community: Where are we now?

In a second part of this thesis, a continuous flow cell experiment fed with 1 µg/L BAM was performed **to identify 'invasion' genes that encode for key processes occurring during the survival of MSH1 when invading the sand filter environment, using three independent high-throughput approaches.**

For all three approaches, BAM-degradation and biofilm formation were verified. It is observed that the BAM-degradation results correspond well with the BAM-degradation results of the invasion – co-colonization experiment (more residual BAM present in effluent from flow cells with ASFC). Effluent from flow cells inoculated with the MSH1 promotor probe library had a higher percentage of BAM remaining after 35 days compared to the other cultures. A possible explanation for the higher amount of residual BAM for flow cells with the MSH1 promoter probe library, is that those MSH1 vector cells carry an extra plasmid resulting in a slower growth rate and consequently in a lower amount of biomass in the biofilms.

For a first approach, called **comparative transcriptomics**, MSH1 wild type (wt) was inoculated after 14 days in six sterile flow chambers (monospecies) and six flow chambers colonized by an ASFC biofilm (multispecies). From the results of reverse transcription qPCR, used to quantify the MSH1 specific 16S rRNA copies in the purified RNA samples, the total amount of RNA that was extracted from one biofilm was estimated. The total amount of RNA that was extracted from a monospecies biofilm was 167.0 ± 20.5 pg. From a multispecies biofilm 176.1 ± 32.3 pg of total RNA was extracted. Since six flow chambers were used for each condition (monospecies and multispecies), 1.002 ± 0.123 ng and 1.057 ± 0.194 ng total RNA was available for sequencing for the monospecies and multispecies condition respectively. ScriptSeq library preparation for RNA-Seq (Illumina) is preferred since in this procedure rRNA removal is performed together with first strand cDNA synthesis from mRNA transcripts and cDNA amplification. A quantity of 100 ng total RNA is given as minimal amount for library preparation for ScriptSeq sequencing. As a result, an insufficient amount of total RNA was extracted from the continuous flow cell experiment.

After sequencing, mRNA transcripts need to be compared between monospecies and multispecies conditions to identify 'invasion' genes. Those 'invasion' genes have elevated numbers of mRNA transcripts in multispecies condition compared to the monospecies condition.

For the second approach, called differential fluorescence induction, a MSH1 promoter probe library of approx. 30 000 reporters was previously constructed by introducing random fragments (1-2 kb) of gDNA of MSH1 into a promoter vector in front of a promoterless *gfp* gene. Next, these various vectors were introduced in MSH1 cells making them reporters of the expression of its own genes. When a promoter on this fragment was activated, the cell became GFP fluorescent as the promoterless *gfp* gene was transcribed. The promoter probe library was grown in the absence and presence of the sand filter community. The biofilms were harvested from flow chambers at the end of the experiment and the harvested cells were sorted by FACS into GFP and non-GFP MSH1 reporters pools. Since only 10^3 GFP-labelled events could be separated for biofilms in the presence of the sand filter community, it can be concluded that the use of a continuous flow cell system mimicking the sand filter environment is reaching the lower limit of the differential fluorescence induction approach. Since only 1000 of the 30 000 vectors were isolated using FACS, the results will be biased.

Next, all sorted cell populations of the promoter probe library were selectively grown based on gentamycin resistance and when the OD reach 0.2 gDNA and plasmids were extracted. Before sequencing could be performed, an entire work flow must be completed. First, inserts (1-2 kb) from the vectors present in the cell cultures were amplified using PCR. These fragments were analysed on AGE, which shows that in addition to the fragments of interest, fragments with a size of 300 bp are present. Since we are only interested in the fragments between 1-2 kb of the amplicons of the inserts, these fragments (1-2 kb) were extracted and purified. An explanation for the presence of fragments with a size of 300 bp is the presence of vectors carrying no insert. These fragments are consequently short and easily amplified.

The next step of the work flow is shearing the inserts to insert fragments of average 300 bp. 100 ng of 1000-2000 bp fragments are required for this shearing. The results of DNA measurements of gel-extraction samples with amplicons of inserts of proper size (±1500 bp), which was confirmed on AGE, show that the DNA-concentrations after gel-extraction and purification are sufficient, for the available volume, to get 100 ng of 1000 – 2000 bp insert fragment library for each sample. After the shearing, another gel-extraction was performed to obtain a 250-350 bp fragment library. The final steps in the work flow, PCR amplification with standard Illumina primers, purification and quantification with qPCR, belong to a library preparation prior to Illumina sequencing.

After sequencing, active promoters in the absence and presence of the artificial community need to be compared to identify 'invasion' genes which are specifically expressed as a response to the artificial community can be identified. Thus, genes that were expressed (GFP-labelled) in the presence of the artificial community but not expressed (Non-GFP labelled) in the absence of the artificial community are 'invasion' genes.

A last approach used, is transposon mutagenesis. A transposon mutant library of MSH1 of approx. 15 000 mutants, created by a mini-Tn5 transposon system with a promoterless *gfp* reporter gene that knocked out the genes of *Aminobacter* sp. MSH1 by randomly introducing a transposon in the genomic DNA, was inoculated in sterile flow chambers (monospecies) and flow chambers colonized by an ASFC biofilm (multispecies). Biofilms were harvested from flow chambers and harvested cells were sorted by FACS into a GFP-labelled and non-GFP-labelled pool. Next, all sorted cell populations of the promoter probe library were selectively grown based on kanamycin resistance. gDNA was extracted from all sorted mutant pools and mutants pools from before cell sorting. Before sequencing could be performed, an entire work flow must be completed. First, the extracted gDNA had to be sheared in fragments of average 350 bp and this required 10 µg gDNA. The results of DNA measurements after gDNA extraction showed that sufficient gDNA was extracted to obtain a DNA concentration of 10 µg in total in 50 µL. This DNA concentration was acquired by taking the required amount of volume for each sample. The next step in the work flow is a gel-extraction and purification of the sheared fragments of appropriate size. The extracted DNA fragments were checked for size on AGE, which revealed that DNA fragments with a size of 100-600 bp were extracted. The final steps in the work flow, PCR amplification with specific forward primer and custom Illumina reverse primer, purification and quantification with qPCR, belong to a library preparation prior to Illumina sequencing. For this purpose, 100 ng of 250-350 bp fragment library was required. The results of DNA measurements of gel-extraction samples with DNA fragments of proper size (100-600 bp), show that the DNA-concentrations after gel-extraction and purification are sufficient, for the available volume, to get 100 ng of 250-350 bp insert fragment library for each sample.

Mutants with a disrupted gene essential for 'invasion' will be lost in the mixed-species condition but will be present in mono-species conditions. Consequently, these genes will be considered as essential 'invasion' genes. After sequencing, these 'invasion' genes can be identified by comparing the mutant pools from before cell sorting in the absence and presence of the artificial community. In addition, this technique also provides information on non-essential 'invasion' genes. When the promoter of the knocked out gene is activated, the cell will be GFP fluorescent as the promoterless *gfp* gene is transcribed. After sequencing, these non-essential 'invasion' genes can be identified by comparing active promoters in the absence and presence of the artificial community using the sorted mutant pools.

Non-essential 'invasion' genes are knocked out genes from which the promoter was activated in multispecies condition but inactivated in monospecies conditions.

The mineralization stability of *Aminobacter* sp. MSH1 in biofilms grown in oligotrophic conditions is also important for the gene identification experiment because the MSH1 promoter probe library and the MSH1 transposon mutant library were both obtained starting from a MSH1 culture of which 1% of the cells is able to mineralize BAM. When an enrichment of these mineralizing MSH1-cells would occur due to a selective advantage by growth on BAM, only 1% of the library would be investigated anymore. This would result in a bias in the identification of 'invasion' genes. But from the results of the stability experiments, we observed that an enrichment of mineralizing MSH1-cells only occurred at BAM-concentrations above 10 µg/L. The BAM-concentration of MS medium used for the gene identification experiment is 1 µg/L resulting in no enrichment of mineralizing MSH1-cells. But a loss of the mineralizing MSH1 cells could occur according to the results of the stability experiments, since 100 µg/L AOC was present in MS medium used for the gene identification experiment, but this will not lead to an excessive bias because only 1% of the cells may be lost.

In the HGT experiment, performed in this thesis, was demonstrated that the biofilms formed in continuously fed flow cells mimicking the oligotrophic conditions of the sand filter environment, to a large extent consisted of dead cells. This observation is a major concern for the comparative transcriptomics approach since it is not known whether and for how long rRNA remains stable after cell death. If rRNA remains stable after cell death, this would result in an overestimation of the amount of mRNA present in each sample for the comparative transcriptomics approach. However, further research is needed to verify this assumption.

Although, continuously fed flow chambers used for gene identification mentioned above mimic the sand filter environment, e.g. oligotrophy, shear stress, biofilm formation and species interactions, some important mechanisms might be missed. Therefore, last objective of this thesis, was the identification of 'invasion' genes in MSH1 using comparative transcriptomics when MSH1 invades labscale sand filters in the absence and presence of the sand filter community. MSH1 wild type (wt) was inoculated after 30 days in columns with sterile sand filter material (monospecies) and columns with sand filter material colonized by an ASFC biofilm (multispecies). At the end of the experiment, RNA was extracted from the sand filter material. First, the effect of using or omitting the separation column of the Powersoil Total RNA Isolation Kit was tested for two sand filter samples from columns of the labscale setup. From the results of reverse transcription qPCR, used to quantify the MSH1 specific 16S rRNA copies in the purified RNA samples, the total amount of RNA that could be extracted from one labscale sand filter column was estimated for both techniques. The total amount of RNA that could be extracted from one labscale sand filter column by using the separation column was estimated to be 1.7×10^5 pg. When the separation

column was omitted, it was estimated that 1.9×10^4 pg of total RNA could be extracted. Consequently, it can be concluded that more total RNA can be extracted when the separation column is used.

Next, RNA was extracted from all remaining sand filter samples for comparative transcriptome analysis. The total amount of RNA that was extracted from a monospecies lab scale sand filter column was 10.6 ± 4.51 ng. From a multispecies lab scale sand filter column 13.6 ± 9.87 ng of total rRNA was extracted.

Conclusion

The main metabolite of the broad-spectrum nitrile herbicide dichlobenil, 2,6-dichlorobenzamide (BAM), is an important contaminant of groundwater which is used for drinking water production. *Aminobacter* sp. MSH1 is capable of mineralizing BAM at environmentally relevant concentrations till below the limit of 0.1 µg/L, making this bacterial strain a promising candidate for bioaugmentation in sand filters of DWTPs as a sustainable alternative for the expensive GAC filtration. The main goal of this thesis was to gain insight in key processes occurring during the survival of MSH1 when invading the sand filter environment and identify genes that encode for these processes using different lab scale experiments.

We showed that the MSH1 population used for bioaugmentation is composed of two subpopulations: one pBAM2-carrying MSH1 subpopulation capable of BAM-mineralization and one pBAM2-deficient MSH1 subpopulation which simply converts BAM to DCBA as end product. In oligotrophic conditions, the presence of residual AOC and the concentration of BAM largely determines the abundance of each subpopulation. At low environmentally relevant BAM concentrations, where both subpopulations grow on AOC, BAM-mineralizing cells are only a small fraction. While when BAM concentrations increase, BAM mineralizing cells increase due to the selective advantage of this subpopulation when growing on BAM and not due to the exchange of pBAM2 between both subpopulations. The low incidence of BAM-mineralizing MSH1 cells showed the accumulation of DCBA at relevant conditions comparable to the sand filter environment and raises questions about whether BAM will be mineralized or just converted to a metabolite. So far, DCBA accumulation was not yet detected in pilot scale sand filter experiments performed in Danish and Belgian waterworks.

Next, we showed that *Aminobacter* sp. MSH1 is able to colonize surfaces both as a pioneer and as a successor in the presence of an indigenous sand filter community. We demonstrated that in case of co-colonization, MSH1 was negatively affected by the co-colonizing sand filter community and likely indicates that during initial biofilm formation competitive effects lead to a lower survival and, hence a lower BAM removal efficiency. When the surface is colonized by a sand filter community and MSH1 has to invade this existing biofilm a positive effect was observed. From sequencing results, it could be concluded that upon infiltration of MSH1 into the biofilm other sand filter bacterial species start to thrive and might indicate MSH1 provides a beneficial effect for them. This seems to be mutual as also MSH1 shows a better BAM removal efficiency. This possible cooperation between MSH1 and other sand filter bacteria was also observed by CLSM where MSH1 was covered by sand filter bacteria which is an indication for metabolic cooperation. This has large implications for the bioaugmentation strategy as based on this result MSH1 should be best inoculated when the sand filter material is conditioned by the groundwater and colonized by the groundwater bacteria instead of inoculating MSH1 into a pristine

sand filter bed. In addition, we demonstrated that the BAM removal efficiency was positively affected by the presence of additional AOC. This implicates that BAM removal from surface water and groundwater with additional AOC was more efficiently compared to the BAM removal from groundwater with no additional AOC. This is likely due to the increased viability of MSH1 inside the biofilm and the increase in MSH1 cell numbers inside the biofilm which directly leads to a higher BAM removal rate.

Finally, we made progression in the essential steps to 'social gene' identification. Gene identification in bacteria growing in oligotrophic conditions is challenging as only little biomass is available for e.g. RNA extraction for RNA-Seq. As such we currently rely on three different strategies to identify social genes in MSH1 when invading a sand filter biofilm. All three approaches have an advantage or disadvantage over the other. By applying them in parallel we hope to complement the outcome of these approaches. We were able to extract sufficient total RNA (for comparative transcriptomic analysis), insert fragments (for differential fluorescence induction) and gDNA (for transposon mutagenesis) from a continuously fed flow cell system for sequencing. Since we are reaching the lower limit of the comparative transcriptomics and differential fluorescence induction approaches, it is possible that the sequences for these approaches will be insufficient to identify 'invasion' genes. However, we will only know this for certain when we actually compare the sequences.

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Appendix 1: Flow cell setup assembly and preparation

Medium bottles were connected with the pump using a Teflon tubing in the bottle which was connected to a rubber tubing with an outer diameter of 5 mm and an inner diameter of 3 mm (3 x 5) used to make a branch. The different parts of tubing were linked with connectors. Each end of the branching was connected to a different channel of the pump. A rubber tubing with an outer and inner diameter of 3 mm and 1 mm respectively (1 x 3) was used in the pump in order to withstand constant friction. In order to prevent the tubing being pulled into the pump, a sphere of autoclaving tape was applied at the influent side of the tubing. Using 1.5 x 3 rubber tubing, bubble traps were connected after the pump. The bubble traps were used to catch air bubbles avoiding them to reach the flow cells, where they could disrupt the biofilm. This bubble traps consist of a 5-6 mL syringe placed on a holder. Next, the flow cells, each consisting of three channels, were attached, with 1.5 x 3 rubber tubing as connecting pieces. Using silicone and microscope cover glass, the flow cells were sealed. Both bubble traps and flow cells were placed on a platform to protect the entire setup whenever there was a leak. Eventually the effluent from the flow cells collected in plastic pots using 1.5 x 3 rubber tubing. Before the entire assembly was put together on a large metal tray, all tubing was autoclaved for 20 minutes at 121°C. Everything was connected as mentioned above closely to the flame to avoid contamination.

When everything was attached, it was connected to a two litre Erlenmeyer flask with approximately one litre of 0.5% javel (sodium hypochlorite). Using clamps, the tubing between the bubble traps and the flow cells were tightened. Thereafter, the pump was put at maximum speed. When all bubble traps were flooding, the pump was turned off and the bubble traps were sealed using insulin syringe caps, which were stored in 100% ethanol. The clamps were untied, followed by turning the pump back on. As a result, the flow cells were filled in their turn. By tapping gently on the flow cells, the air bubbles were removed. The pump was turned off and the arrangement should stand at least three hours to decontaminate. After a minimum of three hours, the Bunsen burners were lit to work sterile and the syringe caps were removed. The javel drained into the waste cups until the setup was empty. Subsequently, all steps of tightening the clamps, filling the bubble traps, placement of the syringe caps, filling the flow cells and finally draining the setup were repeated tree times again but now with MS medium used for the experiment which could contain BAM. The third time, the flow cells were held vertically during the filling so that no bubbles are present. This last time, no draining was done. At that point, tubing in front of the flow chamber was clamped, flow chambers were turned with the clover glass side downward and the various bacterial cultures with the correct cell density were inoculated using an insulin needle (Terumo) lifting the effluent side of the chamber upward to avoid air leaks on the influent side. The effluent side of the flow chamber was clamped and cells were allowed to attach

for one hour, chambers were turned with the cover glass facing upward and clamps were removed. The pump was started generating a flow of 3.5 mL/hour (1.2 rpm) and the setup was incubated at 25°C.

Before inoculation, the flow cells were attached upside down so that a biofilm could form on the coverslip. In addition, the pump was shut down and the tubing between the bubble trap and the flow cell was pinched using clips. Next, the end tubing was hold vertically during inoculation to prevent the flow cells to drain resulting in air bubbles entering the flow cells. The insulin syringes should be free of air bubbles while used for inoculation to avoid disruption of the biofilm, possibly present in the flow cell. After inoculation, also the end tubing was pinched and the flow cell must remain upside down for at least one hour. After this hour, the flow cells were re-rotated, the clamps were released and the pump was turned on again.

Appendix 2: Powersoil Total RNA Isolation Kit protocol

Experienced User Protocol

Wear RNase-Free Gloves (1556) at all times and remove RNase from the work area using Lab Cleaner (12095) for RNase Removal. Both of these products are available from MO BIO. Please see the "Products recommended for you" section at the end of this manual.

1. Add up to 2 g of soil to the 15 ml Bead Tube (provided).

   Note: Please refer to Hints and Troubleshooting Guide for information regarding the amount of soil to process.
2. Add 2.5 ml of Bead Solution to the Bead Tube followed by 0.25 ml of Solution SR1 and 0.8 ml of Solution SR2.

From step 3 until step 6, phenol:chloroform:isoamyl alcohol is used, which had to be handled under the fume hood for safety reasons.

3. Add 3.5 ml of phenol:chloroform:isoamyl alcohol (pH 6.5 – 8.0, [User supplied]) to the bead tube, cap and vortex to mix until the biphasic layer disappears.
4. Place the Bead Tube on the Vortex Adapter (MO BIO Catalog # 13000-V1-15) and vortex at maximum speed for 15 minutes.
5. Remove the Bead Tube from the Vortex Adapter and centrifuge at 2500 x *g* for 10 minutes at room temperature.
6. Remove the Bead Tube from the centrifuge and carefully transfer the upper aqueous phase (avoiding the interphase and lower phenol layer) to a clean 15 ml Collection Tube (provided). The thickness of the interphase will vary depending on the type of soil used. Discard the phenol: chloroform:isoamyl alcohol in an approved waste receptacle.

   Note: The biphasic layer will be thick and firm in soils high in organic matter and may need to be pierced to remove the bottom phenol layer.
7. Add 1.5 ml of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 4°C for 10 minutes.
8. Centrifuge at 2500 x g for 10 minutes at room temperature. Transfer the supernatant, without disturbing the pellet (if there is one), to a new 15 ml Collection Tube (provided).
9. Add 5 ml of Solution SR4 to the Collection Tube containing the supernatant, invert or vortex to mix, and incubate at room temperature for 30 minutes.

   Note: The previous protocol instructions were to incubate at -20°C for 30 minutes. If you've used the -20°C incubation before and know that your soil type yields good results at that temperature, you may continue to follow that protocol.
10. Centrifuge at 2500 x *g* for 30 minutes at room temperature.
11. Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 5 minutes.

    Note: Depending on soil type, the pellet may be large and/or dark in color.

12. Shake Solution SR5 to mix. Add 1 ml of **Solution SR5** to the 15 ml Collection Tube and resuspend the pellet completely by repeatedly pipetting or vortexing to disperse the pellet. **Note**: Depending on the soil type, the pellet may be difficult to resuspend. Resuspension may be aided by placing the tubes in a heat block or water bath at 45°C for 10 minutes, followed by vortexing. Repeat until the pellet is resuspended.
13. Prepare one **RNA Capture Column** (provided) for each RNA Isolation Sample:
    a. Remove the cap of a **15 ml Collection Tube** (provided) and place the **RNA Capture Column** inside the **15 ml Collection Tube**. The column will hang in the **15 ml Collection Tube**.
    b. Add 2 ml of **Solution SR5** to the **RNA Capture Column** and allow it to gravity flow through the column and collect in the 15 ml Collection Tube. Allow **Solution SR5** to completely flow through the column (**Optional**: The Collection Tube may be emptied after Solution SR5 has completely flowed through the column. **Note**: DO NOT ALL OW THE COLUMN TO DRY OUT PRIOR TO LOADING THE RNA ISOLATION SAMPLE.)
14. Add the RNA Isolation Sample from Step 12 onto the **RNA Capture Column** and allow it to gravity flow through the column. Collect the flow through in the 15 ml Collection Tube.
15. Wash the column with 1 ml of **Solution SR5**. Allow it to gravity flow and collect the flow through in the 15 ml Collection Tube.
16. Transfer the **RNA Capture Column** to a new **15 ml Collection Tube** (provided). Shake Solution SR6 to mix and then add 1 ml of **Solution SR6** to the **RNA Capture Column** to elute the bound RNA into the **15 ml Collection Tube**. Allow **Solution SR6** to gravity flow into the **15 ml Collection Tube**.
17. Transfer the eluted RNA to a **2.2 ml Collection Tube** (provided) and add 1 ml of **Solution SR4**. Invert at least once to mix and incubate at -20°C for a minimum of 10 minutes.
18. Centrifuge the **2.2 ml Collection Tube** at 13,000 x *g* for 15 minutes at room temperature to pellet the RNA.
19. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 minutes to air dry the pellet.
20. Resuspend the RNA pellet in 100 µl of **Solution SR7**.

Appendix 3: RNeasy ® MinElute Cleanup Protocol: For RNA cleanup and concentration with small elution volumes.

1. **Adjust the sample to a volume of 100 µl or 200 µl with RNase-free water. Add 350 µl or 700 µl Buffer RLT, and mix well.**
   If starting with an RNA pellet, be sure that the pellet is dissolved in the RNase-free water (supplied) before adding Buffer RLT.
2. Add 250 µl or 500 µl of 96–100% ethanol to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer the sample (700 µl) to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through. For samples >700 µl, transfer the remaining sample (up to 700 µl) and repeat the centrifugation. Discard the flow-through.
4. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 5. Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.
5. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.
   Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
6. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube. To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise). It is important to dry the spin column membrane since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.
7. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA. As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water as the spin column membrane will not be sufficiently hydrated.

Appendix 4: gDNA-extraction using the CTAB-lysozyme method

Materials

- Cell culture

- GTE solution

- Lysozyme solution

- 10 % SDS

- 10 mg/ml proteinase K

- 5 M NaCl

- CTAB solution

- 24:1 (v/v) chloroform/isoamyl alcohol

- Isopropanol

- 70 % ethanol

- TE buffer

- 15-ml polypropylene conical tubes

- Refrigerated tabletop centrifuge

- 2-ml microcentrifuge tubes

- Microcentrifuge

GTE solution

25 mM Tris·Cl, pH 8.0 10 mM EDTA 50 mM glucose. Autoclave and store 1 year at room temperature.

CTAB solution

Dissolve 4.1 g sodium chloride in 90 ml water and while stirring on a magnetic stirrer, add 10 g cetrimide (hexadecyltrimethylammonium bromide). Incubate in 65°C water bath to dissolve cetrimide into solution. Store 1 year at room temperature.

Protocol

1. (Optional) Twenty-four hr before harvesting cells for genomic DNA preparation, add glycine to a late log culture to a final concentration of 1 % using a 10 % (w/v) glycine stock. Incubate 24 hr at 37°C. *The glycine weakens the cell wall and for some strains will lead to a higher yield of DNA.*
2. Transfer 10 ml culture to a 15-ml conical tube and centrifuge in a tabletop centrifuge 10 min at 2000 × *g*, room temperature.
3. Discard supernatant, resuspend cell pellet in 450 µl GTE solution, and transfer to a 2-ml microcentrifuge tube containing 50 µl of a 10 mg/ml lysozyme solution.

4. Mix gently and incubate overnight at 37°C.
5. 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.
6. Add 200 µl of 5 M NaCl and mix gently. *NaCl blocks the binding of DNA to cetrimide.*
7. Preheat CTAB solution to 65°C, add 160 µl, and mix gently. Incubate 10 min at 65° C.
8. Add an equal volume (~1 ml) 24:1 (v/v) chloroform/isoamyl alcohol, shake vigorously to mix, and microcentrifuge for 5 min.
9. Transfer 900 µl aqueous layer to a fresh 2-ml microcentrifuge tube.
10. Repeat extraction with 24:1 (v/v) chloroform/isoamyl alcohol, shake vigorously to mix, and spin in microcentrifuge for 5 min.
11. Transfer 800 µl to fresh 2-ml microcentrifuge tube. *CAUTION: For BSL3 organisms, dip tube in a disinfectant such as Vesphene IIse to disinfect outer surface. From this point on, the supernatant can be processed in a BSL-2 laboratory (see Strategic Planning).*
12. To 800 µl aqueous layer, add 560 µl (0.7 vol) isopropanol, mix gently by inversion until the DNA has precipitated out of solution.
13. Incubate 5 min at room temperature. Microcentrifuge for 10 min, room temperature.
14. Aspirate supernatant and add 1 ml of 70 % ethanol to wash DNA pellet. Mix gently by inversion and microcentrifuge 10 min at room temperature.
15. Carefully aspirate supernatant, avoiding the pellet, and air-dry DNA pellet for 15min. Do not overdry.
16. Add 50 µl TE 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. *RNase A can be added to TE (1 µg/ml) to reduce RNA contamination.*

Appendix 5: Adjusted Guanidinium thiocyanate RNA extraction method.

Solution

Solution D:

4 M guanidinium thiocyanate

25 mM sodium citrate

0.5% (w/v) sodium lauryl sarcosinate

0.1 M β-mercaptoethanol (add just before using)

Solution D is chaotropic and very toxic. It must be handled with gloves and safety goggles.

Adjusted Guanidinium thiocyanate RNA extraction

Start by adding the correct amount of β-mercaptoethanol (0.1 M final concentration) to the volume of solution D that you need during the RNA extraction.

1. Add 2 mL of solution D to each sample and incubate for 15 min at room temperature
2. Add 1 mL of 1:1 chloroform:phenol (pH 4) to each sample and vortex vigorously for 2 min at maximum speed
3. Incubate on ice for 15 min
4. Transfer the upper phase (don't take the last 10%, don't touch the interphase) to a cryotube and centrifugate at 4°C and max. speed for 5 min
5. Extract the upper phase (if interphase is visible) to a new cryotube
6. Add an equal volume of chloroform, vortex briefly and centrifugate at 4°C and max speed for 5 min
7. Carry over the supernatant to a nuclease-free 1.5 mL Eppendorf tube

Starting at step 8, work should be done in the RNA-cabinet.

8. Add an equal volume of isopropanol and incubate for 30 min at -20°C
9. Centrifugate at 4°C and max. speed for 45 min
10. Decant the supernatant and wash the RNA pellet with 1 mL of 70% ethanol
11. Centrifugate at 4°C and max. speed for 5 min
12. Decant the supernatant and wash with 1 mL of 100% ethanol
13. Centrifugate at 4°C and max. speed for 5 min
14. Decant the supernatant and air-dry the RNA pellet on a KimWipe (approx. 15 min)
15. Dissolve RNA in 25 µL of RNA storage solution (Ambion)

Appendix 6: QIAquick® PCR Purification Kit

The QIAquick PCR Purification Kit (cat. nos. 28104 and 28106) can be stored at room temperature (15–25°C) for up to 12 months. For more information, please refer to the *QIAquick Spin Handbook*, *March 2008*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Add 1:250 volume pH indicator I to Buffer PB. The yellow colour of Buffer PB with pH indicator I indicates a pH of =7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the colour of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The colour of the mixture will turn yellow.
2. Place a QIAquick column in a provided 2 ml collection tube or into a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column centrifuge for 30–60 s or apply vacuum. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the centre of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the centre of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix 7: DNA Clean & Concentrator™-25

Protocol

Note: All centrifugation steps should be performed between 10,000 - 16,000 x g.

1. In a 1.5 ml microcentrifuge tube, add 2-7 volumes of **DNA Binding Buffer** to each volume of DNA sample (see below). Mix briefly by vortexing.

Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (>2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl
ssDNA (*e.g.* cDNA, M13 phage)	7 : 1	700 µl : 100 µl

2. Transfer mixture to a provided **Zymo-Spin™ Column** in a **Collection Tube**.

3. Centrifuge for 30 seconds. Discard the flow-through.

4. Add 200 µl **DNA Wash Buffer** to the column. Centrifuge at for 30 seconds. Repeat the wash step.

5. Add ≥ 25 µl **DNA Elution Buffer** or water directly to the column matrix and incubate at room temperature for one minute.Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge at for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use.

Appendix 8: AGE image of amplicons of the inserts from promoter probe library for each sorted GFP pool

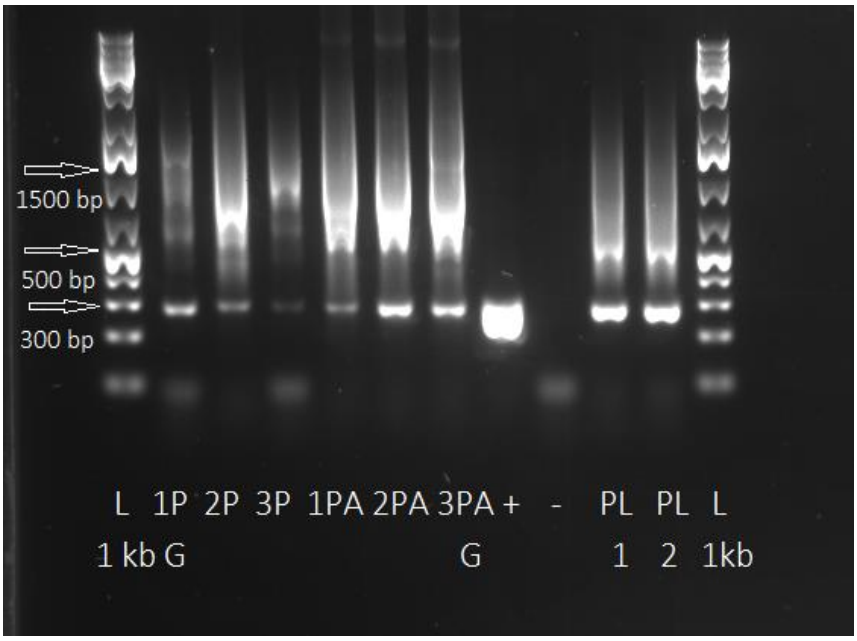

Figure 29: Verification of amplified inserts before gel extraction by AGE (1.5% gel, 90V, 1h); 1P-3P: three replicas of the MSH1 promoter probe library in monospecies condition; 1PA-3PA: three replicas of the MSH1 promoter probe library in the multispecies condition with ASFC; L: ladder, 1 kb: Generuler 1 kb plus ladder; G: cells sorted by FACS as green fluorescent.

Vulgariserende samenvatting

Het grootschalige gebruik van pesticides en farmaceutische producten en nauwkeurigere analytische methodes heeft de laatste jaren de aandacht voor de aanwezigheid van micropolluenten in drinkwater doen stijgen. Dichlobenil, bijvoorbeeld, is een nitril herbicide dat wereldwijd gebruikt werd, maar in 2008 werd het gebruik van dichlobenil verboden in de EU omdat het gemakkelijk afbreekt tot zijn belangrijkste metaboliet, 2,6-dichloorbenzamide (BAM). Aangezien BAM zeer mobiel is, wordt deze micropolluent vaak aangetroffen in het drinkwater met concentraties boven de limiet van 0.1 µg/L, opgelegd door de EU. Op dit moment wordt gebruik gemaakt van granulaire actieve koolfilters om BAM uit het grondwater te verwijderen maar deze techniek is duur en verplaats BAM enkel van water naar de actieve kool in plaats van het effectief af te breken tot CO_2 en H_2O of onschadelijke metabolieten. *Aminobacter* sp. MSH1 is in staat om BAM te mineraliseren bij ecologisch relevante concentraties tot onder de grens van 0.1 µg/L, waardoor MSH1 een hoopvolle kandidaat is voor bioaugmentatie van zandfilters van drinkwater zuiveringsinstallaties als een kosteneffectief en duurzaam alternatief voor granulaire actieve kool. Maar het succes van deze bioaugmentatie hangt af van het vermogen van MSH1 om te integreren in de inheemse bacteriële gemeenschap van de zand filter. Daarnaast moet MSH1 ook kunnen overleven in de voedselarme condities van de zandfilter. In deze thesis worden de belangrijkste processen die een invloed hebben op de overleving van MSH1 tijdens het infiltreren van de zandfilter omgeving onderzocht. Deze belangrijke processen worden gecontroleerd door ‘invasie’ genen. Aangezien de expressie van deze genen gekoppeld kan worden aan het succes van de bioaugmentatie wordt gekeken naar de mogelijkheid om deze genen te identificeren.