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Research Article

Antibiotic resistance genes and the association with bacterial community in biofilms occurring during the drinking water granular activated carbon (GAC) sandwich biofiltration

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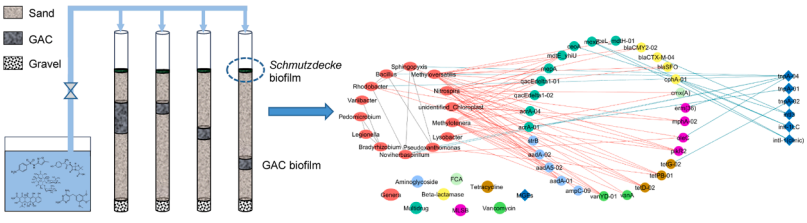
HIGHLIGHTS

- Bench-scale GAC sandwich biofilters were set-up.
- Both surface sand (the *schmutzdecke*) and GAC biofilms were targeted.
- ARG categories decreased in richness along the filter bed.
- Drinking water biofilms harbour high abundance and diversity of ARGs.

GRAPHICAL ABSTRACT



GAC sandwich biofiltration system

Network analysis revealing the co-occurrence patterns between ARGs and bacterial taxa

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ABSTRACT

The granular activated carbon (GAC) sandwich modification to slow sand filtration could be considered as a promising technology for improved drinking water quality. Biofilms developed on sand and GAC surfaces are expected to show a functional diversity during the biofiltration. Bench-scale GAC sandwich biofilters were set-up and run continuously with and without antibiotic exposure. Surface sand (the *schmutzdecke*) and GAC biofilms were sampled and subject to high-throughput qPCR for antibiotic resistance gene (ARG) analysis and *16 S rRNA* amplicon sequencing. Similar diversity of ARG profile was found in both types of biofilms, suggesting that all ARG categories decreased in richness along the filter bed. In general, surface sand biofilm remained the most active layer with regards to the richness and abundance of ARGs, where GAC biofilms showed slightly lower ARG risks. Network analysis suggested that 10 taxonomic genera were implicated as possible ARG hosts, among which *Nitrospira*, *Methyloversatilis* and *Methylotenera* showed the highest correlation. Overall, this study was the first attempt to consider the whole structure of the GAC sandwich biofilter and results from this study could help to further understand the persistence of ARGs and their association with the microbial community in drinking water biofiltration system.

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1. Introduction

Sand biofiltration is often regarded as an efficient and stable technology for drinking water treatment. The major function of the sand biofilter occurs at the surface layer (known as the *schmutzdecke*) of the sand bed in which biological activities are the highest [1]. A typical *schmutzdecke* consists of a gelatinous biofilm matrix made up of bacteria, fungi, protozoa, plankton, diatoms, rotifers and algae and their extracellular material [2]. There are generally two accepted definitions of *schmutzdecke* [3,4]: 1) a slime layer above the sand, and an attached biologically active zone within the sand bed; or 2) the slime layer only. Introducing a layer of granular activated carbon (GAC) to the traditional sand biofilter (GAC sandwich biofilter) has been widely used by Thames Water for drinking water purification [5]. The GAC sandwich biofilter was first studied by Bauer et al. to remove pesticides [6]. Recent studies based on bench-scale GAC sandwich biofilter have shown its capacity to remove pharmaceutical and personal care products (e.g. paracetamol, caffeine, triclosan, sulfamethoxazole and trimethoprim) from the source water [7,8]. This enables the GAC sandwich biofiltration a promising and energy-efficient process for the removal of trace level micropollutants.

Aquatic biofilms are long-term reservoirs for antibiotic resistance genes (ARGs) in the environment and they have been shown to facilitate horizontal transfer of ARGs under environmental conditions because of the nutritional richness and high bacterial density and diversity [9]. In particular, ARGs have been observed to establish and proliferate in drinking water biofilms. For instance, Schwartz et al. have demonstrated that a vancomycin resistant gene, *vanA*, was detected in drinking water biofilms in the absence of bacterial host *enterococci*, suggesting possible gene transfer to autochthonous drinking water bacteria [10]. Farkas et al. reported that biofilm community in a drinking water treatment plant is a reservoir of class 1 integrons, suggesting that drinking water biofilm has the potential to accumulate resistance determinants [11]. Recent high-throughput ARG analysis has revealed a promotion in antibiotic resistome after the drinking water biological activated carbon treatment, with 29 ARGs identified as biofilm source [12].

While antibiotic resistome has been investigated separately in sand and GAC biofilms from previous studies [12,13], the accumulation of antibiotics within the GAC layer on the behaviour of resistance genes remains unknown; the role the antibiotic plays in the variation of ARGs during biofiltration needs further clarification. For the GAC sandwich biofiltration specifically, biofilm forms on both sand and GAC surfaces. Surface sand biofilm (*schmutzdecke*) is expose to all of the components (nutrients, oxygen, micropollutants *etc*.) from the source water and the respiration of bacteria consumes oxygen and degrades organics. When reaching the GAC layer, in addition to the biodegradation, micropollutants and oxygen could be further adsorbed by GAC due to its high surface area and unique pore structure. Biofilms formed on the GAC layer provide an ideal habitat for resistance gene exchange - the unique pore structures could capture more bacterial cells and facilitate cell-to-cell contact, contributing to the dissemination of ARGs in the biofilms [14]. In addition, bacteria can be mobilised between the biofilm and water (e.g. biofilm detachment) during biofiltration, which further complicates the dynamics of antibiotic resistome in GAC biofilms.

Despite of the effective removal of antibiotics, our previous studies showed that the GAC sandwich biofilter did not contribute greatly to the elimination of ARGs from the source water and the GAC media could facilitate horizontal transfer of ARGs in biofilms [8,14]. In light of this, a sound understanding of microbial community structure of biofilm and its association with ARG profiles may provide insights into the mechanism of persistent bacterial antibiotic resistance in the GAC sandwich biofiltration. In the present study, two sets of GAC sandwich biofilters were set-up at bench scale, with one set spiked with antibiotics (SMX sulfamethoxazole, CTM clarithromycin, AMOX amoxicillin, OTC oxytetracycline, and TMP trimethoprim) and another run as control (no antibiotic spike). The selection of the target antibiotics was based on their 1) presence in surface waters used as source of drinking water; and 2) differences in physico-chemical properties. This study explores the performance of GAC sandwich biofilter and the removal of antibiotics with different thicknesses and positions of the GAC layers. In addition, both surface sand (*schmutzdecke*) and GAC biofilms were collected and subject to high-throughput qPCR (HT-qPCR) and microbial community structure analysis. For HT-qPCR, a total of 296 primer sets were used, including 285 ARGs conferring resistance to all major classes of antibiotics, eight transposases; *16 S rRNA* gene; *intI 1*; and the clinical *intI 1* (*cintI1*). Overall, this study examines the hypothesis that GAC sandwich biofiltration could provide benefits for an improved quality of drinking water and enhanced removal of antibiotics; while drinking water biofilms may harbour high abundance and diversity of ARGs, contributing to the spread of ARGs during the biofiltration process.

2. Methods and Materials

2.1. Biofilter design and operation

Biofilters were constructed using eight 62 cm lengths of acrylic (Plastic Shop, UK) columns with a 36-mm inner diameter and 2-mm wall thickness. The eight biofilters comprising four types of GAC sandwich were set-up at bench scale. The design of the GAC sandwich biofilters is shown in Fig. 1. All biofilters had 5 cm of under-drainage (0.6–3 mm gravel) to allow free drainage of filtered water from the columns. A dual head peristaltic pump (Watson-Marlow 323 U) with 8 channels was used to simultaneously deliver feedwater to biofilters from the reservoir. The fine sand was purchased from Mineral Marketing (UK) and had an effective size of 0.20 mm and a uniformity coefficient of 1.82. GAC with a particle size of 0.62 – 1.60 mm was purchased from Chemviron Carbon (UK). More details on the surface characteristics of the filter media can be found in our previous study [14]. GAC sandwich biofilter configurations are shown in Fig. S1 (Supplementary Information).

A total of 15 L raw water was collected from the River Thames twice a week from June to September 2018. Raw water was diluted with dechlorinated tap water at a ratio of 1:2 and used as feedwater to reduce biofilter clogging. A filtration rate of 0.06 m/h was used per each filter throughout this study, which was within the typical range of 0.04–0.4 m/h for slow sand filtration [15,16]. The system was initially operated under identical conditions for 3 weeks (maturation stage) until the biofilm reached maturity, when total coliforms and *Escherichia coli* (*E. coli*) achieved 99% reduction [2]. Then, the system was divided into Sets A and B, each consisting of four biofilters with different GAC thickness or position (as shown in Fig. 1). Set A was operated with the addition of antibiotic mixture (SMX, CTM, AMOX, OTC, and TMP) at 10 μg/L for each compound, while Set B was operated as control (without the addition of antibiotics). The spike concentration was set at a higher level in order to increase the detectability of the target antibiotics in the effluent. Details on the preparation of antibiotic stock solutions and dilutions can be found elsewhere [8]. After maturation, the system run continuously for another 8 weeks (experimental stage).

2.2. Biofilter sampling

During the entire course of the system run, influents and effluents were taken weekly for the determination of general water quality parameters, including pH, conductivity, turbidity, dissolved organic carbon (DOC), chemical oxygen demand (COD), specific ultraviolet absorbency (UV_{254}), dissolved oxygen (DO), total coliforms and *E. coli*. During the experimental period, only influents and effluents collected from Set A were processed for the analysis of antibiotic removal. Raw water from the River Thames without antibiotic addition was used as environmental background.

Schmutzdecke and middle GAC layer samples were collected after 11-weeks' operation. A sterile pipette was used to randomly take the *schmutzdecke* samples from five points of the cross section and then

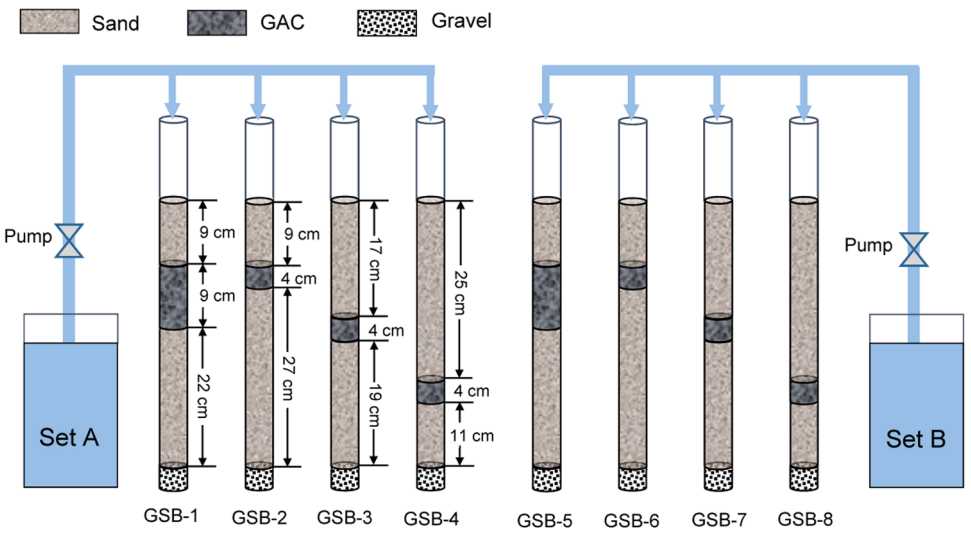

Fig. 1. Schematic of the GAC sandwich biofilter (GSB) composition. Set A was spiked with antibiotics during the experimental period; Set B run as control.

mixed as one sample. Excess water was carefully removed from the *schmutzdecke* samples using a needle syringe and stored at - 20 ºC. All biofilm samples were divided as duplicate for the subsequent process prior to DNA extraction. To separate bacterial cells from media particles, the GAC samples were added to sterile saline (NaCl, 0.85 g/L) and ultrasonicated at 38 kHz, 600 W three times with 20 min exposure and 5 min intervals to suspend the biofilm's DNA in saline [13]. The biofilm suspensions were then filtered through 0.22 μm membrane filters (Millipore, UK) by a vacuum filtration apparatus to capture bacteria. All membranes were stored at - 20 ºC until DNA extraction.

2.3. Analytical methods

2.3.1. General water quality parameters

Determination of general water quality parameters followed standard methods (APHA) [17]. For the analysis of DOC, aqueous samples were pre-filtered through 0.45 μm mixed cellulose esters (MCE) membrane (Millipore, UK). Shimadzu TOC-L machine (UK) was used to quantify the concentration of DOC in aqueous samples. Standard membrane filtration method was used for the enumeration of *E. coli* and coliform bacteria following ISO 9308–1:2014. Details on the methods can be found elsewhere [8].

2.3.2. Quantification of Antibiotics

All antibiotic standards (purity ≥ 99.0%) were purchased from Sigma-Aldrich, UK. HPLC grade acetonitrile and methanol were obtained from Fisher Scientific, UK. Individual stock standards were prepared in methanol at 1 mg/mL, except for amoxicillin, which was dissolved in acetonitrile/water (50:50, v/v) at 1 mg/mL. A working solution was prepared by diluting the stock solutions in ultrapure water into 1 mg/L. All the stock solutions were stored at − 20 ºC and working solutions were stored at 4 ºC.

Solid phase extraction (SPE) technique was used to extract the target antibiotics from aqueous samples. Prior to the extraction, samples were filtered through 0.45 μm membrane filter and acidified to pH 3.0 with hydrochloric acid. Na_2EDTA was added to samples at 0.5 g/L. Then, all samples were subjected to SPE using 500 mg Oasis HLB cartridges (Waters, UK). An Accela 1100 HPLC system coupled to a LTQ ion-trap mass spectrometer (Thermo Finnigan LTQ) was used for the detection of target antibiotics. Further details of the SPE method and mass spectrometer parameters can be found in a previous study [8]. Method validation showed that, except for AMOX (26–31% recovery), the recoveries of the remaining antibiotics ranged from 77% to 120% for reference river water samples (Table S1). Method detection limit (MDL) ranged between 2 and 50 ng/L (Table S1) and the overall method precision (relative standard deviation) was determined to be within the range of 2 –13% for all target antibiotics.

2.3.3. High-throughput qPCR for ARGs

Genomic DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, UK) according to the manufacturers' instructions. For *schmutzdecke* samples, 0.5 g (wet) was used for extraction; for GAC biofilm samples, DNA was extracted directly from the membrane as described in Section 2.2. After extraction, duplicate DNA samples were pooled as a single extract to minimise the biased caused during sample pre-treatment and extraction. The concentration of the purified DNA was quantified spectrophotometrically using the NanoDrop and stored at − 20 ºC until further analysis.

High-throughput qPCR (HT-qPCR) analysis was conducted in the Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences. The SmartChip Real-time PCR System (Warfergen Inc., USA) was used to perform HT-qPCR as previously described [13,18]. A total of 296 primer sets were used, including 285 ARGs; eight transposases; *intI 1*; the clinical *intI 1* (*cintI1*) and *16 S rRNA* gene. PCR mixtures (100 nL per well) consisted of 1 × LightCycler 480 SYBR Green I Master (Roche Applied Sciences, Indianapolis, IN), nuclease free PCR-grade water, 1 mg/mL bovine serum albumin (New England Biolaboratories, Beverly, MA), 500 nM of each primer and a DNA template of 5 ng/μL. The qPCR conditions included initial enzyme activation at 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s for amplification. The melting process was automatically generated by Wafergen software and the qPCR results were analysed using SmartChip qPCR Software. A threshold cycle of 31 was used as the detection limit, and only samples with three replicates amplified were regarded as positive.

2.3.4. Bacterial 16 S rRNA amplicon sequencing

For the bacterial community structure analysis, 16 biofilm DNA samples were sent for high-throughput sequencing using the Illumina Hiseq2500 platform (Novogene, Beijing, China). V3-V4 region of *16 S rRNA* gene was selected for amplification with primers 341 F: CCTAYGGGRBGCASCAG and 806 R: GGACTACNNGGGTATCTAAT [13]. Raw pair-end reads were assembled after the filtering adaptor, low-quality reads, ambiguous N, and barcode to generate clean joined reads capturing the complete V3-V4 region of the *16 S rRNA* gene by FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). The generated high quality sequences were processed and analysed using Quantitative Insights Into Microbial Ecology (QIIME, V1.7.0, http://qiime.org/index.

html). The open-reference operational taxonomic unit (OTU) picking was performed following the online instruction of QIIME. Sequences with ≥ 97% similarity were assigned to the same operational taxonomic unit (OTU).

2.4. Statistical analysis

Removals of DOC, turbidity, UV_{254}, total coliforms, *E. coli* and antibiotics were determined based on influent and effluent concentrations. The absolute abundance of ARGs was defined as the ARG copies per gram in medium samples (copies/g). The relative abundance of ARG was defined as the normalised ARG copies to the *16 S rRNA* gene copies. The richness of ARGs was defined as the number of the detected ARGs. Mean and standard deviation calculations were performed with Microsoft Excel 2016. One-way analysis of variation (ANOVA), Pearson correlation analysis and heatmap were performed using OriginPro 2018.

Principal coordinate analysis (PCoA) based on Bray-Curtis distance was used to evaluate both the ARGs and bacterial community profiles between different biofilm samples. Redundancy analysis (RDA) was performed to analyse the correlation between the abundance of detected ARGs and bacterial communities. Variation partitioning analysis (VPA) was performed for the determination of the contributions of bacterial communities and integrons to the variations of persistent ARGs. PCoA, RDA and VPA were performed using Canoco 5.0 software (USA). Venn diagram analysis was performed to assess the numbers of shared and unique ARGs/OTUs in biofilm samples. OriginPro 2018 was used to draw histogram, line graphs and Venn diagram. Network analysis of the co-occurrence patterns (similarity of location) among ARGs and bacterial taxa was performed using an online analysis pipeline at http://ieg4.rccc.ou.edu/mena/main.cgi and Cytoscape 3.7.1 was used to visualise the network graphs [19,20].

3. Results and discussion

3.1. Biofilter performance

Raw water showed variations in all water quality parameters (Table S2), however no statistical differences ($p > 0.70$) were found across the sampling period. During the maturation stage, the effluents of all biofilters showed similar values for conventional chemical parameters (Table S3 and Fig. S2) such as pH, conductivity and DO but differed slightly with respect to turbidity and organics-related parameters (i.e. DOC, UV_{254} and COD).

General water quality parameters during the experimental stage are listed in Table S4. UV_{254} removal efficiencies ranged from 50.0% to 90.5%, averaging 72.9%, while DOC removal efficiencies varied from 41.8% to 74.3% with an average of 54.6% (Fig. S3). It is interesting to note that the biofilters with 4 cm GAC in the middle of the column showed better removal of organics, albeit not significant, than in the upper or lower sand layers (Fig. S3). Wan et al. found that the upper layer of sand bed (1–10 cm) provided the maximum DOC removal and consumed the most of oxygen from the influent, while the deeper layer of sand (10–50 cm) had limited effect on DOC removal [13]. When reaching the middle layer GAC, the levels of oxygen and nutrients in the planktonic phase may be more favourable for the growth of organics-degrading bacterial communities within the biofilm, contributing to the additional removal of organics when the water flowed.

3.2. The removal of antibiotics

Testing for antibiotic removal began in week 4, allowing an initial 3-week maturation period for the biofilm to establish within the top sand layer. All sandwich biofilters achieved > 90% antibiotic removal (Table S5), which is consistent with the observations in a previous study using lake water as biofilter feed [8]. The overall mean removal of the five antibiotics over the entire course of the experiment were 98.5 ± 1.6%. No difference in removal was found between biofilters with different GAC thickness (9 cm or 4 cm GAC), or between biofilters with 4 cm of GAC at different depths. AMOX was only detected occasionally in the effluent, the values below the quantification limit in the filtered water were taken as half of the LOQ value [21]. It should be noted that the percentage removal calculated may not accurately reflect the actual removal in cases where AMOX was not detectable in the effluent. Removal mechanisms of the antibiotics by the sandwich biofilters could be attributed to both biodegradation and adsorption. This might be particularly the case for AMOX, where chemical hydrolysis or cleavage of the unstable β-lactam ring by β-lactamases could be expected in the aquatic environment [22]. According to our previous study, average removal of the target antibiotics was 20.3% by pure sand biofilter, with TMP being the most efficiently removed antibiotic (55.5%) followed by OTC (20.0%), AMOX (13.6%), CTM (6.7%), and SMX (5.8%) [8]. In addition, further adsorption kinetics of antibiotics on GAC showed > 90% removal when reached equilibrium concentration [8]. However, the contribution of biodegradation could not be differentiated from GAC adsorption in the sandwich biofilter as only final effluent was collected. Further desorption of antibiotics on filter media is needed to clarity the relative roles of sorption and biodegradation.

3.3. Behaviour of ARGs

3.3.1. Richness and diversity of ARGs

Results of HT-qPCR indicated that a total of 155 and 141 ARGs were detected in the *schmutzdecke* biofilm (B-*schm*) and GAC biofilm (B-GAC), respectively. The richness of detected ARGs was generally identical in the B-*schm* replicates in each set, ranging from 125 to 134 in Set A and from 104 to 116 in Set B, respectively (Fig. 2). Specifically, the richness and Shannon diversity of ARGs were higher in Set A compared to the control system. Beta-lactamase resistance genes contributed the most (from 86 to 110 in total) to the increased richness in the B-*schm* in Set A, probably due to continuous exposure to AMOX during the experimental period. GAC biofilms hosted a similar diversity of ARG profiles compared to B-*schm* (Shannon index on average: B-GAC = 1.90 and B-*schm* = 1.92), suggesting that all ARG categories decreased in richness along the filter. The number of detected ARGs classified based on the mechanism of resistance is shown in Fig. S4. Antibiotic deactivation and efflux pump were the two dominant resistance mechanisms, accounting for 81.9% of all ARG subtypes. The number of detected ARGs classified as encoding antibiotic deactivation was slightly higher when exposed to antibiotics, and accordingly, the percentage of efflux pump mechanism was higher in the control biofilms. In particular, numbers of detected efflux pump ARGs became predominant (43.7%) in B-GAC in Set B, mainly due to the contribution of *tet* genes.

3.3.2. Relative abundance of ARGs

Data in Fig. 3 show the relative abundance of ARG category (normalised to *16 S rRNA*) of each sample in order to minimise the variations caused by the background bacterial population. Multidrug resistance genes were predominant in all samples. B-*schm* exposed to antibiotics represented the highest risks of ARG pollution. Compared to the control system, aminoglycoside, MLSB (Macrolide-Lincosamide-Streptogramin B), sulfonamide and vancomycin resistance genes were significantly enriched ($p < 0.01$) when exposed to antibiotics, among which *ereA* and *ermF*, two macrolide resistance genes achieved the highest enrichment (112-fold and 44-fold, respectively). *AadA-01* was the most enriched (37-fold) aminoglycoside resistance gene in B-*schm* in Set A. It should be noted that the co-selection of aminoglycoside and vancomycin resistance gene (*van*) was observed in B-*schm*, with their relative abundance enriched significantly ($p < 0.001$) in Set A. The cluster of genes encoding high-level resistance to vancomycin are typically located on transposons of the Tn*1546* type [23] and *vanA* can be transferred together with MLSB resistance genes *ermB* and *vatE* [24]. The co-transfer

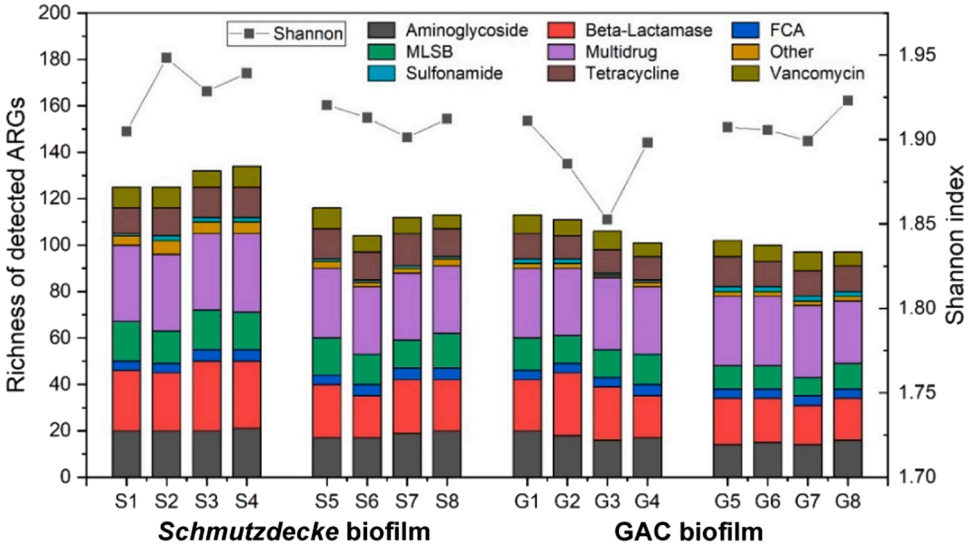

Fig. 2. Richness and Shannon index of detected ARGs in *schmutzdecke* and GAC biofilms. 1–4: biofilters exposed to antibiotics; 5–8: biofilters unexposed to antibiotics. MLSB = Macrolide-Lincosamide-Streptogramin B resistance genes; FCA = fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes.

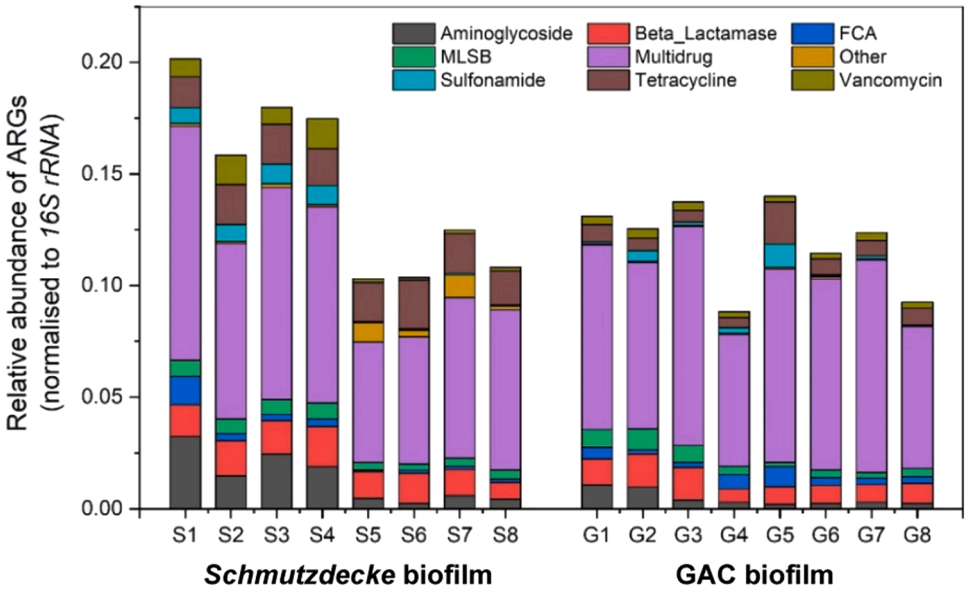

Fig. 3. Relative abundance of ARGs in *schmutzdecke* and GAC biofilm samples. 1–4: biofilters exposed to antibiotics; 5–8: biofilters unexposed to antibiotics. MLSB = Macrolide-Lincosamide-Streptogramin B resistance genes; FCA = fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes.

of vancomycin- and MLSB- resistance genes may occur at the same time in B-*schm* under selective pressure from clarithromycin. Furthermore, a significant positive correlation of the relative abundance of ARGs was found between vancomycin and MLSB ($r = 0.89$, $p < 0.0001$) in B-*schm*. As vancomycin is recognised as a 'last-resort' life-saving antibiotic [25], the enhanced relative abundance of this gene category in the biofilms of drinking water biofilters may pose a risk to human health.

Comparisons of ARGs among B-GAC suggested that the biofilms collected at 10–20 cm (G1-G3, G5-G7) represented similar levels of the relative abundance of ARGs, while the biofilms at 30 cm (G4 and G8) showed the least risks of ARG pollution. In the control biofilters, the GAC biofilm revealed a similar level of relative abundance of ARGs compared to the *schmutzdecke* layer, which is generally considered as the most biologically active layer in slow sand filters. GAC biofilms exposed to antibiotics presented higher abundance of aminoglycoside, beta-lactamase and MLSB resistance genes but was less abundant in tetracycline resistance genes compared with the control biofilms. The behaviour of tetracycline resistance genes observed in this study is conflicting with the previous research as OTC exposure has generally been associated with an increased occurrence and diversity of *tet* genes in environmental water or soil samples [26,27]. This could be explained in two ways: 1) the host bacterial community of *tet* may be a strong competitor within biofilms unexposed to OTC; and 2) the effects of OTC on biofilm communities in the *schmutzdecke* and GAC biofilms occurred to various extents. The adsorption of OTC by GAC layer has led to an accumulation of this compound in GAC biofilms, which may consequently inhibit the growth of specific bacterial hosts for *tet* genes.

The relationships between the biofilm samples were further explored using the PCoA approach (Bray-Curtis distance) according to the relative abundance of resistance types (Fig. 4-a). The structures of ARGs in Set A or Set B were clustered together. B-*schm* exposed to antibiotics were clearly distinct from those in the control biofilms. In contrast, B-GAC in

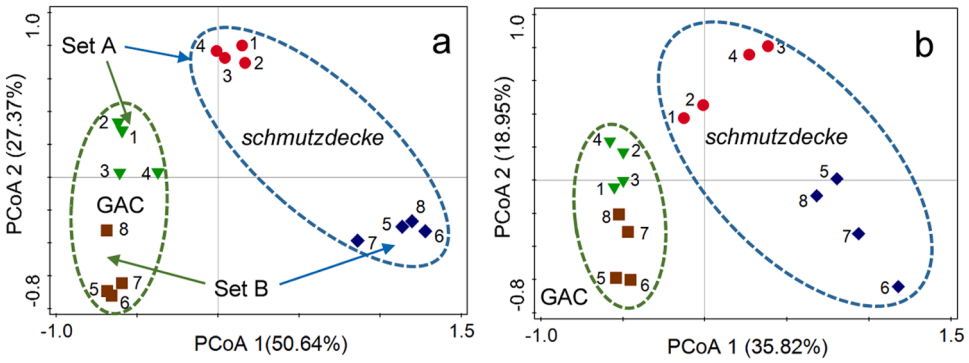

Fig. 4. Principal coordinate analysis based on Bray-Curtis distance showing the overall distribution of (a) ARGs and (b) bacterial communities in *schmutzdecke* and GAC biofilm samples. Set A: biofilter 1–4 exposed to antibiotics; Set B: biofilter 5–8 unexposed to antibiotics.

Sets A and B were grouped close to each other due to the similarity of their antibiotic resistance profiles. The number of detected ARGs in *schmutzdecke* and GAC biofilms samples was compared to ascertain the variation in sources of ARGs. A total of 98 ARGs were core ARGs that persisted in all biofilm samples, accounting for 60.5% and 71.6% of the total number of detected ARGs in Set A and Set B, respectively (Fig. S5). In total, 20 and 11 unique ARGs were detected in B-*schm* and B-GAC in Set A, respectively. As all ARGs present in biofilms originated from the same source water, considering no other potential sources, target antibiotics spiked to the system were the main driving factors for the selection of ARGs during biofiltration. This is further supported by the evidence that among all of the 42 unique ARGs detected in Set A, the top three ARG categories were beta-lactams, MLSB, and tetracycline which contain the target antibiotics of AMOX, CTM and OTC, respectively.

3.4. Bacterial communities in schmutzdecke and GAC biofilms

A total of 1763,413 tags with average of 123,177 and 71,317 high quality tags per B-*schm* and B-GAC sample were obtained, respectively (Table S6). The top 10 largest taxonomic phyla in B-*schm* and B-GAC are shown in Fig. 5. Seeded from the River Thames, biofilms harboured a large diversity of bacterial phyla with an average of 38 ± 3 and 40 ± 2 phyla detected in B-*schm* and B-GAC, respectively. *Proteobacteria* (62.1%) was dominant in all 16 biofilm samples, followed by *Firmicutes* (10.1%), *Actinobacteria* (7.9%), *Acidobacteria* (4.4%) and *Bacteroidetes* (4.2%), accounting 88.7% of the total bacterial communities. At the class level, bacterial community differences became more divergent (Fig. S6). For instance, *Betaproteobacteria* was most abundant in B-GAC exposed to antibiotics, while in control biofilms, the most abundant class shifted to *Alphaproteobacteria*. The distribution of B-*schm* in Sets A and B clearly indicated the effect of antibiotic exposure, as evidenced by PCoA (Fig. 4-b). Under the selective pressure of antibiotics, the proportion of *Firmicutes* in B-*schm* was significantly reduced ($p < 0.01$) from 22.89% ± 9.72% in Set B to 6.27% ± 2.18% in Set A, respectively, and consequently affected its proportion in the underlying GAC biofilms, albeit not significantly. On the contrary, B-*schm* collected from Set A were more abundant in the phylum *Chlorobi* (6.61% ± 1.55%) compared with Set B (0.23% ± 0.13%). The Venn diagram of OUT number (based on the number of effective sequences in every sample) shows that 2165 OUTs were shared between all biofilms (Fig. S7), accounting 58.9% and 54.8% of the total OTUs in B-*schm* and B-GAC, respectively. More unique bacteria were identified in B-*schm* unexposed to antibiotics.

To establish a more detailed view on the bacterial community, Fig. 6 depicts the abundance of 80 major genera (> 0.5% in at least one sample). *Sulfuritalea* (7.3% on average) and *Bacillus* (5.2% on average) were the most abundant genera. *Sulfuritalea* was more abundant in biofilms exposed to antibiotics, especially within the GAC biofilms. Previously, *Sulfuritalea* species were found to be a major component of the planktonic bacterial community in nitrate-depleted hypoxic water [28]. *Bacillus* showed a much higher relative abundance (18.4%) in the control B-*schm*. Genera associated with the nitrogen cycle present in biofilms. For instance, *Bradyrhizobium*, a well-known nitrogen fixer, showed a slightly higher percentage in B-GAC. *Nitrospira* is a globally distributed group of nitrite oxidisers and usually exist in the interiors of

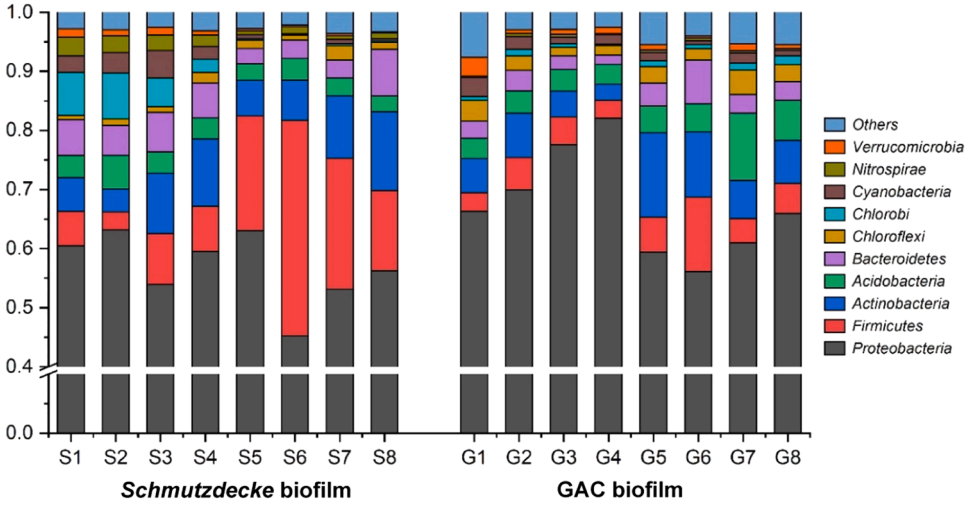

Fig. 5. Bacterial community composition in *schmutzdecke* and GAC biofilms based on the average percentage of the top 10 largest taxonomic phyla. 1–4: biofilters exposed to antibiotics; 5–8: biofilters without the addition of antibiotic.

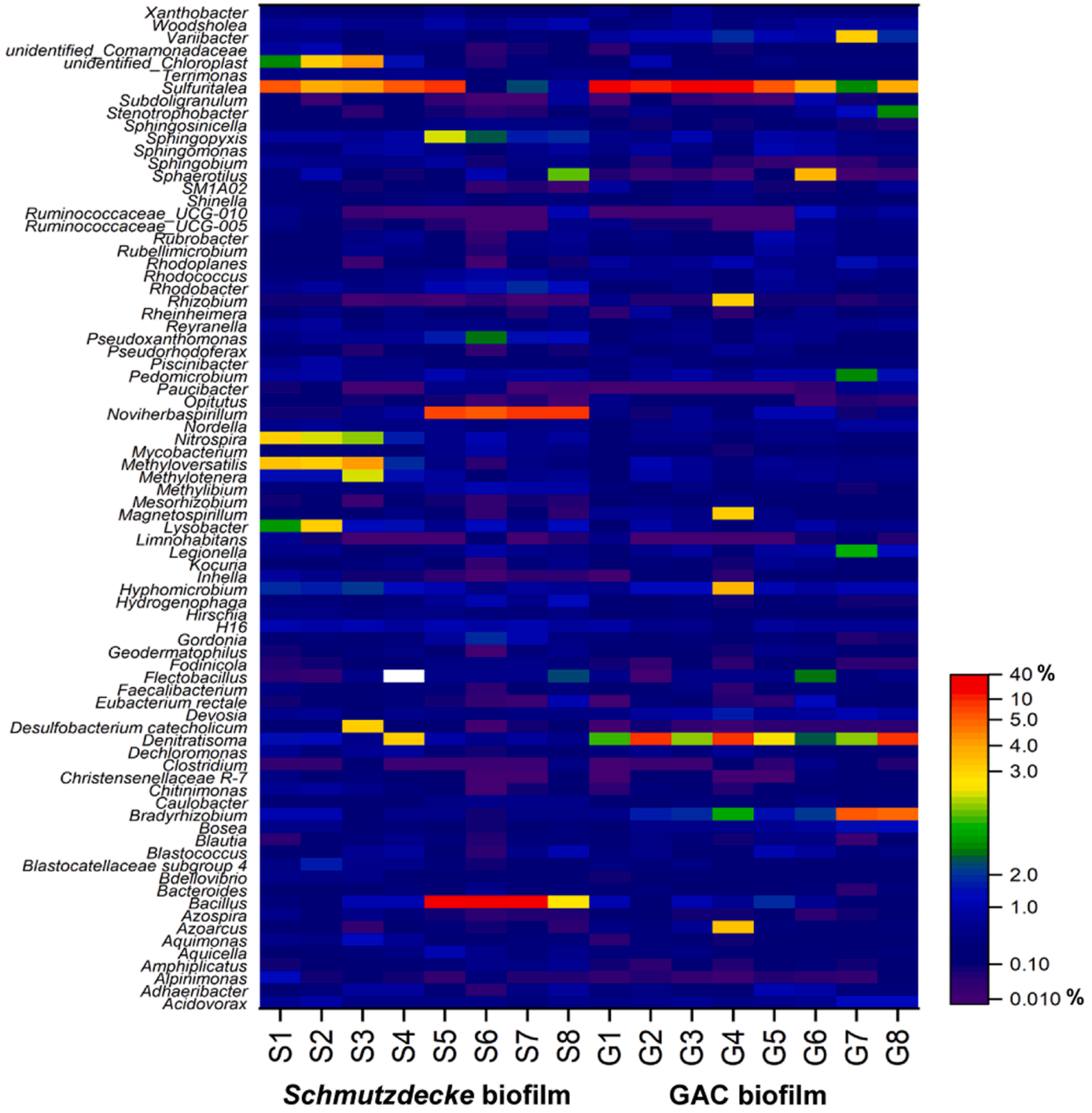

Fig. 6. Heatmap showing the distribution of major genera (relative abundance > 0.5% in at least one sample) in *schmutzdecke* and GAC biofilms.

biofilms and flocs [29,30]. The higher percentage of *Nitrospira* in surface biofilms indicated a greater extent of nitrification. On the contrary, *Denitratisoma*, which is related to denitrification and is involved in nitrate reduction [31], was found to be more abundant in GAC biofilms. *Noviherbaspirillum*, another genus associated with denitrification [32], was sensitive under exposure to antibiotics and more prone to inhabit the surface biofilm (7.0%) than GAC (0.7%). High abundance of genera *Hyphomicrobium*, *Nitrospira*, and *Bradyrhizobium* has also been found in drinking water or in GAC biofilters [33–36], indicating that they may be common inhabitants in biofiltration systems. The listed genera in Fig. 6 exhibit a variety of metabolic capabilities such as nitrogen fixation, nitrification, denitrification, photosynthesis, degradation of carbon compounds etc., indicating the potential functional diversity of the GAC sandwich biofilters. Furthermore, this also implies that the functional redundancy within the community may make up for the loss of sensitive bacteria under antibiotic pressure. Typical genera associated with opportunistic human pathogens were observed in this study. The genera of *Bacillus*, *Legionella*, *Mycobacterium*, and *Pseudomonas* were present in all biofilm samples, and *Bacillus* showed the highest abundance (up to 34.9%) in one of the B-*schm* samples (Tables S7 and S8).

3.5. Correlation between bacterial community and antibiotic resistome

Bacterial genera were considered as the environmental factors

affecting the ARG variation in redundancy analysis (RDA). RDA showed that a total of 73.33% of the difference in the relative abundance of ARG types in biofilms could be explained by variations in the bacterial community (Fig. 7-a). It is clear that the variations of resistome in the *schmutzdecke* or GAC, or when exposed to antibiotics, are associated with different bacterial communities. More specifically, the genera *Methyloversatilis*, *Hyphomicrobium*, *Nitrospira*, and *Lysobacter* significantly ($p < 0.05$) contributed to the relative abundance of ARGs in B-*schm* (with antibiotic), while the genera *Bacillus*, *Noviherbaspirillum* and *Sphingopyxis* were significantly ($p < 0.05$) correlated to the ARG abundance in B-*schm* (without antibiotics). Genera *Denitratisoma* and *Bradyrhizobium* were significantly ($p < 0.05$) correlated with ARGs in GAC biofilms.

In this study, the spike of antibiotics to the biofilter feed directly affected the bacterial community structure in the *schmutzdecke* and GAC biofilms and indirectly affected the antibiotic resistome. Eight out of ten most abundant genera were significantly correlated with the relative abundance of ARGs (Table S9). Aminoglycoside, beta-lactamase, MLSB, sulfonamide and vancomycin resistance genes were significantly correlated ($p < 0.05$) to the main genera in B-*schm* in Set A. By contrast, tetracycline resistance genes (*tet*) were related to the genera *Bacillus*, *Noviherbaspirillum* and *Sphingopyxis* in the control B-*schm*. Genus *Bacillus*, which was a strong competitor (accounting for 18.4%) within the B-*schm* (without antibiotic), was likely to be one of the main hosts for *tet*-carrying resistant bacteria and contributed to the persistence of *tet* in the absence of antibiotic selective pressure. Previous studies have found that members of *Firmicutes* were potential hosts of ARGs; among which genus *Bacillus* was found to be associated with *tetW* during manure composting [37].

The enhanced levels of the relative abundance of aminoglycoside and vancomycin (*van*) resistance genes without exposure to the corresponding antibiotic indicated the co-occurrence of these two ARG types. Both aminoglycoside and vancomycin ARGs were strongly and significantly correlated to beta-lactamase, MLSB, and sulfonamide (Pearson's $r = 0.62–0.72$, $p < 0.05$), and with *Methyloversatilis*, *Nitrospira*, and *Lysobacter* (Pearson's $r = 0.66 – 0.83$, $p < 0.01$) (Tables S9 and S10). Ma et al. have identified *Methyloversatilis* as one of the main genus hosts of multidrug resistance genes in tap water samples based on a large scale survey in seven countries and regions [35]. The genera *Nitrospira* and *Lysobacter* have also been identified as the major hosts of ARGs in surface water [38]. The observation in this study suggested that the differences in bacterial community structure are correlated with the changes in the resistome.

Although the RDA suggested a positive association between integrons and the five ARG types, paired Pearson's correlations only showed significance ($p < 0.001$) between integrons and MLSB resistance genes (Table S10). By contrast, the transposase gene was positively correlated to tetracycline resistance genes. To differentiate the effects of bacterial communities and mobile genetic elements (MGEs), including transposases and integrons, on the variation of antibiotic resistome in biofilms, the VPA showed that a total of 72.9% of the variance of ARGs could be explained by the selected variables in biofilm samples (Fig. 7-b). Bacterial community and MGEs explained 45.1% and 8.5% difference in ARG patterns, respectively. 19.3% of the variation could be attributed to the interactions between bacterial communities and MGEs. These results indicated that the bacterial community was the main factor driving changes in ARG profiles in biofilms.

To further visualize the connections between the bacterial community and ARGs, a co-occurrence network was constructed between the bacterial taxa (at the genus level) and ARGs. Among the 295 ARGs tested, only ARGs that occurred in all biofilm samples (77 ARGs in total) were used to construct the network. This preliminary data process could remove those poorly represented ARG subtypes in order to reduce the artificial association bias [39,40]. The top 20 most abundant microorganism populations at the genus level were selected for network construction. The network analysis is based on the hypothesis that the non-random co-occurrence patterns between ARGs and bacterial taxa could be used to provide new insights into ARGs and their possible hosts if the ARGs and the co-existing bacterial taxa possessed a strong and significantly positive correlation (Pearson's $r > 0.8$, $p < 0.01$) [35,40]. After construction, the entire network consisted of 48 nodes and 272 edges. Fig. 8 showed the co-occurrence patterns among ARG subtypes, MGEs and bacterial taxa, among which ten bacterial genera could be possible ARG hosts. It can be seen that more ARG subtypes belonging to multidrug resistance (20 edges in total) were carried by the identified bacterial genera. Genus *Nitrospira* had the most edges (15) with the ARGs, followed by *Methyloversatilis* (14) and *Methylotenera* (12). *Bacillus* and *Sphingopyxis* were possible hosts for the tetracycline resistance gene *tet*PB-01 (Fig. S8). *Lysobacter* was observed to be the host of *van*YD-01 (vancomycin) and cmx(A) (FCA). *Noviherbaspirillum* only carried *ole*C (MLSB), whereas *Nitrospira* and *Methyloversatilis* carried more diverse ARGs, including genes encoding resistance to all ARG types. MGEs co-occurred with multidrug, beta-lactamase, MLSB and tetracycline resistance genes. Similar co-occurrence among MGE and ARG subtypes was also reported in various environmental samples, including lettuce [40], natural waterbodies [18], soil [41], and drinking water [42], indicating that the dissemination of those ARG groups was associated with transposons and integrons. In particular, *tnpA*-02, *tnpA*-04 and *intI* 1 showed more frequent co-occurrence with ARGs in various environmental matrix.

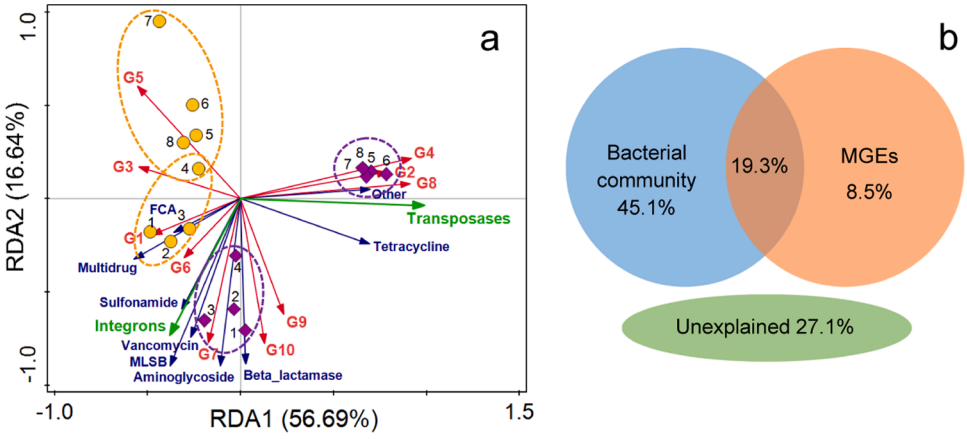

Fig. 7. (a) Redundancy analysis (RDA) of the correlation between major genera (top 10) and relative abundance of ARGs types in biofilm samples. G1: *Sulfuritalea*; G2: *Bacillus*; G3: *Denitratisoma*; G4: *Noviherbaspirillum*; G5: *Bradyrhizobium*; G6: *Hyphomicrobium*; G7: *Methyloversatilis*; G8: *Sphingopyxis*; G9: *Lysobacter*; G10: *Nitrospira*. Purple and diamond scatters 1–4: B-*schm* exposed to antibiotics; 5–8: B-*schm* unexposed to antibiotics; Yellow and circle scatters 1–4: B-GAC exposed to antibiotics; 5–8: B-GAC unexposed to antibiotics. MLSB = Macrolide-Lincosamide-Streptogramin B resistance genes; FCA = fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes. (b) Variation partitioning analysis (VPA) differentiating effects of bacterial community and MGEs (mobile genetic elements) on the variations of ARGs in biofilms.

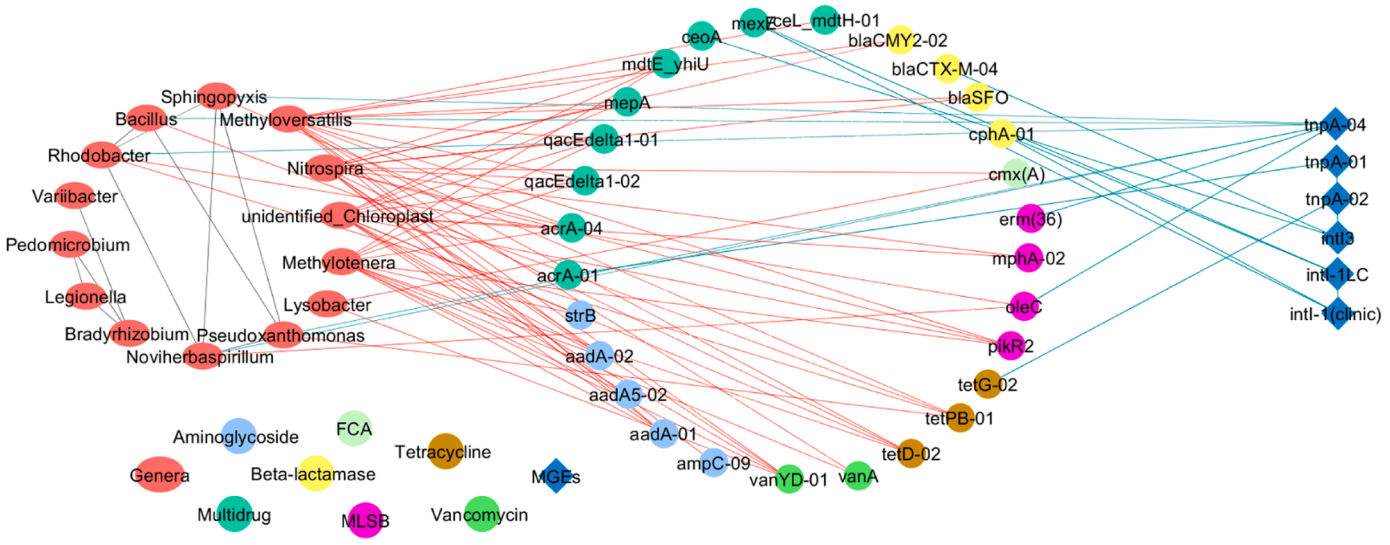

Fig. 8. Network analysis revealing the co-occurrence patterns between ARG subtypes, MGEs and bacterial taxa (genus level). The nodes were coloured according to ARGs types. The connection between ARGs and bacterial taxa represents a strong (Pearson's $r > 0.8$) and significant ($P < 0.01$) correlation. Red edges indicate the connection between bacterial genus and ARG subtypes; black edges are the connections among bacterial genus; and green edges indicate the connection between MGEs and ARG subtypes/bacterial genus. MLSB = Macrolide-Lincosamide-Streptogramin B resistance genes; FCA = fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes. MGEs: mobile genetic elements, including transposons and integrons.

3.6. Limitations

A difficulty for the study of amoxicillin in the aquatic environment is its poor stability; and the high polarity of the compound also implies difficulties to achieve its optimal extraction from aqueous sample [43]. Due to the high removal efficiency by GAC media and restrictions in analytical method for a poor recovery of amoxicillin, the concentration spiked at 10 μg/L for each antibiotic in the biofiltration system was not representative. This could introduce bias on the selection of both phenotypic and genotypic resistance, especially for those above minimal selective concentration (MSC). The MSC for resistant bacteria was predicted at 2 μg/L for amoxicillin and clarithromycin; 4 μg/L for oxytetracycline; 8 μg/L for trimethoprim; and 125 μg/L for sulfamethoxazole [44]. Phenotypic analysis could be included in a future study as the trace level of antibiotics may induce genotypic resistance but may not be sufficient to induce phenotypic resistance due to the lack of gene expression. Nevertheless, those genes (e.g. aminoglycoside and vancomycin ARGs) enriched without the exposure of the antibiotic to which they confer resistance complicates their dissemination in the environment.

For the elimination of the target antibiotics, the present research only investigated the antibiotics in their original form. Biodegradation pathways and metabolites could be further studied to understand the antibiotic degradation mechanisms. This can be difficult due to the typically low concentration of antibiotic residues in the feed and high background organic matter which could interfere with product identification and quantification [45]. Adsorption/desorption of antibiotics on filter media and the inclusion of biodegradation products in the analysis could help to differentiate adsorption and biodegradation as well as to clarity the relevant pathways in drinking water biofilters.

Combined with network analysis tools, the co-occurrence patterns between ARGs and microbial taxa could be assessed in complex environmental samples. The network analysis used in this study was based on correlation analysis, therefore, the correlation between the two nodes (ARG subtype and bacterial taxa) merely depends on their presence and abundance. Similar to this study, the combination of HT-qPCR and *16 S rRNA* sequencing approach has been used previously to explore ARGs and their possible hosts in various types of samples (e.g. soil and sewage sludge; lettuce; sand and GAC biofilms) [12,13,40,46,47]. Metagenomic analysis, on the other hand, is a more powerful tool which can be used to explore the entire antibiotic resistome and therefore could improve the robustness of the network analysis in predicting ARG hosts [20,39,48]. In addition to the benefits of metagenomics, metatranscriptomics could further provide a functional profile by analysing which ARGs are actively being expressed by the community [49]. The combination of metagenomics and metatranscriptomics can be utilised to specifically link ARGs to their transcripts and genetic context, providing a comprehensive insight into the abundance, diversity, expression and hosts of ARGs in complex environmental matrix [50,51]. For future study, the application of the above mentioned techniques could help to further explain how the microbiome react to antibiotic stress within biofilms and how the expression of ARGs relate to antibiotic selection pressure during the biofiltration process.

4. Conclusions

This study was the first attempt to consider the GAC sandwich biofilter as a whole and investigated the behaviour of ARGs and its association with bacterial community in the biofilms with and without the antibiotic selection pressure. Main findings are listed below:

- The diversity and abundance of ARGs in the *schmutzdecke* biofilms were clearly affected by the addition of the target antibiotics. In particular, the relative abundance of aminoglycoside, MLSB, sulfonamide and vancomycin resistance genes was significantly enriched in the *schmutzdecke* biofilms when exposed to antibiotics.
- *Schmutzdecke* biofilms remained the most active layer regarding the richness and abundance of ARGs, where GAC biofilms showed slightly lower ARG risks regardless the thickness and position of GAC layer in the filter bed.
- Among all bacterial phyla identified, *Firmicutes* and *Chlorobi* were significantly affected by antibiotics. Further analysis at class level revealed that *Bacilli* and *Chlorobia* contributed the most to the observed differences.
- Bacterial community, mobile genetic elements and their joint effects were the dominant mechanisms governing the variability of the distribution characteristics of ARGs in the *schmutzdecke* and GAC biofilms. Further network analysis suggested that 10 taxonomic genera were implicated as possible ARG hosts.

Overall performance of the GAC sandwich biofilter could provide useful information for optimising or updating the biofiltration process for industry. This research may help to further understand the persistence of ARGs in drinking water biofiltration system. Land application of drinking water waste products may act as an environmental exposure route for trace level ARGs and introduce a source for diffuse pollution in previously unexposed regions.

CRediT authorship contribution statement

Like Xu: Conceptualisation, Methodology, Formal analysis, Data Visualization, Writing – original draft, Writing – review & editing. **Melisa Canales:** Methodology, Writing – review & editing. **Qizhi Zhou:** Resources, Writing – review & editing. **Kersti Karu:** Methodology, Resources. **Xinyuan Zhou:** Methodology, Writing – review & editing. **Jianqiang Su:** Resources, Writing – review & editing. **Luiza C. Campos:** Conceptualisation, Methodology, Supervision, Writing – review & editing. **Lena Ciric:** Conceptualisation, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgement

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132511.

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