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Combining reverse osmosis and microbial degradation for remediation of drinking water contaminated with recalcitrant pesticide residue

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ABSTRACT

Groundwater contamination by recalcitrant organic micropollutants such as pesticide residues poses a great threat to the quality of drinking water. One way to remediate drinking water containing micropollutants is to bioaugment with specific pollutant degrading bacteria. Previous attempts to augment sand filters with the 2,6-dichlorobenzamide (BAM) degrading bacterium *Aminobacter niigataensis* MSH1 to remediate BAM-polluted drinking water initially worked well, but the efficiency rapidly decreased due to loss of degrader bacteria. Here, we use pilot-scale augmented sand filters to treat retentate of reverse osmosis treatment, thus increasing residence time in the biofilters and potentially nutrient availability.

In a first pilot-scale experiment, BAM and most of the measured nutrients were concentrated 5–10 times in the retentate. This did not adversely affect the abundances of inoculated bacteria and the general prokaryotic community of the sand filter presented only minor differences. On the other hand, the high degradation activity was not prolonged compared to the filter receiving non-concentrated water at the same residence time. Using laboratory columns, it was shown that efficient BAM degradation could be achieved for >100 days by increasing the residence time in the sand filter. A slower flow may have practical implications for the treatment of large volumes of water, however this can be circumvented when treating only the retentate water equalling 10–15% of the volume of inlet water. We therefore conducted a second pilot-scale experiment with two inoculated sand filters receiving membrane retentate operated with different residence times (22 versus 133 min) for 65 days. While the number of MSH1 in the biofilters was not affected, the effect on degradation was significant. In the filter with short residence time, BAM degradation decreased from 86% to a stable level of 10–30% degradation within the first two weeks. The filter with the long residence time initially showed >97% BAM degradation, which only slightly decreased with time (88% at day 65). Our study demonstrates the advantage of combining membrane filtration with bioaugmented filters in cases where flow rate is of high importance.

1. Introduction

Clean drinking water is one of the Earth's most important resources, and yet the quantity and quality of water available for human consumption is increasingly deteriorated due to anthropogenic activities (UNESCO, 2018). The production, use and disposal of numerous chemicals in industry, private households and agriculture is now recognized to lead to a widespread occurrence of organic micropollutants in the environment, including groundwater used for drinking water production (Barbosa et al., 2016). In addition to known groundwater pollutants, new screening methods using non-target high resolution mass spectrometry (Kiefer et al., 2021) will most likely reveal hazardous chemicals that exceed threshold limits for drinking water, for example 0.1 µg/L for pesticides and relevant degradation products in the European Union. Not all contaminants can be removed by conventional technologies and not all technologies are equally acceptable due to high
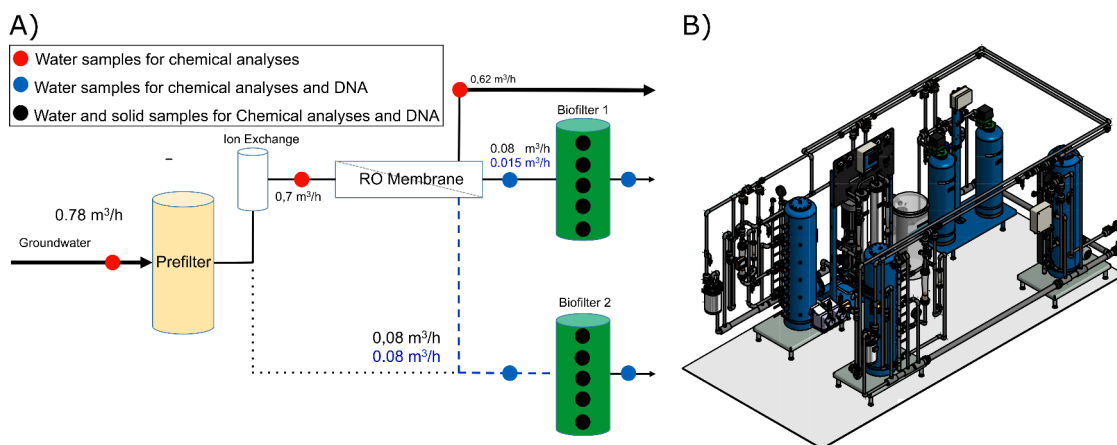
Fig. 1. Pilot water work for water treatment by RO membrane and bioaugmentation of sand filters. A) Scheme of the pilot waterworks showing ports for sampling of water and filter material. Black dotted lines are the piping during the first pilot experiment. Blue punctured line is piping during the second pilot experiment where both biofilters received membrane concentrate. B) Technical drawing of the final pilot waterwork prior to production. The flow in all filters was top down with a moderate pressure. All piping was made of hard PVC.

energy consumption or other drawbacks, such as unwanted by-product formation, incomplete mineralization or generation of chemical waste. Hence, a continuous need exists, to provide remediation solutions to remove low concentrations of organic micropollutants, such as recalcitrant pesticide residues, from drinking water.

Rapid sand filters at waterworks are known to contain a substantial diversity of bacteria (Albers et al., 2015a; De Vet et al., 2009) which may have the potential to degrade some organic micropollutants (Hedegaard and Albrechtsen, 2014; Richter et al., 2008; Zearley and Summers, 2012). However, those organic micropollutants that compromise groundwater quality are often recalcitrant and are thus not readily degradable by the natural microbial populations in sand filters. The introduction of specific micropollutant degrading bacteria into rapid sand filters at waterworks has therefore been suggested as a treatment technology for polluted groundwater (Benner et al., 2013). Recently, such a strategy was found to be very promising for degradation of the pesticide metaldehyde in sand filters (Castro-Gutierrez et al., 2022). Metaldehyde is a common surface water contaminant but rarely detected in groundwater aquifers, presumably due to its relatively high degradability (typical half-life in soil of 5 days, http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/446.htm). 2,6-dichlorobenzamide (BAM) is an example of a widespread recalcitrant organic micropollutant (pesticide residue) in groundwater, where a bacterial degrader organism, *Aminobacter niigataensis* MSH1 (Nielsen et al., 2021) has been isolated and grown in the laboratory (Sørensen et al., 2007). The MSH1 strain has been shown to have the ability to adhere to sand (Albers et al. (2014), and the genes involved in degradation of BAM have been well characterized (Nielsen et al., 2021; Raes et al., 2019). MSH1 is therefore a promising candidate to provide a proof of concept of bioaugmentation for treatment of drinking water. Previous attempts to add MSH1 to sand filters were, however, only partially successful due to loss of bacteria after an initial period with substantial BAM degradation (Albers et al., 2015b; Horemans et al., 2017).

Reverse osmosis (RO) is a well-known membrane separation technology for producing clean water (permeate stream) as a result of rejecting and consequently concentrating organic micropollutants including pesticide residues as well as inorganic nutrients in a 'waste' stream (retentate stream) of a smaller volume (Fini et al., 2019, 2020; Jamil et al., 2019; Madsen and Søgaard, 2014; Urtiaga et al., 2013). By combining membrane filtration with bioaugmented sand filters less water must be bioremediated, opening for a longer water residence time in the filter and thereby longer time for the pollutants to be degraded. Furthermore, as nutrients are also concentrated in the retentate, the growth and survival of the augmented bacteria may be improved. In the

present study, we therefore made series of pilot and laboratory scale experiments to explore the effect of reverse osmosis and water flow conditions on the long-term survival and BAM-degrading activity of MSH1 in a combined membrane-biofilter treatment system. If successful, the purified retentate could even be used to remineralize the permeate from the filters, hence leading to a remediation technology where the micropollutant is eliminated completely without any loss of water.

2. Material and methods

2.1. Field site description

A pilot waterworks with a RO membrane and two biofilters was installed next to an abandoned drinking water abstraction well with BAM contaminated water (varying over time from 0.3 to 1.7 µg/l). The contamination comes from an old farmyard used for loading and cleaning of sprayers. The well is located at 55°30N; 8°27E.

2.2. Pilot waterworks

The pilot waterworks (Fig. 1) was custom designed for this project and was placed in a portable trailer (l: 401 cm, w: 185 cm, h: 205 cm). The raw groundwater was pumped into a prefilter consisting of a closed steel cylinder filled with a chalk filter material (Nevtraco®, Silhorko-Eurowater A/S, Denmark) in which iron was precipitated after aeration using a compressor. From there the water was split in two lines, one with and one without membrane filtration. The membrane filtration line included an ion exchange unit to remove calcium and magnesium ions to prevent scaling and a reverse osmosis membrane unit consisting of two spiral wound FilmTec XLE-4040 (Dupont, USA) membrane elements in series providing 16.2 m^2 membrane area. The retentate of the filtration being concentrated approx. 10 times was led to a 86 L steel cylinder (h: 135 cm, ø: 30 cm) containing Nevtraco filter material (biofilter 1). The line without membrane filtration was coupled to a similar steel cylinder (biofilter 2), but this could be changed so that both filters received retentate.

Water could be sampled at multiple points along the water flow and water and filter material could be sampled at five different depths of the filters, ~30 cm, ~50 cm, ~70 cm and ~90 cm from the top (Fig. 1A). To sample representative samples the water was left running for at least 200 ml, with as low flow as possible avoiding changing the pressure and flow in the system. Sampling of filter material was done by stopping the flow and inserting a brass pipe through a ball vial with costume made

insert with O-ring to enclose the brass tube. At the end of the brass tube a piece of silicon tube was added with a small clamp, to stop the water for floating out. At each depth a total of 3 times 10 gs was sampled and kept cold until storage in the lab at $-20\ °C$ until extraction. Samples were taken at day 1, 3, 8, 22, 55 and 77. With regards to microbial analyses, we consider the period between day 1 and day 3 to be the beginning of the treatment, from day 4–21 is considered middle and day 22–77 is considered the end of the treatment.

Two experiments were conducted at the pilot waterworks to test the effect of preconcentration using RO membrane and to test the effect of residence time, respectively. Before each experiment the two biofilters and the prefilter was filled with fresh filter material with an overhanging water table of 10 cm. This corresponds to 75 kg of Nevtraco® in each filter. To remove smaller particles all filters were back washed until exiting water was clear. Inoculation with *Aminobacter niigataensis MSH1* was carried out basically as described in Albers et al. (2015b). In brief, MSH1 was grown in 5 L baffle bottle from freeze batch. MSH1 cells were then diluted with sterile tap water in 10 L bottles, to a bacterial number of $\sim 10^{13}$ cells, and transported to the pilot waterworks. The culture was added from the top of the filter, and left for two hours before starting the flow. Final cell density was 10^8 cells/ g of Nevtraco® in each of the biofilters. Before running the pilot waterworks with groundwater the bioaugmented biofilter was treated with nutrient addition for the first 48 h as an improved inoculation strategy developed by Ellegaard-Jensen et al. (2020). For more details on the inoculation, see Supplementary Material. During the period of nutrient addition, the outlet was sampled in order to measure the loss of MSH1 in the inoculation phase (See Section 2.6), this was only done in the second pilot waterwork experiment. During the first 48 h, there was a loss of bacterial cells of 79% and 84% for biofilter 1 and 2 respectively.

We did not include a sterile or non-inoculated control biofilter, since it is well known that BAM is not degraded abiotically and does not adsorb to any significant degree, neither to different soils (Clausen et al., 2004) nor sand filter material (Albers et al., 2015b). We have also tested if any sorption to the Nevtraco material exists, and the sorption coefficient (K_d) was $<0,01$ L/kg (unpublished data), meaning no significant adsorption occurs.

The membrane unit performed successfully during the two pilot waterworks experiments, running for 99 and 100 days, respectively. During operation, the inlet and outlet pressure as well as permeate and retentate flow rates were monitored at each sampling time. The inlet pressure was kept at 16 bar, while the outlet pressure varied within the range of 15.5 to 15.9 bar. The influent water flow rate was 780 L/h in both pilot tests producing 700 L/h permeate and 80 L/h of retentate resulting in a 90% recovery throughout the first pilot test. During the second pilot test, as a result of fouling, recovery slightly dropped, starting with 80 L/h retentate (90% recovery) and ending up with 120 L/h (85% recovery) during the last ~ 60 days.

2.3. Laboratory column experiments

Small column experiments were setup to investigate the influence of MSH1 cell density and residence time on bioaugmented biofilter performance. The small column experimental setup was as the biofilters in the pilot waterwork, except water inflow was bottom up. Three cell densities (10^8, 10^7 and 10^6 MSH1 per gram of Nevtraco®) and four different residence times (~ 14, ~ 28, ~ 53 and ~ 133 min) were tested. The column experiments ran for 104 and 88 days for the cell density and the residence time experiments, respectively. Detailed description can be found in the Supplementary Material. During the first 47 h with an influx of nutrients there was a loss of cells ranging from 15 to 50% of the inoculated MSH1.

In an additional experiment with triplicate columns operated with two different residence times (28 and 133 min.), the influence of short-term changes in residence time on BAM removal was tested after running for 104 days by taking four sets of samples, each sampled after

exchange of at least 6 pore volumes, in the following sequence:

1. Before changing the flow, so at residence times 28 and 133 min.
2. After changing the residence time to 28 min in both sets of columns.
3. After changing the residence time to 133 min in both sets of columns.
4. After changing the flow to the original residence times of each set (28 and 133 min).

2.4. BAM analysis using LC-MS/MS

At the first pilot waterworks experiment, the quantification of BAM was done as described in Ellegaard-Jensen et al. (2020), with the modification of larger sample volume (20 ml) used for solid phase extraction. For the remaining experiments, the sample was simply filtered and injected directly on the LC-MS/MS, see description in the Supplementary Material.

In all experiments, BAM was analyzed as the sole compound because several studies have shown that metabolite formation is not of concern, when inoculating sand filters with MSH1 (Albers et al., 2015b, 2014). It has long been known that the MSH1 strain is capable of mineralizing BAM completely to CO_2 and chloride (Sørensen et al., 2007) and the complete degradation pathway of BAM by MSH1 was recently published (Raes et al., 2019). Also in augmented sand filters, MSH1 was found to mineralize BAM completely (Albers et al., 2014) and in a pilot water-work study with a sand filter bioaugmented with MSH1 no detection of the known BAM metabolites, 2,6- dichlorobenzoic acid (2,6-DCBA), ortho-chlorobenzamide, and ortho-chlorobenzoic acid were detected at any point in time (Albers et al., 2015b).

2.5. Additional water analyses

Water was sampled at selected timepoints for the analysis of different water chemistry during operation of the pilot waterwork. Just after sampling, the water was filtered (0.45 μm, Q-Max polyethersulfone, Frisenette, Denmark) and stored at $-20\ °C$ until analysis. Dissolved organic carbon (DOC), NH_4^+, F^-, Cl^-, Br^-, NO_3^-, PO_4^{3-} (from HPO_4^{2-} and $H_2PO_4^-$,), SO_4^{2-}, Mg^{2+}, K^+, and Ca^{2+} were all measured during both pilot waterworks experiments. DOC was analyzed on a TOC-analyzer (TOC-Vcph, Shimadzu, Japan). Ammonium was analyzed by flow injection analysis on a FIAstar 5000 (FOSS, Sweden) according to ISO 11732:2005 with a quantification limit of 0.005 mg/L. The anions were analyzed by anion chromatography (Metrohm 819 IC detector with a Metrosep A 150/4.0 column, Metrohm, Switzerland). Mg^{2+}, K^+, and Ca^{2+} were analyzed by cation chromatography (Metrohm 819 IC detector, Metrohm, Switzerland).

For the performance of the RO membrane, water was analysed for trace metals by a commercial accredited laboratory (Eurofins, DS/EN ISO 17294m:2016 Inductively coupled plasma mass spectrometry (ICP-MS)).

During operation of the pilot waterwork, dissolved oxygen and pH were measured from the different water taps using a Universal multi-parameter portable meter (Multi 3620, WTW, Germany).

2.6. Total cell count using flow cytometry

Total cell count was measured in the effluent of the two laboratory and the second pilot waterwork experiments. For the two laboratory experiments 2.7 mL samples were taken the first 48 h, during nutrient addition and added 0.3 ml 30% formaldehyde. Samples were stored at 4 °C until analysis, no more than 60 days. A subsample of 1 ml was transferred to 2 ml eppendorf tubes with 1 μl of 1000x SYBR green 1 (SYBR green I, Invitrogen, Life Technologies Corporation, USA) and incubated for 20 min at 30 °C. After staining, samples were measured on Accuri C6 (Becton Dickinson, Lyngby, Denmark) equipped with a 488 nm solid-state laser. Green fluorescence was collected in the FL1 channel at 533 nm with a manual gate setting using side scatter and FL1 selecting

Table 1

Selected water chemistry from the pilot waterworks experiments. Full table can be seen in supplementary material.

Date	Days of operation	Sample tap	NO$_3^-$ (mg/L)	PO$_4^{3-}$ (mg/L)	NH$_4^+$ (µg/L)	DOC (mg/L)
First pilot waterworks experiment						
20/06/19	1	Raw water	68.7	0.043	<6	0.475
20/06/19	1	After ionexchange	68.8	<0.005	<6	0.449
20/06/19	1	Permeate	15.6	<0.005	<6	<0.2
20/06/19	1	Before biofilter 1	501	0.050	<6	3.19
20/06/19	1	After biofilter 1	504	12.1	16	3.51
20/06/19	1	Before biofilter 2	68.3	0.033	<6	2.21
20/06/19	1	After biofilter 2	71.0	4.02	20	0.341
27/06/19	8	Raw water	77.4	<0.005	<6	0.615
27/06/19	8	After ionexchange	75.1	<0.005	<6	0.565
27/06/19	8	Permeate	15.5	<0.005	<6	0.244
27/06/19	8	Before biofilter 1	500	0.076	<6	3.48
27/06/19	8	After biofilter 1	497	0.371	12	3.22
27/06/19	8	Before biofilter 2	67.5	0.023	<6	0.320
27/06/19	8	After biofilter 2	66.8	1.15	24	0.601
04/09/19	77	Raw water	64.9	<0.005	<6	0.716
04/09/19	77	After ionexchange	64.9	<0.005	<6	0.479
04/09/19	77	Permeate	10.4	<0.005	<6	<0.22
04/09/19	77	Before biofilter 1	419	0.059	<6	3.13
04/09/19	77	After biofilter 1	421	<0.005	<6	2.89
04/09/19	77	Before biofilter 2	65.4	<0.005	<6	0.461
04/09/19	77	After biofilter 2	64.6	0.058	<6	0.271
26/09/19	99	Raw water	63.7	0.028	<6	0.666
26/09/19	99	After ionexchange	63.8	<0.005	<6	0.592
26/09/19	99	Permeate	11.1	<0.005	<6	0.234
26/09/19	99	Before biofilter 1	447	<0.005	<6	3.46
26/09/19	99	After biofilter 1	447	<0.005	6	3.44
26/09/19	99	Before biofilter 2	64	0.020	<6	0.660
26/09/19	99	After biofilter 2	64	0.025	<6	0.452
Second pilot waterworks experiment						
20/08/20	1	Raw water	32.9	<0.005	<6	0.734
20/08/20	1	Permeate	6.11	<0.005	<6	0.320
20/08/20	1	Before biofilter 1	229	0.090	<6	2.96
20/08/20	1	After biofilter 1	221	24.2	104	3.05
20/08/20	1	After biofilter 2	224	5.65	15	2.42
27/08/20	8	Raw water	33.7	<0.005	<6	0.365
27/08/20	8	Permeate	5.60	<0.005	<6	<0.2
27/08/20	8	Before biofilter 1	221	0.075	<6	1.91
27/08/20	8	After biofilter 1	220	6.77	206	1.95
27/08/20	8	After biofilter 2	217	0.414	28	1.53
23/09/20	35	Raw water	35.1	0.020	<6	0.375
23/09/20	35	Permeate	5.63	<0.005	<6	<0.2
23/09/20	35	Before biofilter 1	210	0.090	<6	1.39
23/09/20	35	After biofilter 1	211	0.975	7	1.84
23/09/20	35	After biofilter 2	211	0.120	15	1.62

for bacterial cells and used as template for all cell count analysis. Data were processed using the Accuri C6 software. Total cell loss within the first 48 h was calculated by plotting cell concentration against total water volume, and then calculating the area under the curve.

### 2.7. DNA extraction of water and filter material for molecular analyses

Water samples were collected in 100 ml of volume from taps along the reactors, subsequently filtrated through 0.2 µm pore size Micro-Funnel filter units (Pall Corp., Ann Arbor, MI) and the filter were stored in freezer at −20 °C until DNA extraction. Nevtraco samples were collected from the reactors in sterile 15 ml falcon tubes and stored at −20 °C until DNA extraction. The DNA extraction of water samples was done by transferring the filters in the bead tubes supplied with the DNeasy PowerWater Kit (Qiagen, DK) following manufacturer's instructions. For Nevtraco samples, 250 mg was placed in the lysis tube provided with DNeasy PowerLyzer PowerSoil Kit (Qiagen, DK) and DNA was extracted according to the manufacturer's instructions. After the DNA extraction we measured the DNA concentration with Qubit fluorometer (Thermo Fisher Scientific) and the clarity with Nanodrop 2000 (Thermo Scientific). All the DNA extractions, from water and Nevtraco samples, were performed along with negative controls of the extraction kit and of non-inoculated samples.

### 2.8. Quantification of total bacteria and Aminobacter niigataensis MSH1

All the bacterial quantifications based on molecular analyses were performed through qPCR assay with AriaMx Real Time PCR System (Agilent, Denmark) using 10 µl Brilliant III Ultra-Fast SYBR Green Low ROX qPCR Master Mix (Agilent) in 20 µL reactions containing, 1 ng of DNA template, a final concentration of 400 nM for forward and reverse primers.

The primer sequences used in this study are summarized in Table S1. For the total quantification of bacteria the same primer set were used for the amplicon sequencing approach, 341F-806R. The presence of MSH1 was measured by targeting the *bbdA* gene on MSH1 plasmid pBAM1, using the primer set bbdA-F – bbdA-R, and a specific prophage-insertion-region on MSH1 genome (Nielsen et al., 2018) using the primers MSH1-F and MSH1-R, developed in a previous study by Ellegaard-Jensen et al. (2020). All the amplifications were performed with the following qPCR program: 95 °C for 3 min followed by 40 of one step cycles of 95 °C for 15 s, 55 °C for 30 s and followed by a melting curve obtained by increasing the temperature of 0.5 °C increments every 5 s from 55 °C to 95 °C to confirm the specificity of the PCR product.

A calibration curve, to measure absolute amount of *Aminobacter niigataensis* MSH1 was performed, relative to microscopy counts, by serially diluting two standards obtained by DNA extraction from
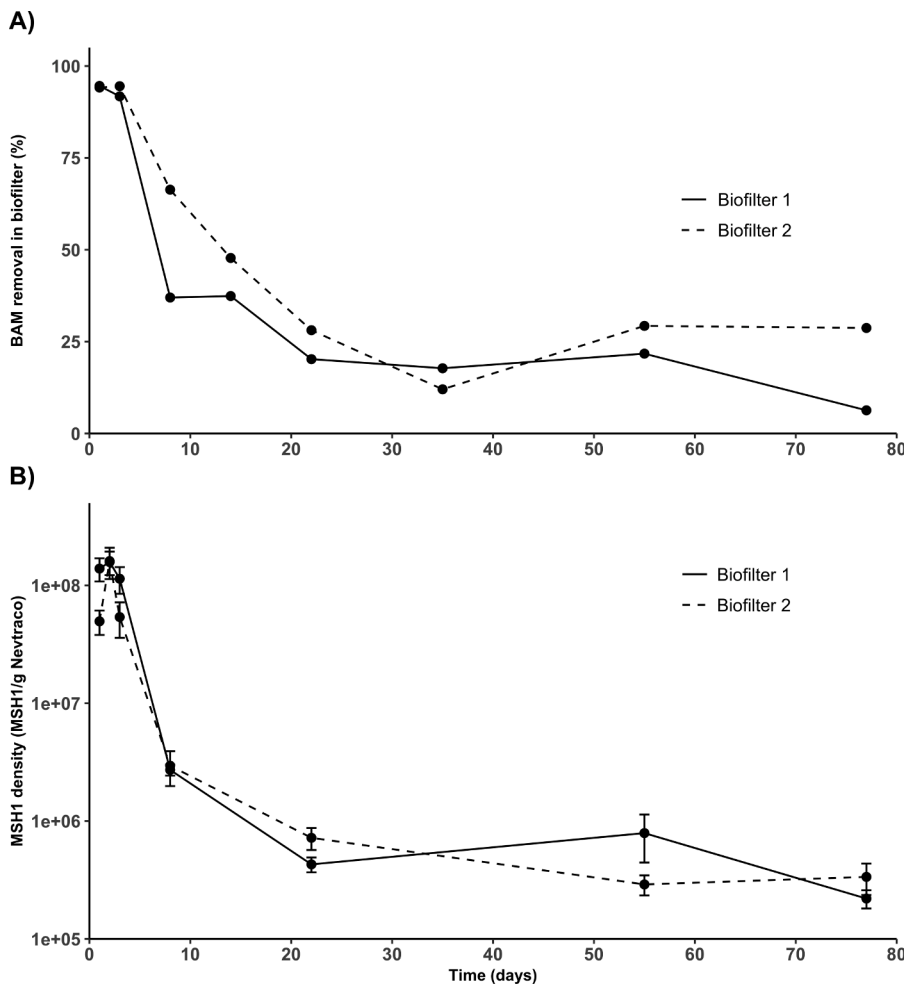
Fig. 2. BAM degradation and density of *Aminobacter niigataensis* MSH1 cells in the first pilot-scale experiment. A) BAM removal efficiency (outlet concentration divided by inlet concentration) in the two biofilters. Biofilter 1 received membrane retentate water while biofilter 2 received non-concentrated water. B) MSH1 density determined by qPCR analysis using specific MSH1 primers, as an average over the five depth samples in each biofilter. Both x-axes represent days after start of the flow. Error bars represent standard error of the mean (*n* = 15).
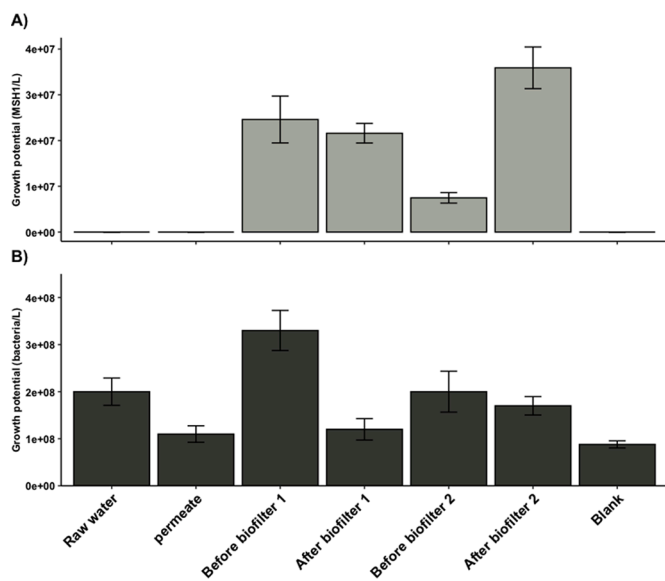
Fig. 3. Growth potential for general water bacteria and *Aminobacter niigataensis* MSH1 at day 99. A) Growth potential for *Aminobacter niigataensis* MSH1 in sterile filtered water from six different locations in the pilot waterworks. B) Growth potential for general water bacteria in sterile filtered water from six different locations in the pilot waterworks.

Nevtraco and water filtrate, respectively. Undiluted standard for water filters contains 6.85×10^7 cells/μl, while for Nevtraco the undiluted standard contains 6.85×10^6 cells/μl. All reactions were run along with negative controls.

During both pilot waterworks experiments no significant difference was observed in the total number of bacteria or the total number of MSH1 (ANOVA p-value = 0.465) between the same depth of the two biofilters, despite a non-homogeneous presence of MSH1 in all the different layers of the reactors. So bacterial number will be presented as an average of the whole biofilter.

2.9. Amplicon sequencing and bioinformatics

DNA samples extracted from Nevtraco and filtered water were used for metabarcoding library preparation on the V3-V4 hypervariable regions of the 16S rRNA gene. Library preparation was carried out by a two-step PCR according to Feld et al. (2016), see supplementary material for details. The samples were sequenced on an Illumina MiSeq using the V3 kit with paired-end (Illumina Inc. SanDiego, US) resulting in 2 × 300 bp reads.

The sequencing dataset produced was analysed using QIIME 2 v. 2019.7 (Bolyen et al., 2019) with an analogous pipeline described by Gobbi et al. (2019). After demultiplexing the reads, these were processed with DADA2 (Callahan et al., 2016). To obtain the phylogenetic tree a multiple sequence alignment was performed with MAFFT (Katoh and Standley, 2013) and the phylogenetic tree was built using FastTree (Price et al., 2010). Diversity analyses were done using the plugin q2-diversity. Phylogenetic Diversity (PD) from Faith (1992) and
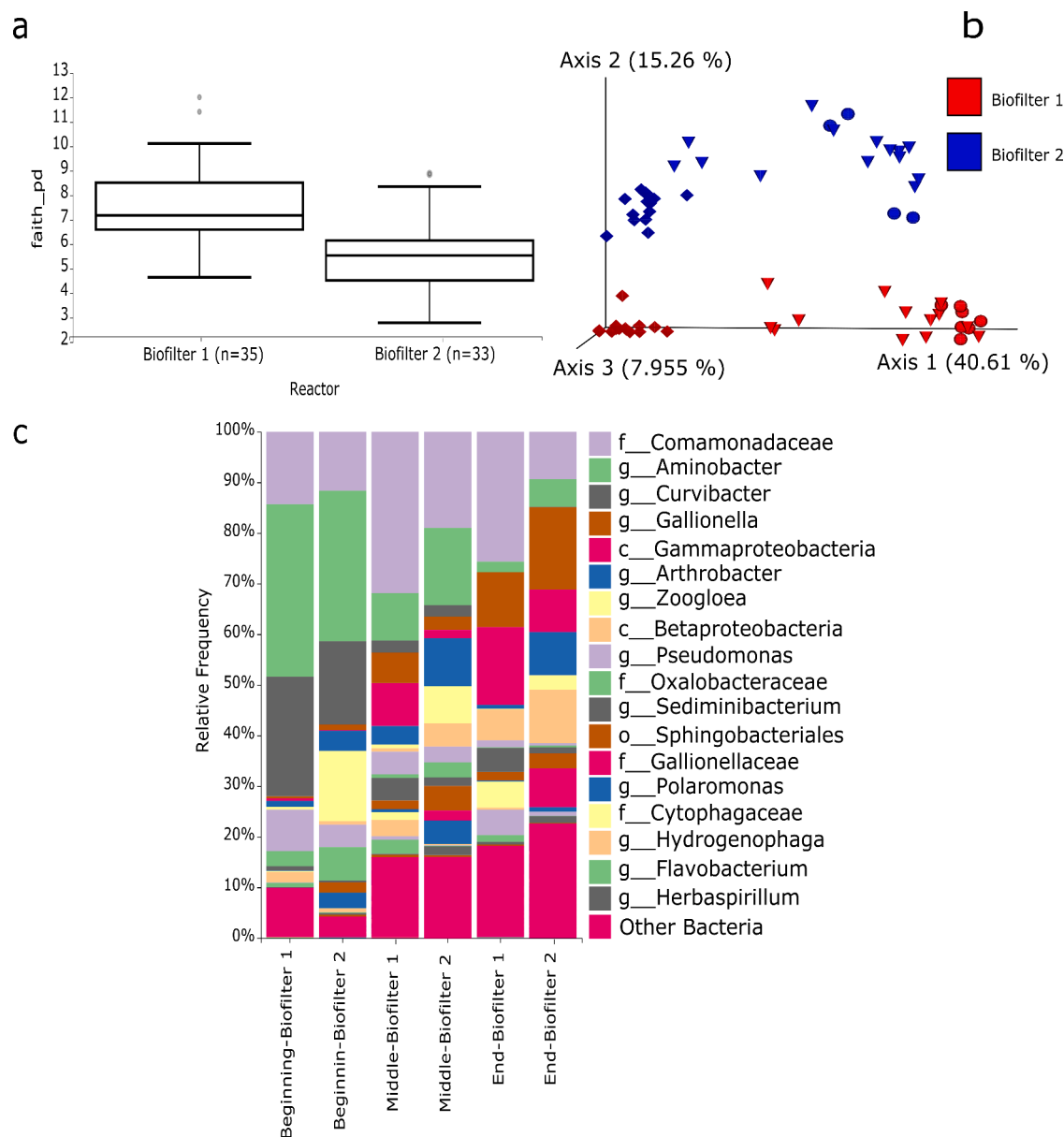
Fig. 4. Results from the 16S rRNA gene amplicon sequencing of filter material from the two biofilters. A) boxplots of phylogenetic diversity variation (measured with Faith-PD) of the two biofilters from the pilot-scale experiment. B) PCoA plot colored by the reactor displaying the beta-diversity between groups. The addition of different shapes identifies the beginning (diamond), middle (cones) and end (spheres) of the treatment. C) Taxa bar plots with the top 18 taxa displayed in the legend in order of decreasing relative abundance. Beginning represents day 1–3, Middle day 4–21 and end day 22–77.

evenness from Pielou (1966) were chosen to measure alpha-diversity, while beta-diversity was measured using Bray-Curtis Dissimilarity and visualized through PCoA plots obtained using Emperor (Vázquez-Baeza et al., 2013). Taxonomy assignments were done using qiime feature-classifier classify-sklearn (Bokulich et al., 2013) with a pre-trained Naïve-Bayes classifier with Greengenes v_13.8 (DeSantis et al., 2006). The raw reads are available on SRA (Study Accession Number (*in progress - to be provided upon final submission*))

2.10. Growth potential – assimilable organic carbon (AOC)

In order to estimate the effect of potential additional nutrients from the membrane treatment prior to the biofilter, that could lead to bacterial growth, a bacterial and MSH1 growth potential assay was performed on the different water samples across the pilot water plant. This was done by sampling 250 ml water in AOC free 250 ml redcap bottles (PTFE-coated cap liner), from seven different water taps sampled at day

99 from the first pilot waterwork experiment. This assay is based on the growth assay by Hammes and Egli (2005), with few modifications. For details see supplementary material. In brief, growth potential was estimated by adding either groundwater microbiome (Water bottle inoculum; Aqua D'Or, Brande, Denmark) or MSH1 to a density of 5000 cells/ml to sterile filtered water samples from seven different water taps at the pilot waterworks into AOC free Wheaton 50 ml glass serum bottles (DWK Life Sciences, USA). The growth of bacteria is assumed to only come from AOC within the water sample. Bacterial growth was measured by flow cytometry after 12 and 13 days of incubation for groundwater microbiome and MSH1 compared to initial added bacteria, respectively.

2.11. Statistical analysis

Statistical analyses of the residence time change for the small column experiments were done with the *multcomp* package in R (R Core Team,
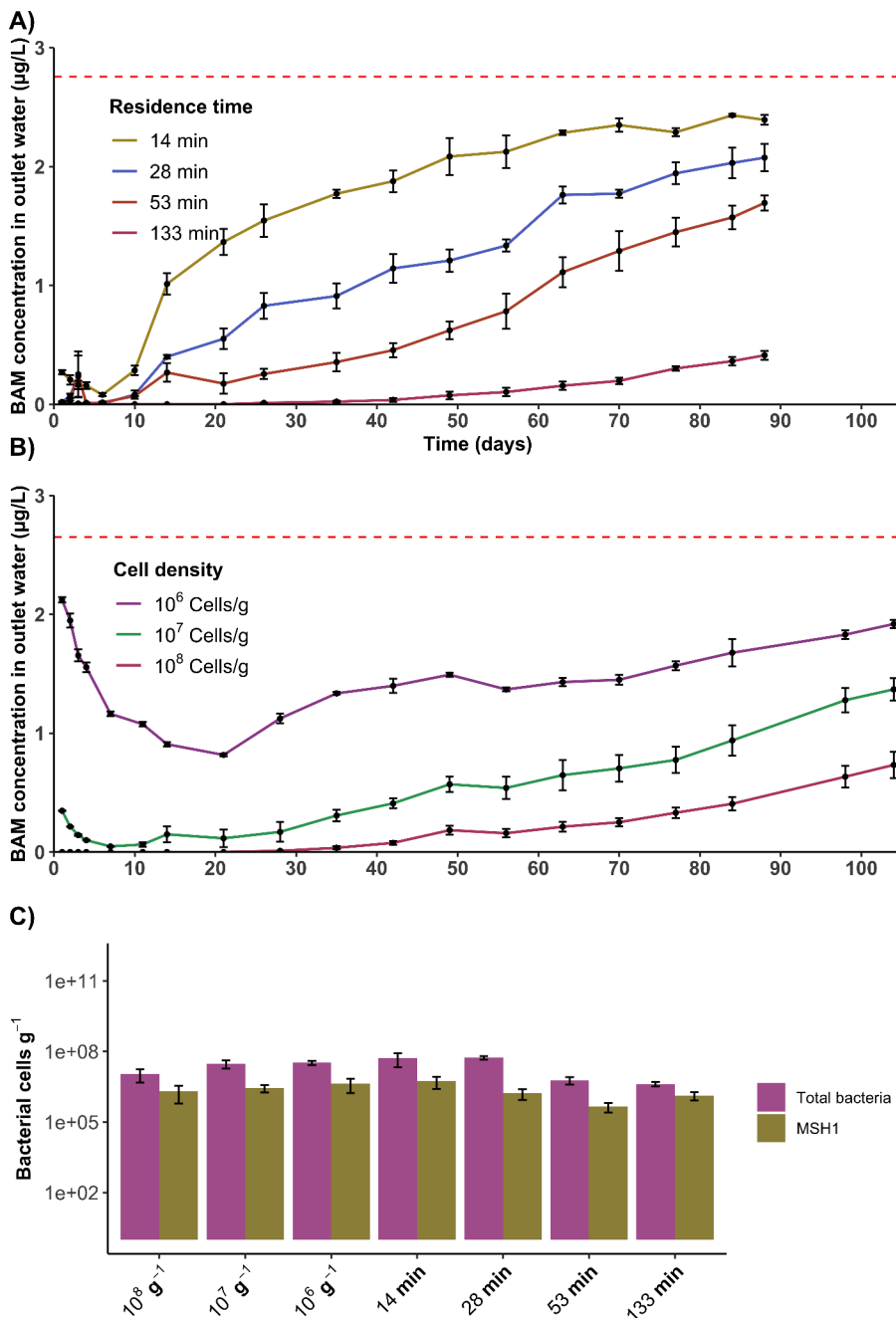
Fig. 5. BAM degradation in small lab-scale columns as a function of cell density and residence time. A) The long-term influence on BAM removal using four different residence times. 10^8 MSH1 were added per g of filter material. Red dashed line indicates inlet BAM concentration. B) The long-term influence on BAM removal using different inoculation densities of MSH1. Residence time was 133 min. Red dashed line indicates inlet BAM concentration. C) Bacterial cell densities within the biofilter at the end of the experiment, presented as both total bacterial and MSH1 numbers.

2013) using ANOVA with a post-hoc Tukey HSD correction test ($p < 0.05$). Unless otherwise stated, data are presented as mean ± standard error of mean (SEM).

The statistical evaluation of these results was performed separately for qPCR and the sequencing data. Regarding qPCR dataset, all statistical analyses and visualisations of the qPCR data were performed on Microsoft Office Excel 2010 using ANOVA and *t*-test.

Statistical analysis performed on the sequencing dataset was analysed through QIIME2 v. 2019.7. After QIIME 2 pipeline processing, the sequencing dataset was evaluated using the Kruskal-Wallis test for alpha diversity and PERMANOVA with 999 permutations for Beta-Diversity. Finally, differentially abundant Amplicon Sequence Variants (ASVs) were detected through the analysis of composition of microbiomes (ANCOM) from Mandal et al. 2015. This test is very conservative as it is based on the assumption that only few ASVs change in a statistical way between the samples and it controls for False Discovery Rate.

3. Results and discussion

3.1. Effect of preconcentration using RO membrane for BAM removal

In the first experiment with the pilot waterworks, biofilter 1 received concentrated water and biofilter 2 non-concentrated water. Residence times were identical in the two filters (28 min). During 77 days, BAM was on average concentrated 8.8 ± 0.6 times by the RO membrane, which is in line with laboratory scale studies using the same XLE membrane where a rejection of around 90% BAM was observed (Fini et al., 2019; Hylling et al., 2019). A seven to ninefold increase in the concentration of most nutrients, metal ions and other water solutes was found throughout both pilot experiments (See 'Before Biofilter 1' data in Table 1, Table S1 and Fig. S3). A slight reduction in the concentration of species over time can be a result of membrane fouling which is more pronounced in the case of pilot experiment 2. BAM degradation in both
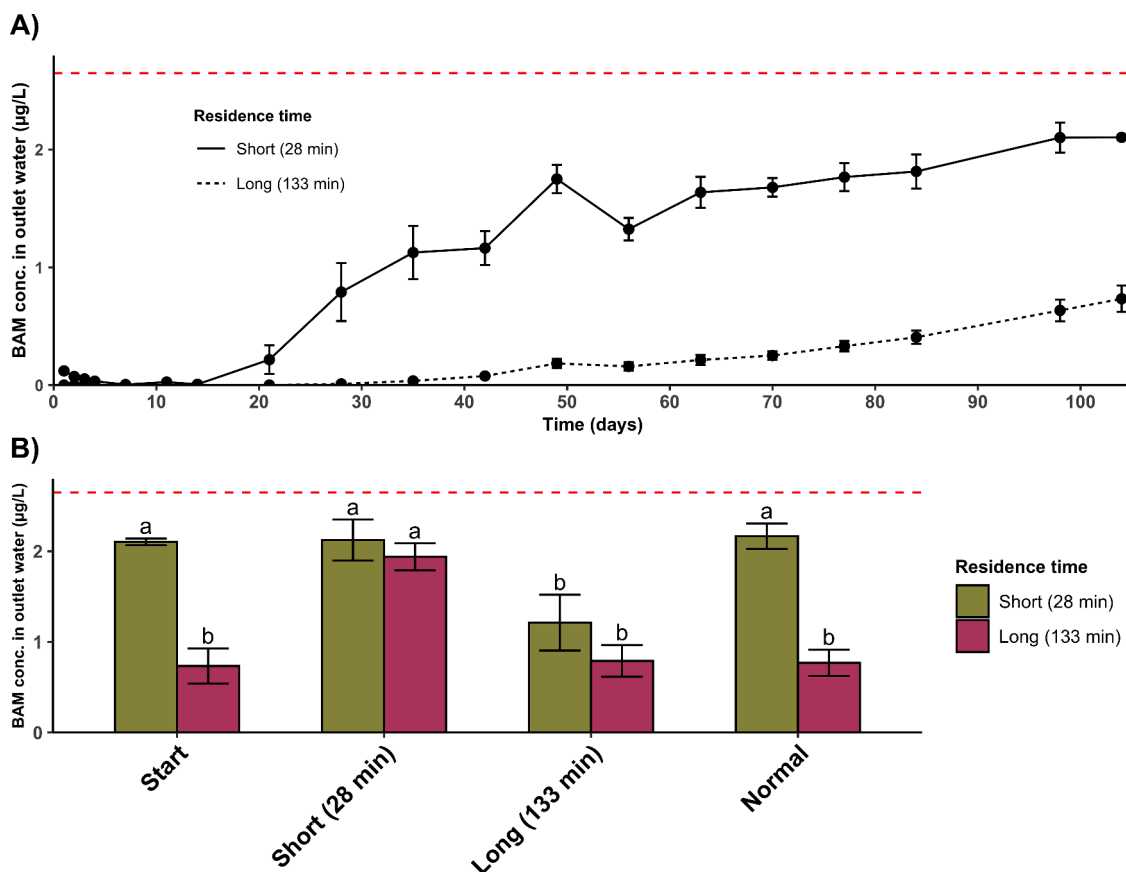
Fig. 6. Influence of short-term changes in residence time on BAM removal in lab-scale columns. A) Repetition of the long-term influence on BAM removal using two different residence times (28 and 133 min) in lab scale columns. B) Influence of BAM removal with short-term change in residence time. This short-term experiment was performed at day 104. At first, the columns with long residence time were changed to have the short residence time, followed by a change for all columns to the long residence time, and finally back to the initial residence times. Letters denote differences at $P < 0.05$ for a post hoc Tukey HSD correction test.

biofilters showed 92–96% removal within the first three days, followed by a fast decline in degradation efficiency to 37% and 66% at day 8 for biofilter 1 and 2, respectively (Fig. 1a). Hereafter the BAM degradation gradually decreased and at the end of the experiment only 6% and 28% BAM was removed in biofilter 1 and 2, respectively. To get below the legal threshold limit (≤ 0.1 μg/l) more than 72% degradation would have been needed. So, in summary the pilot waterworks were able to degrade BAM to levels below the legal threshold limit for less than a week, regardless of whether the biofilter received membrane retentate or non-concentrated groundwater.

The decrease in BAM degradation, correlated with the amount of MSH1 attached to the filter material (Fig. 1B). The attached amount of MSH1 within the biofilters was high for the first 3 days, $1.3 \times 10^8 \pm 8.7 \times 10^6$ and $1.1 \times 10^8 \pm 6.4 \times 10^6$ MSH1 per gram of filter material for biofilter 1 and 2, respectively. Then the MSH1 density within the biofilters dropped to $2.7 \times 10^6 \pm 2.7 \times 10^5$ and $3.0 \times 10^6 \pm 6.9 \times 10^5$ for biofilter 1 and 2, and then slowly decreased to around 4×10^5 MSH1 per gram filter material. qPCRs were repeated targeting both the genome of MSH1 (with the primers MSH1-F and MSH1-R) and the *bbdA* gene carried on the plasmid (using bbdA-F and bbdA-R primers) and the results in terms of abundance are similar throughout the whole treatment for both biofilters. The inoculated bacteria thus did not lose their ability to degrade over time, and the observed decrease in degradation efficiency must simply be caused by the significant decrease in MSH1 abundance ($\sim 10^8$ to $\sim 10^6$ cells/g), since it has been demonstrated that the genes responsible for BAM degradation are constitutively expressed (T'Syen et al., 2015). The reason for the massive decrease, as also observed and discussed by (Albers et al., 2015b), is difficult to assess with certainty and some of the previous suggestions (protozoan grazing and bacterial

starvation) are possible also in our system, while others, like the influence of backwashing, are not relevant here.

There were no significant differences in MSH1 densities between the two biofilters (ANOVA p-value = 0.683). A 5–10x higher content of most nutrients in the permeate thus does not counteract leaching of MSH1 from bioaugmented sand filters by supporting growth of MSH1 or improving adhesion abilities. On the other hand, there was no negative impact of membrane retentate on MSH1 numbers and degradation activity, which is also important.

Carbon for energy and growth has previously been speculated to be a limiting parameter for bioaugmentation in biofilters (Albers et al., 2015a; Horemans et al., 2017). We observed a DOC rejection of 78% to 85% equivalent to 4.4 to 7.1x increase in total DOC using the RO membrane (Table 1). Only a small fraction of this DOC, however, can be readily assimilated by microorganisms in the biofilters. The fraction of DOC that can be used for potential growth, is often named the assimilable organic carbon (AOC) (Hammes and Egli, 2005; Van Der Kooij et al., 1982). AOC represents 0.03–0.36% of the total DOC in groundwater samples (Van Der Kooij et al., 1982). To evaluate the potential of the RO membrane to retain AOC, we measured the growth potential of MSH1 and general groundwater bacteria in the different water taps in the pilot waterworks (Fig. 3).

The growth potential for MSH1 was increased by the RO membrane, being 2.5×10^7 MSH1/L in water sampled before biofilter 1 and 7.5×10^6 MSH1/L before biofilter 2, indicating the RO membrane increased the growth potential for MSH1 roughly by a factor of 3. The same factor for the general bacterial populations seemed to be lower (below 2) with 3.3×10^8 bacteria/L before biofilter 1 and 2.0×10^8 bacteria/L before biofilter 2. We did observe bacterial growth in some the blank samples
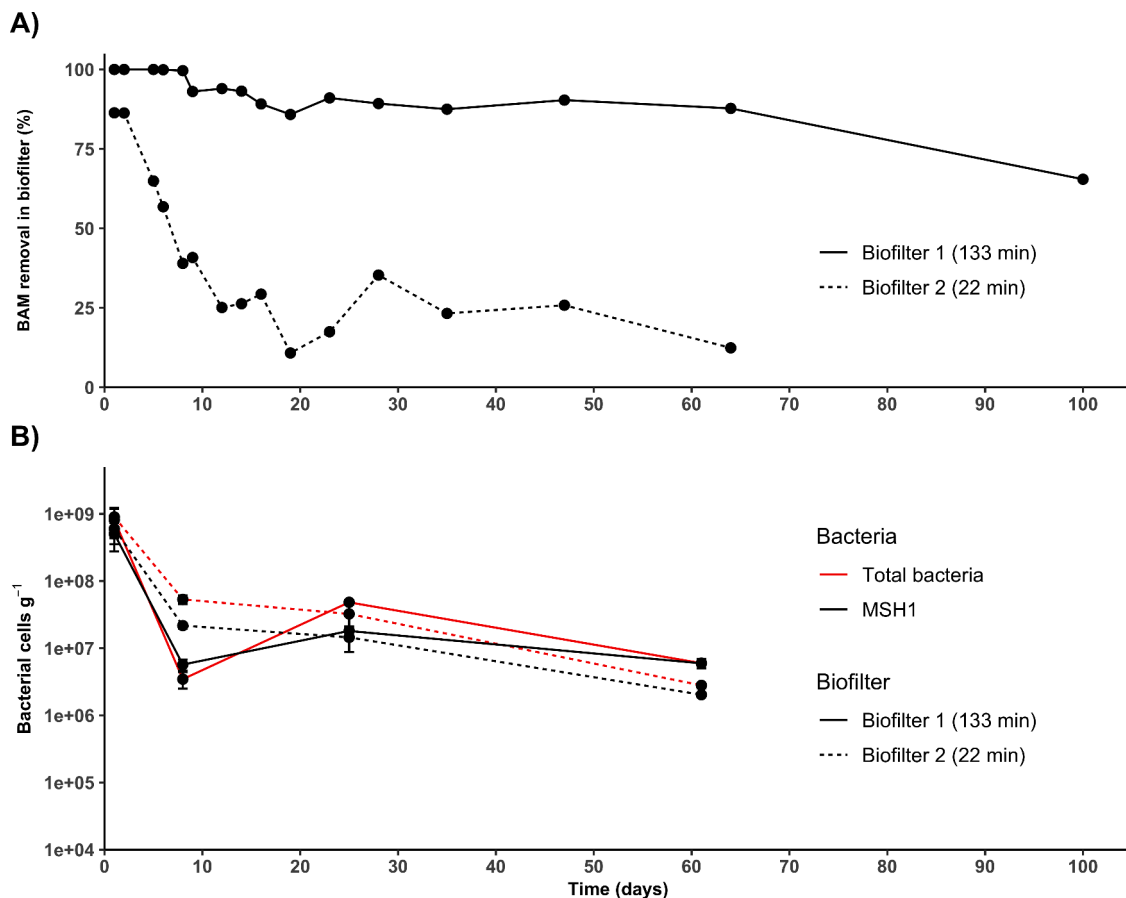
Fig. 7. BAM removal and the bacterial concentration within the biofilters in the second pilot-scale experiment using two different residence times. The two biofilters received the same membrane treated (retentate) water. A) Removal of BAM. Biofilter 2 was operated for 65 days while biofilter 1 continued for another month to see if the high degradation persisted. >90% removal was needed to get below the legal threshold limit of 0.1 μg/L. B) Total bacterial and MSH1 density as determined by qPCR analysis on total 16S rRNA gene and on the MSH1 genome. Numbers are presented as an average over five depths for each biofilter.

(Fig. 3B). This could reflect the lower limit of the method but could also potentially have been caused by bacteria smaller than 0.2 μm and thus not retained by the sterile filtration (Wang et al., 2007). The increase in AOC after membrane filtration was clearly lower than the increase in DOC and inorganic nutrients, indicating that if the system is carbon limited, the increase in inorganic nutrients would have only a small effect on bacterial growth. How this small increase in growth would be distributed between inoculated and indigenous bacteria is unknown. Such an evaluation would require an assay where the growth of total bacteria and degrader strain was evaluated within the same water sample, which might be possible using for example GFP marked MSH1 (Sekhar et al., 2016) combined with Flow Cytometry. Comparing AOC and DOC rejection in the pilot waterworks, results are in line with previous studies concluding that AOC can be rejected by NF and RO membranes to a lesser extent compared to DOC (Escobar et al., 2000; Meylan et al., 2007). This can most likely be due to a smaller molecular size of AOC in combination with the presence of cations in the water shielding negative charges of the membrane surface, that would otherwise repulse negatively charged AOC (Escobar et al., 2000; Nghiem et al., 2006; Soriano et al., 2019).

In order to determine the effect of the membrane on the microbial community within the biofilters, a microbial composition analysis was conducted throughout the operation of the pilot waterwork. From the sequencing dataset we obtained 481 932 high quality reads; they correspond to 1 257 unique ASVs divided in 110 sequenced samples of filter material and water. On average we obtained 15,500 reads/sample. After denoising we retained an average of 4381 ASVs/sample. For the analyses we included the samples that had at least 1350 ASVs each.

Based on this criteria 18 samples including all negative controls were discarded due to insufficient sequencing coverage. The overall taxonomy accounts for 17 ASVs assigned to Archaea while the remaining 1239 were assigned to Bacteria. Despite some differences in relative abundance the community is overall conserved in the dataset and mostly composed by *Aminobacter*, Comamonadaceae, *Pseudomonas, Zooglea, Curvibacter, Gallionella, Arthrobacter*, and *Sediminibacterium*, which are commonly found in waterworks sand filters (Albers et al., 2015a; Ellegaard-Jensen et al., 2020; Gülay et al., 2018; Hu et al., 2020). The major visible difference is due to the depletion of *Aminobacter* from Nevtraco after Day 3 (Beginning in Fig. 4c).

When focusing on the differences, in terms of biofilter microbial community, that can be ascribed to the presence of a preconcentration membrane before biofilter 1, the results are summarized in Fig. 4. Biofilter 1 generally displays a higher phylogenetic diversity compared with biofilter 2, when samples from all timepoints and depths are included (Fig. 4a; p-value <0.001). The PCoA plot based on Bray-Curtis Dissimilarity displays along Axes 2, a distinct clustering of the samples belonging to the two biofilters (Fig. 4b), which is statistically significant when tested with PERMANOVA (p-value < 0.001). These differences can be further observed looking at the taxonomic barplots, (Fig. 4c) which compares the prokaryotic community composition the two biofilters along the whole treatment. To test the effect of the membrane retentate on the microbial community of Nevtraco, we applied ANCOM and the result shows that 16 taxa (only 8 assigned to genus-level) are differentially abundant between biofilters; at genus level, we found that there was a differentially abundance of *Zooglea, Polaromonas, Flavobacterium, Arthrobacter, Sphingopyxis, Rubrivivax, Cupriavidus* and

Algoriphagus. Of these it appears that *Flavobacterium, Sphingopyxis, Cupriavidus* and *Algoriphagus* are more abundant in biofilter 1 together with some other taxa assigned to Cytophagaceae, Chitinophagaceae, Xanthomonadaceae, and Holophagaceae. Some of the organisms relative more abundant in biofilter 1 have been linked to oligotrophic water environments (Jogler et al., 2013; Mijnendonckx et al., 2013; Sack et al., 2011), indicating that it is most likely not the increase in nutrients from the membrane that drives the differences in abundance. *Aminobacter* is not included in this list of differences between biofilters, supporting the qPCR data (Fig. 2b) that the membrane did not have a positive affect on the dominance of *Aminobacter niigataensis* MSH1.

In summary, the RO preconcentration prior to biofiltration did not change the overall composition of the microbial community within the biofilters, but we did observe an increase in the microbial diversity. Increased microbial diversity has been linked to improved performance of waterwork sand filters (Haig et al., 2015) and it most likely increases the microbial gene pool, which potentially could be beneficial for the degradation of micropollutants found in groundwater.

3.2. Small column experiments

The fact that MSH1 degrades BAM equally well with RO retentate as inlet water opens up possibilities to increase residence time of augmented sand filters without having to install very large filter units. To explore the effect of residence time on BAM degradation, we made a series of laboratory column experiments with residence time varied from 14 to 133 min. Since MSH1 density seems to be very important for degradation, we included columns with different initial MSH1 cell densities (10^6 to 10^8 cells/g of filter material) in the experiment. Increased cell density showed a clear positive effect, but the effect diminished somewhat over time, Fig. 5b. The effect of residence time was much larger and consistent over time, Fig. 5a. The period in which 90% of BAM could be removed was 6, 10, 26, 70 days for residence times of 14, 28, 53, 133 min, respectively. This fits the general perception that longer residence time increases the degradation of organic micro-pollutants in soil (Meckenstock et al., 2015), sediments (Weatherill et al., 2018) and biofilters/bioreactors (Haest et al., 2011; Montes et al., 2012). The pattern of complete BAM degradation for the first 21 days and more than 90% for 70 days was observed in two independent experiments with 10^8 cells/g filter material and 133 min residence time (Figs. 5a and 6a).

At the end of each of the small column experiments, filter material was sampled for quantification of total bacteria and MSH1 using qPCR, Fig. 4c. For the experiments with varying densities, there was no significant difference in the density of MSH1 on the biofilters at the end of the experiment despite there being a 100-fold different MSH1 inoculation density (10^8 vs. 10^6). The degradation was 1.5–2.5 times higher in the columns where 10^8 cells/g were added, so probably the MSH1 cell density was still higher at the end of experiment, but the relatively minor difference in degradation and a difference in cell numbers smaller than can be detected by qPCR, demonstrates that over time, merely adding more cells does not make a big difference, when dealing with an oligotrophic system like a groundwater treating sand filter. This is different compared to what was recently concluded for the degradation of the pesticide metaldehyde in bioaugmented sand filters (Castro-Gutierrez et al., 2022), so the potential effect of adding more cells is probably strain and/or system specific. Additionally, it has to be mentioned that the abundance of MSH1 was quantified by DNA, which makes it difficult to calculate the activity of the bacteria within the filters, because DNA is extracted from both living and dead cells.

The fraction of MSH1 in relation to the total microbial community ranged from 3 to 32% across all columns, with the highest dominance in the columns with the longest residence time (133 min). This is a smaller dominance compared to comparable laboratory biofilter experiments (70–95% MSH1 dominance, (Ellegaard-Jensen et al., 2020)). The difference between the two experiments could be due to the longer runtime

in our experiment, probably increasing the effect of death of MSH1 due to starvation.

The short-term (instantaneous) influence of the residence time on BAM removal in biofilters was investigated in small biofilters after 104 days of operation as described in Section 2.3. When equalizing the residence time to 28 min, equal BAM removal of ~30% was observed, with no significant difference between the two sets of biofilters (Fig. 6b). When both sets of columns then were shifted to have a residence time of 133 min, all columns removed ~80%. Finally, the two sets of biofilters were set to their original flows corresponding to 28- and 133-minutes residence time respectively, and their BAM removal capacities were then the same as before changing the flow. Together with the qPCR results (Fig. 5C), this short-term manipulation illustrates that the long-term differences in degradation capacity at different residence times are not due to different abundance or activity of MSH1 cells. In other words, the amount and activity of MSH1 cells seem quite robust towards differences in residence time during operation with almost equal loss of biomass and hence degradation activity over time.

A full or almost full degradation of an organic micropollutant like BAM for a few months before reinoculation could be operational for a waterworks, but as the laboratory experiments show, this would require long residence time, which again would require large biofilters. However, as demonstrated during our first pilot experiment, the filters seem to work at least as well with membrane concentrate as with raw water treated only to remove dissolved iron (Fig. 2) and thus membrane filtration could be a way to minimize the volume needed to be treated by the biofilters. To test this, we set up a second pilot filter experiment applying only membrane retentate to the biofilters.

3.3. Second pilot waterworks experiment

In this experiment, the two biofilters received similar water (RO membrane retentate with on average of 7.3 ± 0.2 times preconcentration of BAM compared to the raw water), but with different flow and hence residence time (133 vs 28 min. in biofilter 1 and 2, respectively). The BAM concentration in the raw water was higher during the second experiment (~1 µg/L) than during the first experiment (~0.4 µg/L) and hence >90% removal was needed to get below the legal threshold limit of 0.1 µg/L. The residence time in biofilter 2 was the same as in the first pilot experiment, and indeed, the BAM degradation pattern was quite similar as well, with high removal during the first few days and then decreasing to only little removal during the following few weeks, followed by a long relatively stable period with 10–30% removal (Figs. 7a and 2a). Biofilter 1, on the other hand, showed complete BAM removal for approximately 10 days followed by a very slow decrease in degradation, which was still close to 90% at day 65 and 70% at day 100 (Fig. 7a). In both filters most of the initially adhered MSH1 cells were lost within the first week followed by a slight decrease in MSH1 density during the following two months (Fig. 7b).

At day 64, a short-term equalization in flow was made, similar to what we did with the laboratory filters (Fig. 6b) although we could only decrease the flow in the fast filter, not increase in the slow filter due to deficits in water supply from the membrane. The test showed that when run at similar residence times, the two biofilters had similar degradation capacity for BAM (Sup. Fig. S5), exactly as was observed in the laboratory (Fig. 6b). Analysis of MSH1 using qPCR also showed similar numbers in the two biofilters (Fig. 7b). The flow test and the qPCR altogether strongly suggest that the large difference in degradation is not caused by different MSH1 activities, but rather that the amount of MSH1 cells that can be sustained in the filters is enough for a high degradation of BAM at the slowest of the two tested flow rates, but not at the faster flow. Overall, the second pilot experiment thus confirms results from the laboratory columns that a high degradation of BAM can be achieved for a long time at a relatively long residence time, but also that this is not due to a higher survival or lower detachment of bacteria at lower flow, but simply because too few MSH1 cells can be sustained at the

oligotrophic conditions of a groundwater fed filter to cope with shorter residence times. In other words, a relatively long residence time is needed to equal the reaction time obtainable with *Aminobacter niigataensis* MSH1 in groundwater treating sand filters. Whether residence time could be increased further to obtain even higher or more prolonged degradation remains an open question for future studies, but there will be a maximum residence time beyond which no further improvement can be obtained due to diffusion-limited mass transfer from the water to the attached bacteria through the boundary layer surrounding individual sand grains (Simoni et al., 2001). In principle one should be able to predict the optimal flow based on porous flow models that include diffusion through stagnant boundary layers using specific BAM degradation rates for MSH1 that have been published for batch studies (Schultz-Jensen et al., 2014; Simonsen et al., 2012). However, specific BAM degradation rates have been found to be up to two orders lower in flow-through sand filters than in batch (Albers et al., 2014, 2015b) so until a better understanding of this phenomenon is achieved, actual experiments demonstrating relationships between residence time and degradation would be preferred.

All in all, the combination of RO membrane filtration and biofilter opens new possibilities of removing organic micropollutants in groundwater through microbial degradation. In the case of the recalcitrant pollutant BAM, the biofilters need to be inoculated with a degrading strain such as MSH1 to achieve degradation. In other cases, with more easily degradable micropollutants, degrading populations might evolve in the biofilter with the groundwater microbiome as "inoculum" (Feld et al., 2016).

4. Conclusion

Here we show a long-time removal (>2 Months) of BAM from contaminated groundwater, using a combination of RO membrane and augmented biofilters in a large-scale pilot waterworks. By increasing the residence time 10x using RO membrane retentate, we could obtain a removal of the groundwater contaminant BAM for a much longer period and residence time is probably a crucial parameter no matter if one attempts to achieve biodegradation of organic micropollutants through inoculation or by "natural" degrader bacteria.

The use of RO membrane prior to biofiltration showed no negative effect on either MSH1 survival or the overall microbial community within the biofilter. Membrane filtration could thus be the way to increase residence time in biofilters at relatively low cost and without compromising water quality for the degrading bacteria as well as for the subsequent use of the treated water.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118352.

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