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Microbiome, resistome and mobilome of chlorine-free drinking water treatment systems

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ABSTRACT

Drinking water treatment plants (DWTPs) are designed to remove physical, chemical, and biological contaminants. However, until recently, the role of DWTPs in minimizing the cycling of antibiotic resistance determinants has got limited attention. In particular, the risk of selecting antibiotic-resistant bacteria (ARB) is largely overlooked in chlorine-free DWTPs where biological processes are applied. Here, we combined high-throughput quantitative PCR and metagenomics to analyze the abundance and dynamics of microbial communities, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs) across the treatment trains of two chlorine-free DWTPs involving dune-based and reservoir-based systems. The microbial diversity of the water being treated increased after all biological unit operations, namely rapid and slow sand filtration (SSF), and granular activated carbon filtration. Both DWTPs reduced the concentration of ARGs and MGEs in the water by about 2.5 log gene copies mL^{-1}, despite their relative increase in the disinfection sub-units (SSF in dune-based and UV treatment in reservoir-based DWTPs). The total microbial concentration was also reduced (2.5 log units), and none of the DWTPs were enriched for antibiotic resistant bacteria. Our findings highlight the effectiveness of chlorine-free DWTPs in supplying safe drinking water while reducing the concentration of antibiotic resistance determinants. To the best of our knowledge, this is the first study that monitors the presence and dynamics of antibiotic resistance determinants in chlorine-free DWTPs.

1. Introduction

Access to safe water and sanitation is a key Sustainable Development Goal (United Nations, 2015) and a central objective of the Water Action Decade (United Nations, 2018) of the United Nations. Drinking water treatment plants (DWTPs) are used to remove water contaminants and deliver safe water for consumption. The origin and nature of contaminants depend on several factors, such as the water source (Yu et al., 2018), geographical location (UNEP - United Nations Environment Programme, 2016), season (Kumpel et al., 2017), and type of anthropogenic activity in the water basin (Khatri and Tyagi, 2015). Contaminants can be divided into physical-chemical (*e.g.*, suspended particles, iron, ammonia) and biological (*e.g.*, pathogens, antimicrobial resistances – AMR) agents (United States Environmental Protection Agency, 2021).

The process configuration of DWTPs is mainly dictated by the water source, either groundwater or surface water. While groundwater is generally microbiologically safe, surface water may contain pathogenic organisms that must be eliminated (Smeets et al., 2009). Chemical disinfectants, such as chlorine, are usually applied to disinfect drinking water (i.e., inactivate pathogenic microorganisms) and/or to prevent microbial (re)growth in the distribution network (Sedlak and von Gunten, 2011). However, the use of disinfectants can generate by-products with mutagenic and carcinogenic effects (Rook, 1976) and selects for antibiotic-resistant bacteria (ARB) (Shi et al., 2013). A few countries (*e.g.*, The Netherlands, Denmark, or Switzerland) ceased disinfectants use and rely on strict source-to-consumer production standards and engineering solutions for drinking water supply (Smeets et al.,

2009). For the chlorine-free drinking water production from surface water, two main DWTP configurations (dune-based and reservoir-based; detailed description in Materials and Methods) are employed in the Netherlands. In both cases, a large fraction of the treatment consists of biological biofilm-based unit operations such as dune infiltration, rapid sand filtration (RSF), slow sand filtration (SSF), or granular activated carbon (GAC) filtration, which combine biological and physical-chemical processes. In these systems, chemical and biological water contaminants are converted by microbial communities (Mouchet, 1992; Tekerlekopoulou et al., 2013), which shape the microbiome of the drinking water that reaches consumers (Pinto et al., 2012). Therefore, the biological safety of the microbial communities harbored in DWTPs is of utmost importance for public health.

In contrast to wastewater environments (Calderón-Franco et al., 2022; Miłobedzka et al., 2022; Pallares-Vega et al., 2019), few studies focus on the fate and removal of ARGs and ARB in DWTPs. Biofilms are known reservoirs of ARB and antibiotic resistance genes (ARGs) (Balcázar et al., 2015). Little is known about the impact of biofilm-based DWTPs on the generation and/or persistence of ARB in drinking water. While antibiotic concentrations are very low or non-existent (Stackelberg et al., 2004), the generation of ARB in biofilms by horizontal gene transfer (HGT) is a well-known phenomenon (Farkas et al., 2013). Therefore, the effect of biofilms present in DWTPs operational-units on ARB development needs to be uncovered. To date, molecular studies of microbial communities, ARB, and ARGs in DWTPs have been limited by the low biomass concentration present in these systems for DNA extraction, sample collection logistics, and sampling standardization (Ma et al., 2017). The results often rely on either lab-scale experiments (Stange et al., 2019; Wan et al., 2019) or specific treatment processes such as biological activated carbon filters (Wan et al., 2021) or

tertiary treatments such as chlorine, UV, or a combination of them (Destiani and Templeton, 2019; Shi et al., 2013). Therefore, information about how biological treatments affect the fate of ARGs and MGEs in full-scale DWTPs from an integral consideration of the treatment train and different geographical areas is missing.

Most integrative studies have been carried out in China (Hu et al., 2019; Jia et al., 2020, 2015; Su et al., 2018; Xu et al., 2016; Zhang et al., 2019, 2016), *i.e.*, one of the largest antibiotic-producing and consuming countries world-wide (Huang et al., 2019). The studies have used qPCR (Hu et al., 2019; Su et al., 2018; Zhang et al., 2016), high-throughput qPCR (Xu et al., 2016) or sequencing methods like amplicon sequencing and metagenomics (Jia et al., 2020, 2015; Zhang et al., 2019) to investigate the concentration and richness of ARGs in full-scale DWTPs. Sevillano et al. (2020) have compared the effect of disinfection systems on antimicrobial resistance determinants on tap water samples in DWTPs from The Netherlands, UK, and USA. Yet, no detailed information about individual process units was provided. Moreover, none of these studies have combined qualitative (metagenomics) and quantitative (HT-qPCR) approaches for their analysis to get information about the total amount and diversity of AMR determinants in chlorine-free DWTPs.

In this work, we qualitatively and quantitatively resolve the role of chlorine-free DWTPs in the control of ARB and ARGs throughout the entire treatment train of two full-scale DWTPs in a low-antibiotic-consuming country. Specifically, we aim at deciphering how the biofilms in biological unit operations shape the resistome and mobilome of the drinking water. To do so, we compared the contribution of different methods for water storage, physical-chemical

113 contaminant removal, and disinfection in one dune-based DWTP and one reservoir-based
114 DWTP.

2. Material and methods

2.1. Sampling of two full-scale DWTPs

Water samples were collected from two different chlorine-free DWTPs supplying drinking water to the South Holland and Zeeland provinces in the Netherlands (Figure 1). The dune-based DWTP in this study infiltrates pre-treated river water into the sand dunes for storage and water quality improvement (e.g., disinfection). Subsequently, dune water is abstracted with wells and treated with pellet softening, powdered activated carbon (PAC), and rapid sand filtration (RSF) to remove hardness, organic (micro)contaminants, iron, and ammonium. Slow sand filtration (SSF) is deployed as a final disinfection step, as well as to ensure the biological stability of the water (i.e., remove trace nutrients). The reservoir-based DWTP in this study stores water in open reservoirs and uses a treatment train of coagulation-flocculation and RSF to eliminate physical-chemical contaminants, UV treatment for disinfection, and granular activated carbon (GAC) to remove organic contaminants (e.g., colour, odour, pesticides).

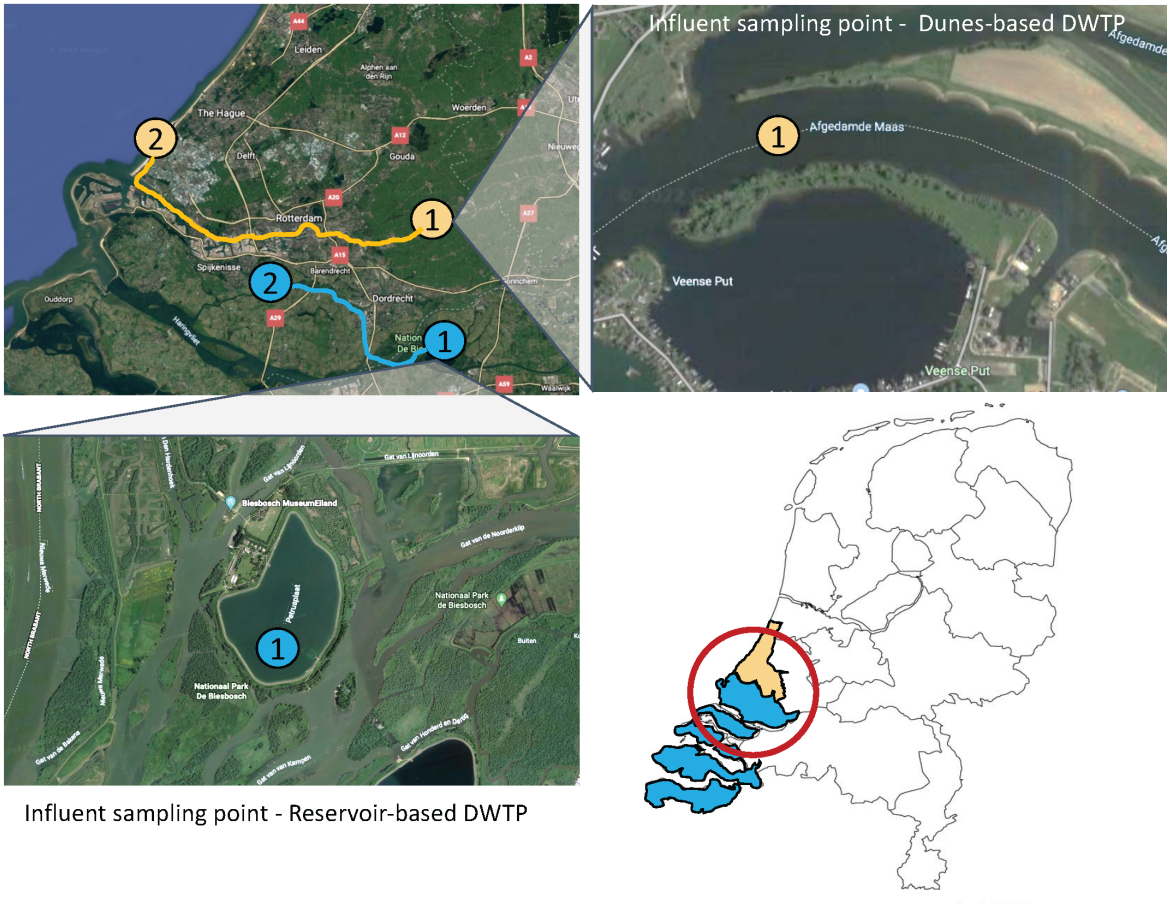


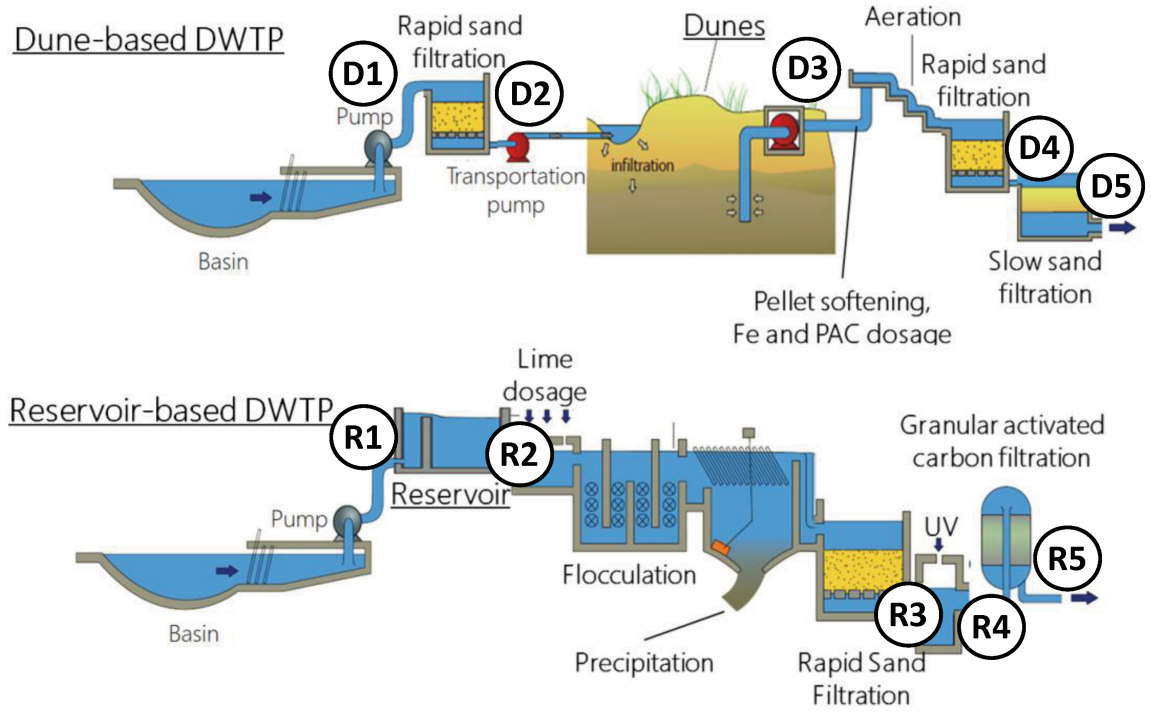
Figure 1. Geographic map of sampling sites. Numbering in the figure: (1) represents the river or reservoir from which the influent sample to the DWTP was taken, and (2) represents the location of the DWTP.

The first, dune-based DWTP(N 52° 7' 1.9992; E 4° 18' 23.9184) treats surface water from the Meuse River. The second, reservoir-based DWTP (N 51° 48' 44.4132; E 4° 20' 0.2112) processes surface water from the Meuse River as well (The Netherlands), after storage in a reservoir. Five sampling process stages per DWTP were targeted for water collection across their process stages (Figure 2,

Table 1).

The dune-based DWTP samples consisted of (D1) influent from the Meuse River water (N 51° 55' 41.7288; E 4° 46' 15.7404), (D2) outlet of the first rapid sand filtration, (D3) dune outlet (3 months hydraulic residence time), (D4) outlet of the second rapid sand filtration, and (D5) outlet of the slow sand filtration. The reservoir-based DWTP samples consisted of: (R1)

144 influent of the reservoir from the Meuse River water (N 51° 45' 39.3228; E 4° 46' 8.6664), (R2)
145 a sample of water after being stored in the reservoir for 3 months, (R3) rapid sand filtration
146 treatment outlet, (R4) UV treatment outlet, and (R5) GAC outlet. Water quality parameters
147 were provided by the DWTPs (Figure S2). The volume of each water sample depended on the
148 expected biomass concentration at each stage, based on the author's experience and
149 knowledge of DWTP personnel.



150

151 Figure 2. Schemes of the dune-based and reservoir-based drinking water treatment processes (DWTPs). The dunes and the
152 reservoir are the storage water steps (underlined). The dune-based DWTP consists of a first rapid sand filtration (RSF1) of
153 the Meuse River water followed by infiltration and storage in dunes (HRT = 3 months). Subsequently, the dune water is
154 processed by pellet softening to regulate hardness, and iron (Fe) and powdered activated carbon (PAC) is dosed to improve
155 color, odor and the performance of the second rapid sand filtration (RSF2). Finally, the water is disinfected via slow sand
156 filtration (SSF). In the reservoir-based DWTP, the Meuse River water is stored in a reservoir (HRT = 3 months) followed by
157 lime dosage to regulate hardness, flocculation, precipitation, and rapid sand filtration to reduce turbidity and disinfection
158 with UV and taste and odor correction with granular activated carbon (GAC). The overall hydraulic residence times of the
159 waters across the treatment trains amount to circa 3 months in both processes. Sampling points are represented by numbers

D1-D5 for the dune-based DWTP (*top*) and R1-R5 for the reservoir-based DWTP (*bottom*). Figure adapted from (van Halem and Rietveld, 2014).

Table 1. Water samples were collected from the different stages of the dune-based and reservoir-based DWTPs processing surface water from the Meuse River and Meuse River, respectively, in the Netherlands. The metadata for the molecular biology analyses are given.

DWTP system	Sample collection date	Sample description	Water volume extracted (L)	DNA extract quality (A260/A280, ±0.1)	DNA concentration in extract (ng/µL)	Metagenomic analyte identifier	BioSample IDs
Dune-based	2020-10-26	Meuse River influent	5.8	1.8	71	INF-DB-DWTP	SAMN31143551
	2020-10-26	RSF1 outlet	9	1.8	27	RSF1-DB-DWTP	SAMN31143552
	2020-11-30	After dune infiltration	1200	1.8	30	DUNE-DB-DWTP	SAMN31143553
	2020-12-14	RSF2 outlet	1200	1.8	15.5	RSF2-DB-DWTP	SAMN31143554
	2020-12-16	SSF outlet	1200	1.8	98	SSF-DB-DWTP	SAMN31143555
Reservoir-based	2020-12-19	Meuse river influent	1.5	1.8	34	INF-RB-DWTP	SAMN31143556
	2021-02-24	Reservoir outlet	1.5	1.8	10	RES-RB-DWTP	SAMN31143557
	2021-03-02	Before UV	100	1.8	11	BefUV-RB-DWTP	SAMN31143558
	2021-03-03	After UV	100	1.8	22	AftUV-RB-DWTP	SAMN31143559
	2021-03-05	GAC outlet	210	1.8	11	GAC-RB-DWTP	SAMN31143560

2.2. DNA extraction

Each water sample was immediately filtered on the DWTP site through a 0.22 µm polyethersulfone membrane via vacuum filtration. The membrane containing the biological retentate was folded and introduced into the DNA extraction tubes. Total DNA was extracted using the DNeasy PowerWater DNA extraction kit (Qiagen, The Netherlands) following the manufacturers' instructions. DNA qualities of the extracts were measured as absorbance ratio at 260 and 280 nm using a NanoDrop spectrophotometer. DNA concentrations were measured with a Qubit4 fluorometer (Thermo Fisher Scientific, USA). The DNA quality and concentration obtained for each sample is given in Table 1.

177 **2.3. Library preparation, sequencing, quality control, and assembly**

178

179 **Preparation of metagenome libraries.** The DNA analytes were sent to Novogene (Cambridge,
180 United Kingdom) for metagenome library preparation and sequencing. A total amount of 1
181 µg DNA per sample was used as input material to prepare libraries that were generated using
182 the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's
183 instructions; index codes were added to attribute sequences to each sample. In short, the
184 DNA sample was fragmented by sonication into fragment sizes of 350 bp; the DNA fragments
185 were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing
186 with further PCR amplification to add the sequence adapters; the PCR products were purified
187 on AMPure XP magnetic beads (Beckman Coulter, USA).

188

189 **Sequencing of libraries.** The library preparations were sequenced with an Illumina HiSEQ
190 PE150 system. Ten raw sequencing files with 150 bp paired-reads were obtained, with an
191 average of 5.6 Gb per sample (41 million reads). More details are given in Table S1.

192

193 **Quality control of sequenced reads.** The quality of the sequenced raw reads was assessed by
194 FastQC (version 0.11.7) with default parameters (Andrews, 2010) and visualized with MultiQC
195 (version 1.0) (Figure S1). Low-quality paired-end reads were trimmed and filtered by
196 Trimmomatic version 0.39 on the paired-end mode (Bolger et al., 2014).

197

198 **Assembly of sequence reads**. Clean reads were assembled into contigs using MetaSPAdes
199 (version 3.13.0) with a contig length between 300 and 2000 bp (Nurk et al., 2017). The number

of contigs obtained was 37,744.2 on average. Altogether, they had a total length of 180,840 kb, on average (Table S1).

2.4. Microbiome profiling obtained from dune and reservoir-based DWTPs

Taxonomic classification of raw reads was performed to profile the microbiome from each sample using the standard Kraken2 (version 2) database (uses all complete bacterial, archaeal, and viral genomes in NCBI Refseq database) with default parameters (Wood et al., 2019). Raw reads, divided into k-mers (substrings of length *k* contained within a biological sequence, determined by Kraken2), were matched with the NCBI database (Agarwala et al., 2018). The absolute abundance of each taxonomic group was indicated as the number of k-mers aligned to a specific taxonomic group. The relative abundance is the normalization of the total number of k-mers aligned in each sample.

Species richness (S) was measured as the number of different species detected in the raw datasets. The Shannon (H') diversity index was calculated with the following equation:

$$H' = -\sum_{i=1}^{S} p_i \cdot \ln p_i$$

where *pi* represents the relative abundance of species *i* with respect to the total amount of species (S). Microbial community distance estimation was calculated using MinHash in Mash v2.3.(Ondov et al., 2016) with "-k" 18, the minimum value required for distance estimation.

2.5. Resistome and mobilome profiling of DNA analytes obtained from both DWTPs

ARGs were annotated by aligning the assembled contigs > 500 bp to the ResFinder 4.0 resistance gene database using the BLASTn (version 2.6.0) nucleotide alignment tool with a cut-off E-value $<10^{-5}$ and sequence identity above 90% (Bortolaia et al., 2020). The richness of ARGs was defined as the number of different detected ARGs.

The mobilome was analyzed on the same set of contigs >500 bp using BLASTn (version 2.6.0) with the following specific databases of MGEs with sequence identity >95% and an e-value $<10^{-20}$. The presence of plasmids was studied with the PLSDB database (Galata et al., 2019). Integrons were detected with the INTEGRALL database (Moura et al., 2009). The ISfinder database was used to identify bacterial insertion sequences (Siguier et al., 2006). The ICEberg database (version May 2, 2018) detected bacterial integrative and conjugative elements (Liu et al., 2019). For all queries, the ARG or MGE identified with the best score was selected to annotate the query.

Co-occurrence (or co-localization) of MGEs and ARGs within the same contig was identified. It was checked with the BLASTn outputs if a contig contained both ARGs and MGEs. Contigs >500 bp that simultaneously included hits from the ResFinder 4.0 database and at least one of the different MGE databases were considered to have co-localized. Afterward, a specific Kraken2 taxonomic analysis was performed with these contigs to identify the potential microbial host that might carry the co-localized ARG and MGE.

2.6. Functional analysis of antibiotic-producing microorganisms in water samples

247 Functional analysis of the metagenomes was performed to detect antibiotic synthesis
248 pathways within microorganisms present in the microbial communities of the water samples.
249 The assembled contigs were transcribed to coding sequences using Prokka (version 1.14.5)
250 with default parameters (Seemann, 2014). Output files were introduced in GhostKOALA
251 (version 2.2.) to assign protein functionality in the Kyoto Encyclopaedia of Genes and Genome
252 s(KEGG) sequence library of "*genus_prokaryotes*" + "*family_eukaryotes*" (version 97.0). The
253 mapping onto the KEGG pathway map was performed to obtain the antibiotic-resistance
254 metabolic profile of the metagenome (Mitra et al., 2011).
255
256 **2.7. High-throughput quantitative PCR analysis**
257
258 Aliquots of the DNA extracts were sent in parallel to Resistomap (Helsinki, Finland) for high-
259 throughput quantitative PCR (HT-qPCR) to detect and quantify the presence and abundance
260 of 295 genes (listed in Table S2). These genes belonged to ARGs (238 genes), MGEs (51), and
261 pathogens (6). A concentration of 2 ng DNA μL^{-1} in a reaction volume of 0.05 µL was used to
262 obtain the number of gene copies of the different biomarkers. HT-qPCR results were
263 corrected (detailed explanation in supplementary material**)** to get the number of gene copies
264 existing per volume of filtered water from the DWTPs sampling points.
265
266 The gene abundance results were expressed in different ways. The absolute abundance of
267 ARGs and MGEs was calculated as a number of gene copies per mL of filtered water as done
268 in Xu et al. (2016). The absolute abundances of ARGs and MGEs sorted by antibiotic class and
269 MGE type were averaged over all genetic components belonging to each group. The relative
270 abundance of ARGs and MGEs was calculated based on the ARG or MGE copies per number

of 16S rRNA gene copies. Abundance values were logarithmically transformed for comprehensive data calculation and visualization.

2.8. Statistics and data visualization

Graphs were made with RStudio (version 1.3.1093). Microbiome absolute and relative abundances were calculated by Pavian (Breitwieser and Salzberg, 2020). Linear correlations between absolute abundances of ARGs and MGEs were analyzed using Pearson correlation coefficient ("ggpubr" R package) at value < 0.05. Pearson correlations between ARGs and each specific type of MGE (plasmid, insertion sequence, integron, and transposon) were also calculated. This gives the first-hint proxy for examining the co-localization of ARGs and MGEs.

3. Results

3.1. Microbial community composition

3.1.1. Richness and alpha diversity of the water metagenomes

The metagenomes of the microbial communities present at the different sampling points across the dune-based and reservoir-based DWTPs were sequenced to obtain first their taxonomic profiles. DNA extracted from 10 water samples was sequenced, resulting in 41 ± 5 million paired-end reads per sample (Table S1). All sequenced samples had high-quality rates (quality rate per sequence base > 30; Q = -10 x log10(P), where P is the probability that a base call is erroneous) (Figure S1). In analogy to engineered ecosystems, and likely owing to current databases incompleteness, an average of 24.6 ± 6.5 % of the raw reads were taxonomically classified.

The alpha diversity of the water microbiomes was assessed using the richness and the Shannon index (Figure 3A). The former measures the number of different populations (at the genus level) in the community, and the latter accounts for the number, relative abundance, and evenness of species (Hill et al., 2003). Richness was stable throughout both DWTPs, ranging between 7027 and 7959 different classified species detected from the water metagenomes (Figure 3). The richness of the influent of the reservoir-based DWTP was 7% higher than the dune-based DWTP influent water. Rapid sand filtration (RSF1 and RSF2), slow sand filtration (SSF), and granular activated carbon (GAC) increased the number of species by 2.9 ± 1.7%. On the contrary, dune infiltration, reservoir, and UV disinfection decreased it by 5.2 ± 2.1%. The Shannon H' diversity index ranged between 4.9 and 7.7 across all samples

(*i.e.*, equivalent to 134 to 2208 virtual equi-abundant populations). The dune-based DWTP gradually increased from 5.3 to 7.7 throughout the plant. Equal H' diversity values were found in the influent (6.7) and effluent (6.6) of the reservoir-based DWTP despite its oscillating trend.

The distances between the microbial community compositions were calculated using the MinHash dimensionality-reduction technique in Mash (Figure 3B). Higher distance indicates the larger dissimilarity between the microbial community at each sampling point and the influent. The dissimilarity significantly increased after every step in both DWTPs (p-value < 0.05, expect for After Reservoir where p-value = 0.09). Overall, the differences in microbial community compositions across the process train of the dune-based DWTP were higher than in reservoir-based DWTP.

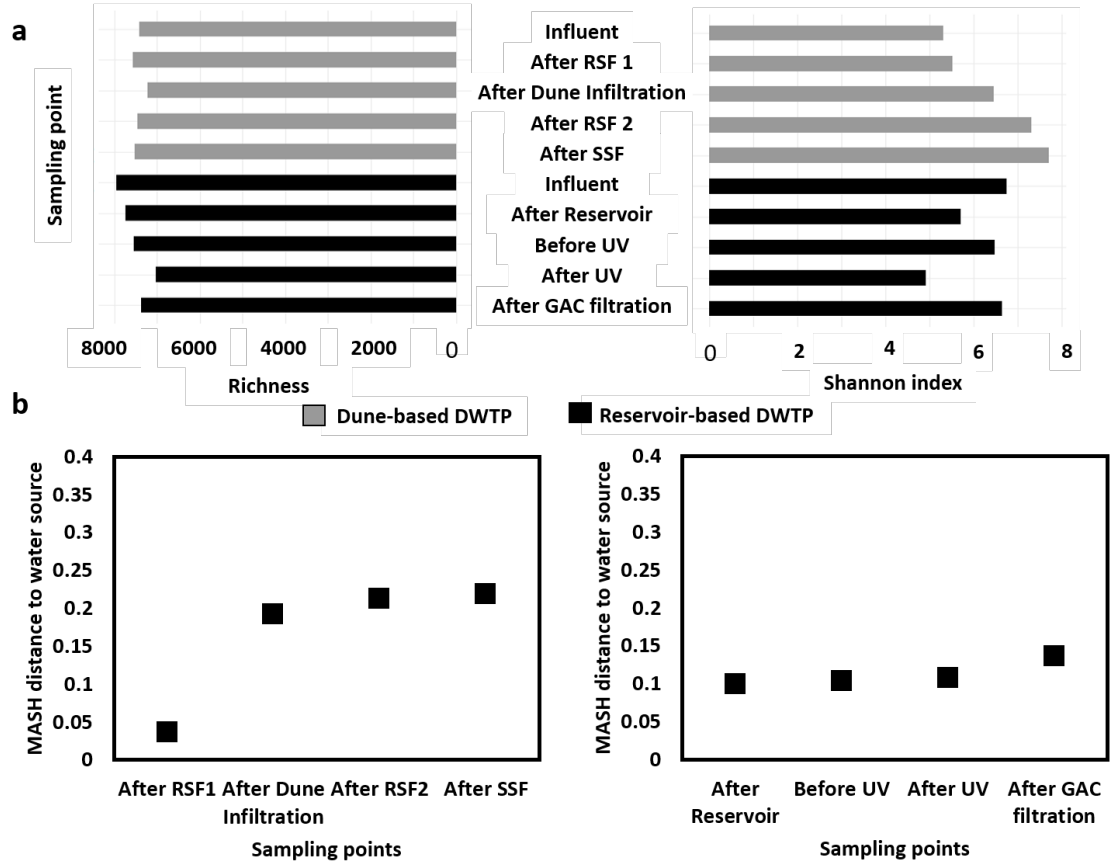


Figure 3. A) Richness and Shannon diversity indices (*x-axes*) of the taxonomically classified metagenomics datasets of the waters sampled at the different locations (*y-axes*) within the dune-based and reservoir-based DWTP trains. B) Microbial community distance estimation with MinHash between each sampling point and the influent (p-value < 0.05). RSF: rapid sand filtration; SSF: slow sand filtration; GAC: granular activated carbon filtration.

3.1.2. **Taxonomic classification of microbial communities**

The relative abundance of the detected prokaryotic populations across the DWTPs at phylum and genus levels is shown in Figure 4. The river-influent water of both DWTPs had similar compositions. At phylum level, *Proteobacteria* (68.1 ± 5.5% in dune-based DWTP vs. 76.9 ± 10.2% in reservoir-based DWTP), *Actinobacteria* (17.1 ± 6.5% vs. 11.9 ± 5.6%) and *Bacteroidetes* (6.2 ± 3.8% vs. 4.9 ± 2.6%) dominated the microbial communities of both

DWTPs. At genus level, the freshwater genera *Limnohabitans* (24.6% vs. 6.4%), "*Candidatus* Planktophila" (8.2% vs. 7.5%), and *Flavobacterium* (6.8% vs. 6.1%) were the main populations detected in both river waters. Their relative abundance decreased throughout the DWTP processes. Interestingly, we found several microbes in the effluent water that were absent in the influent, with most of them appearing after dune infiltration, SSF, and GAC filtration.

In the dune-based DWTP, every sand filter decreased the relative abundance of members of the phylum *Bacteroidetes* (Figure S2) and its most abundant genus *Flavobacterium.* This population decreased from 6.8% to 0.8% in RSF1, from 5.4% to 1% in RSF2, and from 1% to 0.2% in SSF. In contrast, no other genus systematically increased after all sand filtration steps. The most notorious changes were the increase in the relative abundance of *Pseudomonas* (3.5%) and *Acinetobacter* (0.9%) in RSF1 and *Streptomyces* (4.5%) in SSF. Overall, the water infiltration in dunes had the highest impact on the microbial community composition: (i) it substantially decreased the relative abundance of genera that were abundant in the influent, namely *Limnohabitans* (from 6.1 to 0.2%) and of "*Ca.* Planktophila" (from 12.8 to <0.1%); and (ii) increased the relative abundances of other genera like *Sphingophyxis* (from 0.1 to 12.1%) and *Sphingobium* (from 0.2 to 12.7%).

Unlike dune infiltration in the dune-based DWTP, the water storage step in the reservoir-based DWTP did not drastically modify the microbial community of the water. In this DWTP, the most significant change took place in the disinfection step, UV disinfection. The relative abundance of *Pseudomonas* increased from 7.6 to 60%, and *Sphingopyxis* raised from 4.0 to 7.5%. Concomitantly, the presence of the other genera decreased. In the following unit operation, GAC filtration, the relative abundance of *Pseudomonas* decreased to 22.1%, whilst

that of other genera such as *Massilia* (3.7%), *Polaromonas* (1.3%) and *Flavobacterium* (2.4%) increased.

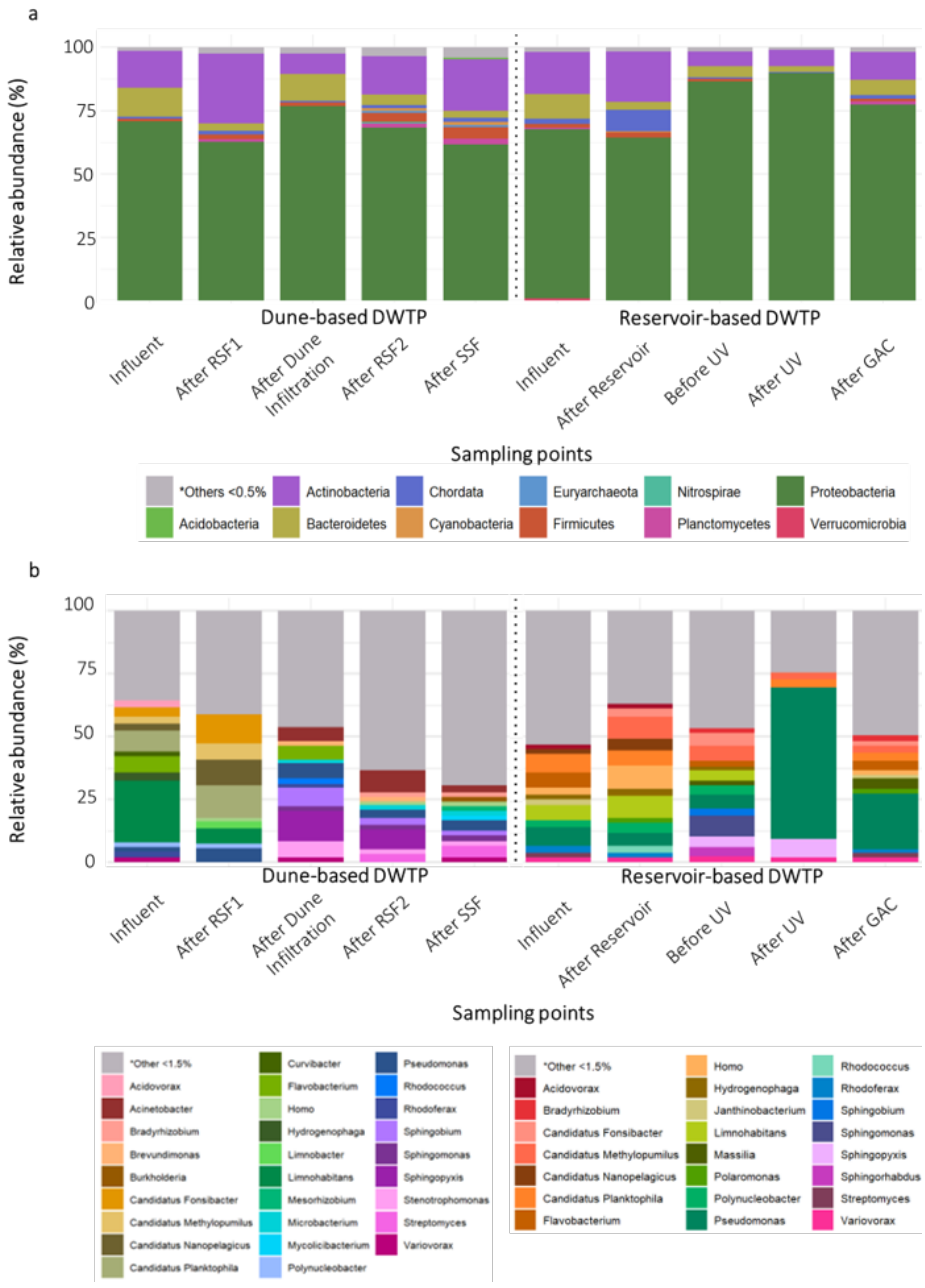


Figure 4. Microbial community composition at phylum (a) and genus (b) level of dune-based and reservoir-based DWTPs, as measured by metagenomics. The relative abundance of classified genera (Y-axis) is represented in the different sampling points (X-axis). Genera with less than 1.5% abundance in all samples were grouped as others.

3.2. Pathogenic bacteria decreased across both chlorine-free drinking water treatment plants.

HT-qPCR was used to detect the presence of *Acinetobacter baumannii, Pseudomonas aeruginosa,* and Enterobacteriaceae*,* the three most critical antibiotic-resistant pathogenic bacteria as designated by the World Health Organization (Tacconelli and Magrini, 2017). Overall, the absolute abundance of the pathogenic bacteria detected by qPCR was low (< 10^6 gene copies mL^{-1}) and further reduced along the two DWTPs. *Acinetobacter baumanii, Pseudomonas aeruginosa*, and *Enterococci* were detected (>10^2 genes copies mL^{-1}), while *Klebsiella pneumoniae*, *Campylobacter*, and *Staphylococci* were not. *A. baumanii* was detected across both plants. *P. aeruginosa* was recalcitrant across the treatment train of the dune-based DWTP but was not detected after UV disinfection in the reservoir-based DWTP. Interestingly, *Enterococci* was found only after RSF1 in the dune-based DWTP.

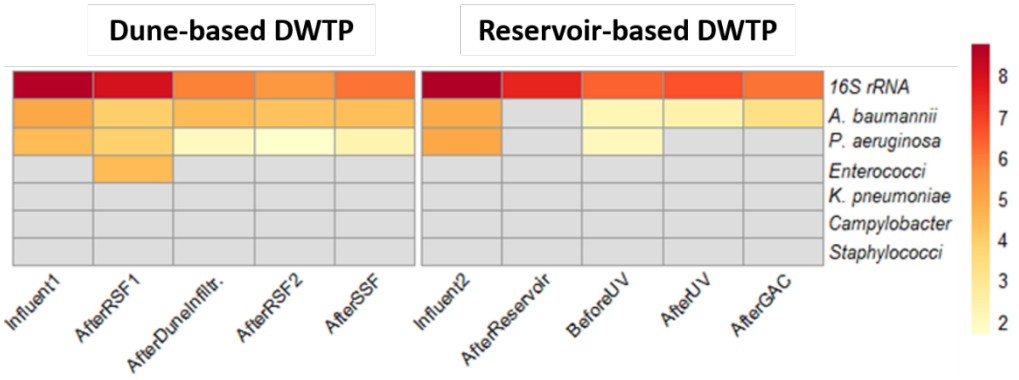


Figure 5. Heat map of absolute gene abundances (number of gene copies mL^{-1}) of 6 pathogenic microorganisms in dune-based and reservoir-based DWTPs, displayed in logarithmic scale. Y-axis represents the abundance of the pathogens in the different sampling points of both DWTPs. In X-axis, the 16S rRNA absolute abundance is provided. RSF: rapid sand filtration; SSF: slow sand filtration; GAC: granular activated carbon filtration.

3.3. Gram-negative bacteria as potential carriers of ARGs in DWTPs

386 The resistance determinants from the two DWTPs exhibited a large diversity of ARGs,
387 highlighted by both qualitative (metagenomics) and quantitative (HT-qPCR) analyses.
388
389 The resistome richness ranged from 3 to 20 different ARGs detected per sample. In total, 34
390 different ARGs were detected in the water of the dune-based DWTP and 58 in the water of
391 the reservoir-based DWTP (Figure 6). The most abundant ARGs related to resistance against
392 macrolides (MLSB, 57 different ARGs), followed by beta-lactams (13), aminoglycosides (10),
393 quinolones (7), sulfonamide (2), tetracycline (2), and trimethoprim (1). Some ARGs were
394 recalcitrant across the DWTPs: notably, the *msr(D)_2_AF27302* gene conferring macrolide
395 resistance remained in the treated water of the reservoir-based DWTP. In the dune-based
396 DWTP, the dune infiltration step was most prominently increasing the diversity of ARGs, likely
397 due to the drastic shift in microbial community (Figure 4). In the reservoir-based DWTP, the
398 rapid sand filtration (before the UV step) and the GAC filtration introduced the highest
399 variability in the resistome profile.
400
401 We linked ARG contigs to potential microbial origins by assigning taxonomies to contigs
402 carrying ARGs. The results of this analysis at genus level are given in Figure S3. Generally,
403 contigs containing ARGs mainly affiliated with *Limnohabitants* in the influent water samples
404 of both DWTPs. Other genera included *Paracoccus* and "*Ca.* Fonsibacter" in reservoir-based
405 DWTP and *Polynucleobacter, Acidovorax, Hydrogenophaga,* and "*Ca.* Fonsibacter" in dunes-
406 based DWTP. Most of these populations but "*Ca.* Fonsibacter" decreased across the
407 treatment train in reservoir-based DWTP, while "*Ca.* Fonsibacter" and *Limnohabitans*
408 persisted within the dune-based DWTP.
409

In the reservoir-based DWTP, the last GAC filtration step mostly increased the number of hosts carrying ARGs. This promoted the release of bacteria potentially carrying ARGs, such as *Pseudomonas, Kaistella, Microbacterium, Cellulosimicrobium, Caulobacter, Methylobacterium, Rhodoplanes, Messorzhibium,* and *Rhodoferax*, among others. In the dune-based DWTP, the dune infiltration step introduced the potential hosts carrying ARGs in the drinking water treatment train. *Acinetobacter, Rhodoferax,* and *Pseudomonas* were the microbial genera that persisted throughout the process after infiltration in the dune.

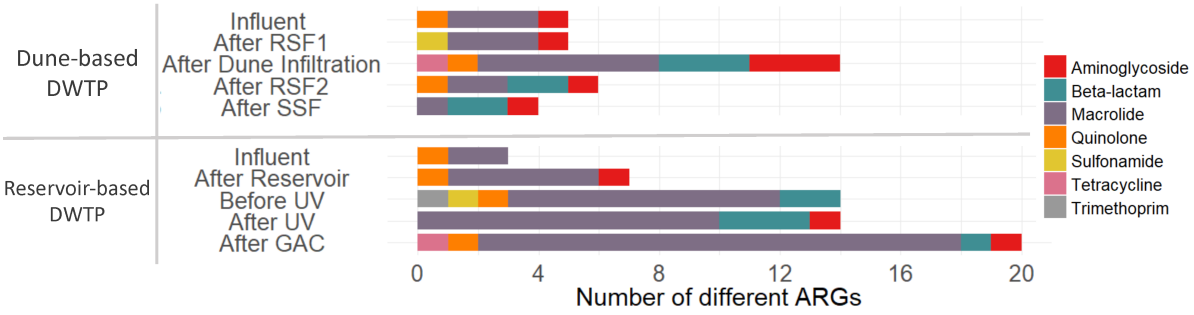


Figure 6. Resistome profile of dune-based and reservoir-based DWTP microbiome sorted by antibiotic class. The number of the different ARGs sorted per antibiotic class is represented in the different sampling points. The dotted line discriminates the results coming from each of the analyzed DWTPs.

3.4. Chlorine-free DWTPs achieve 2-3 logs removal of ARGs and MGEs

HT-qPCR was used to assess the ARG and MGE removal efficiencies from both DWTPs by quantifying the number of gene copies per volume of filtered water in each sampling point. The absolute concentration of ARGs decreased along the treatment trains down to 2.2 log gene copies mL^{-1} (dune-based DWTP) and 2.6 log gene copies mL^{-1} (reservoir-based DWTP) (Figure 7a). MGEs decreased by 2.7 log gene copies mL^{-1} (dune-based DWTP) and 2.6 log gene copies mL^{-1} (reservoir-based DWTP) (Figure 7b). Similarly, the bacterial proxy *16S rRNA* gene decreased by 2.5 (dune-based DWTP) and 2.6 (reservoir-based DWTP) log gene copies mL^{-1}.

The influent water samples from both DWTPs contained similar ARG concentration: 6.4 ± 0.9 (dune-based DWTP) and 6.8 ± 0.9 (reservoir-based DWTP) log ARG copies mL^{-1}. Across the dune-based DWTP (Figure 7a), the concentration of ARGs evolved from 6.0 ± 0.9 (after the first rapid sand filtration) to 3.9 ± 0.9 (after dune infiltration), 3.5 ± 0.9 (after the second rapid sand filtration), and 4.2 ± 0.9 (after last slow sand filtration) log ARG copies mL^{-1}. In the reservoir-based DWTP, the ARG concentration evolved from 5.7 ± 0.7 (after 3 months in reservoir) to 4.5 ± 0.9 (before UV treatment), 4.8 ± 0.8 (after UV treatment), and 4.2 ± 0.9 (after GAC filtration) log ARG copies mL^{-1} .

The MGEs concentration in dune-based DWTP was 7.1 ± 1.2 log MGE copies mL^{-1} in the influent, 6.2 ± 1.1 log MGE copies mL^{-1} after RSF1 step, 4.2 ± 1.1 log MGE copies mL^{-1} after the dune infiltration, 3.7 ± 1.1 log MGE copies mL^{-1} after the RSF2 and 4.5 ± 1.1 log MGE copies mL^{-1} after the last SSF step. In the reservoir-based DWTP case, the MGE concentration in the influent was 7.1 ± 1.1 log MGE copies mL^{-1}, followed by 6.0 ± 0.9 log MGE copies mL^{-1} after the 3 months' time in the reservoir, 4.8 ± 1.2 log ARG copies mL^{-1} before UV treatment, 4.9 ± 1.1 log MGE copies mL^{-1} after UV treatment and 4.4 ± 1.2 log ARG copies mL^{-1} after GAC filtration (Figure 7b).

Some process stages increased the concentration of ARGs and MGEs in water, such as the slow sand filtration in the dune-based DWTP (22% in ARGs and 20% in MGEs) and the UV treatment in the reservoir-based DWTP (7% in ARGs and 2% in MGEs). However, the decrease in the concentration of ARGs and MGEs was progressive across both DWTPs. Detailed

454 information on the ARGs and MGEs reduction throughout the processes per sampling point
455 is given in Table S2.
456
457 Several ARGs persisted across both DWTPs. The *aadA7* (aminoglycoside resistance; 6.0 ± 1.2
458 log gene copies mL^{-1}), *mexF* (multi-drug resistance; 5.6 ± 1.2) and *fox5* (beta-lactam
459 resistance; and 5.5 ± 1.3 log gene copies mL^{-1}) genes were the 3 most abundant ARGs in both
460 DWTPs (Figure S4). Other ARGs were not present in the influent but appeared across the
461 DWTPs such as *blaTEM*, *blaPAO* and *vanWG*. From the 238 ARGs tested, 72 (*i.e.*, 30%) were
462 not detected in any sampling point.
463
464 Regarding MGEs, the integron genes were the most abundant in both DWTPs (5.8 ± 1.7 log
465 gene copies mL^{-1}): the *intI1_1* (integron), *repA* (plasmid), *intI3* (integron), and *Tn5403*
466 (transposon) genes were the most abundant (Figure S5). The conjugative plasmid sequences
467 such as *IncP_oriT* and *trbC and* promiscuous plasmid *IncQ_oriT* gene sequences were also
468 abundant.
469
470 Despite the reduction in ARGs and MGEs concentration in water, the ratios of ARGs and MGEs
471 to the *16S rRNA* gene remained stable throughout both DWTPs (Figure 7c). This indicates that
472 the DWTP process did not enrich for bacterial populations carrying ARGs or MGEs.

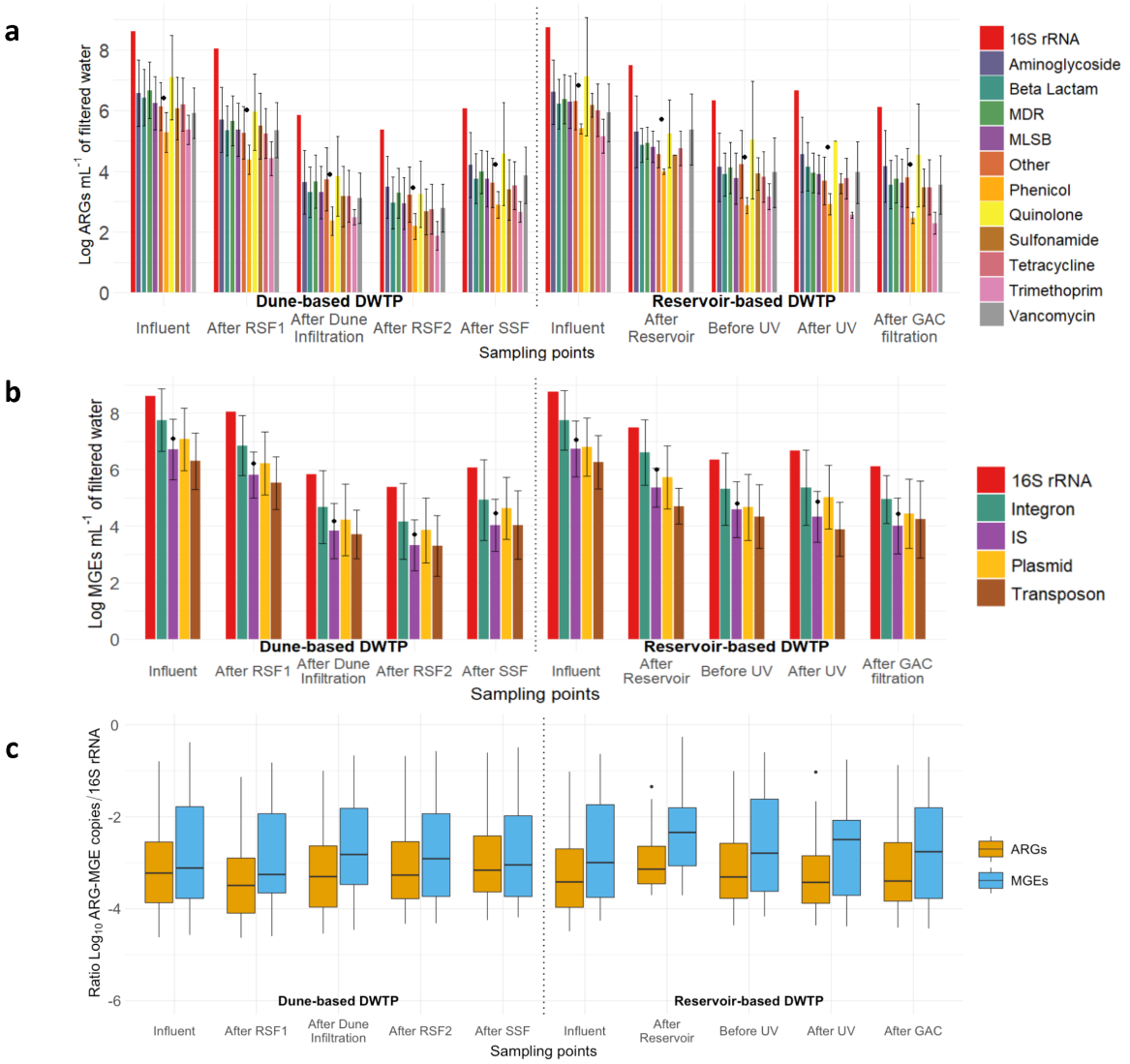


Figure 7. (a) Absolute abundance of difference ARGs mL^{-1} (sorted by antibiotic class) from both DWTPs. Values are represented on a logarithmic scale. Black dots indicate the average ARGs abundance per sampling point (b) Absolute abundance of difference MGEs mL^{-1} (sorted by antibiotic class) from both DWTPs. Values are represented on a logarithmic scale. Black dots indicate the average ARGs abundance per sampling point. (c) The ratio of ARGs or MGEs */16S rRNA* in both DWTPs. Each boxplot represents (from top to bottom) maximum, upper quartile, median, lower quartile, and minimum values. Note: RSF: rapid sand filtration; SSF: slow sand filtration; GAC: granular activated carbon.

3.5. ARGs and MGEs co-localized on contigs of DWTP metagenomes

When ARGs and MGEs co-localize on the same genetic fragment, there is an increased chance that the fragment can be transferred between bacterial cells. Since facilitating the transfer, conjugation, integration, and transposition of genes in genomes, MGE can pose a risk for the dissemination of ARGs. Sets of 7 (dune-based DWTP) and 12 (reservoir-based DWTP) events of co-localization of ARGs and MGEs were detected on contigs retrieved from the sequenced metagenomes. A detailed description of the co-localization events is provided in Table S3. Co-localizations were detected in all sampling points from both DWTPs, except for the influent of the reservoir-based DWTP. The ARGs involved coded for mainly aminoglycosides and beta-lactam resistance.

For instance, the *ant(3'')-Ia* gene is an aminoglycoside resistant gene broadly described in *Klebsiella pneumoniae*. This persistent ARG was annotated from the metagenome of the dune-based DWTP influent and in the outlets of the RSF1, reservoir, UV, and GAC units. This ARG was embedded in plasmids, integrons, and bacterial integrative conjugative elements (ICEs), affiliating with *Polynucleobacter* and *Pseudomonas* genera. The *blaVIM* ARG against last-resource beta-lactamases (carbapenem antibiotics) was embedded in a plasmid, between insertion sequences, and in conjugative elements in different stages of the process: in the reservoir-based DWTP, it taxonomically affiliated with *Sphingobium* and *Sphingomonas* before UV, and with *Pseudomonas* after UV and GAC.

Other ARGs only appeared once. The *sul2* and *blaOXA-287* genes appeared after RSF1 and dune infiltration, respectively, both affiliating with *Acinetobacter*. The *sul2* gene was carried by plasmid, integrons, and bacterial integrative and conjugative elements. A plasmid carried the blaOXA-287 gene. The *sul1* gene appeared before UV, linked to *Sphingobium*. The *blaTEM-*

507 *181* gene after UV linked to *Bacillus*. Both *sul1* and *blaTEM-181* genes were potentially carried
508 by plasmids, integrons, and bacterial integrative and conjugative elements. The *srm(B)* gene
509 after GAC was already assigned in *Rhodoferax*. The *mef(C)* and *mph(G)* genes appeared after
510 GAC as well, with *Kaistella* as the potential host. These 2 ARGs were detected on the same
511 contig (NODE_16870), which implied the possible existence of a plasmid co-containing
512 multiple resistance genes.

4. Discussion

4.1. Operational units shape the structure of the drinking water microbiome

In order to study the impact of each unit operation on the dynamics of the water microbiome, we applied shotgun metagenomics on water samples collected along the treatment train of two chlorine-free DWTPs, namely a dune-based and a reservoir-based plant. The alpha diversities of both effluents, calculated as H' Shannon diversity indices, were varied from 4.9 to 7.7 (average 6.3 ± 0.8). These values are comparable to other chlorine-free DWTPs (4.37 ± 0.1 – 6.02 ± 0.4; (Palomo et al., 2016), and significantly higher than the ones in plants with chemical disinfection (*ca*. 2 – 4) (Tiwari et al., 2021; Waak et al., 2019)(Dai et al., 2019). Noticeably, the alpha diversity increased after every biological filter (RSF1, RSF2, SSF, and GAC filtration), a likely consequence of direct seeding from biofilm detachment (Velten et al., 2011) (Lautenschlager et al., 2014).

Proteobacteria, *Actinobacteria,* and *Bacteroidetes* were the most abundant phyla in both DWTPs. This matched with previous observations (Lin et al., 2014; Oh et al., 2017; Su et al., 2018), and with the presence of *Actinobacteria* and *Bacteroidetes* in freshwater ecosystems (Neuenschwander et al., 2018; Warnecke et al., 2004). Interestingly, the microbial community after the first operational unit of both DWTPs, namely RSF in dune-based DWTP and reservoir in reservoir-based DWTP, was similar to the influent. However, the similarity decreased in the downstream stages of the DWTPs (Figure 3B). This aligned with the observations of Pinto et al. (2012), who highlighted that even though the source water seeds the drinking water microbiome, the unit operations shape the structure of the effluent microbial community.

536 The conditions within biological sand filters have different impacts on microorganisms fitness
537 (Hu et al., 2020), yet Webster & Fierer (2019) postulated that changes in community
538 composition before and after lab-scale biological sand filters are largely predictable. In this
539 line, we found higher abundances of *Actinobacteria* the RSF and SSF effluents as compared to
540 their influents in the dune-based DWTP (Figure 4a), in analogy to the high *Actinobacteria*
541 abundance in bench-scale sand filters (Xu et al.(2020)). In contrast, the relative abundance of
542 *Bacteroidetes* decreased after every biological sand filtration unit, similar to earlier reports
543 (Mukherjee et al., 2016; Pfannes et al., 2015). Another example are the common freshwater
544 bacteria *Limnohabitans*, "*Ca*. Planktophila", "*Ca.* Nanopelagicus" and *Rhodoluna* (Hahn, 2016;
545 Kasalický et al., 2013; Neuenschwander et al., 2018), abundant in the water influent but
546 almost absent in the DWTPs effluents. Overall, our findings showcase common patterns in
547 the effect of biological operational units unit on the water microbiome dynamics of full-scale
548 DWTP, paving the way to predict and modulate the microbial community in the drinking water
549 effluent.
550
551 **4.2. Chlorine-free DWTPs remove antibiotic resistance determinants**
552 To the best of our knowledge, this is the first study in which the fate of ARG and ARB is
553 monitored throughout the treatment train of chlorine-free DWTPs. Both DWTPs effectively
554 reduced the concentration of ARGs by *ca.* 2.5 log gene copies mL^{-1}. These removals are
555 comparable to the highest reported in chlorine-amended DWTPs, between <0.1 log ARG
556 copies mL^{-1} (Su et al., 2018; Zhang et al., 2016) and 2.4 log ARG copies mL^{-1} (Hu et al., 2019).
557 Moreover, the total ARG concentration in the water effluent of both chlorine-free DWTPs was
558 *ca.* 4 log ARG copies mL^{-1}, similar to what Hu et al. (2019) found in a chlorine-amended
559 DWTPs. Overall, both chlorine-free DWTP proved at least as effective as chlorine-amended

DWTP at reducing ARGs. Additionally, the decrase in ARGs and MGEs concentration was linearly correlated with that of *16S rRNA* (Figure 7c, Figure S6), proving that none of the biological unit operations in chlorine-free DWTPs selected for ARB, *i.e.* the ARB/16S ratio did not increase.

The water storage steps yielded the highest ARG and ARB removal in both plants (2.1 and 1.6 ARG copies mL^{-1} in dune- and reservoir-based DWTPs, respectively), likely due to biomass decay and plasmid degradation due to the high hydraulic retention times and low nutrient availability in these systems (Amarasiri et al., 2020; Griffiths et al., 1990; Zhang et al., 2021). Likewise, RSFs reduced the ARG and ARB concentration by decreasing biomass concentration, as previously reported (Hu et al., 2019; Su et al., 2018; Zhang et al., 2016). Unexpectedly, GAC filtration also decreased the concentration of ARGs (Figure 7a), in contrast to previous studies describing GAC filtration as the critical step where resistance determinants increase (Su et al., 2018; Wan et al., 2021; Zhang et al., 2019). However, the decrease in ARGs concentration in this study contrasted with the increase in ARG richness (Figure 6), which suggests that the microbiome in the GAC effluent is seeded by the GAC biofilm.

The final treatment step before discharge to the environment is disinfection, which is intended to suppress or inactivate harmful microorganisms and prevent the regrowth of opportunistic bacteria (National Research Council Safe Drinking Water Committee, 1980). However, the SSF (0.75 log gene copies mL^{-1}) and UV treatments (0.32 log gene copies mL^{-1}) in this study increased the concentration ARGs (Figure 7a-b). The fate of ARG in SSF has not been directly studied before. However, Xu et al. (2020) showed that SSF hardly decreases the concentration of antibiotics in water, and Ciric (2022) reported that while their SSF removed

584 most of the microorganisms, those in the effluent were more prone to resistance to
585 antibiotics. In the case of UV treatment, previous studies proved its efficacy for cell reduction
586 (plate counting) but not for ARGs removal (Chen and Zhang, 2013; Stange et al., 2019). Gram-
587 negative bacteria, and specifically *Pseudomonas*, tolerate UV by efficient repair mechanisms,
588 high growth rates, or the use of low-molecular-weight organic carbon (generated by UV
589 illumination) as an energy source (Chen et al., 2020). This can explain the drastic rise in
590 relative abundance of *Pseudomonas*, a common multi-drug resistant bacteria in drinking
591 water systems (Su et al., 2018), and the quantitative increase in the *16S rRNA* gene marker
592 after UV disinfection (Figure 7a). Nevertheless, despite the intermediate increase of ARGs,
593 MGEs, and pathogenic bacteria after disinfection, DWTPs successfully reduced their effluent
594 concentration (Figure 5, Figure 7, Figure S7).
595
596 **4.3. Clinical implications of gene transfer in chlorine-free DWTPs**
597 In concert with wastewater treatment plants (WWTPs), DWTPs are the ultimate barriers
598 preventing the spread of waterborne diseases, and the release of antibiotics, ARB, and ARGs
599 into water systems (Collignon and McEwen, 2019; Miłobedzka et al., 2022). One crucial aspect
600 is the presence of ARGs against last-resort antibiotics such as carbapenems or colistin.
601 Carbapenem resistance genes like *blaIMP or blaVIM* (class B beta-lactamases resistant genes)
602 have been described in pathogenic bacteria such as *Pseudomonas*, *Acinetobacter,* or
603 *Enterobacteriaceae* (Nordmann et al., 2012; Shanthi Amudhan et al., 2012). Carbapenem is a
604 beta-lactam antibiotic with a broad antimicrobial spectrum and administered as a last resort
605 for treating drug-resistant bacterial infections. However, the number of carbapenem-
606 resistant bacteria has steadily increased (WHO, 2017), and represents a primary concern in
607 drinking water. *blaIMP* genes were rarely detected along both DWTPs. However, *blaVIM* was

608 detected along the dune-based DWTP and in the effluent (after GAC treatment) of the
609 reservoir-based DWTP (Figure S4). The taxonomic annotation of the contigs containing
610 *blaVIM* genes revealed their potential co-localization with multiple plasmids affiliating with
611 *Sphingobium* in the dune-based DWTP, and with *Sphingobium* (plant pathogen) and
612 *Pseudomonas* in the reservoir-based DWTP after GAC filtration. The carbapenem-resistant
613 *Pseudomonas* is accounted by WHO within the list of critical priority pathogens for which new
614 antibiotics are required (Tacconelli and Magrini, 2017). Colistin resistance genes, such as *mcr1*
615 variants, were also highly abundant in both the dune-based and the reservoir-based DWTPs.
616 However, this is not unique to chlorine-free DWTPs as multiple last-resort ARGs have also
617 been identified in conventional DWTP (with chlorine use) as well as in tap water (Dias et al.,
618 2020). Importantly, the *mcr1* gene load decreased significantly along the water treatment
619 trains and neither co-localized with any MGE nor affiliated with any pathogenic bacteria.
620 Further research should underpin the regrowth capacity of such pathogens in chlorine-free
621 DWTP effluents.

5. Conclusions

In this work, we characterized for the very first time the abundance and dynamics of microbial communities, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs) across the treatment trains of two chlorine-free drinking water treatment plants. The in depth analysis of the metagenomes and resistomes led to the following main conclusions:

1. Chlorine-free DWTPs do not select for antibiotic resistant bacteria, as supported by the linear correlated between ARGs and MGEs, and the *16S rRNA* concentrations.
2. The measured reduction in ARGs concentration by *ca.* 2.5 log gene copies mL^{-1} in both chlorine-free DWTPs is comparable to the highest removals reported so far for chlorine-amended DWTPs.
3. Water storage systems alone reduced the abundance of the *16S rRNA* gene, ARGs, and MGEs by *ca*. 1.6 log gene copies mL^{-1}, and dune infiltration achieved the highest removal.
4. Despite a *ca*. 2.5 log *16S rRNA* gene copies mL^{-1} reduction, the effluent microbial diversity increased likely due to the seeding from the biofilms actively growing in the rapid and slow sand filters, and the granular activated carbon ones.
5. Despite the overall ARG decrease in the DWTP, disinfection (slow sand filtration and UV radiation) internally increased the concentration of ARGs, MGEs, and 16S rRNA genes by *ca*. 0.5 log gene copies mL^{-1}, yet with no impact on overall reduction.

Overall, our findings confirm the effectiveness of chlorine-free DWTPs in providing safe drinking water and reducing the load of antibiotic resistance determinants, offering the Water

645 Authorities the possibility to establish centralized risk management around these specific

646 treatment steps.

6. Data availability

Metagenome sequencing data were deposited in the NCBI database with the BioProject ID: PRJNA886863.

7. Authors' contributions

DCF and FCR designed the study with inputs of MvL, DvH, ML, and DGW. Fieldwork was supported by BP and DdR. DCF, FCR, and MCS performed the experimental investigations. DCF and FCR wrote the manuscript with direct contribution, edits, and critical feedback by all authors. DCF and FCR are first co-authors of this manuscript.

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