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Research Paper

Anthropogenic pollution may enhance natural transformation in water, favouring the spread of antibiotic resistance genes

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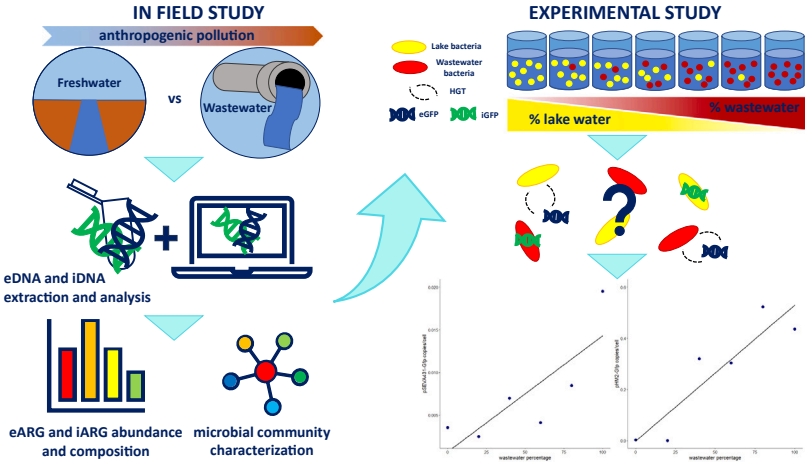
HIGHLIGHTS

- iDNA exhibits a higher antimicrobial resistome richness than eDNA.
- Anthropogenic pollution influences the microbial community and associated resistome.
- Anthropogenic pollution positively influences the uptake of eDNA plasmids.

GRAPHICAL ABSTRACT



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ABSTRACT

Aquatic ecosystems are crucial in the antimicrobial resistance cycle. While intracellular DNA has been extensively studied to understand human activity's impact on antimicrobial resistance gene (ARG) dissemination, extracellular DNA is frequently overlooked. This study examines the effect of anthropogenic water pollution on microbial community diversity, the resistome, and ARG dissemination. We analyzed intracellular and extracellular DNA from wastewater treatment plant effluents and lake surface water by shotgun sequencing. We also conducted experiments to evaluate anthropogenic pollution's effect on transforming extracellular DNA (using Gfp-plasmids carrying ARGs) within a natural microbial community. Chemical analysis showed treated

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wastewater had higher anthropogenic pollution-related parameters than lake water. The richness of microbial community, antimicrobial resistome, and high-risk ARGs was greater in treated wastewaters than in lake waters both for intracellular and extracellular DNA. Except for the high-risk ARGs, richness was significantly higher in intracellular than in extracellular DNA. Several ARGs were associated with mobile genetic elements and located on plasmids. Furthermore, Gfp-plasmid transformation within a natural microbial community was enhanced by anthropogenic pollution levels. Our findings underscore anthropogenic pollution's pivotal role in shaping microbial communities and their antimicrobial resistome. Additionally, it may facilitate ARG dissemination through extracellular DNA plasmid uptake.

1. Introduction

The environment plays a central role in the local as well as in the global cycle of antimicrobial resistance (AMR). Specifically, aquatic ecosystems are among the most important sites for the selection and spread of AMR [1]. Wastewater treatment plants (WWTPs) are considered to be hot-spots for the concentration, selection, and release into the environment of antimicrobial resistant bacteria and genes (ARB, ARGs) of anthropogenic origin. Those sum up to other ARGs of natural origins, commonly present in the water and soil microbiomes [2,3]. Consequently, the occurrence of ARGs is common and widespread, extending from highly anthropogenically influenced environments to less impacted ones [4]. The presence and persistence of ARB as well as the horizontal transfer of ARGs in aquatic ecosystems are influenced by a multitude of factors, with anthropogenic pollution potentially playing a leading role.

Several studies investigated the impact of anthropogenic pollution on composition and abundance of the antimicrobial resistome (i.e., total content of ARGs) within aquatic microbial communities by shotgun sequencing [4–7]. However, there is still very limited knowledge regarding the simultaneous analysis of intracellular DNA (iDNA) and extracellular DNA (eDNA) in the same environment. eDNA is ubiquitous in nature [8] and is primarily a product of cell death and lysis, often occurring naturally during predation or through secretion from live cells, but also during wastewater disinfection [9]. Certainly, eDNA has been well-documented as a carrier of ARGs, thereby potentially contributing to the AMR spread within aquatic ecosystems [10–12]. Additionally, eDNA was found as mainly composed by mobile genetic elements (MGEs) [13] suggesting its involvement in different horizontal gene transfer (HGT) mechanisms.

HGT can occur by conjugation, transformation, transduction, and "non-canonical" processes, the latter involving pseudo-phages, vesicles and tiny pilus-like structures [14], serving as the primary route for the transmission of ARGs among bacteria [15]. Various MGEs, such as plasmids, could also contribute to the persistence of ARGs within microbial communities in waters [16]. The role of anthropogenic pollution as selection factor facilitating the spread of ARGs through eDNA transformation is yet to be elucidated.

Recognizing the critical public health concern posed by the dissemination of AMR, the present study is mainly focused to investigating the role of anthropogenic pollution in the spread of ARGs with a focus on MGEs. The study is structured in two distinct parts. The first one focuses on field activities involving the analysis of waters from systems that are characterized by different levels of anthropogenic pollution: WWTP effluents and lake surface water (LW). These samples were processed to extract iDNA and eDNA and to characterize the microbial community (also in case of the eDNA the taxonomic composition was called microbial community to simplify the representation of the comparative results), the antimicrobial resistome, and the high-risk ARGs (rank I and II ARGs [17]) by shotgun sequencing aiming at identifying any variations attributable to anthropogenic pollution. Furthermore, the potential mobility of the AMR was investigated by exploring the genetic context of ARGs, annotating MGEs. In the second part of the study, a series of experiments was designed to replicate a gradient of anthropogenic pollution. This was achieved by mixing pre-disinfected wastewater (NTWW) and LW in different proportions, and then spiking different Gfp-plasmids carrying ARGs under antibiotic selective pressure. The aim was to assess the influence of anthropogenic pollution on the rate of transformation and selection of the tested eDNA.

2. Materials and methods

2.1. Study sites and sampling activities

To evaluate the role of anthropogenic pollution (considering both the chemical and microbiological components) in the shaping of antimicrobial resistome, we selected three lakes and three WWTPs for sampling. The three lakes were:

- Lake Maggiore (Lake Maggiore, WGS84: 45° 55′ 19.6″ N, 8° 32′ 53.9″ E), a large subalpine lake, contributing, along with the other subalpine lakes, to Europe's primary freshwater resources and currently in an oligo-mesotrophic state [18];
- Lake Orta (45° 52′ 24.9″ N, 8° 24′ 28.4″ E), another subalpine lake, with a history of relevant industrial pollution but successfully restored, currently in an oligotrophic state [19];
- Lake Mergozzo (45° 57′ 39.6″ N, 8° 27′ 09.9″ E), a smaller subalpine lake, subject to low anthropogenic impact due to limited anthropogenic activities in its watershed, and currently in an oligotrophic state (summarized in [20]).

Furthermore, to assess the potential impact of environmental changes on the antimicrobial resistome, Lake Maggiore was sampled four times over the course of one year.

The three WWTPs used for this study were: the WWTP of Verbania (45° 56' 10.0'' N, 8° 33' 34.2'' E) serving a Population Equivalent (PE) of 51,000, equipped with chlorination as disinfection process, the WWTP of Cannobio (46° 4' 29.7'' N, 8° 41' 33.1'' E), which serves a PE of 15,000, equipped with peracetic acid treatment (PAA) as final disinfection, and the WWTP of Gravellona Toce (45° 56' 10.2'' N, 8° 25' 56.8'' E) serving a PE of 18,000, which also employs PAA as disinfection. From the three lakes, surface water was collected (5 L) in sterile glass bottles through March 2022 (on 3rd, 14th and 24th for Lake Mergozzo, Lake Orta and Lake Maggiore respectively) and only for Lake Maggiore other three samples were collected on 6th of June, 13th of September and 30th of November 2022. Post-disinfected wastewater (TWW) was collected in sterile glass bottles (5 L) as three-hour integrated samples on May 3, 2022, at the WWTPs of Verbania and Gravellona Toce, and on May 10, 2022, at the WWTP of Cannobio. All the samples were collected under conditions of no rain during the sampling and for at least 48 h before the sampling and temperature was measured. Subsequently, samples were stored in a cold room at 5 °C, in the darkness, until processing.

2.2. Chemical analyses of water samples

An aliquot from each sample was processed to measure the following chemical parameters: pH (potentiometry); Ammonium (N-NH_4), Reactive Phosphorus (RP), Total Phosphorus (TP), Total Nitrogen (TN), Reactive Silica (Si) (UV–VIS spectrophotometry); Total Organic Carbon

(TOC) (High Temperature Catalytic Oxidation); Chloride (Cl), Sulfate (SO_4), Nitrate (NO_3) (ion chromatography); a range of trace metals including Aluminum (Al), Arsenic (As), Boron (B), Barium (Ba), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Lithium (Li), Manganese (Mn), Nickel (Ni), Lead (Pb), Platinum (Pt), Selenium (Se), Strontium (Sr), Thallium (Tl), Vanadium (V), and Zinc (Zn) (ICP-OES with ultrasonic nebulizer). All the analyses were performed with standard methods for freshwater samples [21], and the laboratory followed established quality assurance and quality control (QA/QC) procedures.

2.3. Sample processing and DNA extraction

Each water sample was prefiltered through 50 μm mesh-size net to remove large particles. For the iDNA extraction an aliquot of prefiltered water (from 0.3 to 0.8 L) was filtered on 0.22 μm polycarbonate filters (Merck Millipore, Germany) and stored at −20 °C until molecular processing. iDNA was extracted using the commercial kit DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. For the eDNA extraction an aliquot of prefiltered water (from 0.5 to 1.5 L) was first pre-treated with NaH_2PO_4 (0.12 M, pH 8.0) and polyvinyl polypyrrolidone (PVPP) and shaken at 250 rpm, at room temperature for 10 min [22]. Then, it was centrifuged at 4 °C, at 7000 g for 10 min and the supernatant was collected. Another 4 mL of NaH_2PO_4 was added to the remaining pellet, and the procedure was repeated twice. The supernatant was, then, filtered through a sterile 0.22 μm polycarbonate filters (Merck Millipore, Germany). Thus, the filtrate was used to extract DNA using cetyltrimethylammonium bromide (CTAB) as previously described [23,24]. Both fractions of DNA were analysed to measure the concentration using the Qubit dsDNA Assay kit (Thermo Fisher Scientific, Waltham, USA). They ranged between 11 and 100 μg mL^{-1} for iDNA and from 2 to 33 μg mL^{-1} for eDNA. The DNA samples were stored at – 20 °C until sequencing. In parallel, negative controls (n = 3) (using sterilized MilliQ water), were processed and, since both iDNA and eDNA resulted not quantifiable, they were not sequenced.

2.4. Shotgun sequencing and bioinformatic analyses

DNA samples were sent to an external service (IGA Technology Services, Udine, Italy) for metagenomic shotgun sequencing. Celero™ DNA-Seq Library Preparation Kit (Tecan, Switzerland) was used for library preparation following the manufacturer's instructions. The NovaSeq 6000 (Illumina, USA) platform was used, with the S1, 2 × 150 bp paired-end kit (Illumina). Read quality were assessed using FastQC (version 0.11.9; [25] and MultiQC (version 1.12) [26]. The assessment considered the overall quality of the sequenced reads, their average length, the duplicates, and an overview of the GC content present in each sample. The quality scores and the other metrics were used to guide the required parameterization of the metagenomic profiling procedure. Trimmomatic (version 0.39) [27] was used to remove Illumina adapters (options: leading:3 trailing:3 slidingwindow:4:15 minlen:36). Merging of paired reads was done with vsearch (version 2.17.1) [28] with default parameters. Samples contained on average 29.5 M ± 6.2 M reads. On average, more than 96.3 % of raw reads passed the quality filtering step and of those, an average of 42.8 % successfully merged. Raw reads were publicly.

available in NCBI under the accession number PRJNA1066593. https://dataview.ncbi.nlm.nih.gov/object/PRJNA1066593?reviewer=phm985t5shqj5ihh1ao1o7lgj2 Read quality filtering and merging are summarized in Supplementary Table 1.

Taxonomic and resistome profiling is detailing described in the Supplementary Text.

Assembly of Metagenome-assembled genomes (MAGs): To retrieve MAGs, raw sequence reads were trimmed of low-quality bases and adapter sequences with TrimGalore! version 0.4.4 (https://github.com/FelixKrueger/TrimGalore), which is a wrapper for Cutadapt [29] and FastQC. Overlapping reads were merged with FLASH version 1.2.11 [30] using a maximum allowed overlap of 150 bp. Processed reads were assembled using spades version 3.15.5 to produce eighteen assemblies, using the 'meta' preset for large and complex communities and a minimum contig length of 1000 bp. MAGs were recovered using a previously described method [31]. Summarizing, the processed reads were mapped to the co-assembled metagenomic contigs using BWA-MEM algorithm version 0.7.15-r1142 with default parameters [32]. Genomes were recovered with MetaBAT version 2.12.1 using default MetaBat2 settings [33]. The resulting bins were refined using RefineM version 0.0.23 [31] with default parameters. Furthermore we identified and removed contaminating contigs from each MAG using Magpurify version 2.1.2 [34] with the modules: '*phylo-markers*', which finds taxonomically discordant contigs using archaeal and bacterial single-copy taxonomic marker genes from the PhyEco database; '*clade-markers*', which finds contaminating contigs using the MetaPhlAn2 database for clade-specific prokaryotic marker genes; '*tetra-freq*', which uses principal component analysis to identify contaminating contigs with outlier tetra-nucleotide frequency; '*known-contam*', which identifies contigs matching a database of known contaminants, such as the human genome; and '*gc-content*', which identifies contaminating contigs with outlier GC content. The resulting bins were finally checked using CheckM version 1.0.11 [35]. Bins with a quality score (defined by Parks et al., 2017) as the estimated completeness of a genome minus five times its estimated contamination) greater than 50 were considered 'high-quality' and they were counted and considered MAGs. However, given that this investigation was contig-focused, in the output table we reported the belonging of ARG-containing contigs to the bin independently of the quality of the bin and report the CheckM quality score output. The taxonomic assignment, the ARG and MGE content of the retrieved MAGs is detailed described in the Supplementary Text.

All genomic maps were built with Proksee (https://proksee.ca). The tools Alien Hunter and Phigaro available in Proksee were used to respectively highlight putative HGT regions of the contigs identified as "alien" therefore considered originated from HGT [36] and find putative prophagic regions in the sequences [37]; Phigaro tool also inferred the taxonomy of the prophagic regions based on the sequence match with the pVOGs (prokaryotic Virus Orthologous Groups) [38].

2.5. Experimental design

To assess the impact of the anthropogenic pollution (considering both the chemical and microbiological components) on the rate of transformation and selection of eDNA within microbial communities in water, two experiments utilizing distinct plasmids were conducted. Two broad host range plasmids were used, i.e., pHM2-Gfp and pSEVA431-Gfp. The first one, pHM2-Gfp (~10.0 kb), carries the *aph* kanamycin resistance gene and a *gfp* cassette [39]. Plasmid pSEVA431-Gfp (~4.3 kb), carrying the *aad*A streptomycin resistance gene, was constructed by cloning a *gfp* cassette in the restriction site *SpeI* of the plasmid pSEVA431 (http://seva-plasmids.com/find-your-plasmid/), as described in [39]. To create a gradient of anthropogenic pollution encompassing both chemical and microbiological pollutants, we mixed pre-filtered surface water from Lake Maggiore and NTWW of the WWTP of Gravellona Toce in varying proportions. In detail, batch cultures (final volume, 250 mL) formed a dilution gradient in six steps: 100 % NTWW, 80–20, 60–40, 40–60, 20–80 % and 100 % Lake Maggiore water. From each batch an aliquot of 2 mL was collected and fixed with formalin (1 % final concentration) to enumerate bacterial cells by flow cytometry (Cytoflex, Beckman Coulter, USA) following the protocols previously described by Horňák and Corno [40]. For each batch culture a known concentration of plasmid, 1×10^9 plasmid copy mL^{-1}, was added. To maintain the selective pressure, 10 mg L^{-1} of streptomycin (the double concentration proven to be selective for resistant bacteria in wastewater) [41] or 20 mg L^{-1} of kanamycin [11] were supplemented to the experimental waters, in case of GFP-pSEVA431 or GFP-pHM2 plasmid, respectively. To

prevent the degradation of the eDNA (plasmid), which is estimated to occur within 48 h [42], the experiments were run for approximately two days. At the end of the experiments, an aliquot of 2 mL of each sample was analysed to measure the bacterial cell numbers by flow cytometry and the remaining volume was filtered onto 0.22 μm polycarbonate filters (Merck Millipore, Germany) to extract iDNA using the commercial kit DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, and quantify the plasmid copy number by quantitative Real Time PCR (qPCR).

2.6. Intracellular plasmid quantification

The plasmid copy number was estimated by quantifying the copies of the *gfp* gene by qPCR using the RT thermocycler CFX Connect (Bio-Rad, Hercules, USA) and the following primer set: GFP_RT_f CACTGGAGTTGTCCCAATTC and GFP_RT_r GCCATGGAACAGGTAGTTT [43]. The qPCR assays were carried out in a volume of 20 μL containing 2 μL of DNA sample, 0.5 μM of each primer, 10 μL of SsoAdvanced universal SYBR Greensupermix (Bio-Rad, Hercules, USA), and filtered and autoclaved MilliQ water (Merck Millipore, Germany) up to the final volume. The qPCR program was as follow: 95 °C for 30 s, 35 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 10 s. Melt curve production and melting peak analysis were performed as previously described [44]. The specificity of the reaction was further tested by electrophoresis run. Standard calibration curves were prepared as described in Di Cesare et al. [45]. Each DNA sample was tested in three technical replicates. The limit of quantification (LOQ) of the reaction was assessed as previously described [46] and was 16.98 gene copy μL^{-1}. The mean value ± standard deviation of the efficiency and R^2 were 94.6 ± 0.7 and 0.996 ± 0.004, respectively.

2.7. Statistical analysis

The statistical analyses were made in the R environment v 4.1.2 [47] with the aim to assess how different levels of anthropogenic impact could influence the dynamics of the microbial community and antimicrobial resistome in both DNA fractions. For the field study, we analysed three datasets: the whole microbial community, the antimicrobial resistome, and a subset of ARGs, considered at the highest risk level (rank I and II) for human health, as proposed by Zhang et al. [17]. We calculated the richness (as number of different genera or ARGs) of each dataset sample, as an index of alfa diversity, and constructed a matrix of dissimilarities using the abundance-based Bray-Curtis dissimilarity index, as an estimator of sample composition (beta diversity). The matrix was also used to plot samples in a tree after hierarchical linkage analysis. First, we tested if the differences among samples were related to the type of water (wastewater vs. lake water, representing two different levels of anthropogenically impacted waters) and origin of DNA (iDNA vs. eDNA). We applied a linear mixed effect model (LMEM), given the non independent nature of the data, on richness (log--transformed), using the sampling site as random effect variable in the model, and a permutational multivariate ANOVA (PERMANOVA) on beta diversity, using the sampling sites as strata in the model. In this case, for Lake Maggiore, we considered only the samples collected on March to be consistent with the samples of the other lakes. Moreover, for high-risk ARGs, we calculated the total abundance for each sample and tested it, after an arcsine square root transformation of values (since they represent proportion data), by LMEM. To prove that the two types of water had a different temperature and chemical composition, a dissimilarity matrix among samples, based on Gower's distance index, was computed starting from the measured parameter values and, then, used to depict sample composition in a tree. As done for biological variables, the matrix was analysed by PERMANOVA, using the type of water as explanatory variable. Moreover, a linear model (LM) was applied to investigate which parameter differed between the two waters. A mantel test was used to correlate distances in terms of Beta-diversity (Bray-Curtis dissimilarity) to distances in terms of chemical composition (Gower's distances). Given the very low variability in terms of chemical parameters, whole microbial community and antimicrobial resistome for the temporal samples of Lake Maggiore, the sampling season was not investigated as a statistical factor. The richness of whole microbial community, antimicrobial resistome, and high-risk ARGs over time was correlated by Pearson's correlation analysis.

When analysing data from the experiments, a generalized linear model (GLM) was applied to evaluate the effect of anthropogenic pollution level (i.e., increasing percentage of NTWW) on the transformation and selection of the eDNA (i.e., Gfp-plasmids), under antibiotic pressure. In this case, the *gfp* copies per mL were related to the number of cells per mL, via *cbind* function with a binomial error structure of the GLM, to take in account the dissimilarity in cell abundances among samples, and used as response variable in the model; the percentage of NTWW was used as explanatory variable.

3. Results

3.1. Field study

3.1.1. Chemical parameters

Data of water temperature and chemical parameters resulting from the analyses are reported in the Supplementary Table 3. Out of 19 heavy metals evaluated, As, Cd, Li, Pt, Se, Tl, and V were below the limit of detection (LOD) in all the samples, regardless the type of water. Co was quantifiable only in one sample. LW and TWW samples demonstrated to have a different chemical profile, since, in the tree depicting sample composition, they clustered in two different branches following the type of water (Supplementary Fig. 1). Indeed, the type of water explained 67.9 % of the variance in chemical composition of the different samples (Supplementary Table 4). Moreover, the temperature, the content of RP, TP, TN, B, Fe, Mn, Pb, Si, and, Zn were significantly higher in TWW than in LW (LM: $p \leq 0.0494$) (Supplementary Fig. 2, Supplementary Table 5). The chemical composition of samples resulted highly correlated to their composition in terms of microbial community, antimicrobial resistome and high-risk ARGs content in the iDNA (Supplementary Table 6). No significant correlations were found for the eDNA (Supplementary Table 6).

3.1.2. Microbial community

In total, 405 genera from 187 different families were identified. In the iDNA lake samples, the most represented genera were *Candidatus Nanopelagicus* and *Candidatus Planktophila* (Fig. 1A, Supplementary Table 7). In eDNA lake samples, a higher variability was observed with *Candidatus Nanopelagicus*, *Candidatus Planktophila Candidatus Methylopumilus, Achromobacter* and *Stenotrophomonas* having the highest abundance per sample (Fig. 1A, Supplementary Table 7). The most abundant genera in iDNA TWW samples were *Aliarcobacter* and *Acinetobacter* (Fig. 1A, Supplementary Table 7). In eDNA TWW samples, the most represented genera were *Aeromonas* and *Acinetobacter* (Fig. 1A, Supplementary Table 7).

3.1.3. Antimicrobial resistome and high-risk ARGs

ARGs were quantified in all the analysed samples. Through read annotation, we found 256 different genes, covering 22 classes of resistance (Fig. 1D, Supplementary Table 8). In lake samples, the most abundant ARGs were *rpo*B2 and *bac*A, regardless of the origin of DNA, either iDNA or eDNA (Fig. 1D, Supplementary Table 8). In case of TWW samples, *mph*D, *bac*A, and *tet*39 genes had the highest abundance in iDNA, while, *mph*D, *bac*A, and bla_{OXA} genes in eDNA (Fig. 1D, Supplementary Table 8). When looking at the high-risk ARGs, *bac*A was the most abundant gene in LW samples from both iDNA and eDNA (Fig. 1G, Supplementary Table 8). For TWW, we found *bac*A as intracellular ARG with the highest abundance in all the samples (Fig. 1G, Supplementary Table 8), while, *bac*A and *aad*A genes were the most abundant in eDNA

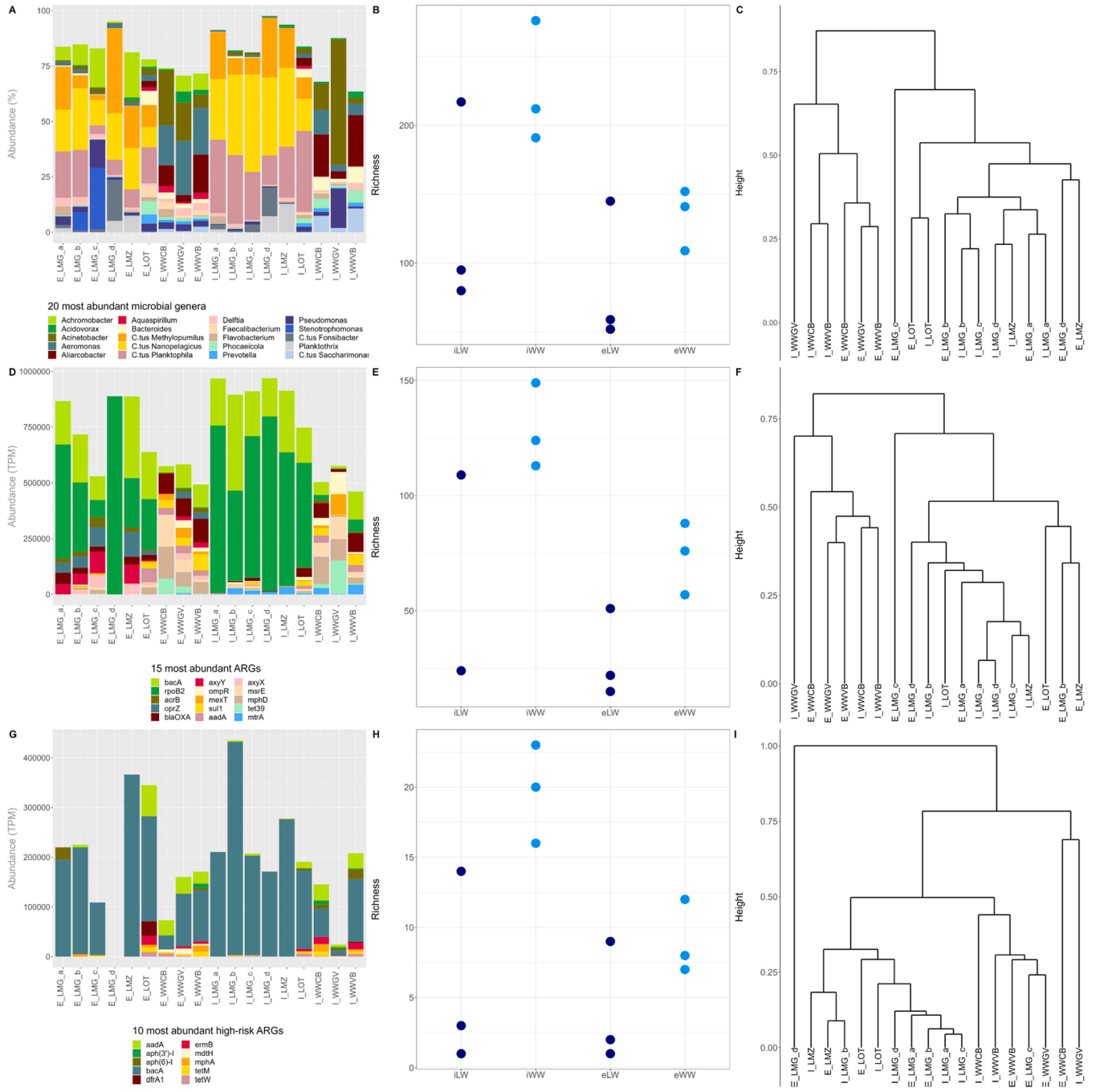
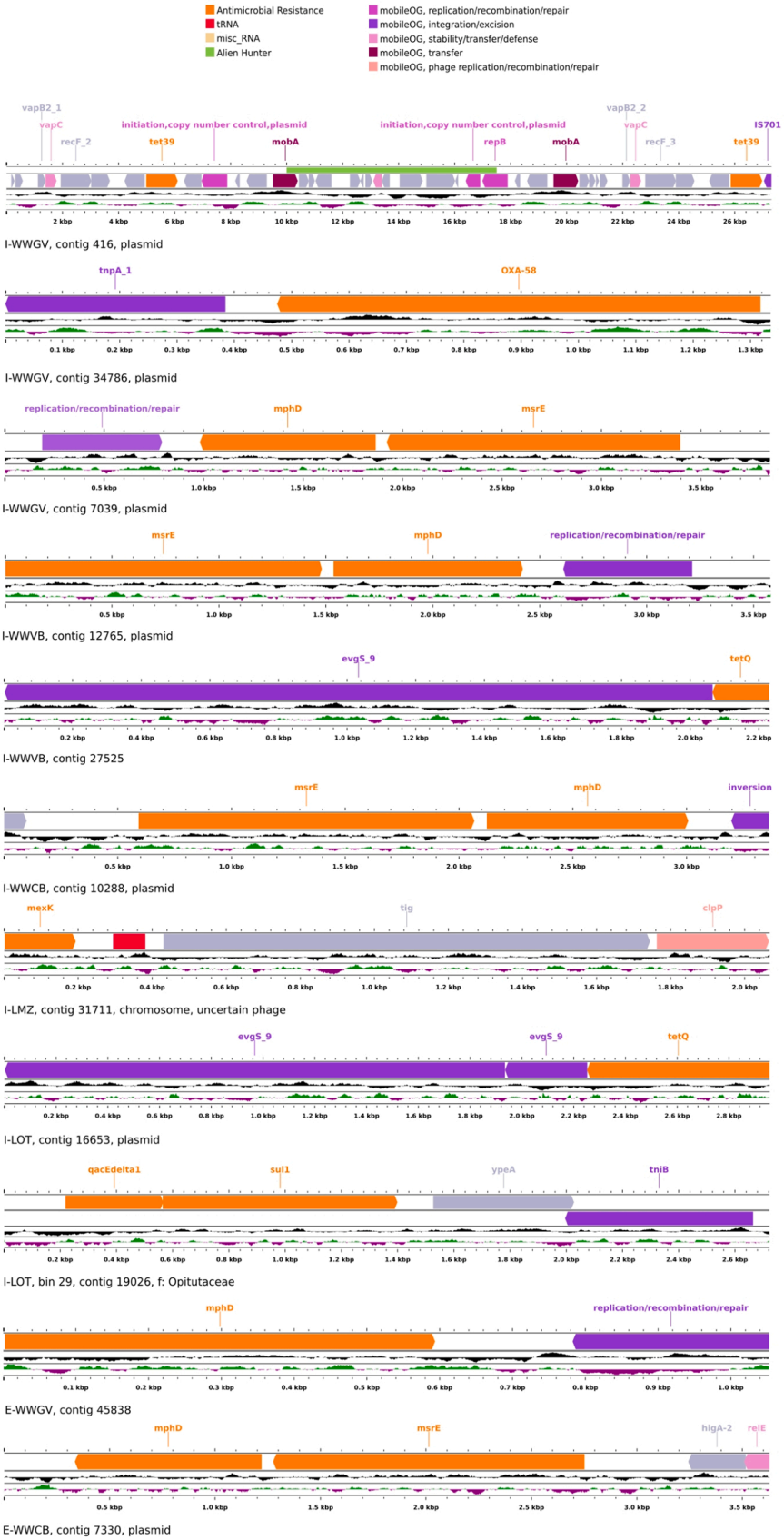


Fig. 1. Microbial community, antimicrobial resistome and high-risk ARGs. Most abundant genera of A) microbial community, and genes of D) antimicrobial resistome and G) high-risk ARGs. In case of microbial community, the abundances were expressed as percentage for each sample. For the ARGs, the abundances per sample were normalized on Transcripts Per Million (TPM). Richness of B) microbial community, E) antimicrobial resistome, and H) high-risk ARGs in the iDNA from lake water (iLW), iDNA from wastewater (iWW), eDNA from lake water (eLW), and eDNA from wastewater (eWW). Sample composition, based on Bray-Curtis dissimilarities, of C) microbial community, F) antimicrobial resistome, and I) high-risk ARGs. Abbreviations: I= iDNA, E= eDNA, LMG= Lake Maggiore, LMZ= Lake Mergozzo, LOT= Lake Orta, WWCB= wastewater from Cannobio, WWGV= wastewater from Gravellona Toce, WWVB= wastewater from Verbania. For Lake Maggiore samples, also the sampling date was indicated: a= March, b= June, c= September, d=November.

samples (Fig. 1G, Supplementary Table 8). When it comes to contig-based analysis, a total of 38 contigs were identified as carrying ARGs (Supplementary Table 9). Among these, 13 were found to be positive for both ARGs and MGEs, with 12 of them shown in Fig. 2 and two in Fig. 3. Of these 13 contigs, seven containing ARGs were localized on plasmids, four on the chromosome, and the location of two could not be definitively determined. In three out of the four contigs that harbor ARGs within MAGs, prophages were also annotated.

We obtained 146 MAGs from the water metagenomes, whose coverage ranged between 3.8 % and 15.1 % mapped reads for eDNA samples and between 8.3 % and 42.9 % mapped reads for iDNA samples (Supplementary Table 10). Only four MAGs (three from iDNA and one from eDNA) tested positive for ARGs (Supplementary Table 9). Two MAGs, deriving from the Gravellona Toce WWTP iDNA sample, were annotated as *Acinetobacter* spp. (a potential pathogenic bacterium). They represented 39.5 % of mapped reads of the Gravellona Toce WWTP iDNA sample and tested positive in several other samples, i.e., Lake Orta, Cannobio, Gravellona Toce, and Verbania WWTP samples both for



(caption on next page)

Fig. 2. Linear representation of ARG-containing contigs and mobilome genes. Focus on the contigs showing the presence of ARGs in eleven bins, a further bin containing ARGs and MGE genes is represented in focus in Fig. 3. The genomic map was obtained with Proksee (https://proksee.ca). The outer track indicates previous HGT putative regions predicted by Alien Hunter, forward and reverse ORFs are represented in stacked tracks when the contig length was bigger than 3 kbp or if needed for clarity, the underlying tracks show the content in GC along the sequence and the GC skew (lowest level). The specific MAG, the contig(s) reference and the putative taxonomy of MAGs elaborated with GTDB_tk are indicated under each genomic map together with the Viralverify putative classification (plasmid, chromosome). Abbreviations: I= iDNA, E= eDNA, LMZ= Lake Mergozzo, LOT= Lake Orta, WWCB= wastewater from Cannobio, WWGV= wastewater from Gravellona Toce, WWVB= wastewater from Verbania.

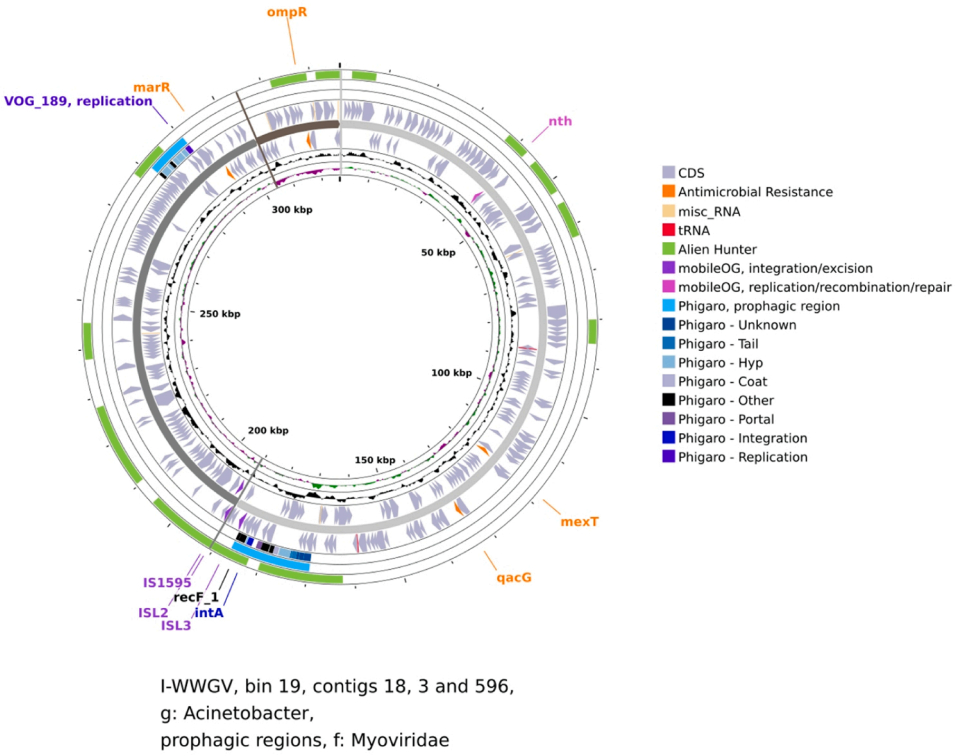


Fig. 3. Circular representation of ARG-containing contigs in the potential pathogen Acinetobacter sp. from intracellular DNA. The genomic map was obtained with Proksee (https://proksee.ca) and the contigs represented do not belong to closed bins, and their order in the figure is clockwise longest to shortest. The outer track indicates previous HGT putative regions predicted by Alien Hunter, followed by a track indicating the extension of the prophagic regions and a further track with the genomic detail of putative prophages (Phigaro tool in Proksee). Forward and reverse ORFs are represented in stacked tracks, the underlying track shows the content in GC along the sequence and the GC skew (most internal layer). When more than one ARG-containing contigs are found for the same MAG, those are distinguishable by the light-, dark- and darker-gray color of the genomic map backbone. The specific MAG, the contig(s) reference and the putative taxonomy of MAGs elaborated with GTDB_tk are indicated under each genomic map, for Acinetobacter, together with the putative taxonomy of the prophagic regions (Myoviridae). Abbreviations: I= iDNA, WWGV= wastewater from Gravellona Toce.

eDNA and iDNA and Lake Maggiore samples collected in March, June and September only for iDNA (Supplementary Table 2). One of these MAGs tested positive for ARGs and MGEs (Fig. 3) and represented 13.4 % of mapped reads of the Gravellona Toce WWTP iDNA sample (Supplementary Table 10).

3.1.4. Effect of anthropogenic impact on microbial community and antimicrobial resistome

To explore the impact of varying levels of anthropogenic pollution on microbial communities and antimicrobial resistomes within both DNA fractions (iDNA and eDNA), we initially examined distinct water types, i.e. TWW and LW, each reflecting different degrees of anthropogenic pollution as demonstrated by the chemical analysis (Supplementary Figs. 1, 2, Supplementary Table 5). The richness of the microbial community and antimicrobial resistome was significantly higher in the TWWs than in LWs (LMEM: $p \leq 0.0302$) and in iDNA than in eDNA ($p \leq 0.0001$) (Fig. 1B, E, Supplementary Table 11). High-risk ARGs had a significant greater richness in TWW than in LW ($p = 0.0146$); however, no significant differences in the diversity of high-risk ARGs were observed between iDNA and eDNA samples ($p = 0.0898$) (Fig. 1H, Supplementary Table 11).

In terms of beta-diversity, the largest part of the variance was explained by the difference between TWW and LW for overall taxonomic composition (PERMANOVA: R2 = 56.6 %), general ARGs (50.3 %) and high risk ARGs (43.2 %) (Fig. 1C, F, I, Supplementary Table 12). Only 8.8 %, 8.6 % and 3.5 % of the variance were attributed to the DNA origin (iDNA and eDNA) for taxonomic composition, ARGs and high risk ARGs, respectively (Fig. 1C, F, I, Supplementary Table 12). High-risk ARG abundances were significantly more abundant in LW (LMEM: $p = 0.0151$) (Fig. 4, Supplementary Table 13). This result was primarily influenced by the very high *bac*A abundances in LWs (Supplementary Fig. 3): indeed, after excluding this gene from the dataset, there was no difference between samples ($p = 0.1370$, Supplementary Table 13). High-risk ARGs were found as much in eDNA as iDNA, whether considering *bac*A ($p = 0.3602$) or not ($p = 0.4472$) (Supplementary Table 13).

Furthermore, we assessed whether iDNA and eDNA ARGs followed changes in abundance over four months that were related to the microbial community as a total, by sampling Lake Maggiore during each season. Despite the limited number of samples, overall, the richness

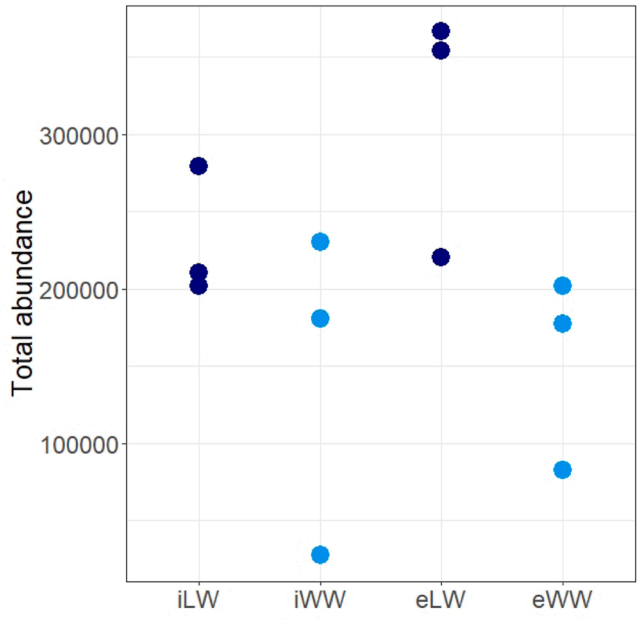


Fig. 4. Abundances of high-risk ARGs in lake and wastewaters. Total abundances of high-risk ARGs in the iDNA from lake water (iLW), iDNA from wastewater (iWW), eDNA from lake water (eLW), and eDNA from wastewater (eWW). The abundances per sample were normalized on Transcripts Per Million (TPM).

across the four datasets exhibited a tendency to increase during the warmer months. The diversity of genera and genes, in general, was higher in iDNA compared to eDNA (Supplementary Fig. 4). Indeed, the richness of the taxonomic composition, antimicrobial resistome, and high-risk ARGs in the temporal samples in Lake Maggiore were found to be strongly and significantly correlated ($R \geq 0.78$, $p \leq 0.0228$) (Supplementary Fig. 4).

3.2. Experimental study

The bacterial concentrations ranged from 1.70×10^5 to 2.17×10^6 cells mL^{-1} at T0 and from 2.28×10^5 to 5.73×10^6 cells mL^{-1} at the end of the incubation of the bacterial community with the pSEVA431-gfp plasmid. They were between 2.15×10^6 and 3.61×10^6 cells mL^{-1} at T0 and between 1.46×10^6 and 1.62×10^7 cells mL^{-1} at the end of the experiment where the pHM2-gfp plasmid was introduced. Following incubation, the intracellular *gfp* gene was quantified in all samples from both experiments. For pSEVA431-gfp plasmid, its concentration ranged from 2.45×10^{-3} to 1.95×10^{-2} copies $cell^{-1}$; while for pHM2-gfp plasmid, it was comprised between 2.79×10^{-4} and 5.23×10^{-1} copies $cell^{-1}$ (Fig. 5). In both cases, the transformation and selection of the spiked plasmids significantly increased with growing percentages of TWW (GLM: $p \leq 0.026$) (Supplementary Table 14).

4. Discussion

In this study, we conducted a comprehensive investigation of the impact of anthropogenic pollution on shaping the microbial community and the associated antimicrobial resistome with particular focus on MGEs involving the analysis of iDNA and eDNA using a shotgun metagenomic approach.

The samples collected within the WWTPs, as expected, were more polluted than those from the lakes. This assumption is based on the chemical measurements, which showed significantly higher concentrations of TP, RP, TN and several trace metals in the TWW than in the LW samples. We thus categorized the samples into two distinct types of water: TWWs and LWs. The microbial community of samples from Lake Maggiore exhibited a seasonal pattern, consistent with observations in various aquatic ecosystems [48,49]. Nevertheless, all the lake samples and the TWWs belonged to two distinct branches of the compositional tree, underscoring that the diversity in both types of water is primarily influenced by anthropogenic pollution. This demonstrates that anthropogenic pollution serves as a significant factor shaping the microbial community in LW, aligning with findings from several other aquatic ecosystems [4,50]. Consistent with previous research focused on the study of the two fractions of DNA, our findings confirm that, whilst not all taxonomic groups are found in eDNA compared to iDNA, there is still a large diversity of genes that persists in the eDNA fraction [24,51].

Similarly to what we observed for the microbial community, anthropogenic pollution resulted to be important in shaping the antimicrobial resistome. The richness of the antimicrobial resistome was significantly higher in the TWWs compared to the LWs, a trend that held true even when focusing solely on high-risk ARGs. This result perfectly aligns with prior research findings [52] and further underlines the role of WWTPs as significant hotspot for the release of ARGs into surface waters [53]. Interestingly, among the three most prevalent ARGs, regardless of the water type under consideration, the presence of *bac*A,

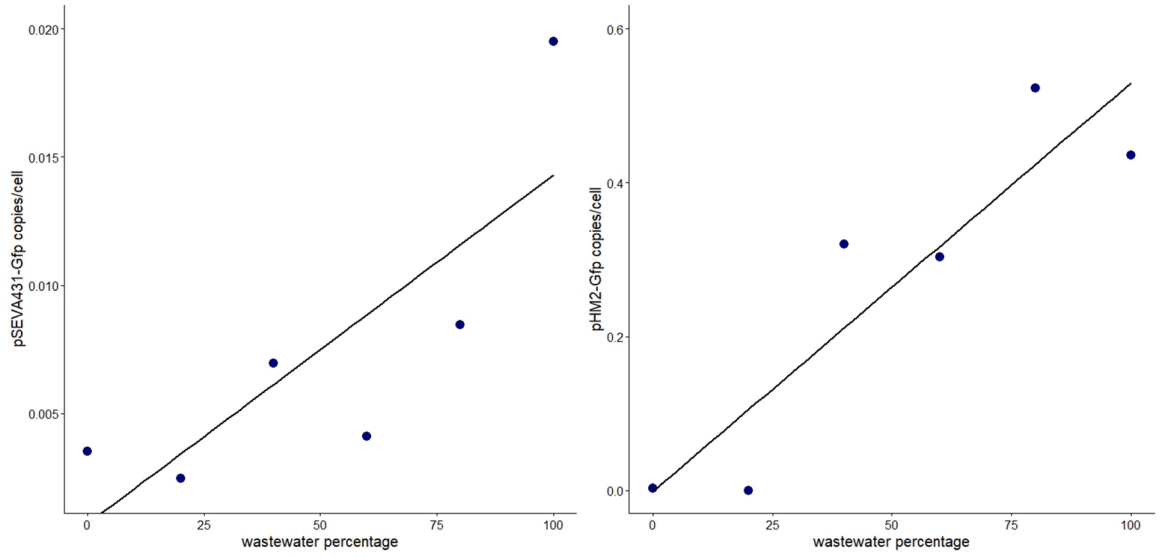


Fig. 5. Gfp-plasmid uptake by natural transformation. Copies of pSEVA431-Gfp and pHM2-Gfp plasmids per cell with increasing percentages of wastewater.

encoding for bacitracin resistance, was consistent. Moreover, being prevalent also among the high-risk ARGs, this gene also contributed to their greater abundance in LWs compared to TWWs. This finding confirms the relatively high abundance of this gene within the antimicrobial resistome associated with the aquatic microbial communities [54,55] and claims for a deeper understanding of the role of the anthropogenic pollution to its spread in the water environments and the factors that allow for its persistence within the aquatic environment. The other abundant ARGs exhibited distinct patterns between the two types of waters, suggesting that while TWWs are relevant carriers of ARGs, their persistence within the aquatic microbial communities is limited. This is possible attributable to the resistance of the autochthonous bacterial community against the invasion by allochthonous bacteria from WWTPs [56]. When comparing the two DNA fractions in terms of the antimicrobial resistome, it is evident that iDNA exhibited higher richness, in agreement with prior findings [24]. However, the same pattern did not hold for the abundance of high-risk ARGs, which remained similar between iDNA and eDNA. This strongly suggests that eDNA is an important source of high-risk ARGs and should be studied with more care for the risk assessment of freshwater.

Consistent with the findings obtained by the read annotation, our results were reinforced by making the assembly and binning, where it became clear that iDNA is the primary carrier of ARGs. Only a small fraction of MAGs tested positive for ARGs, and notably, one of them annotated as a putative pathogenic bacterium affiliated with the genus *Acinetobacter*, which encompasses several nosocomial pathogens prone to resist various antimicrobials and sterilization agents [57]. This MAG was also abundant in the sample from which it was retrieved and widespread across the analysed samples, especially those from WWTPs. This result aligns with previously published data showing this bacterial genus as abundant in wastewaters [58]. Upon analysing the genetic context of the annotated ARGs within this MAG, we identified different MGEs, a prophagic region linked to the *Myoviridae* family and several genomic regions indicating the likelihood of previous HGT events. These findings combined with the documented propensity for its natural transformation [59] suggest that *Acinetobacter* may serve as a carrier of ARGs [60], acquired and potentially transmissible through HGT in aquatic ecosystems.

Exploring the genetic context of all the other ARGs annotated in the contigs, it becomes evident that many of them were linked to MGEs. Notably, there was a prevalence of plasmids, which are known for their relevant role in disseminating ARGs within aquatic environments [61]. Phages were also annotated in the same contigs where ARGs were detected, suggesting that, albeit less frequently in comparison to plasmids, they could play a role in spreading ARGs within the environment, as it has been previously reported [62,63].

Based on earlier observations from our fieldwork, which highlighted plasmids as potentially significant players in the dissemination of ARGs we opted to assess experimental investigations. Our aim was to determine whether anthropogenic pollution, resulting from the increasing of both chemical and microbiological pollutants by mixing NTWW with LW, could affect the uptake of eDNA specifically containing plasmids harboring ARGs in aquatic environments. Our findings demonstrated that, irrespective of the type of ARG-positive plasmids used in the experiments, anthropogenic water pollution had a positive influence on the rates of transformation and selection of extracellular antimicrobial resistant plasmids. This result aligns with previous research findings showing an increase in transformation rates to various anthropogenic factors, generally assessed on the model species *A. baylyi* or few additional ones [64]. This represents the first evidence that overall anthropogenic pollution, involving both chemical and microbiological factors by choosing wastewater prior to disinfection, significantly contributes to the transformation of ARG-positive plasmids, calling for further research to investigate the underlying reasons and mechanisms behind these findings.

5. Conclusions

The data presented in this study unequivocally demonstrate the significant impact of anthropogenic water pollution on the diversity of the whole microbial community and associated antimicrobial resistome and abundance of the high-risk ARGs. This underscores the crucial role that MGEs within both fraction of DNA (iDNA and eDNA) could play in the dissemination of ARGs. Furthermore, our findings provide a first experimental evidence of the contribution of anthropogenic pollution to the spread of AMR via HGT, in particular, by transformation, involving eDNA and environmental microbial communities.

Environmental implications

Within the One Health paradigm, the aquatic environment plays a central role in the antimicrobial resistance (AMR) cycle. Antimicrobial resistance genes (ARGs) are found in both intracellular and extracellular DNA, constituting emerging micropollutants that pose a significant threat to human health. Both forms of DNA contribute to the AMR profile within aquatic ecosystems, with anthropogenic pollution positively influencing its diversity. Moreover, anthropogenic pollution also facilitates the natural transformation of extracellular DNA carrying ARGs, potentially enhancing the spread of AMR in aquatic ecosystems.

CRediT authorship contribution statement

Ester M. Eckert: Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Andrea Di Cesare:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Elena Crotti:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Francesca Mapelli:** Writing – review & editing, Supervision, Resources, Conceptualization. **Sara Borin:** Writing – review & editing, Supervision, Resources. **Andrea Lopez Pilar:** Writing – review & editing, Methodology, Formal analysis. **Gianluca Corno:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Diego Fontaneto:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Data curation. **Giulia Borgomaneiro:** Writing – review & editing, Methodology, Formal analysis. **Michela Rogora:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation. **Periyasamy Sivalingam:** Writing – original draft, Methodology, Formal analysis. **Raffaella Sabatino:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tomasa Sbaffi:** Writing – review & editing, Visualization, Validation, Software, Methodology, Formal analysis, Data curation.

Declaration of Competing Interest

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

Data availability

The sequence data have been submitted to NCBI and the link to access them is provided in the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.134885.

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