Final report

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1 EXECUTIVE SUMMARY

BIOTREAT is an EU financed research project (FP7) that started 1st January 2011 with 11 project partners including research institutions, waterworks and small and medium-sized enterprises (SME's). The primary aim of BIOTREAT was to develop new technologies for bioremediation of drinking water resources contaminated with micropollutants such as pesticides and pharmaceuticals. The technologies are based on the introduction and efficient exploitation of degrader bacteria into for example existing sand filters at waterworks. The project has been structured in 8 well-integrated work packages (WPs) each headed by a WP leader.

In **WP1** new isolation techniques were developed and degrading bacteria were selected to be used in the other WPs. Furthermore, techniques for immobilisation of degrader bacteria on specific carriers was developed improving degradation efficiency and preventing loss of the bacteria from the treatment filters. **WP2** focused on degradation kinetics and pathways at relevant micropollutant concentrations being in the ng to µg/l range. A method for measuring assimilabe organic carbon (AOC) was developed and it was shown that the content of AOC in the water was of decisive importance influencing degradation efficiency. **WP3** examined the surrounding environment of the systems, focusing on the effect of sand filter ecology on introduced degrader bacteria. Tools to analyse molecular fingerprinting results in order to provide ecological parameters including richness (Rr), dynamics (Dy) and functional organization (Fo) were developed. Protozoa naturally living within waterworks sand filters was shown to predate on the degrader bacteria added, thereby limiting contaminant degradation. In **WP4** knowledge gained in WP1-3 was integrated in mathematical modelling to improve the technology predictability and our understanding of the degradation process in general. This allowed the design of *in situ* and *in reactor* applications of microbial remediation necessary for the large scale applications carried out in WP5. Models were developed for both metabolic primary pollutant removal and cometabolic oxidation of micropollutant by ammonium oxidizing bacteria (AOB). **WP5** was devoted to large-scale application of the developed metabolic and cometabolic remediation technologies. Up to 75% removal of 0.2 µg/l 2,6 dichlorobenzamide (BAM) was achieved in sand filters inoculated with *Aminobacter* sp. MSH1, degrading BAM metabolically. Immobilisation of MSH1 improved degradation efficiency for a longer period as it diminished loss of MSH1 from the filters. The cometabolic strategy, using ammonium oxidizing bacteria (AOB) was less promising at large-scale conditions probably due to the short hydraulic retention time of the filter. **WP6** analysed the regulatory, safety, financial and environmental aspects of the developed technologies using cost-benefit analysis (CBA) and life cycle impact assessment (LCIA) approaches. The CBA and LCIA showed that the BIOTREAT metabolic strategy was competitive to granular activated carbon treatment being the most likely competitor technology to the BIOTREAT technology.

The main outcome of BIOTREAT has been disseminated to a broader audience of stakeholders, managers, expert and scientists within the water supply at the BIOTREAT open-end users meetings and at international conferences, symposia and workshops. BIOTREAT has contributed to the education of a new generation of environmental scientists as several early stage researchers have been educated with funding from BIOTREAT including both PhDs and postdoc fellows. More information about the project can be found at the BIOTREAT homepage (www.biotreat-eu.org).

2 A SUMMARY DESCRIPTION OF PROJECT CONTEXT AND OBJECTIVES

Millions of tonnes of organic xenobiotics are used each year worldwide, for example the pesticides used in agricultural production and applied to paved urban areas, along railways and roads, and to farmyards. Pharmaceuticals are considered to be emerging contaminants that enter the environment via the application of manure and sewage sludge to farmland, via effluent from wastewater treatment plants and via accidental spillage during various industrial applications. As a result of these extensive environmental inputs, European water bodies such as rivers, lakes and groundwater that are used as drinking water resources have become contaminated with a wide range of organic micropollutants. There is, however, a striking contrast between the input levels (up to several kg per hectare for pesticides) and the contaminant concentrations detected in water bodies, which are normally in the microgram to nanogram per litre range (Schwarzenbach et al., 2006). The EU limit value for individual pesticides in drinking water is 0.1 µg/l, while that for multiple pesticides is 0.5 µg/l (Directive 98/83/EC). These concentrations are very low, and many water bodies have therefore had to be abandoned as drinking water resources (Stockmarr, 2005).

The incredible capacity of soil and subsurface microbial communities to degrade a wide range of xenobiotic compounds is well known. In most cases where microorganisms are used for bioremediation, however, the biodegradation processes operate as “black box” systems, and detailed knowledge of the microorganisms and the metabolic processes and pathways involved is lacking. As complete mineralisation of organic micropollutants may require specialised strains that are rarely present in the indigenous microbial community (e.g. Topp et al., 2000; Sørensen et al., 2007), bioaugmentation with degradative bacteria has been proposed as a strategy for remediation of contaminated drinking water resources. It is believed that bioaugmentation has a great but unexplored potential for drinking water remediation as decontamination of the water will take place in contained controlled-flow systems such as those used for conventional water treatment (e.g. sand filters and activated C filters). Development of successful bioremediation-based strategies for treating drinking water resources is in its infancy, however, and the low concentration at which micropollutants are generally present in freshwater environments adds to the challenge.

The overall objective of BIOTREAT was to develop new technologies for bioremediation of drinking water resources contaminated with micropollutants such as pesticides and pharmaceuticals. The technologies are based on the introduction and efficient exploitation of degrader bacteria in existing sand filters at waterworks. Once developed the technologies may also be transferred to other types of submerged biofilter systems such as mobile biofilters placed close to groundwater abstraction wells, sand barriers between surface waters and abstraction wells and subterranean protective barriers established to prevent micropollutants from entering into aquifers. To ensure efficient and reliable pollution degradation, the BIOTREAT technologies will make use of bacteria degrading target pollutants by either metabolic or cometabolic degradation pathways. The availability of suitable degrader bacteria and the mixture of micropollutants to be targeted in the individual applications will determine the choice of removal strategy. A strategy based on **metabolic** processes will be exploited for micropollutants where metabolically degrading bacteria are available. For target pollutants where specific degrading bacteria have not been described, an alternative strategy relying on unspecific **cometabolic** degradation by methane or ammonium oxidising bacteria will be exploited.

The BIOTREAT project has been structured into 8 well-integrated work packages, each headed by a WP leader. WP1, WP2, and WP3 aimed at exploring the "black box" of bioaugmentation, providing in-depth knowledge about degradation, growth and survival of microorganisms introduced into sand filters. Together WP1-3 brought progress to front-line research in bioaugmentation technology which in the subsequent WPs was translated into novel applied technologies for water treatment.

The focus of **WP1** was on the **microorganisms**, their physiology and their interactions with the matrix environment. The aim was to characterise existing metabolically and cometabolically pollutant-degrading bacteria regarding degradation of micropollutants at low concentrations and to exploit new isolation and enrichment strategies for degrader bacteria adapted to degradation at low contaminant concentrations. New micropollutant-degrading bacteria were isolated and characterized in details. Based on the research degrader bacteria were selected to be used within other WPs. Furthermore, it was an aim to develop new carrier and encapsulation technologies for introducing degrader bacteria into biofilters/barriers ensuring their long-term survival and activity.

WP 2 emphasised the **degradation processes**, providing insight into metabolic pathways and metabolite formation. The aim was to study microbial growth and degradation kinetics at low contaminant concentrations and to identify rate-limiting factors for growth in waterworks sand filters. It was also an aim to identify contaminant degradation pathways and to elucidate the metabolite pattern for both metabolic and cometabolic degradation processes. Environmental factors being bottlenecks for degradation were also identified, including the availability of assimilabe organic carbon shown to limit growth and degradation in waterworks sand filters.

WP3 examines the surrounding environment of the **systems**, focussing on the effect of sand filter ecology on introduced degrader organisms. The aim was to study how the dynamics of microbial community structure influences the degradation of contaminants and how this can be controlled to increase the predictability of the degradation process. In addition it was an aim to study the effect of protozoan predation of degraders on degradation efficiency in drinking water facilities. To achieve the aims tools for interpretation of molecular fingerprinting pattern, including the range-weighted richness (Rr), dynamics (Dy) and functional organization (Fo) parameters for the different experimental setups were developed.

The focus on **WP4** was on **models and predictability**, where the knowledge gained in WP1-3 was integrated in mathematical modelling to improve technological predictability. The aim was to develop generalised models for both primary pollutant removal (metabolic) and cometabolic transformation. Low contaminant concentrations being in the microgram/l range typically found in polluted drinking water was emphasised in the modelling.

WP 5 focused on **application and reliability** through large-scale applications of the developed technologies, including laboratory-scale reactors and column experiments carried out in the laboratory or at waterworks. The aim was to transfer the developed metabolic and cometabolic bioaugmentation technologies from laboratory-scale experiments to submerged biofilter systems at a waterworks and finally to field-scale implementation. The up-scaling effect on the performance of the remediation technology was determined and the system was further adjusted to improve degradation efficiency.

WP6 contained an **integrated assessment and performance validation** of the developed technologies. The regulatory, safety, financial and environmental aspects of the technologies were analysed using a life cycle impact assessment (LCIA) approach as well as cost benefit analyses (CBA). The aim was to use the LCIA and CBA to compare the BIOTREAT technologies to other technologies including granular activated carbon (GAC) filtration to help decision makers to select the best strategy for remediation of contaminated drinking water resources.

WP7 focused on **dissemination** of the project results, further **exploitation** of the technologies and the development and continuous improvement of the technology action plan containing the overall exploitation strategy.

WP8 was devoted to project management.

3 A DESCRIPTION OF THE MAIN S&T RESULTS/FOREGROUNDS

3.1 WP1 ORGANISMS – IDENTIFICATION AND CHARACTERISATION OF MICROBIAL CULTURES

WP 1 was focused on the degrader organisms, their physiology and their interactions with the matrix environment. Several contaminant degrading bacteria were characterised and new bacteria were isolated including bacteria degrading phenoxy acid herbicides. Surprisingly certain waterworks sand filters were shown to have an inherent potential for degradation of selected pesticides including MCPA, bentazone, and BAM. The BAM degrading amidase gene of *Aminobacter* sp. MSH1, *bbdA* was characterized in details and shown to have a very high affinity for BAM degradation (low K_M value) explaining the high efficiency of this bacterium for BAM degradation at low concentrations. Attempts have also been done to immobilise degrading bacteria on specific carrier materials. Incorporation of cells, suspended in alginate, into pores of inorganic strong and rigid but porous material (intermediate carriers) were most efficient regarding 1) the highest degrading biomass immobilised, 2) the lowest bacterial release in column systems and 3) best performance in contaminant degradation tests. Based on this the intermediate carriers were selected to be further tested in WP5.

3.1.1 Selection and identification of microbial cultures

Metabolically degrading cultures. New methods for enrichment and isolation of bacteria mineralising low contaminant concentrations were developed. The methods were tested with enrichments from a groundwater aquifer exposed to either high (25 mg/l) or low (0.1 mg/l) concentrations of the herbicide MCPA. Isolation of MCPA-degrading bacteria from the enrichments were then attempted by spreading cultures either directly on Petri dishes or on so called Low Flux Filters (LFF; 2.5 cm diameter, 0.22 µm pore size) overlaying the plates, thereby allowing a constant flux of low herbicide concentrations to the bacteria. Several MCPA degrading bacteria were isolated by the method and then further identified and characterised. Purity verification, catabolic gene contents and characteristics in regards to high/low MCPA concentrations was then undertaken (table 1).

Table 1. Biodiversity of five MCPA-mineralizing bacteria isolated from different sediment extracts or enrichment cultures of sediment extracts. Species were isolated from sediment extracts (EXT), low enrichment cultures (LEC) and/or high enrichment cultures (HEC) and identified using 16S rRNA gene sequencing. The match with the closest cultivated strain and presence of the known catabolic genes *tdfA*, *tfdAα* and *cadA* are shown.

Species information		Isolation source			Closest cultivated type strain		Functional genes		
Isolate	Accession number	EXT	LEC	HEC	Type strain [accession no.]	% homology (bp)	*tfdA*	*tfdAα*	*cadA*
ERG1	1519188	X			*Achromobacter piechaudii* strain TZ4 [GQ927161.1]	99 (1393/1396)	X	X	X
ERG2	1519190		X		*Pseudomonas fluorescens* strain S16 [DQ095904.1]	99 (1420/1426)	X		X
ERG3	1519193			X	*Variovorax paradoxus* strain B57 [EU169160.1]	99 (1401/1420)	X		X
ERG4	1519197		X		*Cupriavidus basilensis* strain AU4546 [AY860224.1]	99 (1393/1398)	X		X
ERG5	1519198			X	*Sphingomonas sanxanigenens* strain T12AR21 [JF459935.1]	97 (1322/1358)	X		X

Furthermore, enrichment cultures were established using filter material from different waterworks sand filters. Sand filter material was assessed for the mineralisation of ^{14}C-labeled MCPA, bentazone, and BAM. Table 2 summarizes the characteristics and results for the sampled waterworks. Mineralization of BAM was only found in sand filter material from Kluizen and Eeklo waterworks. Bentazone was mineralized in sand filter material from Kluizen, Eeklo, AWW, and Snellegem waterworks. In contrast, all tested samples, except samples from De Blankaart waterworks, showed mineralization of MCPA. We concluded that the tested samples show interesting pesticide mineralization activities. Especially, the occurrence of bentazone mineralization is of interest since pure cultures of bentazone mineralizing bacteria have not yet been reported.

Cometabolically degrading cultures. Several ammonia and methane oxidizing bacterial cultures were enriched and screened for their ability to degrade a selected group of micropollutants via cometabolic degradation pathways. Mainly sulfamethoxazole and benzotriazole were found to be degraded by methane oxidizing bacterial (MOB) enrichment cultures. The degradation, however, was always most rapid when no methane monooxygenase inhibitors or antibiotics were added, confirming the degradation being due to cometabolic activity by MOB having broad spectred methane oxidizing enzymes. Test for cometabolic degradation of micropollutants were also performed with several different commercially available ammonia oxidizing bacterial (AOB) cultures. The compounds tested were atenolol, BTZ, sulfamethoxazole (SMX), Chloro-BTZ (Cl-BTZ), mecoprop and diclofenac. These tests showed removal efficiencies for most of the compounds, but only partly due to cometabolic degradation (figure 1). For BTZ a removal efficiency of > 90% could be achieved in the biological treatment without inhibition of nitrification, but as the test with inhibitor present also showed a degradation of around 60%, it can be concluded that roughly 30% of the removal was due to cometabolic degradation by the present AOB. Some compounds such as atenolol showed heterotrophic degradation being the only relevant pathway for the disappearance of this compound, but SMX did only show removal when nitrification was taking place.

Table 2. Overview of the extent of BAM, bentazone and MCPA mineralization in sand filter samples from different waterworks.

	Sample characteristics				Mineralization after 50 days ± st.dev.		
	waterworks	Company	SW/GW	Sampling date	BAM	Bentazone	MCPA
K1	Kluizen SF24	VMW	SW + GW	8/11/2011	34% ± 4%	10% ± 1%	37% ± 1%
K2 ZF21	Kluizen SF21	VMW	SW + GW	12/06/2012	65% ± 4%	20% ± 1%	39% ± 7%
K2 ZF22	Kluizen SF22	VMW	SW + GW	12/06/2012	58% ± 13%	21% ± 2%	41% ± 8%
K2 ZF26	Kluizen SF26	VMW	SW + GW	12/06/2012	62% ± 7%	18% ± 1%	48% ± 3%
K3 ZF21	Kluizen SF21	VMW	SW + GW	03/04/2013	64% ± 3%	24% ± 3%	55% ± 8%
K3 ZF26	Kluizen SF26	VMW	SW + GW	03/04/2013	62% ± 3%	23% ± 3%	53% ± 5%
E1	Eeklo SF2	VMW	GW	9/11/2012	6% ± 1%	11% ± 3%	48% ± 2%
E2 ZF1	Eeklo SF1	VMW	GW	13/06/2012	7% ± 1%	14% ± 0%	47% ± 4%
E2 ZF2	Eeklo SF2	VMW	GW	13/06/2012	6% ± 1%	11% ± 2%	42% ± 2%
E2 ZF3	Eeklo SF3	VMW	GW	13/06/2012	8% ± 0%	14% ± 1%	52% ± 4%
Z1	Zele	VMW	GW	10/05/2011	nm	Nm	39% ± 3%
Z2	Zele	VMW	GW	28/06/2012	1% ± 0%	3% ± 1%	40% ± 3%
Si0	Sinaai	VMW	GW	10/05/2011	nm	nm	27% ± 5%
Si1	Sinaai	VMW	GW	10/11/2011	nm	nm	27% ± 1%
Si2	Sinaai	VMW	GW	28/06/2012	2% ± 1%	3% ± 0%	29% ± 1%
Sn	Snellegem	VMW	GW	28/06/2012	8% ± 6%	5% ± 1%	57% ± 2%
DB1 ZF1	De Blankaart SF1	VMW	SW	27/06/2012	2% ± 1%	2% ± 1%	43% ± 7%
DB1 ZF3	De Blankaart SF3	VMW	SW	27/06/2012	nm	nm	4% ± 1%
DB1 ZF4	De Blankaart SF4	VMW	SW	27/06/2012	nm	nm	4% ± 1%
DB2 ZF1[a]	De Blankaart SF1	VMW	SW	02/04/2013	1% ± 0%	2% ± 0%	3% ± 0%
DB2 ZF3	De Blankaart SF3	VMW	SW	02/04/2013	nm	nm	1% ± 0%
DB2 ZF4[a]	De Blankaart SF4	VMW	SW	02/04/2013	nm	nm	3% ± 0%
StL	Saint Léger	VMW	GW	13/06/2012	1% ± 0%	2% ± 0%	14% ± 1%
AWW	AWW	AWW	SW	13/06/2012	4% ± 1%	8% ± 0%	45% ± 5%

SF = sand filter
VMW = De Watergroep
AWW = Antwerpse Waterwerken
GW = groundwater
SW = surface water
nm = not measured

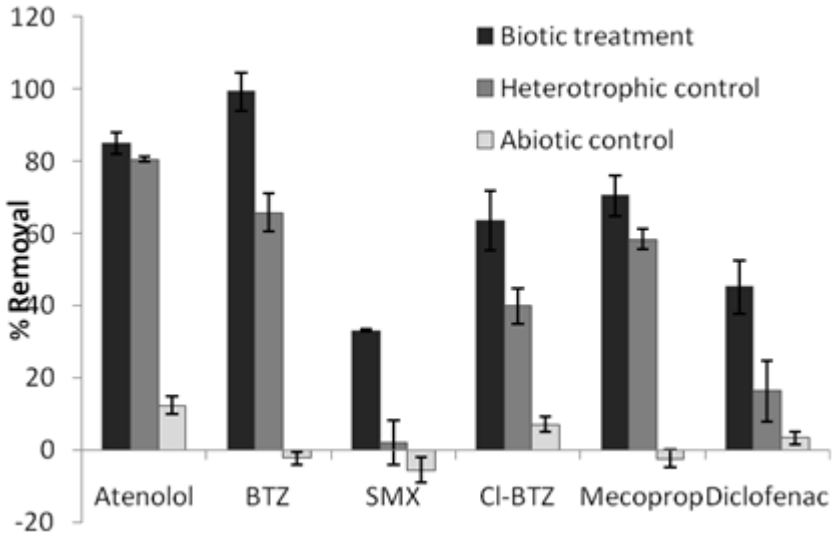


Figure 1. Micropollutants removal efficiencies by the culture HANDS for biotic treatment, heterotrophic control (presence of C_2H_2 and allythiourea as inhibitors) and abiotic control (heat-killed biomass) analysed by LC-MS after 7 days.

Based on the results the following bacteria and bacterial enrichment cultures where selected to be used for the development of the BIOTREAT remediation technologies:

- *Aminobacter* sp. MSH1 (BAM)
- *Variovorax* sp. SRS16 (Linuron)
- *Sphingobium herbicidovorans* MH (mecoprop)
- *Novosphingobium* sp. *KN65.2* (carbofuran)
- Methane oxidising bacterial culture enriched from soil (MOB_{soil})
- Methane oxidising bacterial enrichment culture ($MOB_{bourgoyen}$)
- HANDS (ammonium oxidising enrichment culture)

3.1.2 Physiological and genetic characterisation of microbial cultures

The BAM degrading strain *Aminobacter* MSH1 was selected as a model bacterium in BIOTREAT and its physiological and genetic features have been characterized in details.

The genetic characterization and elucidation of the BAM degradation pathway in *Aminobacter* sp. MSH1 was initiated by identifying the amidase gene function performing the first step in BAM degradation. Moreover, a draft genome sequence of *Aminobacter* sp. MSH1 was obtained (figure 2 and 3).

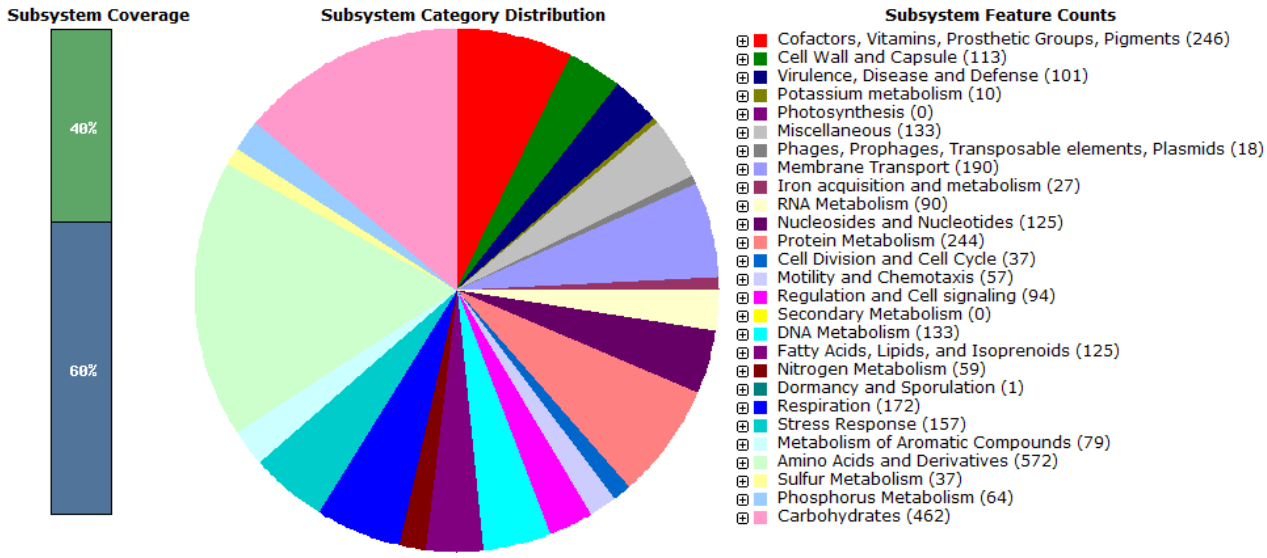


Figure 2: Overview of the distribution of the putative genes of *Aminobacter sp.* MSH1 in subsystems by RAST.

Figure 3: Schematic presentation of the ORF organization of the identified 8200 bp contig containing the BAM amidase gene *bbdA* in the MSH1 genome sequence. From left to right: bp 232-1269: putative regulatory protein; bp 1559-1933: Transcriptional regulatory protein MarR; bp 2047-3714: AMP-dependent synthetase and ligase; bp 3732-4457: Nitroreductase; bp 4470-4874: Transposase IS4; bp 4825-5187: Transposase; bp 5630-7189: *BbdA*; bp 7368-8018: Conjugal transfer protein TrbE. The direction of the arrows indicates the transcription direction. Red arrows indicate putative regulatory genes, the blue arrow the synthetase/ligase gene; the green arrow the nitroreductase gene; the purple arrows the IS elements; the brown marked gene is *bbdA* and the yellow gene *trbE*.

This information is important in elucidating the genetics of BAM degradation beyond 2,6-dichlorobenzoate and to elucidate functions involved in BAM degradation and its ability to cope with realistic low BAM concentrations at oligotrophic sand filter conditions. However, plasmid sequences were underrepresented in the draft genome and further sequencing (454 pyrose-quencing) of isolated plasmids of strain MSH1 was done. A draft sequence of the plasmid, pBAM2 was obtained by Illumina sequencing and annotation with RAST (Rapid Annotation us-ing Subsystem Technology) and is shown in figure 4.

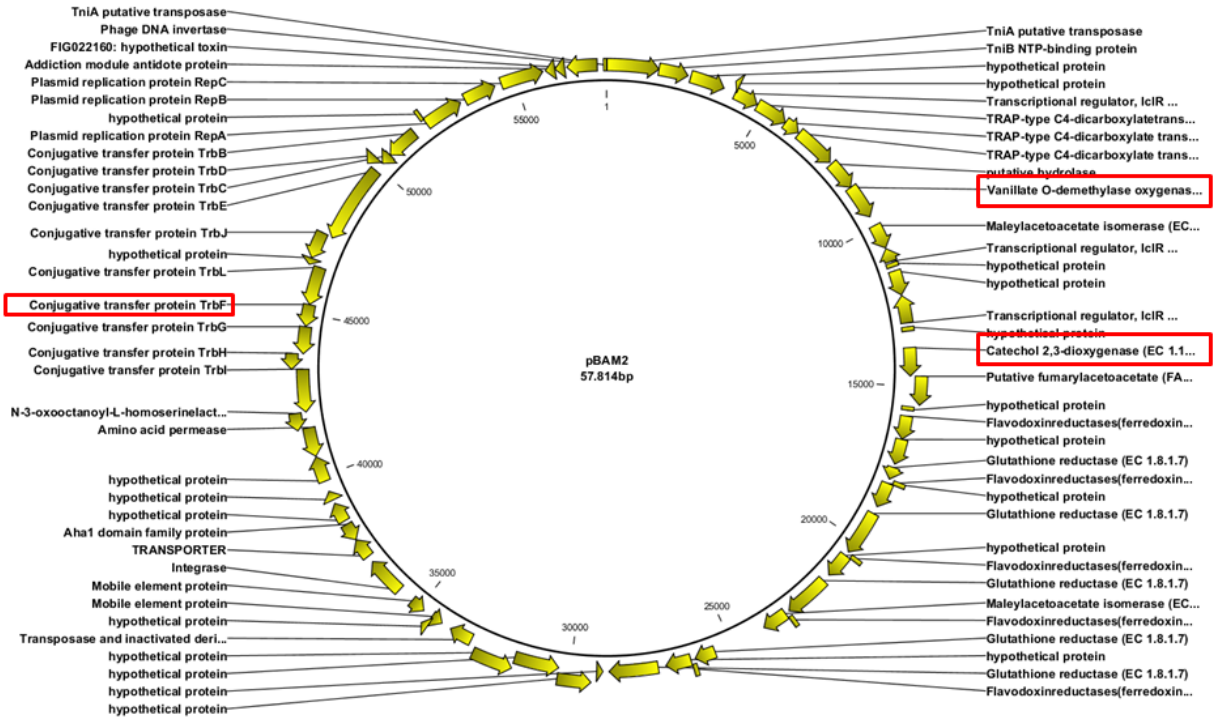


Figure 4: Schematic representation of the draft sequence of plasmid pBAM2 (approximately 57.8 kb), suspected of encoding dichlorobenzoic acid (DCBA) catabolic genes. Putative gene functions are shown based on annotation done with RAST (Rapid Annotation using Subsystem Technology). PCR reactions were designed targeting three putative genes indicated by the red boxes.

A BAM amidase gene (*bbdA*) was identified being responsible for the first step in the BAM deg-radation pathway in *Aminobacter* sp. MSH1. The BAM amidase has a size of 55.7 kDa and shows only limited homology to other known proteins. The most similar enzyme known is an enanti-oselective 2-phenylpropionamide amidase of *Agrobacterium tumefaciens*. The BAM degrading activity of *bbdA* was confirmed by cloning the *bbdA* gene in a pEXP5-CT/TOPO vector (Invitro-gen) and expressing the protein in *E. coli* BL21(DE3)pLysS (figure 5).

The Michaelis constant (K_M) of *bbdA* was estimated by non-linear regression analysis using a Michaelis Menten equation as model and was calculated to be 0.69 µM. This value can be con-sidered as a measure for the affinity of the enzyme for its substrate BAM. This K_M value is very low compared to other amidases, indicating that *bbdA* has a very high affinity for BAM. The high affinity explains why *Aminobacter* MSH1 degrades BAM so efficiently at very low environ-mentally relevant concentration.

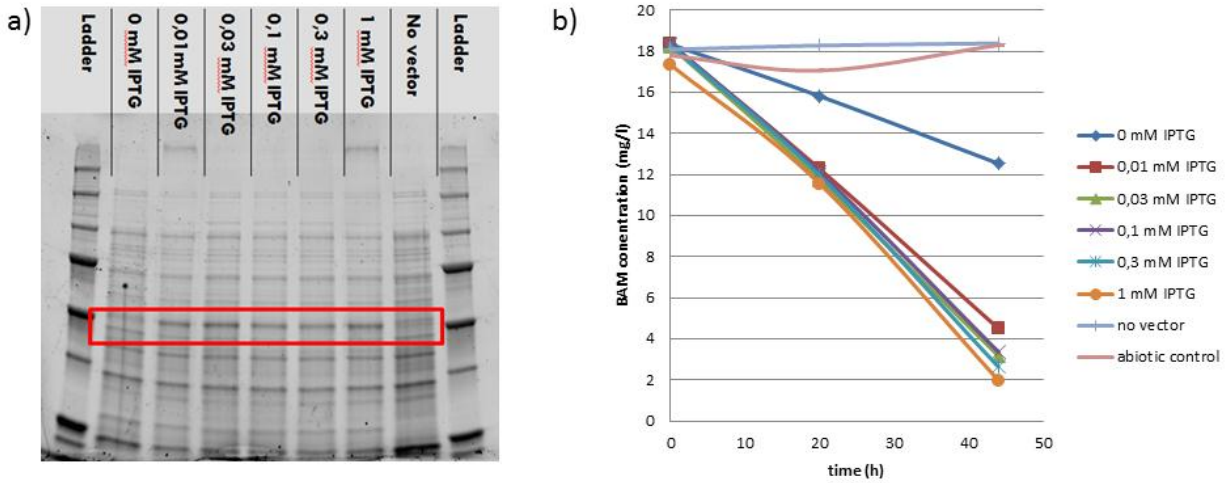


Figure 5: Heterologous expression of *bbdA* in *E. coli* BL21(DE3)pLysS induced with different IPTG concentrations. a) SDS-PAGE analysis of *bbdA* expression in *E. coli* BL21(DE3)pLysS. The BAM amidase (55.7 kDa) is indicated by the red rectangle. b) Degradation of BAM by *E. coli* BL21(DE3)pLysS expressing *bbdA*. Most of the BAM is degraded after 44 h in the IPTG induced samples, while the *E. coli* without vector or the abiotic control showed no degradation.

At equimolar substrate levels the BAM amidase showed a 23.2 times higher conversion of OBAM and even a 50.3 times higher conversion of benzamide compared to BAM. These numbers indicate that *bbdA* actually has a higher activity on benzamide and OBAM than on BAM, suggesting that this enzyme might actually be a benzamide amidase able to degrade BAM.

A method for quantifying the amidase gene using qPCR was developed and used in other WPs. Primers specific for the amidase gene were designed and tested on a dilution series of the gene and on samples with *bbdA* added to soil and sand filter DNA extracts - as low as 300 *bbdA* copies per PCR reaction could be detected using the developed method.

In conclusion the biochemical characterization of the BAM degrading amidase of *Aminobacter* sp. MSH1, *bbdA* has been characterized in details. This enzyme has a very low K_M value, indicating a high affinity for BAM and interestingly also shows a higher activity towards benzamide and OBAM than towards BAM. Several proteins were upregulated in strain MSH1 in the presence of BAM, many of which were also found to be absent in the dichlorobenzoic acid (DCBA) degradation impaired mutant M6.100g. This lead to the identification of 12 genes which we believe has a high chance of being involved in the BAM catabolic pathway. One of these genes was identified as *bbdB*, encoding the NADH dependent DCBA degradative protein *bbdB*.

3.1.3 Designing of carriers for cell immobilisation

The most simple bioaugmentation strategy is addition of degrader bacteria as a liquid culture to the environment. More advanced strategies using immobilised degrader bacteria may, however, be superior protecting the bacteria from predation by protozoa or preventing them from wash out from the environment. In the BIOTREAT project attempts have been done to immobilise degrader bacteria on different carrier materials to improve degradation efficiency of inoculated waterworks sand filters.

In general the carriers should not be toxic neither to humans or the entrapped bacteria. Several non-toxic carrier materials have been tested including alginate, chitosan and cellulose natural polymers and inorganic carriers as expanded clay, carbonates and nanosol gel matrixes based

on silicone. A method has been developed to change the surface charge of bacteria facilitating efficient cell immobilisation. No significant change in the ratio between death and live *Aminobacter* MSH1 or linuron degrading *Variovorax* SRS16 cells were observed following immobilisation.

In general three principles for development of carriers can be determined: Bacteria can be attached on the surface of carriers, embedded in the matrix or they can be physically stabilized with specific techniques like freeze drying. According to the survivability and relatively easy upscaling of the procedure, the embedding into the matrix is preferable. Similarly, stabilisation by modification of the bacterial surface by a Layer by layer (LBL) approach is according to our previous experiments also a very good method for preserving the activity of cells.

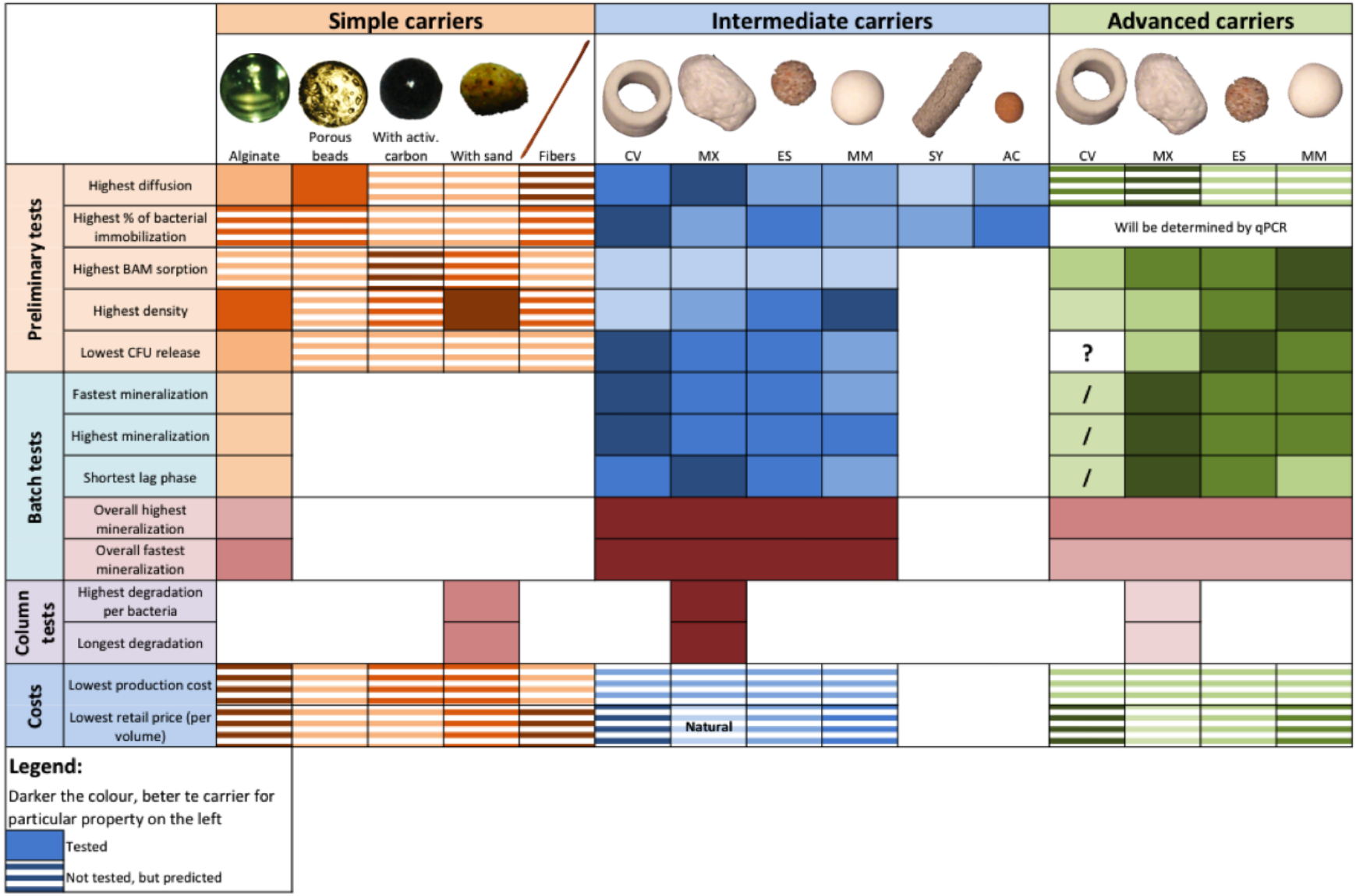
Based on alginate matrix and LBL approaches different carriers have been prepared and were divided into three groups based on their complexity of the preparation: **simple**, **intermediate**, and **advanced** carriers.

The **simple carriers** were prepared exclusively based on the alginate matrix, which was modified to increase strength, diffusion, specific density, and sorption capabilities. The best alginate beads were formed when extruding 2% alginate solution drop wise into 0.2 M $CaCl_2$. The **intermediate carriers** were prepared by incorporation of cells, suspended in alginate, into pores of inorganic strong and rigid but porous material. These carriers were prepared, because higher strength and durability may be needed to sustain higher pressures and abrasions in sand filters. Different porous mineral matrixes that have been used in aquarium filters were selected for development of such carriers. The **advanced carriers** were based on the LBL method for deposition of polymers. On such functionalized surface the bacterial cells were able to attach with strong electrostatic interactions.

Based on results from batch tests a selection of carriers were further tested for their suitability in a flow-through column system. The results from the column experiments are suggesting that the **intermediate carrier** (Seachem Matrix ^{TM} (MX)) is the best to be used in the waterworks sand filter. Also the simple carriers with quartz sand were effective for degradation of BAM. The same can be concluded from a financial perspective that preparation of the simple carriers is less expensive than the other procedures. Although the intermediate Sera Siporax (CV) carrier was not tested in the column system, they were the best at the immobilizing the largest amount of bacteria, they had the lowest bacterial release and they performed best in batch mineralization tests. Thus intermediate CV carriers should also be considered as a prominent carrier (see Table 3).

Briefly, the procedure of preparing intermediate carriers is based on the suspension of cells dispersed in alginate solution of variable percentage (from 1% to 3%). The solution of alginate and bacteria is then forced to get incorporated into the pores of mineral matrix (e.g. MX) by the variation of pressure (from 0 – 20% of the normal atmospheric pressure). To stabilize the incorporated alginate-cell solution inside the mineral pores of mineral matrix the solution of $CaCl_2$ is poured over the carriers or carriers are soaked into the solution.

Table 3: Overview of properties of carriers prepared in the BIOTREAT project



3.2 WP2 PROCESSES – KINETICS OF DEGRADATION AND METABOLITE FORMATION

The overall aim of this part of BIOTREAT was to provide insight into the degradation processes focusing on metabolic pathways and metabolite formation especially at the low micropollutant concentrations found in groundwater and surface waters exploited as drinking water resources. A sensitive method for the analysis of assimilable organic carbon (AOC) at low concentrations was developed as this constituent may influence contaminant degradation efficiency. For the degrader bacteria studied it was demonstrated that parameters of substrate utilization kinetics estimated at high concentrations could accurately predict substrate utilization at lower concentrations under AOC-restricted conditions. Tentative degradation pathways have been established for several contaminants including sulfamethoxazole (SMX), Oxcarbazepine (OXC) and carbofuran. Threshold concentrations below which degradation is either slow or not occurring may hamper degradation at low contaminant concentrations, but only for some bacteria as to example a high threshold value was observed for carbofuran degradation by *Novosphingobium* sp. KN65.2, while such a threshold concentration could not be detected for BAM degradation by *Aminobacter* MSH1.

3.2.1 Microbial growth at low concentrations

Easily degradable organic carbon or as it is called assimilabe organic carbon is an important constituent that may control contaminant degradation. At one hand the AOC may serve as a growth substrate for the degrading bacteria thereby stimulating the degradation process on the other hand the AOC may be preferred as substrate at the expense of the contaminants. A method has been developed within BIOTREAT to estimate the AOC concentrations in water. The method has been applied to a system in which pure bacterial strains were growing on a target pesticide as the sole carbon source. In applying this method, growth on external sources of AOC was minimized and thereby enabling precise estimates of microbial growth and substrate utilization parameters for each strain-substrate pair. We first sought to validate the applied method by investigating growth and substrate utilization in high-concentration incubation experiments that paralleled conventional biodegradation assays (figure 6). We then applied the method to a series of incubation experiments conducted at increasingly lower initial substrate and inoculum concentrations to determine the limits of microbial growth and substrate utilization. The bacteria studied included the previously identified strains: *Sphingobium herbicidovorans* MH (mecoprop); *Variovorax* sp. *SRS16* (linuron); *Novosphingobium* sp. *KN65.2* (carbofuran); and *Aminobacter* sp. *MSH1* (BAM).

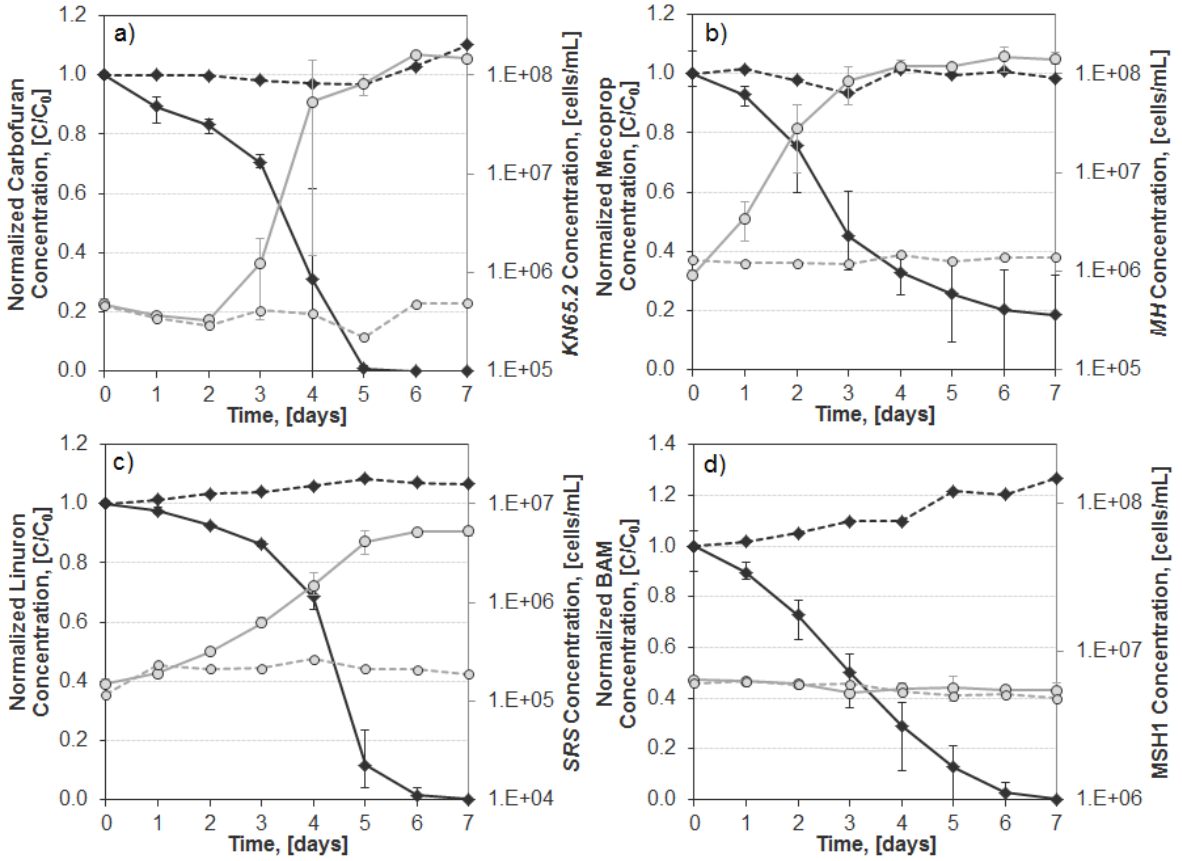


Figure 6: Growth and substrate utilization in high-concentration incubation experiments for: (a) *Sphingomonas* sp. KN65.2 + carbofuran; (b) *S. herbicidovorans* strain MH + mecoprop; (c) *Variovorax* sp. SRS16 + linuron; and (d) *Aminobacter* sp. MSH1 + BAM. Solid lines represent the average of three replicate experiments for cell growth (grey) and substrate utilization (black). Error bars are the minimum and maximum measurements of three replicates. The dashed lines are the negative controls for no cells (black) and no substrate (grey).

The effects of increasingly lower substrate and inoculum concentrations on microbial growth and substrate utilization were unique for each strain. This is a fascinating and important result with respect to practical application of these strains in bioaugmentation processes; it is apparent that bacterial strains must be investigated on an individual basis and there is not a generalizable rule for bacterial growth at low concentrations of individual substrates. For strain SRS16, the initial substrate and inoculum concentrations had no effect on linuron degradation; all experiments resulted in removal of linuron to below the limit of detection within 14 days with a lag phase only apparent at the highest substrate (10 mg/l) and lowest inoculum concentrations (10^3 cells/ml). Growth of strain SRS16 was measurable in incubation experiments where the yield of cells was higher than the inoculum concentration. For strain KN65.2, both the initial substrate and cell concentration had an apparent effect on the degradation of carbofuran. At cell inoculum concentrations of 10^3 and 10^4 cells/ml, no degradation of carbofuran was observed in incubations with initial substrate concentrations below 1 mg/l and 0.1 mg/l, respectively. At all other sets of initial conditions, degradation of carbofuran was complete to a concentration below the limit of detection within 28 days. Growth of strain KN65.2 was likewise measurable in those incubation experiments where the yield of cells was higher than the inoculum concentration. For strain MSH1, BAM disappeared rapidly in incubations in which the inoculum concentration was high and the initial BAM concentration was low. However, as with the high-concentration incubation experiments, no cor-

responding growth was observed while a persistent peak formed in the UV chromatogram putatively corresponding to 2,6-dichlorobenzoic acid suggesting a single, cometabolic biotransformation reaction (data not shown). For strain MH, lowering the mecoprop concentration to below 10 mg/l nearly completely inhibited metabolic activity. Only at the highest initial substrate and inoculum concentrations was partial mecoprop utilization observed within 28 days.

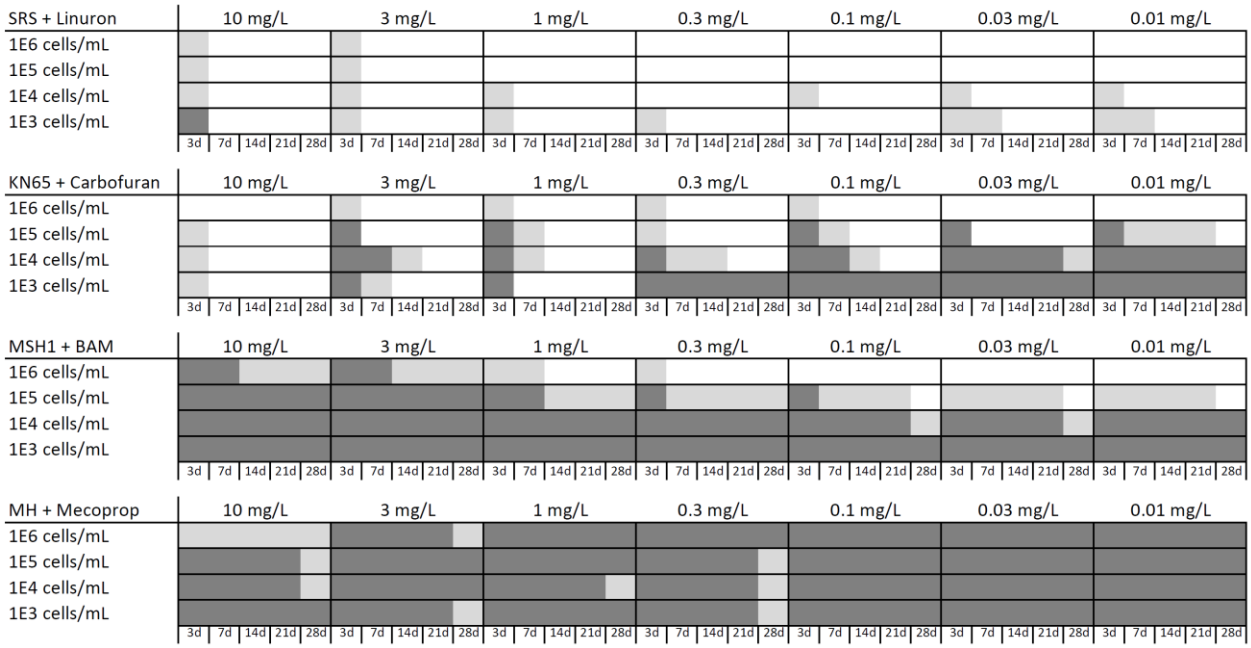


Figure 7*:* Summary of experiments conducted at increasingly lower inoculum and initial substrate concentrations. Dark block (■) indicates no degradation. Grey block (▪) indicates degradation in progress. White block (□) indicates complete degradation.

Using the data presented in figure 6, we estimated the kinetic parameters describing microbial growth and substrate utilization assuming Monod growth kinetics. The goal of the modelling exercise was to use a simple model to estimate kinetic parameters for each strain-substrate pair that could subsequently be used to simulate biodegradation under varying sets of initial conditions. No kinetic parameters were estimated for strain MSH1 because no growth was observed. In table 4 we present the maximum specific substrate utilization rate ($\hat{q}$), the maximum growth rate ($\hat{\mu}$), and the substrate concentration giving one-half the maximum rate (*K*) for the remaining three strains. The estimated parameters were highly variable among the strains with $\hat{q}$ varying over an order of magnitude and *K* varying over three orders of magnitude, indicating the unique kinetics of each growth process.

The next step was to determine whether or not microbial growth and substrate utilization kinetics estimated from high concentration incubation experiments could be used to predict strain behaviour at environmentally relevant concentrations. We hypothesized that these observed shifts in kinetics are the result of a shift from single-substrate to mixed-substrate utilization kinetics as the concentration of the target substrate approaches the concentration of background assimilable organic carbon (AOC). We reasoned that our method to measure substrate utilization and microbial growth under AOC-restricted conditions would eliminate mixed-substrate utilization and could resolve these kinetic issues.

Table 4. Growth parameters of each strain estimated at high concentration

Strain	SRS16	KN65.2	MSH1	MH
Substrate	linuron	carbofuran	BAM	mecoprop
x_0, [cells/ml][1]	$1.5x10^5 \pm 1x10^4$	$4.7x10^5 \pm 6x10^4$	$6.4x10^6 \pm 4x10^5$	$9.2x10^5 \pm 9.1x10^4$
$x_{dry\ weight}$, [10^{-13} g/cell]	5.8	3.4	2.2	1.1
s_0, [mg/l][1]	10.9 ± 0.1	65 ± 0.7	7.3 ± 0.6	48.9 ± 3.3
$\hat{q}$, [µg substrate/µg cells-day]	2.02	1.26	NA[2]	2.83
$\hat{\mu}$, [day^{-1}]	0.57	1.0	NA	1.1
K, [mg substrate/l]	0.010	0.96	NA	2.2
Threshold, [mg/l]	0±0.01	<0.001	NA	9.1±8.1

x_0 = inoculum concentration; $x_{dry\ weight}$ = dry weight of cells; s_0=initial substrate concentration
[1]all data reported as average and standard deviation of triplicate experiments
[2]No growth was measured for strain MSH1 and therefore kinetic parameters could not be estimated

We first aimed to make robust estimates of Monod kinetic parameters for strains *Variovorax* sp. *SRS16* utilizing linuron and *Novosphingobium* sp. *KN65.2* utilizing carbofuran. The optimal dataset for kinetic parameter estimation was identified at an initial cell density of 10^5 cells ml^{-1} and initial substrate concentrations of 1 mg L^{-1} and 10 mg L^{-1} in experiments with strains SRS16 and KN65.2, respectively. The estimated parameter values and standard deviation of the marginal distributions are provided in Table 5.

Table 5: Estimated and measured parameters for each strain

Strain Substrate	SRS16 linuron	KN65.2 carbofuran
	Estimated Kinetic Parameters	
μ_{max}, [day^{-1}]	1.3±0.1	7.8±1.4
K_s, [C-mM]	0.0029±0.001	0.54±0.2
$Y_{C/C}$, [C-mol X C-mol S^{-1}]	0.08±0.01	0.39±0.01

μ_{max} = the maximum specific growth rate; K_s = the half-saturation constant; $Y_{C/C}$ = yield on the basis of C moles of cells per C moles of substrate

In general our results demonstrate that parameters of substrate utilization kinetics estimated at high concentrations can accurately predict substrate utilization at lower concentrations under AOC-restricted conditions. Multiphasic kinetics was not observed. While our data cannot disprove the existence of multiple, concentration-dependent uptake and transformation systems in bacteria that lead to observed shifts in substrate kinetics at low concentrations, our data is consistent with our hypothesis that observed shifts in kinetics could be the result of shifts from single substrate utilization to mixed substrate utilization. Published data suggest that shifts to mixed-substrate utilization can likewise result in shifts in kinetics.

3.2.2 Metabolite patterns at low substrate concentrations

The research about metabolite pattern at low substrate concentrations has primarily focused on 1) BAM degradation by *Aminobacter* MSH1, 2) carbofuran degradation by *Novosphingobium* strain KN65.2, 3) sulfamethoxazole (SMX) degradation by a methan oxidizing bacteria (MOB) and 4) Oxcarbazepine (OXC) degradation in laboratory incubations with materials of waterworks sand filters.

BAM degradation: BAM is a highly stable metabolite from the herbicide dichlobenil and this residue is a well-known water contaminant in area where dichlobenil is or has been used. In order to determine mineralization of the compound at low concentrations, work has been done using radioactively labelled BAM. A Thin Layer Chromatography method with radiochemical detection was established, which made it possible to also look at the formation of BAM metabolites. An unknown metabolite was discovered and a relationship between the time needed for complete degradation of BAM and the extent of metabolite production was revealed as shown in figure 8. The degradation rate of BAM by *Aminobacter* sp. MSH1 can be controlled by adding different amounts of cells. Figure 9 shows an example of slow BAM mineralisation, where the production of the unknown metabolite is below detection. Faster degradation could be obtained by using more MSH1 cells, but under these conditions the unknown polar metabolite is produced in higher levels (figure 8 and figure 10), which could be a problem when bioaugmentation of drinking water resources is attended.

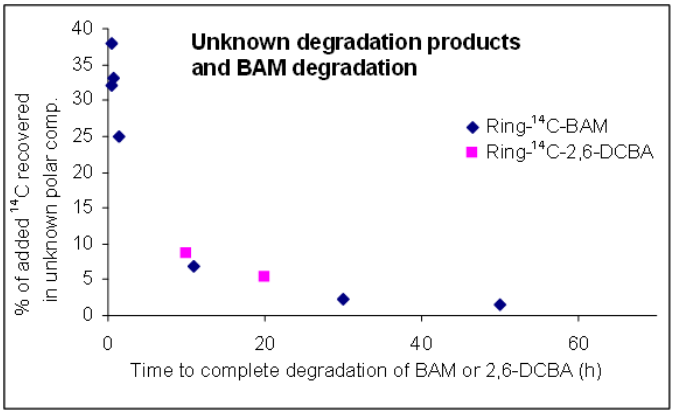


Figure 8: Possible negative relationship between formation of unknown polar ^{14}C-containing compounds during degradation of [Ring-U-^{14}C]-BAM and the time until the added BAM or [Ring-U-^{14}C]-2,6-DCBA is completely transformed. The concentration of the tested compounds were approximately 75 µg/l.

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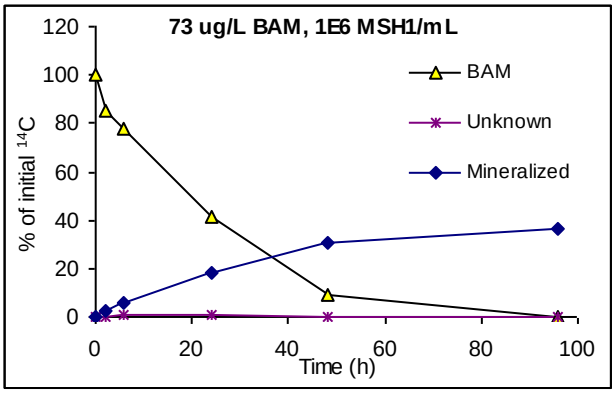


Figure 9: Degradation and mineralisation of BAM (73 µg l^{-1}) by *Aminobacter* sp. MSH1 in initial densities of 10^6 ml^{-1}.

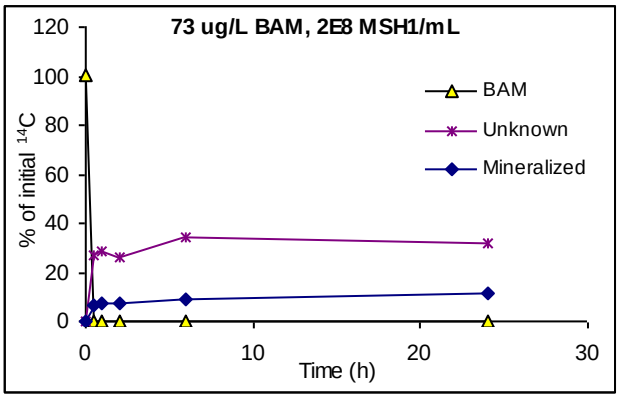


Figure 10: Degradation and mineralisation of BAM (73 µg l^{-1}) by *Aminobacter* sp. MSH1 in initial densities of 2 x 10^8 ml^{-1}.

Attempts to identify the unknown metabolite failed, but the metabolite was shown to be degraded completely in waterworks sand filters as demonstrated in a survey including 6 different waterworks sand filters. Based on these results it was concluded that the metabolite would not be a problem during bioaugmented remediation of BAM polluted groundwater.

Carbofuran degradation: In addition to BAM the degradation pathway of carbofuran by *Novosphingobium* strain KN65.2 was also elucidated. Several *Novosphingobium* mutants were produced and following incubation metabolite formation was characterized by high-resolution mass spectrometry. Several transformation product structures were proposed and a single persistent metabolite was identified as 1-(2-benzoic- acid)-(1H,3H)-quinazoline-2,4-dione (BaQD) (figure 11).

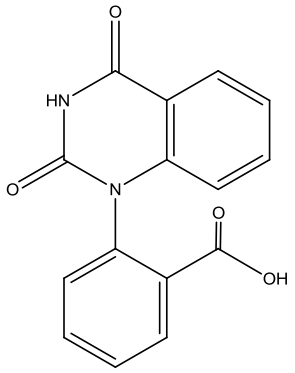
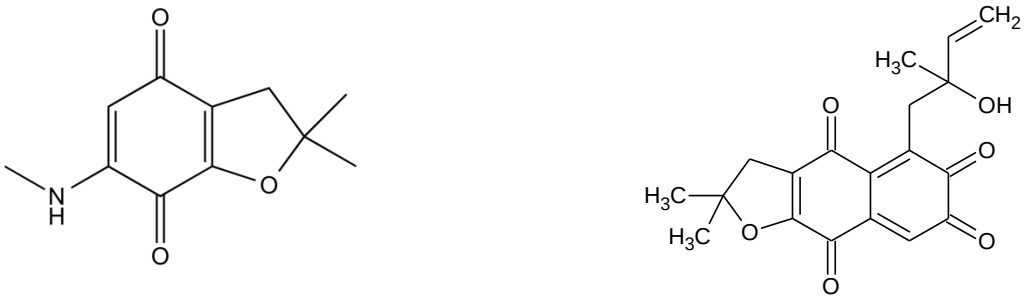


Figure 11: Proposed structure for TP282.

Besides these metabolites it was also observed that several incubations turned reddish following prolonged incubations. One of the mutants was found to produce a more vibrant red colour in the incubations than the other or the wild type and from this incubation we were able to isolate several red metabolites. A chemical structure of two of the metabolites were suggested based on mass spectrometry and NMR spectroscopy (figure 12)



CAR 208 CAR 343

Figure 12: Proposed structure of purple (CAR 208) and red (CAR 343) metabolite of carbofuran

Sulfamethoxazole degradation: In addition metabolite formation from sulfamethoxazole (SMX) by a methan oxidizing bacterium (MOB) was studied in details. 4-nitrosulfamethoxazole (4-Nitroso-SMX) and 4-N-acetyl-SMX were detected, whereas in the experiments with heterotrophic bacteria only the formation of 4-N-acetyl-SMX was observed. Presumably these transformations do not significantly change the antibiotic efficiency as it is known that at least 4-N-acetyl-SMX has a similar affinity as SMX to dihydropteroate synthase, the bacterial enzyme blocked by SMX.

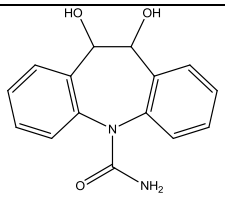
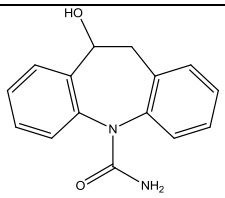
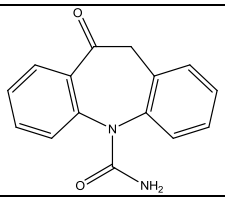
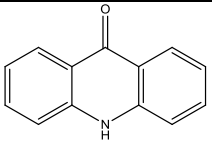
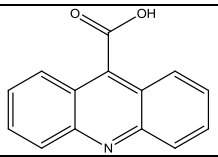
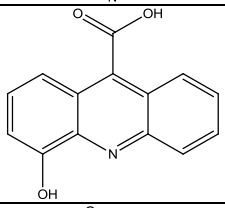
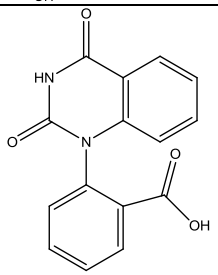
Oxcarbazepine (OXC) degradation: OXC exhibited a half live of only 1.3 days in incubations with sand filter materials. Transformation products were isolated and enriched using semi-preparative HPLC/UV and measured via high-resolution mass spectrometry and NMR. Interestingly, the same transformation products were observed irrespectively of whether OXC or two carbamazepine pharmaceuticals (10OHCBZ and DiOHCBZ) were used as target compounds (Table 6).

3.2.3 Mechanisms of contaminant transformation at low substrate concentrations

The goal of this work was to describe mechanisms of transformation for model contaminants as related to the BIOTREAT project. Here, we present pathways and mechanisms based on plausible chemical and biochemical reactions. First, we examine the mineralization pathway for carbofuran. Carbofuran is utilized as a growth substrate for *Novosphingobium* sp. KN65.2 and several metabolites have been described (see above). Second, we examine the transformation pathway of oxcarbazepine and two metabolites of carbamazepine and oxcarbazepine by a mixed-community derived from a drinking water treatment plant sand filter. In presenting these two examples, we provide data and mechanisms for metabolic and co-metabolic processes.

Carbofuran: Based on identification of carbofuran degradation products, we proposed a tentative pathway to carbofuran mineralization by *Novosphingobium* sp. KN65.2 (figure 13)

Table 6. Formation of transformation products from DiOHCBZ, 10OHCBZ and OXC

name	MW $[M+H]^+$ (error in ppm)	proposed structure	proposed fragmentation	^{1}H NMR peaks (ppm)
DiOHCBZ target compound	271.1076 (0.44) $C_{15}H_{15}N_2O_3$		271.11 $[M+H]^+$ 253.10 $[M+H -H_2O]^+$ 236.07 $[M+H -H_2O -NH_3]^+$ 210.09 $[M+H -H_2O -CHON]^+$ 208.08 $[M+H -H_2O -NH_3 -CO]^+$ 182.10 $[M+H -H_2O -CHON -CO]^+$ 180.08 $[M+H -H_2O -NH_3 -CO -CO]^+$	7.52 d (H-4) 7.77 dd (H-1) 7.24 dt (H-2,3) 5.58 s (H-10) 5.43 d (HO) 4.69 bs NH (353.5 K)
10OHCBZ target compound	255.1128 (-0.02) $C_{15}H_{15}N_2O_2$		255.08 $[M+H]^+$ 237.10 $[M+H -H_2O]^+$ 194.10 $[M+H -H_2O -CHON]^+$ 192.08 $[M+H -H_2O -NH_3 -CO]^+$	7.52-7.18 m (arom. H) 5.59 s (H-10) 5.49 d (HO) 5.04 bs (NH) 3.31 d (H-11') 2.90 s (H-11)
OXC target compound	253.0970 (-0.81) $C_{15}H_{13}N_2O_2$		253.10 $[M+H]^+$ 236.07 $[M+H -NH_3]^+$ 210.09 $[M+H -CHON]^+$ 208.08 $[M+H -NH_3 -CO]^+$ 208.08 $[M+H -NH_3 -CO - CO]^+$	
TP195 ADON	196.0757 (0.10) $C_{13}H_{10}NO$		196.08 $[M+H]^+$ 178.07 $[M+H -H_2O]^+$ 168.08 $[M+H -CO]^+$ 167.07 $[M+H -CHO]^+$	11.81 s (NH) 8.23 dd (H-4) 7.73 dt (H-2) 7.55 d (H-1) 7.25 t (H-3)
TP223 9-CA-ADIN	224.0705 (-0.42) $C_{14}H_{10}NO_2$		224.07 $[M+H]^+$ 196.08 $[M+H -CO]^+$ 180.08 $[M+H -CO_2]^+$ 167.07 $[M+H -CO -CHO]^+$	8.18 d (H-4) 8.08 d (H-1) 7.88 t (H-3) 7.67 dt (H-2)
TP239	240.0657 (0.58) $C_{14}H_{10}NO_3$		240.07 $[M+H]^+$ 222.05 $[M+H -H_2O]^+$ 194.06 $[M+H -H_2O -CO]^+$ 166.07 $[M+H -H_2O -CO -CO]^+$	8.32 dd (H-3) 8.22 dd (H-1,5) 7.69 dt (H-7) 7.53 d (H-8) 7.21 (H-2) 7.17 (H-6)
TP282 (BaQD)	283.0712 (-0.58) $C_{15}H_{11}N_2O_4$		283.07 $[M+H]^+$ 265.06 $[M+H -H_2O]^+$ 240.07 $[M+H -CHON]^+$ 222.06 $[M+H -CHON -H_2O]^+$ 196.08 $[M+H -CHON -CO_2]^+$	13.04 bs (COOH) 11.67 s (H-3) 8.13 dd (H-13) 8.03 dd (H-5) 7.80 t (H-11) 7.76 t (H-12) 7.5 d, t (H-7,10) 7.22 t (H-6) 6.33 d (H-8)

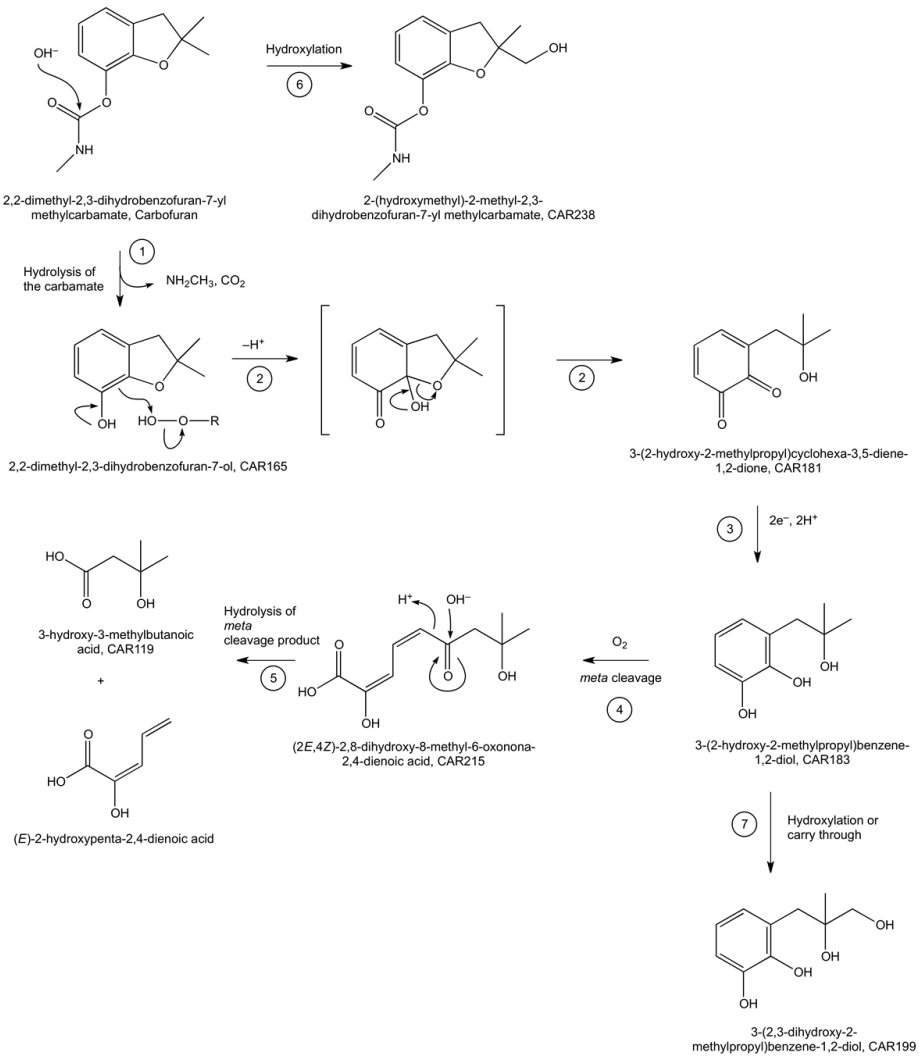


Figure 13. Tentative pathway proposed for the degradation of carbofuran. (1) Hydrolysis of carbamate. (2) Monooxygenation of dihydrobenzofuranol. (3) Reduction of the *ortho* quinone to the corresponding catechol. (4) *meta* cleavage of the 3-substitued catechol. (5) Hydrolysis of *meta* cleavage product. (6) and (7) Unspecific hydroxylations.

The first step [1] in the proposed pathway is the hydrolysis of the carbamate to the corresponding alcohol, methylamine and carbon dioxide. As a second step [2], we suggest an electrophilic monooxygenation reaction analogous to *ipso* substitution reactions with *para* alkoxyphenols. Such a reaction can potentially be catalysed by flavin or cytochrome P450 dependent monooxygenases. Note that R-OOH represents the hydroperoxo-iron of a P450-dependent monooxygenase or the C4a-hydroperoxy-flavin intermediate of a flavin dependent monooxygenase. Step three [3] must be a reduction of the quinone (most likely NAD(P)H dependent) to the corresponding catechol analogous to reductions described for *ortho* quinones to hydroquinones. For further metabolism, we suggest *meta* cleavage [4] of the substituted catechol with subsequent hydrolysis [5] of the *meta* cleavage product to yield 3-hydroxy-3-methyl butanoic acid and 2-hydroxypenta-2,4-dienoic acid in analogy to a pathway described for the metabolism of 2-alkylphenols. Reaction [7] seems to be an unspecific hydroxylation reaction not necessarily involved in the productive pathway. Reaction [8] could be similar to reaction [7] or metabolite CAR199 could be a descendent of metabolite CAR238 carried through the pathway.

Oxcarbazepine: Interestingly, degradation of oxcarbazepine, 10OHCBZ and DiOHCBZ lead to the formation of the same TPs, even though differences in abundances of individual TPs were observed. This leads to the proposal of two main transformation pathways for DiOHCBZ, 10OHCBZ and OXC (figure 14).

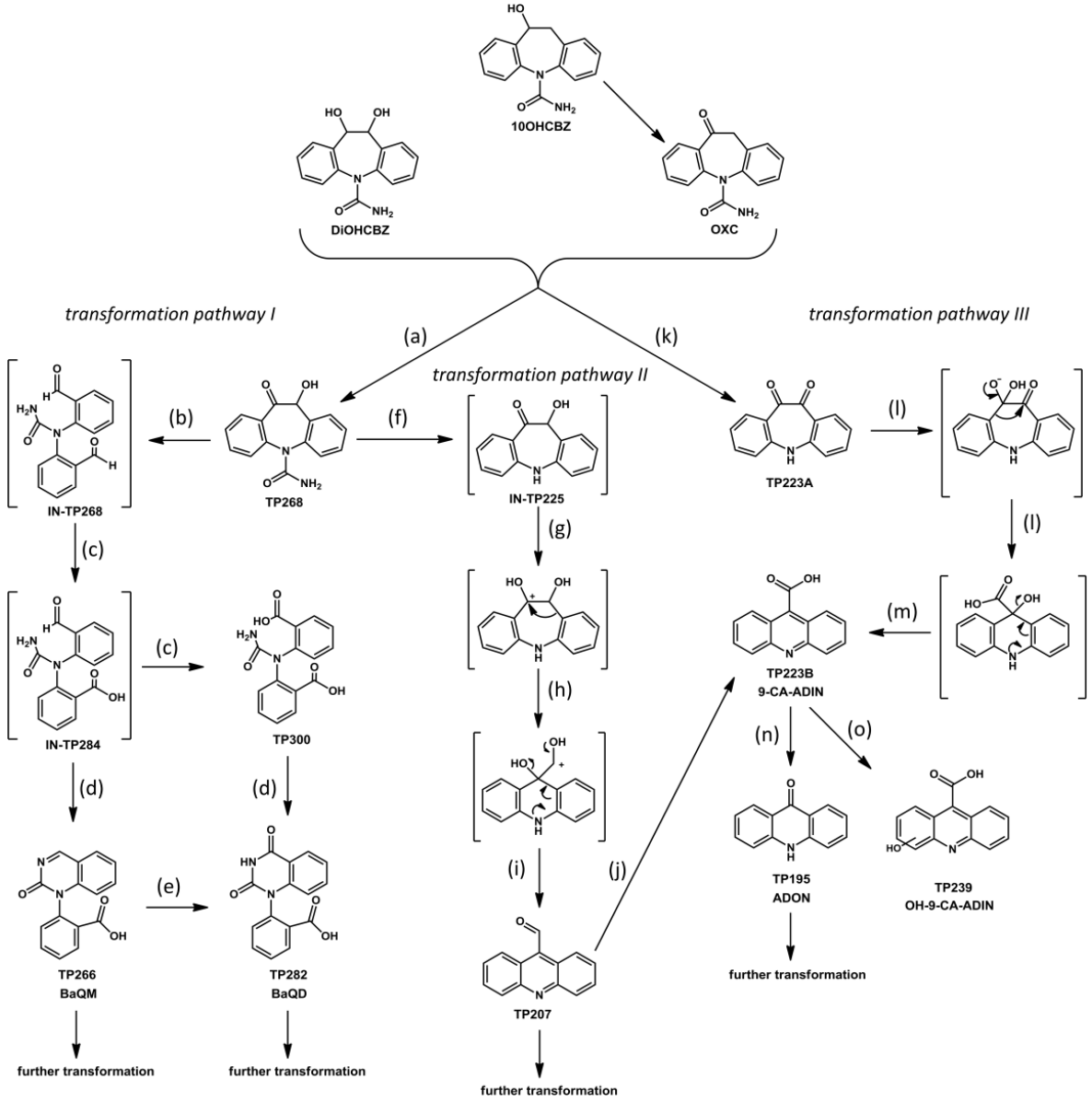


Figure 14: Proposed transformation pathway of DiOHCBZ, 10OHCBZ and OXC, respectively. Compounds in brackets are hypothetical intermediates

In general, transformation is initiated by dehydrogenation of DiOHCBZ, hydroxylation with subsequent dehydrogenation for 10OHCBZ as well as a hydroxylation for OXC, giving rise to the formation of TP269A and/or TP269B (10,11-dihydoxy-carbamazepine and its hydroxyl ketone analogue). The formation of TP269A is succeeded by the cleavage of the C10-C11 bond leading to TP300. This dioxygenase-mediated dissociation has been also described in the biodegradation of catechol as well as phenanthrene. Subsequent ring closure via intramolecular reaction of the ni-

trogen of the carbamoyl moiety with the carboxylic acid group and the loss of water leads to the formation of BaQD. Formation of TP269B is also followed by cleavage of the C10-C11 bond, leading to the respective dialdehyde, and oxidation of one aldehyde to a carboxylic acid. Subsequent ring closure proceeds via intramolecular reaction of the primary amine with the remaining aldehyde group which is followed by dehydrogenation (BaQM) and oxidation (BaQD).
Furthermore, the loss of the carbamoyl group of OXC, DiOHCBZ and 10OHCBZ was observed followed by oxidation (for DiOHCBZ) or hydroxylation followed by oxidation (for OXC and 10OHCBZ) at the C10 and/or C11 position leading to the formation of TP223B. From TP223B a benzilic acid rearrangement leads to a ring contraction. The consecutive elimination of the hydroxy group results in the formation of 9-CA-ADIN. Decarboxylation, hydroxylation and oxidation of formed hydroxyl-group to the respective ketone lead to the formation of ADON. As no further TPs were detected in the transformation experiments with ADON, mineralization and/or microbial uptake can be assumed for this TP. In addition, hydroxylation of one of the aromatic ring of 9-CA-ADIN gives rise to the formation of 4-OH-9CA-ADIN.

3.2.4 Threshold concentrations

Threshold concentrations below which degradation is either slow or not occurring were determined for *Sphingobium herbicidovorans* MH, *Novosphingobium* sp. KN65.2, *Variovorax* sp. SRS16 and *Aminobacter* sp MSH1 degrading mecoprop, carbofuran, linuron and BAM.

Sphingobium herbicidovorans MH: In high-concentration incubation experiments, strain MH degraded mecoprop rapidly to a residual concentration of 9.1±8.1 mg/l; the high standard deviation was the result of triplicate experiments yielding residual concentrations of 0.1, 11.7, and 15.5 mg/l. In low-concentration incubation experiments, little or no mecoprop degradation was observed at initial concentrations below 10 mg/l. Based on the residual concentrations observed in the high-concentration incubation experiments, this apparent threshold concentration could have been expected. However, even when cells from the high-concentration incubation experiment yielding a residual concentration of 0.1 mg/l were inoculated into low-concentration incubation experiments, little or no mecoprop degradation was observed (data not shown). We suggest here that average residual concentrations observed in high-concentration experiments can serve as a valuable predictor of threshold concentrations in equivalent systems. This suggestion is predicated on the assumption that the fundamental mechanism resulting in both residual and threshold concentrations is the same. We argue that this mechanism is biological in nature and related to an apparent dependence of the induction and expression of part of the metabolic pathway on the substrate concentration.

Variovorax sp. SRS16 and Sphingomonas sp. KN65.2: Strains SRS16 and KN65.2 at least partially degraded their respective substrates in the low-concentration incubation experiments. No threshold concentrations were evident for strain SRS16, while an apparent threshold concentration that was dependent on both the initial carbofuran and cell concentration was observed for strain KN65.2. In an effort to explain these observations, we used the kinetic parameters reported in table 4 to simulate the theoretical time course of substrate utilization for each strain at varying sets of initial substrate and inoculum concentrations.

In figure 15a, we provide the simulated and measured data for strain KN65.2 utilizing 0.01 mg/l of carbofuran at varying initial cell concentrations. The simulated data shows that an inoculum of 10^6

cells/ml should theoretically degrade carbofuran completely after approximately seven days. Lower inoculum concentrations will theoretically result in residual carbofuran concentrations ranging between 20% and 100% after 28 days. On a purely conceptual level, the measured data align with this theoretical expectation rather well, with complete utilization of carbofuran observed at an inoculum of 10^6 cells/ml and no utilization observed at inoculum concentrations of 10^4 and 10^3 cells/ml. In figure 15b, we provide the simulated and measured data for 10^3 cells/ml of strain KN65.2 degrading carbofuran over a range of initial concentrations. The simulated data show that the rate of the process is strongly dependent on the initial carbofuran concentration within this range; carbofuran concentrations ranging between 1 and 10 mg/l are predicted to degrade relatively rapidly while those in the range of 0.01 to 0.1 are predicted to degrade very slowly. Again, on a conceptual level the measured data align very well with the simulated data; very rapid degradation was observed in the range of 1 to 10 mg/l and no degradation was observed in the range of 0.01 to 0.03.

We also compared the simulated versus measured linuron utilization by strain SRS16 (results not shown). As with strain KN65.2, the measured data agreed very well with the theoretical simulated data on a conceptual level. Despite the disparate behaviour of strains SRS16 and KN65.2 at low initial substrate and inoculum concentrations, the experimental data could be explained by the unique substrate utilization kinetics estimated for each strain at high concentrations. This is an exciting and important observation in that growth and substrate utilization can be predicted at environmentally relevant concentrations with parameters estimated from relatively simple experiments conducted at substrate and cell concentrations that can be easily measured with common analytical techniques. Also, for strain KN65.2, we conclude that unique substrate utilization kinetics is the primary factor for the observed reduction of metabolic activity at low substrate concentrations, and that a threshold concentration was not attained.

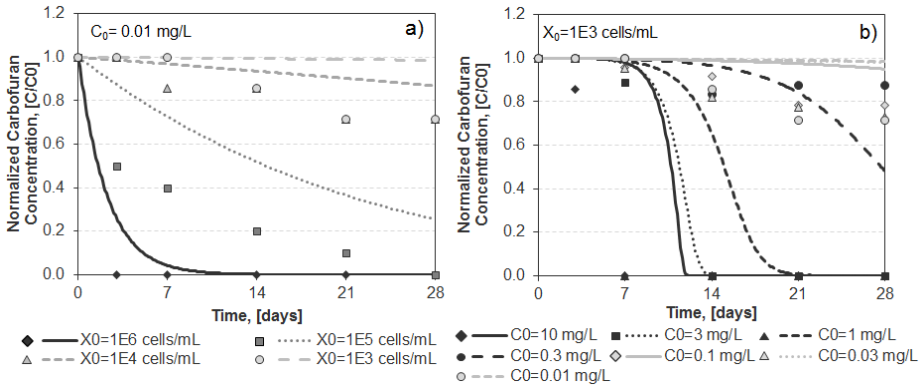


Figure 15: Simulated and measured data showing utilization of carbofuran by *Novosphingobium* strain KN65.2 at (a) an initial concentration of 0.01 mg/l and a variety of inoculum concentrations and (b) an inoculum concentration of 10^3 cells/ml and a variety of initial linuron concentrations. Data were generated by using estimated growth parameters provided in Table 4. Simulated utilization is given as lines and measured data is given as points.

Aminobacter sp MSH1: It has been shown that BAM degradation is initiated by an amidase. This enzyme is constitutively expressed and a threshold concentration for induction hampering BAM degradation is therefore not a major issue.

3.3 WP3 Systems – Microbial interactions with the biofilter environment

This part of BIOTREAT examines the surrounding environment of the systems, focusing on the effect of sand filter ecology on introduced degrader bacteria. Tools to analyse molecular fingerprinting results in order to provide ecological parameters including richness (Rr), dynamics (Dy) and functional organization (Fo) have been developed. It was concluded that BAM mineralization by MSH1 in a sand filter ecosystem can be affected by the presence of other sand filter bacteria but that this depends on the diversity. Even at relevant trace pollutant concentrations *Aminobacter* MSH1 was revealed to produce extracellular polymeric substances (EPS) which may necessary for biofilm formation. Furthermore, protozoa naturally living within waterworks sand filters was shown to predate on the MSH1 cells added the filters, thereby limiting BAM degradation. Copper (Cu) was demonstrated to control cometabolic degradation of several contaminants by methane-oxidizing cultures including chloropropham, metazachlor and benalaxyl. Cometabolic degradation of sulfamethoxazole (SMX) and carbamazepine (CBZ) by ammonium oxidizing enrichment cultures was also demonstrated.

3.3.1 Tools to describe the diversity of microbial communities in treatment filters

A microbial ecosystem, such as a waterworks sand filter consists of a microbial community interacting with its environment, including also the introduced contaminant degrading microbial species. The structure of microbial communities is commonly unravelled using molecular fingerprinting patterns and tools for interpretation such pattern, (e.g. PCR-DGGE) including the range-weighted richness (Rr), dynamics (Dy) and functional organization (Fo) parameters for the different experimental setups have been developed. Such interpretation provides an ecological and predictive value to the analysis of the structure and diversity of a microbial community in a given environment, enabling us to compare both different communities as well as the behaviour of a community facing different kinds of stress. An image analysis protocol optimised for the characterisation of biofilms composition and spatial structure and architecture (Ar) has been developed. Biofilm composition and architecture is typically analysed by use of cytostains combined with confocal scanning laser microscopy (CSLM). We have identified essential parameters to be extracted from the individual micrographs of the CSLM in order to describe spatial structure and architecture. The parameters includes biovolumes, substratum coverage, surface to volume ratios, thickness, roughness, relative population abundances, and population co-localization (table 7). Different image analysis software packages used to analyse CLSM micrographs were tested and ranked in relation to their ability to extract these parameters from individual micrographs (table 8). Among several programs, the Daime protocol was selected as the best-available package to be used. Daime is a scientific image analysis and visualization program for microbiology and microbial ecology offering tools for analyzing 2D and 3D microscopy datasets of microorganisms stained by FISH with rRNA-targeted probes or other fluorescence labelling techniques. Daime is a freely distributed stand-alone program, generated directly by the microbial ecology scientific community. It is the most comprehensive, compared to other tested packages, in terms of contained analysis features (Table 8). It is relatively easy to use, and it combines image analysis with 3-D visualization functionality. Using the DAIME manual as a guideline, we have developed a user-friendly DAIME – based protocol to conduct image analysis on micrographs obtained with CSLM – combined with cytostains. The Daime user instructions were further modified to better describe the spatial structure of microbial biofilms of waterworks sand filters.

Table 7 Explanation of Core Parameters Describing Biofilm Composition and Structure

Parameter	Unit	Description
Bio-volume	μm^3	Represents the overall volume of the biofilm, and also provides an estimate of the biomass in the biofilm.
Area occupied by bacteria in each layer	%	Reflects how efficiently the substratum is colonized by bacteria of the population
Thickness distribution max. and mean thickness	µm	Locates the highest point (µm) above each (*x*,*y*) pixel in the bottom layer containing biomass. Hence, thickness is defined as the maximum thickness over a given location.
Identification and area distribution of micro-colonies at the substratum	μm^2	Total number of identified micro-colonies, the area size of each micro-colony (μm^2) and the mean micro-colony area (μm^2). The number and area sizes of micro-colonies at the substratum provide valuable information about the organization of the biofilm community
Volumes of micro-colonies identified at the substratum	μm^3	Calculates the volume (μm^3) of each of the micro-colonies identified above and the mean micro-colony volume (μm^3)
Roughness coefficient.		Biofilm roughness provides a measure of how much the thickness of the biofilm varies, and is an indicator of biofilm heterogeneity. Dimensionless.
Surface to volume ratio	%	Reflects what fraction of the biofilm is in fact exposed to the nutrient flow, and thus may indicate how the biofilm adapts to the environment
Population relative abundances	%	Relative abundance of each population in the observed bacterial community.
Co-localisation of 2 bacterial populations (*g(r)*)	<1,1,>1	*g(r)* is 1 if the populations are randomly distributed, greater than 1 if they cluster together at distance *r*, and less than 1 if they repulse each other at distance *r*

Table 8. Considered Image Analysis Packages and Their Supported Features

	Biovolume ($\mu m^3/\mu m^2$).	***Substratum coverage (%)***	***Surface to volume ratio (%)***	***Thickness (Av; max; µm)***	***Roughness***	***Brightness***	***Co-localisation***	***Morphometry objects***	***Motion analysis***
COMSTAT(1)	✓	✓	✓	✓	✓				
Phlip (2)	✓	✓	✓	✓	✓		✓		
DAIME (3)	✓	✓	✓	✓	✓	✓	✓	✓	
BioImage_L (3)	✓	✓	✓	✓	✓				
Metamorph (4)	✓	✓	✓	✓	✓	✓	✓	✓	✓
Imaris (5)	✓	✓	✓	✓	✓	✓	✓	✓	✓

(1) (Heydorn et al., 2000), (2) (Mueller and Brouwer 2006), (3) (Daims et al., 2006), (4) (Chavez de Pas and Resin 2011), (5) (Molecular Devices).

3.3.2 Microbial community composition facing abiotic stress

Metabolical degradation processes Biofilm formation by the BAM-degrading *Aminobacter* MSH1 has been studied in details to better understand abiotic factors controlling surface colonisation of introduced bacteria. It has been shown that the bacterium produce extracellular polymeric substances (EPS) even at relevant trace pollutant concentrations. EPS are high-molecular weight compounds secreted by microorganisms into their environment and known to be involved in biofilm formation. Various fluorescent stains have been tested for EPS labelling. By using the EPS stain ConA (Concanavalin A-specific) which binds to α-glucosepyranosyl residues and α- mannopyranosyl residues, at least part of the EPS was visualized in *Aminobacter* sp. MSH1 biofilm. A relatively larger volume of EPS was indeed visualized in biofilms fed with 1 µg/l and in biofilms fed with media containing no other C source (figure 16) than in biofilms developed at higher BAM concentrations.

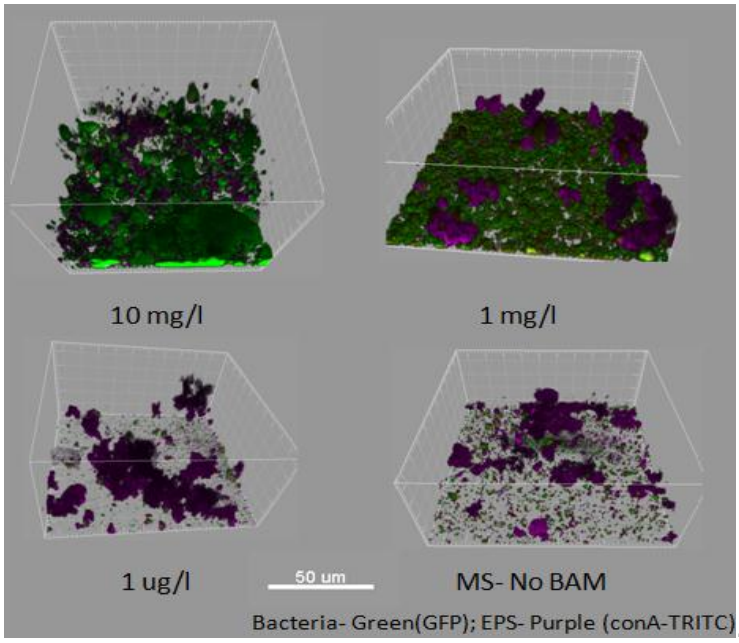


Figure 16. EPS detection in MSH1-GFP biofilms fed with minimal medium containing BAM at concentrations of 10 mg/l, 1 mg/l, and 1 µg/l and without BAM. Biofilms were stained with EPS stain ConA TRITC (purple).

Then the question raised was whether BAM removal in biofilms fed at micropollutant concentrations was not due to abiotic loss. To examine this, biofilms were intervened with two spontaneous mutants of the BAM degrader *Aminobacter* sp. MSH1. The first mutant was M6 100g, a mutant that degrades only BAM and not DCBA; the second mutant was M1 100g, a mutant which can degrade DCBA but not BAM. Both mutants were GFP labelled. By growing the MSH1-GFP that degrades both BAM and DCBA and the two mutants in biofilms, we were able to investigate and to provide evidence about the role of sorption and other abiotic removal processes on BAM removal, both in the flow chambers and on the link of EPS with BAM presence/degradation. The corresponding BAM/DCBA degradation curves are shown in figure 17. As expected, the strain MSH1-GFP degraded both BAM and DCBA, while the M6 100g-mutant degraded BAM but not DCBA. The M1 100g-mutant did not degrade BAM. BAM was clearly not adsorbed by the EPS.
Clear differences existed in the number of cells of MSH1-GFP, M1 100g and M6 100g in the biofilms (figure 18). MSH1-GFP, which metabolizes BAM and DCBA, had the largest number of cells. The BAM concentrations in the feed did not influence the number of cells in the effluent.

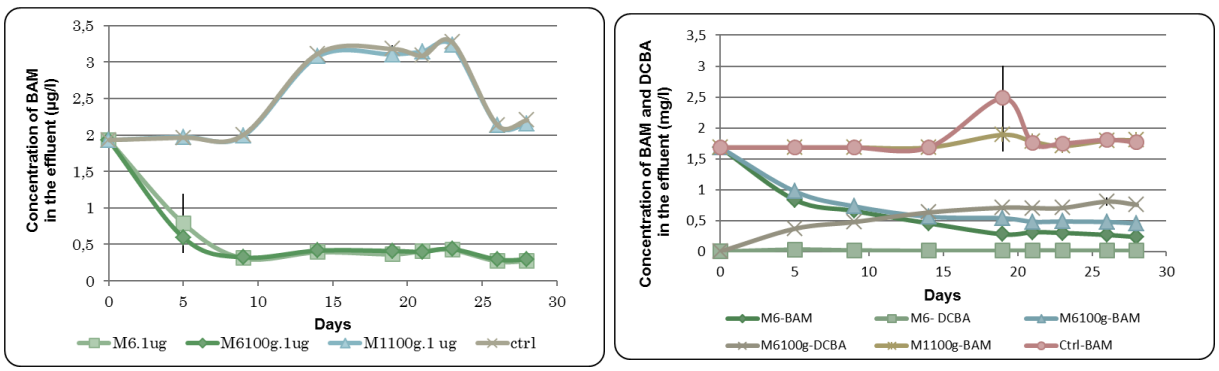


Figure 17. BAM/DCBA degradation curves obtained with biofilms grown with strain MSH1-GFP (M6) and mutants M6 100g and M1 100g in case of feeding fed with 1 µg/l(left) and 1 mg/l (right).

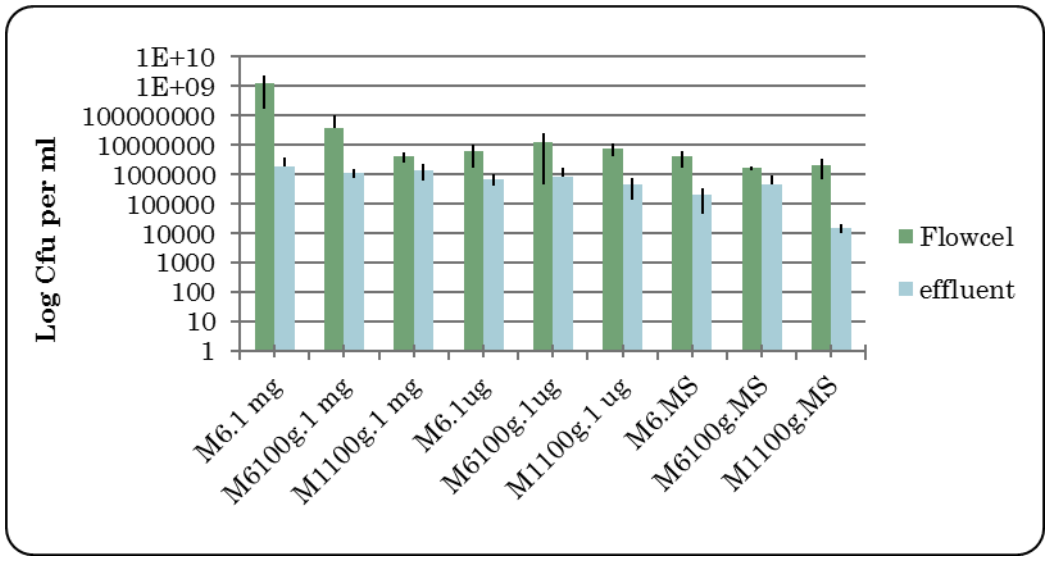
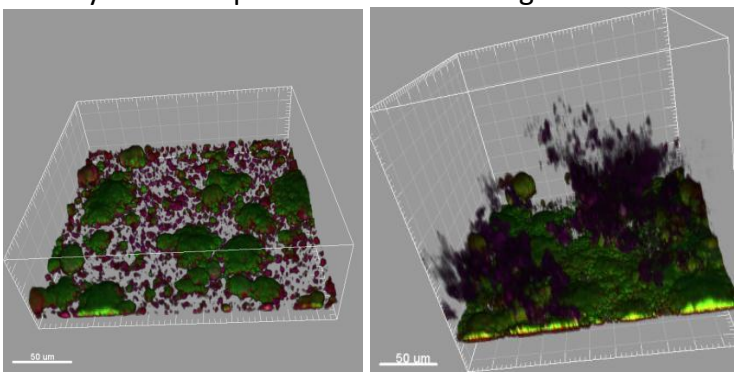


Figure 18. Comparison of the number of cells in the biofilm measured in the flow chambers at the end of the experiment, and the cells in the effluent at one time point for strain MSH1-GFP (M6) and mutants M6 100g and M1 100g, and at different BAM concentrations.

Confocal scanning images of the biofilms show that the biofilm structure depends not only on the BAM concentration but also on the composition of the medium (figure 19). Even when the MSH1 was fed with artificial groundwater media, MSH1 formed a biofilm and degraded BAM. The amount of extracellular polysaccharides though was less and was less loose, i.e., more closely associated with the colonies and attached to the glass surfaces. EPS was produced in biofilms without BAM indicating that they were not produced from the degradation of BAM.



A B

Figure 19. Three-D projection of MSH1 biofilms fed with 1 mg/l BAM in artificial groundwater with amended phosphate (A) or in MS minimal medium (B). Green cells: GFP labelled MSH1 cells. Red cells: syto62 stained MSH1 cells. Purple: EPS labelled with ConA-TRITC.

Cometabolical degradation processes. The effect of copper (Cu) on cometabolical degradation of iopromide, bentazone, mecoprop, diclofenac, CBZ, and BTZ were studied in methane oxidizing cultures (MOB). Cu-limitation has previously been shown to stimulate co-metabolic degradation of organic chemicals by MOB. Significant cometabolic removal (81%) was only observed in Cu-starved cultures degrading BTZ. A linear correlation between the consumption of CH_4 [mmol/l medium] versus the removed BTZ [mmol/l medium] was seen (figure 20) showing a clear link between BTZ degradation and CH_4 oxidation activity of the culture.

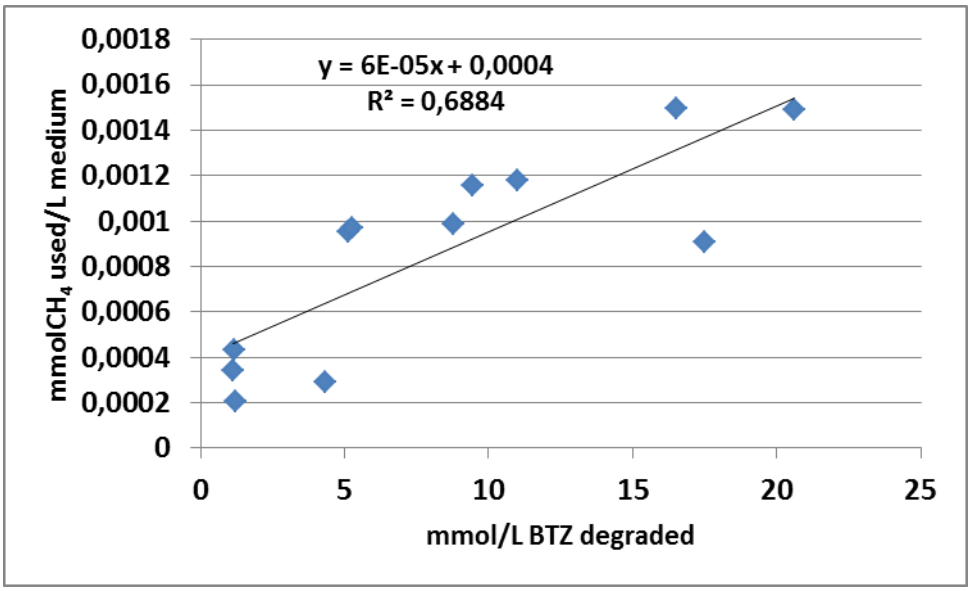


Figure 20. Correlation of BTZ degradation with the CH_4 oxidation activity of a methane oxidising culture

Cometabolic degradation of metazachlor and benalaxyl by a MOB culture (Bourgoyen) expressing particle bound methane mono oxygenase (pMMO) was also demonstrated.

The microbial community of the $MOB_{bourgoyen}$ culture was identified using DNA extraction, followed by PCR and illumina sequencing and compared to an methane oxidizing culture enriched from soil (MOB_{soil}), The community structures of the investigated cultures were completely different. The main constituents of the MOB_{soil} culture originate from the *Flavobacteriaceae, Chitinophagaceae, Bradyrhizobiaceae, Methylocystaceae, Methylophylaceae, Moraxellaceae* and *Xanthomonadaceae* families while the main constituents of the $MOB_{bourgoyen}$ culture were *Flavobacteriaceae*, *Chitinophagaceae*, *Methylophylaceae*, *Comamonadaceae* and *Methylococcaceae* (figure 21). For both cultures; all OTUs classified as methanotrophic *Proteobacteria* were classified into two MOB families: either *Methylococcacae* (Gammaproteobacteria or type I MOB) or *Methylocystaceae* (*Alphaproteobacteria* or type II MOB). However, while the *Methylocystaceae* family was an abundant community constituent of the MOB_{soil} (up to 8.8% of the total OTU count), it was not dominant in the $MOB_{bourgoyen}$ (0.07% of the total OTU count). On the other hand, the *Methylococcaceae* family was a very important community constituent of the $MOB_{bourgoyen}$ (25% of the total OTU count) while it was less important in the soil culture (0.28% of the total OTU count).

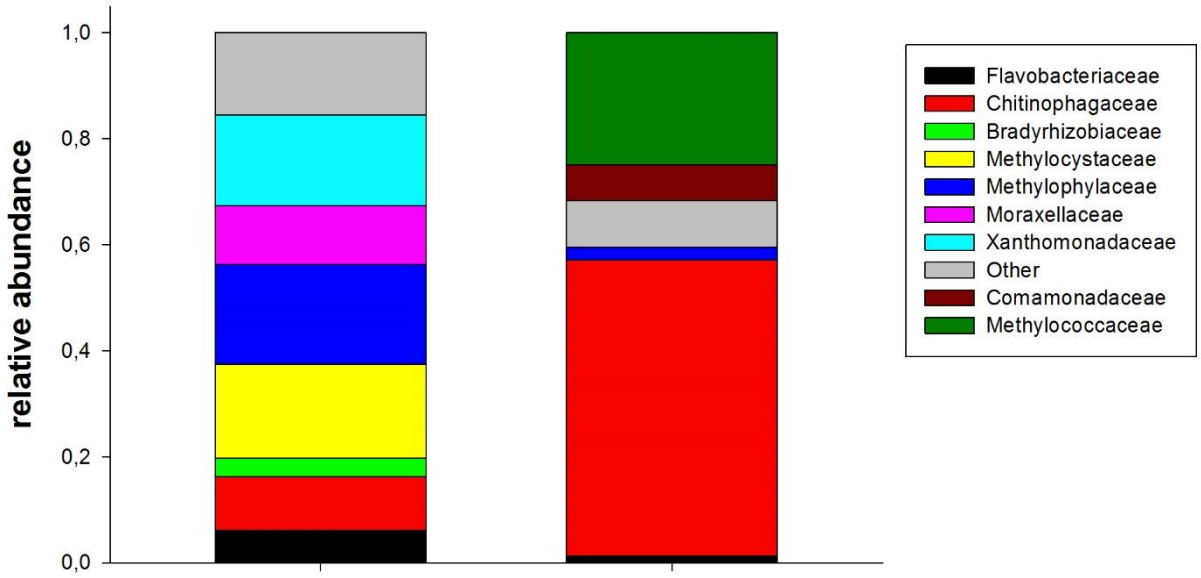


Figure 21: Relative abundances of taxa in MOB_{soil} and $MOB_{bourgoyen}$. All taxa accounting for more than 1% of the total sequence count are displayed. The RDP classifier, reference set and taxonomy were used. The deepest possible classification is given up to the family level. Relative abundances were calculated after summing the sequence counts of the OTUs that could be classified on the family level.

The MOB_{soil} culture was enriched and used in the experiments with a copper concentration which allows sMMO expression and prevents pMMO expression (no added copper; Choi et al., 2003; Hanson et al., 1996) On the other hand, the $MOB_{bourgoyen}$ culture could oxidize methane at elevated Cu^{2+} concentrations of 10 µmol L^{-1} which is much higher than the sMMO inhibition concentration of 1 µmol L^{-1} reported by Begonja et al. (2001) and should eliminate any possible sMMO formation. Under these specific conditions, both cultures were able to co-metabolically degrade metazachlor but at different pesticide degradation efficiencies. The sMMO expressing culture (MOB_{soil} culture) has a 7 times higher degradation efficiency than the pMMO expressing culture ($MOB_{bourgoyen}$ culture). It has been reported that the degradation rate of VC, t-DCE and TCE is much faster for sMMO expressing cells of *M. trichosporium* OB3b compared to pMMO expressing cells (Lee et al., 2006; Lontoh et al., 1998 and Oldenhuis et al., 1991; Han et al., 1999). Also considering the higher maximum inhibition concentration observed for sMMO expressing cultures (2.5 times higher than pMMO), one might prefer the use of the sMMO expressing cultures over the use of pMMO expressing cultures. However, Lee et al. (2006) observed faster growth of pMMO expressing cells and faster degradation of VC, t-DCE and TCE when pollutant concentrations were higher than 100 µM. When pollutant concentrations were lower, sMMO was the preferred enzyme. Furthermore, it should not be forgotten that MOB only catalyse the oxidation step, which leads to the formation of unknown oxidation by products which can also have an effect on the maximum inhibition concentration (Han et al., 1999; Lontoh et al., 1999; Oldenhuis et al., 1991; Oldenhuis et al., 1989). The pMMO expressing MOB culture was also able to co-metabolically degrade chloropropham, benalaxyl and metazachlor. Until recently, oxidation of aromatic compounds has only been observed for sMMO. While pMMO is present in almost all methane oxidising bacteria, sMMO is

mainly limited to type II MOBs. Both cultures contain representatives of type I as well as type II MOB, allowing these cultures to perform in a broader range of circumstances as type I and type II MOB show distinct ecophysiological features (Hanson et al., 1996) and have even been suggested to possess different life strategies (Ho et al., 2013). However, our illumina results show that type II MOB are much more dominant in the sMMO expressing soil MOB culture suggesting that sMMO is indeed expressed under the given limited Cu^{2+} concentrations. On the other hand, the pMMO expressing culture contains more type I MOB.

In conclusion, this study demonstrates for the first time the successful cometabolic degradation of chloropropham, metazachlor and benalaxyl by methane-oxidizing cultures. Both sMMO and pMMO expressing cultures seem to be able to degrade these components although the pesticide degradation efficiency for pMMO degrading cultures is lower at the tested pollutant concentrations. As MMO has a much lower substrate specificity compared to heterotrophic bacteria, the usage of methanotrophic bacteria to remediate contaminated water seems promising.

Cometabolic degradation of SMX and CBZ by ammonium oxidizing enrichment cultures deriving from either 1) sludge of a conventional municipal waste water treatment plant (WWTP), 2) activated sludge of a waste water treatment plant of a hospital (Hospital WWTP), 3) soil (SOIL), or 4) water from the canal Coupure (COUPURE). Figure 22 shows the degradation of SMX and figure 23 of CBZ in all tested inocula, including also another test using HANDS as nitrifying culture for comparison.

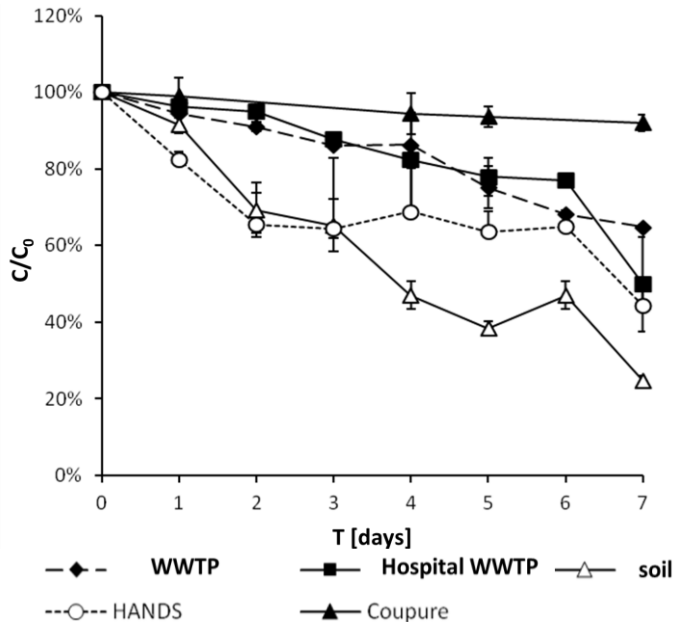


Figure 22: Degradation of SMX within 7 days of new AOB enriched cultures.

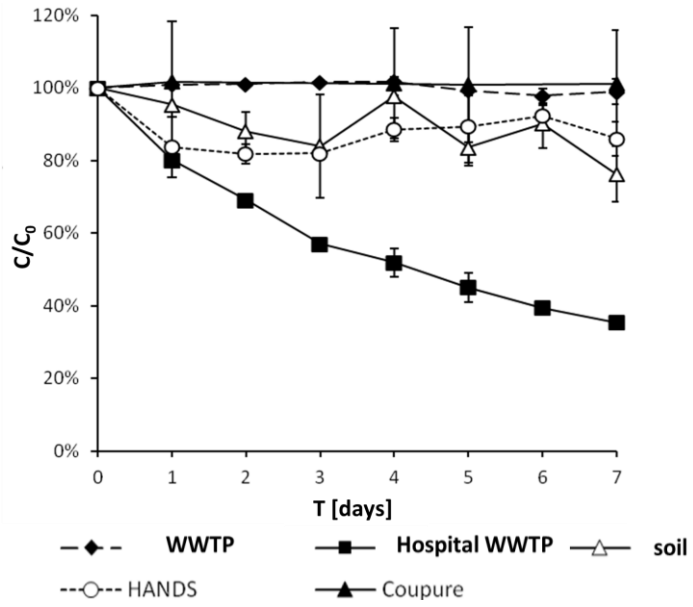


Figure 23: Degradation of CBZ within 7 days of new AOB enriched cultures.

The culture originating from the soil sample showed up to 75% removal of SMX within 7 days, however, additional tests showed that a lot of the removal was also due to sorption (data not shown). The Hospital WWTP culture showed very good potential for degradation of CBZ with up to 64.6 ± 0.2% in 7 days.

3.3.3 Microbial community composition facing biotic stress

Degrader bacteria added to waterworks sand filters are faced with biotic stress in the form of competition with indigenous bacteria and grazing by protozoa. Much work in BIOTREAT aimed at applying the BAM-degrading bacterium *Aminobacter* sp. MSH1 to waterworks sand filters. We therefore study the protozoan grazing of MSH1 added to sand filter material.

The density of the bacteria added might have an effect on the protozoan numbers. In experiments added 10^7 MSH1 cells g^{-1} to filter sand, with an indigenous microbial community, resulted in a maximum of $5x10^4$ protozoa ml^{-1}. Adding $5x10^8$ MSH1 cells g^{-1} resulted in a much higher density of protozoa implying that MSH1 was used as a food source for the protozoa in the sand filters (figure 24).

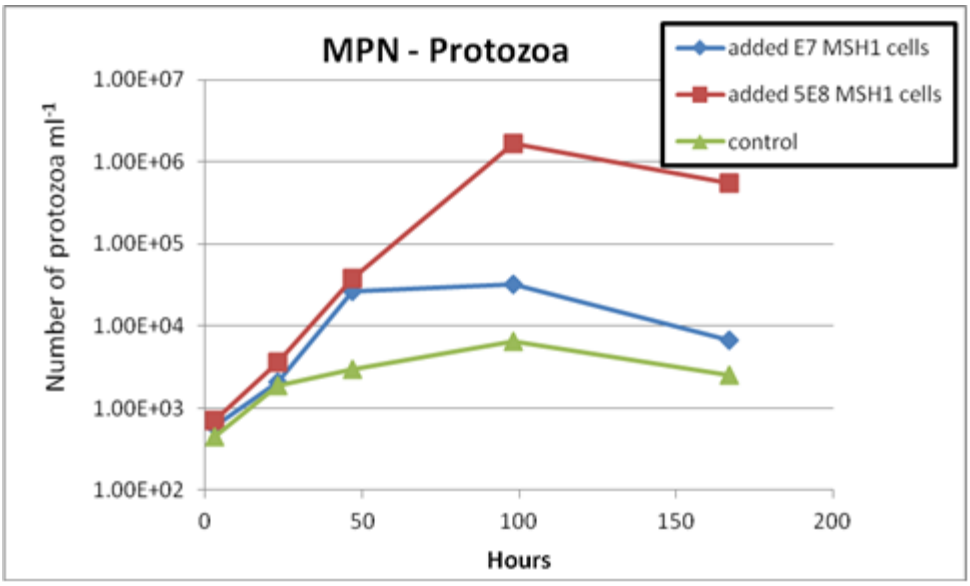


Figure 24. Number of protozoa in the filter sand after addition of 10^7 and $5x10^8$ MSH1 cells g^{-1}. The number of active protozoa was determind by the MPN method.

A column experiment was also conducted in which MSH1 was inoculated in sand filter material that was either sterilized or containing the natural microbial community (i.e. bacteria and protozoa) of a sand filter. The results showed that BAM was removed more efficiently in the columns containing the sterile sand compared to the natural sand (figure 25). Correspondingly, the number of *Aminobacter* cells found in the sterilized sand was also higher, after the first 48 hours of the experiment, than the number of *Aminobacter* cells in the natural sand added MSH1 (figure 26).

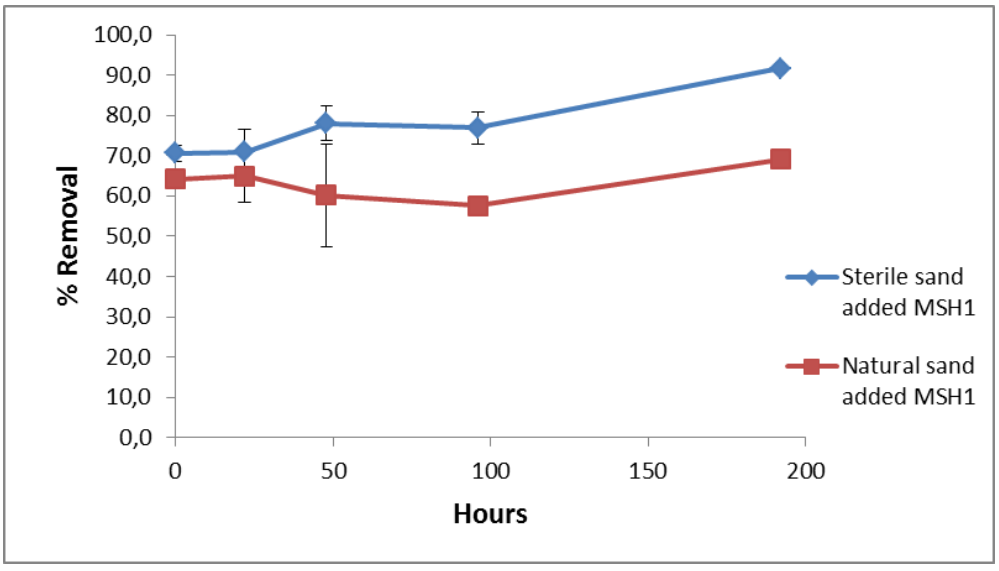


Figure 25. Removal of BAM in the columns with the sterile and the natural sand added MSH1.

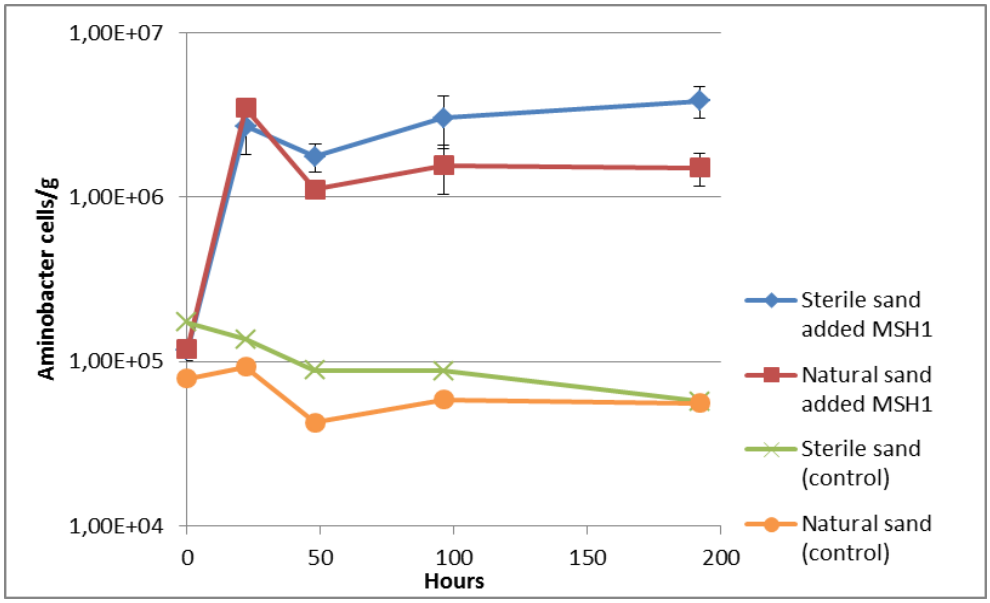


Figure 26. Number of Aminobacter cells in the sterile and the natural sand added MSH1 as well as the control columns.

In conclusion the results suggest that the protozoa naturally living within waterworks sand filters not only predate on the MSH1 cells added the filters, but also limit BAM degradation by MSH1.

3.3.4 Successful species invasion for bioaugmentation

The primary goal of this part of BIOTREAT was to point out the role of the structure of the whole microbial community in counteracting the effect of a new degrader species invasion and how this can influence specific ecosystem functionality. Still the basic process occurring during invasion of an existing community are not yet fully understood. To investigate the role of the evenness on invasion, artificially prepared communities with a constant diversity but varying evenness were used together with *gfp*-labelled model-invaders. The more uneven a community was the higher was the degree of invasion. So for bioaugmentation, i.e. the desired invasion of species, a lower degree of evenness of the receiving community would seem favourable. However, in the tested system, invasion also decreased the indigenous functionality as such, which in a lot of cases could be problematic for implementation. Similar tests but this time using sand filter isolates supported the earlier findings that invasion was in general more successful in less even communities, but the degree of invasion also showed a high dependency to the actual dominant species. Most probably this is due to the level of direct competition between the invader and the dominant species. This needs to be kept in mind when considering a certain species for bioaugmentation.

Biofilm experiments were also performed to examine whether the BAM degrading strain *Aminobacter* sp. MSH1 was able to invade a sand filter microbial biofilm community. To study this, biofilms of the *gfp*-labelled variant of MSH1 in the presence of a microbial community extracted from an operational sand filter in Sinaai (Belgium) were developed in flow chambers continuously fed with a medium with different concentrations of BAM (figure 27). At all tested conditions a MSH1 mono-species biofilms developed and biomass of MSH1 decreased with decreasing BAM concentrations. In the presence of the sand filter community, the numbers of MSH1 cells appear lower compared to that found in mono-species MSH1 biofilms. In the flow chambers inoculated with MSH1 and the sand filter community which was continuously fed with 1 mg L^{-1} BAM, MSH1 formed small isolated colonies within the multi-species biofilm of sand filter bacteria. This contrasted with the corresponding mono-species biofilms of MSH1 in which the MSH1 cells spread out over the entire surface. MSH1 cells appear to avoid contact with the sand filter bacteria.

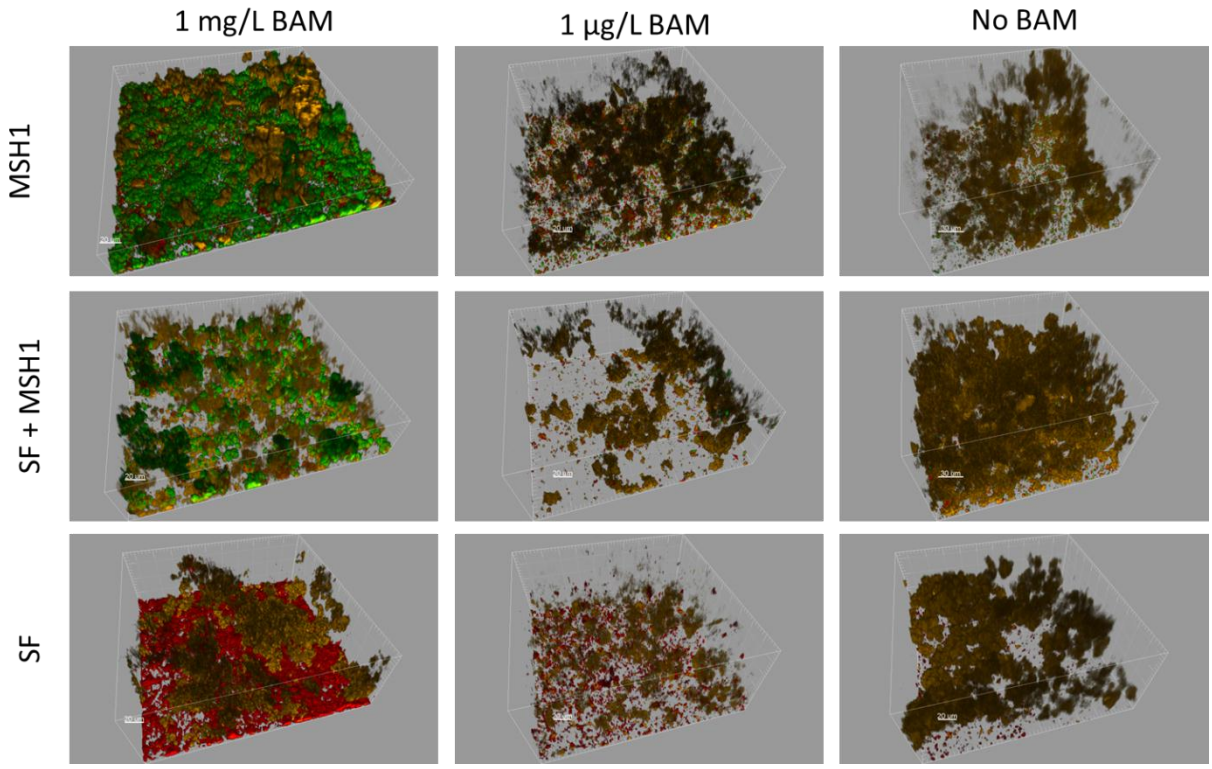


Figure 27. Three-D projection of MSH1 biofilms that developed in flow chambers fed with MS medium supplemented with 1 mg L^{-1} and 1 µg L^{-1} BAM and MS medium without BAM. Images are 3D reconstructions acquired with CLSM showing living MSH1 cells (green), dead/inactive MSH1 cells (red), sand filter bacterial cells (red) and ConA stained EPS (brown).

No BAM degradation was observed in the flow chambers containing only sand filter bacteria (figure 28). This coincided with the inability of the Sinaai sand filter community to mineralize BAM. BAM degradation was observed in flow chambers inoculated with only MSH1 and in flow chambers inoculated with both MSH1 and the sand filter community. The extent of BAM degradation at steady-state conditions was however on average 10-20% lower in the mixed biofilms compared to the mono-species MSH1 biofilms indicating that either the BAM degrading activity and/or the growth of MSH1 was affected in the mixed species biofilm systems.

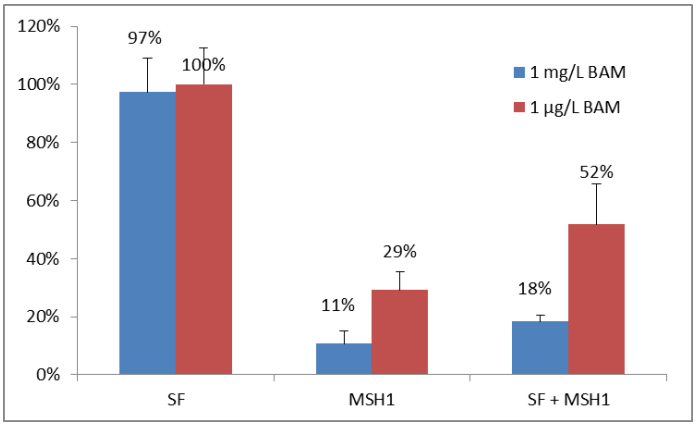


Figure 28. Steady state residual concentration of BAM in the effluent expressed as percentage of the influent BAM concentration (1 µg L^{-1} and 1 mg L^{-1}) in flow chambers inoculated with either the sand filter community (SF), MSH1 and both MSH1 and the sand filter community (SF+MSH1).

We conclude that we have first indications that BAM mineralization by MSH1 in a sand filter ecosystem can be affected by the presence of other sand filter bacteria but that this depends on the diversity. A higher richness of sand filter bacteria seems to wipe out effects observed in the presence of single strains of sand filter bacteria. We can also conclude that MSH1 is able to establish in biofilms of sand filter communities and perform its BAM degrading functionality even at 1 µg/l influent concentrations although the presence of the community affects degradation negatively.

3.4 WP4 Models and predictability – Modelling and prediction of microbial degradation of micropollutants

Mathematical modelling of biodegradation kinetics can improve our understanding of the reactions and allows the design of *in situ* or *in reactor* applications of microbial remediation. In this part of BIOTREAT we have chosen, validated and parameterised appropriate bio-kinetics expressions for 1) metabolic primary pollutant removal of trace pollutant concentrations and 2) cometabolic oxidation of trace pollutant concentrations by ammonium oxidizing bacteria (AOB).

3.4.1 Choose, validate, and parameterize appropriate biokinetic expressions for cometabolic oxidation of micropollutants by AOB and MOB

In this part of BIOTREAT a generalized model review for cometabolic transformation of micropollutants was conducted. We successfully generated the first set of cometabolic models based on the metabolic relationship of non-growth and growth substrates. We chose three criteria to evaluate the cometabolic models for the considered use: ability to fit experimental data, identifiability of parameters and suitability of the model size and complexity. The ability to fit to experimental data and the identifiability of the model parameters are of high importance for any model to be valuable. To make decisions about the most suitable models for the future, we evaluated the following five models for their ability to fit the synthetic experimental observations and whether they would return reasonable parameter estimates: A 1st-order model, a Monod model, a reductant model, a competition model and a combined mode (see deliverable 4.1 for details).

Simulated batch experiments were designed to resemble actual cometabolic substrate degradation experiments. These observation were generated by using the most complex model structure and assumed parameters based on the literature reported range. The weighted sum of squared residuals (WRSS) of the reductant model in the parameter/initial concentration domain is consistently lower than in the other three models. WRSS are used to find parameter values that minimise a measure of badness of fit originating from the fact that model equations are generally nonlinear forming a non-linear optimization problem. Akaike information criterion (AIC) and Bayesian information criterion (BIC) results show that the first-order model was smaller than the other models because it has less parameters. Besides the first-order model, the reductant model was much better than the other models for the AIC and BIC criteria. AIC and BIC are criteria used to rank models in accordance to complexity and size. The identifiability of the reductant model was better in the higher initial cometabolic substrate concentration zones, while in the lower initial cometabolic substrate concentration zone, the first-order model was much better than the other models. From our simulations and model comparison, we can choose the best suitable model for different parameter/initial substrate concentration condition domain. While the first-order model is at times superior (for it has fewer parameter than other models), it yields poor weighted sum of squared residuals. We conclude that the Reductant model is most adequate to describe observations across the considered experimental domain.

3.4.2 Choose, validate, and parameterize appropriate biokinetic expressions for primary pollutant removal at 'micro g/l' range

The concentration of easily assimilable organic carbon (AOC) largely determines the microbiological stability of drinking water (Egli 2010). Residual concentrations can be caused by diffusion limitations, maintenance energy demand of the organisms, a threshold concentration for enzyme induction, and environmental factors, like the presence of additional substrates. With respect to pesticide removal strains, many previous studies focused on their survival and/or die-off with pesticide as the sole carbon source, whereas only little knowledge is available on factors affecting their growth under environmental conditions, i.e. growth at low cell/nutrient concentrations with mixtures of substrates, e.g. groundwater (~10 µg/l AOC), shallow stream (~100 µg/l AOC), and stagnant pond water (~1000 µg/l AOC). In this research part, we developed a comprehensive general dynamic model framework including growth/survival state of the specific pesticide degrading strains in the environment with the target trace pollutant, an aquatic background AOC and background bacterial community. The model describes different reactions and different relationships between the specific pesticide degrading bacterium and the background bacteria under different background AOC that could support growth. We carried out the model framework to explain the competition between special strain and the background community; the competition between the AOC and the transformation products (TP) and also the relationship between the special strain with the AOC and TP, which were difficult explaining experimentally.

3.4.3 Incorporate Biokinetic Rate Expressions in Continuum Biofilm Models to infer Reactor Scale Models for Cometabolic Micropollutant Oxidation

A continuum biofilm-bioreactor model that describes nitrification in a pilot-scale rapid sand filter has been developed (Deliverable D4.2). This model has been enhanced by explicit inclusion of appropriate biokinetic terms to capture co–metabolism. The model is presented in table 9.

Table 9 – Petersen Matrix Summarizing the Stoichiometry and Rate Expressions for Two step Ammonium Oxidation and Cometabolic Trace Pollutant Removal by Ammonium Oxidizers

Component → / Process ↓	S_O	S_C	S_{NH4}	S_{NO2}	S_{NO3}	$X_{B,AOB}$	$X_{B,NOB}$	Rates
Growth of $X_{B,AOB}$	$-\frac{3.43-Y_{AOB}}{Y_{AOB}}$		$-\frac{1}{Y_{AOB}}$		$\frac{1}{Y_{AOB}}$	1		$\mu_{AOB}\frac{S_{NH4}}{K_{NH_4}^{AOB}+S_{NH4}}\frac{S_{O2}}{K_{O_2}^{AOB}+S_{O2}}X_{B,AOB}$
Growth of $X_{B,NOB}$	$-\frac{1.14-Y_{NOB}}{Y_{NOB}}$			$-\frac{1}{Y_{NOB}}$	$\frac{1}{Y_{NOB}}$		1	$\mu_{NOB}\frac{S_{NO2}}{(K_{NO_2}^{NOB}+S_{NO2})}\frac{S_{O2}}{(K_{O_2}^{NOB}+S_{O2})}X_{B,NOB}$
Cometabolic Degradation		-1				$-T$		$k_{AOB}\frac{S_C}{K_C^{AOB}+S_C}X_{B,AOB}$
Decay of $X_{B,AOB}$						-1		$b_{AOB}X_{B,AOB}$
Decay of $X_{B,NOB}$							-1	$b_{NOB}X_{B,NOB}$

The relevant additional process is described in the third row, entitled 'cometabolic degradation'. In this process cometabolic substrate, S_C, is removed at the expense of ammonium oxidizing biomass, $X_{B,\,AOB}$ and this dependency is characterized by a transformation yield. 'T. T' quantifies the units of biomass consumed per unit of cometabolic substrate removed. It is further, assumed that Monod kinetics are applicable to describe the removal kinetics.

The model was encoded in the free open source software Aquasim (www.aquasim.eawag.ch). For model implementation, specific operation and design parameters for the pilot-scale rapid sand filtration were taken from Albers et al. (2015). The pilot column had a diameter of 30 cm and a depth of 80 cm sitting on top of 30 cm of support material. The filters were operated at an average hydraulic loading rate of 3.7 m h^{-1}. Based on our earlier experience, the pilot scale column was modelled as three completely mixed biofilm reactor compartments in series. Estimates of biofilm area, biofilm thickness, and other important system biokinetics and hydrodynamic parameters were based on available knowledge or best estimates. All details are provided in Deliverable 4.4. Typical output of the model is presented in figure 29.

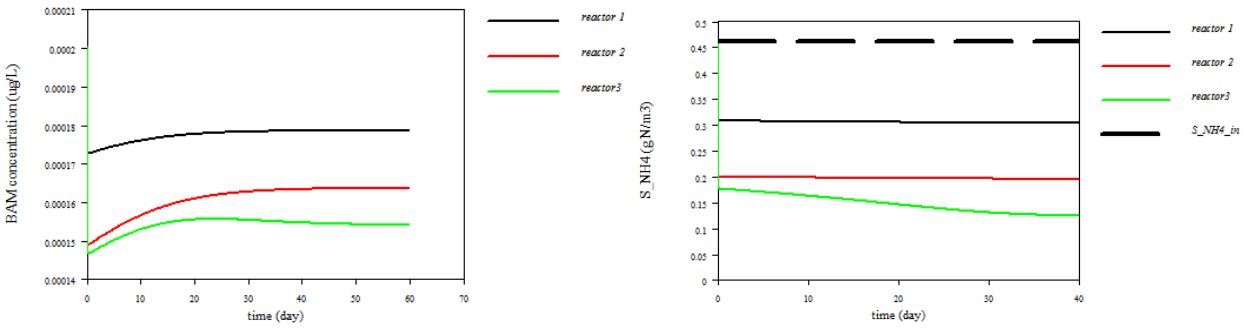


Figure 29. Model predicted effluent concentrations of cometabolically removed pollutant (BAM, left) and the primary growth substrate (ammonium-N, right) as a function of time, at different depths of the pilot-reactor.

3.4.4 Incorporate a Model for Oligotrophic Growth on micropollutant with a Biofilm Dynamics model in a multipopulation reactor-scale process model

The above mentioned continuum biofilm-bioreactor model that describes nitrification in a pilot-scale rapid sand filter (deliverable D4.2) were also enhanced by explicit consideration of an additional process: an oligotrophic strain that specifically degrades a trace pollutant. The model is presented in the matrix below (table 10).

Table 10 – Petersen Matrix Summarizing the Stoichiometry and Rate Expressions for Two step Ammonium Oxidation and Trace Pollutant Removal by as Specific Degrader Strain

Component→ / Process ↓	S_O	S_{BAM}	S_{NH4}	S_{NO2}	S_{NO3}	$X_{B,AOB}$	$X_{B,NOB}$	$X_{B,MSH1}$	Rates
Growth of $X_{B,AOB}$	$-\frac{3.43-Y_{AOB}}{Y_{AOB}}$		$-\frac{1}{Y_{AOB}}$		$\frac{1}{Y_{AOB}}$	1			$\mu_{AOB}\frac{S_{NH4}}{K_{NH_4}^{AOB}+S_{NH4}}\frac{S_{O2}}{K_{O_2}^{AOB}+S_{O2}}X_{B,AOB}$
Growth of $X_{B,NOB}$	$-\frac{1.14-Y_{NOB}}{Y_{NOB}}$			$-\frac{1}{Y_{NOB}}$	$\frac{1}{Y_{NOB}}$		1		$\mu_{NOB}\frac{S_{NO2}}{(K_{NO_2}^{NOB}+S_{NO2})}\frac{S_{O2}}{(K_{O_2}^{NOB}+S_{O2})}X_{B,NOB}$
Growth of $X_{B,MSH1}$		$-\frac{1}{Y_{MSH1}}$						1	$\mu_{MSH1}\frac{S_{BAM}}{K_{BAM}^{MSH1}+S_{BAM}}X_{B,BAM}$
Decay of $X_{B,AOB}$						-1			$b_{AOB}X_{B,AOB}$
Decay of $X_{B,NOB}$							-1		$b_{NOB}X_{B,NOB}$
Decay of $X_{B,MSH1}$								-1	$b_{MSH1}X_{B,MSH1}$

The relevant additional process is described in the third row, entitled 'growth of '$X_{B, MSH1}$'. In this process the trace pollutant substrate, S_{BAM}, is removed by the specific degrader $X_{B, MSH1}$, and this

removal results in growth of $X_{B, MSH1}$ captured by a growth yield Y_{MSH1}. Y_{MSH1} quantifies the units of biomass produced per unit of substrate processed. It is further, assumed that Monod kinetics are applicable to describe the removal kinetics. In addition to growth, the strain is subject to loss (last row in Matrix): this term includes all possible loss mechanisms such as loss via endogenous decay, biomass detachment, activity loss, protozoan grazing, etc.

This model was encoded in the free open source software Aquasim (www.aquasim.eawag.ch) and the model implementation, specific operation and design parameters for the pilot-scale rapid sand filtration were taken from Albers et al. (2015) as described above. In Albers et al. (2015), the BAM degrading *Aminobacter* MSH1, was inoculated in the columns to stimulate BAM degradation via bioaugmentation. This strain was initially mixed in the top 20 cm of the column; hence the top 20 cm was modelled as two equally sized completely mixed biofilm reactor compartments of 10 cm depth, followed by a third reactor of 60 cm depth. Estimates of biofilm area, biofilm thickness, and other important system biokinetics and hydrodynamic parameters were again based on available knowledge or best estimates.

All details are provided in Deliverable 4.3 and Deliverable 4.4. Typical output of the model is presented in figure 30.

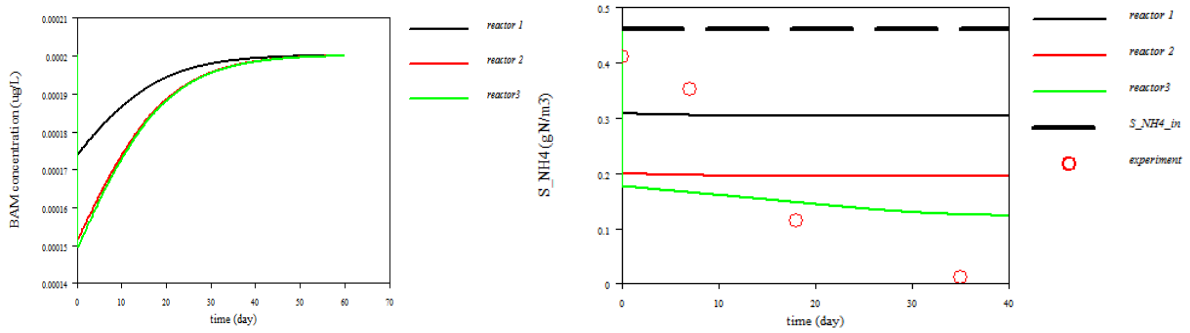


Figure 30. Model predicted effluent concentrations of trace pollutant (BAM, left) and the primary pollutant (ammonium-N, right) as a function of time, at different depths of the pilot- reactor. Clearly, removal of BAM only happens transiently, as the BAM degrader only transiently survives in the pilot-reactor.

3.5 WP5 Application and reliability – Evaluation and adjustment of BIOTREAT systems for large scale applications

The focus of this part of BIOTREAT was on large-scale application of the developed technologies. The research included laboratory-scale reactors and column experiments carried out in the laboratory and at waterworks. The metabolic remediation strategy used the BAM-degrading *Aminobacter* sp. MSH1 as model organism for remediation of BAM-polluted water, while the cometabolic strategy used the ammonium oxidizing bacterial (AOB)-mixed culture HANDS to remediate water polluted by carbamazepine (CBZ), sulfamethoxazole (SMX) and benzotriazole (BTZ). Inoculation with the BAM-degrading *Aminobacter* sp. MSH1 resulted in up to 75% removal of 0.2 µg/l BAM giving concentrations in the purified water well below the 0.1 µg/l legal threshold limit. Though, it was difficult to maintain efficient degradation for longer time periods due to loss of MSH1-bacteria. Loss of bacteria was diminished by immobilising the degrader bacteria on the 'intermedi-

ate carriers developed within WP1. No clear decrease in micropollutant concentrations was observed at the large-scale cometabolic facility added the HANDS culture. This was attributed to the short contact time (20 – 30 min) and the relatively low N/micropollutant ratio of waterworks sand filters.

3.5.1 Larger scale immobilised systems for treating pesticide-contaminated groundwater at waterworks for drinking water production by means of metabolic processes

In this part of BIOTREAT several pilot scale sand filter systems have been in operation to test the efficiency of the BIOTREAT metabolic strategy for remediation of pesticide polluted drinking water. The system established in Denmark consists of an aeration basin followed by two rapid sand filters that could be operated in parallel or series (figure 31). Ports for water sampling were placed before aeration for raw water analysis; between the aeration basin and filter 1 (inlet water), after filter 1 (filter 1 outlet), and after filter 2 (filter 2 outlet). Backwashing could be performed at desired time intervals.

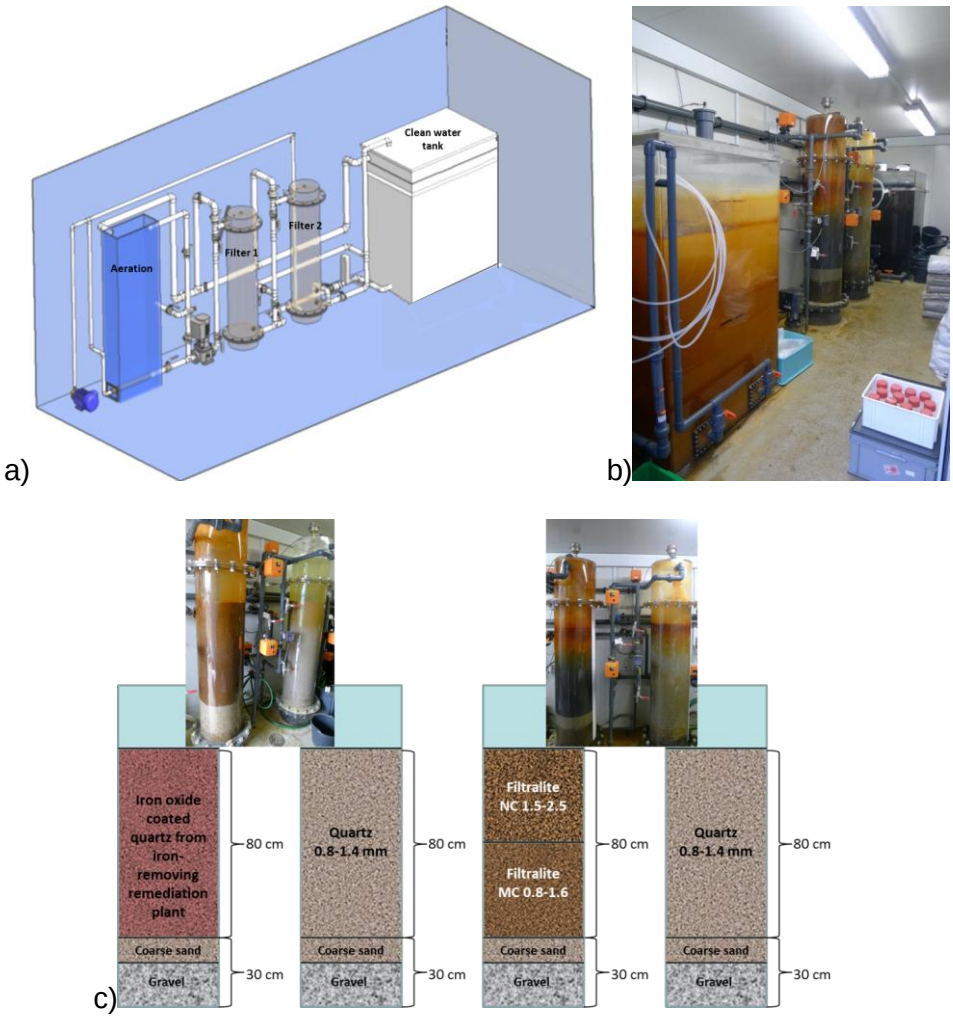


Figure 31. A) Schematic view of the pilot waterworks established in Denmark. B) Photo of the pilot waterworks. C) Filter composition during experimental phase 1 (left) and phase 2+3 (right). The diameter of the filters is 40 cm.

The pilot waterworks was established 15 km west of Copenhagen and received anaerobic ground water from two waterworks abstraction wells, both polluted by BAM (0.13-0.22 µg/l BAM).

In the first experimental phase, quartz sand from the remediation plant in operation with a natural population of microorganisms (filter 1) was compared to fresh quartz sand (filter 2; figure 32). Inoculation with MSH1 resulted in an immediate drop in BAM concentrations from 0.15 to 0.08 µg/l in both filters (figure 32a) corresponding to a reduction of 50% during the first two days of operation (figure 32b). With time, however, the capacity to degrade BAM decreased and after 23 days only 5-11% of the inlet BAM concentration was degraded, most in filter 1. At the same time that the capacity for BAM degradation disappeared, the numbers of MSH1-bacteria in the filters also decreased (figure 32c).

In the second experimental phase, removal of BAM in a filter with the high porosity products Filtralite NC and Filtralite MC (filter 1) was compared to filter 2 with fresh quarts sand similar to the sand used in filter 2 in phase 1. As observed in phase 1, inoculation with MSH1 resulted in an immediate reduction in the outlet BAM concentration to well below the legal threshold limit (figure 32a). The, BAM removal was most efficient in the Filtralite filter, to which also sorption occurred. In both filters the capacity to remove BAM declined with time, most rapidly in the filter with the Filtralite products. As in the first phase this decline coincided by a significant reduction in MSH1-bacteria, especially from the top part of the filters were also the highest densities of bacteria were seen (figure 32c). Predation on MSH1 cells was indicated by a twofold increase in the protozoan density (figure 32d).

In phase 3, backwashing was avoided in filter 2 by changing from parallel to serial operation mode, where precipitated iron oxides were removed by backwashing in filter 1 only. This was done to investigate whether loss of MSH1-bacteria could be diminished ensuring longer maintenance of the capacity for BAM degradation. Following inoculation, the BAM concentration decreased to below 0.1 µg/l in the outlet water of filter 1 corresponding to a reduction of about 50%. A further 50% reduction was achieved in filter 2 giving overall removal efficiency in the system of 75%. With time, however, the BAM-removal capacity decreased, though most rapidly in the backwashed filter 1. Also the number of protozoa increased after MSH1 inoculation. In filter 1 (filter material not exchanged between phases 2 and 3) the response was fastest with most of the increase occuring within a day after inoculation in both top and depth. Hereafter the number of protozoa decreased along with the decrease in MSH1-bacteria.

In conclusion inoculation with the BAM-degrading *Aminobacter* sp. MSH1 resulted in up to 75% removal of 0.2 µg/l BAM giving concentrations in the purified water well below the 0.1 µg/l legal threshold limit. In addition no unwanted BAM degradation products were observed and no effects of inoculation on important filter processes like oxidation of iron and ammonium were seen. Though, it was difficult to maintain efficient degradation for longer time periods due to loss of MSH1-bacteria. Significant losses of bacteria were especially observed during backwashing. By avoiding backwash procedures the degradation was prolonged but bacteria and hence degradation activity was still lost with time.

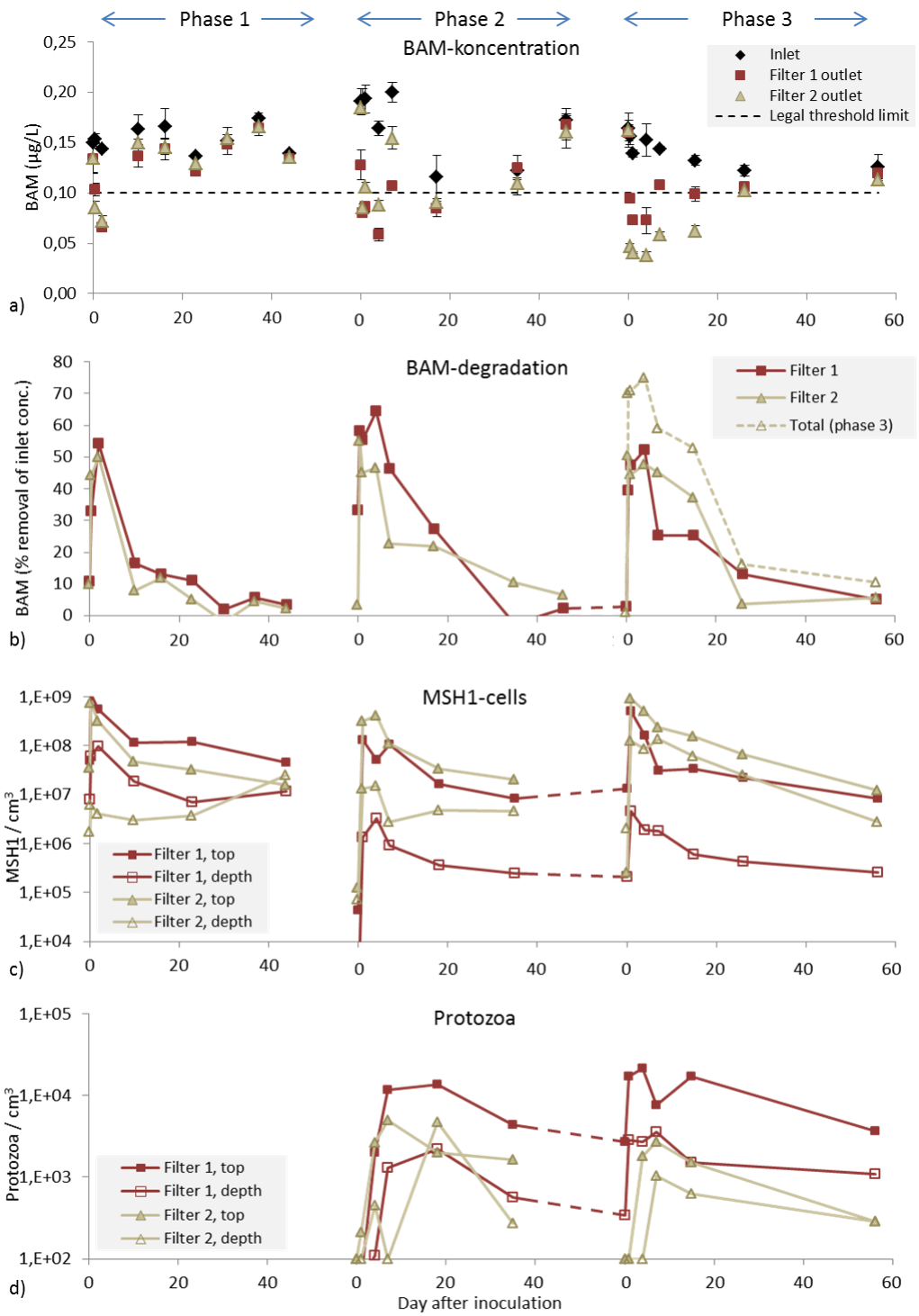


Figure 32. BAM degradation, density of *Aminobacter* sp. MSH1 and growth of protozoa in the pilot waterworks sand filters established in Denmark. A) Concentration of BAM in in- and outlet water from the two filters; B) BAM removal efficiency in % of inlet BAM concentration; C) *Aminobacter* MSH1 density and D) protozoan density. During phase 3, the filters were operated in series, and the BAM concentration shown for the outlet of Filter 2 therefore represents the concentration after passage through both filters. Error bars are standard deviation of three replicate water samples. Protozoan densities below the detection limit of $2\times10^2/cm^3$ are set to half the detection limit. The first data point in each phase is just before inoculation.

Figure 33: Picture of the pilot scale sand filter system operated in Egenhoven (Belgium)

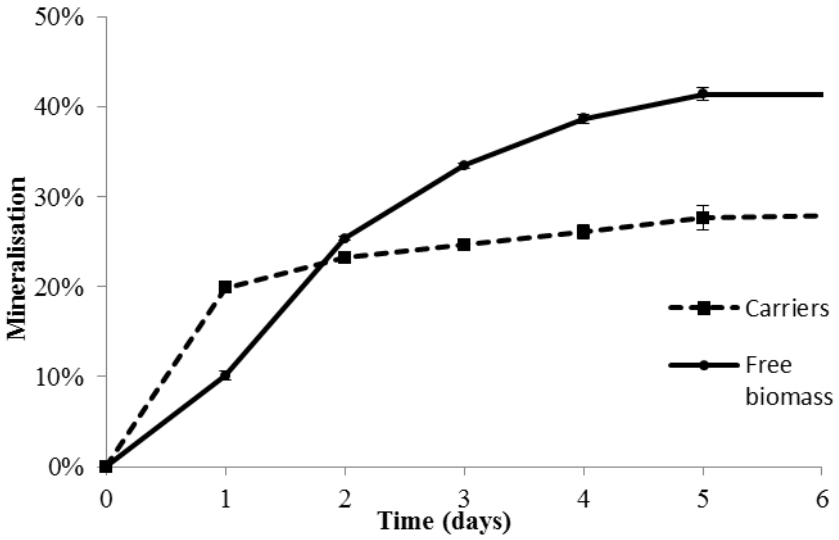


Figure 34. Mineralization of ^{14}C-BAM by *Aminobacter* sp. MSH1 as free suspended cells (solid line) or immobilized in carriers (dashed line) which were used for bioaugmenting columns 2 and 3, respectively at Egenhoven. Values are averages (n=3) with standard deviation.

A pilot scale system was also established at a drinking water production plant of 'De Watergroep' in Egenhoven, Belgium (figure 33). Similar to the Danish facility, the bioremediation strategy was based on bioaugmentation with the BAM degrading *Aminobacter* sp. MSH1. In this facility it was investigated whether bacteria entrapped in alginate and immobilized on a porous carrier (Seachem Matrix; input from WP1; see chapter 3.1.3) improved the BAM degrading efficiency.

Before inoculation, the MSH1 cell suspension and MSH1 containing carriers were tested for BAM mineralizing activity (figure 34). BAM mineralization was much faster for the MSH1 carriers as after one day already 20% of the added ^{14}C-BAM was mineralized as opposed to 10% for the free suspended cells. However, the BAM mineralization percentage increased to a greater extent the four following days for the free suspended cells compared to the carriers.

BAM in the influent and the effluent of the four columns of the pilot scale system was regularly measured (figure 35). In the first pilot scale experiment, the column 2, bioaugmented with free suspended MSH1 cells, showed clear degradation of BAM during the first two weeks after bioaugmentation, reaching in the first week even 84.8% degradation. However, starting from day 17 after bioaugmentation, the difference between the BAM concentration in the influent and the effluent became gradually smaller. In the column 3, containing the MSH1 containing carriers, residual BAM concentrations in the effluent were lower compared to the influent indicating also degradation of BAM. Moreover, during the 44 operational days, the BAM concentration in the effluent was always below the norm of 0.1 µg/l (figure 35a). As time progressed, an increasing trend in BAM concentration in the effluent of column 3 with regard to the influent concentration was observed. However, on day 44 there was still 52.3% degradation in column 3 as opposed to only 18.7% degradation in column 2. BAM analysis for control columns 1 and 4 resulted in no statistically significant difference from the results of the influent concentration. It is therefore concluded there was no BAM degradation in these columns.

In the second pilot experiment, column 3 showed the same BAM removal as observed in the first experiment (figure 35b). Interesting is that the MSH1 carriers were already 6 months old (stored at 4°C) and apparently did not diminish in BAM-degrading activity as the freshly made. Column 2 showed improved BAM removal, i.e., 50% compared to the first experiment (30%), which could be related to an improved BAM mineralization capacity and/or the recirculation.

The effluent of the two inoculated columns (column 2 and 3) was regularly sampled and loss of *Aminobacter* cells from the filters was determined by quantifying the numbers of the BAM catabolic genes *bbdA* and *bbdB* by qPCR in the effluent (figure. 36).

Samples of the sand filter material at the top (20 cm) of the columns were regularly taken after bioaugmentation. For column 3, these samples contained some MSH1 carriers; however, at this point DNA was only extracted from sand and not from carriers. The sand filter samples were used to quantify the presence of MSH1, *bbdA* and *bbdB* over time with qPCR (figure 37).

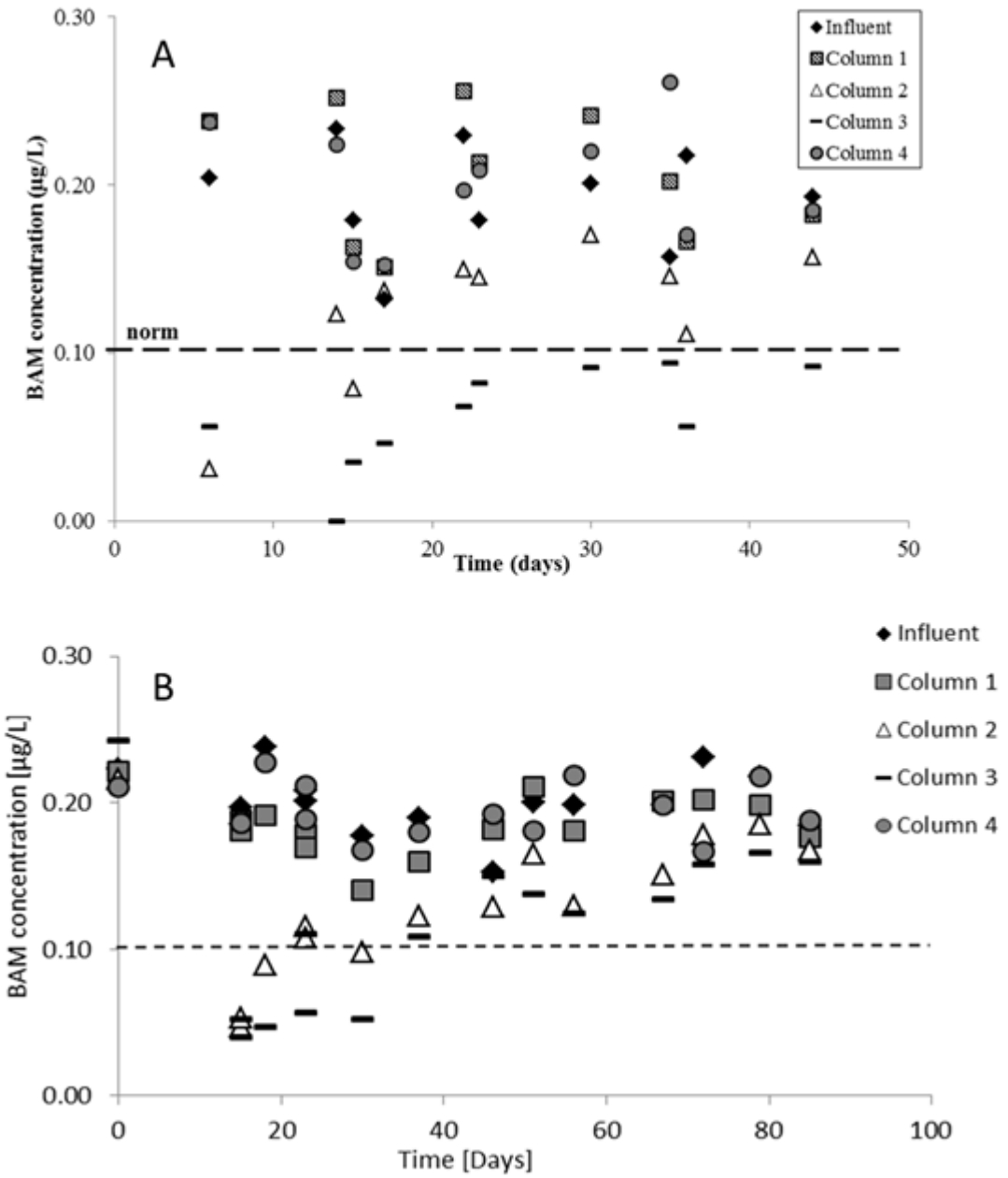


Figure 35. BAM concentrations in the influent and effluent of the four columns after bioaugmentation of column 2 and 3 in the (A) first pilot experiment and the (B) second pilot experiment. The dashed line indicates the drinking water norm of 0.1 µg/l for pesticide residues.

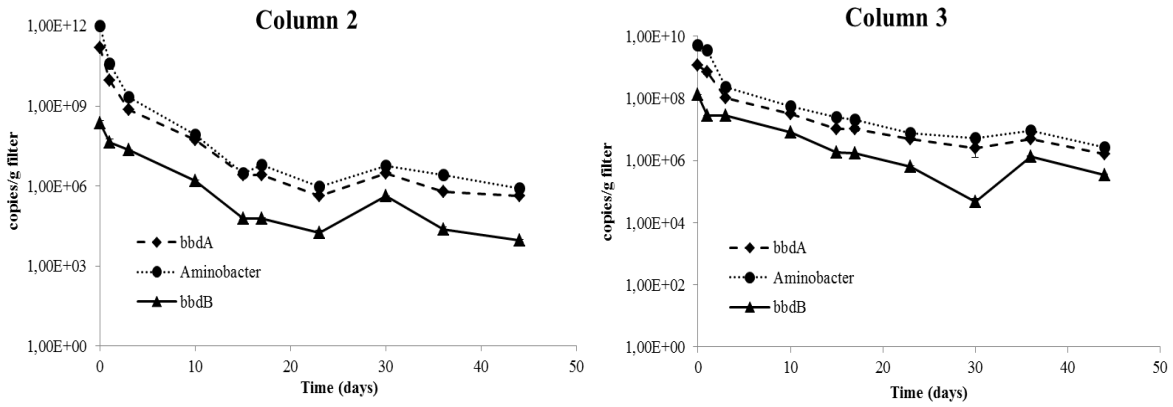


Figure 36. Number of copies of the 16S rRNA gene of *Aminobacter*, and of *bbdA* and *bbdB* genes in the effluent from column 2 (left) and column 3 (right). Time 0 is the day of inoculation. Values are averages (n=2) and maximum-minimum values are included on the graph.

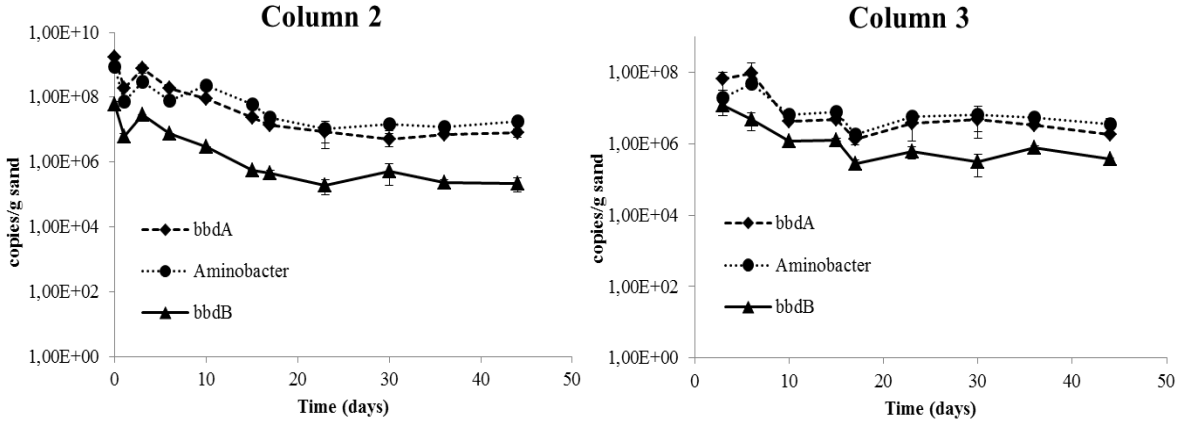


Figure 37. Number of copies of the 16S rRNA gene of *Aminobacter*, and *of bbdA* and of *bbdB* genes in the sand filter samples taken from column 2 (left) and column 3 (right). Day 0 is the day of inoculation. Values are averages (n=4) with standard deviations.

To conclude, it was observed that the presence of MSH1 in the top of the columns decreased significantly during the first two weeks of operation but to a lesser extent for column 3. The MSH1 cells in column 3 have a higher BAM mineralization capacity than those in column 2, since *bbdB* was proportionally more present.

It can be concluded that the bioaugmentation with MSH1 in the sand filter columns by using MSH1 immobilized carriers showed most promising results. Degradation of BAM to concentrations below the norm were achieved for at least a period of 4-5 weeks in both pilot scale experiments for column 3 at an average of 70%. In column 2, to which free MSH1 cells were added, BAM was only degraded efficiently in the first two weeks in the first experiment with an average BAM removal of 30% in the first 30 days of operation. In the second pilot scale experiment, BAM removal was improved to 50% by recirculating the inoculum for 5 days over the column before starting normal operations. The inoculation of free suspended cells in the sand filters is too inefficient at this point as most cells are immediately lost due to shear and bad initial attachment of MSH1 cells to sand. Carriers provided considerable improvement for reducing the loss of cells during inoculation. However, design of the carriers and the pilot system should be further improved to guarantee a long term (6 – 12 months) operation.

3.5.2 Immobilised systems for treating water contaminated with pharmaceuticals by means of cometabolic processes

Medium-scale sand filter reactors were also operated to investigate cometabolic degradation of micropollutants by ammonium-oxidizing bacteria (AOB). One sand filter acted as a control reactor, other sand filters were test reactors in which the co-metabolic conversion of micropollutants by means of AOB's was tested under different operation conditions. Both reactors were always fed with artificial water, i.e., local tap-water with addition of micropollutants and, for the test reactor, with addition of 2 to 4 mg ammonium-N/l.

At the start, the test sand filters were seeded with an AOB-mixed culture designated HANDS. The culture was previously tested in batch lab-scale experiments and showed the capacity for co-metabolic conversion of certain micropollutants. Three micropollutants were selected and added to the influent of the sand filters, namely carbamazepine (CBZ), sulfamethoxazole (SMX) and benzotriazole (BTZ). SMX and BTZ were previously shown to be co-oxidized by AOBs while no conversion was obtained with CBZ (acting as a negative control in the sand filter tests).

No problems were ever encountered with the nitrification in the test sand filter: the latter remained stable and complete under the different process conditions tested (continuous feeding with artificial water, containing 6 to 8 mg O_2/l). The nitrification was not only demonstrated by means of the frequent N analyses (> 95% NH_4^+ conversion), but also by means of a clear drop in dissolved oxygen (DO) and in pH (compared to the control reactor).

The operation parameters of the two sand filters that were altered in the course of the experiment were the influent flow rates and hence the hydraulic retention times (HRT), and the micropollutant concentrations (table 11). Since it considered rapid sand filters, a short HRT of 20 to 40 min was aimed at. The dosages of the micropollutants were started at relatively high concentrations (100 µg/l) as also used in the lab-scale batch tests. During the test, the micropollutant concentrations in the influents were stepwise lowered down to 0.3 µg/l (of each micropollutant). Since the ammonium-N concentration in the influent of the test sand filter could not be increased because of the limited amount of oxygen in the influent water (in the range of 8 mg O_2/l), a change in influent flow rate was always accompanied by a change in N-load. The results of the micropollutant concentration analyses of influents and effluents of different test periods are summarized in Table 12.

Table 11. Overview of the most important process parameters of the different successive test periods of the two medium scale sand filter reactors

	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Period 7	Period 8
Test period (days)	12 – 44	45 - 50	51 – 76	77 – 83	84 – 103	104-144	145-253	254-293
Reactor volume (L)	2.2	2.2	2.2	2.2	2.2	2.2	2.2 ↓1.5**	1.5
Influent NH_4^+-N concentration (mg/l)	2	2	2.5	2.1	3.0	Alteration° < 0.015 3.8	3.0	4.8
Micropollutant concentration* (µg/l)	100	100	10 – 15	10 – 15	3 – 5	3 – 5	3 – 5	0.3 – 0.9
HRT (hours)	1	3.6	9	21	5	5	5 ↓ 3.5	3.5
N load (mg N/d)	± 110	± 34	± 14	± 5	± 37	± 38	± 38	± 49
Micropollutant load* (µg/d)	5500	1390	55 – 85	25 - 35	30 – 50	30 - 50	30 – 50	3 – 9
N/Micropollutant ratio***	7	8	67	56	308	475	475	4083

*of each micropollutant added, namely SMX, CBZ and CBZ
** due to several samplings of colonized sand
*** for the total of micropollutants added (3 types until period 5; afterwards 2 types)
°Alteration of 1 week with no N addition and 1 week with N addition

Although it could be demonstrated in several batch tests that the biomass in the test pilot sand filter reactor still had the capacity for co-metabolic conversion of the micropollutants tested, no clear decrease of these could ever be measured in the continuous sand filters. Hence, it had to be concluded that the specific process conditions in these sand filter reactors did not allow for the co-metabolic conversion of the micropollutants investigated. Limiting factors for this co-metabolic conversion in a continuous sand filter reactor are most probably the short contact time (20 – 30 min) and the relatively low N/micropollutant ratio. Further batch tests confirmed that the colonized sand still had co-metabolic activity for the removal of SMX. Depending on the influent N-concentration and, consequently, on the N-load and N/micropollutant ratio, the co-metabolic conversion was relatively low and ranged between 6% and 22%. A comparison between the conditions in the batch tests and the applied operational parameters in the pilot scale test showed that, even if the very long contact times of 4 days would be feasible, the theoretical co-metabolic removal of the investigated micropollutants would still be too low to give removal efficiencies which would justify any effort to apply this technology in practice. Therefore, it was concluded that, although the biomass in the test sand filter still contained co-metabolic activity, the overall process conditions of a sand filter did not allow for co-metabolic conversion of the micropollutants tested.

Table 12. Overview of the micropollutant concentrations in the influents and corresponding effluents of the two medium scale sand filter reactors (average values and standard deviations)

CONTROL sand filter	**Period 1b**	**Period 2**	**Period 3**	**Period 5**	**Period 6**	**Period 8**
INFLUENT	n = 5	n = 3	n = 6	n = 4	n = 5	n = 6
SMX (µg/l)	84 ± 5	106 ± 15	13.6 ± 0.9	4.9 ± 0.3	4.2 ± 0.1	0.39 ± 0.11
CBZ (µg/l)	82 ± 4	102 ± 13	13.8 ± 0.6	3.4 ± 0.1	4.8 ± 0.1	0.37 ± 0.12
EFFLUENT						
SMX (µg/l)	83 ± 4	109 ± 7	11.0 ± 0.5	4.1 ± 0.2	3.7 ± 0.5	0.37 ± 0.12
CBZ (µg/l)	84 ± 1	103 ± 17	13.4 ± 1.1	3.9 ± 0.2	5.3 ± 0.1	0.39 ± 0.22
TEST sand filter	**Period 1b**	**Period 2**	**Period 3**	**Period 5**	**Period 6**	**Period 8**
INFLUENT	n = 5	n = 3	n = 6	n = 4	n = 5	n = 6
SMX (µg/l)	83 ± 11	95 ± 24	14.0 ± 1.6	5.4 ± 0.3	3.9 ± 0.2	0.86 ± 0.03
CBZ (µg/l)	81 ± 10	97 ± 21	14.1 ± 0.8	3.8 ± 0.3	4.6 ± 0.1	0.88 ± 0.04
EFFLUENT						
SMX (µg/l)	92 ± 3	114 ± 13	10.9 ± 1.2	4.3 ± 0.6	3.4 ± 0.1	0.77 ± 0.13
CBZ (µg/l)	90 ± 3	111 ± 13	14.4 ± 0.8	4.1 ± 0.2	5.0 ± 0.1	0.86 ± 0.07

n = amount of samples

3.6 WP6 INTEGRATED ASSESSMENT AND PERFORMANCE VALIDATION

With the view to developing a water purification technology that is also competitive it is necessary to analyse both the financial and the environmental aspects of its further exploitation. In BIOTREAT financial and environmental aspects of the developed technologies have been assessed using cost-benefit analysis (CBA) and life cycle impact assessment (LCIA) approaches. It is concluded that well relocation is the cheapest option also having the lowest environmental impact. This option, however, depends on the distance to unpolluted water resources and is often not possible. The CBA showed that the BIOTREAT metabolic technology, especially used in combination with the BIOTREAT carrier is the most attractive technology especially for small scale drinking water production plants due to the relatively low investment costs. The BIOTREAT metabolic technology had an environmental impact similar to granular activated carbon GAC treatment. Immobilisation of degrading bacteria on carriers increased the environmental impact, due to the additional materials needed, but probably the impact could be reduced using other carrier materials as to example ordinary sand.

3.6.1 Performance and cost analysis

The final goal of the BIOTREAT project was the development of a prototype system ready for commercialisation. The cost-benefit analysis (CBA) was performed to check whether commercialisation is a viable option.

The first step in the CBA was the collection of data including inputs from the small-scale tests carried out mainly in WP3 and the large scale prototype test carried out mainly in WP5 (see chapter 3.3 and 3.5). Financial data were gathered from the drinking water production experts mainly from the drinking water companies involved in the project. The next step was to build in the collected data in a CBA model. The model was then used to calculate the costs for the following four scenarios:

- **BIOTREAT bioaugmentation technology**: in this scenario a Danish waterworks is upgraded with the bio-augmentation technology under development in this project. In particular, sand filters are inoculated with BAM-degrading bacteria. After the sand filter, a UV disinfection step is added, to make sure the number of bacteria leaving the filters does not exceed the legal threshold. Every other aspect of the waterworks operation remains unchanged.
- **BIOTREAT bioaugmentation technology + carrier:** This scenario is essentially equivalent to the one above, with the difference that carrier materials are used to improve the stability of the bacterial population. The materials used are a mineral substrate and sodium alginate.
- **Granular activated carbon (GAC) filter**: GAC has been identified as the most likely competitor technology for BIOTREAT. In fact, several waterworks in Denmark already apply this technology in order to remove micropollutants. In this scenario, a GAC filter bed is installed and operated in an existing Danish waterworks, followed by a UV disinfection system, to ensure microbiological safety. Every other aspect of the waterworks operation remains unchanged.
- **Well relocation**: a possibility for a waterworks that risks exceeding the legal limits for micropollutants is to close the contaminated wells and open new ones elsewhere, with concentrations of micropollutants under the legal limits. In this scenario, the waterworks operation is not changed with any additional process, but it involves dismantling the existing wells and opening new ones.

Part of the data interpretation was the sensitivity analysis by which we could identify the factors that influence the costs the most. Giving answer to the questions:

- How do the four scenarios relate to each other financially?
- Is the BIOTREAT technology financially competitive with alternative approaches?

The results of the CBA, calculated for a drinking water production plant producing 800,000 m^3 per year, are given as investment costs/yearly costs, as well as yearly operating costs, see table 13 and 14.

Table 13. Investment costs and yearly costs of the four scenarios.

Scenario name	Investment costs	Yearly costs
BIOTREAT bio-augmentation technology	€ 80,000	€ 150,000
BIOTREAT bio-augmentation technology + carrier	€ 170,000	€ 80,000
Granulated active carbon (GAC) filter	€ 840,000	€ 60,000
Well relocation	€ 390,000	€ 0

Table 14. Yearly operating costs

Scenario name	Operating costs per year	Operating cost per m^3 drinking water
BIOTREAT bio-augmentation technology	€ 160,000	€ 0.20
BIOTREAT bio-augmentation technology + carrier	€ 90,000	€ 0.11
Granulated active carbon (GAC) filter	€ 120,000	€ 0.15
Well relocation	€ 30,000	€ 0.04

Based on the sensitivity analysis it is concluded that the calculated values for the BIOTREAT bio-augmentation technology scenario and BIOTREAT bio-augmentation technology + carrier scenario cannot be given within the 30% deviation range. Further research is needed to get more reliable data on the amount of inoculum needed per m^3 drinking water, the costs of one litre high-density BAM-degrading bacteria and the (regeneration) costs of the carrier.

Based on the available data it is concluded that the well relocation scenario is the cheapest scenario. But because the length of the transportation pipe has a large effect on the costs of the well replacing scenario, the well replacement scenario is only cheaper if the new well is placed within a few kilometres from the water production plant (3.5 kilometres for the 800,000 m^3 per year scenario). If clean water is more than a few kilometres away, implementing a BAM removal strategy becomes the best solution.

For the larger drinking water production volumes the GAC filter is the cheapest BAM removal technique. The BIOTREAT technology, especially the BIOTREAT bio-augmentation technology + carrier is the most attractive technology for small scale drinking water production plants due to the relatively low investment costs.

Because the data for the CBA is not reliable enough, it cannot be concluded that the BIOTREAT technology is ready for commercialisation. However, the technology looks promising from a financial point of view. If further commercialisation is attempted, it is advised to focus on relative small drinking water production plants where clean water is more than a few kilometres away.

In general, the amount and quality of primary data available for the CBA was insufficient to deliver an accurate picture of the BIOTREAT technology. This is not the fault of the project, since lack of data and uncertainty is typically inherent to any CBA applied to an emerging technology. There is no experience of applying this technology in waterworks, and the field tests carried out were limited. As a consequence, many assumptions had to be made in the study, and more often than not, expert judgement rather than recorded data had to be used. This is the reason why in the conclusions summarised in the previous sections we generally abstain from providing quantitative assertions, but instead give qualitative judgements.

We think nevertheless that this study has shed light on the potential environmental impact and the market potential of the BIOTREAT technology, benchmarking it against current alternatives and pointing out key areas where either more knowledge is needed or attention should be put in order to minimize the environmental impacts and costs of this technology.

3.6.2 Elaboration of Life Cycle Assessment (LCIA)

The life cycle impact assessment (LCIA) for the four scenarios (see chapter 3.6.1) under study, according to 15 impact categories is shown in figure 38. The basis for comparison, or functional unit, is the provision of 1 m^3 of drinking water with BAM levels under the legal threshold of 0.1 µg/l. The graph shows the LCIA scores in relative numbers, whereby for each impact category the highest scoring alternative is normalized to a score of 1, and the remaining ones, are expressed in relation to that score. In terms of interpreting the graph, the higher the LCIA score the higher the environmental impact.

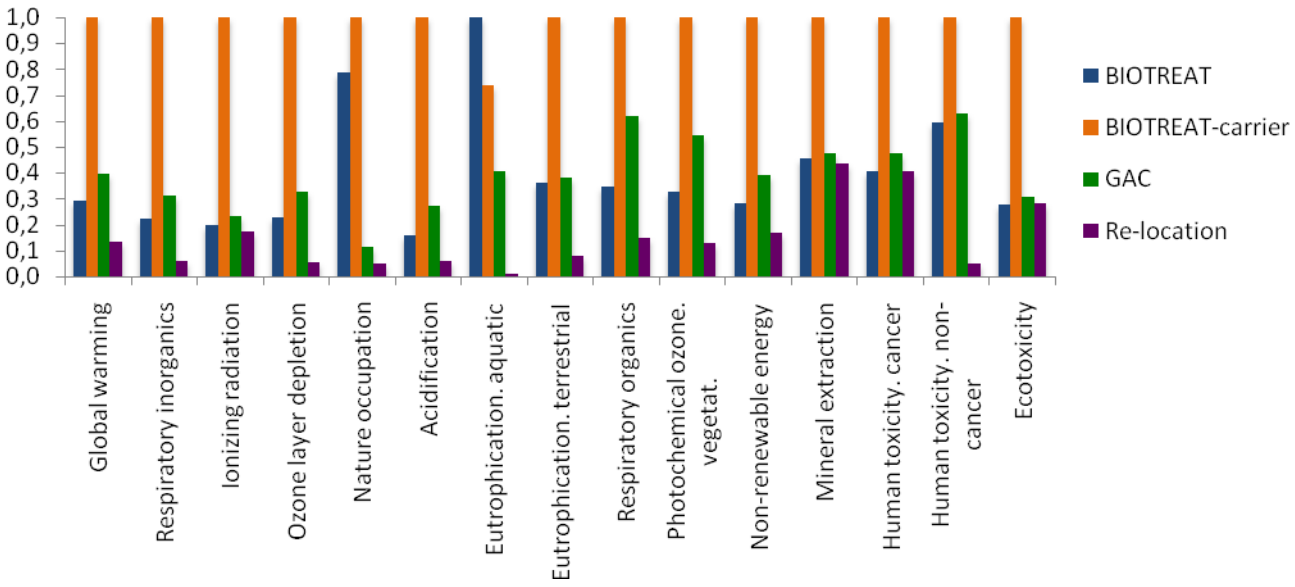


Figure 38. Life cycle impact assessment results.

The main aspects to highlight from figure 38 are the following:

- The BIOTREAT-carrier alternative appears as the one with the highest environmental impact, in all categories but the one on aquatic eutrophication, where BIOTREAT has a higher environmental impact. The relatively high impact of BIOTREAT-carrier is associated with production of the mineral carrier material.
- The BIOTREAT alternative obtains somewhat lower impact scores than the GAC alternative, with the exception of the impact categories of land occupation and aquatic eutrophication. The higher impact in these categories is mainly related to the production of the bacterial inoculum.
- In all 15 impact categories, the best performance is attributed to the well-relocation alternative. This can be explained by the fact that even though shutting down a well and opening a new one is material- and energy-intensive, it is a single operation where the lifespan of the

new well is 50 years, and as opposed to the other alternatives, it does not involve any additional and continuous input of energy or chemicals to treat polluted water.

At this level, i.e. midpoint, where all impact categories are assessed separately, we can identify well re-location as the best alternative from the ones assessed. From the remaining alternatives, which involve tackling water pollution rather than just avoiding it, it is interesting to see that BIOTREAT is approximately at the same impact level as GAC, as long as carriers are not used. We delve into the carriers issue in next the section.

Sensitivity analysis on carrier materials

As we have seen, in most impact categories the use of carrier materials leads to the BIOTREAT-carrier alternative being the least desirable. It must be taken into account that assessing this alternative leads to uncertain results, given that:

- At this early stage of technology development it is not clear what type of mineral carrier would be used in a full-scale application.
- The amount of mineral carrier, alginate, and $CaCl_2$ needed per m^3 of treated water is based on laboratory tests, which are far from being optimized when compared to a full-scale application.

We have checked the influence in the results of considering a different carrier material. Based on conversations with Institut za Mikrobioloske Znanostiin Techologije who have developed the carriers, it was decided that the most environmentally friendly option would be to use sand (instead of expanded clay) extracted from the waterworks site. In this way, we use a less energy-intensive material and avoid the transport step. The results of this sensitivity analysis are shown in figure 39.

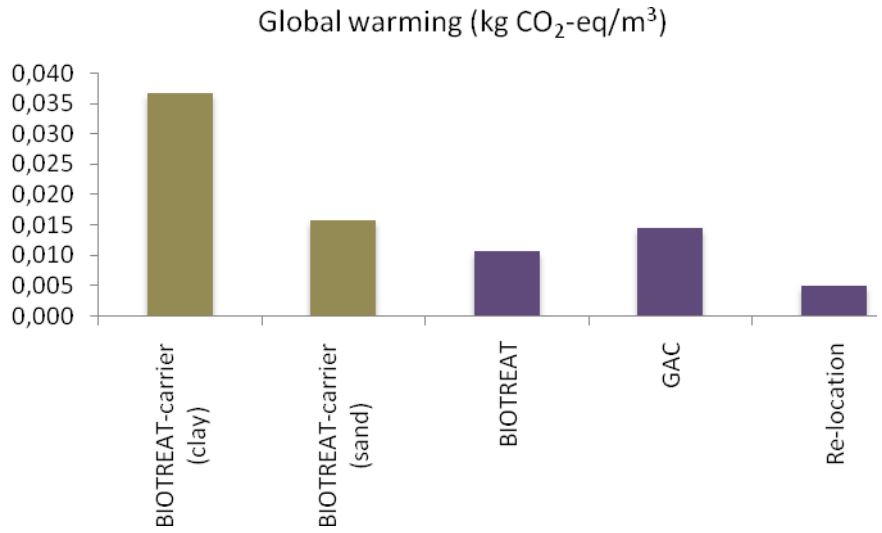


Figure 39. Greenhouse-gas emissions (GHG) of BIOTREAT-carrier alternative considering sand as carrier material. Comparison with clay as carrier material (default) and with the other alternatives.

The results of this sensitivity analysis are shown for greenhouse-gas emissions (GHG) in figure 39. In this figure the first bar shows the overall emissions for BIOTREAT-carrier, with our initial assumption on expanded clay, whereas the next one shows the emissions when local sand is considered instead. The remaining bars show the emissions for the other alternatives, for reference. It can be seen that the choice of carrier material has a substantial effect on the GHG-intensity of BIOTREAT-carrier. When local sand is considered, the GHG emissions are reduced by more than 50%. At this point, BIOTREAT-carrier is approximately at the same level of impact than BIOTREAT and GAC.

Finally, a glimpse of the implications that applying the BIOTREAT technology would have on a typical Danish waterworks, in terms of GHG emissions is provided. The question we try to answer was how much would the carbon footprint of drinking water production increase by applying the concepts developed in BIOTREAT. In order to do this we consider the life cycle impact of a waterworks (excluding distribution to the consumer) and add on top of it the impact of either BIOTREAT or BIOTREAT-carrier (sand). The result can be seen in figure 40.

This figure shows that the BIOTREAT concept would have a rather small impact as far as drinking water production is concerned. BIOTREAT would increase GHG emissions by less than 4% whereas the use of carriers would lead to an increase of above 5% over current emissions. Most of the impacts associated with drinking water production are related to the electricity used for operation, and the additional emissions associated with BIOTREAT are comparatively small.

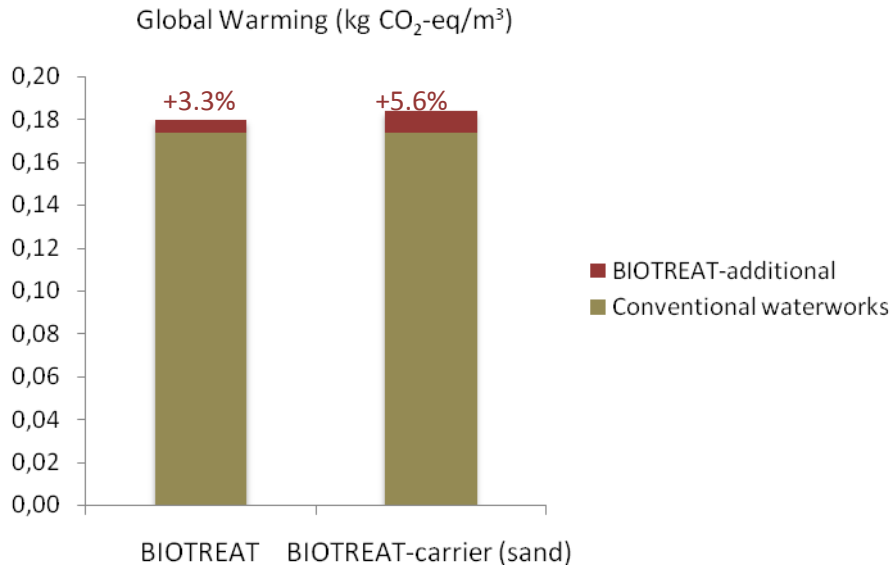


Figure 40. Additional GHG emissions in the waterworks life cycle caused by implementing BIOTREAT in a Danish waterworks.

The following overall conclusions of the LCIA can be drawn:

- The environmental impact of applying the BIOTREAT technology is related to the production of the bacterial inoculum and to the energy used by the additional UV system that needs to be installed in the waterworks.
- When compared to GAC adsorption, BIOTREAT has an impact of a similar magnitude, with the exception of land use and aquatic eutrophication, where its performance is poorer. In the other impact indicators the actual scores are very often lower than GAC, implying a lower impact, but this cannot be taken at face value, given the uncertainty involved in the study.
- The performance of BIOTREAT is poorer in all impact indicators when compared to the alternative of well re-location. However this is only the case if we assume that the re-located wells will have the same pumping energy needs than current ones.
- Inoculation frequency can be increased without jeopardizing the environmental performance. This is true as long as bacteria are produced at large-scale, with efficiency similar to that of industrial yeast production.
- If the inoculum were produced at a small scale and inefficiently, as done in the pilot plant tested near Copenhagen, the environmental impact of BIOTREAT would be much higher than that of GAC. It is therefore of the utmost importance to make sure bacteria are produced efficiently.
- Applying the BIOTREAT concept in a Danish waterworks would increase the carbon footprint of drinking water production by less than 5%. If water distribution to consumers were accounted for, this percentage would be even lower.

- The use of carriers increases the impact of the BIOTREAT technology, due to the additional materials needed: mineral carrier, sodium alginate and calcium chloride. The impact of producing these materials and replacing them on an annual basis does not compensate for the reduced inoculation frequency. As we have seen, producing the inoculum involves a relatively low impact if done at industrial scale.
- The impact of this option is very sensitive to the inoculation frequency, with the impact quickly increasing if augmentation is required more than once per year.
- But it must be mentioned that if augmentation is kept to once per year, and instead of a high-value material it is decided to use sand mined in the waterworks area, the impact of using carriers is substantially reduced, making it competitive – in environmental terms – with GAC adsorption.
- The point above, added to the fact that the data used to assess carriers was insufficient (from small lab-scale experiments), prevents us from advocating against carriers use. Fur-

ther research is encouraged in order to get a better picture of its feasibility and benefits, especially if it turns out to be the only way of stabilizing the bacteria in the filter.

- Applying BIOTREAT with carriers in a Danish waterworks would increase the carbon footprint of drinking water production by around 5-6%, if an optimistic scenario is considered. If water distribution to consumers were accounted for, this percentage would be even lower.

4 POTENTIAL IMPACT

Millions of tonnes of organic xenobiotics are used each year worldwide, for example the pesticides used in agricultural production and applied to paved urban areas, along railways and roads, and to farmyards. Pharmaceuticals are considered to be emerging contaminants that enter the environment via the application of manure and sewage sludge to farmland, via effluent from wastewater treatment plants and via accidental spillage during various industrial applications. As a result of these extensive environmental inputs, European water bodies such as rivers, lakes and groundwater that are used as drinking water resources have become contaminated with a wide range of organic micropollutants. Clean drinking water is a limited resource, not only in Third World countries but also in many European regions. It is becoming increasingly difficult to meet the quality standards of the European Drinking Water Directive regarding chemical residues of pesticides and other micropollutants and many potential drinking water resources have been abandoned due to exceedance of the EU limit values for these micropollutants. The EU limit value for individual pesticides in drinking water is 0.1 µg/l, while that for multiple pesticides is 0.5 µg/l (Directive 98/83/EC). These concentrations are very low, and many water bodies have therefore had to be abandoned as drinking water resources (Stockmarr, 2005). There is therefore an urgent need to develop new sustainable water treatment technologies that satisfy the EU quality standards for drinking water. The aim of BIOTREAT was to develop new sustainable treatment technologies that exploit the potential of microorganisms to mineralise a range of pollutants without the accumulation of unwanted degradation products. The impacts of the project on water supply and science are discussed below.

4.1 SCIENTIFIC IMPACTS

The incredible capacity of soil and subsurface microbial communities to degrade a wide range of xenobiotic compounds is well known. In most cases where microorganisms are used for bioremediation, however, the biodegradation processes operate as “black box” systems, and detailed knowledge of the microorganisms and the metabolic processes and pathways involved is lacking. As complete mineralisation of organic micropollutants may require specialised strains that are rarely present in the indigenous microbial community (e.g. Topp et al., 2000; Sørensen et al., 2007), bioaugmentation with degradative bacteria has been proposed as a strategy for remediation of contaminated drinking water resources.

BIOTREAT has provided essential knowledge opening the “black box” of bioremediation of drinking water polluted by micropollutants. Furthermore, the project has filled the gap between laboratory studies and full-scale application. Selected main findings are listed below:

- The finding of a hitherto unknown potential for degradation of micropollutants, including bentazone, MCPA and BAM, but only in in certain waterworks sand filters.

- Development of Low Flux Filters to be used for isolation of bacteria degrading contaminants at trace concentrations.
- Complete genetic characterization of the BAM degrading bacterium *Aminobacter* MSH1, including biochemical characterization of the BAM-degrading amidase *bbdA* gene.
- Development of techniques for immobilization of degrader bacteria on specific carriers to be used for bioaugmentation of waterworks sand filters to improve degrading efficiency.
- Determining the effect of assimilabe organic carbon on degradation of target micropollutants.
- Elucidation of the carbamazepine degradation pathway in sewage sludge and waterworks sand filters, including accumulation of recalcitrant transformation products.
- Identification of protozoa in waterworks sand filters and clarification of their role as predators feeding on introduced bacteria.
- Development of tools for interpretation of molecular fingerprinting pattern (e.g. PCR-DGGE), including the range-weighted richness (Rr), dynamics (Dy) and functional organization (Fo) parameters for the different experimental setups.
- The development of a model framework to describe growth-linked biodegradation of trace-level pollutants in the presence of coincidental carbon substrates and microbes.
- The development of a continuum biofilm model to describe metabolic and cometabolic trace pollutant removal scenarios in waterworks sand filter systems.
- Demonstration of the BIOTREAT metabolic strategy in pilot-scale sand filters inoculated with the BAM degrading *Aminobacter* bacterium.
- Defining degrading kinetics constrains limiting the cometabolic degradation strategy at pilot-scale systems simulating waterworks sand filters.
- The development of a prospective environmental and economic assessment for biotreatment of micropollutants in drinking water resources.

These main scientific findings have all been published in high-ranking international journals with peer review or are submitted or in preparation for publication. All together 39 scientific articles have been published, 2 have been submitted, 10 are in preparation for publication and several more are foreseen. In addition the findings have been disseminated at international conferences, symposia and workshops.

4.2 Output to stakeholders, managers and experts within the water supply

Within BIOTREAT, new and much needed sustainable biotechnologies have been developed for remediating contaminated water from subterranean and surface drinking water resources. The technologies focus on remediation of waters polluted by organic chemicals at trace pollutant concentrations typically at the µg to ng range. The basis of the proposed technologies is bioaugmentation, which in the present context is the introduction of specific degrading microorganisms or microbial consortia into existing sand filters at waterworks. The basic idea is that once the technology has been developed for waterworks sand filters it can easily be transferred also to other types of submerged biofilter systems such as mobile biofilters placed close to groundwater abstraction

wells, sand barriers between surface waters and abstraction wells and subterranean protective barriers established to prevent micropollutants from entering into aquifers (figure 41).

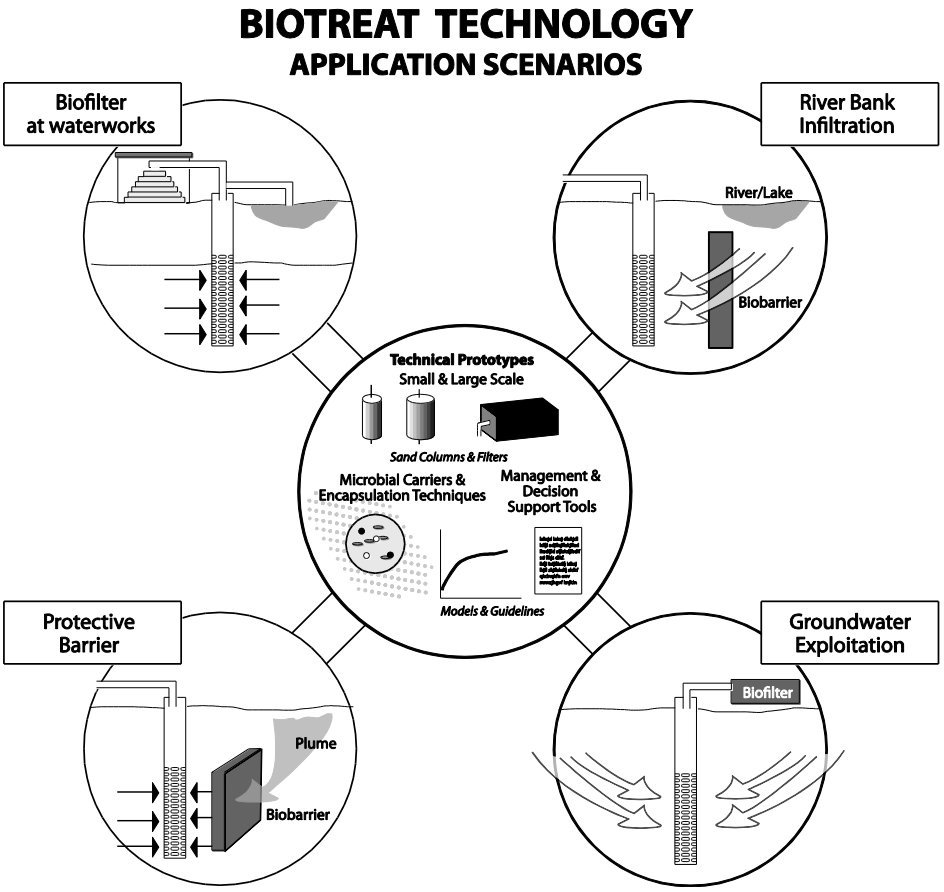


Figure 41. Possible applications of the BIOTREAT filter technologies

Little is known about degradation at trace pollutant concentrations and bioaugmentation of waterworks sand filters, as a technology to remediate polluted drinking water has not been attempted previously. BIOTREAT has exploited the potential of both metabolic and cometabolic processes to remediate water resources abandoned or in danger of being abandoned due to the presence of low concentrations of micropollutants and thereby enable them to meet the EU quality criteria for drinking water. Addition of degrader bacteria to waterworks sand filters as a technology to remediate drinking water polluted by trace concentrations is not trivial, but it is of great interest. Several laboratory studies have shown that such technology may be feasible, but until now no one has striven to investigate this in pilot-scale simulating real waterworks sand filters. The challenges going from laboratory-scale to full-scale are many. Firstly, the short retention time of sand filters leaves very short time for degradation to occur, secondly pollutant concentrations may be in the ng to µg/l range and thus too low to support growth of degrader bacteria at the prevailing flow conditions, and thirdly at real filter conditions loss of bacteria may occur either because of detachment of degrader bacteria due to the rapid flow across the filters, because of predation by native protozoa in the filters, or due to loss during waterworks operation procedures. These challenges have all been experimentally addressed in BIOTREAT and suggestions for further improvement of the technology have been put forward.

In general the metabolic strategy showed to be the most effective. Pilot scale field studies examining the potential for bioaugmentation with the BAM-degrading *Aminobacter* sp. MSH1 in sand filters operated at the rapid flow conditions prevailing in waterworks filters resulted in up to 75% removal of 0.2 µg/l BAM, giving concentrations in the purified water below the 0.1 µg/l legal threshold limit. In addition, no BAM degradation products were observed and no adverse effects of inoculation on important filter processes such as oxidation of iron and ammonium were observed. Though, it was difficult to maintain efficient degradation for longer time periods due to loss of MSH1-bacteria which occurred mainly during backwashing operation. By avoiding backwash procedures, the degradation was prolonged, but bacteria, and hence degradation activity, were still lost with time.

The exploitation of the cometabolic strategy was less convincing. Although laboratory experiments showed cometabolic degradation of several trace pollutants this could not be demonstrated in a test pilot sand filter reactor. Based on this it was concluded that the specific process conditions in waterworks sand filters do not allow for co-metabolic conversion, at least not for the micropollutants investigated in the project.

The above research has been disseminated to a broader audience of stakeholders, managers and expert within the water supply at the BIOTREAT open-end users meetings, with partition of the BIOTREAT end-users board. At the meetings BIOTREAT partners gave presentations of the main findings followed by intensive discussions. At the first meeting which was held 4th December 2012 in Leuven, Belgium, the end-users still had the possibility to influence the project in relation to immediate needs in water supply. To example it was emphasized by the end-users to carry out experiments at real trace pollutant concentrations and at conditions simulating real waterworks operational procedures, including realistic hydraulic retention times and backwashing. The end-users were also asked to contribute to the selection of model pollutants of practical relevance in European water supply. The second open-end user meeting were the main outcome of the project were given was held 4th November 2014 in Copenhagen, Denmark. At this meeting there was a thorough discussion with the end-users about next steps to be taken for further exploitation of the developed technologies in practice.

The BIOTREAT technologies has also been disseminated to a broader audience of stakeholders, managers, experts and scientists involved in bioremediation and biotechnology in general at the following meetings:

- 5th European Bioremediation Conference, 4-7th July Chania, Crete, Greece. (Special session about KBBE)
- Environmental Microbiology and Biotechnology – In the frame of the knowledge-based bio and green economy (EMB2012) 10-12th April 2012, Bologna, Italy
- “Bioremediatie van pesticiden” Leuven, Belgium 27/3/2014. A mini-symposium on pesticide biodegradation for Flemish stakeholders including talks on general biodegradation of pesticides and results from the BIOTREAT project.

A technology action plan including a business plans has been developed for 1) The BIOTREAT bioaugmentation technology focusing on remediation of BAM in waterworks sand filters and 2) the BIOTREAT carrier technology for immobilization of degrader bacteria. The technology action plan identifies the main competing technologies being granular activated carbon (GAC) treatment or

more simple well relocation. The latter is in many cases a more obvious solution, but is often not an option because clean drinking water resources may not be available in the vicinity. A comparison of the operating costs of the BIOTREAT techniques with the operating costs of the traditional GAC technology shows that the BIOTREAT techniques are mainly financially interesting for small water suppliers.

Three steps have been envisaged in the process towards the development of a commercial prototype where the funding of BIOTREAT has covered the pre-competitive phase. The next step will be the innovative phase involving further development of the technology focusing on more competitive issues. The innovative phase will include large-scale demonstration of the technology emphasizing specific applications for the treatment of contaminated water using e.g. 1) existing sand filters at waterworks 2) mobile biofilters placed close to groundwater abstraction wells, 3) sand barriers between surface waters and abstraction wells and 4) subterranean protective barriers established to prevent micropollutants from entering into aquifers. The innovative phase also includes a survey of regulatory and legislative issues related to the use of microorganisms in water treatment processes. Funding for this phase may be sought through the EUREKA network for market-oriented R&D, and/or private sources. The final step will be Commercialisation where specific bio-augmentation applications and encapsulation technologies are launched on the market (figure 42). It is currently discussed by the BIOTREAT partners how to go from the pre-competitive phase to the innovative phase.

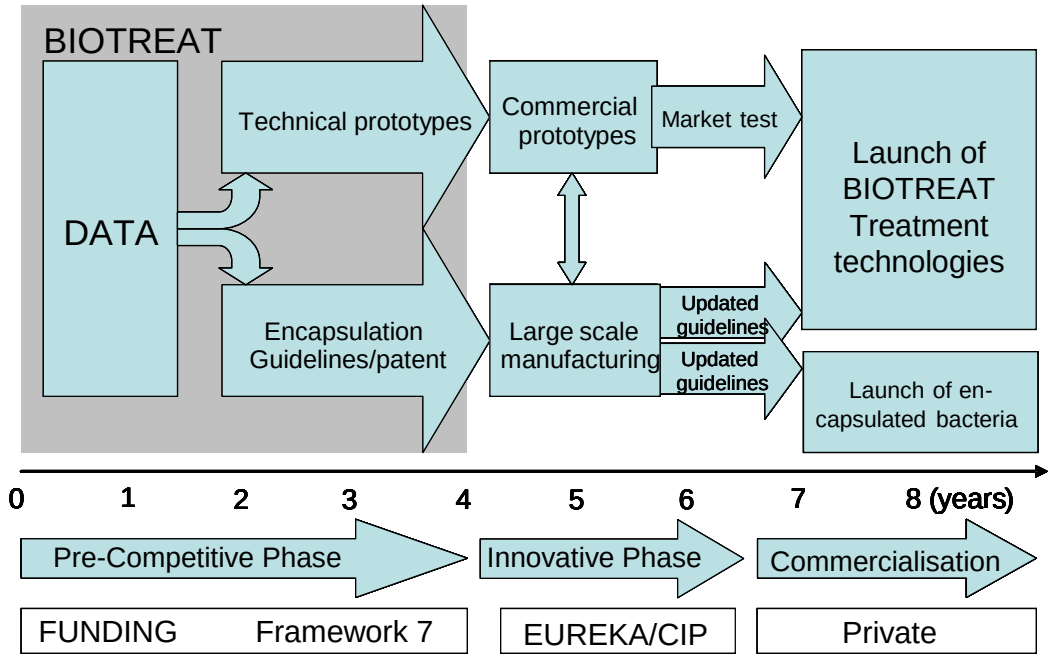


Figure 42. From research impact to economic impact

4.3 OUTPUT TO THE GENERAL AUDIENCE

The general public has been informed about the project through the BIOTREAT homepage (www.biotreat-eu.org) and through a project leaflet which was distributed broadly at different meetings and events. Both the homepage and the leaflet give broad introduction to the project.

4.4 EDUCATION OF EARLY STAGE RESEARCHERS

The education of a new generation of environmental scientists able to meet problems of tomorrow concerning the increased pollution of water resources by organic chemicals is urgent. Several early stage researchers have been educated with funding from BIOTREAT including both PhDs and postdoc fellows. BIOTREAT has therefore contributed to the education of the next generation environmentalists having specific expertise in bioremediation technologies. Three young fellow workshops have been held within BIOTREAT to improve teamwork and to provide a long lasting network for the fellows also beyond BIOTREAT. The workshops were arranged by the fellows themselves and included sessions about knowledge sharing, dissemination and improvement of presentation skills.

5 CONCLUSIONS

The multidisciplinary BIOTREAT consortium has been working for more than 48 month on the development of new water treatment technologies to be used for remediation of drinking water polluted by pesticides, pharmaceuticals and other micropollutants. Two remediation strategies have been examined; a strategy based on **metabolic processes** for micropollutants where metabolically degrading bacteria were available and a **cometabolic strategy**, relying on unspecific degradation by methane or ammonium oxidizing bacteria, for target pollutants where specific degrading bacteria have not been described. The results of the metabolic strategy was most promising as up to 75% of the investigated pollutant (BAM) was degraded at realistic waterworks flow conditions with a hydraulic retention time of about 20 minutes. The technology may be improved by measures preventing loss of degrader bacteria from the filter as it was difficult to maintain efficient degradation for longer time periods. Immobilisation of degrader bacteria on carriers was a promising technology as it diminished loss of degrader bacteria from the filter and thereby prolonged the period of effective degradation. The cometabolic strategy was less promising as no micropollutants removal was observed going from laboratory scale experiments to large-scale filters operated at waterworks sand filter conditions. Cost-benefit analysis and life cycle impact assessments showed that the BIOTREAT metabolic strategy was competitive to granular activated carbon treatment being the most likely competitor technology to the BIOTREAT technology. Both cost and environmental impact increased using the BIOTREAT carriers in combination with the BIOTREAT metabolic remediation strategies. However, cost and environmental impact could possibly be lowered selecting other carrier materials as to example ordinary quartz sand.

6 PROJECT PUBLIC WEBSITE & CONTACT DETAILS

Project website: www.biotreat-eu.org

Table 15: List of participants: List of participants:

No	Organisation Name (Legal Entity)	Organisation Short Name	Participant	Country
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