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Prevalence of antibiotic resistance and virulence genes in the biofilms from an aquifer recharged with stormwater

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ABSTRACT

An improved understanding of the diversity and composition of microbial communities carrying antibiotic resistance genes (ARGs) and virulence genes (VGs) in aquifers recharged with stormwater is essential to comprehend potential human health risks from water reuse. A high-throughput functional gene array was used to study the prevalence of ARGs and VGs in aquifer biofilms (n = 27) taken from three boreholes over three months. Bacterial genera annotated as opportunistic pathogens such as *Aeromonas, Burkholderia, Pseudomonas, Shewanella,* and *Vibrio* were ubiquitous and abundant in all biofilms. Bacteria from clinically relevant genera, *Campylobacter, Enterobacter, Klebsiella, Mycobacterium, Mycoplasma,* and *Salmonella* were detected in biofilms. The mean travel time of stormwater from the injection well to P1 and P3 boreholes was 260 and 360 days respectively. The presence of ARGs and VGs in the biofilms from these boreholes suggest a high spatial movement of ARGs and VGs in the aquifer. The ARGs with the highest abundance were small multidrug resistance efflux pumps (SMR) and multidrug efflux (Mex) followed by β-lactamase C genes. β- lactamase C encoding genes were primarily detected in *Enterobacteriaceae, Pseudomonadaceae, Bacillaceae*, and *Rhodobacteraceae* families. The VGs encoding siderophores, including aerobactin (*iro* and *iuc* genes), followed by pilin, hemolysin, and type III secretion were ubiquitous. Canonical correspondence analysis suggested that Total Organic Carbon (TOC), Dissolved Organic Carbon (DOC), turbidity, and Fe concentration has a significant impact on the microbial community structure of bacteria carrying ARGs and VGs. Post abstraction treatment of groundwater may be prudent to improve water security and reduce potential health risks.

1. Introduction

Groundwater replenishment via Managed Aquifer Recharge (MAR) with alternative water sources such as stormwater, surface water, and treated sewage effluent is increasingly used for the production of non-potable and indirect potable water in Australia, USA, and Europe (Dillon et al., 2010). The presence of pathogens, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in stormwater has been reported previously (Garner et al., 2017; Sidhu et al., 2012; Zhang et al., 2016). The environmental resistome comprises both the natural antibiotic-resistance gene pool, the "intrinsic resistome", and that resulting from anthropogenic activities. The introduction of ARB and ARGs into groundwater via recharge water may pose a potential threat to water security and public health which remains un-quantified.

Bacterial pathogens, even present in low numbers in the recharge water may get embedded in the biofilms, which may, in turn, prolong their survival by providing enhanced resistance to adverse environmental conditions (Wingender and Flemming, 2011). The presence of pathogenic and opportunistic bacteria in the groundwater and biofilms have been reported previously (Richards et al., 2018). Biofilms have been suggested as an ideal setting for horizontal gene transfer (HGT) due to the presence of high bacterial cell density, increased genetic competence, and accumulation of mobile genetic elements (MGEs) (Fux et al., 2005). However, the extent of transfer of ARGs originating from anthro-

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pogenic sources to groundwater autochthonous microorganisms in biofilms remains unknown.

Intrinsic antibiotic resistance in bacteria comprises a diverse range of mechanisms related to structural, physiological, and biochemical functions of bacteria such as insensitivity or reduced permeability to antibiotics, efflux systems, and metabolic functions (Baquero et al., 2013). Multidrug resistance (MDR) efflux pumps encoded by gram-positive and gram-negative bacteria have been known to impart resistance to aminoglycosides, β-lactams, macrolides, phenicols, lincosamides, quinolones, streptogramins, and tetracyclines (Davies and Davies, 2010). MGEs including plasmids, insertion sequences, transposons, integrons, genomic islands, integrating conjugative elements, and bacteriophages are also involved in HGT among bacteria in clinical and environmental settings (Frost et al., 2005). The presence of ARGs and VGs on MGEs enables their horizontal transfer. The spatial and temporal distribution of bacteria carrying ARGs and VGs in aquifers and biofilms is essential to determine the extent of potential human health risks from abstracted water use.

Recent advances in molecular methods such as microarrays and next-generation sequencing have ushered in new opportunities for the assessment of antibiotic resistome in the environment. Functional gene arrays (FGAs) are a high-throughput tool that allows a specific, sensitive, and quantitative profiling of microbial community diversity, structure, composition, and metabolic activity (He et al., 2010). FGAs have been previously used to analyze ARG profiles in different environments such as urban watershed (Low et al., 2016) and wastewater (Zhang et al., 2013).

To achieve a comprehensive understanding of the prevalence of clinically relevant bacteria, ARGs, and VGs in groundwater, biofilm samples from aquifer recharged with stormwater were screened with GeoChip 4.2. The main aim of the study was to investigate the prevalence and diversity of bacteria carrying ARGs and VGs in the biofilms developed in a subsurface aquifer environment. In particular, we were interested in (i) temporal and spatial distribution and abundance of ARB and ARGs in the aquifer recharged with stormwater (ii) environmental gene pool of clinically relevant virulence factors (iii) influence of biogeochemical factors on the distribution and abundance of ARGs and VGs.

2. Materials and methods

2.1. Stormwater catchment and aquifer recharge

The investigated Parafield stormwater harvesting and Managed Aquifer Recharge (MAR) site is located at Parafield in Adelaide, South Australia. The Parafield stormwater catchment has an area of 1,590 ha and is primarily urban (73%). It is composed of mainly residential (36%) but also has vacant land (13%) and industrial areas (8%) (Page et al., 2014).

The urban stormwater collected from the catchment through the weir on the Parafield drain passes through a series of two 50 ML detention basins and a 2 ha constructed wetland prior to direct injection into the tertiary aquifer (T2). Two separate MAR and water recovery options are available at this site; (i) Aquifer Storage and Recovery (ASR) where recharged water is recovered from the injection boreholes; (ii) Aquifer Storage Transfer and Recovery (ASTR) systems where recharged water is recovered from four separate recovery boreholes (Fig. 1). A detailed description of the operational parameters of the ASR and ASTR scheme was reported previously (Page et al., 2014). There are three observation boreholes (P1, P2, and P3) at the ASTR site located 10 m, 20 m, and 30 m respectively from the injection borehole (IW). The mean travel time of injected stormwater from IW to P1 and P3 is 260 and 360 days respectively (Page et al., 2014). We used borehole P1, P3, and ASR for the experimental work. The aquifer was constantly recharged during the investigation period (91 days).

2.2. Groundwater water quality profiling

Groundwater geochemical parameters (pH, temperature, redox potential, dissolved oxygen, electrical conductivity, and turbidity) were measured in the field using a field lab analyzer (TPS-90FL, TPS Pty. Ltd. Australia). Groundwater geochemical parameters were recorded at the beginning of the experiment, then at a monthly interval at the time of retrieving chambers from the boreholes. Groundwater grab samples (1L) were also collected at the monthly intervals and sent to a commercial laboratory (Australian Water Quality Centre, South Australia) for chemical analysis (nutrients, metals, TOC and DOC).

2.3. In-situ *biofilm study set up*

Teflon chambers (25 mm diameter) filled with sterile silica wool (SC0006 - Sercon, Crewe, UK) were used to provide a physical matrix for the development of biofilm and colonization of microorganisms (Fig. 2). The Teflon chambers were fitted with metal mesh discs (3 mm pore diameter), which allowed the free flow of water through the silica wool inside the chamber as outlined in our previous study (Sidhu and Toze, 2012). Prior to the assembly of chambers, all parts of the diffusion chambers were sterilized by autoclaving (20 min). Diffusion chambers (n = 27) were assembled in a laminar-flow cabinet and nine chambers were suspended on a stainless steel wire and lowered into each of the three boreholes (P1, P3, and ASR) so that the uppermost chambers were suspended at 1 m below the water table (at a depth of ~170 m) in the slotted section of the borehole to intercept the flow of groundwater. Three chambers from each of the three boreholes were removed at 1, 2, and 3 months respectively that allowed the development of biofilms and colonization of biofilms by the bacteria from recharged stormwater. Collected chambers (three from each borehole) were placed in zip lock bags for transportation to the laboratory in an insulated container at 4°C . The silica wool from each chamber was removed aseptically, weighed, and transferred into sterile tubes and stored at -80°C till DNA extraction.

2.4. DNA extraction from biofilms

DNA was extracted from the biofilm developed over the silica wool (4.5 g) using a PowerMax® Soil DNA isolation kit (MoBio Laboratories, Inc) following the manufacturer's protocol. Extracted DNA from replicate chambers was pooled, which resulted in a total of nine samples (three samples at a monthly interval from each of the three boreholes). Lyophilised DNA was then shipped to the Institute of Environmental Genomics, the University of Oklahoma for microarray analysis.

2.5. Microarray analysis of biofilm samples

The GeoChip 4.2 has very high hybridization specificity, containing 83,992 50-mer oligo probes covering 152,414 gene variants in 410 gene categories involved in biogeochemical, cellular, and ecological processes, metal resistance, antibiotic resistance, and virulence (Wang et al., 2014). The GeoChip 4.2 analysis was performed as outlined previously (Cong et al., 2015). Lyophilised DNA was re-hydrated in sterile MilliQ water and the DNA was quantified using a PicoGreen dsDNA Assay kit (Invitrogen, CA, USA). To produce consistent hybridizations from all samples, whole-community genome amplification was used to generate approximately 3.0 µg of DNA from 50 ng of template DNA using the TempliPhi Kit (GE Healthcare, Piscataway, NJ) and following a modified protocol

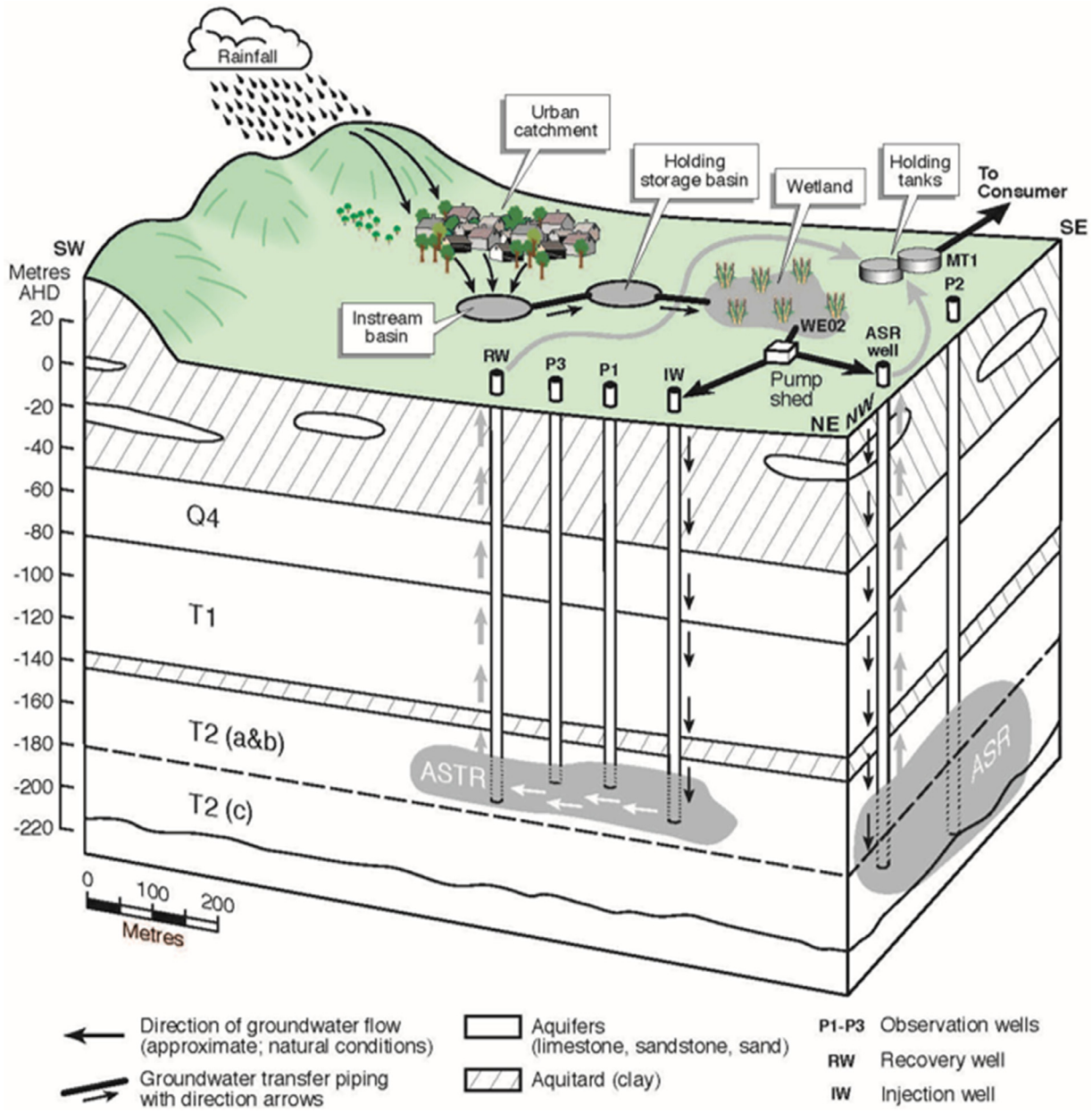

Fig. 1. Schematic diagram of the Parafield stormwater harvesting system, Aquifer Storage and Recovery (ASR) and Aquifer Storage Transfer and Recovery (ASTR) sites; Note ASR, P1 and P3 boreholes.

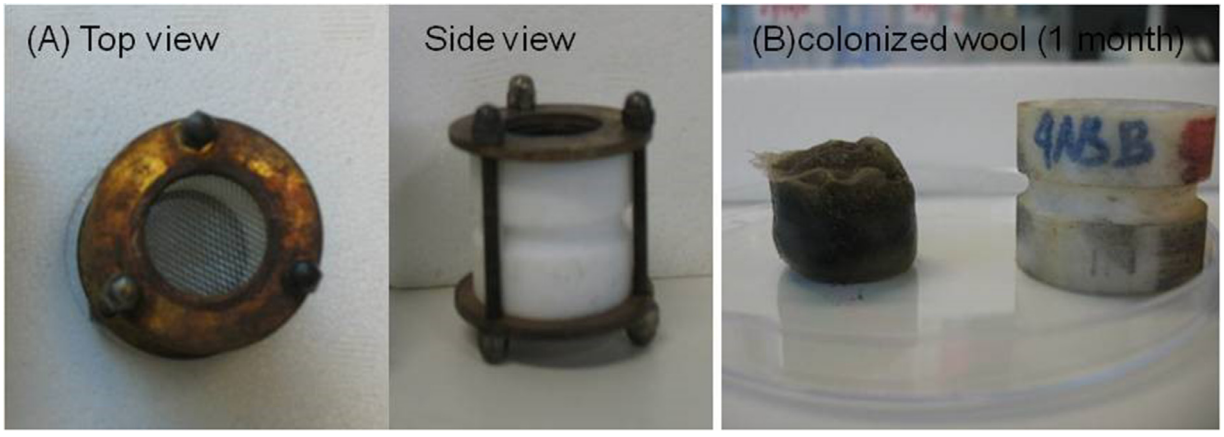

Fig. 2. Teflon biofilm chambers (A) and the silica wool matrix (B) colonized by aquifer water microbial communities.

(Wu et al., 2006). For each sample, 500 ng of amplified DNA was labeled with the fluorescent dye Cy-3 (GE Healthcare, CA, USA) by random priming as described previously (He et al., 2010). Samples were then hybridized on a Maui hybridization station (BioMicro Systems, Salt Lake City, UT, USA) at 42°C and 40% formamide for ~16 hours. The hybridized arrays were scanned with a NimbleGen MS 200 Microarray Scanner, images were extracted and quantified using NimbleScan software (Roche NimbleGen, Madison, WI, USA), followed by data processing as outlined previously (Azarbad et al., 2015). The genes detected in only one of three replicates samples were removed before further analysis.

2.6. Data analysis

The scanned images of hybridized GeoChips were processed using NimbleScan software (Roche Nimblegen, Madison, WI, USA). Microarray Data Manager (http://ieg.ou.edu/microarray/) was used for the statistical analyses of GeoChip data as described previously (Cong et al., 2015). Statistical differences in the functional categories and subcategories across sites were analyzed by a one-way ANOVA ($P = 0.05$) followed up with Tukey's test as a post hoc test. The sum of the normalized signal intensity values (total abundance) for each gene category was used for ANOVA. The ARGs and VGs diversity was calculated by using the Shannon index and Simpson index. Bray-Curtis coefficient was used to construct dissimilarity matrices of communities and Nonmetric multidimensional scaling (NMDS) ordination was used to visualize Bray-Curtis similarities. Significant differences in community structure were tested for different borehole biofilms with Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) and Analysis of Similarity (ANOSIM) (Clarke and Ainsworth, 1993) using Hellinger transformed data and a Bray-Curtis dissimilarity matrix constructed using PRIMER-E (Primer-e v7; www.primer-e.com). We also analyzed the abundances of bacteria families (top 50) and distribution of ARGs and VGs categories in response to fixed variables (site and maturity of biofilms) with multivariate generalized linear models using the function manyglm, and ANOVA within the MVABUND (v3.13.1) (Wang et al., 2012) package of R (v1.0.143) (Team, 2013). p-values were calculated using 500 resampling iterations via PIT-trap resampling, to account for correlation in testing. Canonical correspondence analysis (CCA) was used to determine the ARGs and VGs distribution and abundance that can be explained by the measured groundwater geochemical parameters in P1, P3, and ASR boreholes.

3. Results

3.1. Groundwater characteristics

Groundwater water samples were collected on a monthly interval from P1, P3, and ASR boreholes and tested for the geochemical parameters. The average water temperature across three boreholes varied from 15.60-19.80°C, pH from 7.35-8.00 and dissolved oxygen of remained low at ~0.8 mg/L (Table 1). TOC and DOC were highest in the ASR borehole 6.35 and 3.85 mg/L respectively. The turbidity of water was highest in the ASR boreholes (29 NTU) and reduced with distance from the injection boreholes P1 (19 NTU) and P3 (3 NTU). Groundwater was fresh with the lowest EC in the ASR (317 µS/cm) and highest (592 µS/cm) in the P1 boreholes.

3.2. Overview of microbial diversity based on Geo Chip 4.2

A total of 954 probes (out of 3334) provided a positive signal for ARGs and 1025 probes (out of 3738) for VGs from the biofilm samples collected from all three boreholes. To assess the α-diversity of microbial communities carrying ARGs and VGs

Table 1
Recorded groundwater geochemical parameters in three boreholes during the experiments.

Parameters	P1	P3	ASR
pH	7.90 (±0.1)	8.00 (±0.1)	7.35 (±0.2)
Temp (°C)	18.30 (±1.5)	19.80 (±1.4)	15.60 (±0.1)
DO (mg/L)	0.80 (±1.2)	0.80 (±1.2)	0.76 (±1)
EC (µS/cm)	592 (±44)	357 (±18)	317(±57)
Eh (mV SHE)	-145 (±70)	-148 (±55)	-202 (±53)
Turbidity (NTU)	19 ±(3)	3 ±(3)	29 (±6)
Ca (mg/L)	42.27 (±6)	24.74 (±7)	5.12 (±7)
Mg (mg/L)	8.53 (±0.4)	11.43 (±2)	8.03 (±0.7)
Fe (mg/L)	0.18 (±0.1)	0.10 (±0.1)	5.42 (±0.3)
K (mg/L)	4.72 (±1)	3.27 (±0.4)	3.91 (±0.1)
NO_3^- (mg/L)	<0.05	<0.05	<0.05
SO_4^{2-} (mg/L)	27.00 (±2)	12.00 (±7)	8.85 (±6)
DOC (mg/L)	1.90 (±0.9)	1.60 (±0.7)	3.85 (±1)
TOC (mg/L)	1.80 (±0.9)	1.60 (±0.4)	6.35 (±0.5)

All parameters recorded at the beginning of the experiment and then at the monthly interval (n=4); mean (± standard deviation)

Shannon-Weaver index (H), Simpson's index and evenness were calculated (Table S1). The value of H was very similar across all sites and within the sample, ranging from 7.28 to 7.42. Simpson's index and evenness also did not show significant differences among sites. A nonmetric multidimensional scaling (NMDS) plot of Bray-Curtis similarities for the ARGs and VGs gene profiles suggests similar diversity within sub-samples (Figure S1). The relative overlap of ARGs and VGs across sites varied between 66-76%. Whereas, high similarity (>80%) among the biofilm samples collected from the same borehole was observed.

3.3. Taxonomic identity of bacteria carrying ARGs and VGs

GeoChip 4.2 utilizes highly specific probes targeting functional gene DNA gyrase subunit B (gyrB) for determining diversity. The gyrB gene has been reported to have higher evolutional rates than 16S rRNA genes in bacteria and hence more useful to achieve higher taxonomic resolution at the species-strain level (Scheler et al., 2014). At a high taxonomic level of phylum or class, *Proteobacteria* followed by *Actinobacteria, Firmicutes,* and *Bacteroidetes* were the major carriers of ARGs and VGs in the biofilms . Top ten bacterial families carrying ARGs and VGs included; *Enterobacteriaceae, Burkholderiaceae, Pseudomonadaceae, Vibrionaceae, Rhodobacteraceae, Streptomycetaceae, Rhizobiaceae, Comamonadaceae, Neisseriaceae*, and *Bacillaceae* (Fig. 3). The following genera of putative pathogenic bacteria were identified in the biofilms: *Acinetobacter, Aeromonas, Bacillus, Bacteroides, Bartonella, Bordetella, Brucella, Burkholderia, Campylobacter, Carnobacterium, Chlamydia, Citrobacter, Clostridium, Corynebacterium, Edwardsiella, Enterobacter, Enterococcus, Escherichia, Gordonia, Haemophilus, Helicobacter, Klebsiella, Lactococcus, Legionella, Leptospira, Listeria, Micrococcus, Mycobacterium, Neisseria, Nocardia, Ochrobactrum, Pasteurella, Photobacterium, Propionibacterium, Proteus, Pseudomonas, Rhodococcus, Salmonella, Serratia, Shewanella, Shigella, Staphylococcus, Stenotrophomonas, Streptococcus, Vibrio,* and *Yersinia*.

To determine the influence of sites on the occurrence of ARGs and VGs, data were subjected to ANOSIM and follow on PERMANOVA analysis. The influence of the site on the occurrence of ARGs and VGs was significant (ANOSIM R:1, $p = 0.004$), 10% of the observed variation was due to sites (PERMANOVA). As evident from the heatmap in Fig. 3, bacterial populations carrying ARGs and VGs were influenced by the site. The dominant bacterial families at site P1 were distinctly different compared to the sites P3 and ASR. Similarly, at site P1, bacteria carrying β-lactamase C, MFS and ABC efflux, iron oxidation, and hemolysin genes were more prevalent (Fig. 4).

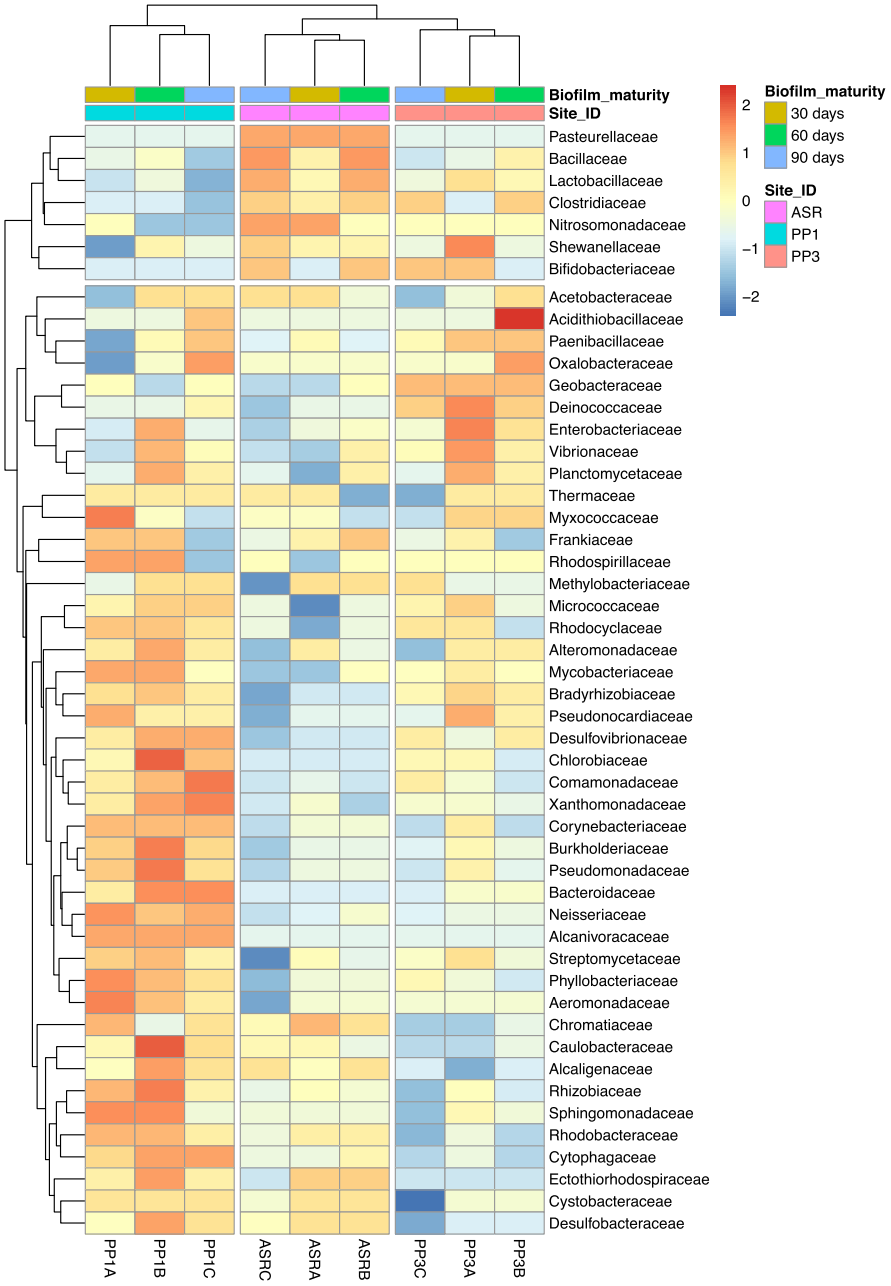

Fig. 3. Heatmap **and dendrogram of** bacterial families (top 50) that were significantly different across sites. The color key represents relative abundances as $\log_{10}$ transformed signal intensity data. The top half of the heatmap shows those bacterial families over-represented in ASR and P3 boreholes. The bottom half are those over-represented in P1 borehole.

3.4. Antibiotic resistome of biofilms

Out of 3334 probes specific for 12 ARGs categories, 987 probes showed positive hybridization signals, including five transporter genes (ATP-binding cassette (ABC), multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS), multidrug efflux (Mex), and small multidrug resistance efflux pumps (SMR). On average, 790, 726, and 720 positive probes were detected from the biofilm samples taken from the P1, P3, and ASR boreholes, respectively. The major phyla which carried ARGs across all three sites (P1, P3, and ASR) included; Proteobacteria (63-64%), Actinobacteria (14-16%), Firmicutes (9-11%) and Bacteroidetes (3-3.3%) (Table S2). Bacteria and archaea belonging to 138 families carrying ARGs were detected in the biofilms (Table S3). *Enterobacteriaceae* (13–14%) followed by *Burkholderiaceae* (6.7-7.5%), *Streptomycetaceae* (5-5.1%), *Rhodobacteraceae* (4.70-5.50%), *Rhizobiaceae* (4.6-5.1%), *Pseudomonadaceae* (3.6-4.4%), *Mycobacteriaceae* (2.1-2.5%) and *Vibrionaceae* (2.2-2.5%).

The relative abundances and numbers of all detected ARGs were similar within the site but some variation across sites was observed (Fig. 5A). Among the significantly changed ($P < 0.05$) ARGs categories across three sites were SMR, MFS, and β-lactamase C. Antimicrobial efflux transporters, genes encoding for the SMR family were widely distributed across sites (89 families), with high prevalence in medically important genera including *Pseudomonas, Burkholderia, Aeromonas, Streptomyces, Escherichia, Enterobacter, Yersinia, Vibrio, Citrobacter, Proteus, Salmonella, Klebsiella, Mycobacterium* and *Bacillus* (Fig. 6). The genes encoding for the MFS family were frequently detected in bacteria (45 families), with prevalence recorded in the genera *Pseudomonas, Burkholde-*

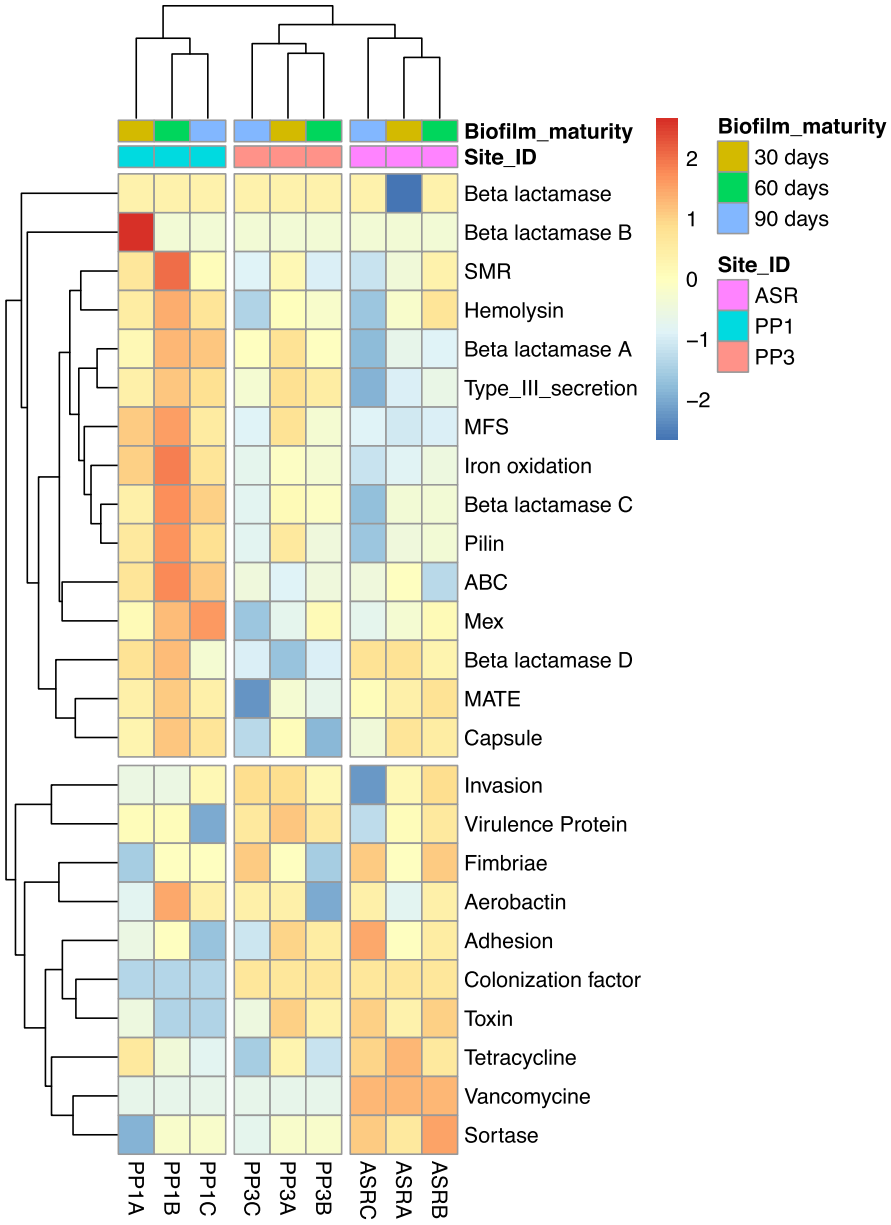

Fig. 4. Heatmap **and dendrogram of** ARGs and VGs categories that were significantly different across sites. The color key represents relative abundances as $\log_{10}$ transformed signal intensity data. The top half of the heatmap shows those ARGs and VGs categories over-represented in P1 borehole. The bottom half are those over-represented in ASR and P3 boreholes.

ria, Aeromonas, Escherichia, Klebsiella, Vibrio, Yersinia, and *Bacillus.* The MATE family genes were less abundant (22 families) and primarily detected in genera *Pseudomonas, Burkholderia, Aeromonas, Escherichia, Yersinia, Vibrio, Klebsiella*, and *Bacillus.* Similarly, ABC transporter genes were also less frequently detected (16 families) and often found in the genera *Streptomyces, Escherichia, Enterobacter, Citrobacter*, and *Klebsiella*. The genes encoding for the RND family (Mex genes) were less frequently detected and were most prevalent in the genera *Pseudomonas*.

Among β-lactamase resistance genes, β-lactamase C encoding genes were frequently detected (42 families) in the biofilms with an observed high prevalence in the members of genera *Pseudomonas, Burkholderia, Aeromonas, Streptomyces, Escherichia, Enterobacter, Yersinia, Citrobacter, Klebsiella, Mycobacterium,* and *Bacillus*. The genes encoding for β-lactamase A were the second most common and prevalent in the genera *Pseudomonas, Burkholderia, Streptomyces, Yersinia, Vibrio, Clostridium*, and *Mycobacterium*. The genes encoding for β-lactamase D were comparatively rare and mostly detected in the genera *Pseudomonas and Aeromonas*.

Of the 324 tetracycline resistance genes probes on the GeoChip, 70 genes from 25 families were detected in the biofilm samples across sites P1, P3, and ASR. Tetracycline resistance encoding genes were more frequently detected in the genera *Burkholderia, Streptomyces, Yersinia, Clostridium*, and *Bacillus.* The genes encoding for vancomycin resistance had very low prevalence with three genes detected in the families *Clostridiaceae* and *Paenibacillaceae*.

3.5. Prevalence of virulence genes

Out of 3729 probes specific for 13 VGs categories, in total 1011 probes showed positive hybridization signals belonging to three major phyla including, Proteobacteria (72-76%), Actinobacteria (8-9%), and Firmicutes (6-9%) (Table S4). Bacteria and archaea belonging to 140 families carrying VGs were detected in the biofilms.

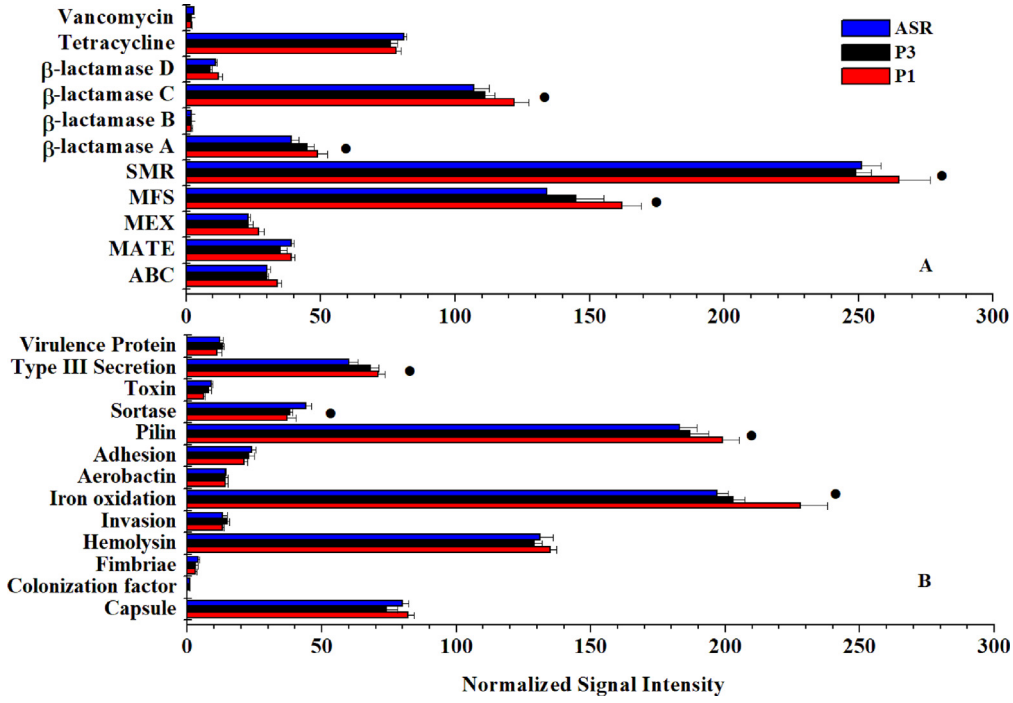

Fig. 5. Normalized signal intensity of detected key antibiotics resistance (A) and virulence (B) gene categories (gene abundance) involved in virulence. The signal intensity for each functional gene is the average of the total signal intensity from three monthly samples from each site. All data are presented as mean ± SE. Circles (•) indicate a significant difference of $P < 0.05$ (One-way ANOVA).

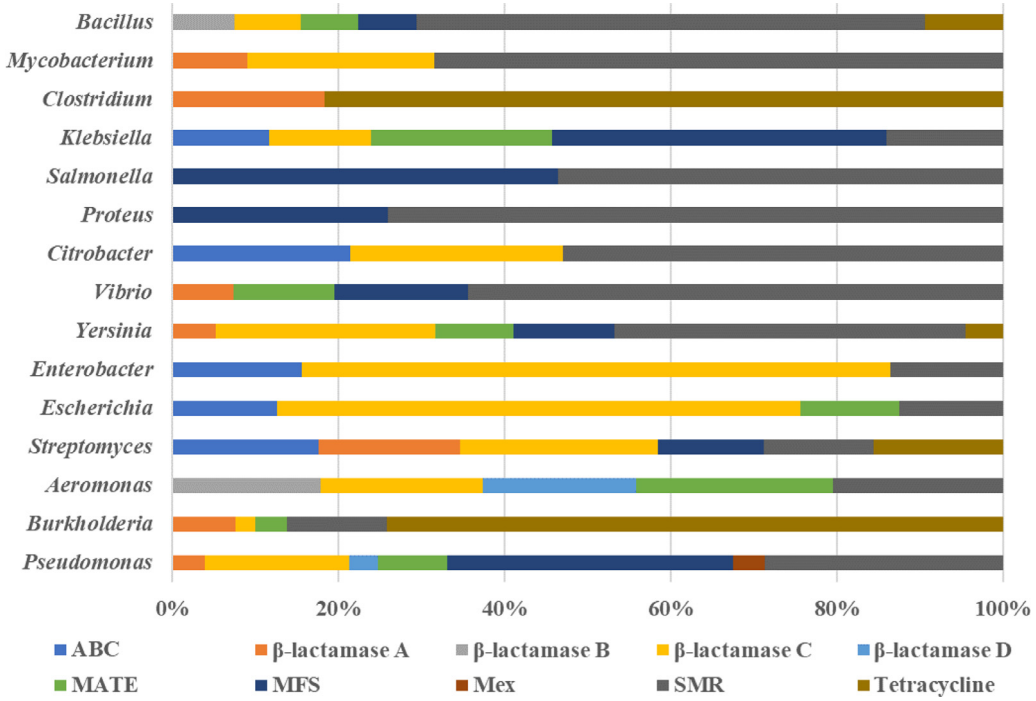

Fig. 6. Proportion of most abundant antibiotic resistance genes in the clinically relevant bacterial genera. ABC = ATP-binding cassette, MATE = multidrug and toxic compound extrusion, MFS = major facilitator superfamily, Mex = multidrug efflux, and SMR = small multidrug resistance efflux pumps

Members of the family *Enterobacteriaceae* (11%), *Burkholderiaceae* (10%), *Pseudomonadaceae* (9%), *Vibrionaceae* (8%), *Comamonadaceae* (4.7%) and *Neisseriaceae* (3%) were the major carriers of VGs (Table S5).

The relative abundances and numbers of all detected VGs were similar within the site but some variation across sites was observed (Fig. 5B). Among the significantly changed ($P <$ 0.05) VGs categories across three sites were toxins, pilins, adhesions, invasions, and fimbriae. Genes encoding siderophores, including aerobactin (*iro* and *iuc* genes), followed by pilin, hemolysin, and type III secretion were more commonly detected in all samples. Whereas VGs linked to colonization factor, toxins and virulence were less abundant. Siderophore related genes were widely distributed in bacteria from the genera including *Pseu-*

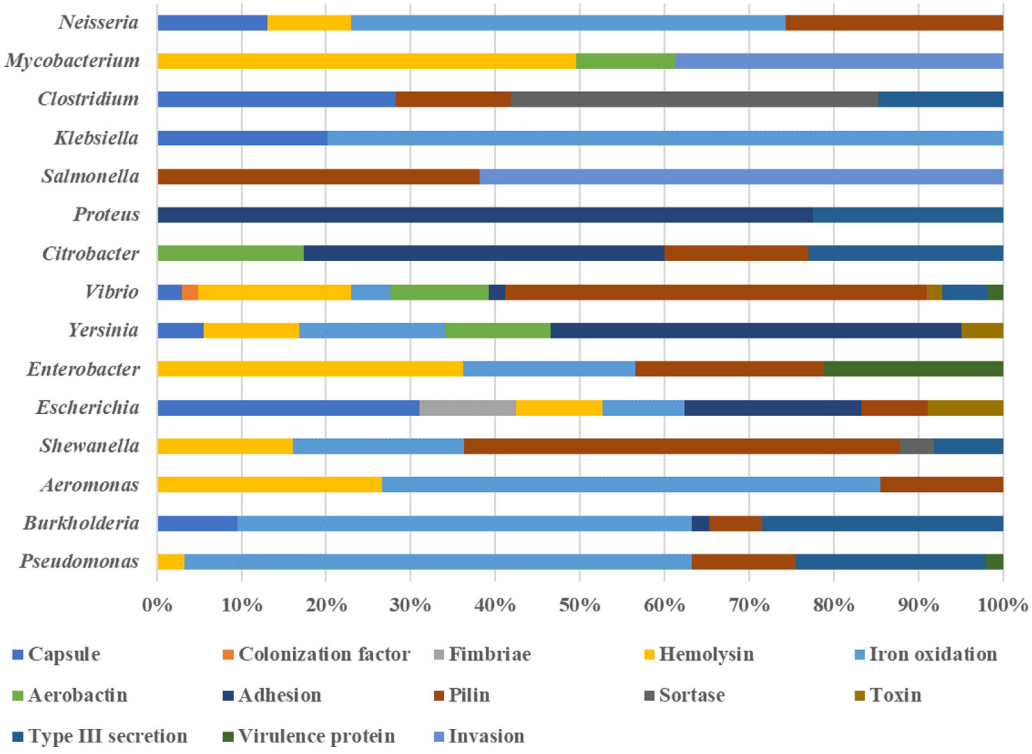

Fig. 7. Proportion of most abundant virulence genes in the clinically relevant bacterial genera.

domonas, Burkholderia, Aeromonas, *Shewanella*, *Escherichia, Enterobacter, Yersinia, Vibrio, Citrobacter, Klebsiella,* and *Neisseria* (Fig. 7). The *hyl* genes were most commonly associated with the members of genera *Pseudomonas, Aeromonas*, Shewanella, *Escherichia, Enterobacter, Yersinia, Vibrio, Mycobacterium,* and *Neisseria.* Pilin genes were widely prevalent in the genera *Pseudomonas, Burkholderia, Aeromonas*, *Shewanella*, *Escherichia, Enterobacter, Yersinia, Vibrio, Citrobacter, Proteus, Salmonella, Klebsiella, Clostridium, Mycobacterium, Neisseria*. Type III secretion related genes were associated with the genera *Pseudomonas, Burkholderia*, *Shewanella*, *Vibrio, Citrobacter, Proteus*, and *Clostridium.* Adhesion genes (*pap*) were carried by the genera *Burkholderia, Escherichia, Yersinia, Vibrio, Citrobacter,* and *Proteus.* Virulence proteins genes (*vip*) were less prevalent and carried by bacteria in the genera *Pseudomonas, Enterobacter*, and *Vibrio.*

3.6. Relationship between ARGs, VGs and physicochemical factors

Pearson's correlation analysis of ARGs and VGs data from P1, P3, and ASR sites showed several strong positive correlations (R>0.8), (Table S6). The most noticeable correlation (R>0.8) was between β-lactamase C and *hyl, iro*, pilin, and type III secretion genes. β-lactamase C also had a strong correlation (R>0.8) with efflux pumps Mex, MFS, and SMR. Virulence gene pilin also showed a noticeable strong correlation (R>0.8) with β-lactamase A, β- lactamase C, MFS, *hyl,* and *iro* genes. A siderophore related *iro* gene exhibited a strong correlation (R>0.8) with antibiotic transporters ABC, MFS, SMR, and β-lactamase C genes. The *hyl* genes also exhibited a strong correlation (R>0.8) with *iro*, antibiotic transporters Mex, SMR, and β-lactamase C genes.

Results from CCA analysis of normalized signal intensity data indicated the environmental variables that best-explained patterns of similarity in ARGs and VGs profiles among the three boreholes (P1, P2, and ASR) (Fig. 8). The first two axes of the CCA described 67.30% and 32.37% of the variation, respectively, and the model was significant ($P \leq 0.05$). Temperature, DO, pH, and Mg concentration showed a strong negative correlation with both the first and second axes whereas, turbidity, TOC, DOC, and Fe concentration showed a strong positive correlation with both the first and second axes. EC, SO_4, and K concentration showed a positive correlation with the second axis and a negative correlation with the first axis. The presence of bacteria carrying ARGs and VGs in the ASR borehole had a positive relationship with TOC, DOC, turbidity, and Fe concentration whereas, in the boreholes P1 a positive relationship with SO_4 and K concentration was observed.

4. Discussion

At a high taxonomic level of phylum or class, bacteria belonging to phyla *Proteobacteria (Gammaproteobacteria, Betaproteobacteria,* and *Alphaproteobacteria), Actinobacteria, Firmicutes,* and *Bacteroidetes* were ubiquitous in all biofilms. This is in agreement with the previously reported dominance of bacteria belonging to the phyla *Proteobacteria, Actinobacteria, Bacteroidetes,* and *Firmicutes* in the aquatic environment (Chen et al., 2018; Smith et al., 2012).

The α-diversity analysis of microbial communities carrying ARGs and VGs in the biofilm samples from the P1, P3, and ASR boreholes showed a high degree of similarity (H = 7.20-7.40). High similarity in ARGs and VGs (>80%) in time separated (1-3 months) biofilm samples from the same borehole suggest a relatively quick formation of the biofilm (i.e. within one month) and relatively stable bacterial population afterward. The relative overlap of ARGs and VGs across sites (66-76%) suggests that the presence of temporal variability of the bacterial communities within the aquifer. This is expected as diversity and functional capacity of microbial communities in aquifers vary in response to bio-available nutrients and geochemistry (Smith et al., 2012) which is influenced by the anthropogenic activities (Griebler and Lueders, 2009).

CCA indicated a positive correlation between ARGs, VGs, and biogeochemical factors (Fe, TOC, DOC, and turbidity) which is potentially due to the influence of stormwater with high turbidity, bio-available carbon and Fe on the groundwater microbial com-

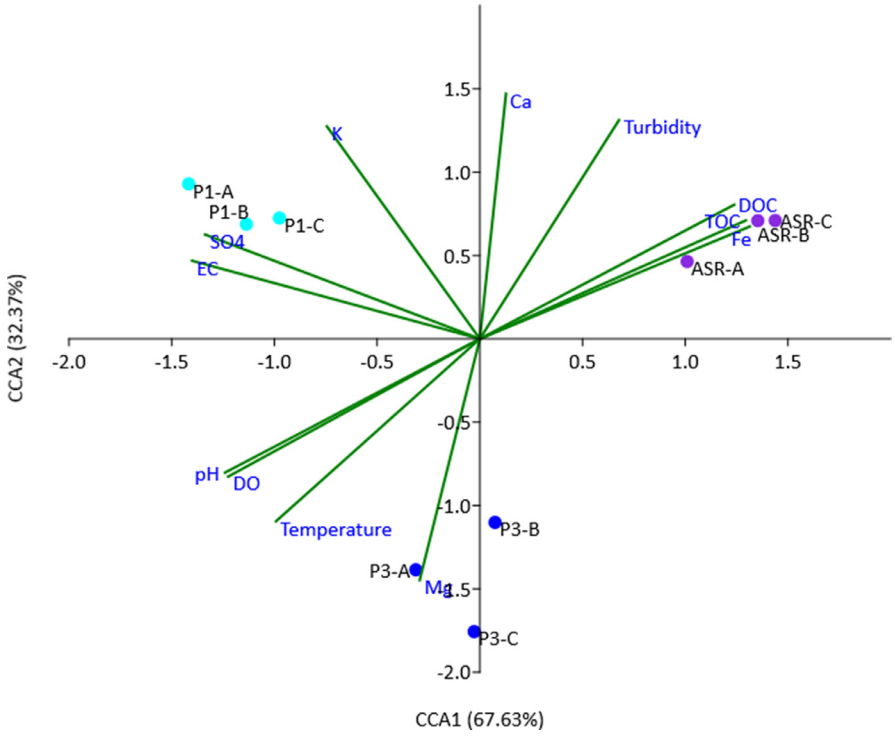

Fig. 8. Canonical correspondence analysis (CCA) of functional genes detected by GeoChip 4.2 and environmental variables (vectors). Filled circles represent borehole samples. Purple, ASR; blue, P3; light blue, P1. Letters after borehole ID indicate samples A, B, and C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

munities closer to the injection boreholes. A positive relationship between SO_4 and K concentration and ARGs and VGs in borehole P1, which is located ~ 260 m from the injection boreholes, could be explained by the low level of available nutrients and prevailing reducing conditions as recharged water moves from the injection boreholes (Table 1). At the genera level, putative pathogenic bacteria such as *Campylobacter, Enterobacter, Klebsiella, Mycobacterium, Mycoplasma, Salmonella,* and *Staphylococcus* were detected in all biofilms. The presence of pathogenic bacteria in the stormwater (Sidhu et al., 2012; Steele et al., 2018), aquifers (Levantesi et al., 2010), and biofilms (Emtiazi et al., 2004; Sun et al., 2014) is well documented. The present study, however, suggests that bacteria carrying ARGs and VGs can find their way into the groundwater via recharge water. It is worth mentioning that the travel time for the stormwater from the injection borehole (IW) to P1 and P3 is 260 and 360 days, respectively. This also suggests that bacteria normally present in the mammalian gut such as from the family *Enterobacteriaceae,* could survive in the groundwater for a long-time embedded in the biofilms. Long-term survival of pathogenic bacteria in drinking water biofilms has also been reported previously (Flemming et al., 2016; Wingender and Flemming, 2011).

Medically relevant genera including *Klebsiella, Escherichia, Citrobacter, Enterobacter, Pseudomonas, Campylobacter, Enterobacter, Mycobacterium, Mycoplasma,* and *Salmonella* were ubiquitous in the biofilms. Bacteria belonging to these genera have been previously reported as carriers and potential vectors for ARGs and virulence factor transfer (Vaz-Moreira et al., 2014). Consequently, there is always a potential for ARGs and VGs residing within MGEs to be transferred to pathogenic and opportunistic bacteria resulting in an *in-situ* development of ARB (Boehm et al., 2009). Transfer of NDM-encoding plasmids from *Enterobacteriaceae* to *P. aeruginosa* and *A. baumannii* has been demonstrated in biofilms (Tanner et al., 2017). In this study, we did not directly explore the occurrence of HGT in the groundwater biofilm. The role of bacteria carrying ARGs and VGs within biofilms as potential disseminators of antibiotic resistance is not clear and needs to be further investigated.

The observed high prevalence of MDR efflux pumps in the biofilms can be attributed to their presence in many microorganisms, sometimes in multiple copies within a single cell (Lubelski et al., 2007). Moreover, efflux pumps associated with MDR are reported in both pathogenic and opportunistic pathogens (Piddock, 2006; Webber and Piddock, 2003). The high prevalence of bacteria carrying genes coding for MDR efflux pumps in the aquatic environment has also been reported previously (Low et al., 2016; Ng et al., 2017). Also, bacterial cells can carry multiple efflux pumps e.g., the presence of more than one type of Mex efflux pump in a single *P. aeruginosa* cell and expression of more than one type of *Acr* efflux pump in a single of *E. coli* has been reported previously (Piddock, 2006).

The SMR family encoding genes have been reported in a range of bacteria including human pathogens (Bay et al., 2008), and can be easily disseminated due to their presence on R plasmids (Schuldiner et al., 2001). The SMR family efflux pumps are reported to provide resistance to a variety of antibiotics including aminoglycosides, chloramphenicol, erythromycin, and tetracyclines(Bay et al., 2008). The observed high prevalence of SMR efflux pumps from bacteria in the medically relevant genera *Pseudomonas, Burkholderia, Aeromonas, Streptomyces, Escherichia, Enterobacter, Yersinia, Vibrio, Citrobacter, Proteus, Salmonella, Klebsiella, Mycobacterium* and *Bacillus* suggested the presence of pathogenic bacteria in the biofilms.

MFS efflux pumps are known to be ubiquitously distributed in bacteria (Reddy et al., 2012) and involved in MDR, in particular fluoroquinolone, lincosamides, novobiocin and rifampin resistance (Mokracka et al., 2012). In this study, MFS efflux pumps were the second most prevalent efflux systems among bacteria belonging to the genera including *Pseudomonas, Burkholderia, Aeromonas, Escherichia*, and *Klebsiella.* MFS efflux pumps e.g., EmrAB of *E. coli*,

which are associated with macrolides resistance, have been previously reported in groundwater (Bockelmann et al., 2009) and surface water (Stoll et al., 2012).

The MATE family efflux pumps are the most recently categorized among the five multidrug efflux transporter families and believed to be universally present in all living organisms (Poole, 2007). The genes encoding for the MATE family were less abundant (22 families) and primarily detected in bacteria from genera *Pseudomonas, Burkholderia, Aeromonas, Escherichia, Yersinia, Vibrio*, and *Klebsiella*.

β- lactams, most commonly administered to humans and in animal husbandry, account for approximately two-thirds of total antibiotics use worldwide (Lachmayr et al., 2009). The high prevalence of β- lactamase genes in the groundwater biofilms suggest that external input via stormwater recharge is potentially the path of flow of β-lactamase genes. High prevalence of β-lactamase genes in confined (Caltagirone et al., 2017) and unconfined aquifers (Smith et al., 2012) has been reported previously. β- lactamase genes are frequently located on MGEs and are reported to coexist with other ARGs working on other classes of antibiotics such as aminoglycosides and fluoroquinolones (Marti et al., 2014). The ubiquitous presence of β-lactamase C and A genes in a wide range of gram-negative bacteria including members of *Enterobacteriaceae* and *Pseudomonadaceae* families combined with a high probability of transfer to a range of bacteria via plasmids or other transferable elements (Poole, 2004) is the most likely reason for their high occurrence in biofilms. The role of biofilm-bound bacteria in the dissemination of β- lactamase genes remains unclear and needs further investigation.

Tetracycline resistance is mediated by three mechanisms, antibiotic efflux pumps, target modification with ribosomal protection proteins, and antibiotic inactivation (Roberts, 2005). In this study, tetracycline resistance genes were detected in all biofilms in a diverse range of bacteria from the genera *Burkholderia, Streptomyces, Yersinia, Clostridium*, and *Bacillus* suggesting diverse gene pool of tetracycline resistance. Previous reports suggest a high prevalence of *tet* genes in the aquatic environment (Stoll et al., 2012; Tao et al., 2010) and abstracted water from the aquifer (Bockelmann et al., 2009).

Pathogenicity in bacteria is determined by several virulence factors such as adherence, colonization, invasion, secretion system, immune evasion, toxin production, and iron uptake (Kaper et al., 2004). In gram-negative bacteria, pili and S-fimbriae are crucial virulence factors (Hospenthal et al., 2017). The results of this study are in agreement with the previously reported wide prevalence of pili in gram-negative bacteria from the *Pseudomonadaceae, Burkholderiaceae, Vibrionaceae*, and *Neisseriaceae* families (Hospenthal et al., 2017).

Production of siderophores is considered essential for the acquisition and transport of iron across the cell membrane in many pathogenic and environmental bacteria (Dobrindt et al., 2004). Bacteria belonging to the genera including *Pseudomonas, Burkholderia, Aeromonas*, *Shewanella*, *Escherichia, Enterobacter, Yersinia, Vibrio, Citrobacter, Klebsiella,* and *Neisseria* were the main carriers of siderophore-encoding genes including aerobactin production. The observed high prevalence of genes coding for siderophores and aerobactin production is expected due to the high prevalence of siderophores in both pathogenic and non-pathogenic bacteria (Miethke and Marahiel, 2007).

Exotoxins such as α-hemolysin and enterotoxins are important virulence factors in gram-negative bacteria that result in host cell lysis (Gal-Mor and Finlay, 2006). Hemolytic genes are widely reported in both pathogenic and non-pathogenic strains of bacteria (Kim et al., 2015; Sidhu et al., 2013). Hemolysin encoding genes were most commonly associated with members of the genera *Pseudomonas, Aeromonas*, *Shewanella*, *Escherichia, Enterobacter, Yersinia, Vibrio, Mycobacterium,* and *Neisseria.* The type III secretion system (T3SS), a crucial virulence factor in the gram-negative bacteria, and reported in several pathogenic and opportunistic bacteria including *Salmonella, Shigella, Yersinia, P. aeruginosa* and B. pseudomallei (Kuhle and Hensel, 2004; Thibault et al., 2004). The observed high prevalence of T3SS encoding genes in biofilms is most likely due to the high prevalence of bacteria belonging to genera *Pseudomonas* and *Burkholderia*.

MDR efflux pumps have been reported to not only export antimicrobial agents from bacterial cells but also virulence determinants, such as toxins, adhesions, and colonization proteins which are important for the infection and colonization of human and animal cells (Piddock, 2006). For instance, in *V. cholerae* the activity of the RND efflux pumps is vital for antimicrobial resistance, production of toxins, and colonization factors (Bina et al., 2008). Moreover, ARGs and VGs can also be carried on the same plasmid e.g., in *Klebsiella pneumoniae,* R-plasmid carries extended-spectrum β-lactamases TEM-5, along with the non-fimbrial protein CF29K gene, aerobactin (Hennequin and Robin, 2016). Therefore, the correlation between ARGs and VGs is expected to a certain extent.

Several strong correlations between efflux pumps and virulence factors were detected e.g., a strong correlation ($R>0.80$) was observed between hemolysin and iron uptake, which is expected, as previously demonstrated in the case of *E. coli,* where hemolysis of erythrocyte can make iron available for absorption (Opal et al., 1990). In *P. aeruginosa*, overexpression of efflux pumps and β – lactamase genes along with porin loss has been reported to lead to high levels of drug resistance (Tomas et al., 2010). A strong correlation ($R>0.80$) was also observed between β – lactamase C and efflux pumps Mex, MFS, and SMR. Similar results have been reported previously in the aquatic environment (Escudeiro et al., 2019; Liang et al., 2020). In the case of *K. pneumoniae* extended-spectrum, β-lactamases genes, aerobactin, and its ferric aerobactin receptor and non-fimbrial protein CF29K gene are located on a relatively large R-plasmid (Hennequin and Robin, 2016). The observed strong correlation ($R>0.80$) between β – lactamase C and several virulence factors such as *hyl, iro, pilin*, and type III secretion genes may be due to co-existence of these genes on MGEs.

5. Conclusions

A better understanding of the diversity and ecology of bacteria carrying ARGs and VGs in freshwater aquatic ecosystems may provide a better understanding of the dissemination pathways of antibiotic resistance to and from humans. The presence of bacteria non-native to the aquatic environment belonging to putative pathogenic bacterial genera in the biofilms underscores the importance of stormwater as a source of human and animal-derived resistomes in the groundwater. Despite some inherent limitations (analysis for pre-defined sequences and an inability to distinguish between active and inactive genes), FGAs are an indispensable molecular tool for large-scale screening of resistome in the aquatic environment. The use of high-throughput FGA allowed relative quantification of ARGs and VGs biofilm samples. This study highlights the diversity and abundance of putative pathogens, ARGs, and VGs in groundwater biofilms, which together offer a higher probability of dispersal and HGT. The role of such bacteria as potential disseminators of ARGs and VGs remains unclear and needs further investigation. The presence of putative pathogens and bacteria carrying ARGs and VGs in the biofilms can contaminate potable reuse water and pose a public health hazard. To reduce potential health risks, post abstraction treatment may be required.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116269.

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