FACULTY OF BIOSCIENCE ENGINEERING

BIO-AUGMENTATION OF ACTIVATED CARBON FOR ENHANCED REMOVAL OF 2,6-DICHLOROBENZAMIDE (BAM)

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Dankwoord

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Contents

List of Abbreviations

AC	Activated carbon
AS	Activated sludge
BAC	Biological activated carbon
BAM	2,6-dichlorobenzamide
BW	Backwash water
COD	Chemical oxygen demand
DOC	Dissolved organic carbon
DWTP	Drinking water treatment plant
GAC	Granular activated carbon
HESI	Heated electrospray ionization
HRT	Hydraulic retention time
MTZ	Mass-transfer zone
OD	Optical density
PAC	Powder activated carbon
Pidpa	Provincial and Intermunicipal Drinking Water Society of the Province of Antwerp
RIWA	Association of River Water Supply Companies
SEM	Scanning electron microscopy
SPE	Solid-phase extraction
TOC	Total organic carbon
UHPLC-DAD	Ultra high performance liquid chromatography diode array detection
UHPLC-MS/MS	Ultra high performance liquid chromatography tandem-mass spectrometry
VMM	Flemish Environmental Agency
WWTP	Wastewater treatment plant

Abstract

BAM, or more specific 2,6-dichlorobenzamide is the main metabolite originating from dichlobenil, or 2,6-dichlorobenzonitril. Dichlobenil is a broad-spectrum herbicide which was used for weed control in the aquatic environment. Since 2008, the pesticide dichlobenil was banned. However, the herbicide is relatively persistent and therefore remains in the environment for a long time via sorption to soil and sediment. Dichlobenil is rather hydrophobic, but when it degrades, the more hydrophylic pesticide residue BAM appears in the groundwater. In the groundwater used by drinking water utility Pidpa to prepare drinking water, concentrations up to 0.3 μg L^{-1} BAM are detected. Since the maximum allowed concentrations of pesticides, including their degradation products, in groundwater set by the European Commission is 0.1 μg L^{-1}, efficient BAM removal is needed to provide drinking water which meets the drinking water standards. In this thesis, research is performed for enhanced BAM-removal using bio-augmentation of activated carbon. Two pathways are investigated: bio-augmented GAC filters and microbiological regeneration of GAC. Decisive results of the actual improvement of BAM degradation by bio-augmentation in combination with GAC adsorption are not found. Since successful results are seen in other research concerning bio-augmentation, for example in sand filters bio-augmented with BAM-degrading bacteria or biological activated carbon filters for removal of other pollutants augmented with other bacteria, new pathways may be investigated leading to an effective bio-augmented BAM removal strategy.

Samenvatting

BAM, of meer specifiek 2,6-dichloorbenzamide, is het belangrijkste degradatieproduct van dichlobenil, of 2,6-dichloorbenzonitril. Dichlobenil was een alomtegenwoordig gebruikte herbicide voor onkruidbestrijding in het aquatisch milieu. In 2008 was dichlobenil verbannen, maar de herbicide is relatief persistent en blijft daarom langdurig in het milieu door sorptie aan bodem en sediment. Dichlobenil is eerder hydrofoob, echter wanneer het degradateert verschijnt het meer hydrofiele residu BAM in het grondwater. In het grondwater, dat door de drinkwatermaatschappij Pidpa wordt gebruikt voor drinkwaterproductie, worden concentraties tot 0.3 μg L^{-1} BAM gedetecteerd. Omdat de maximaal toegestane concentraties van pesticiden, inclusief hun afbraakproducten, in de bodem 0.1 μg L^{-1} is, vastgelegd door de Europese Commissie, is er nood aan doeltreffende BAM-verwijdering om drinkwater te bekomen die voldoet aan de drinkwaterstandaarden. In deze thesis wordt onderzoek uitgevoerd naar de mogelijkheid om bio-augmentatie toe te passen op geactiveerde koolstof voor verbeterde verwijdering van BAM. Twee methodes worden onderzocht: bio-geaugmenteerde GAC filters en microbiologische regeneratie van GAC. Besluitende resultaten van verbeterde verwijdering door BAM-afbraak via bio-augmentatie in combinatie met GAC adsorptie is niet gevonden. Aangezien succesvolle resultaten bekomen worden in ander onderzoek betreffende bio-augmentatie, bijvoorbeeld bio-geaugmenteerde zandfilters voor BAM verwijdering of biologisch actieve koolfilters voor verwijdering van andere polluenten geaugmenteerd met andere bacteriën, kunnen nieuwe methodes onderzocht worden leidend tot een effectieve bio-geaugmenteerde BAM verwijderingsstrategie.

1

Literature Study

1.1 Introduction

In Flanders, the Integrated Water Policy is organised by the Flemish Environmental Agency (VMM). The drinking water supply is provided by eight intermunicipal entities, including the Provincial and Intermunicipal Drinking Water Society of the Province of Antwerp (Pidpa) (Pidpa, 2013). Nowadays, the five largest utilities supply 92% of the drinking water. Approximately 25% of the drinking water distributed in Flanders comes from Wallonia. In Flanders, 51% of the water originates from surface water and 49% from groundwater (De Watergroep, 2016). In Flanders, groundwater reserves are able to supply drinking water societies such as Pidpa. Between 1983 and 1997, the production of drinking water rose from 272 million m^3 to 349 million m^3 without a noticeable increase in number of subscribers. Since 1998, the total water production remains more or less constant influenced by two opposite phenomena. The first phenomenon is the reduction of consumption per household by a growing awareness of the consumer. Water utilities and the government make people aware of the fact that one should use precious drinking water in a sustainable manner (De Bruyne et al., 2006). On the other hand, the amount of households are increasing.

Pidpa is a water company with experience in: production and distribution of drinking water; process water, management sewage system and small wastewater treatment plant (WWTP). Groundwater (raw water) is used to make drinking water which needs to meet the physical, bacteriological and chemical quality standards (Pidpa, 2013). In some groundwater sources Pidpa has the presence of pesticide residue 2,6-dichlorobenzamide (BAM). Removal of BAM is complicated, which is why pidpa is currently employing activated carbon treatment. Although carbon works well for

BAM removal, carbon regeneration in the conventional manner (thermal/chemical) is expensive and energy-intensive. Therefore, this thesis will investigate an alternative way of regenerating the carbon and enhance the removal of BAM in activated carbon columns. The thesis is a part of the Resource Recovery Technology (R2T) consortium where academic and industrial partners are co-operating on a project. This thesis is in collaboration with the drinking water utility Pidpa and Ghent University (CMET and PaInT).

1.2 BAM in the environment & related problems

BAM (2,6-dichlorobenzamide) is the main metabolite of the pesticide dichlobenil (2,6-dichlorobenzonitril) (Björklund et al., 2011) (Figure 1.1). Dichlobenil is a broad-spectrum pesticide which has been used for submerged weed control in the aquatic environment, in particular in association with aquaculture such as fish hatcheries and ponds for fish production (Barrett and Makepeace, 1999; Makepeace and Glaisher, 1999). Furthermore, dichlobenil has been used in non-agricultural areas, such as industrial sites and motorways but also in forest plantations and fruit orchards. This pesticide has been banned for use in Belgium since 2008 (Vlaamse Milieumaatschappij, 2003). The pesticide is relatively persistent and has remained in the environment via sorption to soil and sediment (Sheets et al., 1968) as dichlobenil has a log K_{ow} (i.e. the octanol-water partition coefficient, representing the logarithmic ratio of the concentration of a solute in an apolar solvent (octanol) to a polar solvent (water)) of 2.70. Besides, dichlobenil has a low solubility in water of 18 mg L^{-1} (Table 1.1). In contrast, BAM has a low log K_{ow} (0.77) and high solubility in water (2.7 g L^{-1}). Furthermore, it has been demonstrated that BAM is very persistent with low degradation rates in aquifers and is therefore more mobile than dichlobenil (Vlaamse Milieumaatschappij, 2012). As such, BAM remains in the water body over time and therefore it is necessary to evaluate

Figure 1.1: Left: Structural formula of 2,6-dichlorobenzonitril (dichlobenil). **Right**: Structural formula of 2,6-dichlorobenzamide (BAM).

the possible risks to the health of humans when groundwater is treated for use as drinking water. Potential risks for the general population can be caused by two different ways: exposure *via* food and *via* drinking water. For BAM, as well as dichlobenil, acute toxicity to mammals is noticed upward from several hundred mg kg^{-1} of body weight. Björklund et al. (2011) calculated the margin of safety and concluded that the risk to humans is extremely low for acute toxicity. Besides, due to the low log K_{ow} of 0.77 for BAM (Table 1.1), it is reasonable to assume that BAM will be rapidly removed by the kidneys and excreted in the urine once it is taken up in the body, leaving only little time for the compound to exert any toxic effect (Björklund et al., 2011). Moreover, effects on chronic exposure of low concentrations have not yet been determined (Björklund et al., 2011). In conclusion, risks of toxicity are very limited since drinking water utilities are obliged to ensure they are providing drinking water which meets the drinking water standards. In this way, BAM has never exceeded the drinking water standard in treated drinking water and does not pose hazards.

Table 1.1: Relevant properties of dichlobenil and BAM concentrations (Linders et al., 1994; Nakagawa et al., 1992; Clausen et al., 2004).

	Dichlobenil	BAM
Molecular weight (g mol^{-1})	172.01	190.03
Vapour pressure (mPa)	88 (20 °C)	Not found
Solubility in H_2O (mg L^{-1})	18 (20 °C)	2700 (20 °C)
DT_{50} (days)	15	660
Log K_{ow}	2.70	0.77
K_{oc} (L kg^{-1})	896	33

Groundwater is the most sensitive and the largest body of freshwater in the European Union and specifically, also a main source of public drinking water supplies in many regions (European Union, 2006). The maximum allowed concentrations of active substances of single pesticides, including their relevant metabolites, degradation and reaction products in groundwater set by the European Commission, is 0.1 μg L^{-1}. The sum of all individual pesticides, including their relevant metabolites, degradation and reaction products must not exceed 0.5 μg L^{-1} (European Union, 2006). For several years, many countries have been monitoring BAM in groundwater due to its high mobility, solubility and persistence. For instance, in Denmark and Greenland BAM has been

reported in thousands of samples from groundwater wells (Jørgensen, 2003). Despite the extensive use of dichlobenil, the BAM concentration is however below the threshold limit of 0.1 μg L^{-1} in 78% of these samples. Concentrations above 1 μg L^{-1} are rare. The highest concentration of BAM in groundwater ever reported was 560 μg L^{-1}, measured in one extreme case in Denmark (Jørgensen, 2003). The pesticide dichlobenil itself has also been identified in groundwater with a maximum concentration of 2.8 μg L^{-1} which is much lower in comparison with BAM (Jørgensen, 2003), emphasising the persistence of the microbial degradation product. In the Netherlands, an annual report is provided by RIWA-Rhine (Association of River Water Supply Companies), that use surface water for the production of drinking water. BAM is analysed monthly at three locations of the Rhine. For Nieuwegein, there is no detection of BAM during the entire year. For Amsterdam-Rhine Canal Water at Nieuwersluis, BAM is detected in four months in a range between the detection limit (0.01 μg L^{-1}) and 0.014 μg L^{-1}. In the IJsselmeer water at Andijk, the detection limit is exceeded each month with a maximum concentration of 0.026 μg L^{-1}. In Flanders, half of the sources of the drinkwater utilities contain BAM and in more than 10% of the drinkwater utilities, the drinking water standard of 0.1 μg L^{-1} is exceeded (Vlaamse Milieumaatschappij, 2012). In the groundwater used by Pidpa, concentrations up to 0.3 μg L^{-1} BAM are detected. Besides BAM concentrations, its metabolites may also cause concerns, e.g. the partial dechlorination of BAM can yield 2-chlorobenzamide, which is a potential carcinogen (Holtze et al., 2007a). Figure 1.2 gives an overview of measured BAM concentrations in Flanders in 2010 (Vlaamse Milieumaatschappij, 2012).

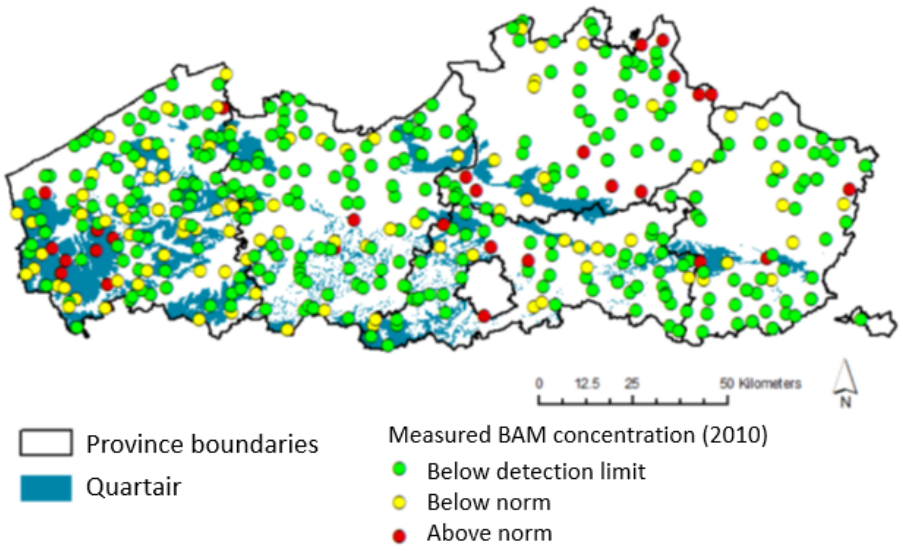


Figure 1.2: Measured BAM concentrations in Flanders (Vlaamse Milieumaatschappij, 2012).

1.3 Micro-biological BAM removal

As mentioned in Section 1.2, there is a need to remove BAM. The first removal technique which will be discussed is bio-degradation. In the context of removing BAM through bio-degradation, bio-remediation is considered, which is the application of micro-organisms to degrade or neutralise hazardous contaminants into less harmful forms (Ractliffe, 2014). Micro-organisms are characterised by their metabolic versatility and their capacity to adapt to changing environmental conditions (Meckenstock et al., 2015), and have potential to remediate contaminants in soil and water resources (Schultz-Jensen et al., 2016). In many cases, micro-organisms feed on the chemical pollutant deriving nutrition for respiration. However, the efficiency of micro-organisms in remediation of contaminants is dependent on many factors, particularly the pollutant's chemical structure and concentration, along with its bio-availability and the physico-chemical characteristics of the environment (e.g. temperature, pH, dissolved oxygen, etc.) (El Fantroussi and Agathos, 2005). The redox potential is one of the most important factors. It influences the degradation of pollutants since micro-organisms are dependent on it (e.g. oxidised degradation) (Benner et al., 2013), and determines which degradations can occur and which not. The potential of a microbial population to degrade organic pollutants within an environmental matrix can however be enhanced, either through stimulation (e.g. by adding substrates and nutrients) or by the addition of specific micro-organisms to the local population (bio-augmentation) (El Fantroussi and Agathos, 2005). Bio-augmentation is relevant when the local population is not able to degrade the contaminant. It has also been suggested as an economically feasible and environmentally friendly method for contaminant remediation (Benner et al., 2013), as the microbial population can be self-sustaining, using pollutants as nutrition. Additionally, mineralisation of the target substrate abates the risk of that pollutant (Benner et al., 2013). However, in most cases only minor effects on degradation efficiency are obtained due to biotic and abiotic factors. Examples of issues with bio-augmentation are the competition for nutrients and resources, predation by natural micro-organisms and the lack of ability to sustain activity and viability for longer periods (Owsianiak et al., 2010; Acea et al., 1988). An important abiotic factor in degradation of pollutants is oxygen. Pukkilan and Kontro (2014) investigated that micro-organisms and oxygen enhance the dissipation rate of BAM in some deposits resulting in shorter half-lives. Besides, oxygen can serve as an electron acceptor in chemical and biological redox reactions, further enhancing the degradation of pollutants (Benner et al., 2013).

1.3.1 Mineralisation of BAM

The potential for microbial mineralisation of BAM to CO_2 has been reported and investigated by Pukkila et al. (2009); Albers et al. (2014); Sørensen et al. (2007) (Table 1.2). The isolated soil bacterium *Aminobacter* sp. strain MSH1 degrades BAM into five known degradation products (benzamide, 2-chlorobenzamide, 2,6-dichlorobenzoic acid, benzoic acid and 2-chlorobenzoic acid), using BAM as a source of carbon, nitrogen and energy in aerobic conditions (Sørensen et al., 2007; Jensen et al., 2009). Besides, *Aminobacter* sp. strain MSH1 is able to mineralise BAM for up to 60% within 50 days (Simonsen et al., 2006). This isolate, *Aminobacter* sp. strain MSH1, is a promising candidate for use in bio-remediation processes aimed at treating natural waters polluted with low concentrations of BAM or dichlobenil. Sørensen et al. (2007) cited "the high degree of similarity to the isolated BAM mineralising *Aminobacter* sp. strain ASI1 and the fact that closely related *Aminobacter* type strains are capable of degrading nonchlorinated structural analogues add to knowledge of the metabolic capacities of this genus and suggest that this closely related group of *Aminobacter* spp. could have selective advantages in BAM contaminated environments." Still, many challenges have to be overcome, as stated by Knudsen et al. (2013): "i) securing the survival of the introduced strains, ii) development of efficient tools for spreading degrader organisms in the polluted environment and iii) providing access for the micro-organisms to the pollutants sorbed to the sediment and organic matter or trapped in micro-pores in the soil." When strains are introduced in the soil, it is difficult to ensure the survival of the species due to biotic or abiotic stress, such as temperature, moisture content and nutrient availability (Pepper et al., 2002). Furthermore, studies have shown that bacteria applied to soil surfaces rarely are transported more than 5 cm into the soil without help of a transport agent, such as percolating water (Madsen and Alexander, 1982). Another possible bottleneck of bio-augmentation is that the pollutants may be present at low level concentrations with the consequence that the degrading bacteria have difficulties to have access to it (Wick et al., 2007). However, some works showed no adverse effect caused by low concentrations. Mrozik and Piotrowska-Seget (2010) give an overview of several cases using bio-augmentation in soils contaminated with aromatic compounds and concluded that bio-augmentation appears to have a great potential for remediation of aromatic compounds remediation.

Anaerobic degradation of BAM is difficult. Pukkilan and Kontro (2014) investigated BAM degradation using deposits of different groundwater wells and monitoring pipes incubated under anaer-

obic conditions. After 527 days, still no degradation of BAM is observed.

Table 1.2: Summary of bacteria able to degrade BAM (Pukkila et al., 2009; Albers et al., 2014; Sørensen et al., 2007).

Species	Energy source	Features	Initial concentration	Degradation efficiency	Isolation source
Zoogloea ramigera	N-source	Gram-negative aerobic			Groundwater subsurface deposits
Ralstonia basilensis	N-source	Gram-negative aerobic	0.12 +/- 0.03 μg L^{-1}	37.6 +/- 14%	
Rhodococcus erythropolis	N-source	Gram-positive aerobic			
Aminobacter sp. MSH1	N and C-source	Gram-negative aerobic	2.7 μg L^{-1}	$\geq$94%	Pasteur collection
Aminobacter sp. ASI1	N and C-source	Gram-negative aerobic	41.6-1384.7 μg L^{-1}	14.7-64.4%	Dichlobenil treated soil

1.3.2 Metabolic and co-metabolic bio-degradation

Alexander (1981) specified that, when participating in biologically mediated reactions with micro-pollutants, micro-organisms in general employ two catalytic processes. The first process is that micro-organisms can interact with micro-pollutants via metabolic reactions, in which growth-linked processes result in mineralisation of the micro-pollutant (Benner et al., 2013). The second process involves co-metabolic reactions where the degradation of pollutants do not sustain growth of the responsible micro-organisms. Instead, they use enzymes that co-metabolically transform micro-pollutants into compounds which can be consumed as growth substrates by other members of the community (Alexander, 1981). In order to support the freshly augmented microbial population for successful long-term efficacy of micro-pollutant removal, additional dosing of growth substrate may be required. If there is need for supplementary growth substrates, then the co-metabolic bio-augmentation strategy will be more costly and less sustainable, compared to the

metabolic bio-degradation strategy (Benner et al., 2013). In comparison with the metabolic bio-degradation process, a critical factor for success of co-metabolic bio-degradation is integration of the organisms into the existing microbial community. The availability of a growth substrate is also a crucial factor to allow growing of the organisms and active participation of the organisms in co-metabolic reaction processes with a wide range of micro-pollutants. This is in contrast to the metabolic bio-degradation strategy where only one micro-pollutant is degraded per organism. A schematic overview of the metabolic and co-metabolic strategies is provided in Figure 1.3. Enrichment and isolation of the micro-organisms is achieved from environments that are regularly exposed to specific micro-pollutants such as agricultural soils (Holtze et al., 2007b). A critical factor is the ability of the strain to integrate in the existing microbial community and actively participate in metabolic reactions with the pollutants (Sørensen et al., 2007). Besides, the introduced strain or microbial consortium must grow on the very low and fluctuating substrate concentrations present in raw water.

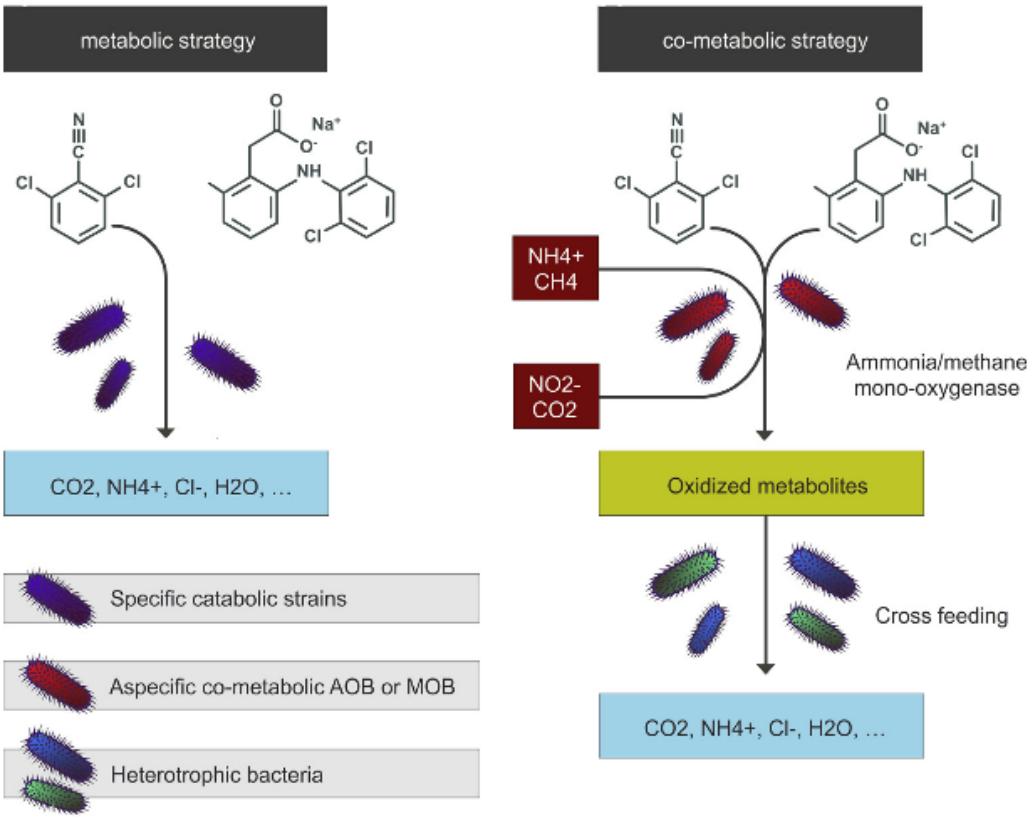


Figure 1.3: Schematic overview of the metabolic and co-metabolic bio-degradation strategies (Benner et al., 2013).

1.3.3 Biosorption

In the context of organic pollutants and micro-organisms, biosorption is a well known phenomenon. Aksu (2005) defines it as ”the passive uptake of pollutants from aqueous solutions by the use of both non-growing or non-living microbial biomass”. Biosorption is a promising alternative technique to remove organic pollutants from wastewaters since it could be possible to recover and to dispose the pollutants on an environmentally friendly manner. A variety of micro-organisms can be considered: fungi, yeasts, bacteria, etc. Biosorption involves a number of metabolism-independent processes: physical and chemical adsorption, electrostatic interaction, ion exchange, complexation, chelation and microprecipitation. The mechanism of binding by inactivated biomass may depend on the chemical nature of the pollutant, the size and ionic charge, the type of biomass and its specific surface properties, and the environmental conditions (e.g. pH, temperature, ionic strength) (Aksu, 2005). Biosorption can occur on different sorption sites of a biofilm, such as extracellular polymeric substances, cell walls, cell membranes and cytoplasm (Aksu, 2005). The kinetics of biosorption consist of several steps. Firstly, the transport of the organic pollutant from the bulk liquid to the boundary film which surrounds the biosorbent. Secondly, the transport of the pollutants from the boundary film to the external surface of the biofilm (film diffusion). This is followed by the transfer of the pollutants from the biofilm's surface to the intraparticular active sites and the uptake of the pollutants by the active sites of the sorbent (Aksu, 2005).

1.4 Physico-chemical BAM removal

Besides the application of micro-biological removal of pollutants, physico-chemical removal of micro-pollutants is also an important application. For example, oxidation and disinfection methods are often applied in drinking water production. Besides, they can stabilise the biological quality of the purified water, specifically they remove color and odor, and inactivate pathogens (Benner et al., 2013). More particularly, oxidative chlorine species can inactivate pathogens (Hebling and Van Briesen, 2007). In some cases, however, these oxidative chlorine species can react with micro-pollutants, leading to the production of halogenated by-products that may have equal or greater toxicity (Buth et al., 2011). An alternative could be the application of ozone, which has a strong affinity to micro-pollutants containing phenol, aniline, or deprotonated-amine substructures (von

Sonntag and von Gunten, 2012). Both techniques are not ideal as they produce a variety of persistent oxidation products that may have equal or even greater toxicity as the parent chemical (Benner and Ternes, 2009). Physico-chemical techniques, which do not modify the chemical structure of the pollutant, are reverse osmosis and activated carbon. Reverse osmosis makes use of a pressure to push the fluid through a membrane where particles with a size of more than 0.1 nm can not pass (EMIS, 2015). Activated carbon filters are highly effective due to their high efficiency of VOC and micro-pollutants removal, simplicity, robustness, simple installation and maintenance, and are suited for discontinuous processes as well (EMIS, 2015). In the following sections, an overview is given of adsorption on activated carbon. Since specific information on adsorption of BAM is limited, a general overview of adsorption is given.

1.4.1 Activated carbon

Activated carbon (AC) is a widely used treatment technology in drinking water treatment plants (DWTPs) for the removal of hazardous organic compounds such as pesticide residues from groundwater by adsorption (see Figure 1.4) (Benner et al., 2013). AC can also be applied in order to remove residual growth substrates and minimise bacterial regrowth in the distribution system (Benner et al., 2013). Regarding organic micro-pollutant removal, caution is needed as highly polar pesticides and pharmaceuticals can break through activated carbon filters (Snyder et al., 2007). The main drawback is that AC is a relatively expensive technique, which is mainly given by the required periodical (and expensive) regeneration (Albers et al., 2015). Nevertheless, the benefits of using AC are decisive of its large scale use.

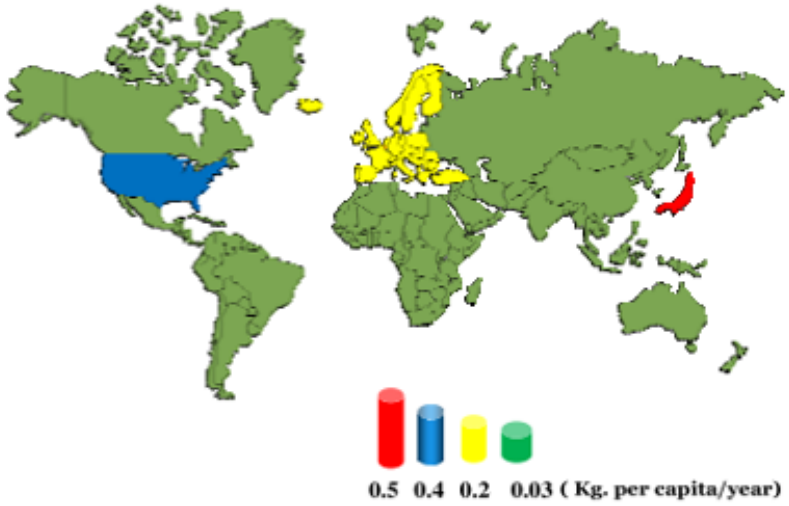


Figure 1.4: The world consumption of adsorbent (Shah et al., 2013).

AC can be used in the form of powdered activated carbon (PAC), granular activated carbon (GAC) or extruded activated carbon pellets (Ayranci and Hoda, 2005). PAC has a larger surface to volume ratio compared to GAC. Pellets are made of PAC and a binder and are extruded into cylindrically shaped blocks with diameters ranging from 0.8 to 130 mm (Sorbos, 2017). GAC and PAC are used for water treatment and pellets and PAC for gas treatment. Since GAC has a lower surface to volume ratio, there is less resistance against the flow and therefore a lower pressure drop in the column can be considered (Verliefde, 2011). Figure 1.5 represents different types of AC delivered by Desotec NV (Belgium). AC is able to capture compounds due to its high specific surface area ranging between 500 and 1500 $m^2\ g^{-1}$ and pore volumes ranging between 0.7 and 1.8 $cm^3\ g^{-1}$ (Çeçen and Aktas, 2011). The final properties of the AC depend on three criteria: the active pores of the carbon, how the AC has been activated and the raw material (Desotec, 2015).

Commercially available AC are produced from natural materials (e.g. wood, coal, lignite, peat, coconut shells) or synthetic high polymers (Verliefde, 2011). A common feature of those materials is the fact that they have a high carbon content. The process of transforming those materials into activated carbon, consists of two phases: carbonisation and activation. The first step, carbonisation, consists of drying and heating of the material in order to remove undesirable by-products (e.g. tar and other hydrocarbons). The material is brought in an oxygen-deficient atmosphere, in order to start pyrolysis and carbonisation within a temperature range of 400-600°C. Here, the volatile low-molecular-weight fraction is removed and the second phase, the activation process,

starts. This can be achieved thermally by the use of oxidative gases (e.g. steam at 800°C or carbon dioxide at 900°C). At this last phase, adsorption properties of carbon are developed. In addition, the AC micro-pores are formed (Çeçen and Aktas, 2011). For specific applications and to increase the removal efficiency, the activated carbon can be impregnated using chemicals such as phosphoric acid, potassium hydroxide, zinc chloride, and others. Impregnated AC adsorbs and holds the specific components long enough to allow the chemical to react with the contamination (chemisorption). Impregnated activated carbon is specially designed to capture chemical components that are difficult to adsorb on AC (EMIS, 2015).

Figure 1.5: Different types of activated carbon delivered by Desotec (Desotec, 2015). The size of the AC from PAC, GAC and extruded AC pellets is respectively around 0.075 mm, 1.65 mm and 4 mm.

Activated carbon is a precious product. Regeneration and reuse of AC is generally required since replacement with fresh AC is (too) expensive. The regeneration can be done using one of the following methods: thermal, chemical and biological regeneration (Lenntech, B.V., 2016). Regeneration is needed to desorb adsorbed material and recover AC in its original state, or to recarbonise the adsorbed material (thermally) (Verliefde, 2011). The selection of which regeneration process will be used depends on the priority of regeneration. In particular, physical methods are preferred when both adsorbate (pollutant which adsorbs on adsorbent) and adsorbent (material on which adsorbate adsorbs on) recovery is desired. If only adsorbent recovery is desired, chemical regeneration is preferred (Shah et al., 2013). Bioregeneration can be used for compounds which readily desorb and bio-degrade. Biological activated carbon systems and bioregeneration are parallel processes in one system (Aktas and Çeçen, 2007). While pollutants are adsorbed and/or degraded on the BAC, microbial degradation regenerates the GAC and makes adsorption sites available again for new pollutants.

1.4.2 Adsorption on activated carbon

Activated carbon removes compounds by the adsorption process: a solid surface attracts solutes and gases using physical forces. The term ”adsorption” is regularly confused with ”absorption”, which takes up and mixes solutes in a liquid phase. The adsorption process consists of an interaction energy in a three-phase system: AC, solute and water (De Ridder et al., 2013). The main physical forces responsible for adsorption are van der Waal interactions. In addition, electrostatic interactions, hydrogen bridges and pi-pi interactions have an influence on the adsorption process as well. Remark that the adsorption process does not change the chemical property of the AC and the adsorbed compound (Desotec, 2015).

Adsorption isotherms are used to describe the adsorption behaviour of target molecules on the adsorbent surface. The Freundlich isotherm (Equation 1.1) is widely used for activated carbon applications (Çeçen and Aktas, 2011):

$$Q = K.C^{1/n}. \tag{1.1}$$

With Q: the total amount of organic compound X adsorbed on AC ($g_X.g_{AC}^{-1}$),
K: Freundlich's constant,
C: adsorbate-concentration C ($g_X.m_{water}^{-3}$),
n: exponent of non-linearity.

The Freundlich equation is an empirically established relation between carbon loading and equilibrium concentration. As a result of the empirical nature of this relationship, extrapolation to extremely high or low concentrations is impossible. The use of this equation is therefore limited to the concentration range under investigation (Verliefde, 2011). Equation 1.1 can be linearised by taking a log-transformation (Equation 1.2):

$$log(Q) = log(K) + \frac{log(C)}{n}. \tag{1.2}$$

The parameters K and n of the Freundlich isotherm define the properties of the AC and adsorbate. Via linear regression, log(K) and n^{-1} are derived. High values of K and n^{-1} point out a relatively good adsorption for the entire concentration range. In contrast, low values of K and n^{-1} point out a

relatively low adsorption for low concentrations and a relatively high adsorption for high concentrations. After establishing a Freundlich isotherm, the amount of adsorbent needed for the removal of a pollutant can be determined. Moreover, adsorption-isotherms are used to compare different adsorbents, adsorbates or adsorption conditions (e.g. pH) (Verliefde, 2011).

In water treatment, the water usually flows continuously through a solid matrix of adsorbent, thus via a continuous process. The mass-transfer zone (MTZ) refers to the zone where exchange between dissolved and adsorbed adsorbate-molecules occurs, and it shifts gradually in the flow direction. Point P2 in Figure 1.6 represents the MTZ which has not yet reached the lower boundary of the column. Points P3 represents breakthrough in the effluent indicated by V_b, the breakpoint volume, or t_d, the time to reach breakthrough of the adsorbate. When the influent concentration is equal to the effluent concentration, the column is completely saturated with adsorbate, with t_s the time to reach saturation (Point P5 of Figure 1.6) (Verliefde, 2011). The length of the MTZ is obtained *via* the following formula:

$$L_{MTZ} = L_{column} . \frac{(t_s - t_d)}{t_s}. \tag{1.3}$$

With L_{MTZ}: length of the mass transfer zone (m),

L_{column}: length of the column (m),

t_s: saturation time (s),

t_d: time to reach breakthrough (s).

The length of the MTZ is preferably as small as possible. However, it strongly depends on the flow, which varies between 3 and 9 m^3 $(h.m^2)^{-1}$. The shape of the breakthrough curve and the length of the MTZ is defined by several factors such as the dimensions of the column, the flow, the type of AC, the composition of the feed, the temperature and the pH. To increase contact time between AC and the water, successive columns which are placed in series, can be used (pulsed bed). In this way, the capacity of the adsorbent is optimally used. The main parameters for adsorption are the contact time between the water, the AC and the configuration of the system (e.g. columns are placed in series or parallel) (Verliefde, 2011).

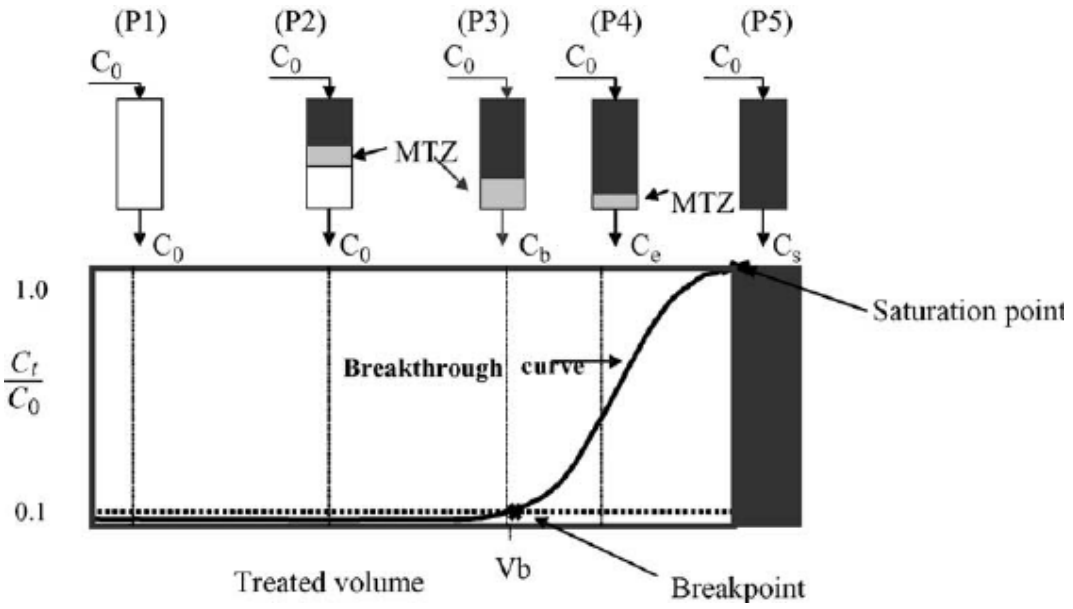


Figure 1.6: Typical breakthrough curve for activated carbon showing the movement of the mass transfer zone according to the throughput volume (Taty-Costodes et al., 2005).

1.5 Bio-augmented physical applications

In the following sections, the use of two physical applications in combination with bio-augmentation is described. Bio-augmentation of physical applications may prolong the overall lifetime, i.e. delay of breakthrough of an activated carbon filter due to microbial degradation in the filter. At first, bio-augmentation of activated carbon will be considered, followed by bio-augmented rapid sand filters.

1.5.1 Biological activated carbon (BAC)

For many years, the removal of dissolved organics has been performed using granular activated carbon (GAC) for water purification (Dussert and Van Stone, 2000). In the early seventies, studies revealed that a fraction of the net removal of organics in the GAC filter is due to bacteria growing inside the filters. At the start, physical adsorption is responsible for most of the removal occurring, while the bacteria are in the acclimation phase. During this period, dissolved organic carbon (DOC) removal ranges between 40% to 90%. In general, 10% to 20% of the DOC is nonadsorbable on GAC. In the next phase, biological degradation starts playing a role next to the adsorption process. Now, the bacteria are acclimated and the role of the adsorption process is gradually decreasing as

saturation of adsorption sites occurs. Finally, a steady-state period is considered. Pre-ozonation is found to enhance the biological activity on GAC. For example, a study performed by Reungoat et al. (2012) revealed that through the combination of ozonation and biological activated carbon (BAC) filtration, 50% removal of DOC and more than 90% of organic micro-pollutant removal can be achieved. In the '80s, the BAC process was implemented in many large water treatment plants by ozonating the GAC (Dussert and Van Stone, 2000) in Europe. An important remark is that the presence of micro-organisms in BAC filters leads to the formation of a biofilm, resulting in an increased pressure drop. Therefore, more frequent and efficient backwashing procedures are required on a regular basis to prevent the growth of higher organisms in the filter and to preserve a low trophic level. A part of the bacterial biomass fixed on GAC is removed during backwashing which temporarily causes a lower removal efficiency of bio-degradable matter after backwashing (Dussert and Van Stone, 2000).

The first factors to be considered in BAC are the macroporous structure, adsorption capacity and surface chemistry of the AC, which increase the suitability of the AC as support media for biological treatment. Secondly, adsorption capacity properties have to be considered, in particular total and organic micro-pollutants adsorption capacity. The last factors to be considered are physical properties such as density, abrasion resistance, hardness and their effect on a reactivated product (Dussert and Van Stone, 2000).

When GAC and bacteria are combined, a biofilm is formed of the bacteria on the GAC. Weber et al. (1978) described the biofilm as a "porous tangled mass of slime matrix". Microbial cells form the biofilm and they can be immobilised at the surface of the GAC or embedded in an extracellular microbial organic polymer matrix (Weber et al., 1999). The biofilm consists of bacterial and fungi cells which secrete extracellular polymeric substances to form a cohesive, stable matrix in which cells are held in a dense agglomeration (Branda et al., 2005). The extracellular matrix consists of polysaccharides, proteins, nucleic acids and lipids (Horan and Eccles, 1986). The total amount of biofilm can be represented by physical (i.e. biofilm thickness, total dry weight) or physico-chemical (total organic carbon (TOC) or chemical oxygen demand (COD)) parameters in order to get information of the composition of the biofilm (Lazarova and Manem, 1995).

Researchers observed that the activity of the microbial biofilm increases enormously upon adherence onto the GAC media. Several hypotheses are set which could contribute to the increased activity of the biofilm. The first hypothesis is that bacterial cells modify physiologically upon attachment to the GAC surface, which is associated with the promotion of certain genes (Dagostino et al., 1991). A second hypothesis is that the bacterial cell surroundings are changed by the biofilm to increase either the local concentration of nutrients, oxygen and enzymes (Hosh et al., 1999) or limit the presence of toxic or inhibiting compounds (Blenkinsopp and Costerton, 1991). As well, an adsorbed microbial biofilm is less affected by changes in environmental conditions (i.e. temperature, pH, nutrient concentrations, metabolic products and toxic substances) than a suspended biofilm (Pederson, 1990).

1.5.2 Bio-augmentation of rapid sand filters

To remove the common groundwater pollutant BAM, previously conducted studies have been performed using *Aminobacter* into bio-augmented sand filters (Albers et al., 2014; Benner et al., 2013). Sand filters have been applied extensively for single purification processes, especially when groundwater is treated for drinking water (e.g. removal of iron manganese and ammonium species). As such, the hypothesis of the aforementioned studies was that when BAM is present in the feed water, the sand can be used as a carrier material for *Aminobacter*. A common problem with sand filters is clogging due to precipitated metal oxides and excess biomass. Therefore, there is a need to backwash the filter regularly (e.g. circumvention of the water flow and air flushing) (Albers et al., 2015). Several studies revealed that microbial populations are able to adapt and to degrade organic micro-pollutants in some circumstances in sand filters, e.g. with limitation in organic carbon and other nutrients (Metz et al., 2006; Zearley and Summers, 2012). However, several issues are still present. For example, in a study performed by Albers et al. (2015): sand filters inoculated with pesticide degrading bacteria are able to remediate polluted drinking water containing trace concentrations of pesticide residues. However, extensive loss of the bacteria immediately after inoculation is observed. More than 90% of the bacteria initially adhered are lost with the effluent within the first weeks, mainly due to backwashing of the filter. The initial BAM degradation efficiency of more than 50% dropped to 20% due to this loss. By avoiding backwash procedures, the degradation efficiency can be maintained for a longer time, however the 90% bacteria (and thus the degradation efficiency) are lost within a month. Besides, backwashing of filters treating anaerobic groundwater is mandatory to remove precipitated metal oxides formed when metal species come

into oxic condition due to aeration (Albers et al., 2015). Additional losses of bacteria were caused by predation by protozoa and starvation due to the remediated water containing limited amounts of easily assimilable organic carbon. Further research found that BAM degradation had taken place without adverse effect on other sand filter processes, such as ammonium and iron oxidation. In addition, degradation rates achieved from laboratory experiments could not be replicated to the large-scale sand filter. Improving degradation rates is thus the main challenge and needs further investigation (Albers et al., 2015).

Bio-augmentation of drinking water treatment processes, with micro-organisms following a metabolic or co-metabolic strategy for removal of micro-pollutants, works effective, cost-effective and sustainable (Benner et al., 2013). However, when considering the implementation of bio-augmentation, technical limitations are forthcoming and must be conquered in advance to implementation. To begin, the hydrodynamics of the process identified for bio-augmentation must be considered. For rapid sand filters standard loading rates are around 1-10 m h^{-1} and for slow sand filters around 0.1 m h^{-1}, as determined by the hydraulic retention times (HRT), being in the order of minutes in rapid sand filters and hours in slow sand filters (Benner et al., 2013). A continuous bioreactor will have a steady-state effluent concentration as a function of the HRT. If this HRT is not long enough this may lead to insufficient micro-pollutant removal (Zearley and Summers, 2012). For this reason, longer hydraulic retention times could be more effective for successful bio-augmentation in a continuous bioreactor, here the sand filter. On the other hand, a shorter retention time (by increasing the loading rate) results in a higher flux of substrates which could improve the growth of the degrading bacteria. Besides, the diffusive boundary layer surrounding each filter particle will reduce and in this way a more efficient degradation will be obtained. Investigation is needed to clarify relations between hydraulic retention time, hydraulic loading rates, and pollutant degradation. Besides, the limitation of mass transfers within the filtration process must also be considered: a strain which is physiologically capable of degrading a specific micro-pollutant at very low concentrations can degrade inefficiently when the process is diffusion limited. At last, the primary function of the process that is being modified by bio-augmentation should be retained. In conclusion, sand filters need regular backwashing periods for their routine operation and maintenance. Individual strains of microbial consortia that are added to these processes should withstand these operations without losing their function (Benner et al., 2013).

1.6 Objectives

Activated carbon is a widely used adsorbent, for instance in the tertiary treatment stage of DWTPs for the removal of hazardous organic compounds or the first treatment stage of raw groundwater. Since activated carbon is an expensive product, prolonging its lifetime is a must. The objective of this study is to create a process where physical and microbial mechanisms interact in parallel. The microbial action needs to assist the physical action in order to enhance the removal of pollutants. In this thesis, the focus lays on BAM, since this pesticide residue present exceeds the drinking water standard of 0.1 μg L^{-1} (European Union, 2006) in several feed waters of drinking water treatment plants in the province of Antwerp. However, BAM never exceeds the drinking water standard in the treated drinking water. Three main parts are considered. In the first part, bio-degradation of BAM is investigated. Here, aspects such as degradation efficiency and survival in mixed cultures are investigated. In the second part, the BAM adsorption behaviour on GAC is investigated. Information about adsorption kinetics and desorption are gathered. In the third part, the adsorptive and bio-degradative removal mechanisms are combined. Pollutant removal in GAC columns is assisted micro-biologically through bio-augmentation of the GAC, and the possibility of biologically regenerating GAC after breakthrough is also investigated.

2

Materials & Methods

2.1 Chemical analysis

In this section, two different methods for chemical analysis were developed, one suitable for samples in a low concentration range and one suitable for samples in a high concentration range. BAM analysis of high concentrations, ranging between 1-200 mg L^{-1}, is performed using an Ultra High Performance Liquid Chromatography equipped with a Diode Array Detector (UHPLC-DAD). The experiments discussed in Section 2.3 are performed using high concentrations of BAM. The second method is developed on an Ultra High Performance Liquid Chromatography Tandem-Mass Spectrometry (UHPLC-MS/MS) for lower, more environmentally relevant, concentrations between 50 ng L^{-1}-100 μg L^{-1}. The following sections describe the development of both methods, while in Section 3.1, the results of these method developments will be discussed. BAM used in the experiments is manufactured by Acros Organics with 97% purity (Belgium).

2.1.1 Ultra High Performance Liquid Chromatography Diode Array Detector

Sample pretreatment

The sample pretreatment involves particulate removal using a syringe filter with a pore size of 0.2 μm. For concentrations higher than 200 mg L^{-1}, serial dilutions of the samples is carried out until the concentration is within the detection range, using the same medium of the samples.

UHPLC-DAD method

The developed UHPLC-DAD method is based on Connick and Bradow (1984), using an Ultimate

3000 UHPLC Focused (Thermo Scientific, San José, USA). A sample volume of 20 μL is injected on a reversed-phase Acclaim 120 C18 (3 μm 2.1 x 150 mm) column (Thermo Scientific, San José, USA), a column suited for relatively hydrophilic compounds such as BAM (Thermofisher Scientific, 2017). Elution is performed using an isocratic mobile phase consisting of 50% acetonitrile HiPerSolv CHROMANORM for HPLC (min. 99.9 % - VWR Chemicals, Belgium) and 50% ultrapure water (Milli-Q) at a flow rate of 0.5 mL min^{-1} with a maximum pressure of 300 bar. A temperature of 20°C is set. For detection, the UltiMate 3000RS Diode Array Detector (Thermo Scientific, San José, USA), set at 205 nm, is used. Instrument control and data processing are performed using Chromeleon 6.8 Chromatography Data System Software (Thermo Scientific, San José, USA).

Calibration method

Before each run, BAM standards are prepared with concentrations between 1 and 200 mg L^{-1} BAM dissolved in various aqueous matrices (tap water, methanol or M9 medium), used for the construction of a calibration curve.

2.1.2 Ultra High Performance Liquid Chromatography Tandem-Mass Spectrometry

Sample pretreatment

The sample pretreatment involves particulate removal, using a syringe filter with a pore size of 0.2 μm.

Solid-phase extraction

The solid-phase extraction (SPE) is performed using Oasis HLB 200 mg, 6 cc, 30 μm (WAT106202) (Waters, UK) cartridges and a solid-phase extraction manifold (Figure 2.1). Oasis HLB is a polymeric reversed-phase sorbent and is an all purpose, strongly hydrophilic, water-wettable polymer with a unique hydrophilic-lipophilic balance (Waters, 2017). Therefore, it maintains a high retention for BAM. In Appendix 5.1, the method for performing SPE is described in detail, using a sample volume of 50 mL.

UHPLC-MS/MS method

The UHPLC-MS/MS procedure is based on Sekhar et al. (2016). The UHPLC-MS/MS is a

TSQ Vantage (Thermo Scientific, San José, USA). A volume of 12.5 μL sample is injected on a reversed-phase Acquity UPLC HSS T3 (1.8 μm 2.1 x 50 mm) column (Waters, UK) and elution is performed using a gradient mobile phase consisting of 90% methanol HiPerSolv CHROMANORM for HPLC (min. 99.8 % - VWR Chemicals, Belgium) and 10% 0.01% formic acid dissolved in ultrapure water (Milli-Q) at a flow rate of 0.3 mL min^{-1}. The MS is equipped with a heated electrospray ionization (HESI-II) source in positive ion mode, operated at a spray voltage at 4 kV; vaporizer temperature of 350°C and capillary temperature of 315°C; sheath and auxiliary gas of respectively 45 and 5 arbitrary units. The S-lens RF amplitude is 63 V. Instrument control and data processing are performed using respectively Tune and Xcalibur Software 2.0.7 SP1 (Thermo Scientific, San José, USA).

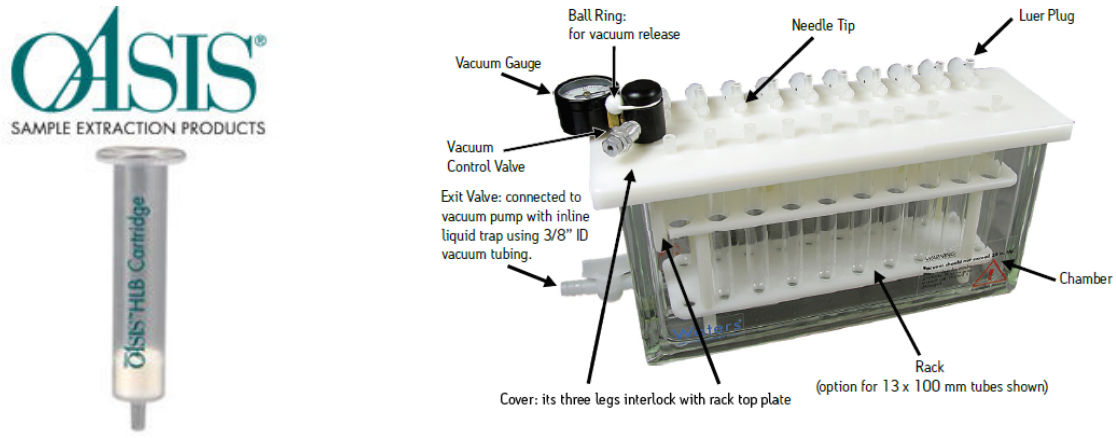


Figure 2.1: Left: Cartridge of Oasis HLB 200 mg, 6 cc, 30 μm (WAT106202) (Waters, 2013).
Right: Representation of Waters 20-position extraction manifold with specifications (Waters, 2013).

Calibration method

A calibration series is prepared with the following concentrations: 0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg L^{-1}. These concentrations are chosen so that the (expected) concentrations in the samples are within the range of the calibration series. The calibration series are a mixture of BAM and four previously reported degradation products of BAM: 2,6-dichlorobenzoic acid, benzamide, benzoic acid and 2-chlorobenzoic acid (Jensen et al., 2009). An internal standard, BAM-d3, is added to every standard and sampled at a concentration of 0.4 μg L^{-1}.

2.2 Microbial analysis

Optical density

The optical density (OD) is measured in order to estimate cell density. A wavelength of 600 nm is set. OD is measured for aerobic and anaerobic cultures using, respectively a MDA Photometer ISIS 9000 (Hach, Germany) and Tecan Infinite 200 PRO Multimode Microplate Reader (Tecan, Switzerland), and a Tecan Sunrise Microplate Reader (Tecan, Switzerland).

Flow cytometric analysis

Flow cytometric analysis is done in order to determine the cell density. The samples are diluted to a maximum of 2.10^3 events μL^{-1}. Staining is done with a nucleic acid, namely SYBR Green I (100x diluted in 0.22 μm-filtered dimethyl sulfoxide), followed by 13 min of incubation in the dark at 37°C. The samples are analysed immediately afterwards on a C6 Accuri^{TM} flow cytometer (BD Biosciences, Belgium).

Scanning electron microscopy

Scanning electron microscopy (SEM) is performed to visualise the granular activated carbon and the possible adsorbed bacteria on its surface. Prior to the sample visualisation, the sample preparation requires essentially three steps. In the first step, the samples are fixed using a fixative glutaraldehyde solution followed by air drying. In the next step, the samples are mounted on an aluminium pin with double sided carbon tape. Finally, the samples are coated with a thin layer of gold using a Sputter Coater 108auto (Cressington Scientific Instruments, UK) to make the samples electrically conductive. A coating time of 45 seconds is set using a current of 30 mA and a pressure of 0.08 mbar argon gas. Sample imaging and element identification is done using respectively a Phenom ProX and the Phenom Pro Suite program (Phenom World, The Netherlands).

2.3 Statistical analysis

Statistical analysis is done to investigate the significance of the results. Firstly, one-way analysis of variance (ANOVA) (significance level of 0.05), performed in Microsoft Excel, is used to determine the significant difference when comparing values of BAM concentrations and cell density within the same experiment. If $F \geq F_{crit}$, then the averages of the populations are significantly different.

Secondly, the t-test 'two-sample assuming unequal variances' (significance level of 0.05) is used to determine similarity of the variances of two populations in the same experiment. If $t \leq -t_{crit}$ or $t \geq t_{crit}$, then the samples are significantly different (Microsoft, 2017).

2.4 Experimental design

The method developed for BAM analysis using UHPLC-MS/MS, is not feasible in order to measure BAM at low concentrations (50 ng L^{-1}-100 μg L^{-1}) (see Section 3.1). Therefore, there are no experiments described in this section using low concentrations of BAM. A summarised overview of the different experiments performed in this thesis is provided in the flow sheet in Figure 2.2. Three main parts are considered. First, the microbial degradation experiments are investigated, followed by the physico-chemical experiments. Finally, the experiments are explained where microbial and physico-chemical removal are joined. In the following paragraphs, the different experiments will be elaborated more in detail.

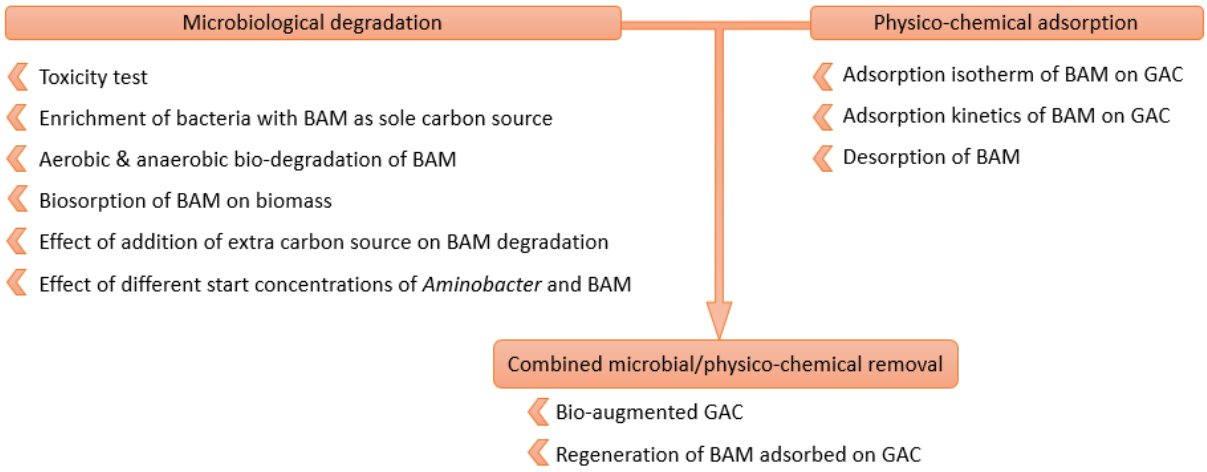


Figure 2.2: The flow sheet representing the contents of the experiments performed.

2.4.1 Microbiological degradation of BAM

All microbial tests are performed using M9 medium (see Section 5.2), except for *Escherichia coli* which is grown in LB Broth.

Toxicity test

The first microbial test involves the investigation of possible toxicity of BAM to micro-organisms in order to have additional information of toxicity discussed in Section 1.2. Four different cultures are tested using concentrations of BAM in between 0 mg L^{-1} and 1 g L^{-1}, at a constant temperature of 28°C. Firstly, a test is performed on *Escherichia coli*, one of the most common bacteria. A second test is performed using activated sludge, consisting of concentrated nitrifying and denitrifying active heterotrophic and autotrophic biomass with a high specific activity, provided by Avecom NV (Belgium) (Avecom, 2016). Both toxicity tests are performed in aerobic conditions. A third toxicity test is performed on the bacteria present in the backwash water, originating from the water used to backwash the full-scale activated carbon filters from Pidpa at the water production center in Westerlo, under anaerobic conditions (obtained by sparging with nitrogen gas). Finally, the growth response of *Aminobacter* sp. MSH1 is investigated. Cell growth is measured with platereaders (see Section 2.2) using 96-well plates and is performed in triplicate.

Enrichment of bacteria with BAM as sole carbon source

In order to grow and enrich aerobic bacteria able to degrade BAM, two mixed culture consortiums are applied for BAM degradation (Table 2.1). The first culture used for enrichment is activated sludge, identically as in the toxicity test (Avecom, 2016). By using BAM as sole carbon source, enrichment of bacteria able to degrade BAM may be obtained. Secondly, backwash water is used, identical as in the toxicity test. The backwash water may contain a culture able to degrade BAM since it originates from GAC filters used for remediation of groundwater impaired with BAM. As such, a natural adaptation of the microbial population may have already taken place. An INNOVA 2300 platform shaker (New Brunswick Scientific, New Jersey, USA) at 125 rpm at a constant temperature of 28°C, is used for incubation. Experiments are performed in triplicate. For 3 weeks, OD measurements are taken 4 times a week, to follow up growth response of the bacteria using the MDA Photometer ISIS 9000 from Section 2.2.

Table 2.1: Enrichment of BAM-degrading bacteria from activated sludge and backwash water in aerobic conditions (n=3).

Activated sludge blanc	Activated sludge
50 mL M9 medium	50 mL M9 medium
1 mL activated sludge	1 mL activated sludge
0 mg L^{-1} BAM	1 mg L^{-1} BAM

Backwash water blanc	Backwash water
50 mL M9 medium	50 mL M9 medium
1 mL backwash water	1 mL backwash water
0 mg L^{-1} BAM	1 mg L^{-1} BAM

Aerobic & anaerobic bio-degradation of BAM

Since Pidpa is looking for ways to improve the current anaerobic GAC filters used for pesticide residue removal, a bacterium which is able to degrade BAM in anaerobic conditions is desirable. Considering the difficulty to isolate bacteria in anaerobic conditions, experiments in aerobic conditions are designed as well. In aerobic conditions, three different compositions of active bacterial cultures are made: activated sludge, *Aminobacter* and a mixture of activated sludge and *Aminobacter* (Table 2.2). As mentioned earlier, activated sludge consists of nitrifying and denitrifying active biomass (Avecom, 2016) and by using BAM as sole carbon source, enrichment of bacteria able to degrade BAM may be obtained. Since *Aminobacter* is able to degrade BAM, as mentioned in Section 1.3.1, the BAM-degradation by *Aminobacter* in a pure culture as well as a mixed culture, in order to observe the influence of the presence of other micro-organisms present in the culture, are investigated. In anaerobic conditions, backwash water, *Aminobacter* and a mixture of backwash water and *Aminobacter* are used. Anaerobic conditions are obtained by flushing penicillin bottles with nitrogen gas, and closing them with a rubber stopper and an aluminum cap. Backwash water is used in anaerobic conditions since it comes from the full-scale GAC filters from Pidpa, treating anaerobic groundwater. The micro-organisms from the backwash water have already come into contact with BAM (present in the groundwater) and may thus be able to degrade BAM. A mixture is made to determine if *Aminobacter* is able to degrade BAM in cooperation with the backwash water since in the one only consisting of *Aminobacter* no degradation is expected. During incubation, the platform shaker at 125 rpm is used, at a constant temperature of 28°C. Over a period of 5 weeks, BAM is analysed using the UHPLC-DAD equipment (see Section 2.1.1).

Table 2.2: Enrichment of BAM-degrading bacteria in aerobic & anaerobic conditions, starting from activated sludge, backwash water and *Aminobacter* (n=1).

Aerobic conditions			Anaerobic conditions		
					
Activated sludge	***Aminobacter***	**Activated sludge + *Aminobacter***	**Backwash water**	***Aminobacter***	**Backwash water + *Aminobacter***
45 mL M9 medium 100 mg L^{-1} glucose	45 mL M9 medium 100 mg L^{-1} glucose	40 mL M9 medium 100 mg L^{-1} glucose	45 mL M9 medium 100 mg L^{-1} glucose	45 mL M9 medium 100 mg L^{-1} glucose	40 mL M9 medium 100 mg L^{-1} glucose
5 mL activated sludge	5 mL *Aminobacter*	5 mL activated sludge + 5 mL *Aminobacter*	5 mL backwash water	5 mL *Aminobacter*	5 mL backwash water + 5 mL *Aminobacter*
100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM

In the next experiment, the influence of different ratios of *Aminobacter* and native micro-organisms, originating from full-scale GAC filters, affecting the BAM-degradation efficiency is investigated in aerobic conditions. Five different ratios of start concentrations of *Aminobacter* and backwash water, in duplicate, are incubated at 28°C (Table 2.3). The cell density of both cultures is 6.00×10^6 cells mL^{-1}. Over a period of 14 days, BAM is analysed in order to follow up the degradation efficiency (see Section 2.1.1).

Table 2.3: Investigation of the influence of different ratios of *Aminobacter* and native micro-organisms, originating from full-scale GAC filters, on the degradation efficiency, indicated by X mL *Aminobacter* (AB) and Y mL backwash water (BW) (n=2).

				
5 AB/0 BW	**4 AB/1 BW**	**1 AB/1 BW**	**1 AB/4 BW**	**0 AB/5 BW**
45 mL M9 medium	45 mL M9 medium	45 mL M9 medium	45 mL M9 medium	45 mL M9 medium
5 mL *Aminobacter*	4 mL *Aminobacter*	2.5 mL *Aminobacter*	1 mL *Aminobacter*	-
-	1 mL BW	2.5 mL BW	4 mL BW	5 mL BW
100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM

Biosorption of BAM on biomass

In order to see if micro-organisms are able to biosorb BAM, a biosorption test is performed. On short (22 hours) and long term (19 days), the possible additional influence of the activity of *Aminobacter* is investigated as well by using living and dead *Aminobacter* at different concentrations. Dead micro-organisms are obtained by autoclaving (15 minutes at 121°C) living *Aminobacter*. The control incorporated in this design is free of bacteria and consists only of BAM in M9 medium. The test is performed in 10 mL tubes in duplo (Table 2.4). The tubes are incubated on the platform shaker at 125 rpm at a constant temperature of 28°C and BAM is analysed during the two terms (see Section 2.1.1).

Table 2.4: Investigation of BAM-biosorption on living and dead *Aminobacter* (n=2).

Control	Living *Aminobacter*	Dead *Aminobacter*
10 mL M9 medium 100 mg L^{-1} glucose	9 mL M9 medium 100 mg L^{-1} glucose	9 mL M9 medium 100 mg L^{-1} glucose
-	1 mL living *Aminobacter*	1 mL dead *Aminobacter*
100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM

Effect of addition of extra carbon source on BAM degradation

In this batch test, the effect of an additional carbon source, i.e. glucose, on the degradation efficiency of BAM by *Aminobacter* is investigated. Two different series are made in duplo in 10 mL tubes with or without glucose (Table 2.5). The start concentrations of *Aminobacter* are varied as well. Incubation of the tubes is done at a constant temperature of 28°C on the platform shaker at 125 rpm. BAM analysis is done at the start and after 1 week (see Section 2.1.1).

Table 2.5: Investigation of the effect of another carbon source (i.e. glucose) on the degradation efficiency of BAM using varying start concentrations of *Aminobacter* indicated by "Low", "Medium", "High" and "Very high" (n=2).

Low(-glucose)	Medium(-glucose)	High(-glucose)	Very high(-glucose)
9.5 mL M9 medium (100 mg L^{-1} glucose)	9 mL M9 medium (100 mg L^{-1} glucose)	8.5 mL M9 medium (100 mg L^{-1} glucose)	8 mL M9 medium (100 mg L^{-1} glucose)
0.5 mL *Aminobacter*	1 mL *Aminobacter*	1.5 mL *Aminobacter*	2 mL *Aminobacter*
100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM	100 mg L^{-1} BAM

Effect of different start concentrations of *Aminobacter* and BAM

This experiment is designed in order to see the influence of different start concentrations of *Aminobacter* and BAM on the degradation efficiency of BAM (Table 2.6). Three different series are made with a different concentration of BAM (50, 100 and 200 mg L^{-1}) in duplicate. Different start concentration of *Aminobacter* are set as well. In Table 2.6, those concentrations are indicated by "Low", "Medium" and "High", respectively $2.28x10^7$, $3.42x10^7$ and $4.82x10^7$ cells mL^{-1}. The flasks are incubated at a constant temperature of 28°C and are shaken at 125 rpm on the platform shaker. Over a period of 21 days, BAM is analysed using the UHPLC-DAD equipment, as described in Section 2.1.1. Secondly, flow cytometry analysis is applied for cell counting as described in Section 2.1, in order to see if there is a difference in cell growth in the various assays.

Table 2.6: Investigation of the influence of different concentrations of BAM (50, 100 and 200 mg L^{-1}) and *Aminobacter* ("Low", "Medium" and "High") on the degradation efficiency (n=2).

50/100/200 mg/L-Low	50/100/200 mg/L-Medium	50/100/200 mg/L-High
50 mL M9 medium	50 mL M9 medium	50 mL M9 medium
$2.28x10^7$ cells mL^{-1} *Aminobacter*	$3.42x10^7$ cells mL^{-1} *Aminobacter*	$4.82x10^7$ cells mL^{-1} *Aminobacter*
50/100/200 mg L^{-1} BAM	50/100/200 mg L^{-1} BAM	50/100/200 mg L^{-1} BAM

2.4.2 Physico-chemical removal of BAM

The following experiments are shaken for the specified time at 150 rpm at a constant temperature of 25°C in a MaxQ 4000 orbital shaker (Thermo-Scientific, Belgium). The fresh GAC is Organosorb 10 (Desotec, Belgium) and the used GAC is Organosorb 10 sourced from a full-scale GAC filter used for groundwater treatment (Desotec, Belgium).

Adsorption isotherm of granular activated carbon

An adsorption isotherm is constructed, using different masses (0, 0.025, 0.06, 0.1, 0.25, 0.6, 1, 2.5, 6, 10 and 15 g) of fresh GAC to which 80 mL of 200 mg L^{-1} BAM (dissolved in distilled water) is added. The bottles are shaken for 6 days and afterwards, BAM analysis is done (see Section 2.1.1).

Adsorption kinetics of granular activated carbon

The adsorption kinetics of BAM on fresh GAC are determined using 75 mg fresh GAC (Organosorb 10) (Desotec, Belgium) added in 80 mL of 200 mg L^{-1} BAM (dissolved in distilled water). BAM analysis, as described in Section 2.1.1, is done at different times, i.e. 0, 0.25, 0.5, 1, 2, 6, 24, 48, 72, 144 and 240 hours with one bottle per measuring point. Afterwards, the best fitted model is selected (Azizian, 2004)

Desorption of BAM

A chemical desorption test is performed in order to investigate the desorption of BAM from GAC. In this way, knowledge is obtained about chemical desorption of BAM, which will be used in Section 2.4.3. Three different series are created. With the first two series, a comparison is made between the adsorption capacity of fresh (Fresh GAC) and used GAC (Used GAC_a). "Used GAC_a" is made in order to determine how much adsorption capacity is remaining in the already used GAC. The third series (Used GAC_b) is a control. This batch test is based on 4 steps explained in detail below.

In step 1, adsorption of BAM on GAC is done, in order to saturate the GAC. For "Fresh GAC" and "Used GAC_a", 80 mL bottles are filled with 75 mg of respectively fresh GAC and used GAC, in 80 mL of 200 mg L^{-1} BAM (dissolved in distilled water). Each series is performed in triplicate. The bottles are shaken for 6 days, after which the GAC is separated from the liquid by using filter

paper.

In step 2, "Fresh GAC" and "Used GAC_a" from previous step are used, and "Used GAC_b" consists of 75 mg used GAC. BAM is desorbed from the GAC by using 80 mL of methanol HiPerSolv CHROMANORM for HPLC (min. 99.8 % - VWR Chemicals, Belgium), in order to determine how much BAM can be desorbed from the activated carbon after BAM adsorption in step 1. Identically as in step 1, the bottles are shaken for 6 days, and separated from the liquid by using filter paper.

The consecutive steps 3 and 4 are similar to step 2, but now respectively acetone (min. 99.5 %) and acetonitrile (min. 99.9 %) HiPerSolv CHROMANORM for HPLC (VWR Chemicals, Belgium) are used. These steps are added to test if complete desorption of BAM can be achieved in case methanol is not sufficient. BAM analysis is done after each step (see Section 2.1.1).

2.4.3 Combined microbial/physico-chemical removal of BAM

In the following experiments, breakthrough curves of GAC will be constructed. In order to limit the runtime of these breakthrough experiments, the GAC is first partially preloaded with BAM. In order to achieve this, a 4 L column with fresh GAC (Organosorb 10) (Desotec, Belgium) is loaded with BAM as presented in Figure 2.3. A BAM concentration of 200 mg L^{-1} (dissolved in tap water) is pumped at a flow rate of 0.6 L h^{-1} through the column and collected in a waste vessel, at room temperature. Because in a continuous process a mass-transfer zone is formed, resulting in an unequal BAM loading throughout the column (see Section 1.4.2), the GAC is thorougly mixed in order to create a homogenous mixture of preloaded GAC. Eventually, an average carbon loading of $5.6x10^{-3}$ g BAM g^{-1} GAC is obtained after desorption with methanol HiPerSolv CHROMANORM for HPLC (VWR Chemicals, Belgium).

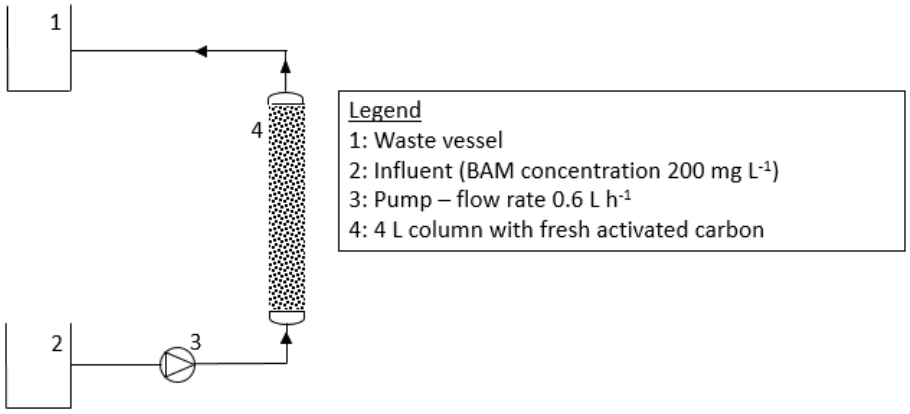


Figure 2.3: Visualisation of the saturation of fresh GAC with indication of the feed and the waste vessel, the pump and the GAC column.

Bio-augmented granular activated carbon

The aim of this experiment is to bio-augment the GAC in order to have enhanced removal of BAM, i.e. BAM adsorption on the one hand, and microbial degradation (through bio-augmentation with *Aminobacter*) of BAM on the other hand. In this way, breakthrough should be delayed and the lifetime of the GAC could be prolonged. GAC with a carbon loading of 5.6x10^{-3} g BAM g^{-1} GAC is used (Figure 2.3), in order to limit the runtime of these breakthrough experiments. Three series of columns with a volume of 10 mL are considered and each series is performed in triplicate (Figure 2.4). Every column consists of 5 g of the preloaded GAC, to enhance the survival of *Aminobacter*, and is fed with 200 mg L^{-1} BAM (dissolved in tap water). Tap water is used in order to investigate if *Aminobacter* is able to survive in non sterile conditions. To inhibit microbial activity, a first control is additionally fed with 0.1% sodium azide (min. 99.5% - Sigma-Aldrich, Belgium) (Lichstein and Soule, 1944; Cabrola et al., 2017). In this way, BAM removal is done in particular *via* physico-chemical adsorption. The GAC columns without inoculum are a second control since only removal through adsorption and through the influence of tap water bacteria will be observed. The enhancement of BAM removal is obtained by inoculating *Aminobacter* in the last series of columns. GAC is bio-augmented with *Aminobacter* on day 0, 17 and 22, with respectively 3.14x10^{9} cells of *Aminobacter* g GAC^{-1} (5 g GAC per column), 2.42x10^{9} cells g GAC^{-1} and 2.00x10^{8} cells g GAC^{-1}, *via* three-way valves at the feed of the GAC columns. This inoculated culture of *Aminobacter*, measured using the flow cytometer, is obtained at this high density by centrifuging an *Aminobacter* culture using a Sorvall RC 6 Plus Centrifuge (Thermo Scientific, Belgium). A phosphate-buffered saline solution is used to transport the centrifugated cells since the cells are maintained in the solution at a constant pH when they are inoculated in the columns

for a short term (Dulbecco and Vogt, 1954). On day 22 (5 days after breakthrough occurred) 100 mg L^{-1} of glucose is added to the influent of the "GAC" and "GAC + *Aminobacter*" for 4.6 days long (3.33 L per column) in order to give a boost to *Aminobacter* as discussed in the experiment investigating the influence of an extra carbon source in Section 3.2. The series "GAC" is fed with glucose as well since a control of the growth of the tap water bacteria is needed. The set-up is set at room temperature and covered from sunlight, since light may influence the growth of the tap water bacteria, and in this way inhibit growth of *Aminobacter*. Samples are taken two times a week for BAM analysis (see Section 2.1.1) to construct the breakthrough curve, and for flow cytometry (see Section 2.2) to follow up the cell density in the effluent. Once a week, the activity of the micro-organisms (respiration) is determined by measuring the dissolved oxygen concentration in the effluent with a Multi 3420 (WTW, Germany).

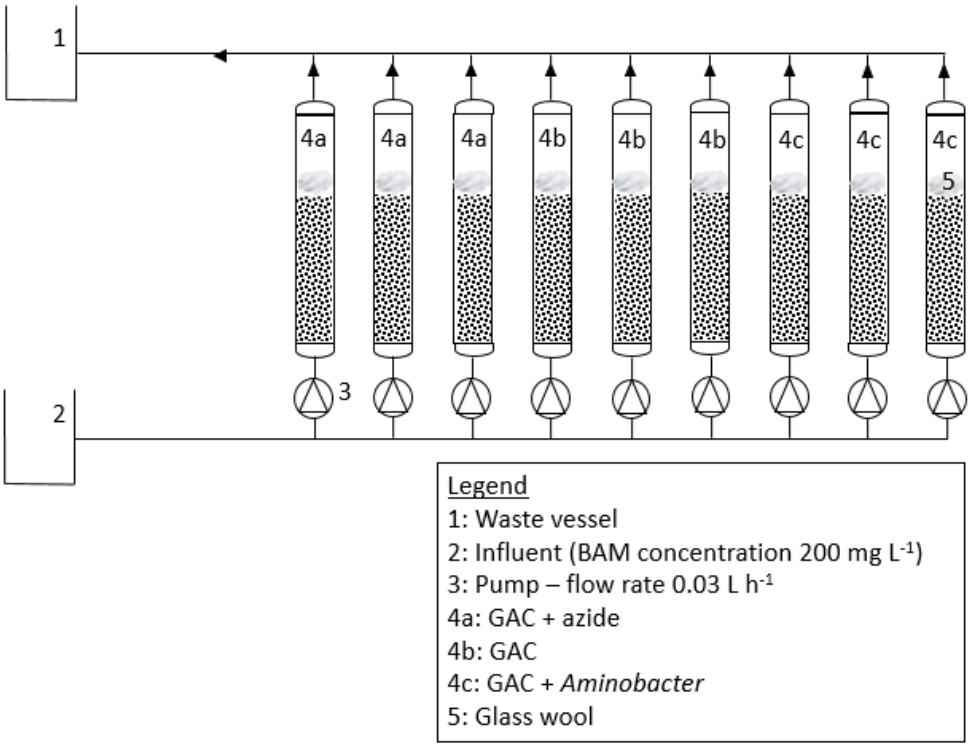


Figure 2.4: Visualisation of the set-up with three different series of columns: "GAC + azide", "GAC" and "GAC + *Aminobacter*" (n=3). Additionally, the feed and the waste vessel, and the pump are represented.

After 32 days, the feeding of the columns is stopped and the GAC of each column is collected for visualisation using scanning electron microscopy (see Section 2.2) and desorption. The GAC is desorbed using two different methods in order to see how much BAM is adsorbed on the GAC, and if *Aminobacter* has degraded a part of the adsorbed GAC. In the first method, 0.5 g of GAC

is contacted in bottles with 250 mL of methanol HiPerSolv CHROMANORM for HPLC (min. 99.8 % - VWR Chemicals, Belgium) for 6 days at room temperature. In the second method, the GAC is first subjected to ultrasonication in order to remove bacteria sorbed on the surface of the GAC. By detaching sorbed bacteria (and possible biosorbed BAM), only the amount of BAM adsorbed on the GAC should remain. A sample of 0.5 g GAC is added in 6 mL of tap water, after which ultrasonication is performed using a Branson Sonifier 250 for 30 seconds at a power of 5 W. Afterwards, the water is decanted and a GAC without attached *Aminobacter* is obtained, from which BAM is desorbed with 250 mL of methanol for 6 days at room temperature. Finally, BAM concentrations are analysed with UHPLC-DAD (see Section 2.1.1) in each method.

Regeneration of BAM adsorbed on granular activated carbon

The second experiment where GAC is bio-augmented, is the biological regeneration of BAM-loaded GAC. The aim is to regenerate the GAC by bio-degrading the adsorbed BAM, in order to reuse the GAC and prolong its lifetime. Regeneration is done using preloaded GAC (at a carbon loading of 5.6x10^{-3} g BAM g^{-1} GAC), derived from the set-up described in Figure 2.3. Three series of 1 L bottles are made, each consisting of 36 g GAC in 1 L M9 medium (Table 2.7). The amount of preloaded GAC is chosen to obtain a final concentration of 200 mg L^{-1} BAM in the medium when all BAM would have been desorbed from the GAC. The "Control" is free of bacteria. The second and third series are inoculated with respectively 1.01x10^7 and 1.01x10^6 cells of *Aminobacter* mL^{-1}, as measured with flow cytometry (see Section 2.2). Twice a week, samples are taken for cell density analysis of the medium using flow cytometry (see Section 2.2). From Figure 3.24 representing the cell density as a function of time (see Section 3.4), it is clear that *Aminobacter* is not growing by not degrading BAM of the medium using flow cytometry (see Section 2.2). On day 20, the "High density" series is reinoculated with 1.50x10^6 cells mL^{-1} of *Aminobacter* and as well 100 mg L^{-1} glucose is added in order to give a growth boost to *Aminobacter*, as discussed in the experiment investigating the influence of addition of an extra carbon source (see Section 3.2). The experiment is performed in triplicate and shaken at 125 rpm at a constant temperature of 28°C.

Table 2.7: Representation of the set-up for the regeneration experiment with the start concentrations of *Aminobacter* using three different series: ”Control”, ”Low density” and ”High density” (n=3).

Control	Low density	High density
36 g loaded AC	36 g loaded AC	36 g loaded AC
1 L M9 medium	1 L M9 medium	1 L M9 medium
-	10^7 cells mL^{-1}	10^6 cells mL^{-1}

After 29 days, the biological regeneration process is ended and the GAC of each column is collected for visualisation using scanning electron microscopy (see Section 2.2), chemical desorption of the GAC and re-adsorption of BAM on the GAC. These last two steps are performed in order to determine the biological regeneration capacity by bio-degradation of BAM, adsorbed on the GAC. For the desorption test, 2 g of GAC (in duplo per bottle) is subjected to desorption in 10 mL of methanol HiPerSolv CHROMANORM for HPLC (min. 99.8 % - VWR Chemicals, Belgium) at 28°C and 125 rpm for 6 days. For the re-adsorption test, 0.5 g of GAC, with potentially *Aminobacter* sorbed on the surface, is added in 0.5 L of M9 medium with 60 mg BAM L^{-1} and shaken at 125 rpm for 6 days at 28°C. Afterwards, for each test BAM analysis is done using UHPLC-DAD (Section 2.1.1).

3

Results & Discussion

3.1 Chemical analysis

3.1.1 Ultra High Performance Liquid Chromatography Diode Array Detector

The method developed with UHPLC-DAD is suitable for analysis of BAM concentrations between 1-200 mg L^{-1} BAM. In order to have a correct measurement of BAM, each run on the UHPLC-DAD (see Section 2.1) includes a calibration series as mentioned in Section 2.1.1, after which the samples are measured. An example is given in Figure 3.1 where a calibration curve is constructed using different BAM concentrations, dissolved in M9 medium. The trendline is a second-order polynomial with a regression coefficient (R^2) of 0.9988. The associated equation is used to determine the concentrations of the samples used for BAM analysis during the experiments.

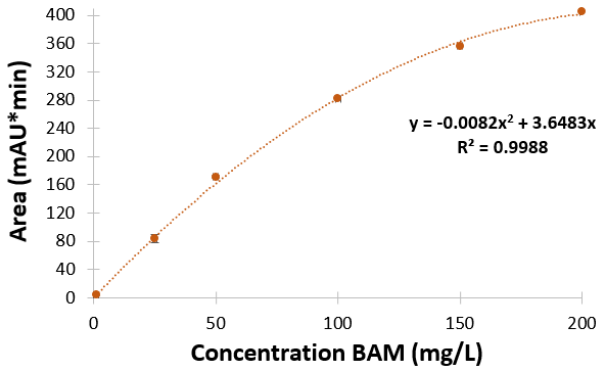


Figure 3.1: Calibration curve using high concentrations ranging in between 1-200 mg L^{-1}, dissolved in M9 medium. The error bars are the standard deviations (n=2).

3.1.2 Ultra High Performance Liquid Chromatography Tandem-Mass Spectrometry

The developed UHPLC-MS/MS method proved not to be suitable for measuring samples at low concentrations. Firstly, this is due to a lot of background being present in the concentrated (i.e. after SPE) samples, indicated in Figure 3.2, where BAM has a retention time of 3.44 min. To illustrate, in Table 3.1, BAM areas are presented of SPE-concentrated samples, i.e. a blanc (distilled water) sample and different GAC samples where initially 20 μg L^{-1} BAM (dissolved in distilled water) was added. The blanc has the highest area despite the fact that no BAM was added. In addition, a clearly decreasing area pattern is not observed for the GAC samples (in fact, it was expected that higher areas than in the blanc would be observed). For these reasons, it was concluded that the method is not suitable to conduct further experiments at these relatively low BAM concentrations.

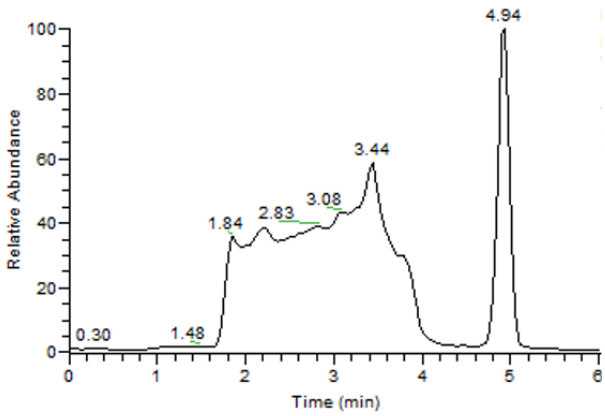


Figure 3.2: Output of BAM analysis (retention time=3.44 min) using Xcalibur Software 2.0.7 SP1.

Table 3.1: Area of samples determined with a blanc (distilled water) sample and different GAC samples where initially 20 μg L^{-1} BAM was added.

Sample	Area BAM
Blanc	8653304
125 mg GAC	2579106
80 mg GAC	6061942
50 mg GAC	2792934

3.2 Microbiological degradation of BAM

Toxicity test

As discussed in Section 1.2, BAM may cause toxicity and therefore this microbial test is done investigating possible toxicity of BAM to micro-organisms. Firstly, an aerobic test is performed on one of the most common bacteria, *Escherichia coli*, and secondly on activated sludge, a mixed culture full of diverse bacteria. Thirdly, toxicity is tested on backwash water in anaerobic conditions which may contain a culture able to degrade BAM since it originates from GAC filters used for remediation of groundwater impaired with BAM and in this way may be less inhibited by BAM. Finally, the growth response of *Aminobacter* sp. strain MSH1 is investigated.

Escherichia coli

An inhibition of growth of *Escherichia coli* is seen for the highest concentration of 1 g L^{-1} BAM since a delay of increasing optical density (OD) is observed, in contrast to the other concentrations (Figure 3.3 Left). The fact that there is an increasing OD in all other samples, i.e. bacterial growth, is due to the presence of nutrients in the LB broth medium wherein *Escherichia coli* is grown previously. The blancs indicate a background OD of 0.1. After 44 hours, a significant difference is seen between 1 g L^{-1} BAM and the other BAM concentrations (Figure 3.3 Right) and therefore it can be stated that there is some BAM toxicity towards *Escherichia coli* at high concentrations.

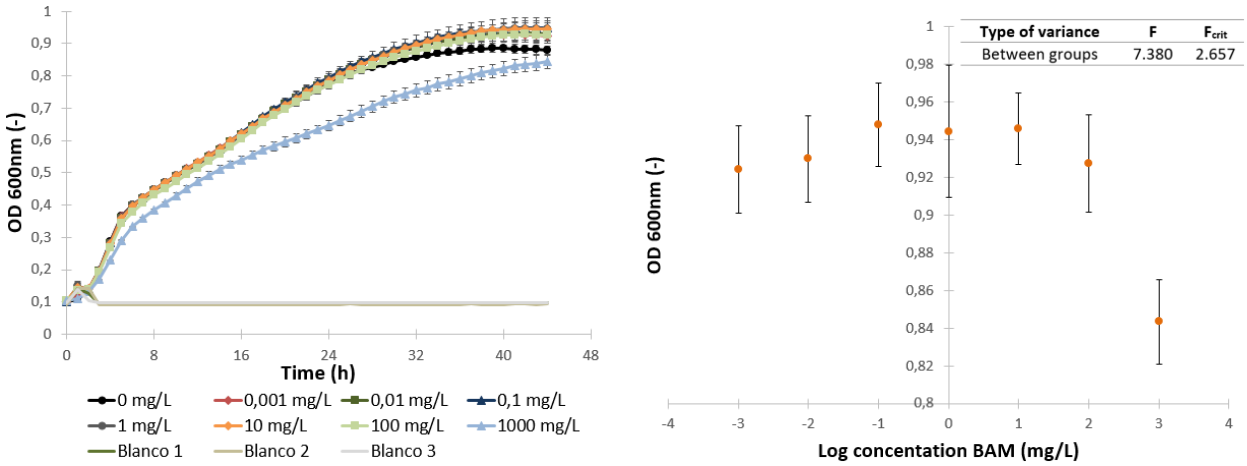


Figure 3.3: Investigation of toxicity of BAM on *Escherichia coli*. The concentrations of BAM used are on log scale between 0 and 1 g L^{-1}. The error bars are the standard deviations (n=3). **Left**: The growth pattern of *Escherichia coli* indicated by the different concentrations of BAM. **Right**: The end values of OD for the different log concentrations of BAM.

Activated sludge

No influence is seen of low concentrations of BAM on the growth of activated sludge (Figure 3.4 Left). Nonetheless, a concentration of 1 g L^{-1} decreases growth of activated sludge. The fact that the bacteria are growing could be due to organic matter available in the activated sludge since it is rich in nitrifying and denitrifying active heterotrophic and autotrophic biomass (Avecom, 2016). Blanc 1 and 2 are sterile and indicate a background OD of 0.09, but blanc 3 is contaminated. After 69 hours, a significant difference in cell density between 1 g L^{-1} BAM and the other BAM concentrations is found and therefore it can be stated that there is some BAM toxicity at high concentrations (Figure 3.4 Right).

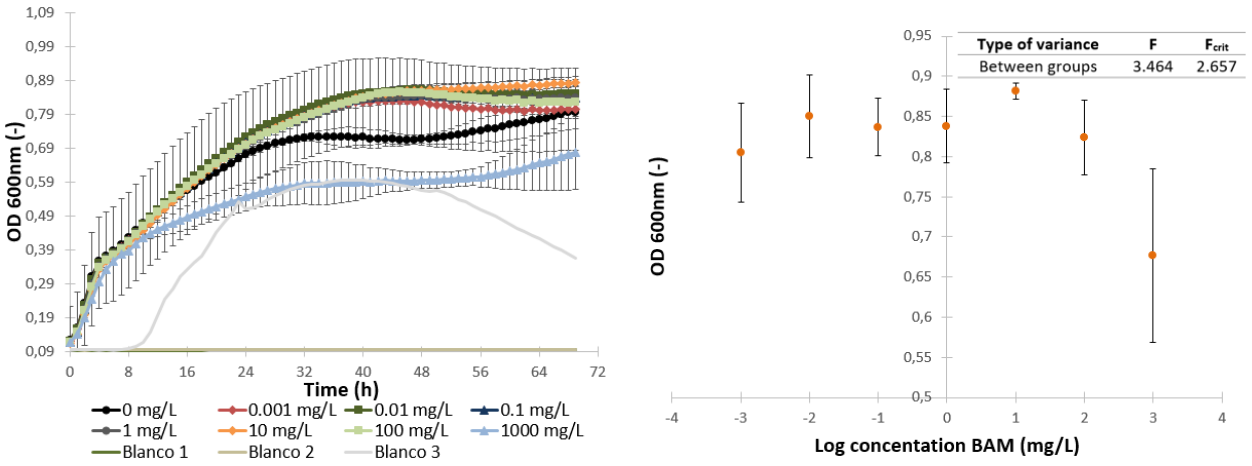


Figure 3.4: Investigation of toxicity of BAM on activated sludge. The concentrations of BAM are on log scale between 0 and 1 g L^{-1}. The error bars are the standard deviations (n=3). **Left**: The growth pattern of activated sludge indicated by the different concentrations of BAM. **Right**: The end values of OD for the different log concentrations of BAM.

Backwash water

The growth responses of 0 mg L^{-1} and 1 g L^{-1} BAM follow a deviated pattern compared to the other BAM concentrations, for which no explanation was found (Figure 3.5, Left). They might be contaminated with other bacteria but no confirmation of this can be done. The other samples seem to have no inhibition of BAM as they follow the same pattern. The blancs indicate an average background OD of 0.09. The reason why the cell density is declining for all samples, independently of the BAM concentration, is because there is less extra carbon source available (a total organic carbon content of 1 mg L^{-1} (Pidpa, 2016)), contrary to the previous cases of *Escherichia coli* and activated sludge.

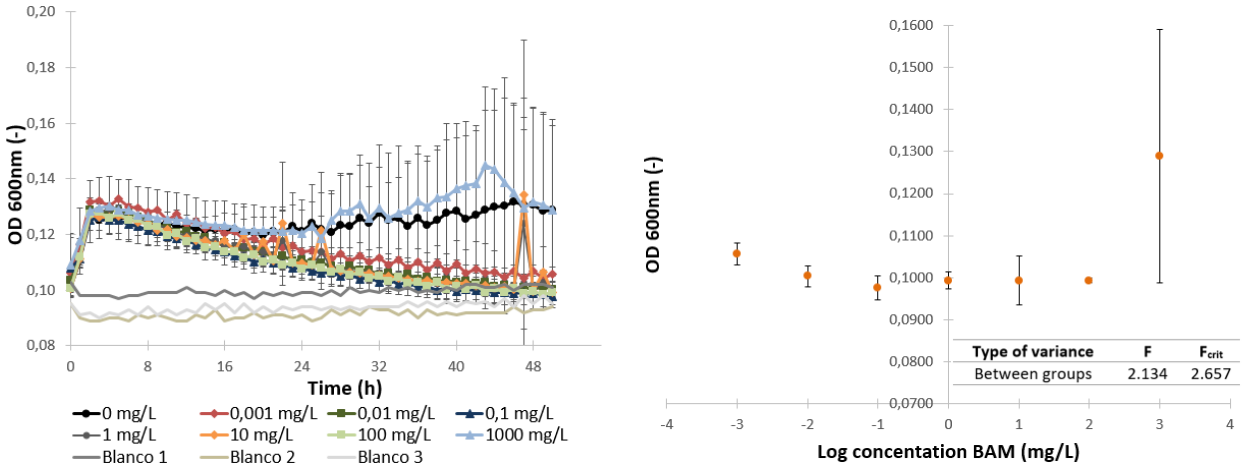


Figure 3.5: Investigation of toxicity of BAM on backwash water originating from a full-scale activated carbon filter, received from Pidpa. The concentrations of BAM are on log scale between 0 and 1 g L^{-1}. The error bars are the standard deviations (n=3). **Left**: The growth pattern of backwash water indicated by the different concentrations of BAM. **Right**: The end values of OD for the different log concentrations of BAM.

Aminobacter sp. MSH1

The highest concentration of 1 g L^{-1} shows a significant increase in growth compared to the other BAM concentrations applied in this assay, although a relatively high standard deviation is observed (Figure 3.6 Left). Other concentrations are following the same pattern independently of the BAM concentration. The blancs indicate an average background OD of 0.09. After 44 hours, a significant difference between 1 g L^{-1} BAM and the other BAM concentrations is observed and therefore it can be stated that BAM has a positive effect on the growth of *Aminobacter* at a macro-concentration of 1 g L^{-1} BAM observed over 2 days (Figure 3.6 Right).

As mentioned in Section 1.2, several hundred mg BAM kg^{-1} body weight gives acute toxicity to mammals. For bacteria, growth inhibition is observed for *Escherichia coli* and activated sludge at 1000 mg L^{-1} BAM. To conclude, *Escherichia coli* and activated sludge exposed to BAM will not be inhibited since environmentally concentrations of BAM do not have an effect on growth. *Aminobacter* is positively affected by the presence of BAM since it degrades BAM using it as a carbon, nitrogen and energy source in this design of the experiment (see Section 1.3.1).

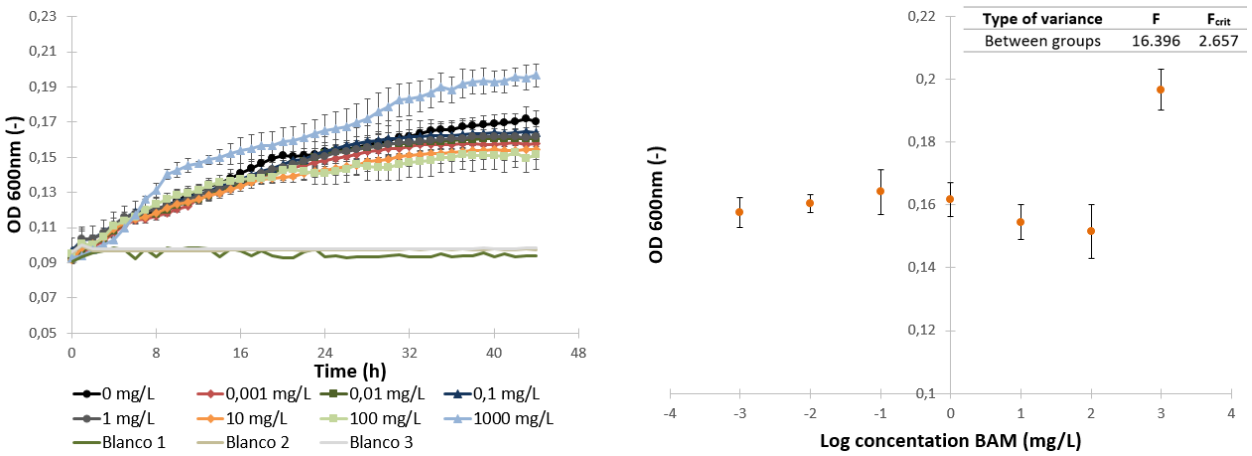


Figure 3.6: Investigation of the influence of different concentrations of BAM, ranging on log scale between 0 and 1 g L^{-1} on *Aminobacter* sp. MSH1. The error bars are the standard deviations (n=3). **Left**: The growth pattern of *Aminobacter* indicated by the different concentrations of BAM. **Right**: The end values of OD for the different log concentrations of BAM.

Enrichment of bacteria with BAM as sole carbon source

In this experiment, the aim is to investigate if microbial growth occurs in mixed cultures through addition of BAM. A concentration of 1 mg L^{-1} BAM is used as a source of carbon, nitrogen and energy for bacteria present in activated sludge, and a blanc without BAM is included. Growth response is measured *via* optical density (OD) for 16 days (Figure 3.7). The OD fluctuates a lot and no extreme growth response is noticed. Small increases can be due to bacteria which die and

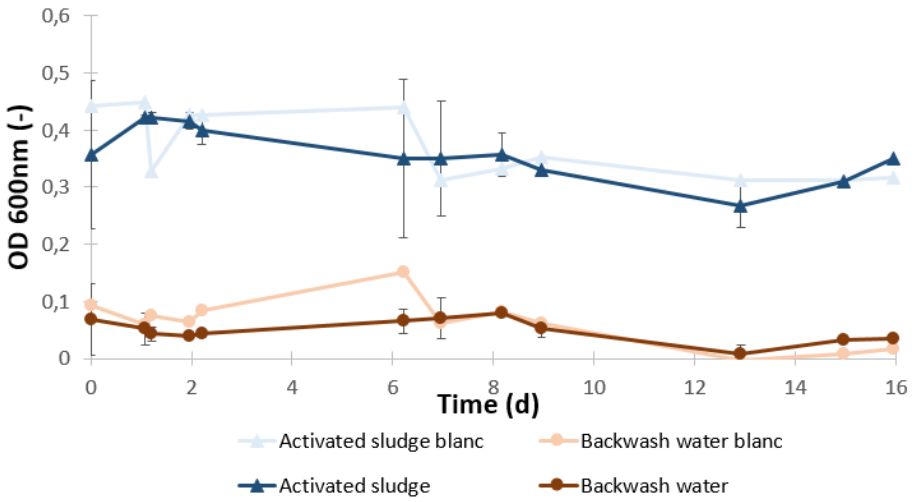


Figure 3.7: Graphic presentation of the optical density, representing growth response, of activated sludge and backwash water. The error bars are the standard deviations (n=3).

become a carbon source for the remaining bacteria. This could be the case with activated sludge consisting of active biomass (Avecom, 2016). For the backwash water, lower OD values are observed indicating that less bacteria are present. There are no indications in the pattern which might refer to a growth response by BAM-degradation in Figure 3.7. To conclude, it is not possible to gather bacteria able to degrade BAM on short term (i.e. 16 days) *via* this batch test.

Aerobic & anaerobic bio-degradation of BAM

In aerobic conditions, the first approach for BAM degradation is by stimulating growth of BAM-degrading bacteria possibly present in activated sludge. The purpose is that by using BAM as sole carbon source eventually only BAM-degrading bacteria will remain. Activated sludge is used since it consists of a very diverse composition of bacteria (Avecom, 2016). In addition, the aerobic BAM-degrading bacterium *Aminobacter* sp. MSH1 is used and a mixture of *Aminobacter* and activated sludge. Figure 3.8 Above shows the BAM concentration over a 5 week period. In the beginning, the BAM concentration is rising which could be due to fluctuations of its solubilisation. It is speculated that microbial exudates act as a surfactant, thus enhancing complete solubilisation of BAM within the first days of the experiment, however this theory needs further investigation. After 30 days, BAM is completely degraded using the pure culture of *Aminobacter*. The activated sludge and the mixed culture are not able to degrade BAM. *Aminobacter* seems to be outcompeted by the diverse mixed culture of activated sludge. This is an important observation which might give difficulties. Because, when bio-augmentation of BAM-degrading bacteria is targeted, they would need to survive in the resident community present in GAC filters of Pidpa. Vandermaesen, J. (2016) investigated the success of bio-augmentation using *Aminobacter* depending on the diversity of the resident community in sand filters. The study revealed that overall a negative trend is observed between the diverse resident community and the survival, and in this way the degrading capacity of *Aminobacter*.

Aerobic bio-degradation of BAM can be applied after the GAC, present in a full-scale granular activated carbon filter, is saturated. In this case, aerobic BAM-degrading bacteria can be applied in an in-situ regeneration process. However, the ideal scenario would be that an anaerobic culture is found for the in-situ BAM-degradation since the BAM-degrading bacteria need to survive in anaerobic conditions, as the full-scale GAC filter exploited by Pidpa is operated in anaerobic conditions, as first step in the drinking water treatment. This GAC is operated in anaerobic conditions

because the influent has a high dissolved iron (Fe(II)) content, and in order to prevent precipitation of iron in the GAC filter, oxygen must be avoided at this stage. Following the hypothesis of in-situ bio-degradation, the bacteria need to find a way to survive in this anaerobic conditions. Similarly as in the aerobic conditions, the aim is to start from a mixed culture, here the backwash water since the bacteria present in the filter have already come into contact with BAM (present in the groundwater) and may thus be able to degrade BAM. Unfortunately, from Figure 3.8 Below no significant response is observed in degradation of BAM using backwash water. No response is seen for *Aminobacter*, which is logical as it is an aerobic bacterium (see Section 1.3.1), nor for the mixture culture of backwash water and *Aminobacter*. To conclude, anaerobic degradation of BAM proves to be difficult. Pukkilan and Kontro (2014) investigated BAM degradation by incubating deposits of different groundwater wells and monitoring pipes under anaerobic conditions. After 527 days, no degradation of BAM was observed.

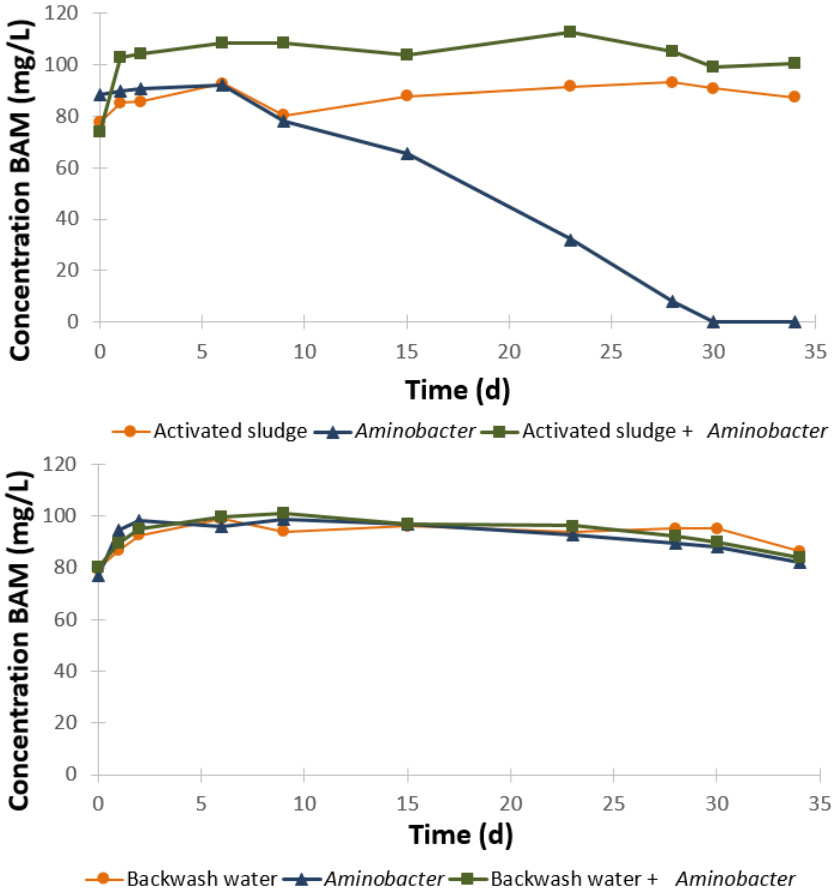


Figure 3.8: Above: BAM degradation in aerobic conditions: activated sludge, *Aminobacter* and a mixed culture of activated sludge and *Aminobacter* (n=1). **Below**: BAM degradation in anaerobic conditions: backwash water, *Aminobacter* and a mixed culture of backwash water and *Aminobacter* (n=1).

In the following experiment, the survival of *Aminobacter* in backwash water is investigated, which is full of bacteria of the resident community (i.e. from the full-scale GAC filter from Pidpa), by using different ratios of *Aminobacter* and backwash water. The survival of *Aminobacter* will be reflected by the BAM degradation efficiency (Figure 3.9). The BAM concentrations of the different ratios on day 9 are significantly different (Table 3.2) and therefore an additional t-test is done of the first three ratios (Table 3.3). The ratio 4 AB/1 BW has the best BAM degradation efficiency. It is reasonable to assume that the backwash water contains other carbon sources (total organic carbon content = 1 mg L^{-1}, Pidpa) which can give *Aminobacter* a boost and accelerate the degradation of BAM. On the other hand, the bacteria of the BW can be competitive as for 1 AB/4 BW, *Aminobacter* needs more time for complete BAM degradation as a delay is observed in the degradation pattern. Vandermaesen, J. (2016) investigated the influence of interactions of the resident communities, present in sand filters, and *Aminobacter*. Some strains are competitive which negatively affects BAM mineralisation while other strains can affect BAM mineralisation on a positive manner. Eventually, interactions between the resident community in GAC filters and BAM-degrading bacteria would determine the survival of the BAM degradable communities and in this way the BAM degradation efficiency.

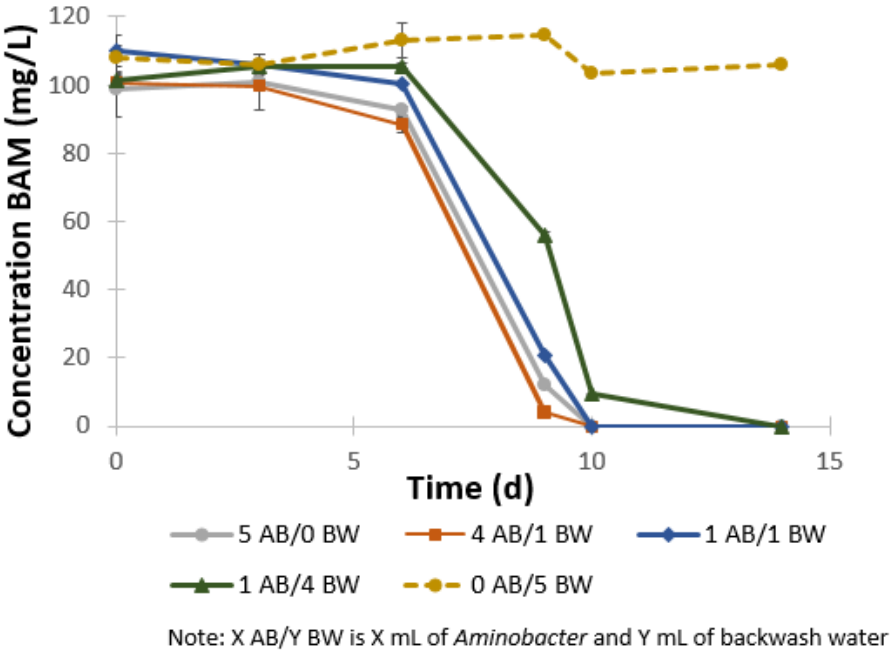


Figure 3.9: The influence of the presence of indigenous bacteria on the degradation efficiency using different ratios of *Aminobacter* and backwash water. The error bars are the standard deviations (n=2). The amount of cells mL^{-1} is for both $6.00x10^{6}$.

Table 3.2: Anova single factor of the results represented in Figure 3.9 on day 9, showing a significant difference between the different ratios (n=2).

Type of variance	F	F_{crit}
Between the different ratios	1737.977	5.192

Table 3.3: t-Test assuming unequal variances of the results represented in Figure 3.9 on day 9 (n=2). A significant difference is seen for all three combinations.

Sample	t	t_{crit}
5 AB/0 BW and 4 AB/1 BW	51.414	12.706
5 AB/0 BW and 1 AB/1 BW	-43.873	4.303
4 AB/1 BW and 1 AB/1 BW	-114.472	12.706

Biosorption of BAM on biomass

The aim of this experiment is to see if living and/or dead cells have an influence on BAM concentration by biosorption. Figure 3.8 showed a slightly rising concentration of BAM during the first 6 days. Therefore, an experiment is conducted to investigate the cause of this fluctuation of BAM, which is possibly affected by the presence of *Aminobacter*, e.g. through biosorption and/or desorption. A comparison is made between living and dead *Aminobacter*, and a control which is free of bacteria. On short term (22 hours), the control has small fluctuations in BAM concentration (Figure 3.10 Above). After 22 hours, a decrease of BAM is noticed in the living *Aminobacter*. It appears that in this experiment the BAM-degradation starts sooner than it did in the previous experiment (Figure 3.8). It seems that in this case *Aminobacter* is already acclimatised to BAM, which is likely because the *Aminobacter* used in this experiments originate from the previously conducted experiment where aerobic degradation pathways were investigated. As such, the *Aminobacter* have already been in contact with BAM before the start of this experiment. For the dead cells, fluctuations are seen particularly at the start which may be a due to noise or an instrumental error. For long term incubation, a period of 19 days is considered (Figure 3.10 Below). After 8 days, the living cells have completely degraded BAM (i.e. until below the detection limit of 1 mg L^{-1}). In this experiment, rising concentrations at the start are limited compared to

Figure 3.8 which may be due to an instrumental error. Therefore, no clear difference is determined towards biosorption of dead or living *Aminobacter*.

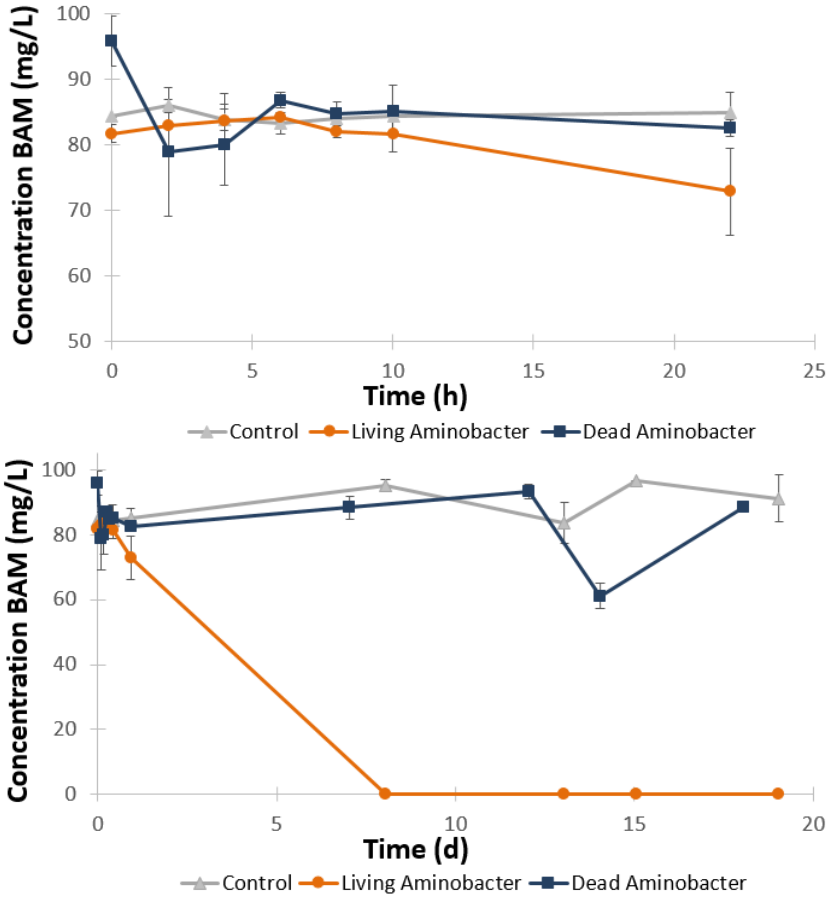


Figure 3.10: Above: Short term analysis of biosorption on living cells of *Aminobacter*, dead cells of *Aminobacter* and the control free of bacteria. The error bars are the standard deviations (n=2). **Below**: Long term analysis of biosorption on living cells of *Aminobacter*, dead cells of *Aminobacter* and the control free of bacteria. The error bars are the standard deviations (n=2).

Effect of addition of extra carbon source on BAM degradation

In this experiment, the aim is to see in which way the degradation efficiency of BAM changes upon addition of an easily degradable carbon source. In the series where glucose has been added, all BAM is degraded after one week, while in absence of glucose, concentrations of BAM decrease faster for a higher number of *Aminobacter* cells being inoculated at the start (Figure 3.11). In Table 3.4, the results from the ANOVA analysis are presented for the series without glucose. Since the F (31.573) is higher than F_{crit} (6.591), the different groups of the series without glucose are significantly different from each other. In other words, the amount of *Aminobacter* added at the start is relevant towards the degradation efficiency. It could be that glucose gives a boost to

Aminobacter and therefore *Aminobacter* is able to grow faster. Eventually, the more dense culture of *Aminobacter* adapts faster to BAM as carbon source. Holtze et al. (2007b) investigated the effect of the addition of an easily degradable carbon source (sodium succinate) on the BAM mineralisation levels in soil slurries. BAM is mineralised extensively in addition of the extra carbon source without the accumulation of metabolites, such as 2,6-dichlorobenzoic acid (see Section 1.3.1). When BAM is the main carbon source, BAM mineralisation levels are below 50%. The addition of alternative carbon sources seems to be an efficient strategy for stimulating and enriching BAM-mineralising bacteria in soils. This strategy may be transferred to the bio-augmentated bacteria in full-scale GAC filters which should eventually be able to degrade BAM.

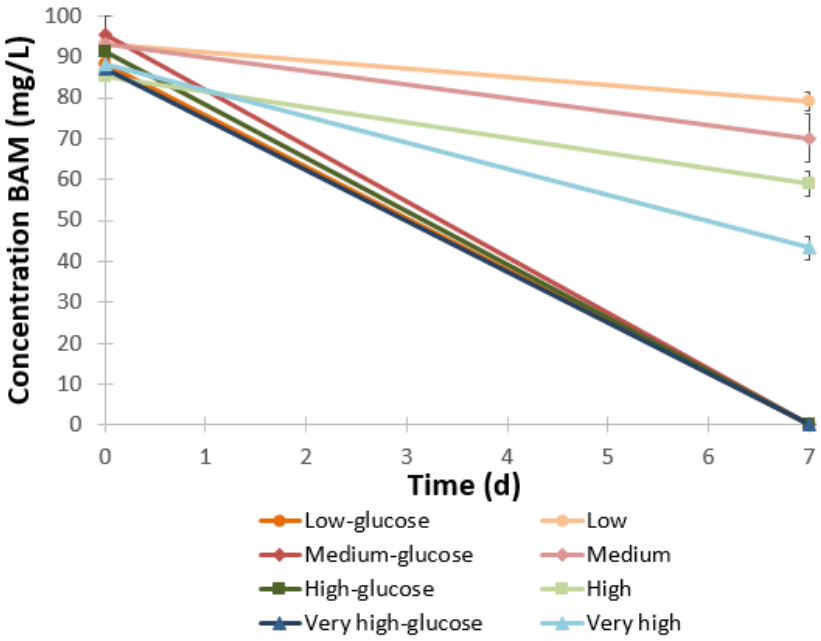


Figure 3.11: Influence of an additional carbon source, e.g. glucose, on the BAM-degradation efficiency. "Low", "Medium", "High" and "Very high", respectively 0.5, 1, 1.5 and 2 mL, indicate the start concentration of *Aminobacter* in the series with and without glucose. The error bars are the standard deviations (n=2).

Table 3.4: Anova single factor showing the significant difference between the groups of the series without glucose and reflecting the importance of the start concentration of *Aminobacter* (n=2).

Type of variance	F	F_{crit}
Between groups without glucose	31.573	6.591

Effect of different start concentrations of *Aminobacter* and BAM

In this experiment, the influence of different start concentrations of *Aminobacter* (e.g. 2.28x10^7, 3.42x10^7 and 4.82x10^7 cells mL^{-1}) and BAM (e.g. 50, 100 and 200 mg L^{-1}) is investigated. It takes 10 days to initiate BAM-degradation, independently of both *Aminobacter* and BAM concentrations (Figure 3.12). This is in contrast with the results found before (Figure 3.10), however in this case, *Aminobacter* comes directly from the -80°C freezer, and is grown directly using glucose without being in contact with BAM. On day 15, only a distinction is observed for the series using 100 mg L^{-1}, although only for "Medium" and "High" *Aminobacter* there is a significant difference in degradation efficiency. On day 15, the series with 50 and 100 mg L^{-1} BAM have the same concentration level of BAM, except for 100 mg L^{-1} - Low.

Figure 3.13 represents the cell density of *Aminobacter* on the last day of the experiment. One should expect that the higher the BAM concentration, the higher the cell density of *Aminobacter* at the end of the degradation since more source for cell growth is available. However, no significant differences can be found (Table 3.5). Sekhar et al. (2016) investigated how trace BAM concentrations affect *Aminobacter* surface colonisation and BAM-degrading activity when inoculated in flow channels (i.e. to simulate filtration units of drinking water treatment plants), continuously fed with macro- and micro-concentrations of BAM dissolved in a nitrogen and carbon limiting medium. The flow channel is colonised at all BAM concentrations but the BAM degradation efficiencies are dependent on the BAM concentration, and are ranging between 70 and 95%. The BAM concentration also had an impact on surface colonisation. It appeared that for BAM concentrations of 100 μg L^{-1} and lower, colonisation is similar to the systems without BAM. Hypothetically, the nitrogen and carbon limiting medium sustain colonisation at BAM micro-concentrations. Since in a following experiment, granular activated carbon columns will be bio-augmented and fed continuously with BAM (see Section 3.4), the results from Figure 3.13 are important to confirm how well *Aminobacter* degrades BAM in these conditions, and its ability to colonise the GAC. Besides, as suggested in the experiment investigating the influence of an extra carbon source, additional easily degradable carbon sources present in the medium are important to sustain growth, specifically when considering environmentally relevant conditions of BAM, e.g. below 0.3 μg L^{-1} as is the case for Pidpa.

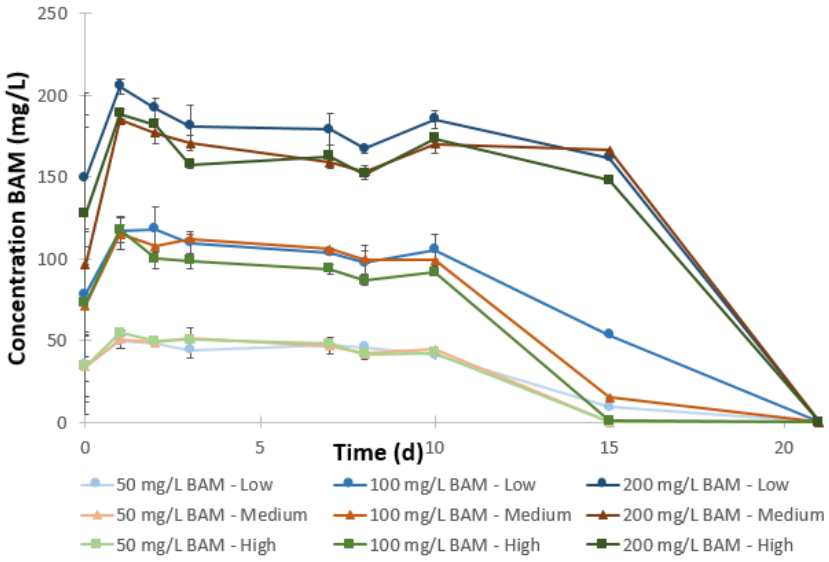


Figure 3.12: Influence of different start concentrations of *Aminobacter* and BAM (50, 100 and 200 mg L^{-1}) on the degradation efficiency. "Low", "Medium" and "High" indicate the start concentration of *Aminobacter*, respectively 2.28x10^7, 3.42x10^7 and 4.82x10^7 cells mL^{-1}. The error bars are the standard deviations (n=2).

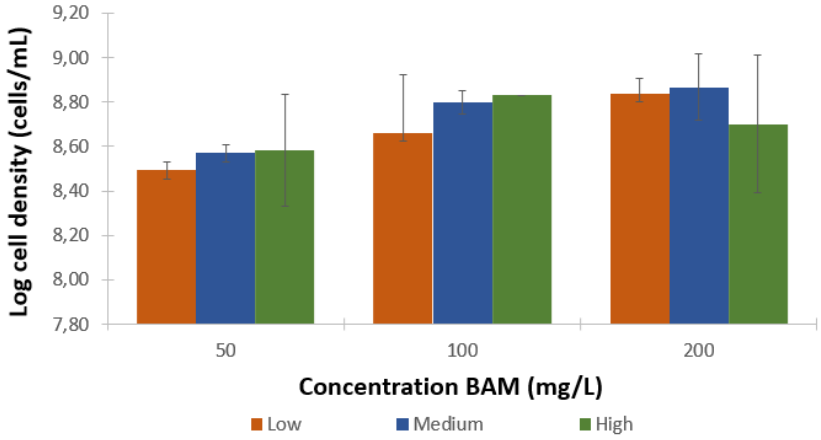


Figure 3.13: Visualisation of the end values of flow cytometric analysis at day 27. "Low", "Medium" and "High" indicate the start concentration of *Aminobacter*, respectively 2.28x10^7, 3.42x10^7 and 4.82x10^7 cells mL^{-1}. The error bars are the standard deviations (n=2).

Table 3.5: Anova single factor of the results of Figure 3.13 showing the insignificant difference between the groups (n=2).

Type of variance	F	F_{crit}
Between all groups	1.230	3.230

3.3 Physico-chemical removal

Figure 3.14 represents several pictures of fresh GAC made with scanning electron microscopy (see Section 2.2). The surface of the GAC has a very varied structure, e.g. pores as well as a robust surface is seen.

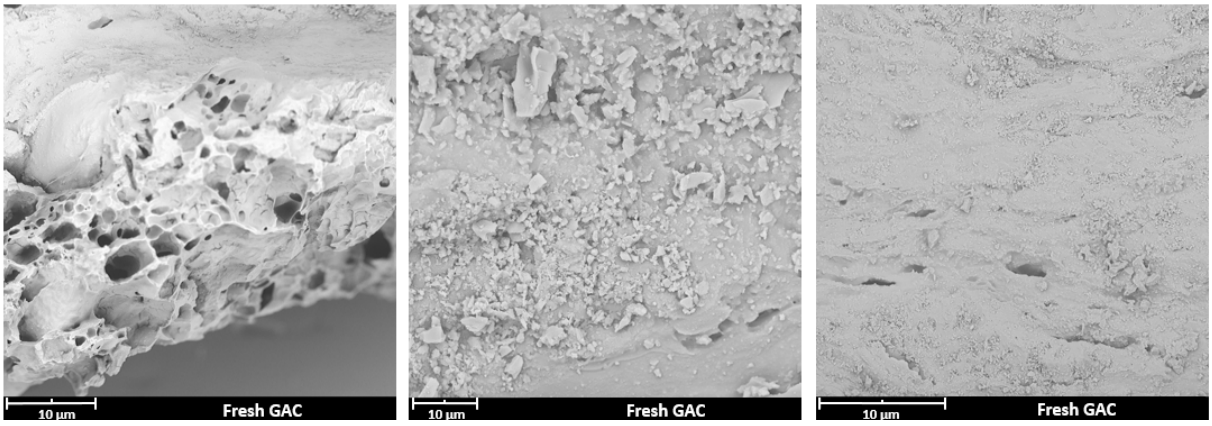


Figure 3.14: Three pictures made using scanning electron microscopy visualising fresh GAC.

Adsorption isotherm of granular activated carbon

An adsorption isotherm is constructed represented in Figure 3.15 with the amount of BAM adsorbed on the activated carbon (g BAM g^{-1} GAC) as a function of the equilibrium concentration remaining in the solution (mg L^{-1}). The adsorption isotherm is modeled using the Freundlich equation: log carbon loading = 0.3039*log concentration - 1.1399 (Çeçen and Aktas, 2011). The determined Freundlich fit has a regression coefficient of 0.945 which may be due to the varied surface to volume ratio of one single GAC leading to a varying adsorption capacity (Desotec, 2015).

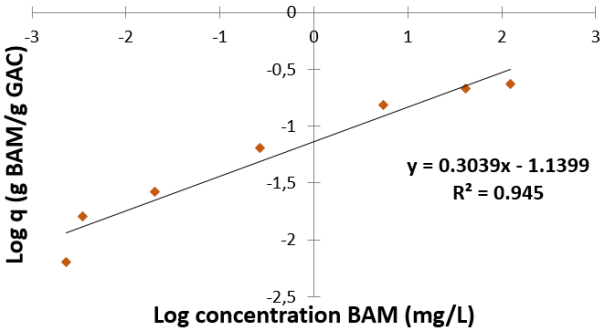


Figure 3.15: The adsorption isotherm with following Freundlich equation: log carbon loading = 0.3039*log concentration - 1.1399 (n=1).

Adsorption kinetics of granular activated carbon

In this experiment the adsorption kinetics of BAM on GAC are investigated. Almost after one day, equilibration is observed: 90% of the initial concentration of BAM is adsorbed on the GAC (Figure 3.16). A pseudo-first order model is found: q = 0.17605(1-exp(-0.12035t)) (Azizian, 2004). Pseudo-first order adsorption kinetics of various organic micropollutants, including some pesticides, are found in the literature. For example, the pesticide aldicarb (log K_{ow} = 1.13) has a pseudo-first order adsorption kinetic as well (Ayranci and Hoda, 2005; Hansch et al., 1995).

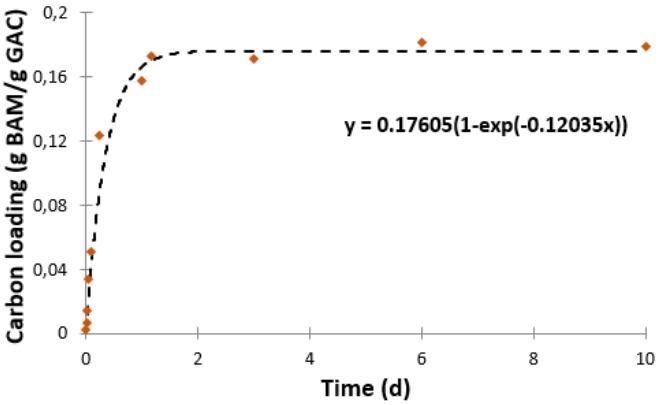


Figure 3.16: The adsorption kinetics given by the carbon loading as a function of time (n=1).

Desorption of BAM

In this experiment, the adsorption and desorption activities are determined using three different chemical solvents. Initially, BAM was adsorbed on fresh GAC and used GAC (as described in Section 2.4.2), yielding carbon loadings of 0.2 and 0.09 g BAM g GAC^{-1} respectively (Figure 3.17). For the fresh GAC, 96% of the BAM is adsorbed, however for the used GAC, only 42% of the BAM is adsorbed which indicates that the remaining adsorption capacity of the used GAC is significantly

lower because it has been used before in a full-scale GAC filter for drinking water production (see Section 2.4.2).

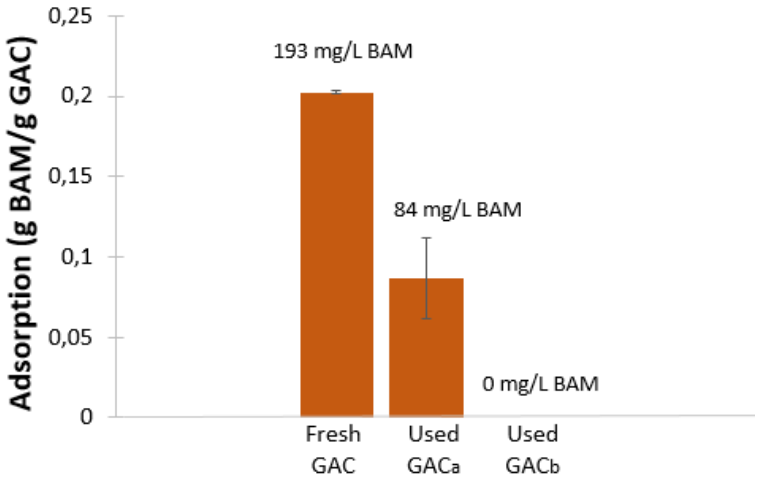
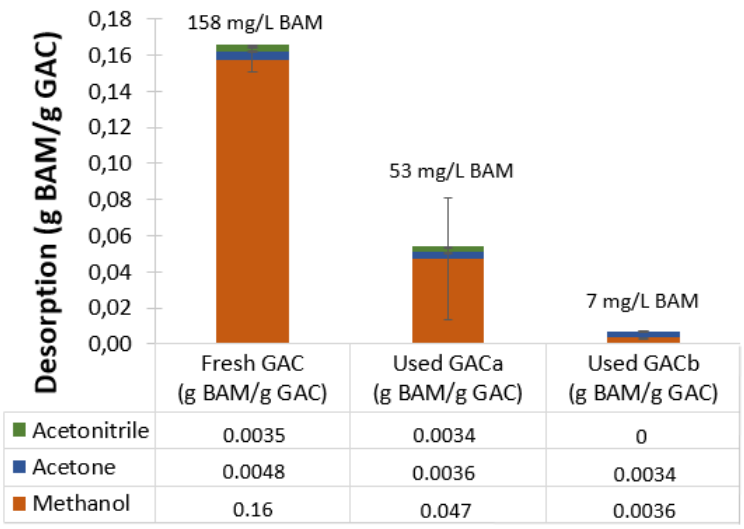


Figure 3.17: The carbon loading represents, for series "Fresh GAC" and "Used GAC_a", the amount of BAM adsorbed on GAC. The error bars are the standard deviations (n=3).

After initial loading of the fresh and used GAC with BAM, desorption is performed using organic solvents as described in Section 2.4.2. These results are shown in Figure 3.18. For all series, methanol (the first solvent used for desorption) is able to desorb the major part of the adsorbed BAM. In the following successive desorptions using acetone and acetonitrile, only a small amount is left to desorb (i.e. 0.26-0.63 mg BAM). For the fresh GAC, 82% of the adsorbed BAM can be desorbed using all three solvents, and 78% by using only methanol. For the used GAC, the amount of desorbed BAM can be originating from the aforementioned adsorption phase, but as well from previous BAM adsorption during drinking water treatment when the GAC was in operation at a water production center in Herentals. Almost 7 mg L^{-1} is desorbed in the "Used GAC_b", which is BAM originating purely from full-scale operation of the GAC. In the experiments of Section 3.4, desorption will only be done using methanol, since these results indicate it is responsible for the vast majority of BAM desorption.



	Fresh GAC (g BAM/g GAC)	Used GACa (g BAM/g GAC)	Used GACb (g BAM/g GAC)
Acetonitrile	0.0035	0.0034	0
Acetone	0.0048	0.0036	0.0034
Methanol	0.16	0.047	0.0036

Figure 3.18: Desorption of amount of BAM from the GAC loaded with BAM performed using the successive organic solvents: methanol, acetone and acetonitrile. The error bars are the standard deviations (n=3).

3.4 Combined microbial/physico-chemical removal of BAM

Bio-augmented granular activated carbon

The first experiment where micro-biological and physico-chemical removal are combined is based on the GAC filters used for groundwater treatment, exploited by Pidpa. As mentioned in Section 1.4.2, regeneration of GAC is expensive and therefore a parallel process of adsorption on the GAC of BAM and BAM-degradation by *Aminobacter* is targeted. In this way, the GAC's lifetime could be extended, i.e. a delay of breakthrough, by degradation of BAM adsorbed on the GAC and/or present in the feed. Three different series are used to study this. The first series is a control GAC assay, which is additionally fed with 0.1% sodium azide to inhibit microbial activity (Lichstein and Soule, 1944), resulting in removal of BAM through physical adsorption only. The series "GAC" might indicate possible growth of bacteria present in tap water (i.e. this GAC is not fed with sodium azide), and BAM removal by sorption. The columns inoculated with *Aminobacter* are biological activated carbon columns.

In Figure 3.19 the cell density in the effluent is represented, measured with flow cytometry, with indication of the three inoculation points and the added glucose, to give a boost of bacterial growth. Overall, the control with azide has a lower cell density, which is logical as biological activity of

bacteria present in tap water is inhibited. Immediately after the first and second inoculation, samples are taken in which a high increase of cells is found. This indicates that a large part of the inoculated *Aminobacter* cells are washed away due to the continuous flow through the column, although high standard deviations are observed. Probably only a small fraction of the inoculated *Aminobacter* is left behind in the columns and it is this small fraction that needs to acclimatise in this new environment (Albers et al., 2015). From the moment that 100 mg L^{-1} glucose is added in "GAC" and "GAC + *Aminobacter*", a small increase of the cell density is observed in both series, indicating a rising activity of bacteria. Unfortunately, flow cytometry cannot distinguish between tap water bacteria and *Aminobacter*, neither living or dead cells since SYBR Green I is used.

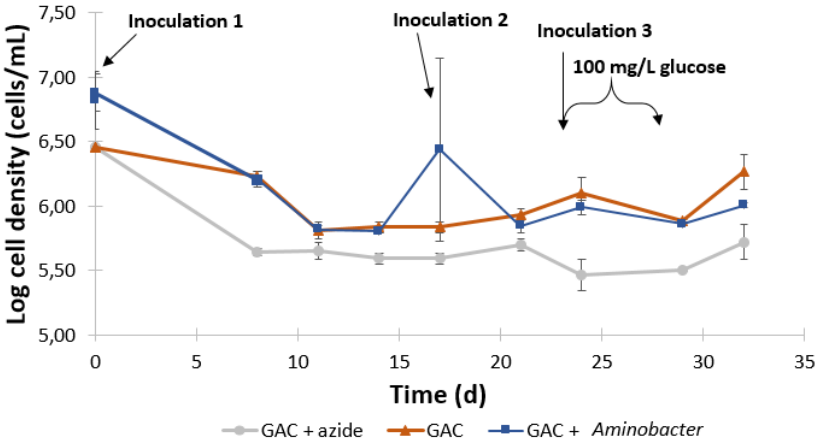


Figure 3.19: Log cell density as a function of time with indication of the three inoculation points of *Aminobacter*, and the glucose addition in series "GAC (+ *Aminobacter*)". The error bars are the standard deviations (n=3).

An extra measurement to follow up activity of aerobic bacteria is done by measuring dissolved oxygen present in the effluent of each column (Figure 3.20). As expected, "GAC + azide" gives constantly higher oxygen concentrations. There is no activity of bacteria consuming oxygen for respiration. From the moment glucose is added, a clear difference is seen for "GAC" and "GAC + *Aminobacter*". The tap water bacteria in "GAC" are responding to the glucose addition, as shown by a lowered dissolved oxygen concentration. In "GAC + *Aminobacter*" the bacteria respond even more which may indicate that *Aminobacter* grows intensively after the last inoculation due to the addition of glucose. The difference is declining after a few days, indicating a decrease in oxygen consumption, which may be due to the inoculated *Aminobacter* (partially) being washed away. Note that the glucose addition is ended at this point as well.

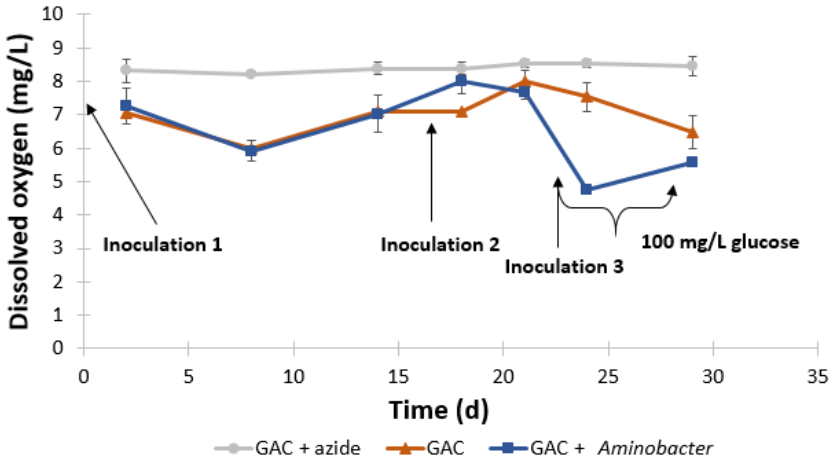


Figure 3.20: Dissolved oxygen as a function of time with indication of the three inoculation points of *Aminobacter*, and the glucose addition in series "GAC (+ *Aminobacter*)". The error bars are the standard deviations (n=3).

Figure 3.21 gives the breakthrough curve of BAM for the three series of activated carbon columns. On day 17, a slightly lower effluent concentration is observed for "GAC + azide" which may be an artifact since at the end, all graphs come together. "GAC" follows the same pattern as "GAC + *Aminobacter*" and comparing with "GAC + azide", it seems that the presence of any microbial activity has no effect on adsorption of BAM. Even the BAM-degrading *Aminobacter* is not able to delay the breakthrough curve since no difference is seen with the other graphs, not even at the end though the recommended contact time of 20 minutes is fulfilled. Simpson (2008) mentions that micro-organisms are able to colonise on the surfaces of the GAC, and may evolve into a biofilm by feeding themselves on the adsorbed organic matter and other micro-organisms present in the medium. The SEM-scans of the GAC of the columns can give more information about the sorption of bacteria on the GAC (Figure 3.22). The first scan (Left) is from "GAC + azide". When comparing with the fresh GAC (Figure 3.14), the surface of the "GAC + azide" is smoother as no rough peaks are seen. Since 0.1% sodium azide is fed for 4 weeks, which is 4.2 g sodium azide g^{-1} GAC, element identification is done focusing on the elements of sodium azide (sodium an nitrogen). This revealed that there is no sorption of these elements on the surface. On the second scan (Middle), from series "GAC", bacteria coming from the tap water are sorbed on the surface. Comparing this scan with the third scan (Right) of "GAC + *Aminobacter*", no differences are seen which is caused by the high loss of *Aminobacter* when it is inoculated at the start. Tap water bacteria and *Aminobacter* can not be differentiated based on these scans. In this test, no conclusions can be drawn relating the role of micro-organisms on breakthrough since no

differences are seen regarding the breakthrough curve, probably due to the low amount of sorbed bacteria on the GAC surface.

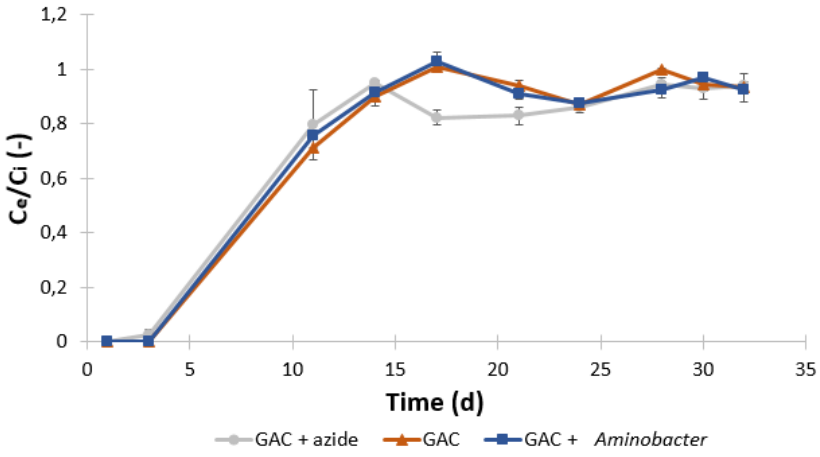


Figure 3.21: The breakthrough curve with the ratio of effluent to influent concentrations as a function of time for the three different series of columns: ”GAC + azide”, ”GAC” and ”GAC + *Aminobacter*”. The error bars are the standard deviations (n=3).

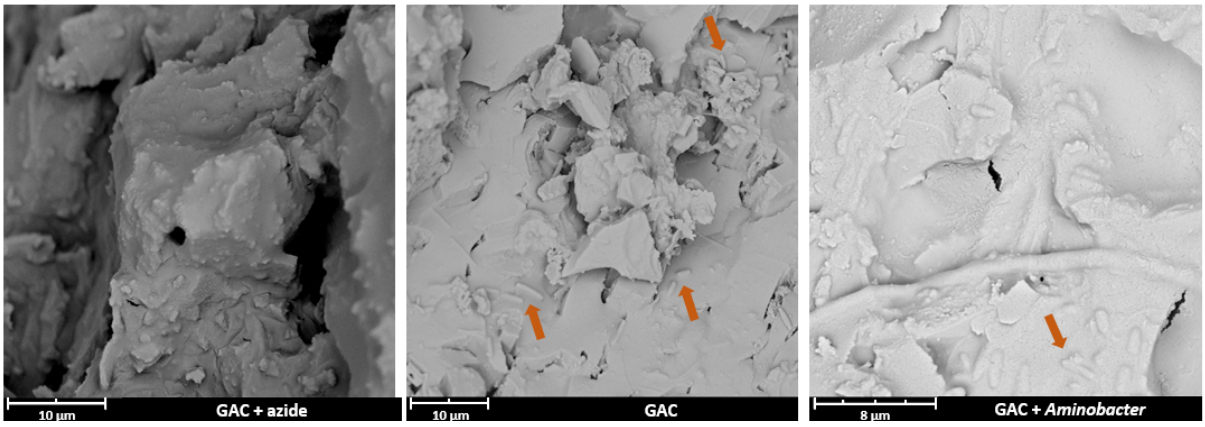


Figure 3.22: Scanning electron microscopy of the columns ”GAC + azide”, ”GAC” and ”GAC + *Aminobacter*”. The arrows indicate the presence of a bacterium.

After the continuous, long-term, experiment, the GAC was removed from the columns, and a desorption test was performed using the procedure as described in Section 2.4.3, in order to determine the amount of remaining (adsorbed) BAM, and thus also the amount of bio-degraded BAM (Figure 3.23). Two desorption methods are performed, one with and one without ultrasonication. Ultrasonication is a technique commonly used for microorganisms and biofilm removal from GAC surfaces (Van der Kooij et al., 1995). With this method it is possible to remove potential biosorbed BAM. The series without ultrasonication is not significantly different between the different types

of carbon, in contrast to the one with ultrasonication (Table 3.6). Therefore an additional t-test is performed for the results with ultrasonication (Table 3.7), whereby it is noticed that only "GAC" and "GAC + *Aminobacter*" are significantly different from each other. To see if this is caused by an unequal biosorption of BAM on bacteria on the surface, a t-test is done comparing, per series, the results with and without ultrasonication (Table 3.8). There are no significantly different results so the fact that "GAC" and "GAC + *Aminobacter*" are considerably different is not due to biosorption of BAM on bacteria. To conclude, no significant impact of BAM sorbed on bacteria is seen, which may be due to the limited amount of bacteria sorbed on the GAC (Figure 3.22). Evidently, Tsezos and Bell (1989) investigated the biosorption of hazardous organic pollutants and concluded that the biosorbed pollutants can also desorb.

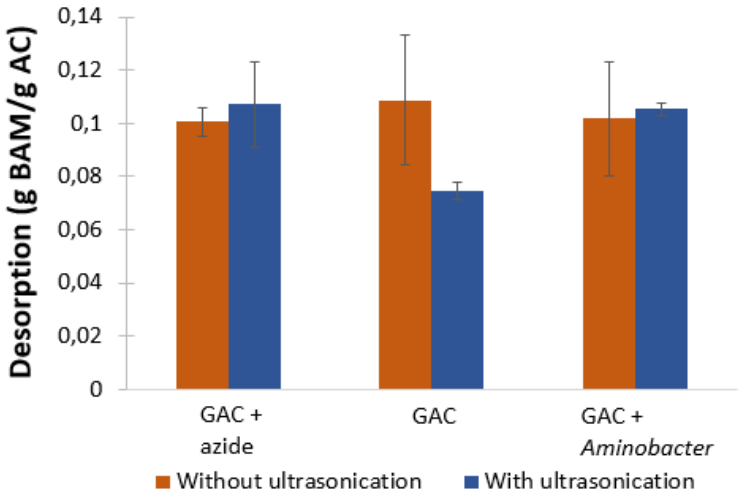


Figure 3.23: Amount of BAM desorbed of GAC using methanol with and without ultrasonication, from columns "GAC + azide", "GAC" and "GAC + *Aminobacter*". The error bars are the standard deviations (n=3).

Table 3.6: Anova single factor based on the results of Figure 3.23. The series without ultrasonication is insignificant different, and the one with ultrasonication is significant different (n=3).

Type of variance	F	F_{crit}
Between groups without ultrasonication	0.164	5.143
Between groups with ultrasonication	10.649	5.143

Table 3.7: t-Test, assuming unequal variances, of the results with ultrasonication represented in Figure 3.23 showing a significant difference between "GAC" and "GAC + *Aminobacter*" (n=3).

Sample	t	t_{crit}
GAC + azide and GAC	3.380	4.303
GAC + azide and GAC + *Aminobacter*	0.189	4.303
GAC and GAC + *Aminobacter*	-13.007	3.182

Table 3.8: t-Test, assuming unequal variances, of the results in Figure 3.23 represented between the series with and without ultrasonication showing no significant differences (n=3).

Sample	t	t_{crit}
GAC + azide	-0.649	4.303
GAC	2.429	4.303
GAC + *Aminobacter*	-0.282	4.303

Regeneration of BAM adsorbed on granular activated carbon

The second experiment where microbiological and physico-chemical removal of BAM are combined in a bio-augmented approach is the regeneration of GAC. This set-up is performed batch wise to biologically regenerate the GAC, using *Aminobacter* which could theoretically degrade BAM adsorbed on the surface of the GAC, and in this way freeing adsorption sites again for BAM adsorption. Three different series are used in order to investigate the possibility of regeneration by *Aminobacter*. The first series is a control which is free of bacteria. The second and third series are inoculated at slightly different concentrations of *Aminobacter* (see Section 2.4.2).

Figure 3.24 represents the cell density as a function of time with indication of the reinoculation point of *Aminobacter* and the additional glucose in the "High density" series. The control without *Aminobacter* represents the background measured with the flow cytometer. From the start, a decrease in the number of cells is observed in the inoculated bottles. *Aminobacter* seems not able to grow by degrading BAM adsorbed on the GAC. Therefore the series "High density" on day 20 is reinoculated with *Aminobacter*, and additionally 100 mg L^{-1} glucose is added to give the

regeneration process an extra boost which lasts till day 25. Series "Low density" stays constant over time.

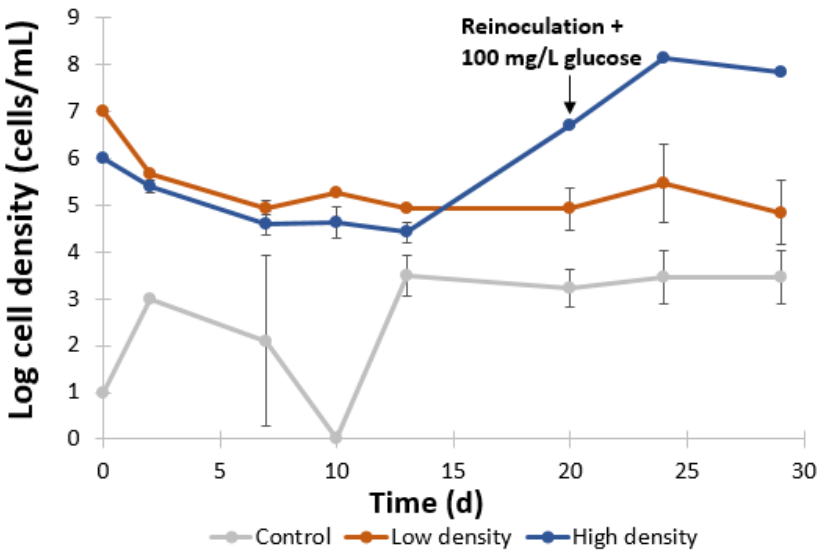


Figure 3.24: Log cell density as a function of time with indication of the reinoculation point and the glucose addition in series "High density". The error bars are the standard deviations (n=3).

Figure 3.25 represents the results of the re-adsorption test which is done in order to see if *Aminobacter* has degraded the BAM on the GAC, and in this way has created available adsorption sites again. Hypothetically, the more BAM degraded by *Aminobacter* in the regeneration step, the more adsorption sites should be available in this re-adsorption test. *Aminobacter* may degrade BAM *via* two different actions. Firstly, *Aminobacter* has been able to degrade BAM adsorbed on the GAC in the regeneration phase and in this way adsorption sites are available again. Secondly, a biofilm made of *Aminobacter* is attached on the GAC in the regeneration phase. In this way, *Aminobacter* is present in the re-adsorption test and degrades BAM present in the M9 medium. From Table 3.9 it is seen that *Aminobacter* did not have a significant impact on regeneration of the GAC. This is more or less in agreement with the results presented in Figure 3.24, indicating the low activity of *Aminobacter*. Besides, the unhomogeneous character of the GAC derived from the continuous saturation of GAC (Figure 2.3) may also have an effect, and may have contributed to high standard deviations. Based on this test no conclusions can be drawn, and it is recommended that this test is repeated using saturated GAC.

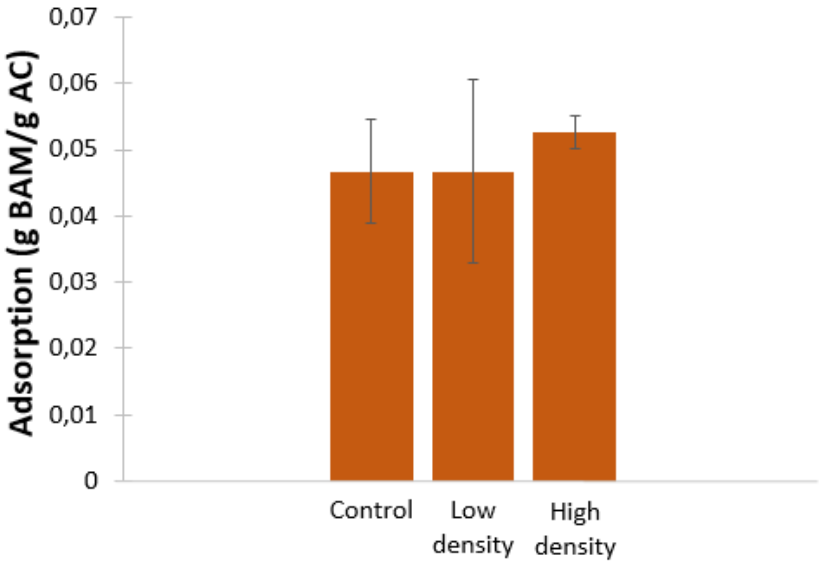


Figure 3.25: Amount of BAM adsorbed on the GAC expressed by q for the "Control", "Low density" and "High density". The error bars are the standard deviations (n=3).

Table 3.9: Anova single factor of the results represented in Figure 3.25 showing an insignificant difference between all groups (n=3).

Type of variance	**F**	**F**$_{crit}$
Between all groups	0.369	5.786

Figure 3.26 represents the results of the desorption test where the non-degraded BAM is being desorbed. Hypothetically, the more BAM has been degraded by *Aminobacter* in the regeneration step, the less BAM should be desorbed in this desorption test. Since the F (1.628) is lower than F$_{crit}$ (3.682), the results are not significantly different between the groups in the desorption test (Table 3.10). The fact that results are not significantly different may be due to the low initial loading of the GAC, which is insufficient to see effects of BAM degradation by *Aminobacter*, since this amount of BAM may be insufficient for survival of *Aminobacter*. A higher activity of *Aminobacter* may be needed as well, e.g. through addition of glucose and/or a higher cell density at the start of an experiment.

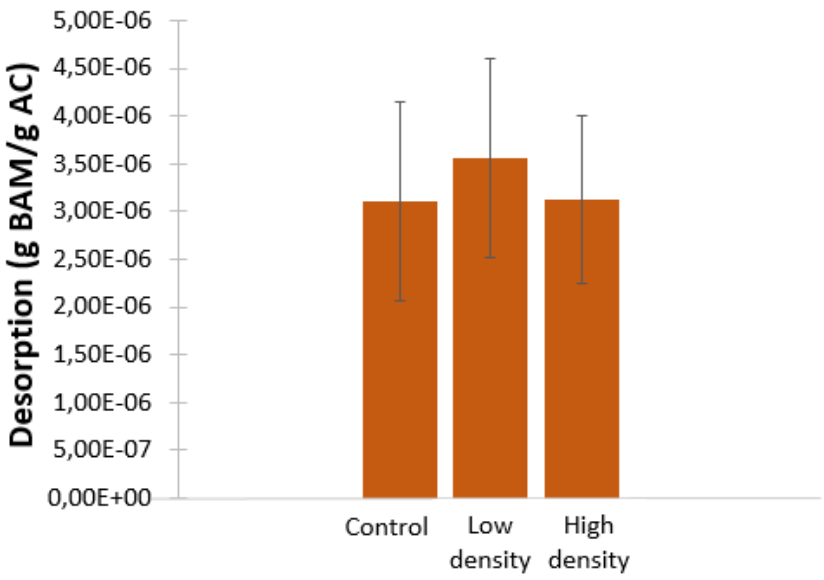


Figure 3.26: Amount of BAM desorbed of GAC using methanol for GAC derived from the control and the series with a low and high density of *Aminobacter*. The error bars are the standard deviations (n=6).

Table 3.10: Anova single factor of the results represented in Figure 3.26 showing an insignificant difference between all groups (n=6).

Type of variance	F	F_{crit}
Between all groups	1.628	3.682

Hypothetically, if the bacteria are able to degrade adsorbed BAM then *Aminobacter* should be located at the surface of the GAC. Figure 3.27 represents the SEM scans of the GAC of the regeneration experiment. Since no bacteria are present to consume the M9 salts, a high amount of crystals is seen in the scan of the control (Left). The scan of "Low density" (Middle) shows several bacteria attached on the surface which should be *Aminobacter*. Less crystals are seen on the surface as *Aminobacter* is present and is able to consume it. On the third scan (Right), no bacteria are seen although more cells are inoculated than in series "Low density". The reason for this might be the fact that only one GAC particle is visualised which may not be representative for all (36 g) the GAC used in the regeneration experiment. On the other hand, *Aminobacter* could prefer to remain in the liquid, since easily degradable glucose is added and they may not have been starving yet at the time the SEM scans were performed. Therefore, they might be less attached on the GAC (i.e. to degrade BAM), but are more present in the liquid (consisting of an easily degradable carbon

source).

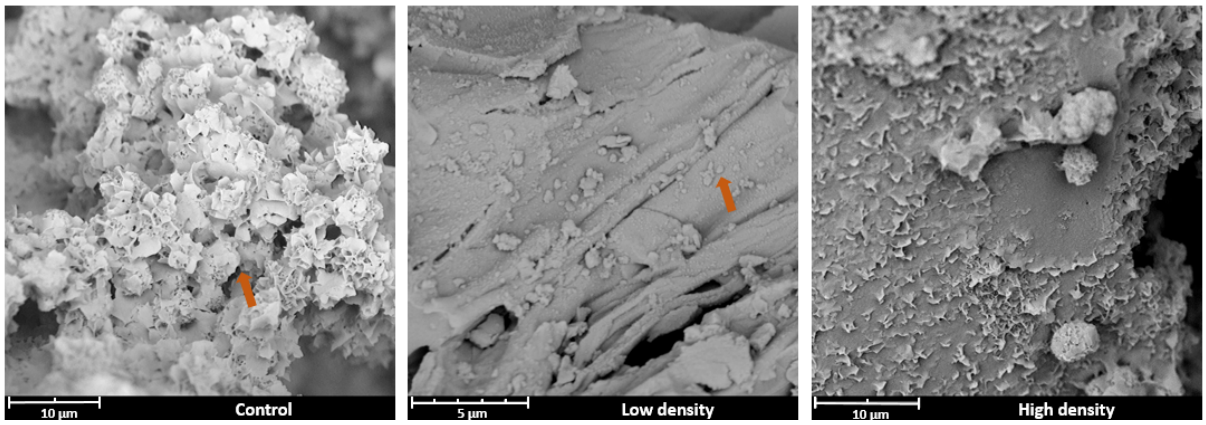


Figure 3.27: Scanning electron microscopy of the GAC from the control and the ones inoculated with a low and high cell density of *Aminobacter*. The arrow in the first scan indicates the presence of salts, and in the second scan it indicates the presence of a bacterium.

4

Conclusion & Further perspectives

The excessive use of the pesticide dichlobenil in the past, resulted in contamination of groundwater sources by its degradation product, BAM. Since drinking water treatment plants use ground water as water source, there is a need to develop efficient and economically feasible pollutant removal techniques. In this thesis, granular activated carbon is used as an effective removal technique. To enhance this removal, research in this thesis is done to investigate two different pathways of bio-augmentation of granular activated carbon for enhanced removal of BAM. It was not feasible to enrich an anaerobic BAM-degrading culture and therefore the focus of this thesis lays on aerobic BAM-degradation by *Aminobacter*. The first pathway investigates the possibility to regenerate BAM-loaded GAC by bio-degradation of BAM in aerobic conditions after the GAC is partly saturated with BAM. The second pathway is based on in-situ bio-augmentation of BAM on the GAC in the first step of the purification process of anaerobic groundwater.

The possibility to reuse used GAC may be possible performing biological regeneration of BAM. In this thesis no significant differences are however seen before and after biological regeneration, which may be due to the low amount of adsorbed BAM which is decisive regarding the survival of *Aminobacter*. For further research, one should use completely BAM-saturated GAC. Although, in practice GAC filters will be saturated with a variety of pollutants, not only with BAM. Then aerobic BAM-degrading bacteria can be applied and may perform an effective regeneration process. The importance of additional carbon sources is indicated in the experiments of the section about microbiological degradation, which may ameliorate the survival of *Aminobacter*. By having successful biological regeneration of the saturated GAC, adsorption sites are freed up again, and as a result the GAC may be modified again in an effective filter product.

The bio-augmentation of GAC filters may be a successful way to combine two different removal techniques in a parallel process: physico-chemical adsorption and microbiological degradation. From the results in this thesis, no impact of bio-augmentation is obtained when using high concentrations of BAM (200 mg L^{-1}). *Aminobacter* seems unable to survive and withstand the continuous flow through the columns, even though the recommended contact time of 20 minutes is fulfilled. A possible reason for this failure may be due to BAM adsorption in the GAC micro-pores, where bacteria may have difficulties to attach on the surface. When translating this situation to full-scale GAC filters, the minimum contact time of 20 minutes is still met to ensure the possibility of microbiological degradation. If in future experiments environmentally relevant concentrations are used, the importance of other carbon sources, present in the groundwater or additionally fed, may have a decisive effect towards the survival of *Aminobacter*. In addition, the influence of BAM-adsorption in micro-pores and the ability of bacteria to sorb in micro-pores, and eventually degrade the adsorbed BAM, needs to be investigated.

Since only BAM removal is considered, this research could by extended by including enhanced removal of the other organic micro-pollutants present in the groundwater, e.g. bromacil, diuron, chloridazon, desphenylchloridazon, bentazon, acrylamide. For every compound, an aerobic or anaerobic bacterium (depending on the application) able to degrade the concerning compound is needed, and ideally they should interact with each other in a positive way (i.e. no inhibited behaviour) in order to have an optimal mineralisation capacity for each compound.

5

Appendix

5.1 Protocol solid-phase extraction

The developed method of the solid-phase extraction is based on Blandin et al. (2016).

1. Conditioning

   In this step, the sorbent is activated or wetted in order to have good phase interface with the sample applied. The SPE cartridges are placed on the little valves in the lid of the SPE manifold (see Figure 2.1). The valves are closed. On each cartridge, 3 mL methanol HiPerSolv CHROMANORM for LC-MS is pipetted. Now all the valves are opened. In order to start the methanol flow through the cartridge, vacuum is shortly applied. Then the methanol flow goes through the cartridge by means of gravity. The valves need to be closed once there is approximately 1 mm methanol remaining on top of the upper frit. The adsorbent cannot be dry.

2. Equilibration

   This step creates a sorbent chemistry environment as similar to the sample as possible. 5 mL of matrix solution (Milli-Q water) is pipetted on each cartridge. The valves must not yet be opened.

3. Loading

   On the SPE manifold, adapters are connected in which the syringes are placed. The syringes have a volume of 50 mL wherein the standards/samples are transferred. Now, the valves can be opened and the extraction can be started. If necessary, the vacuum pump can be

used shortly to induce dripping. When the samples are dripping (rather than a continuous stream) through the cartridge, the contact time between sample and adsorbent is sufficient, and the flow speed is good. Once the extraction is finished, the adapters and the syringes are removed.

4. Washing

   In this step, the sorbent is washed to selectively remove undesired contaminant species from the sorbent, while the target analytes are retained on the sorbent. On each cartridge, 5 mL Milli-Q water is pipetted and flows through the cartridge. Afterwards, the inside of the SPE cartridges need to be dried with paper and the cartridges are air-dried by means of applying a continuous vacuum for 10 minutes, with the valves open.

5. Elution

   In the elution step, the desired analytes are recovered from the sorbent column using a solvent. A rack with eluate vials is placed inside the manifold, directly underneath the SPE cartridges. The SPE cartridges are eluted with 2 times 4 mL methanol. If necessary, the elution can be started by very shortly applying a vacuum. Then the methanol flow goes through the cartridge by means of gravity.

6. Concentration

   The eluate is evaporated with N_2 until dryness, after which 1 mL of 90/10 Milli-Q water (0.01% formic acid)/methanol is added. This results in a concentration factor of (50 / 1 =) 50, 2 μg L^{-1} will become 100 μg L^{-1} in case of 100% extraction recovery. The samples are pipetted in a 1.5 mL vial for UHPLC-MS/MS analysis.

5.2 Composition M9 medium

Table 5.1: Composition growth medium based on M9 minimal medium (standard).

Adjustment: no glucose is added.

*Autoclave and store at room temperature.

Reagent	Amount to add for 100 mL
M9 salts (see Table 5.2)	20 mL
$MgSO_4$ (1 M; Fisher Scientific)*	200 μL
$CaCl_2$ (1 M; Fisher Scientific)	10 μL
Deionized H2O	78 mL

Table 5.2: Composition M9 salts.

Reagent	Amount to add for 1 L
$Na_2HPO_4.H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5 g
Deionized H_2O	until 1 L

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