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Microbial community during the initial stage of biologically active carbon filters' operation and its role in organic matter removal from water

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Abstract: Filtration through biologically active carbon (BAC) filters is an effective method of organic matter removal during drinking water treatment. In this study, the microbial community in the initial period of filters' operation, as well as its role in the organic matter removal were investigated. Research was carried out in a pilot scale on two BAC filters (Filter 1 and Filter 2) which were distinguished by the type of inflowing water. It was observed that the number of heterotrophic plate count bacteria and total microbial activity were significantly higher in water samples collected from Filter 2, which received an additional load of organic matter and microorganisms. Despite the differences in the values of chemical and microbiological parameters of inflowing water, the composition of the microbiome in both filters was similar. The predominant taxon was a bacterium related to *Spongiibacter* sp. (*Gammaproteobacteria*) (>50% of relative abundance). In both filters, the efficiency of organic matter removal was at the same level, and the composition and relative frequency of predicted functional pathways related to metabolism determined using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States Software) at level 3 of KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology – were also similar. The study demonstrated that a 40-day period of filter operation after filling with virgin granular activated carbon, was sufficient to initiate biofilm development. It was proved, that during the initial stage of filter operation, microorganisms capable of biodegradation of various organic compounds, including xenobiotics like nitrotoluene, colonized the filters.

Introduction

Ensuring high-quality drinking water free of contaminants potentially hazardous to human health requires the use of advanced technological solutions in water treatment, e.g., biologically active carbon (BAC) filters. The BAC process eliminates dissolved organic matter (DOM), which includes xenobiotics and substances known as contaminants of emerging concern (CEC) such as pharmaceuticals, pesticides, endocrine disrupting chemicals and personal care products, disinfection by-products and their precursors, water genotoxicity, cyanotoxins, geosmin and 2-methylisoborneol, amines, aliphatic aldehydes, and phenols/chlorinated phenols. BAC filters also efficiently remove some inorganic compounds such as ammonium and manganese(II) (Simpson 2008, dos Santos and Daniel 2020).

The mechanism of DOM removal in BAC filters is based on two processes: adsorption on the grains of granulated active carbon (GAC) and biodegradation by microorganisms living in the biofilm. Biofilm is primarily formed by the heterotrophic bacteria, which have the capability to metabolize various types of organic or inorganic substances. The growth and metabolism of microorganisms depend on many factors, in particular, the concentration of nutrients, dissolved oxygen (DO), pH and temperature of the filtered water. During the initial stage of filter operation, physical sorption on the GAC surface dominates. This phase is known as the acclimation phase, during which microorganisms start to settle down and colonize GAC grains, initiating biodegradation. This phase typically spans 2–3 months. In the next phase, after biofilm formation, the adsorption and biodegradation processes occur concurrently. This leads to a reduction in GAC adsorption sites, resulting in a decline of adsorption within BAC filters. In the third phase, biodegradation predominates in DOM removal, and the biofilm biomass stabilizes at a steady state

(Simpson 2008, Korotta-Gamage and Sathasivan 2017, Mądrecka et al. 2018).

The BAC filters operation, process of biodegradation and microbial community were examined in previous research. Matilainen et al. (2006) investigated the removal of various fractions of organic matter and its transformation during a period of 1 year at full-scale drinking water treatment plant. There are also studies concerning the removal of specific water pollutants (Kennedy et al. 2015, Selbes et al. 2017). In the recent years, molecular biology techniques have also enabled the identification of microorganisms living in the biofilm, and even the description of the entire microbiome of BAC filters. These techniques were applied in many research. They included: study on bacterial community existing in the top layer of the bed of full-scale BAC filters (LaPara et al. 2015), comparison of microbial population growing on GAC and other filtration media (Shirey et. al 2012, Vignola et al. 2018), evaluation of microorganisms' role in organic matter degradation (Oh et al. 2018, Vignola et al. 2018), dynamics of microbial community in drinking water treatment plant (Li et al. 2017), changes in microbial community in pilot scale up-flow or down-flow BAC filtration system (Liao et al. 2012, 2013, 2015), influence of low temperature on bacterial diversity and biomass (Kaarela et al. 2015), searching for microorganisms with specific metabolism e.g., those responsible for ammonia oxidation (White et al. 2012).

Most of the mentioned studies were conducted in full--scale water treatment plant, during the period of steady stage of biofilm development in BAC filters. The advantage of pilot scale studies is the possibility of performing tests in selected water quality conditions and hydraulic parameters. Pilot scale studies on microbial communities in the initial phase of filter operation are quite rare. Servais et al. (1994) investigated the colonization of BAC filters. They observed that the maximum biomass of biofilm was reached after 100 days of filter operation. Velten et al. (2011) investigated biofilm development in a BAC filter by employing flow cytometry and measuring bacterial ATP concentration over a 6-month period of filter operation. The effectiveness of organic matter removal from groundwater was examined in the research conducted by Papciak et al. (2016). They discovered that during the initial 6 months of filter operation, the combined process of adsorption and biodegradation enabled the removal of nearly 100% of organic matter. Shirey et al. (2012) described changes in the composition of bacterial communities during initial colonization and the stable period using T-RFLP analysis. Qi et al. (2019), conducted more comprehensive studies, detailing the impact of carbon filter depth and the backwashing process on the microbial community composition after 1, 3 and 5 months of filter operation employing 16s rRNA sequencing. Ma et al. (2020) investigated the factors influencing microbial communities within filters, along with the influence of biofilters on water quality. They utilized real-time qPCR to estimate total bacterial biomass and 16s rRNA sequencing to characterize the microbiome. The latter analysis was conducted by them after a year of filter operation.

Understanding of the biodegradation process is crucial and valuable for proper BAC filter operation and in the result can increase the efficiency of DOM removal. The aim of this research was to examine the microbial community formation, its taxonomic composition and metabolic functions – particularly DOM removal – during the initial phase of filter operation. The research was performed in the pilot scale with the use of two BAC filters, which differed in parameters of inflowing water, to investigate whether these differences affect the process of biofilm formation and DOM removal.

The research included both physical and chemical analyses of water, as well as microbiological analyses of water and filter beds. The microbiological parameters were analyzed with the use of traditional, culture method – heterotrophic plate count – as well as testing the total microbial activity. New generation sequencing (NGS) – 16s rRNA analyses were conducted to obtain comprehensive information about the taxonomy of bacteria populating the filter bed. The novelty of this study lay in the application of the software package Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Douglas et al. 2020), along with the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (Kanehisa et al. 2021). This allowed for the prediction of metabolic pathways related to microorganisms residing in BAC filters, encompassing processes such as xenobiotic biodegradation and metabolism.

Materials and methods

Pilot installation

The pilot installation comprised two identical rapid filters, denoted as Filter 1 and Filter 2, each with a total height of 300 cm, a total filter bed height of 210 cm, a supporting layer height of 30 cm, and external/internal diameter of 14/10 cm) (Fig. 1). Each filter column was made of polymethyl methacrylate (PMMA), protected against the light by a dark cover, and equipped with a water jacket to ensure an even temperature of about 15°C in the bed throughout the research. The filter medium was granular activated carbon (GAC) – WG-12 (Gryfskand Ltd., Poland). According to the manufacturer's specification, WG-12 is composed of low-ash coal, connected by a binder and activated using water vapor. The material possesses the following parameters: iodine quantity = 1,100 mg g^{-1}, methylene blue adsorption = 0.30 g g^{-1}, specific surface area (BET) = 1100 m^2 g^{-1}, particle size = 1.5–0.75 mm) (Holc et al. 2021). The supporting layer of the filter columns was constructed using gravel quartz. Sampling ports, consisting of metal spigots, were positioned at 45 cm, 85 cm, 125 cm, 165 cm, and 205 cm heights along the bed. The columns were connected to rotameters for flow rate control and piezometers for pressure losses monitoring.

The filters were supplied with the tap water sourced from the municipal water supply network. The raw water used for drinking water production was obtained through artificial infiltration uptake and treated using a conventional iron and manganese removal system, involving aeration and filtration through anthracite-quartz rapid filters. The treated water was disinfected with UV rays and sodium hypochlorite. Throughout the study, the residual chlorine concentration in the tap water was monitored several times and no traces were detected.

Filter 2 was additionally supplied with a biohumus solution to provide an additional load of organic matter and to increase the number and biodiversity of microorganisms in the inflowing water. The added biohumus solution was prepared

by diluting the solution of vermicompost (BIOHUMUS, Ekodarpol, Poland). The volume percentage concentration of the added biohumus solution was 10%. According to the manufacturer's specification, it included products resulting from organic matter decomposition by earthworms, specifically *Eisenia fetida*. The product also contained microorganisms such as *Azotobacter*, *Pseudomonas*, phosphorus accumulating bacteria, bacteria decomposing cellulose, *Actinobacteria* and spore-forming bacteria. The solution was continuously mixed in a tank using an electric stirrer to prevent sedimentation of particles. It was continuously dosed into the water inflow of Filter 2 using a diaphragm pump.

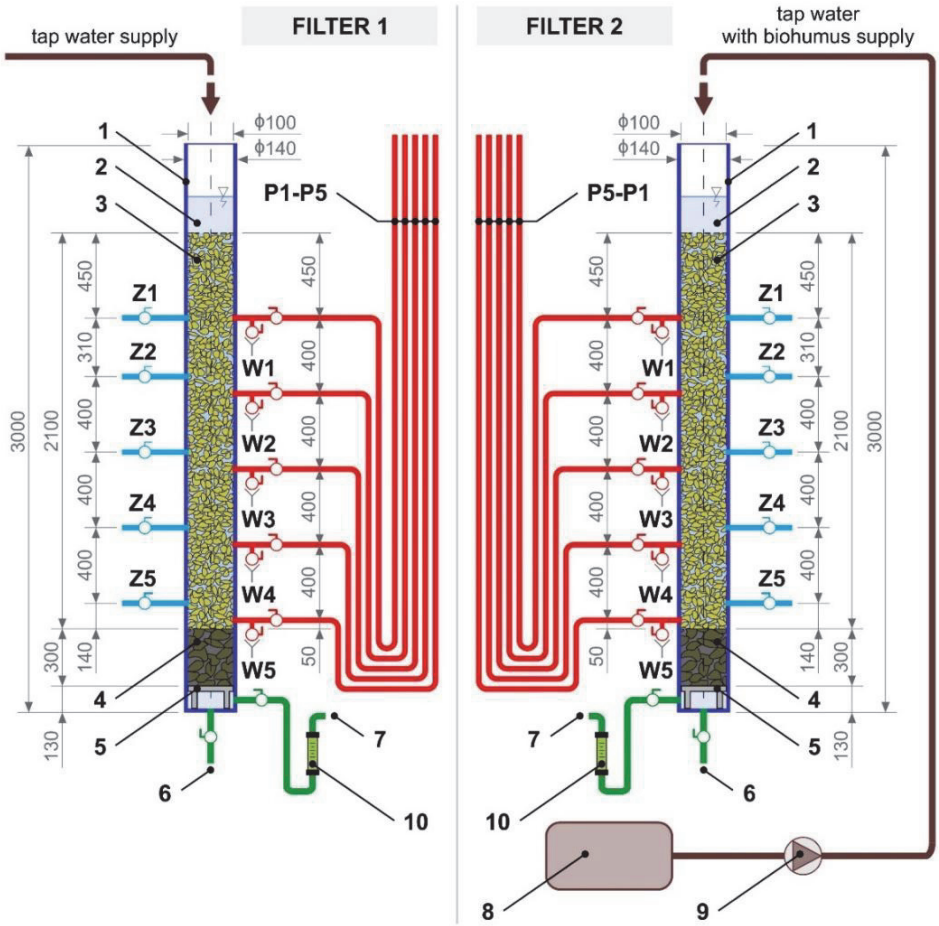
The analyzed filtration run of both BAC filters lasted for 40 days. Filtration commenced after loading the filter columns with new GAC medium. Both filtration columns were operated under the same hydraulic conditions, with a flow rate of 20 L h^{-1}; filtration velocity of 2.55 m h^{-1}; and an empty bed contact time of 50 minutes.

Physical and chemical analyses of water samples

Water samples for assessing physical and chemical parameters were collected from the filter's inflow and outflow five times a week. The parameters under consideration included temperature, pH, electrical conductivity, oxidation reduction potential (redox), turbidity, total alkalinity, ammonia nitrogen ($N\text{-}NH_4$), dissolved oxygen (DO), UV_{254} absorbance, and total organic carbon (TOC). These parameters were analyzed in accordance with the Standard Methods guidelines (APHA 2017). For TOC measurements, a TOC/TN multi NC 3100 instrument (Analytik Jena, Swiss) was used. Chemical oxygen demand (COD_{Mn}) was determined using the acidic permanganate method, following the Polish Standard PN-C-04578-02:1985. Each analysis was performed with a minimum of triplicate samples.

Heterotrophic plate count and total microbial activity analyses

Water samples for culture method and total microbial activity tests were collected from the inflow, outflow and five different depths within the filter columns once a week. The number of cultivated bacteria – heterotrophic plate count (HPC) – was determined on nutrient agar (BTL, Poland) after incubation at 22°C for 72 hours. The total microbial activity (TMA) was measured by fluorescein diacetate (FDA) test. The luminescence intensity of the released fluorescein was measured using PerkinElmer Instruments LS55 luminescence



1 - water jacket, 2 - filtration column, 3 - filter bed, 4 - gravel supporting layer, 5 - drainage, 6 - backwash water, 7 - outlet, 8 - biohumus tank, 9 - diaphragm pump, 10 - rotameter, P1-P5 - piezometers, W1-W5 - water sampling ports, Z1-Z5 - filter bed sampling ports.

Fig. 1. Pilot installation consisted of two filtration columns with GAC (after Holc et al. (2019), changed)

spectrometer, with an excitation wavelength of 433 nm and an emission wavelength of 525 nm. The measurements were conducted with FLWinLab software, following the methodology previously described (Mądrecka et al. 2018). In this method the activity of microorganisms is indirectly measured as the FDA decomposition rate. The results of test are given as an activity increase during 1 minute (A min^{-1}).

Sampling for metagenomic analysis (16s rRNA) and DNA extraction

Bed samples for molecular analyses were collected from Filter 1 and Filter 2 at a depth of 45 cm within the filter beds on the final day of the 40-day filtration run. Samples of pure GAC (WG-12) and biohumus solution were also included in the tests. All samples were stored at -20°C prior to DNA extraction.

Samples of the bed, virgin GAC, and biohumus, each weighing 500 mg were homogenized using the FastPrep-24 homogenizer (MP Biomedicals, LLC). The total genomic DNA from all samples was extracted using the Power Soil DNA Isolation Kit (Qiagen). As negative controls, blank DNA extraction, blank PCR samples and pure GAC samples were sequenced.

Microbial population analysis with a use of NGS

The metagenomic analyses were performed by sequencing the V4 region of 16S rRNA gene. PCR reactions, preparation of the final library, and sequencing using Ion Torrent Personal Genome Machine (Ion PGM) system (Ion Torrent) were performed as previously described (Makowska et al. 2020).

Raw sequence data were pre-filtered by Ion Torrent Suite software version 5.10.1 (Life Technologies, USA) to remove polyclonal and low-quality sequences. Further bioinformatic analyses were conducted using fastq data and custom workflow. Sequence reads shorter than 200-bp were removed from the dataset using Geneious R11.1.5 (Biomatters Ltd.). Leading and trailing low-quality bases or Ns were removed using Trimmomatic version 0.39. Sequences with a minimum of 50% bases with a quality score ≥25 were extracted using the Fastx toolkit. The quality filtered sequences were then sorted by barcodes and trimmed at 5' and 3' ends to remove PCR primers in Geneious R11.1.5. Singleton sequences with fewer than 10 reads were discarded using the -fastx_uniques and -sortbysize algorithms. Chimeric sequences were eliminated using the default settings in UCHIME2 version 4.2.40. Operational taxonomic unit (OTU) clustering at 97% similarity was performed using USEARCH version 11.0.667 (Edgar 2013). Sequences were denoised into zero-radius operational taxonomic units (ZOTUs) and subsequently, a ZOTU table was constructed following the denoising steps. Phylogenetic affiliations were analyzed by USEARCH SINTAX algorithm using confidence threshold of 0.8. OTUs were compared against two microbial databases: the Ribosomal Database Project (RDP) 16S rRNA gene training set version 16 and SILVA database for ARB for small subunit ribosomal RNAs version 138. Approximately 1,000,000 sequence reads were identified for Filter 1 and Filter 2, and around 300,000 for biohumus. The data were normalized before proceeding to the next analyses. Conversely, only approx. 200 sequence reads were detected for GAC, confirming its high purity. The majority of identified OTUs were specific only for the GAC and were predominantly associated with *Actinobacteria* (61.0%), *Bacteroidetes* (23.7%) and *Proteobacteria* (15.2%). These data served as the negative control.

The nucleotide sequences obtained in this study are available in GenBank under accession numbers MW741757-MW741810. Basic Local Alignment Search Tool (BLAST) was used to compare the predominant OTU sequence with the sequences deposited in the GenBank database.

The metagenome's functional content was predicted from the 16S rRNA sequencing data using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States – PICRUSt2 v2.4.1 software package (Douglas et al. 2020). This prediction aimed to define the types of metabolic-related pathways and their percentage shares. Functional prediction was carried out using the rarefied ZOTU table, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2021). The predictions were categorized at KEGG Orthology level 1, 2 and 3 within the KEGG pathway hierarchy. To assess the accuracy of PICRUSt2 prediction, the Nearest Sequenced Taxon Index (NSTI) for each sample was estimated and calculated (Langille et al. 2013). NSTI scores ranged from 0.170 to 0.188, suggesting a high level of confidence in the authenticity of the predicted functional pathways.

Statistical analysis

Statistical analyses were performed with the use of Statistica 13.3 (TIBCO software). Differences in the physical and chemical parameters of inflowing water for the two studied filters were assessed using either *t*-test (2-tailed) or Mann-Whitney test. A p value < 0.05 was considered to be statistically significant. To examine the potential relationship between the number of HPC bacteria and TMA value, Spearman's rank correlation coefficient was applied ($p < 0.05$). For the purpose of identifying shared OUTs between Filter 1, Filter 2 and biohumus, a Venn diagram was created using the web-based tool InteractiVenn (Heberle et al. 2015).

Results and Discussion

Physical and chemical parameters of water

The values of physical and chemical parameters are presented in Table 1. There were no significant statistical differences observed when comparing the values of temperature, pH, conductivity, redox and total alkalinity between the two filters (Table 2). Larger mean concentration values of ammonium, turbidity, COD_{Mn}, TOC and UV_{254} absorbance were noted in the water supplied to Filter 2, while mean DO concentration was higher in the inflow to Filter 1. The statistical tests revealed significant differences ($p < 0.05$) in the values of turbidity, ammonium, DO and COD_{Mn} between both filters (Table 2). These differences were expected and appeared as result of biohumus solution supply to Filter 2.

As a consequence of the filtration process, the concentrations of DOM (expressed as COD_{Mn}, TOC concentration and UV_{254} absorbance), DO, turbidity and ammonia nitrogen decreased in both filters. Notably, the disparities between inflow and outflow were more pronounced in the case of Filter 2. A comprehensive overview of the water quality changes during the filtration process is presented in Table 3.

During the entire operational period, Filter 1 exhibited a mean DO reduction of 79.84%, while Filter 2 displayed a slightly lower reduction of 67.64% (Fig. 2 a–b). Weekly variations in the mean reduction of COD_{Mn}, were noted, hovering around 80% for both filters (Fig. 2 c–d). The average removal efficiency of TOC was 91.93% (Filter 1) and 91.33% (Filter 2). Additionally, for UV_{254} absorbance, the mean removal efficiency reached 41.63% and 37.41% for Filter 1 and Filter 2, respectively. The residual dissolved organic matter in the filtered water was found to be non-adsorbable to GAC. It is consistent with earlier research findings (Yapsakli and Çeçen 2010).

Table 1. Physical and chemical parameters of water supplying the filters

Parameter	Unit	Filter 1				Filter 2			
		Min	Max	Mean	SD	Min	Max	Mean	SD
pH	–	7.37	7.84	7.55	0.12	7.33	7.72	7.51	0.10
Conductivity	μS cm^{-1}	683.3	933.2	790.6	660.7	691.4	865.1	785.4	51.8
Redox	mV	208.8	316.8	272.8	34.7	197.2	317.3	271.2	37.8
Turbidity	NTU	0.13	0.97	0.28	0.20	0.18	0.88	0.46	0.22
Total alkalinity	mval L^{-1}	3.99	4.18	4.08	0.05	3.98	4.18	4.08	0.05
$N\text{-}NH_4$	mg-N L^{-1}	0.074	0.230	0.141	0.052	0.101	0.251	0.172	0.048
DO	mg-O_2 L^{-1}	4.43	5.45	4.92	0.24	3.89	5.34	4.51	0.35
COD_{Mn}	mg-O_2 L^{-1}	3.35	4.92	4.08	0.49	3.74	5.39	4.58	0.49
TOC	mg-C L^{-1}	5.40	5.90	5.67	0.19	5.60	6.20	5.87	0.22
UV_{254}	m^{-1}	0.120	1.220	0.478	0.305	0.180	1.120	0.561	0.299

Table 2. Results for the hypothesis tests of physical and chemical parameters of water inflowing to Filter 1 and Filter 2 (*differences statistically significant at $p < 0.05$)

Parameter	N	Test	p-value
pH	26	Mann-Whitney	0.148
Conductivity	26	*t*-test	0.742
Redox	26	Mann-Whitney	0.898
Turbidity	26	Mann-Whitney	0.0003*
Total alkalinity	26	Mann-Whitney	0.557
$N\text{-}NH_4$	26	Mann-Whitney	0.014*
DO	26	Mann-Whitney	0.00005*
COD_{Mn}	26	*t*-test	0.0005*
TOC	6	*t*-test	0.117
UV_{254}	26	Mann-Whitney	0.284

Table 3. Changes in the water quality during filtration process presented as differences in parameters' values between inflow and outflow ("–" – increase of value during filtration)

Parameter	Unit	Filter 1				Filter 2			
		Min	Max	Mean	SD	Min	Max	Mean	SD
pH	–	-1.14	0.26	-0.04	0.37	-1.13	0.21	-0.08	0.38
Conductivity	μS cm^{-1}	-30.8	59.4	-1.87	20.67	-42.5	26.3	-6.37	18.10
Redox	mV	-11.0	34.5	4.25	12.55	-45.5	36.0	0.53	13.51
Turbidity	NTU	0.03	0.57	0.13	0.13	0.08	0.72	0.29	0.18
Total alkalinity	mval L^{-1}	-0.38	0.08	-0.07	0.13	-0.49	0.16	-0.08	0.15
$N\text{-}NH_4$	mg-N L^{-1}	0.062	0.217	0.101	0.042	0.065	0.237	0.129	0.044
DO	mg-O_2 L^{-1}	2.77	4.57	3.93	0.57	1.64	4.49	3.08	0.78
COD_{Mn}	mg-O_2 L^{-1}	2.65	3.89	3.26	0.39	2.93	4.50	3.69	0.40
TOC	mg-C L^{-1}	4.87	5.51	5.21	0.27	4.89	5.72	5.36	0.35
UV_{254}	m^{-1}	-0.220	1.060	0.258	0.324	-0.140	1.040	0.295	0.344

At the beginning of filter operation, the removal of organic compounds primarily occurs through adsorption process (Korotta-Gamage and Sathasivan 2017). Simultaneously, oxygen is also adsorbed onto the GAC surface (Matsis and Grigoropoulou 2008). Studies involving virgin GAC have shown that a considerable amount of oxygen, over 4.7 mg per gram of GAC, can be abiotically removed through chemisorption (Choi et al. 2008). In the investigated filters, the removal of DO during 1–3 weeks was probably the result of this process. Notably, a decline in the rate of DO reduction became apparent in the 2nd and 3rd week of the run, with this effect being more pronounced in Filter 2 (Fig. 2 a–b). The higher values of COD_{Mn} in the inflow to Filter 2 may have hastened the exhaustion of the adsorption capacity. Subsequent to this period, a decrease in the DO concentration in the filter's outflow decreased again, suggesting the initiation of a biodegradation process around the 4th week of filter operation.

Heterotrophic plate count bacteria and total microbial activity

Throughout the studied period, the number of HPC bacteria in the inflow to Filter 1 remained at a low level, ranging from 3–54 CFU mL^{-1}, with an average of 25 CFU mL^{-1} (Fig. 3a). The average number of HPC bacteria number in the inflow of Filter 2 was approx. 200 times higher, reaching 5.1 × 10^3 CFU mL^{-1} (range: 2.2–8.0 × 10^3 CFU mL^{-1}). Moreover, the bed profile of Filter 2 also exhibited a notably elevated presence of HPC bacteria, ranging between 5.3–7.1 × 10^3 CFU mL^{-1} (mean 5.9 × 10^3 CFU mL^{-1}). In contrast, Filter 1's bed profile showed a lower concentration, ranging from 2.8–4.8 × 10^3 CFU mL^{-1} (mean 4.0 × 10^3 CFU mL^{-1}). Filter 1 consistently exhibited a higher number of HPC bacteria in the outflow compared to the inflow. Conversely, in Filter 2, the reduction efficiency of HPC bacteria ranged from 16.9 to 86.9% (mean 54.5%).

In Filter 1, the total microbial activity (TMA) within the inflow ranged from 0.015 to 0.031 A min^{-1} (mean 0.022 A min^{-1}), while in Filter 2, it fluctuated between 0.018 and 0.047 A min^{-1} (mean 0.035 A min^{-1}) (Fig. 3b). The total microbial activity in the outflow of Filter 1 exceeded that of the inflow in most samples. In contrast, Filter 2 exhibited variations in TMA in the effluent throughout the filter's operation, with both reductions and increases observed. Throughout the entire filter operation, the average TMA in Filter 1's bed profile ranged from 0.019 to 0.035 A min^{-1} (mean 0.028 A min^{-1}), while Filter 2 displayed a range of 0.032 to 0.042 A min^{-1} (mean 0.036 A min^{-1}). The highest TMA values in both filter beds were recorded at the depth of 205 cm during the initial stages of the filters operation (5th day). This occurrence could be attributed to the washing of microorganisms out from the filter beds due to unfavorable conditions for microbial adhesion at this early stage. In Filter 1, a decrease in TMA was observed across all sampling points (inflow, bed, outflow) on the 12th day of the operation subsequently, TMA exhibited a continuous increase from week to week until the end of the filter run. In contrast, Filter 2 did not exhibit a similar tendency.

The analysis of HPC bacteria numbers and TMA indicates a more rapid colonization of bacteria in Filter 2

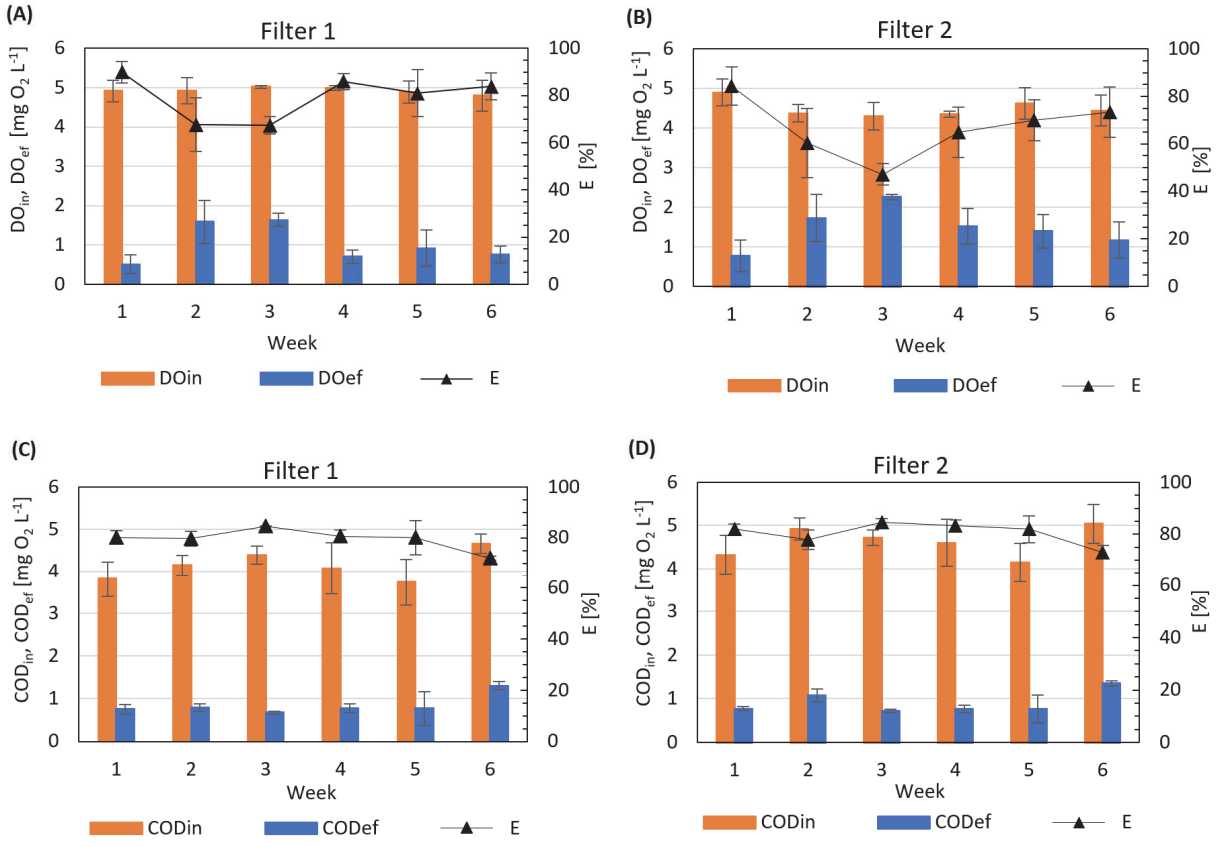


Fig. 2. Average concentration of DO and COD_{Mn} in the influent (DO_{in}, COD_{in}), effluent (DO_{ef}, COD_{ef}), and average reduction efficiency (E) of these parameters after filtration in every week of filter operation

compared to Filter 1. The consistent decrease in the number of HPC bacteria along the inflow-outflow pathway of Filter 2, throughout the filter operation affirmed that this filter provided better conditions for bacteria to settle down on GAC grains, likely due to the influence of biohumus supplementation. The alteration in HPC bacteria numbers and TMA values within both filters did not display the same patterns (Fig. 3). This disparity can be attributed to differences in the methods employed. The advantages and disadvantages of these methods were previously described by Holc et al. (2021). The authors of the mentioned publication highlighted the lack of correlation between HPC bacteria numbers and TMA values, as these parameters reflect distinct bacterial attributes: the capacity to proliferate on nutrient agar at 22°C (HPC bacteria number) and total microbial activity (TMA). Our research further corroborated this finding, as no statistically significant correlation ($p < 0.05$) between HPC bacteria numbers and TMA values was observed in either of the filters.

According to HPC bacteria number results, the biodegradation within the filters started to occur about 26th day of filter operation. On that day, in both filters, a significant decrease in HPC bacteria number at outflow was observed and their values were shown to be lower in the outflow than in the deepest layer of the filter bed. Such decreases were not observed in the case of total microbial activity (Fig. 3b), but the changes of COD_{Mn} and DO concentration confirmed that the biodegradation started to take place within filters after about 4th week of filters' operation.

The previous studies revealed variability in the duration required for microorganisms to acclimate to GAC, as evidenced in diverse studies. Servais at al. (1994) found that it took approximately 100 days for fixed bacterial biomass levels to be attained in their filter setup, whereas Velten et al. (2011) observed a need for around 90 days to establish a stable biofilm on a virgin GAC bed. In our study, the results indicate that the 40-day operational period was too short to achieve full biological stability for the filter beds, although it proved adequate for biofilm development.

Biodiversity and taxonomic composition of microbial community

In Filter 2, the number of predominant OTUs (each accounting for over 1% of the total sequence reads), was found to be lower than that in in Filter 1 (Fig. 4). Notably, 8 predominant OTUs were shared between both filters. Furthermore, a sole predominant OTU was shared between Filter 2 and biohumus sample. As expected, no common predominant OTU was identified between Filter 1 and the biohumus sample.

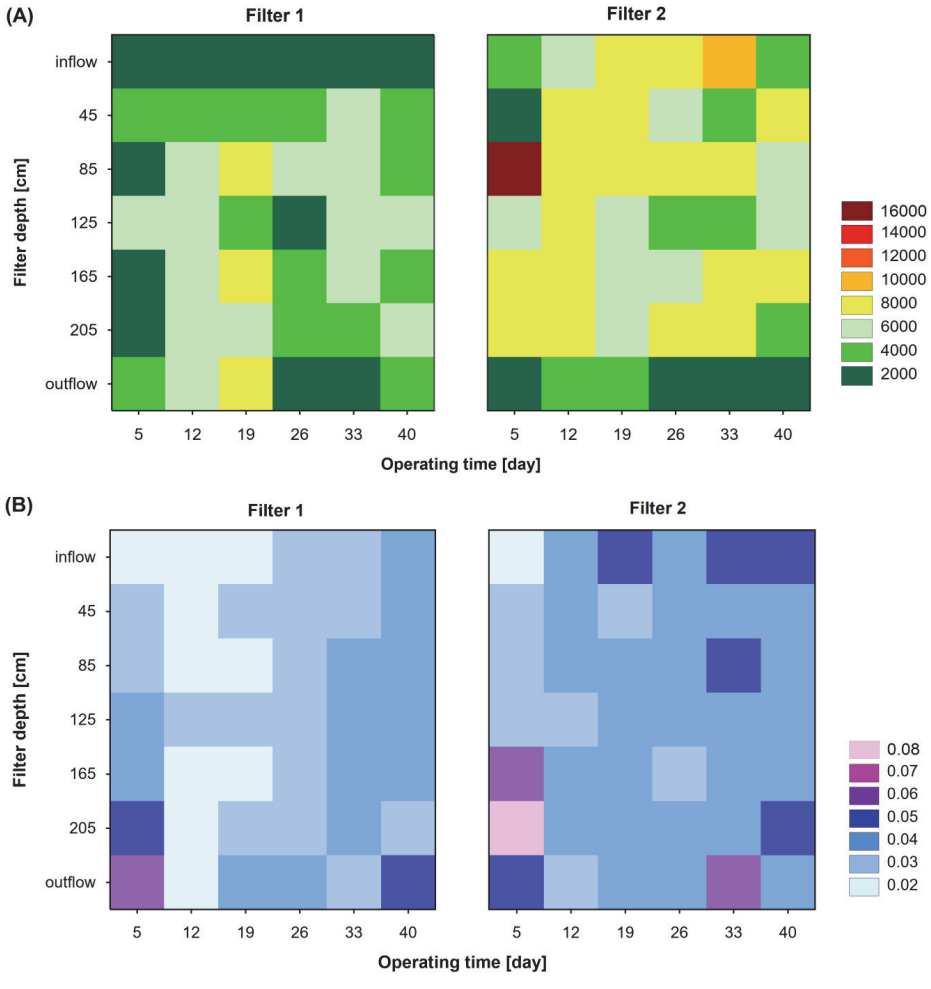


Fig. 3. Number of heterotrophic plate count bacteria (CFU mL^{-1}) (A) and total microbial activity (A min^{-1}) (B) in water samples collected from Filter 1 and Filter 2

The vast majority of the noted *Prokaryotes* within the filters belonged to the domain *Bacteria*. In Filter 1, only 0.001% of sequence reads were classified as *Archaea,* while Filter 2 showed a slightly higher count at 0.086%. Conversely, in the biohumus sample, the relative abundance of *Archaea* amounted to 0.679%. Phylum *Proteobacteria* emerged as the dominant group in both filters, constituting 99.8% of the prokaryotic community in Filter 1 and 97.8% in Filter 2. The analysis of microbial community at class level has shown that the primary classes within each filter bed were *Gammaproteobacteria* (53.4% in Filter 1 and 60.6% in Filter 2), *Betaproteobacteria* (35.8% in Filter 1 and 29.8% in Filter 2) and *Alphaproteobacteria* (10.5% in Filter 1 and 7.3% in Filter 2).

Classes belonging to the *Proteobacteria* are often identified as predominant components in raw water, as well as at various stages of drinking water treatment, including GAC filters operating in pilot or full-scale settings. However, their proportions tend to vary, highlighting the influence of multiple factors on the composition of the microbiome. These factors encompass aspects such as the source of raw water, the employed water treatment technology, and the duration of filter operation (Liao et al. 2015, Su et al. 2018, Oh et al. 2018).

The predominant family of the *Gammaproteobacteria* class was *Spongiibacteraceae* (53.2% of relative abundance in Filter 1 and 60.1% in Filter 2) (Fig. 5). This family was represented by only one OTU. Upon conducting a BLAST analysis, a 100% sequence similarity emerged between its sequence and the sequence of uncultured bacteria identified in GAC filters utilized for drinking water treatment (Table 4). Comparative analysis against sequences of cultured bacteria revealed the highest similarity with *Spongiibacter* (94%). The *Spongiibacteraceae* family comprises bacteria typically found in marine and halophilic environments. These bacteria are known to be strictly aerobic, Gram-negative, and motile or non-motile rods (Jean et al. 2016). The taxonomic position of this predominant bacterium needs further research.

In both filters, the *Betaproteobacteria* class was represented mainly by *Burkholderiales* (19.8% of the relative

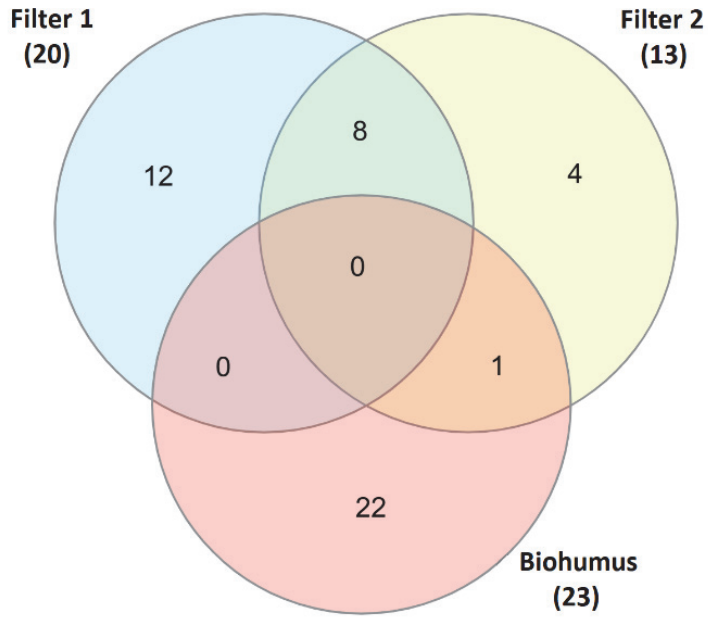


Fig. 4. Venn diagram of predominant OTUs (total number of sequence reads >1%) identified in filters and biohumus

Table 4. Similarity of predominant OTU noted in the filter beds (OTU1_F1_F2_BH; accession no. MW741757) with sequences determined by BLAST nucleotide search

Database	Closest database match (acc. no.)	Description	Sequence identity (%)
NCBI BLAST Standard database including uncultured/environmental sample sequences	KJ615131.1	Uncultured bacterium clone A1F_28 16S ribosomal RNA gene, partial sequence	100
	KJ615128.1	Uncultured bacterium clone A1F_25 16S ribosomal RNA gene, partial sequence	100
	KJ615120.1	Uncultured bacterium clone A1F_17 16S ribosomal RNA gene, partial sequence	100
NCBI BLAST Standard database excluding uncultured/environmental sample sequences	KC169814	*Spongiibacter* sp. CC-AMW-B 16S ribosomal RNA gene, partial sequence	94

abundance in Filter 1 and 22.01% in Filter 2). The main genera affiliated with this phylum and identified in both filters were: *Acidovorax, Hydrogenophaga* and *Rhizobacter,* In Filter 1 also *Methylotenera, Herminiimonas* and *Ferribacterium* were identified and in the case of Filter 2, the main taxa included *Curvibacter* and *Limnobacter thiooxidans* (Fig. 5).

The predominant families of *Alphaproteobacteria* were *Hyphomicrobiaceae* (4.0% in Filter 1, and 2.7% in Filter 2), *Beijerinckiaceae* (2.9% in Filter 1, and 2.3% in Filter 2), *Bradyrhizobiaceae* (2.2% in Filter 1, and 1.5% in Filter 2), and in the case of Filter 1, also *Acetobacteraceae* (1.2%). In both filters, the predominant genera from this class were *Hyphomicrobium*, *Beijerinckia* and *Bradyrhizobium* (Fig. 5).

Many of mentioned genera (*Hyphomicrobium*, *Hydrogenophaga*, *Acidovorax*, *Curvibacter*, *Bradyrhizobium*) have been previously identified in BAC filters (Kaarela et al. 2015, Liu et al. 2018, Oh et al. 2018, Dong et al. 2019). *Hyphomicrobium* is frequently noted in water environments such as freshwater, puddles, sewage treatment plants, and in soil (Garrity 2005), has also been found in biofilm of pipes within drinking water distribution systems (Chan et al. 2019). Its abundance tends to be higher in drinking water with low concentrations of free chlorine, aligning with the undetectable concentration of chlorine in the tap water used in this study (Waak et al. 2019). *Acidovorax* has been noted as predominant taxon in the effluent of GAC filters following 10 months of pilot-scale filter operation (Liao et al. 2015), while the *Hydrogenophaga* was the second predominant genus isolated from GAC filters operating within full-scale water treatment plants (Magic-Knezev et al. 2009). *Curvibacter* identified in a bed of BAC filter preceded by ozone treatment in the pilot-scale study (Dong et al. 2019), is commonly found in oligotrophic environments like wells (Rosenberg et al. 2014). Another genus worth noting, *Rhizobacter*, emerged as the sole predominant genus in both filters that was not present in biohumus sample. This genus encompasses species responsible for plant galls, yet it is also found in soil, biofilms of polluted rivers, and indoor dust. With a wide distribution worldwide, *Rhizobacter* proves challenging to culture (Jin et al. 2016).

In the case of biohumus, the highest relative abundance was observed for the following phyla: *Proteobacteria* (38.3%), *Firmicutes* (16.5%), *Bacteroidetes* (11.4%), *Actinobacteria* (6.5%) and *Chlorobiota* (6.2%). The predominant classes were *Gammaproteobacteria* (23.7%), *Bacilli* (11.3%), *Actinobacteria* (6.4%), *Alphaproteobacteria* (6.3%) and *Ignavibacteria* (6.1%). It is worth noting that a portion of

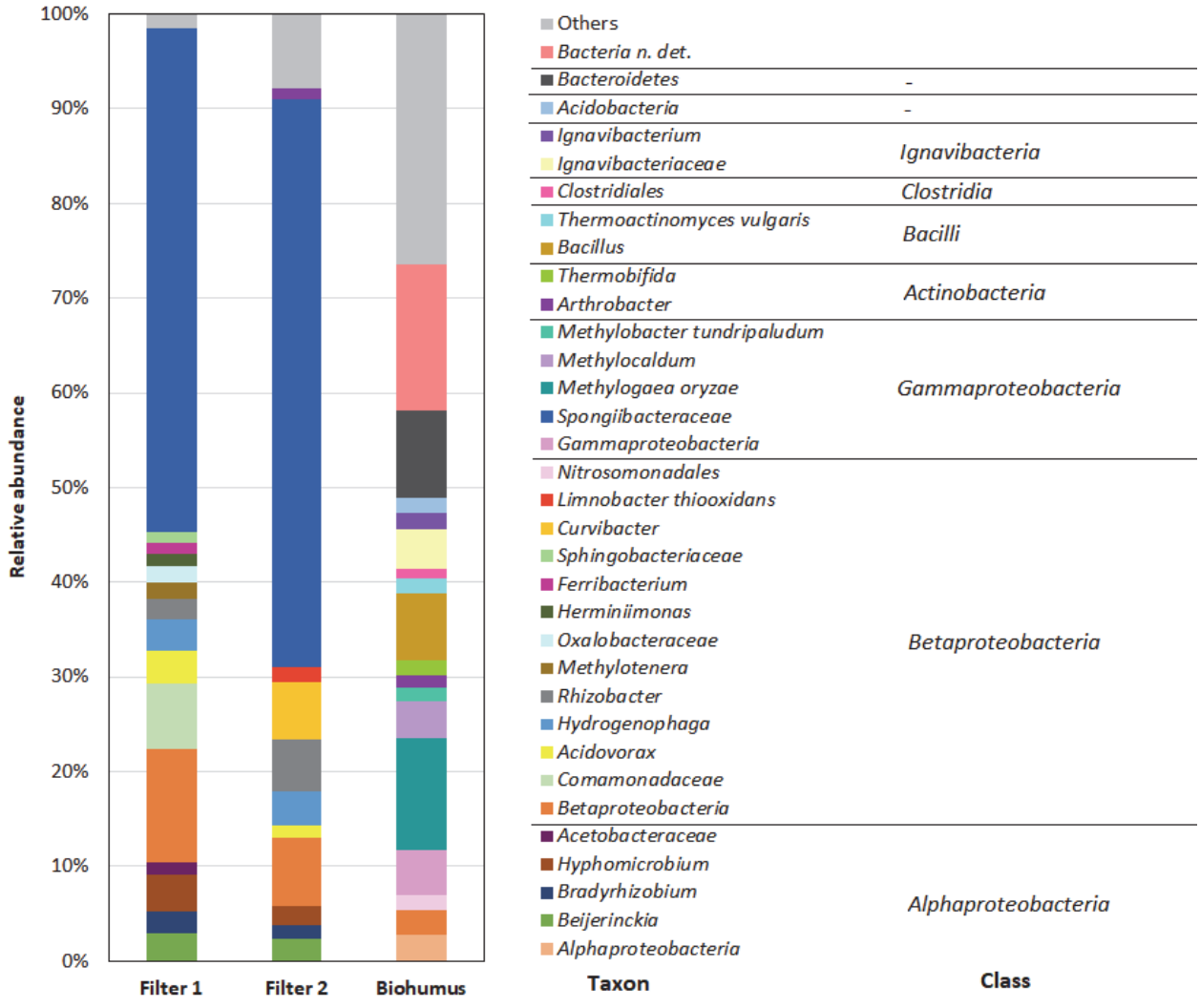


Fig. 5. Relative abundance of predominated taxa (>1% of total number of sequence reads) of bacterial communities of Filter 1, Filter 2 and biohumus ("Others" – taxa with the relative abundance <1%)

approximately 15.5% of biohumus sequence reads could not be classified into any specific phylum or class. Additionally, a substantial relative abundance of the "Others" group comprising taxa with the relative abundance below 1% was noted, making up 26.5% of the composition.

The biohumus community was predominantly represented by the gammaproteobacterial family: *Methylococcaceae* (17.3%), along with representatives from *Firmicutes*: *Bacillaceae* (7.6%) and *Thermoactinomycetaceae* (1.9%). Furthermore, the family *Ignavibacteriaceae* from the *Chlorobiota* phylum constituted 6.1%. Apart from *Arthrobacter*, the predominant genera/species identified in biohumus sample differed from those in filters. These included *Methylogaea oryzae*, *Bacillus*, *Methylocaldum*, *Thermobifida*, *Thermoactinomyces vulgaris*, and *Methylobacter tundripaludum* (Fig. 5).

Arthrobacter, a strictly aerobic bacterium, has been isolated from various environments including marine water and fresh water bodies, activated sludge, human skin, oil, air, sewage, clinical specimens, and cyanobacterial mats. However, its primary habitat is soil (Rosenberg et al. 2014). It is commonly found in agricultural soil and is known for its capability to degrade various environmental pollutants such as triazine herbicides, organophosphate insecticides, benzene and its derivatives, phthalates, polychlorinated biphenyls or some alkaloids. Beyond its impressive pollutant degradation prowess, *Arthrobacter* demonstrates remarkable adaptivity, being capable of enduring diverse environmental stresses including starvation, hypertonic and hypotonic conditions, oxidative stress, heavy metal stress, extreme temperatures and pH values. In fact, its resilience and versatility have led to its application as a valuable biosensor for biodegradation monitoring, as well as its involvement in bioremediation (Guo et al. 2019).

None of bacterial indicators associated with fecal contamination, such as *Escherichia coli, Shigella, Yersinia, Serratia, Salmonella, Enterobacter, Enterococcus,* and *Klebsiella*, were detected in either the filters or the biohumus sample. Additionally, the water environment pathogen *Vibrio* was absent across the samples. However, several bacteria classified as opportunistic premise plumbing pathogens (OPPPs) according to Hayward at al. (2022), were identified in both filters. These included *Arthrobacter*, *Chlamydiales, Bacillus, Legionella, Pseudomonas, Mycobacterium, Sphingomonas, Methylobacterium, Rhodococcus.* Filter 1 also revealed the presence of *Nocardia*, while Filter 2 exhibited *Microbacterium* and *Flavobacterium.* The relative abundance of most of these genera was very low (<0.01%). The exceptions were *Arthrobacter* (1.06% in Filter 2), *Bacillus* (0.12% in Filter 2), *Legionella* (approx. 0.03% in both filters), and *Pseudomonas* (0.03% in Filter 1 and 0.09% in Filter 2). Certain OPPPs have the potential to pose life-threatening risks, therefore, their ability to proliferate within BAF filters should be investigated and controlled.

Metabolic functions and biodegradation abilities of predominant microorganisms

The most predominant taxa observed in filters are chemoorganoheterotrophs and/or chemolithotrophs. These microorganisms belong to a group that biodegrades a wide range of organic compounds. For example, *Beijerinckia* prefers sugars as a carbon source for growth but it can metabolize a wide range of multicarbon compounds as well (Rosenberg et al. 2014). *Bradyrhizobium* can utilize several carbohydrates and organic acids as a carbon source. Furthermore, it exhibits the capability for aromatic degradation (Rosenberg et al. 2014, Oh et al. 2018). *Hydrogenophaga* and *Acidovorax* are proficient in the biodegradation of carboxylic acids, and/or amino acids. However, they may also function as facultative chemolithoautotrophs, utilizing the oxidation of H_2 as an energy source and CO_2 as a carbon source (Garrity 2005).

The analyses conducted using PICRUSt resulted in the generation of 141 functional pathways at level 3, utilizing KEGG pathway metadata. The majority of pathways at level 1 were linked to metabolic processes, accounting for 77.5% in the case of Filter 1 and 77.9% for Filter 2. The most frequently occurring pathways, as defined at level 2 in KEGG classification, were related to carbohydrate metabolism, amino acid metabolism, and the metabolism of cofactors and vitamins (Fig. 6). The relative frequencies of pathways at level 3 in KEGG were generally similar between both filters and biohumus. Only the frequency of the "xenobiotic biodegradation and metabolism" pathway was significantly higher in filters compared to biohumus (Fig. 6).

The analysis of pathways within the "xenobiotic biodegradation and metabolism" category revealed that among the filters, the highest relative frequency was observed in pathways associated with the biodegradation and metabolism of nitrotoluene (Fig. 7). This frequency was approximately 3 times higher in filters than in biohumus. Nitrotoluenes, like other xenobiotics, are substances that do not naturally occur in the environment. These chemical substances find applications in the production of azo and sulphur dyes for cotton, wool, silk, leather and paper. They are also utilized in agriculture, photography, the pharmaceutical industry and rubber production. Nitrotoluenes can be released into the environment, particularly the air and water, during their production and use. They have been detected in wastewater, groundwater, surface water, and drinking water. Removing these substances from aquatic environment poses challenges. The primary natural processes contributing to the removal of 2-nitrotoluene from water are photochemical degradation and biodegradation (IARC 2012, Khan et al. 2022).

Other identified pathways encompassed a range of processes, including drug metabolism involving other enzymes, chloroalkane and chloroalkene degradation, benzoate degradation, styrene degradation, chlorocyclohexane and chlorobenzene degradation, caprolactam degradation, aminobenzoate degradation, fluorobenzoate degradation, atrazine degradation, bisphenol degradation, polycyclic aromatic hydrocarbon degradation, and toluene degradation (the latter found exclusively in Filter 2). The same pathways were also observed in biohumus, but generally at lower relative frequency, with exceptions in pathways related to drug metabolism via other enzymes, chloroalkane and chloroalkane degradation and toluene degradation. The presence of bacteria capable of the above-mentioned biodegradation of xenobiotics in BAC filters indicates water contamination with these substances and requires further research, as they may be harmful to human health.

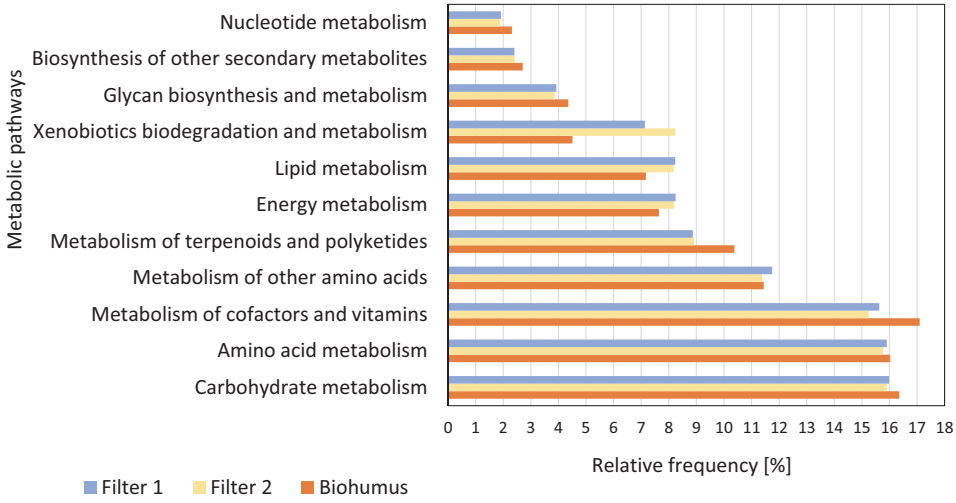


Fig. 6. Type and relative frequency of predicted pathways related to metabolism identified in filters and biohumus

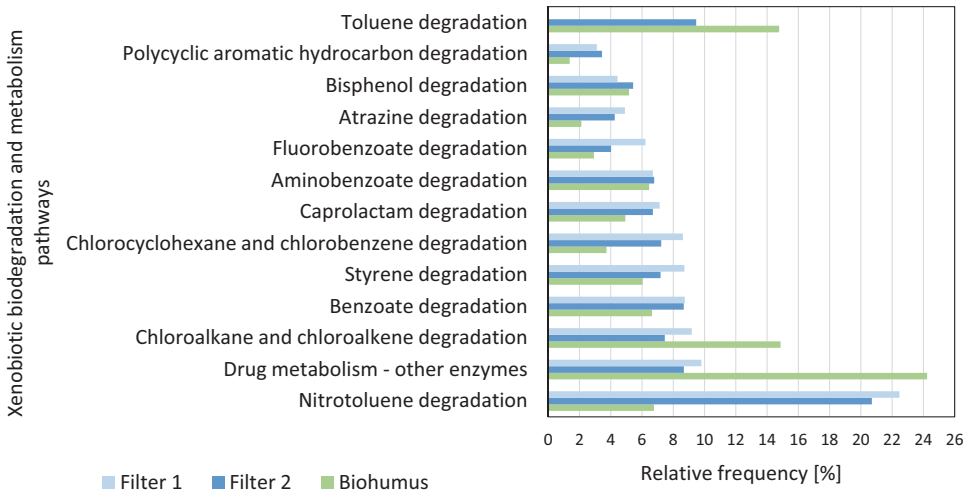


Fig. 7. Type and relative frequency of predicted pathways belonged to the "xenobiotic biodegradation and metabolism pathway" identified in filters and biohumus

Conclusions

The findings of the presented research can be summarized through the following conclusions:

- Biofilm formation in BAC filters can initiate approximately during the 4th week of filters' operation after being filled with the virgin GAC medium.
- Variations in the chemical and microbiological quality of the inflowing water between filters did not have a substantial impact on the efficiency of DOM removal.
- Introducing the biohumus solution resulted in faster colonization of bacteria onto GAC grains. Discrepancies between both filters were evident in terms of HPC bacterial numbers and TMA values, but no statistically significant correlation was detected between these parameters. These two methods of microbial community analysis should be used in a complementary manner.
- Dosing of the biohumus solution to Filter 2 caused the reduction in the biodiversity of predominant OTUs. While variations in the microbial composition between the filters encompassed several taxa with relatively low abundance, only one taxon (with a relative abundance >1%) was shared between Filter 2 and biohumus. This observation suggests that the filters function as microbial sieves and provide specific conditions that support the growth of a restricted group of microorganisms.
- Numerous bacterial taxa detected in BAC filters have been previously found in similar installations. The exception was *Spongiibacter* sp. – the main predominant taxon noted in both filters, which is not a common and well-known bacterium. This underscores the necessity for further updates and advancements in our understanding of the microbiome within BAC filters.
- The study revealed the potential existence of certain opportunistic pathogens within BAC filters. In such cases, in order to prevent any adverse impacts on human health, it is imperative to consistently implement an effective disinfection procedure at the drinking water treatment plant.
- Analysis of the predicted functional pathways showed that BAC filters may be able to remove xenobiotics

through microbial biodegradation and metabolism even at the initial stage of filter operation.

- Employing the PICRUSt software and KEGG Orthology can be helpful in estimating the potential hazard posed by xenobiotics in treated water, as well as evaluating the efficiency of microorganism-mediated removal of these contaminants within BAC filters.

In further research, we want to focus on studying the microbiome of fully activated BAC filters operating on both pilot and full-scale. We want to investigate the role of microorganisms in the biodegradation of organic matter, e.g., xenobiotics such as antibiotics.

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Zbiorowisko drobnoustrojów w początkowej fazie pracy biologicznie aktywnych filtrów węglowych i ich rola w usuwaniu materii organicznej z wody

Streszczenie: Filtracja przez biologicznie aktywne filtry węglowe (BAF) jest skuteczną metodą usuwania materii organicznej podczas uzdatniania wody przeznaczonej do spożycia przez ludzi. W niniejszej pracy zbadano zbiorowisko drobnoustrojów w początkowym okresie eksploatacji filtrów oraz jego rolę w usuwaniu materii organicznej z wody. Badania przeprowadzono w skali pilotowej na dwóch filtrach BAF (Filtr 1 i Filtr 2) różniących się składem dopływającej wody. Stwierdzono, że liczba bakterii heterotroficznych i całkowita aktywność mikrobiologiczna były znacząco większe w próbkach wody pobranych z Filtra 2 – zasilanego wodą wzbogaconą o dodatkowy ładunek materii organicznej i mikroorganizmów. Pomimo różnic w wartościach parametrów chemicznych i mikrobiologicznych dopływającej wody, skład mikrobiomu w filtrach był podobny. W obu filtrach dominującym taksonem była bakteria spokrewniona z *Spongiibacter* sp. (*Gammaproteobacteria*) (>50% względnej liczebności). W obydwóch filtrach efektywność usuwania materii organicznej była na podobnym poziomie oraz skład i względna częstość występowania przewidywanych szlaków funkcjonalnych związanych z metabolizmem, oznaczone przy użyciu PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States Software) na poziomie 3 KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology były również zbliżone. Badania wykazały, że 40-dniowy okres pracy filtrów po napełnieniu świeżym granulowanym węglem aktywnym był wystarczający do rozwoju biofilmu. Udowodniono, że w początkowym okresie pracy złoża filtracyjne zasiedlane były przez mikroorganizmy zdolne do biodegradacji różnych związków organicznych, w tym ksenobiotyków, np. nitrotoluenu.