Microbial communities and processes in biofilters for post-treatment of ozonated wastewater treatment plant effluent

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Abstract

Ozonation is an established solution for organic micropollutant (OMP) abatement in tertiary wastewater treatment. Biofiltration is the most common process for the biological post-treatment step, which is generally required to remove undesired oxida-

tion products from the reaction of ozone with water matrix compounds. This study comparatively investigates the effect of filter media on the removal of organic contaminants and on biofilm properties for biologically activated carbon (BAC) and anthracite biofilters. Biofilms were analysed in two pilot-scale filters that have been operated for > 50,000 bed volumes as post-treatment for ozonated wastewater treatment plant effluent. In parallel, the removal performance of bulk organics and OMP, including differentiation of adsorption and biotransformation through sodium azide inhibition, were carried out in bench-scale filter columns filled with material from the pilot filters. The use of BAC instead of anthracite resulted in an improved removal of organic bulk parameters, dissolved oxygen, and OMP. The OMP removal observed in the BAC filter but not in the anthracite filter was based on adsorption for most of the investigated compounds. For valsartan, however, biotransformation was found to be the dominant pathway, indicating that conditions for biotransformation of certain OMP are better on BAC than on anthracite. Adenosine triphosphate analyses in the media-attached biofilms of the pilot filters showed that biomass concentrations in the BAC filter were significantly higher than in the anthracite filter. The microbial communities (16S rRNA gene sequencing) appeared to be similar with respect to the types of organisms occurring on both filter materials. Alpha diversity also exhibited little variation between filter media. Beta diversity analysis, however, revealed that filter media and bed depth substantially influenced the biofilm composition. In practice, the impact of filter media on biofilm properties and biotransformation processes should be considered for the design of biofilters.

Keywords

Tertiary wastewater treatment, Ozonation, Biofiltration, Adsorption, Biotransformation, Microbial community

1 Introduction

Municipal wastewater treatment plants (WWTP) have been shown to be important point sources for anthropogenic organic micropollutants (OMP) in surface waters (Petrie et al., 2015). To mitigate the contamination of surface waters with OMP, several WWTP in Switzerland and Germany have been upgraded in recent years with advanced treatment technologies such as ozonation or activated carbon adsorption (Benstoem et al., 2017; Bourgin et al., 2018; Itzel et al., 2020). When ozonation is applied, it should generally be combined with a biological post-treatment step to degrade readily biodegradable organic matter as well as potentially toxic oxidation by-products and transformation products, formed by the reaction of ozone with the water matrix (Lee and Von Gunten, 2016; Luo et al., 2014). A broad variety of treatment processes has been used as biological post-treatment. However, several of these solutions such as constructed wetlands (Kharel et al., 2021), fixed-bed or moving-bed bioreactors (Bourgin et al., 2018), or ozonation as an intermediate step in the activated sludge process (Baresel et al., 2016) were applied infrequently and often only at pilot-scale. The most common solution for biological post-treatment is biofiltration in deep-bed filters with different media such as sand, anthracite, or granular activated carbon (GAC) (Reungoat et al., 2012; Sauter et al., 2021a; Zimmermann et al., 2011).

Several biofiltration studies that comparatively investigated GAC and non-adsorptive media (sand or anthracite) demonstrated that the choice of filter material may have an impact on the treatment result. After an acclimation phase for biofilm establishment, biologically activated carbon filters (BAC) exhibited a higher removal of bulk organic matter and dissolved oxygen (DO) than non-adsorptive filters, indicating an enhanced biological activity (Bacaro et al., 2019; Reaume et al., 2015; Reungoat et

al., 2011; Sauter et al., 2021a). Also, improved abatement of OMP has been repeatedly reported, when GAC was used instead of non-adsorptive filter media (Bourgin et al., 2018; Knopp et al., 2016; Reungoat et al., 2011; Sauter et al., 2021a). The superior OMP removal performance of GAC/BAC filters was either exclusively attributed to adsorption processes (Knopp et al., 2016) or an additional involvement of biotransformation processes in the GAC-attached biofilm was hypothesised (Bourgin et al., 2018; Reungoat et al., 2011; Sauter et al., 2021a). Since exhaustion of the GAC adsorption capacity over-time results in a decreasing adsorptive OMP removal, biotransformation of OMP was suggested when a continuous removal was still observed after long-term filter operation (Bourgin et al., 2018; Fundneider et al., 2021; Sauter et al., 2021a).

The differentiation between adsorption and biotransformation in BAC filters is not trivial. Different approaches such as inhibition experiments (Rattier et al., 2012), comparative rapid small-scale column tests (Zhiteneva et al., 2020), and tests with ^{14}C-labeled OMP (Betsholtz et al., 2021) have been applied. However, the question as to why conditions for biotransformation seem to be more favourable in BAC filters than in non-adsorptive biofilters remains largely unanswered. Potential explanations are the surface properties of the GAC grains (roughness, porosity, charge, high specific surface area) and the better availability of adsorbed organic substrates that facilitate biofilm growth, which may result in a higher biomass concentration and/or activity (Basu et al., 2016; Simpson, 2008). Also, the regeneration of GAC adsorption sites by attached microorganisms (bioregeneration) is an important mechanism, maintaining the adsorptive removal performance of BAC filters (Aktaş and Çeçen, 2007). Recent studies also found an impact of filter media on the microbial community composition that may influence the capacity for biotransformation of OMP (Oh et

al., 2018; Vignola et al., 2018; Zhang et al., 2018).

Biotransformation processes, biomass quantity, and biofilm composition have not been investigated for BAC filters in a combined approach so far. Therefore, it is unknown how the enhanced treatment performance of BAC filters compared to non-adsorptive filters is related to the microbial community of the filter material. This study, investigates the removal of organic contaminants (including differentiation of adsorption vs. biotransformation) and biofilm characteristics (quantitative and qualitative) under the same conditions and thus, helps to better understand the interplay of biotransformation processes and the microbial communities. The experiments were performed with a BAC filter and an anthracite filter at pilot scale, both having been operated long-term (> 50,000 bed volumes) as post-treatment of ozonated wastewater treatment plant (WWTP) effluent. The removal of bulk organics and OMP was studied in bench-scale filter columns filled with media from the pilot filters and sodium azide (NaN_3) inhibition was applied to distinguish adsorption and biotransformation. Biofilm characterisation was carried out with filter media samples from three depths of the pilot-scale filters. Active biomass was quantified based on adenosine triphosphate (ATP) analysis. The microbial community compositions were determined using 16S rRNA gene sequencing.

2 Material and Methods

2.1 Experimental setup

2.1.1 Pilot-scale filters

Pilot-scale tertiary treatment with ozonation followed by two dual-media deep bed filters for biological post-treatment was operated on a long term at the full-scale municipal WWTP Schoenerlinde / Germany (Fig. 1). The secondary effluent quality of the WWTP during the trial period is characterised in Table S1. The ozone unit was

run with a targeted specific consumption of 0.7 g O_3/g DOC throughout the trial. For comparability, both pilot filters were designed and operated identically, except for the upper layer of filter media which consisted of GAC (grain size: 1.4−2.4 mm) in one and anthracite (grain size: 1.4−2.5 mm) in the other. The filter beds had a diameter of 0.3 m and a height of approx. 1.8 m (1.2 m GAC or anthracite + 0.6 m sand). The empty bed contact time (EBCT) in the upper layer amounted to approx. 15 min (flow rate: ~ 340 L/h). For additional phosphorus removal, ferric chloride was dosed in both filter influents. Backwash with air and water was carried out daily on workdays to prevent clogging. Further details on pilot plant design and operation as well as the secondary effluent quality of the full-scale plant have been previously published (Sauter et al., 2021a, 2021b).

Filter media cores with a length of approx. 15 cm were taken from three depths of the pilot BAC and anthracite filter to quantify the active biomass and to analyse the microbial communities. As depicted in Fig. 1, the samples from the upper (0−15 cm), middle (45−60 cm), and lower (90−105 cm) layer of the filter beds are abbreviated as BAC-u, BAC-m, BAC-l, and Ant-u, Ant-m, Ant-l. Quantitative indications of the sampling depths refer to the middle of the respective filter media sample, i.e., 7.5 cm, 52.5 cm, and 97.5 cm. Sampling was conducted with a metal pipe with an inner diameter of 2.5 cm that was pushed into the drained filter bed from the top. For the middle and lower layer, a precisely fitting wooden bar was placed in the metal pipe to prevent filter media from entering the pipe until the target depth of 45 cm or 90 cm, respectively, was reached. Subsequently, the wooden bar was removed and the metal pipe was pushed 15 cm deeper into the filter bed to obtain the filter media core. The filter media samples were transferred to sterile plastic bottles and transported to the laboratory on ice.

2.1.2 Bench-scale filters

Previous studies observed a steady removal of several OMP at high treated bed volumes in deep-bed filters with BAC but not with anthracite, which suggested the involvement of biological processes besides adsorption (Bourgin et al., 2018; Sauter et al., 2021a). A bench-scale experiment aiming to inhibit biological processes with NaN_3 was conducted, to elucidate the influence of biotransformation and the role of filter media. Four identical bench-scale filter columns (diameter = 2.6 cm) were filled with a 15.6 cm layer of used media from pilot-scale filters, two filter columns were filled with BAC and two with anthracite. Until the day of the filter media transfer, the BAC and the anthracite material had treated approx. 55,000 and 50,000 bed volumes, respectively. The bench-scale filters were operated at approx. 15 min EBCT (flow rate: ~ 0.33 L/h) in accordance with the pilot filter and backwashed with water when required (approx. twice a week). Until the start of the experiment the BAC and Ant filters reached approx. 65,000 and 60,000 bed volumes, respectively. For the inhibition experiment, one of the identical filters was fed with a 25 mM solution of NaN_3 (dissolved in ozonated secondary effluent) until the bed volume of the filter was exchanged twice. Thereafter, the operation was stopped for 20 hours to provide sufficient time for the inhibition of the filter biofilm. The operation was relaunched and after the filters had treated three bed volumes of regular ozonated secondary effluent without NaN_3, three separate consecutive samplings of corresponding influent and effluent grab samples were performed. As shown in Fig. 1, the sampling points were abbreviated according to the respective upstream treatment process (O_3, BAC, BAC+NaN_3, Ant, and Ant+NaN_3). Samples were analysed for OMP, DOC, $UVA_{254,}$ and DO. As the latter three parameters are good indicators for biodegradation processes, they were used to assess the effectiveness of NaN_3-inactivation.

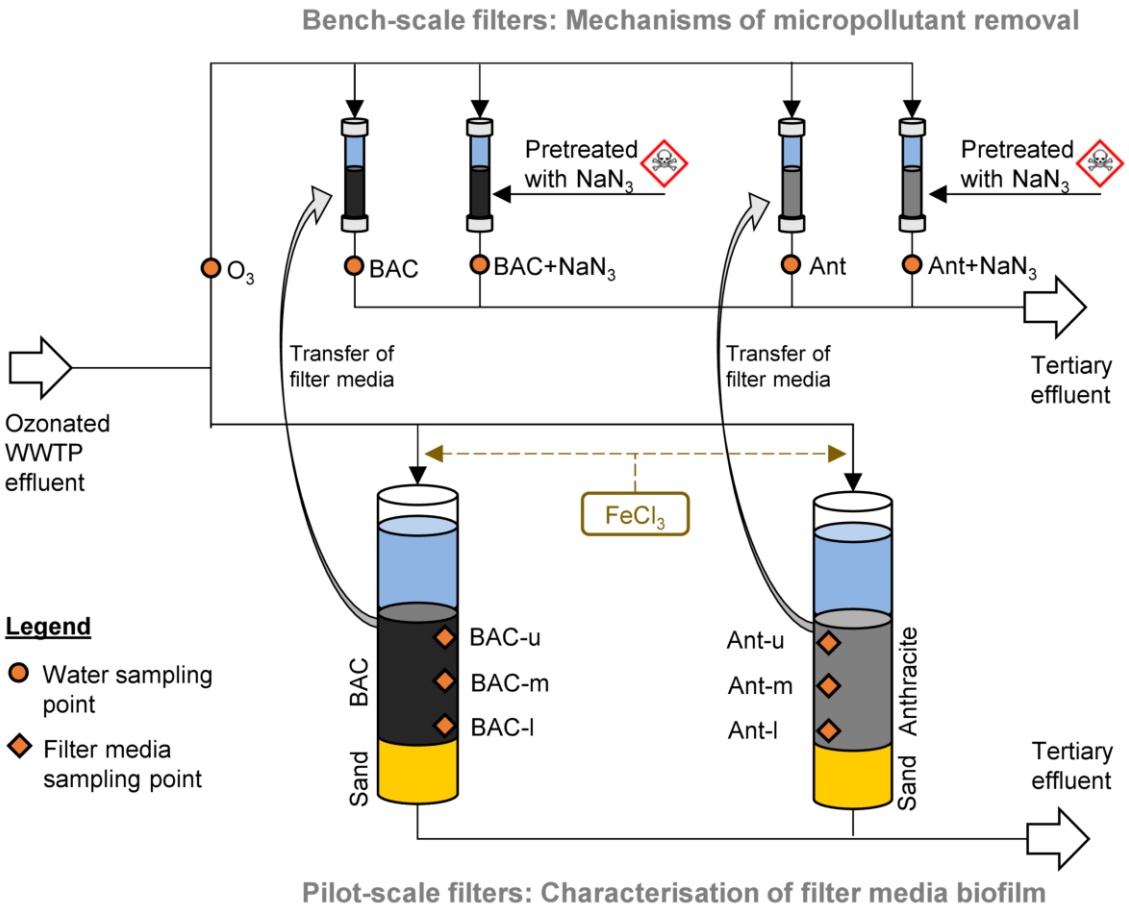

Fig. 1: Scheme of bench-scale and pilot-scale filter column experiments.

2.2 Analytical methods

2.2.1 Water quality parameters

Organic bulk parameters DOC and UVA_{254} were analysed according to DIN EN 1484 (H03) and DIN 38404-C03, respectively. OMP (Table S2) were analysed with high-performance liquid chromatography followed by tandem mass spectrometry following DIN 38407-F47. All OMP were either pharmaceuticals or transformation products thereof. When compounds were detected at concentrations below the limit of quantification (LOQ), values were replaced by 1/2 LOQ. For transparency, they were marked as hatched columns in the presentation of the results. A more detailed description of the methods above can be found in a previous publication (Sauter et al.,

2021a). In addition, DO was measured directly onsite with an optical probe (Fibox 4, PreSens Precision Sensing, Germany).

2.2.2 Active biomass on filter media

Active biomass on the filter media samples of the pilot-scale BAC and anthracite filter was analysed based on ATP measurements. ATP serves as an energy transfer unit in all living cells and therefore is a suitable parameter for active biomass quantification. It has been frequently used for the investigation of biofilters in drinking water treatment (Magic-Knezev and van der Kooij, 2004; Pharand et al., 2014; Velten et al., 2011), in rare cases also in wastewater treatment (Reaume et al., 2015; Sbardella et al., 2018). ATP was quantified directly on the filter media with BacTiter-Glo™ Microbial Cell Viability Assays (Promega, USA) following an established method (Velten et al., 2007). In short: approx. 200 mg of wet filter media sample were mixed with 100 µL phosphate buffer and 300 µL BacTiter-Glo™ reagent (after 3 min) while incubating at 30 °C. After 1.5 min, 200 µL of the supernatants were transferred to a 96-microtiter well plate and luminescence was measured after 30 seconds in a microtiter plate luminescence reader (Tecan Infinite® M200, Tecan Trading AG, Switzerland). For calculation of ATP concentrations, a calibration curve from 0.5 µM to 5 µM ATP was established using fresh autoclaved GAC as filter media. Triplicates of each concentration were analysed and the mean values were used to calculate the linear regression (Fig. S1). ATP concentrations in the filter media samples and the sample blanks (addition of 300 µL phosphate buffer instead of BacTiter-Glo™ reagent) were also determined in triplicates. Values from sample blanks were subtracted from the ATP concentration of the respective sample. Dry matter fractions of all samples were measured following DIN EN 15934. The final result of active biomass analysis was expressed as ATP per filter bed volume, using Equation

S1. This approach is advantageous since it takes into account varying sample moistures and bulk densities of different filter media or grain sizes. Furthermore, the parameter of interest for biofilter design is the filter bed volume rather than the filter bed weight. In the following, ATP concentrations will be referred to as biomass concentration.

2.2.3 16S rRNA gene sequencing for microbial community analysis

DNA was extracted from approx. 250 mg wet filter media sample using a GeneMATRIX Soil DNA Purification Kit (Roboklon, Germany) according to the manufacturer's instructions. 16S rRNA gene sequences were amplified using the universal primer pair 515F (5'-GTGYCAGCMGCCGCGGTAA-3') (Parada et al., 2016) and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015) targeting the V4 region from triplicates of three different levels of the pilot BAC and anthracite filters (Fig. 1). PCR primers 515F and 806R were modified with two linker sequences and barcoded (8 cycles) with a unique dual barcoding (UDB) setup (Pjevac et al., 2021). First-step PCRs for 16S rRNA genes were done in triplicate (12.5 µl vol per reaction) with: 1X DreamTaq Buffer (Thermo Fisher), 2 mM $MgCl_2$ (Thermo Fisher), 0.2 mM dNTP mix (Thermo Fisher), 0.2 µM of forward and reverse primer each, 0.08 mg ml-1 Bovine Serum Albumin (Thermo Fisher), 0.02 U Dream Taq Polymerase (Thermo Fisher), and 0.5 µl of DNA template. Thermal cycling included: 95°C for 3 min, followed by 30 cycles of 30 sec at 95°C, 30 sec at 52°C and 50 sec at 72°C, and finally 10 min at 72°C. Triplicates were combined for barcoding. Amplicon sequencing was performed on a 2 × 300 bp paired-end mode (V3 chemistry) with a MiSeq (Illumina) instrument at the Joint Microbiome Facility (Vienna, Austria) (project ID JMF-2007-2). Sequence reads were quality filtered and trimmed using the model-based Divisive Amplicon Denoting Algorithm of the R-package DADA2 (Callahan et al., 2016a), using a previ-

ously published workflow (Callahan et al., 2016b; Pjevac et al., 2021). Obtained amplicon sequence variants (ASV) were assigned using the SILVA database release 138 (Parks et al., 2018; Quast et al., 2013), and using the naïve Bayesian classification approach implemented in mothur (Schloss et al., 2009). Raw sequencing data are available in the BioProject database of the National Center of Biotechnology Information (NCBI) under the accession number PRJNA885243. Table S3 summarises the number of reads per sample. For comparison with other studies, it must be considered that all *Betaproteobacteria* were reclassified in the SILVA database under the order *Burkholderiales* in the class *Gammaproteobacteria* (Parks et al., 2018), while the NCBI database still holds *Betaproteobacteria* as its own class. The transformation, normalization, and alpha-diversity calculations were carried out with the R-package Rhea (Lagkouvardos et al., 2017). Richness, Shannon index and Inverse Simpson Index were applied for the comparison of alpha-diversities. To assess the beta-diversity, a principal coordinate analysis (PCoA) was performed using a Bray-Curtis dissimilarity matrix including relative abundances. The 'vegan' package (version 2.5-3) was used to construct the plot in R. Analysis of similarity (ANOSIM) of the bacterial community composition was done using the MicrobiomeAnalyst (Chong et al., 2020; Dhariwal et al., 2017). Low abundant features were filtered based on the mean values with the minimum count set at 4, low variance features were removed based on the interquartile range. Data were rarefied to the minimum library size and normalized using the total sum scaling method.

2.3 Statistical data analysis

All statistical data analyses were carried out with the software R and R studio. DOC, DO and UVA_{254} removal data from the bench-scale experiment were tested for significant differences between the BAC vs. BAC+NaN_3 and the Ant vs. Ant+NaN_3. If

normality (Shapiro-Wilk test) and homogeneity of variance (Levene test) of the data were fulfilled (for DOC and DO), an unpaired two-sample t-test was applied. For the UVA_{254} removal data, a non-parametric Mann-Whitney-U test was performed since it did not follow a normal distribution. OMP concentrations at the sampling points of the bench-scale filters were compared with a one-way ANOVA followed by a Tukey post hoc test if the data of a compound exhibited normality (Shapiro-Wilk test on residuals) and homogeneity of variance (Levene test). For compounds that did not comply with these preconditions, a non-parametric Kruskal-Wallis test followed by a Dunn-Bonferroni post hoc test was carried out. The ATP-based biomass data and alpha diversity data of the different filter media samples were also analysed using a one-way ANOVA followed by a Tukey post hoc test, as normality and homogeneity of variance criteria were fulfilled. Differences in the data were considered statistically significant if the test result gave a p-value < 0.05. All obtained p-values are listed in Tables S4, S5, and S6.

3 Results and Discussion

3.1 Bench-scale investigation of OMP removal mechanisms

Bench-scale filters with BAC showed enhanced removal of organic bulk parameters measured as DOC and UVA_{254} compared to anthracite filters (Fig. 2). The removal was combined with increased consumption of DO. This agrees with observations made in pilot-scale filters with the same materials (Sauter et al. 2021a).

The pre-treatment of the BAC filter with NaN_3 resulted in a significant reduction of UVA_{254} and DO removal compared to the untreated BAC filter. UVA_{254} is a widely used surrogate parameter for the removal of OMP during ozonation and activated carbon adsorption (Altmann et al., 2016a; Stapf et al., 2016). The mean DOC removal decreased accordingly. However, the difference between the NaN_3 treated

and untreated BAC filter was not significant for DOC removal, suggesting an incomplete inhibition of the biofilm activity by NaN_3. Other studies observed incomplete inhibition of aerobic microbial processes by NaN_3 (Pires et al., 2013; Rattier et al., 2012). NaN_3 affects microbial growth and activity, however, its efficiency depends on its concentration and matrix effects (Cabrol et al., 2017). Furthermore, not all bacterial groups are equally susceptible to NaN_3 treatment (Lichstein and Soule, 1944), inhibition may be reversible for certain enzymes (Scotto and Lai, 1998), and strategies to overcome inhibition through bypassing affected respiratory chains have been described (Bore et al., 2017). Despite these shortcomings heat treatment as an alternative was not suitable for inactivation experiments in this set-up. High temperatures may cause desorption of OMP from the GAC surface together with a subsequent improvement of the adsorptive removal of GAC (Betsholtz et al., 2021). The incomplete inhibition of the biofilm activity in the BAC+NaN_3 filter hampered a quantitative analysis of the contributions of adsorption and biotransformation to the overall removal of an OMP in the BAC-filters. However, if biotransformation plays a substantial role for a compound, a significantly different abatement of an OMP in the BAC+NaN_3 compared to the non-treated BAC filter can be expected.

For the Ant and Ant+NaN_3 filters no significant difference in the removal of bulk parameters UVA_{254}, DO, or DOC was found, suggesting that the inhibition was ineffective (Fig. 2). The NaN_3 solution may have been washed out faster from non-porous anthracite than from the BAC material, where pores may have retained NaN_3. No relevant abatement of OMP was observed in the pilot Ant filter in a previous study (Sauter et al., 2021a). Therefore, in the following, removal mechanisms of OMP will be analysed for the BAC filters, and Ant filter results (shown in Fig. S2) only serve as a reference and for demonstrating the reproducibility of the pilot-scale results.

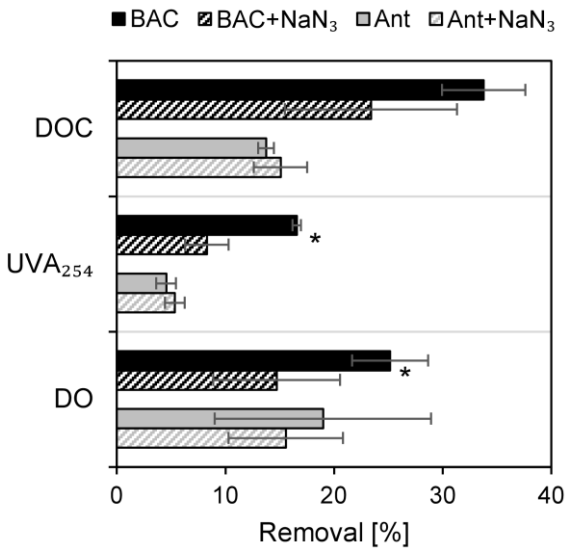

Fig. 2: Mean values with standard deviations (n=3) of the removal of DOC, UVA_{254}, and DO in the bench-scale filters. Asterisks indicate a significant difference between the unharmed and NaN_3-pretreated filters.

Most of the analysed OMP could be grouped according to their behaviour in the bench-scale BAC filters. As depicted in Fig. 3 and Fig. S3, they exhibited no removal at all (grey: no significant concentration change in both filters), adsorptive removal only (blue: significant and comparable concentration decrease in both filters), or removal/formation by biotransformation (green: significantly different filter effluent concentrations).

The concentrations of the compounds candesartan, gabapentin, primidone, and its metabolite phenylethylmalonamide (PEMA) were not impacted by the filter passage, showing that neither adsorption nor biotransformation played a significant role for this group. Removal based on adsorption in the already loaded BAC filters (~65,000 bed volumes) has not been expected for any of these substances, since they are all known to have moderate or poor adsorptive properties and hence exhibit relatively quick breakthrough in GAC filtration (Fundneider et al., 2021; Sauter et al., 2021a;

Zhiteneva et al., 2020). Candesartan, primidone, and PEMA are typically classified as persistent compounds that do not show relevant biotransformation (Filter et al., 2021; Hass et al., 2012; Hellauer et al., 2017). Information on the biodegradability of gabapentin is less consistent and seems to be highly dependent on factors such as redox conditions and organic substrate availability (Filter et al., 2021). Moreover, the influent concentration seems to play a crucial role in gabapentin degradation. Altmann et al. (2016b) reported that gabapentin had almost reached a full breakthrough in a wastewater GAC filter before biodegradation was established, accompanied by a drastic influent concentration increase from 4 to 16 µg/L. This would also explain why studies on GAC filtration of secondary effluent generally found a sustained gabapentin removal of 40−60 % (Altmann et al., 2016b; Fundneider et al., 2021; Zhiteneva et al., 2020) while applications with prior ozonation and thus lower gabapentin concentrations in the GAC filter influents observed long-term abatements of <20 % (Bourgin et al., 2018; Sauter et al., 2021a). Interestingly, gabapentin was constantly removed by >10 % during pilot-filter operation with the same BAC material as in the bench-scale experiments (Sauter et al., 2021a). Since mean gabapentin influent concentrations during the pilot study were more than double than during the bench-scale experiment (0.55 µg/L vs. 0.23 µg/L), again, influent concentration could be the potential driver for the different behaviour. Further research is needed to confirm the concentration dependence of gabapentin biotransformation. In any case, significant removal of gabapentin based on biodegradation should not be assumed for practical applications of BAC filters with prior ozone treatment.

Adsorption-based removal was found for the compounds oxypurinol, lamotrigine, and 10,11-dihydroxycarbamazepine (CBZD). Despite the high treated bed volumes of the BAC filter, they were significantly abated by 67 %, 73 %, and 26 %, respectively. The

very similar behaviour in the inactivated BAC+NaN_3 filter suggests that biotransformation does not contribute substantially to the abatement. None of the three substances exhibited a statistically significant difference between the two filters. Literature confirms their persistence even in treatment systems with long hydraulic retention times of >2,000 min (Filter et al., 2021; Hermes et al., 2019). In accordance with the observed abatements, oxypurinol and lamotrigine have been shown to be well adsorbable (Fundneider et al., 2021; Golovko et al., 2020; Ullberg et al., 2021), while CBZD moderately adsorbs onto activated carbon (Margot et al., 2013). Based on the breakthrough behaviour in BAC filtration, two recent studies hypothesised that biodegradation might play a role in oxypurinol removal in addition to adsorption (Fundneider et al., 2021; Sauter et al., 2021a). In light of the presented bench-scale results, a direct degradation of oxypurinol seems unlikely. However, biodegradation might indirectly contribute to a prolonged adsorption-based oxypurinol removal by degrading adsorbed bulk DOC and biodegradable OMP from the GAC surface, and thus regenerating adsorption sites. An extended GAC lifetime for non-biodegradable compounds due to biodegradation of competing biodegradable adsorbates has been experimentally demonstrated (De Laat et al., 1985). A short-term inactivation experiment, as conducted here, does not allow to uncover this effect since it would take some time for the remaining free adsorption sites to be occupied.

The group of compounds that was impacted by biotransformation consisted of valsartan and valsartan acid. The concentration of valsartan was significantly reduced in both BAC filters. However, the abatement in the untreated BAC filter was significantly higher than in the BAC+NaN_3 filter, indicating biotransformation. As discussed above, the contributions of biotransformation and adsorption could not be quantified due to the incomplete inhibition of the biofilm in the BAC+NaN_3 filter.

However, the influence of adsorption is assumed to be negligible at this stage of filter operation since valsartan has a relatively low adsorbability (Altmann et al., 2015). In the pilot-scale filter, valsartan reached full breakthrough at 10,000−15,000 bed volumes before a sustained removal started to establish (Sauter et al., 2021a). Thus, it is likely that the valsartan abatement in the BAC+NaN_3 filter (24 % on average) was linked to the biological activity that remained despite the NaN_3 pre-treatment. This interpretation is corroborated by the results for valsartan acid, the major biological transformation product of valsartan (Helbling et al., 2010). The filter influent concentration of valsartan acid was slightly reduced by the undisturbed BAC filter, while in the BAC+NaN_3 filter a small concentration increase was observed. The resulting effluent concentrations of the BAC and BAC+ NaN_3 filters were significantly different. The reported biotransformation rate constant of valsartan is considerably higher than of valsartan acid. Therefore, an overall formation of valsartan acid is expected as long as the parent compound is present (Kern et al., 2010; Posselt et al., 2020). Possibly, the remaining transformation of valsartan into valsartan acid in the incompletely inhibited BAC+NaN_3 filter masked the transformation of valsartan acid, while in the untreated BAC filter the transformation of valsartan acid was observable due to the complete depletion of valsartan. A longer EBCT in the BAC filter could have improved the transformation of valsartan acid, suggesting that EBCT is a key parameter for moving towards OMP mineralisation via multi-step biotransformation. It is noteworthy that even though biotransformation was found to be the dominant pathway in the BAC filters in our study, the anthracite filters were not able to effectively attenuate valsartan (Ant: 7 % reduction, Ant+NaN_3: 13 % reduction; Fig. S2). This indicates that GAC media provides better conditions than non-adsorptive filter media for the biotransformation of valsartan and presumably other OMP (Reungoat et al.,

2011). Using radiolabelled OMP, a recent study proved that contaminants previously adsorbed onto the GAC surface are available for degradation by the attached biofilm (Betsholtz et al., 2021). This decoupling effect of biotransformation from the EBCT is a major advantage of GAC compared with non-adsorptive media. Further benefits of BAC filters might originate from the biofilm characteristics. Biomass concentration and the abundance of specialised microorganisms in the biofilm community are potential drivers for OMP biotransformation. Both aspects will be analysed in the sections 3.2 and 3.3.

Several compounds analysed in this study could not be clearly categorised based on the results of the experiment (gabapentin lactam, metformin, metoprolol) (Fig. S3). The concentration of gabapentin lactam, a major transformation product of gabapentin (Henning et al., 2018), increased in both BAC filters, but only in the untreated BAC filter significantly. This could point to a biological formation, however, on a molar basis, the concentration increase of gabapentin lactam in the BAC filter was approx. 6 times higher than the gabapentin decrease (Table S7). Thus, the significant gabapentin lactam increase originates either from desorption processes or from the biotransformation of a parent compound other than gabapentin. Metformin exhibited a significant concentration increase in the BAC filter and no change in the BAC+NaN_3 filter, resulting in significantly different filter effluent concentrations. Although this pattern might indicate a biological formation, we assume that desorption was responsible for the observations. In the pilot-scale BAC filter, relative effluent concentrations of metformin strongly fluctuated between 70 % and 170 % and seemed unpredictable in the complex wastewater matrix (Sauter et al., 2021a). Effluent concentrations of metoprolol were <LOQ in all samples of both BAC filters. This indicates that adsorption was involved in the removal. However, analytical sen-

sitivity did not allow for elucidating the potential additional impact of biotransformation.

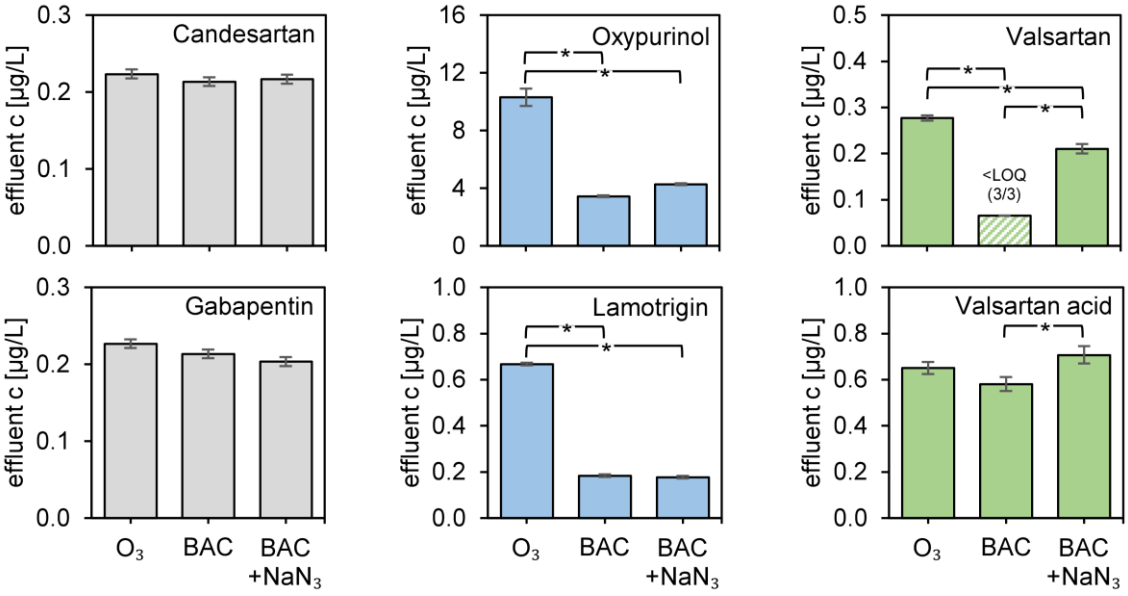

Fig. 3: Mean concentrations with standard deviations (n=3) of selected OMP in the effluents of ozonation (O_3) and the bench-scale filters BAC and BAC+NaN_3. Brackets with asterisks indicate significant differences between mean effluent concentrations. Column colours illustrate the compounds' behaviour: no removal (grey), removal by adsorption (blue), and removal/formation by biotransformation (green).

3.2 Active biomass on filter media

ATP quantification in the filter media biofilms revealed that the levels of active biomass per filter bed volume were significantly higher in the BAC filter (2.2−3.2 µg ATP/cm³) than in the Ant filter (0.8−1 µg ATP/cm³) for all investigated filter bed depths (Fig. 4). There was no pronounced trend with depth, most likely due to the frequent backwash of the filters. The improved colonisation of BAC compared to non-adsorptive media is typically explained by its surface characteristics (porosity, surface area, surface charge, roughness) and the rich supply of adsorbed organic matter (Basu et al., 2016; Simpson, 2008). The higher biomass concentrations on

BAC than on anthracite may result in an increased microbial carbon turnover, and hence, be responsible for the superior removal of DOC, $UVA_{254,}$ and DO observed in the bench-scale filters (section 3.1). Higher organic carbon removal by BAC compared to non-adsorptive filter media was observed in several other studies (Bacaro et al., 2019; Knezev, 2015; Reungoat et al., 2011; Sauter et al., 2021a; Wang et al., 1995). ATP-based biomass concentration has been described as the main driver in biofiltration for global OMP biotransformation (Cao et al., 2022). As described in section 3.1, a significantly improved biotransformation of valsartan was observed in the bench-scale BAC filter compared to the Ant filter. The higher biomass concentrations in the BAC filter compared to the Ant filter could therefore explain the enhanced valsartan removal. Co-metabolic biotransformation is assumed to be the major degradation pathway for many OMP (Alidina et al., 2014; Hellauer et al., 2018; Nguyen et al., 2019; Zhang et al., 2019). A comprehensive review study on influential factors of OMP degradation found a strong correlation between valsartan attenuation and removed DOC in technical biofiltration processes, indicating co-metabolic biotransformation of valsartan (Filter et al., 2021). The fact that the improved biotransformation of valsartan on BAC compared to anthracite appeared together with enhanced DOC removal and higher biomass concentrations supports the hypothesis of a co-metabolic transformation pathway.

ATP-based biomass concentrations reported in literature mostly originate from drinking water biofilters. They typically range from 0.1−10 µg ATP/cm³ (Greenstein et al., 2018; Lautenschlager et al., 2014; Magic-Knezev and van der Kooij, 2004; Pharand et al., 2014; Velten et al., 2011; Zhang et al., 2010, 2017) which is in accordance with our results. Of the few wastewater studies found, only one reported comparable biomass concentrations (Reaume et al., 2015) while the others obtained much high-

er concentrations of several hundred µg ATP/cm³ (Bacaro et al., 2019) and >1,000 µg ATP/cm³ (Sbardella et al., 2018). Hence, drinking water and wastewater biofilters do not appear to generally differ in their biomass concentrations. Operational aspects such as backwash interval, pre-treatment (e.g. pre-ozonation), or hydraulic and organic carbon might be more relevant for biomass concentrations than the influent water quality. Also, it cannot be ruled out that differences in the analytical procedures had an impact on the study results since there is no standardised method for ATP-based active biomass quantification. Concerning the influence of filter media on biomass concentration, there is no consensus in literature. Higher (Bacaro et al., 2019; Basu et al., 2016; Lautenschlager et al., 2014), comparable (Arnold et al., 2018; Greenstein et al., 2018; Magic-Knezev and van der Kooij, 2004), and even lower (Reaume et al., 2015; Zhang et al., 2017) ATP concentrations in BAC filters compared with sand or anthracite filters have been reported. The reason for the different outcomes could not be further elucidated based on the information given in the studies.

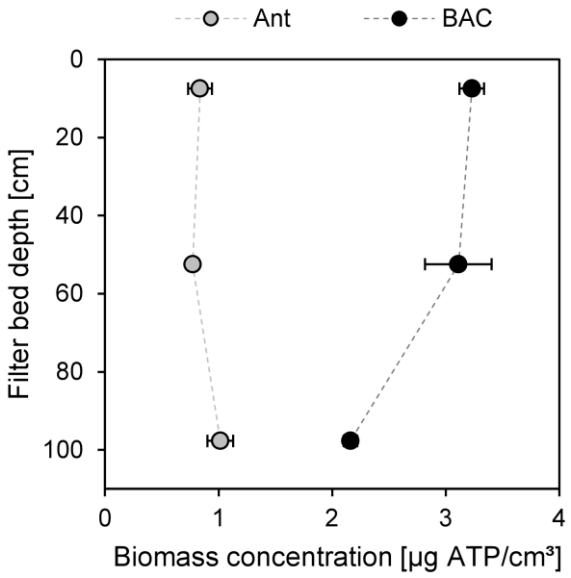

Fig. 4: Mean values with standard deviations (n=3) of ATP-based biomass concentrations at different filter bed depths of the pilot-scale Ant and BAC filter.

3.3 Microbial community analysis

3.3.1 Microbial diversity

Filter media samples from the different depths of the BAC and Ant filter exhibited a similar ASV richness, mostly ranging between 850 and 950 (Fig. S4). No significant trend with sampling depth or filter media was found (Table S6). The Shannon index that also encompasses taxonomic evenness was in the range of 5.4−5.8 for both filters, which is consistent with values between 4.6 and >6 reported for a comparable ozone/biofiltration system (Gerrity et al., 2018). A significantly higher alpha diversity on BAC than on anthracite as observed in drinking water filters (Greenstein et al., 2018), could not be observed. Interestingly, the Shannon index slightly increased with depths in the BAC filter, while it slightly decreased in the Ant filter. Despite the relatively small changes, the difference between the top and bottom samples was statistically significant in both filters (Table S6). The trend in the BAC filter is in agreement with previous studies that observed diversification of the microbial community with advancing infiltration as a consequence of declining biodegradable organic carbon availability (Alidina et al., 2014; Li et al., 2013). For the Ant filter, a lower or no diversity increase with depth would be expected, since organic carbon degradation is lower and the biofilm on the closed anthracite surface is more exposed to shear forces during backwash than on the porous GAC grains. The reason for the depth-dependent decrease of the Shannon index remains unknown so far. The behaviour of the Inverse Simpson index that gives more weight to dominant taxa was in accordance (except for the Ant-u sample) with the Shannon index results (Fig. S4).

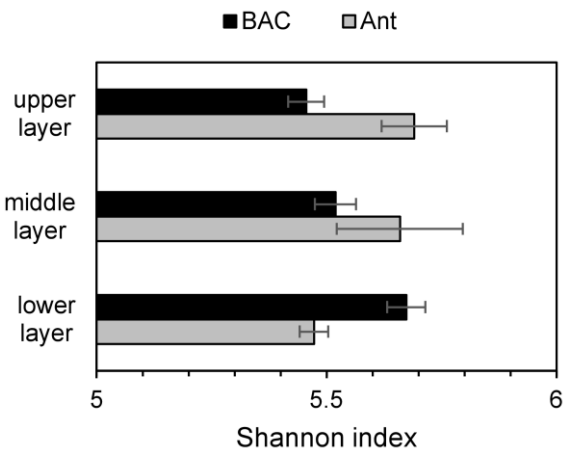

Fig. 5: Mean values with standard deviations (n=3) of Shannon indices at different filter bed depths of the pilot-scale Ant and BAC filter.

Microbial community structures (beta-diversity) clustered by filter material, and within one filter by depths (except for the overlap of Ant-u and Ant-m data points) (Fig. 6). This demonstrates that the choice of filter media and the sampling depth substantially impacted the microbial community structure. ANOSIM results confirmed a significant difference between the microbial communities of the two filter materials and between different depths within the BAC filter (Table S8). Other studies also reported an impact of media type on the microbial community of drinking water and wastewater filters (Gerrity et al., 2018; Oh et al., 2018; Vignola et al., 2018; Zhang et al., 2018). Further research is needed to assess if filter media-based differences in the biofilm composition translate into OMP biotransformation. An indication for the influence of media type was provided by a study that investigated functional genes in biofilters and found an enhanced aromatics degradation metabolism for GAC as compared to sand (Oh et al., 2018). This might be highly relevant for the removal of OMP since many of them contain aromatic moieties.

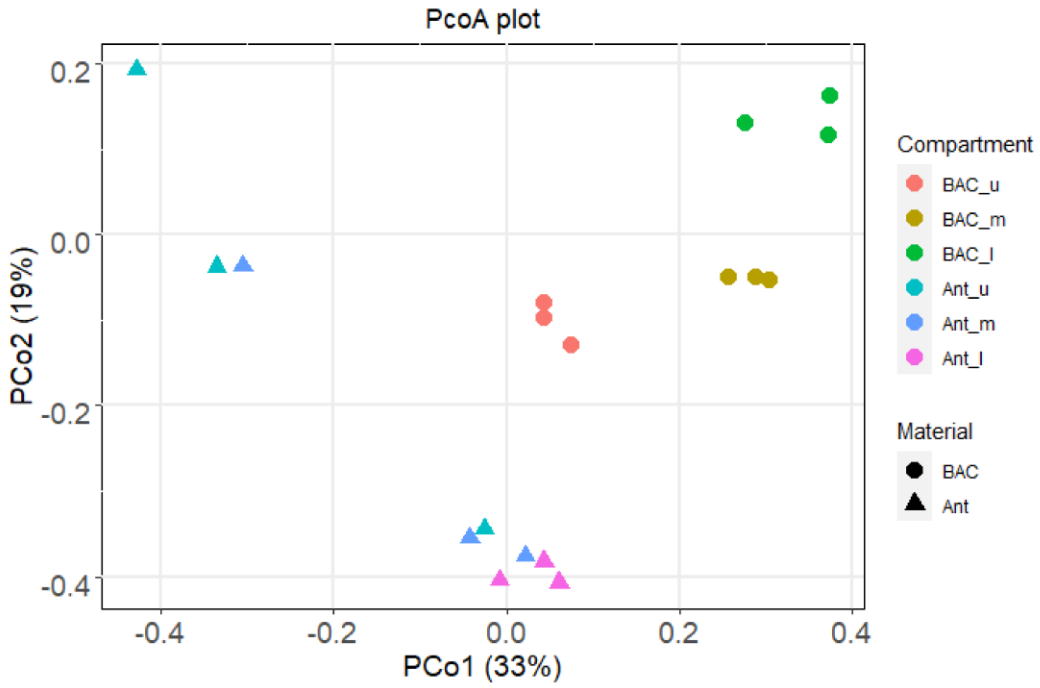

Fig. 6: Principal Coordinate Analysis based on Bray-Curtis dissimilarities of all individual filter media samples from the different depths of the pilot-scale Ant and BAC filter.

3.3.2 Composition of the microbial community

The 16S rRNA gene sequencing results were used to calculate the relative abundances of microbial groups on different taxonomic levels (Fig. 7, Fig. S5). To focus on the most relevant representatives in the biofilms, all taxa with a relative abundance <1 % were assigned as ”others”.

At the phylum level (Fig. 7A), 12 bacterial and archaeal phyla with a relative abundance ≥1 % were found, the three most dominant of which in all samples were *Proteobacteria (Alpha- and Gammaproteobacteria)* (31−40 %), *Bacteroidota* (11−17 %), and *Acidobacteriota* (7−10 %). The remaining phyla (*Planctomycetota*, *Nitrospirota*, *Chloroflexi*, *Thaumarchaeota*, *Actinobacteriota, Verrucomicrobiota, Myxococcota, Gemmatimonadota)* also appeared in all samples mostly at comparable levels, ex-

cept for *Elusimicrobiota* which exceed 1 % relative abundance only in the BAC-I sample. The identified bacterial phyla are in agreement with other studies that analysed the microbial communities of activated sludge (Nascimento et al., 2018; Ouyang et al., 2019; Wolff et al., 2018) or biofilter media (Feng et al., 2013; Gerrity et al., 2018; Li et al., 2021; Zhang et al., 2018). The considerable abundance of the archaeal phylum *Thaumarchaeota* (formerly *Crenarchaeota*) was unexpected. Relevant amounts of these ammonia-oxidising archaea (AOA) have been observed in activated sludge (Park et al., 2006) and drinking water biofilters (Gerrity et al., 2018), but not in wastewater biofilters.

Based on the genus level (in some cases family-level analysis), the microbial community was screened for groups that have been associated with the biotransformation of OMP (Fig. 7B). 17 genera with relative abundances ≥ 1 % could be distinguished and accounted for 31−40 % of the microbial communities. The plots of class, order, and family-level analysis are shown in the supplementary information (Fig. S5).

It was striking that several nitrifying microorganisms (*Nitrospira, Candidatus Nitrosocosmicus, Nitrosomonas, IS_44*) were dominant in the filter media biofilms. Numerous studies found that the biodegradation of OMP was linked to nitrification activity (Helbling et al., 2012; Rattier et al., 2014; Roh et al., 2009; Sathyamoorthy et al., 2013; Xu et al., 2017). Within the nitrifying community, ammonia-oxidising bacteria (AOB) have been demonstrated to be responsible for OMP transformation while the subsequent nitrite-oxidising bacteria (NOB) do not seem to be relevant (Sathyamoorthy et al., 2013; Yu et al., 2018). The key enzyme present in all AOB as well as AOA is ammonia monooxygenase (AMO). It catalyses the ammonia oxidation to nitrite, and, as suggested by various studies, simultaneously causes co-metabolic

oxidation of OMP (Helbling et al., 2012; Roh et al., 2009; Sathyamoorthy et al., 2013; Tran et al., 2013; Yu et al., 2018). Moreover, an abiotic reaction with hydroxylamine, an intermediate formed by all ammonia oxidisers, was shown to additionally contribute to the transformation of several OMP (Yu et al., 2018; Zhou et al., 2019). The AOB genera found in the filter media biofilms of this study were *Nitrospira*, *Nitrosomonas,* and *IS_44*. *Nitrospira* was the only relevant genus within the *Nitrospirota* phylum. Besides normal AOB, *Nitrospira* harbours so called Comammox bacteria, which have been recently discovered and can catalyse the complete oxidation of ammonia to nitrate as a single organism (Daims et al., 2015; Han et al., 2019). It has been demonstrated that selected OMP are biotransformed by Comammox bacteria but not by other tested AOA or AOB (Han et al., 2019). *Nitrosomonas* and *IS_44* belong to the *Nitrosomonadaceae* family, which are typically abundant in the nitrifying community of WWTP and were associated with the biotransformation of different OMP such as 17α-ethinylestradiol, atenolol, or bisphenol A (Khunjar et al., 2011; Sun et al., 2012; Xu et al., 2017). The genus *Candidatus* Nitrosocosmicus was the only representative of the archaeal phylum *Thaumarchaeota* with a relative abundance > 1 %. The occurrence and role of *Candidatus* Nitrosocosmicus in wastewater treatment systems have been recently investigated (Sauder et al., 2017; Xie et al., 2021), however, their potential for OMP biodegradation is still unexplored. Given their ability of AMO-based ammonia oxidation (Sauder et al., 2017), they may transform OMP similarly to AOB. For the AOA *Nitrososphaera gargensis,* originating from the same family of the *Nitrososphaeraceae as Candidatus* Nitrosocosmicus*,* biotransformation efficiency for the two pharmaceutical compounds mianserin and ranitidine was found to be comparable and based on the same reactions as for AOB (Men et al., 2016). In another study, the same species showed even higher biotransformation rates for

several sulphonamides than the AOB and Comammox bacteria tested in parallel (Zhou et al., 2019).

Besides the autotrophic nitrifying community, several taxa with mostly heterotrophic metabolism were identified that have been previously associated with the biotransformation of OMP. The genera *Bradyrhizobium* and *Rhizorhapis* both belong to the most abundant class of *Alphaproteobacteria* (Fig. S5). Nitrilase encoding genes have been found in *Bradyrhizobium* (Zhu et al., 2008, 2007), making it a potential degrader of nitrile-containing OMP such as the pesticides bromoxynil and acetamiprid (Achermann et al., 2019, 2018). The genus *Rhizorhapis* accounted for more than half of the *Sphingomonadaceae* family (Fig. S5), which has been repeatedly related to the biotransformation of OMP, e.g. bisphenol A and polycyclic aromatic hydrocarbons (Ghosal et al., 2016; Oh and Choi, 2019). *Flavobacterium*, a representative of the *Bacteroidota* phylum, has been shown to degrade pentachlorophenol, bromoxynil, and 17β-estradiol (Saber and Crawford, 1985; Topp et al., 1992; Yu et al., 2007). Individual genera of the family *Comamonadaceae* (class *Gammaproteobacteria*) did not exceed 1 % relative abundance, however, they might also be relevant for OMP removal. *Hydrogenophaga* has been described as a potential degrader of several OMP (Gao et al., 2019; Zhou et al., 2022). *Ideonella*, *Rubrivivax*, and *Leptothrix* have been shown to indirectly degrade OMP via formation of reactive manganese oxides (Martínez-Ruiz et al., 2020; Nega, 2020).

The vast majority of the identified taxa occurred in both filters and at similar abundances. Only 32 genera (0.16 % of the community) exclusively appeared in the overall community of the Ant filter, 29 genera (0.26 % of the community) were only present in the BAC filter. The generally small differences between the two filter media also applied to the microorganisms potentially involved in the biotransformation

of OMP. The community of ammonia oxidisers was slightly more abundant in the BAC filter (16 % depth-averaged) than in the Ant filter (14 % depth-averaged). Also, *Rhizorhapis* abundance was slightly higher on BAC (4 % depth-averaged) than on Ant (2 % depth-averaged). Conversely, *Flavobacterium* was more dominant in the Ant filter (4 % depth-averaged) than in the BAC filter (2 % depth-averaged). Considering the significantly higher biomass concentrations on BAC compared to anthracite (section 3.2), it can be assumed that, on an absolute scale, taxa associated with OMP biotransformation are much more abundant in the BAC filter in the Ant filter.

A direct link of the microbial community results to the biotransformation of valsartan, demonstrated in section 3.1, remains speculative. The reaction pathway of the transformation of valsartan to valsartan acid has been described in literature: 1. N-dealkylation, 2. hydrolysis, 3. oxidative deamination (Helbling et al., 2010). It has been shown that ammonium removal in wastewater treatment significantly correlates with the removal of valsartan and several other OMP that undergo N-dealkylation (Helbling et al., 2012), pointing to the involvement of AOB or AOA in valsartan transformation. Another study, however, reported no clear effect of the nitrification inhibitor allylthiourea on valsartan biotransformation in biofilters, while the addition of NaN_3 significantly reduced valsartan transformation (Rattier et al., 2014). This indicates that microorganisms other than nitrifiers or mixotrophic nitrifiers are responsible for valsartan biotransformation. Indeed, the most abundant genus *Nitrospira* consisted almost exclusively of the species *Nitrospira defluvii*, a known mixotrophic nitrifier capable of heterotrophic metabolism via catabolism and assimilation of different organic substrates (Metch et al., 2019). Hence, further research on the role of mixotrophic nitrifiers in the biotransformation of valsartan and other OMP might give important insights into their OMP degradation abilities.

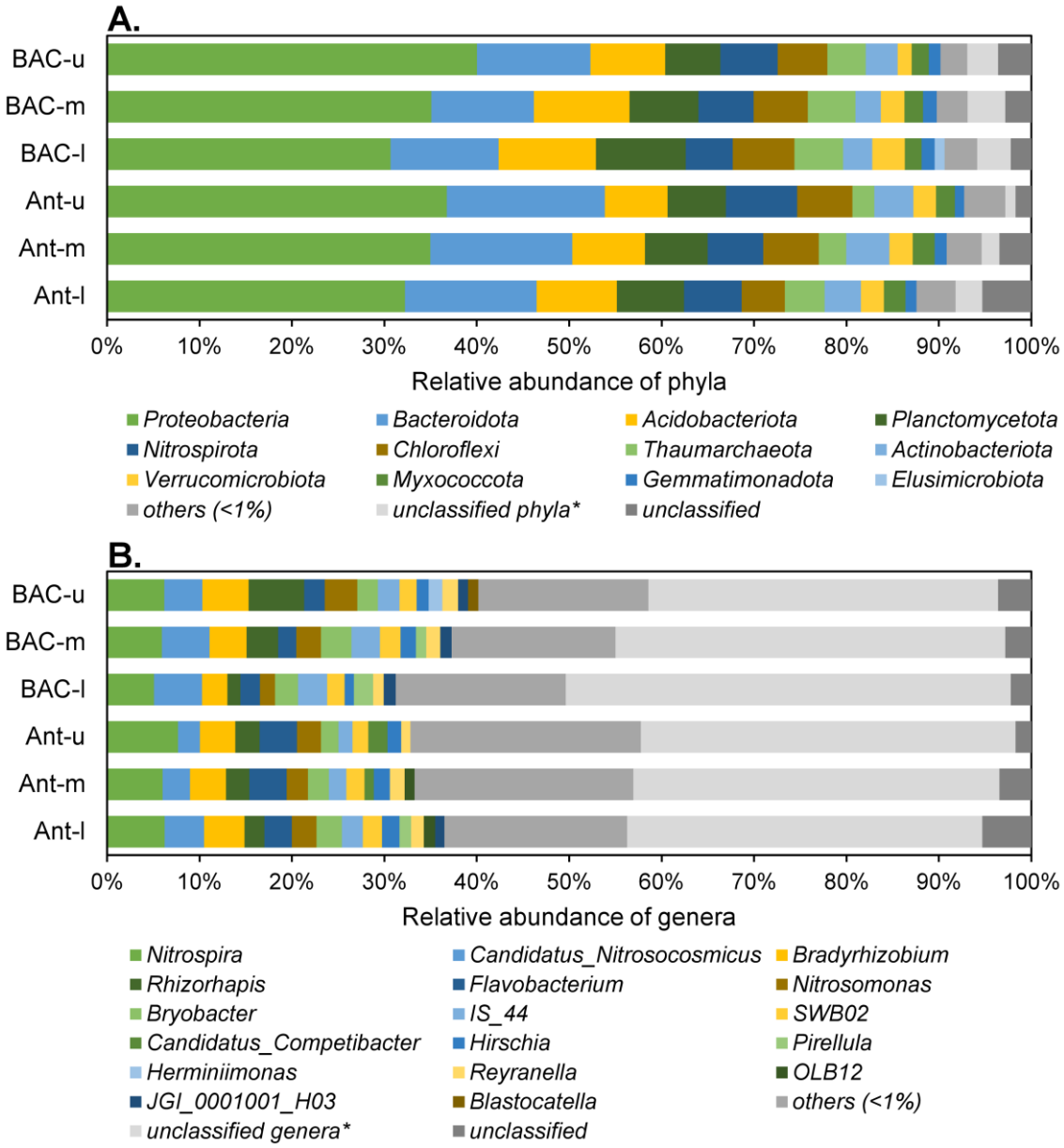

Fig. 7: Mean relative abundances (n=3) at phylum (A.) and genus (B.) level for the different filter bed depths of the pilot-scale Ant and BAC filter. All ASV with relative abundances <1% were summarised as "others (<1%)". * These ASV were classified at a taxonomic rank higher than phylum (A.) or genus (B.), respectively.

4 Conclusions

The bench-scale inhibition experiment with NaN_3 confirmed that BAC filters remove organic bulk parameters more efficiently from ozonated secondary effluent than an-

thracite filters and that the removal is governed by biodegradation. The abatement of OMP was also better in the BAC filters. The concentrations of four of the twelve studied compounds were significantly reduced in BAC filtration but not in anthracite filtration. Three of them (CBZD, lamotrigine, oxypurinol) were removed by adsorption only, while for valsartan biotransformation was the dominant mechanism. These results demonstrate that the benefits of BAC as compared to anthracite are not limited to additional adsorption but also comprise improved conditions for biological OMP abatement.

Biomass concentrations in the pilot BAC filter were significantly higher for all filter bed depths than in the pilot anthracite filter, highlighting that GAC is a more suitable growth media than anthracite. Biomass concentration might be a crucial influential factor for the biodegradation of bulk organic matter and biotransformation of OMP.

The dominant taxa within the biofilms of the two post-treatment options, BAC and anthracite filtration, were similar, and thus did not appear to be significantly influenced by the choice of filter media. Several microorganisms that have been associated with OMP biotransformation have been identified among these dominant taxa, most strikingly ammonia-oxidising bacteria, and archaea. In accordance with the dominant taxa, alpha diversities (richness, Shannon index) of the overall microbial communities were comparable for both filters. Beta diversity analysis, however, clearly revealed an impact of the filter media type and bed depth on the composition of the microbial community. The question if and how this influence is linked to the ability of OMP biotransformation should be addressed in future studies.

The results have practical implications for the design of biofilters as post-treatment of ozonated WWTP effluents. GAC media showed enhanced conditions for biomass development and biotransformation processes. This makes it a promising alternative

to non-adsorptive filter media in biological post-treatment that may achieve long-term benefits for the effluent quality with a one-time investment for the higher costs of GAC.

Acknowledgements

This work was supported by the European Union's Horizon 2020 research and innovation programme (project AquaNES, grant agreement no. 689450). We are grateful to Alexander Loy for his support with sequencing. We thank Petra Pjevac and the Joint Microbiome Facility, Vienna, Austria, for their support with sequencing and sequence data analysis.

CRediT author contribution statement

Daniel Sauter: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing; **Andrea Steuer:** Formal analysis, Investigation, Visualization, Writing – review & editing; **Kenneth Wasmund:** Formal analysis, Investigation, Methodology, Resources, Writing – review & editing; **Bela Hausmann:** Formal analysis, Investigation, Methodology, Resources, Writing – review & editing; **Ulrich Szewzyk:** Resources, Writing – review & editing; **Alexander Sperlich:** Conceptualization, Funding acquisition, Writing – review & editing; **Regina Gnirss:** Conceptualization, Funding acquisition, Writing – review & editing; **Myriel Cooper:** Conceptualization, Formal analysis, Methodology, Visualization, Supervision, Writing – review & editing; **Thomas Wintgens:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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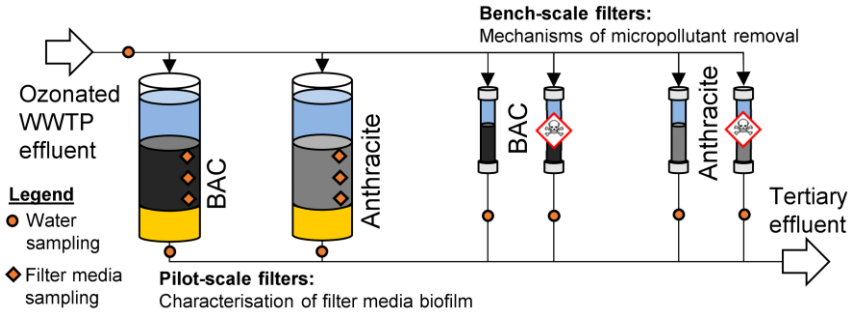
Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

- Filter media choice affects biofilter performance in post-treatment after ozonation
- Organic contaminants better removed by BAC than by anthracite biofilter
- More active biomass per filter bed volume in BAC than in anthracite filter
- Dominant taxa and alpha diversity similar in biofilms of BAC and anthracite filter
- Beta diversity reveals impact of media type and bed depth on biofilm composition