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Impact of fluoroquinolone and heavy metal pollution on antibiotic resistance maintenance in aquatic ecosystems

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Abstract

Background Freshwater pollution with compounds used during anthropogenic activities could be a major driver of antibiotic resistance emergence and dissemination in environmental settings. Fluoroquinolones and heavy metals are two widely used aquatic pollutants that show a high stability in the environment and have well-known effects on antibiotic resistance selection. However, the impact of these compounds on antibiotic resistance maintenance in aquatic ecosystems remains unknown. In this study, we used a microcosm approach to determine the persistence of two fluoroquinolones (ciprofloxacin, ofloxacin) and two heavy metals (copper and zinc) in the Rhône river over 27 days. In addition, we established links between antibiotic and metal pollution, alone and in combination, and the composition of freshwater bacterial communities, the selection of specific members and the selection and maintenance of antibiotic and metal resistance genes (ARGs and MRGs) using a metagenomics approach.

Results Whereas ofloxacin was detected at higher levels in freshwater after 27 days, copper had the strongest influence on bacterial communities and antibiotic and metal resistance gene selection. In addition, heavy metal exposure selected for some ARG-harboring bacteria that contained MRGs. Our research shows a heavy metal-driven transient co-selection for fluoroquinolone resistance in an aquatic ecosystem that could be largely explained by the short-term selection of *Pseudomonas* subpopulations harboring both fluoroquinolone efflux pumps and copper resistance genes.

Conclusion This research highlights the complexity and compound-specificity of dose-response relationships in freshwater ecosystems and provides new insights into the medium-term community structure modifications induced by overall sub-inhibitory levels of antibiotic and heavy metal pollution that may lead to the selection and maintenance of antibiotic resistance in low-impacted ecosystems exposed to multiple pollutants.

Keywords Antibiotic resistance, Heavy metals, Freshwater, Metagenomics, Maintenance, Fluoroquinolone, Aquatic ecosystems

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Background

The continuous emergence of antibiotic resistant bacterial pathogens in humans and animals is one of the major threats to global health and food safety according to the World Health Organization [1]. An urgent response is needed to prevent the further development of antibiotic resistance in clinics and the concomitant increase in mortality [2]. This crisis currently observed in the clinics is fueled in part by the spread of antibiotic resistance in the environment. Therefore, understanding and quantifying the contribution of this spread is a critical aspect in its mitigation [3]. Although antibiotic resistance occurs naturally in the environment, major societal changes (industrialization) and medical breakthroughs (antibiotic discovery) in the 20th century have accelerated the spread of antibiotic resistance to an unprecedented level [4]. Surface waters receive treated and untreated waste from hospitals, industries, drug manufacturing, farms, agricultural fields and the overall population (e.g., wastewater treatment plants). This waste contains a cocktail of chemical pollutants that may have deleterious effects on indigenous bacteria [5]. There is a growing concern that aquatic environments, which are in contact with the human microbiome in both urban and rural settings (drinking water, recreational bathing, crop irrigation), serve as a reservoir of antibiotic resistance genes (ARGs) that might disseminate to human pathogens through horizontal gene transfer (HGT) [6] in the environment. However, the magnitude of this dissemination risk and the mechanisms involved remain unknown.

Anthropogenic waste reaching the environment contains a mixture of pollutants that have been associated with the selection and dissemination of antibiotic resistance genes in environmental settings. Antibiotics and heavy metals are two major pollutants found worldwide with well-known effects on antibiotic resistance selection and dissemination. Metal pollution may co-select for antibiotic resistance through co-resistance (presence of antibiotic and metal resistance determinants on the same genetic element), cross-resistance (antibiotic and metal resistance conferred by the same genetic determinant) and co-regulation of responses to antibiotic and metal stress [7]. Although anthropogenic pollutants are usually found in the environment at doses that are too low to inhibit bacterial growth (*i.e.* "sub-inhibitory"), several studies have shown that, even at those levels, antibiotics and metals may promote antibiotic resistance emergence, selection and dissemination [8–11]. In addition, heavy metals are more widespread than antibiotics, and they are not biodegradable [12]. They are considered to persist longer in the environment than antibiotics, which could lead to a higher impact on the environmental resistome on the long term. Moreover, some antibiotics and heavy metals have synergistic effects on antibiotic resistance selection [13], and their simultaneous presence in aquatic environments might have a stronger effect on the environmental resistome. Although the selective potential of both antibiotics and metals has been widely studied, the extent to which these pollutants contribute to the maintenance of antibiotic resistance in aquatic ecosystems has not yet been elucidated.

This study aimed to determine the persistence of two fluoroquinolones (ciprofloxacin and ofloxacin) and two heavy metals (copper and zinc) in aquatic ecosystems and their effects on river water bacterial communities and their associated antibiotic resistance genes at overall sub-inhibitory concentrations. Fluoroquinolones are one of the most used antibiotic classes worldwide and their efficiency is threatened by the emergence of antibiotic resistant pathogens in clinics [1]. They are widely found in environmental settings and more stable than most antibiotics [14, 15]. Copper and zinc (Cu and Zn) are two of the most prevalent heavy metals found in surface waters and resistance to these metals has been associated with fluoroquinolone resistance [16]. We hypothesized that Cu and Zn are more stable in the environment and have a stronger influence at longer exposure times on the aquatic microbiome and resistome than fluoroquinolones. Using a microcosm approach, we evaluated the persistence in an aquatic ecosystem of these antibiotics and heavy metals alone and in combination and their impact on bacterial communities and their associated antibiotic resistance over 27 days. We established links between pollution levels, the selection of specific members of the community and the abundance of antibiotic and metal resistance genetic traits.

Methods

Sampling and microcosm set-up

River water from the Rhône (Lyon, France) was sampled on December 8th 2022 (45°45'08.1"N 4°50'11.4"E). Samples were left overnight at room temperature before preparing 1 L microcosms in polypropylene containers. Four pollutants were used: two fluoroquinolone antibiotics, ciprofloxacin and ofloxacin (Sigma-Aldrich), and two heavy metal salts, copper (II) chloride dihydrate and zinc sulfate heptahydrate (Sigma-Aldrich). Pollutants were added at 50 ng/ml, an overall sub-inhibitory concentration to Rhône river bacteria *in vitro* for the four compounds alone and in combination (Figure S1A in Supplementary Information). Five conditions were set-up in triplicate: non-polluted controls, samples polluted with ciprofloxacin and ofloxacin once at day 0 (named antibiotics one dose), samples polluted with these two antibiotics at days 0, 3, 6 and 27 (named antibiotics multiple doses), samples polluted with Cu and Zn once at day 0 (named metals) and samples polluted with the two antibiotics at days 0, 3, 6 and 27 and a single dose of

Cu and Zn at day 0 (named antibiotics + metals). Microcosms were incubated protected from light for 27 days at room temperature and agitated at 60 rpm to mimic river currents.

Quantification of antibiotic and heavy metal concentrations in river water

In order to determine the persistence of the four added compounds in the system, the concentrations of ciprofloxacin, ofloxacin, copper and zinc were determined in the 12 polluted samples incubated for 27 days (triplicates of antibiotics one dose, antibiotics multiple doses, antibiotics + metals, metals). In addition, a non-polluted sample obtained at day 0 was measured in parallel to determine background pollution levels in the Rhône. One hundred mL samples were frozen at -20 °C. Prior to antibiotic analysis, samples were melted, vortexed, filtered through PTFE (polytetrafluorethylene) filters (pore size 0.22 µm) and pipetted into 2 ml vials. A LC-MS/MS system was used for the determination of ciprofloxacin and ofloxacin levels. For high concentrations of antibiotics, samples were diluted so that the predicted concentration was within the linear range of the analysis (150-10000 ng/L for ciprofloxacin, 150–5000 ng/L for ofloxacin). The analysis was performed using a LC/HRMS system consisting of the ExionLC system (AB Sciex, Framingham, USA) with QTOF X500R equipped with electrospray ionization (ESI) Turbo V ion spray (AB Sciex, Framingham, USA). The selected antibiotics were separated using the separation column Triart C18 column (100 mm × 2.1 mm I. D., 3 µm, YMC CO., LTD., Kyoto, JP). The mobile phase consisted of 0.1% formic acid (phase A) and methanol (phase B). The flow rate was set at 0.42 mL·min – 1, the injection volume was 50 µL, and the column compartment temperature was set at 40 °C. The analysis lasted 16.2 min. Identification and quantification of ciprofloxacin and ofloxacin were performed in positive mode using scheduled multiple reaction monitoring (sMRM). ESI + settings were a spray voltage of 5500 V and a temperature of 550 °C. Other adjustable instrument parameters were: the curtain gas (35 psi) and collision gas (7 psi). Ion source gas 1 and 2 were set at 30 and 40 psi, respectively. Declustering potential (DP) and collision energy (CE) were optimized for both test substances. The limit of detection (LOD) was 150 ng/L and the limit of quantification (LOQ) was 500 ng/L for both compounds. Copper and zinc quantification was performed in an accredited laboratory number 252/2024 according to EN ISO/IEC 17025:2018. Samples were measured on an ICP-MS (PerkinElmer NexION 300D). Samples were measured without prior adjustment, and the calibration curve was measured using a multi-element standard. The detection limit (LOD) was 1 ng/mL for both compounds.

DNA extraction and estimation of bacterial abundance in the microcosms

DNA was extracted from the microcosms after 0, 3, 6 and 27 incubation days. One hundred mL of river water were filtered on a 0.2 µm pore membrane filter, and DNA extraction was carried out following the Phenol/Chloroform method described by Griffiths et al. [17]. DNA was eluted in 40 µl. Then, the abundance of the bacterial communities was estimated by quantifying the 16S rRNA gene by qPCR using the 341F (5'-CCTACGGGAGGCAGCAG- 3') and 534R (5'-ATTACCGCGGCTGCTGGCA-3') primers [18]. qPCR amplification was carried out using the Corbett Rotor-Gene 6000 (QIAGEN) in a 20 µl reaction volume containing QuantiNova SYBR Green PCR Master Mix (Qiagen), 0.75 µM of each primer and 2 µl of DNA. Two non-template controls were included in the assay. Standard curves for all the assays were obtained using 10-fold serial dilutions of a linearized plasmid pGEM-T Easy Vector (10^8 -10^3 copies) containing the 16 S rRNA gene of *Pseudomonas aeruginosa* PAO1. Cycling conditions for qPCR amplification were 95 °C for 2 min followed by 35 cycles of 95 °C for 5s and 60 °C for 30s. Melting curves were generated by increasing temperature from 60 °C to 95 °C after amplification.

16S rRNA gene (*rrs*) sequencing and analysis

The V4 hypervariable region of the 16S rRNA gene was amplified using forward 515F and reverse 806Rb primers with Illumina overhangs [19]. DNA was amplified by PCR using the Platinum Taq DNA Polymerase (Invitrogen) and the following conditions: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension for 5 min at 72 °C. DNA libraries were prepared from amplified products using the Platinum Taq DNA Polymerase (Invitrogen) and the Nextera XT Index Kit V2 (Illumina) according to the Illumina's protocol for amplicon sequencing library preparation. Paired-end sequencing (2 × 250 bp) of barcoded amplicons was performed using the MiSeq System and the MiSeq Reagent Kit v2 (Illumina). Sequences were treated using the DADA2 [20] pipeline (version 1.22.0) to remove primers, trim the last 10 bases of the forward reads and the last 40 bases of the reverse reads, quality trim the sequences (maxN = 0, maxEE = 2, truncQ = 2) merge forward and reverse reads, remove chimeric reads and obtain amplicon sequence variants (ASVs). Sequencing depth after sequence treatment can be found in Table S1a in Supplementary Information. The 2659 obtained ASVs were annotated taxonomically to the genus level using the Ribosomal Database Project v18 [21]. Then, ASVs that were not annotated to the class level and/or that had less than 10 copies in total were removed, as well as sequences identified as contaminants using extraction and sequencing controls and the "decontam"

package in R (version 1.14.0). The overall impact of the analyzed pollutants on bacterial community composition was determined by a non-metric multidimensional scaling (NMDS) analysis on Bray-Curtis dissimilarity, calculated from ASV abundance using the "vegan" package in R (version 2.6-4). PERMANOVA tests with 999 permutations were applied to each dissimilarity matrix to determine the significance of the impact of pollution on bacterial community composition using the "vegan" package in R. The diversity of ASVs annotated to the class level was measured using the Shannon Diversity Index. Then, ASV abundances were merged by genera to determine changes in the abundance of the most prevalent members of the community. In addition, the abundance of the 25 ASVs associated with the most prevalent genus, *Pseudomonas*, was evaluated to determine the dynamics of subpopulations exposed to antibiotic and metal pollution. ASV and genus abundances were divided by sequencing depth after sequence treatment (relative abundance) and multiplied by the number of copies of the 16S rRNA gene determined by qPCR to obtain total inferred abundances.

Metagenomics sequencing and analysis

Metagenomics libraries were prepared from ≤1 ng of DNA using the Nextera XT Library Prep Kit and Indexes (Illumina) as detailed in Illumina's "Nextera XT DNA Library Prep Kit" reference guide, using 13 amplification cycles for the indexing PCR. DNA sequencing was performed using the MiSeq System and the MiSeq Reagent Kit v2 (Illumina). Sequencing depths obtained from the metagenomic sequencing of water samples can be found in Table S1b in Supplementary Information. Reads were quality-filtered according to the criteria described by Minoche et al. [22]. Forward and reverse short reads were concatenated and annotated using Diamond (version 2.1.7) [23] and the CARD antibiotic resistance gene (version 3.2.9) [24] and the BacMet metal and biocide resistance gene (version 2.0) [25] databases in order to identify antibiotic resistance and metal resistance genes (ARGs and MRGs) in the samples. Results were filtered at a minimum amino acid identity of 60%, a minimum length of 33 amino acids and a maximum e-value of 10^{-5}. The best hit was used, singletons were removed, and gene abundance was normalized by sequencing depth. A list of fluoroquinolone, Cu and Zn resistance genes identified in water metagenomes can be found in Table S2 in Supplementary Information. In parallel, all reads were co-assembled using MEGAHIT [26] and mapped onto the 53,995 obtained contigs using Bowtie2 [27]. Anvi'o profiles were created for each individual sample and merged using the anvi'o [28] metagenomic workflow (anvi'o version 8). Contigs were manually binned based on their differential coverage across samples using anvi'o and the bins were manually refined based on differential coverage and sequence composition to obtain MAGs with a >50% completion and <10% redundancy. Bin taxonomy and redundancy were estimated using checkM2 [29] (version 1.1.0), and bins with <50% completion and ≥10% redundancy were discarded since they were considered low-quality metagenome assembled genome (MAG) drafts according to criteria suggested by Bowers et al. [30]. MAG taxonomy was estimated using the Genome Taxonomy Database (GTDB) as part of the anvi'o workflow. The contigs from the refined MAGs were annotated using Diamond, CARD and BacMet to identify fluoroquinolone, copper and zinc resistance genes. Results were filtered at an amino acid identity percentage of 60%, 150 amino acid length and an e-value of 10^{-5},and the best hit was used. Finally, the relative abundance of each MAG across samples was represented as the percentage of recruitment (percentage of sequences from each individual sample that are binned into a specific MAG).

Statistical analyses

Statistical analyses and graphics were generated using Prism GraphPad v 9.5.0. Each variable was tested using the Shapiro-Wilk test for normal distribution. Two-tailed ANOVA and Tukey post-hoc tests were used to determine statistical differences between normally-distributed variables and Kruskal-Wallis with post-hoc Dunn's tests were used to determine statistical differences between non-normally-distributed variables. p-values were corrected to account for multiple comparisons ($\alpha < 0.05$). Only pairwise comparisons with a p-value < 0.05 are shown in the graphs. Finally, all variables evaluated in this study were correlated using the Pearson correlation coefficient to determine potential links between pollutant doses, ASV and MAG abundance and the abundance of antibiotic and metal resistance genes. Two variables were considered to be correlated at a threshold of ±0.6, and the correlation was considered strong at a threshold of ±0.8.

Results

Persistence of ciprofloxacin, ofloxacin, copper and zinc in Rhône river water

The four pollutants used in this study were quantified at the end of the experiment to determine their persistence in the Rhône river aquatic ecosystem (Fig. 1). Background pollution levels in the Rhône were determined by quantifying a sample before pollution and incubation: ciprofloxacin and ofloxacin were not detected, and Cu and Zn were present at 1.7 and 1.2 ng/mL, respectively. Ciprofloxacin and ofloxacin were detectable after 27 days in all the samples polluted once and multiple times by these compounds. None of the antibiotics was detected in the samples polluted only by Cu and Zn, and

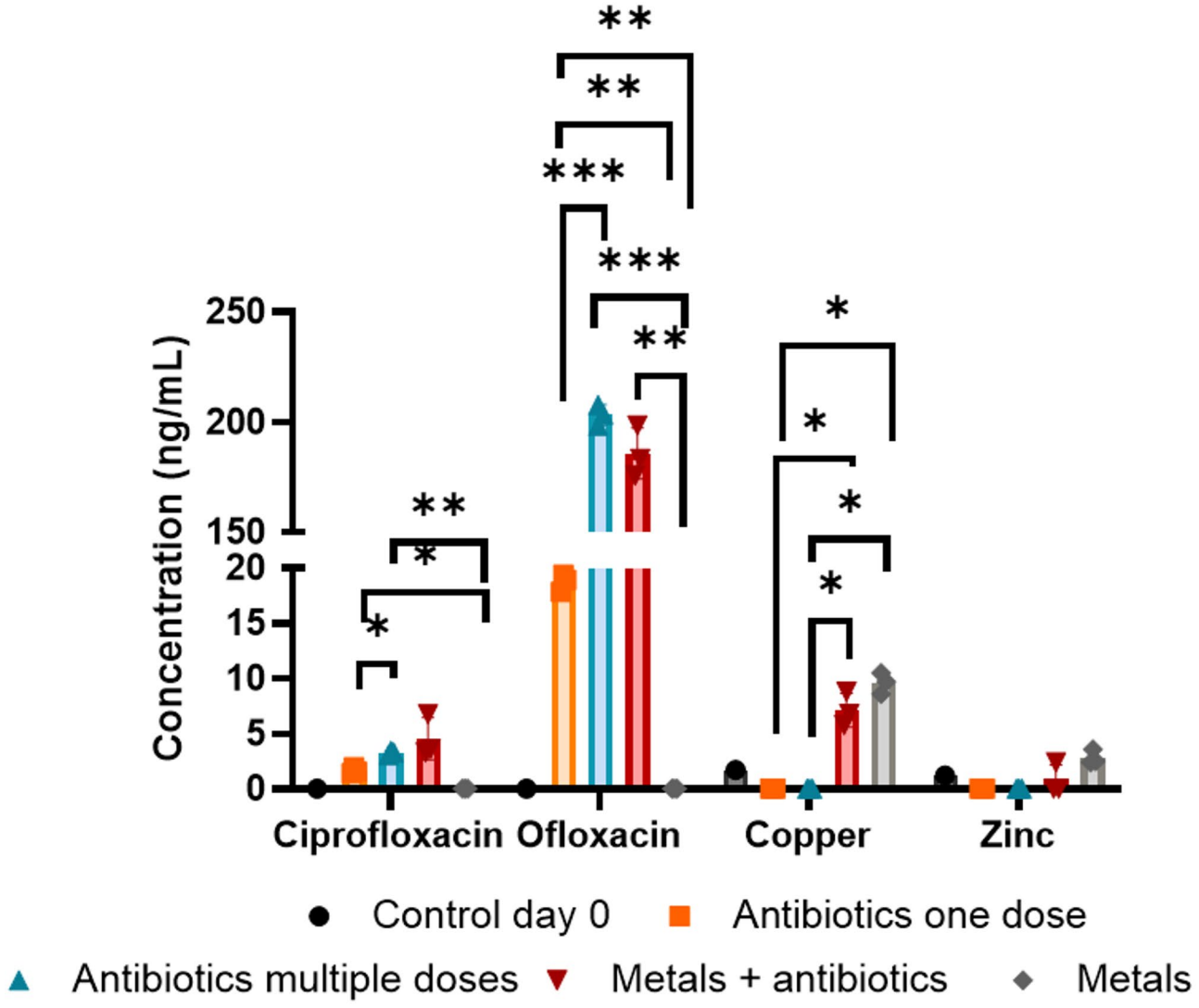

Fig. 1 Concentrations of ciprofloxacin, ofloxacin, copper and zinc measured in Rhône river water after 27 days of exposure to antibiotics, metals and both. *n* = *3*. One non-polluted sample at day 0 (control day 0) was measured to determine background pollution levels (no statistics were performed on this sample). Only significant differences are shown. **p*-value < 0.05. ***p*-value < 0.01. ****p*-value < 0.001

significantly higher doses of both antibiotics were found in the samples polluted with multiple doses of antibiotics than in the samples polluted with a single dose. Ofloxacin was detected at higher levels in the Rhône river aquatic ecosystem than ciprofloxacin: at a single dose of 50 ng/mL at day 0, an average concentration of 18.73 ng/mL of ofloxacin was measured after 27 days, whereas ciprofloxacin was detected at an average concentration of 1.65 ng/mL. In samples polluted four times (200 ng/mL) for 27 days, average concentrations of ofloxacin were 203.2 ng/mL in the absence and 185.75 ng/mL in the presence of metals, and ciprofloxacin average concentrations were 3.27 ng/mL in the absence and 4.54 ng/mL in the presence of metals. After 27 days, copper was detected in all samples polluted by this metal (average concentrations of 9.6 ng/mL in the absence of antibiotics and 7.1 ng/mL in the presence of antibiotics). Copper concentrations were under the detection limit in samples where it was not added. Thus, these three compounds were detected at a significantly higher abundance in the samples where they were added than in the non-polluted samples and showed a consistent persistence in the system after 27 days. On the other hand, zinc was under the detection limit in non-polluted samples and in 2/3 of the samples polluted by antibiotics and heavy metals. It was consistently detected only in the triplicates exposed to Cu and Zn without antibiotics, where its average concentration was 2.8 ng/mL. Contrarily to ciprofloxacin, ofloxacin and copper, dissolved zinc did not show a significant persistence in the polluted systems after 27 days.

Impact of antibiotics and heavy metals on bacterial community composition

Antibiotic and metal pollution did not affect overall bacterial abundance (Figure S1B in Supplementary Information) and diversity (Figure S1C in Supplementary Information) at any time compared to non-polluted controls. At day 3, samples exposed to antibiotics only (one dose and multiple doses) showed lower bacterial abundance than those exposed to metals, and metals reduced bacterial diversity at day 3 compared to samples polluted with one dose of antibiotics. Therefore, although all pollutants were overall sub-inhibitory to river water bacteria, some short-term differences related to the nature of the pollution were observed in terms of bacterial abundance and diversity. In addition, all pollutants affected the overall composition of Rhône river water bacterial communities (Fig. 2). Samples at day 27 clustered slightly apart from samples at days 0, 3 and 6 (PERMANOVA time effect $R^2 = 0.176$, F = 11.98, p-value = 0.001). From day 3, the impact of antibiotic and metal pollution on community composition was observable at an overall scale, and the differences between the conditions increased with time. A PERMANOVA analysis on all polluted samples at day 27 revealed that, amongst the four pollutants, copper concentrations explained most of the differences between samples ($R^2 = 0.31$, F = 15.15, p-value = 0.001), followed by ciprofloxacin ($R^2 = 0.19$, F = 9.31, p-value = 0.001), whereas ofloxacin and zinc had a more limited impact ($R^2 = 0.12$, F = 5.72, p-value = 0.009 for ofloxacin, $R^2 = 0.12$, F = 5.61, p-value = 0.002 for zinc).

At the genus level, the most striking change was the increased abundance of *Pseudomonas* after a 3-day exposure to metals, both in the presence and in the absence of antibiotics (Figure S2 in Supplementary Information). This increase was still observable after 6 and 27 days, although the abundance of *Pseudomonas* was higher in the samples that contained both antibiotics and metals than in samples exposed to metals alone. *Flavobacterium* also increased its abundance in all samples polluted with metals at day 3, but this increase was transient. At day 27, the most prevalent genus in samples polluted with metals alone was *Sphingorhabdus*, whereas *Pseudomonas* remained the most abundant genus in samples exposed to the four antibiotics. *Nevskia* was the most prevalent genus in samples exposed to a single dose of ciprofloxacin and ofloxacin after 27 days, and *Daejeonella* showed the highest abundance in samples exposed to multiple doses of these antibiotics. In addition, similar genera were found for the 25 most abundant ASVs. An ASV associated to *Pseudomonas* was the most abundant one and the one showing the strongest increase under metal exposure (Figure S3 in Supplementary Information). Since *Pseudomonas* was the genus showing the highest abundance and the biggest changes across conditions, its dynamics were evaluated at the ASV level to determine the influence of antibiotic and heavy metal pollution on the selection of *Pseudomonas* subpopulations (Fig. 3). Three ASVs represented 98% of the reads associated with *Pseudomonas*: ASV 1 (Fig. 3A), ASV 25 (Fig. 3B) and ASV 28 (Fig. 3C). All three showed a higher abundance in

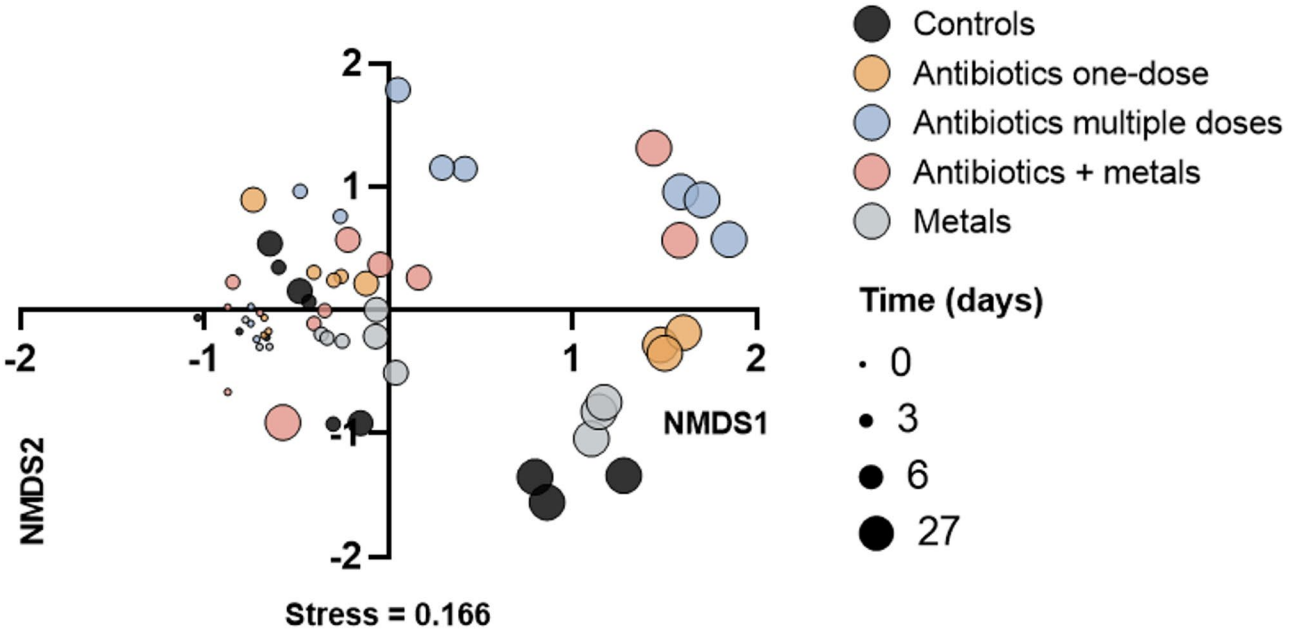

Fig. 2 Non-metric multidimensional scaling of the Bray-Curtis dissimilarity calculated from the relative abundance of ASVs annotated to the class level. Dot color represents the five conditions used in the study, whereas dot size represents exposure time. NMDS stress = 0.166. $n = 3$

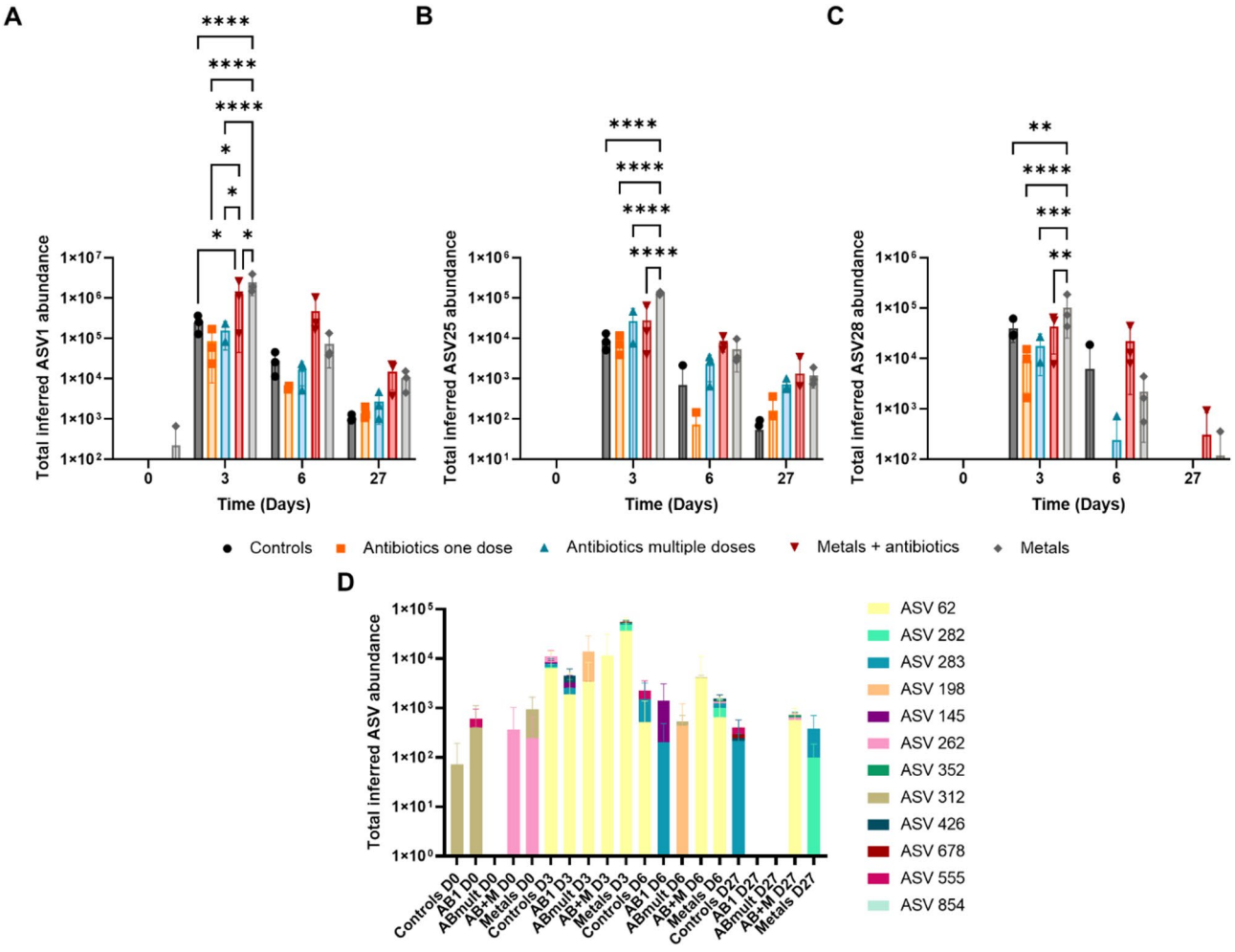

Fig. 3 Population dynamics over time and under different pollution scenarios of the 15 most abundant ASVs associated with the most abundant genus found in river water samples, *Pseudomonas*. A, B, C: total inferred abundance (ASV abundance divided by sequencing depth and multiplied by the number of copies of the 16 S rRNA gene) of the three most abundant ASVs (ASV 1, ASV 25, ASV 28). D: total inferred abundance of the remaining 12 ASVs. AB1: antibiotics one dose. ABmult: antibiotics multiple doses. AB + M: antibiotics + metals. D0: day 0; D3: day 3. D6: day 6. D27: day 27. $n = 3$

samples exposed to Cu and Zn for 3 days than in all other samples at the same time (Fig. 3A-C), although only ASV 1 had a higher abundance in samples exposed to metals and antibiotics than in non-metal polluted samples at day 3 (Fig. 3A). In the three cases, this increase was transient, and no significant differences between conditions were observed from day 6 onward. The abundance of the other ASVs associated with *Pseudomonas* (Fig. 3D) reflected temporal dynamics (loss of ASVs 262 and 312 over time, increase of ASV 62 in all conditions at day 3) and the impact of pollution (increased abundance of ASV 198 in samples exposed to multiple doses of antibiotics for 2 and 7 days, increase in the abundance of ASV 282 over time in samples exposed to Cu and Zn, loss of all ASVs in samples exposed to antibiotics at one and multiple doses at day 27). Overall, the dynamics of *Pseudomonas* subpopulations over time varied with pollution and short-term metal pollution showed the strongest impact on *Pseudomonas* ASV abundance.

Impact of antibiotics and heavy metals on the aquatic resistome

Metal pollution affected the relative abundance of antibiotic and metal resistance genes in non-assembled reads (Fig. 4). Regarding the total abundance of antibiotic resistance genes (which include fluoroquinolone resistance genes), opposite trends were observed at short and long exposure times. Metals alone increased the relative abundance of ARGs after 3 exposure days, and metals in combination with fluoroquinolones increased this abundance after 6 exposure days. However, at day 27, ARG abundance was significantly lower in samples exposed to metals (both in the presence and absence of antibiotics) than in samples exposed to fluoroquinolones alone (Fig. 4A). Metals also increased the relative abundance

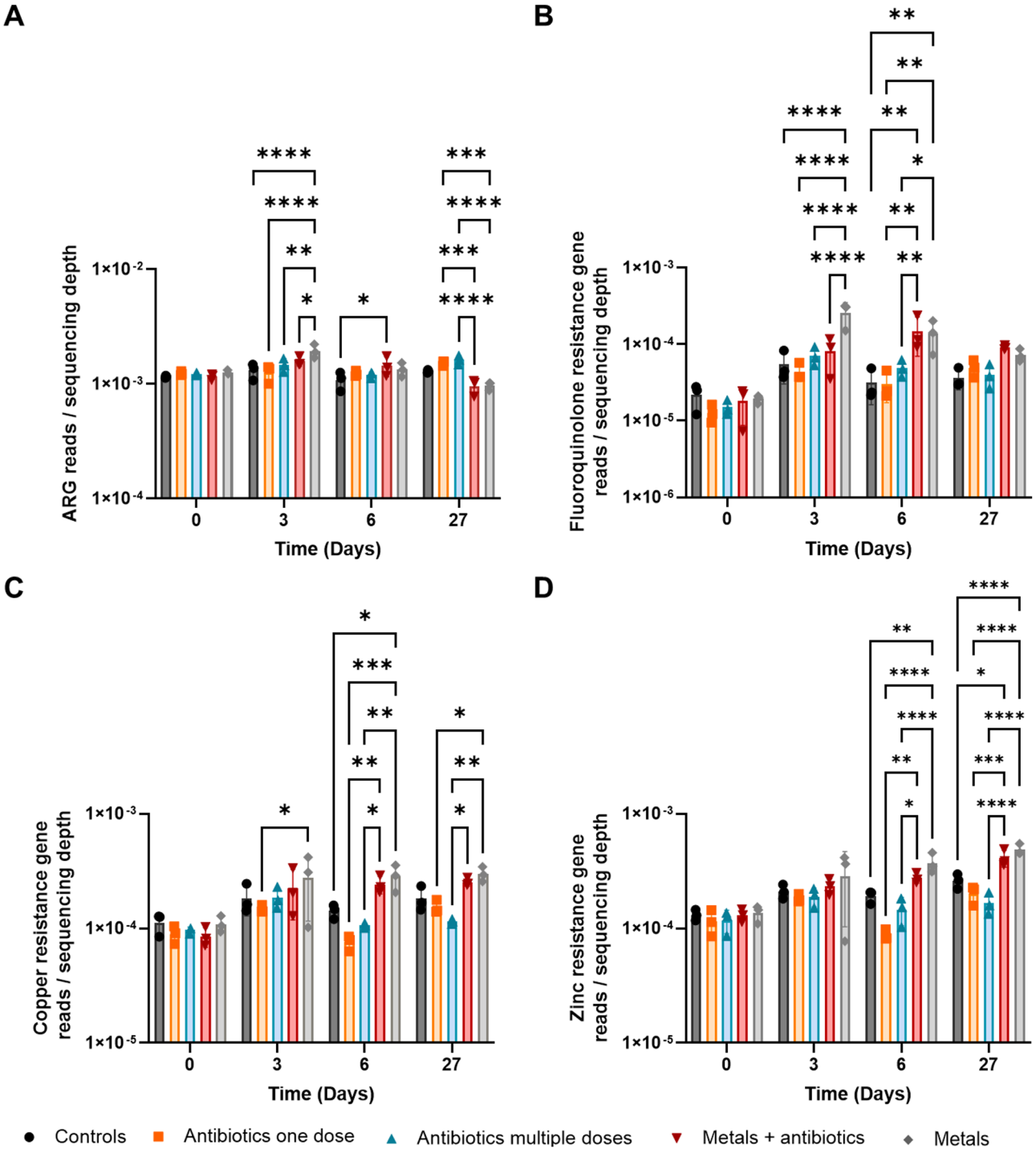

Fig. 4 Relative abundance (normalized by sequencing depth) of antibiotic and metal resistance genes in river water metagenomes. **A**: relative abundance of total antibiotic resistance genes (ARGs). **B**: relative abundance of fluoroquinolone resistance genes. **C**: relative abundance of copper resistance genes. **D**: relative abundance of zinc resistance genes. Only significant differences are shown. **p*-value < 0.05. ***p*-value < 0.01. ****p*-value < 0.001. **** *p*-value < 0.0001. *n* = 3

of fluoroquinolone resistance genes after 3 days (metals alone) and 7 days (alone and in combination with fluoroquinolones), but these differences were no longer significant at day 27 (Fig. 4B). Metal selection for copper and zinc resistance genes increased with time. At day 6, metals alone increased the abundance of copper resistance genes compared to non-metal-exposed samples, and metals combined with antibiotics increased the

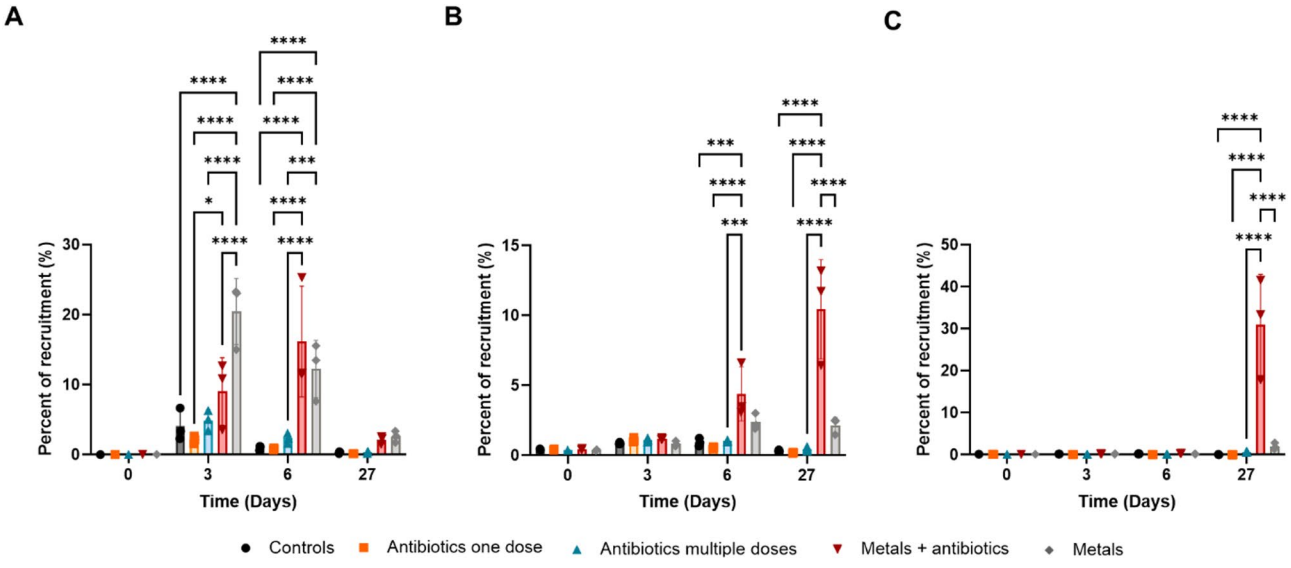

Fig. 5 Relative abundance of metagenome assembled genomes (MAGs) selected by metals in the presence and/or in the absence of antibiotics. **A**: MAG associated with *Pseudomonas*.**B**: MAG associated with *Prosthecobacter*.**C**: MAG associated to *Aquisediminimonas*. Relative abundance was calculated using the percentage of recruitment of each MAG across samples, which represents the percentage of sequences from a sample recruited into a MAG. Only significant differences are shown. **p*-value < 0.05. ***p*-value < 0.01. ****p*-value < 0.001. **** *p*-value < 0.0001. *n* = 3

abundance of these genes compared to samples exposed to antibiotics alone. Most of these differences were still detectable at day 27 (Fig. 4C). Finally, zinc resistance genes were selected in samples exposed to metals (alone and with antibiotics) from day 6 onward (Fig. 4D). Thus, whereas antibiotics did not modify the relative abundance of antibiotic and metal resistance genes, metal pollution led to a transient increase in antibiotic resistance gene abundance (partially explained by the increase in fluoroquinolone resistance gene abundance) and a long-term increase in metal resistance gene abundance in river water metagenomes. The relative abundance of fluoroquinolone resistance genes was correlated to that of copper resistance genes (Pearson = 0.65), and copper and zinc resistance genes showed a strong correlation between them (Pearson = 0.85).

Metagenomes were assembled to establish links between the changes in bacterial community dynamics and the selection of antibiotic and metal resistance genes induced by antibiotic and metal pollution. Seven MAGs were obtained (MAG taxonomy, size, completion, redundancy and ARG/MRG content are shown in Table S3 in Supplementary Information), three of which showed an increased abundance under metal pollution (Fig. 5). Metal pollution, alone and in combination with antibiotics, increased the abundance of a *Pseudomonas* MAG after 3 and 6 exposure days (Fig. 5A). This MAG contained a copper resistance gene, *cueA*, as well as the genes coding for the efflux pump *mexAB-oprM*, which is involved in fluoroquinolone resistance. The relative abundance of *mexAB-oprM* genes both in the metagenomes (non-assembled reads) and within the *Pseudomonas* MAG increased in samples exposed to metals alone for 3 days and to both metals alone and in combination with antibiotics for 6 days (Figure S4 in Supplementary Information). Metal pollution combined with fluoroquinolones increased the relative abundance of a *Prosthecobacter* MAG after 6 and 27 days (Fig. 5B), and this MAG contained a *czcA* zinc resistance gene and an efflux pump gene, *mexF*, which is involved in fluoroquinolone resistance. A third MAG associated with *Aquisediminimonas* showed a significant increase in its abundance under combined antibiotic and metal pollution for 27 days (Fig. 5C), although it did not contain any fluoroquinolone, copper nor zinc resistance genes. In addition, three MAGs associated with *Limnohabitans*, *MAG-120802* and *SYFN01* showed a lower abundance under metal pollution (Figure S5 in Supplementary Information), and one MAG associated with *Fluviicola* decreased in all samples (Figure S6 in Supplementary Information). None of these four partial MAGs contained any fluoroquinolone, copper or zinc resistance genes.

Correlations were evaluated between MAG/ASV abundance and the abundance of resistance genes to determine possible links between key members of the bacterial communities and the antibiotic and metal resistome (Tables S4a, S4b in Supplementary Information). Only the abundance of the *Pseudomonas* MAG showed Pearson coefficients > 0.6 when correlated with total ARGs (Pearson = 0.61), fluoroquinolone resistance genes (Pearson = 0.93), copper resistance genes (Pearson = 0.63) and genes coding for the *mexAB-oprM* efflux pump found in its genome (Pearson = 0.92). Three *Pseudomonas* ASVs were correlated to fluoroquinolone

resistance genes (ASV 1, Pearson = 0.6; ASV 25, Pearson = 0.71, ASV 282, Pearson = 0.67), all of which were selected under the metal-polluted conditions. Thus, the changes in the resistome induced by metal pollution seem to be mostly linked to the selection of *Pseudomonas* in metal-polluted samples. Moreover, pollution levels were correlated to the relative abundance of MAGs, ASVs and resistance genes in samples polluted for 27 days (the only samples where pollutants were measured) to establish possible links between pollutant persistence and the abundance of bacteria and resistance genes (Table S5 in Supplementary Information). Ciprofloxacin had a positive effect on the relative abundance of the *Aquisediminimonas* MAG (Pearson = 0.64), and it was negatively correlated to the abundance of three *Fluviicola* (Pearson = -0.7), whereas ofloxacin did not show any positive or negative correlations. None of the two antibiotics showed any Pearson coefficients > 0.6 when correlated to the abundance of resistance genes and to *Pseudomonas* ASVs. Copper and zinc were positively correlated to *Pseudomonas* (Pearson = 0.92 for copper and 0.72 for zinc) and *Fluviicola* (Pearson = 0.81 for copper and 0.92 for zinc), and negatively correlated to *SYFN01* (Pearson = -0.84 for copper and − 0.64 for zinc). Both metals were positively correlated to copper resistance genes (Pearson = 0.92 for copper and 0.75 for zinc) and zinc resistance genes (Pearson = 0.97 for copper and 0.82 for zinc). Copper alone showed a positive effect on fluoroquinolone resistance gene abundance (Pearson = 0.73) and a negative correlation to total ARG abundance (Pearson = -0.89). Finally, only one *Pseudomonas* ASV abundance was correlated to zinc pollution levels, ASV 282 (Pearson = 0.73). Overall, copper showed the strongest links to the abundance of bacteria and antibiotic and metal resistance genes in Rhône river water after a 27-day exposure period.

Discussion

The simultaneous presence of multiple pollutants in aquatic ecosystems that may persist in the environment for long periods of time raises concerns about their potential to select for antibiotic resistance in environmental bacteria and thus increase human health risk. The objective of this study was to establish links between the persistence of four major pollutants in surface waters, two fluoroquinolone antibiotics and two heavy metals, and the medium-term dynamics of aquatic bacterial communities and their associated resistome. Our results indicate that, despite not being the compound detected at higher levels over time, copper was responsible for most changes observed at the bacterial scale.

Contrary to our hypothesis, metals did not show a higher persistence and lower disappearance over time than antibiotics. Twenty-seven days after a single addition, ofloxacin levels were two times higher than those of copper and almost 10 times higher than those of zinc in the dissolved phase. Our results showed that the fluoroquinolone levels in surface waters after several weeks are antibiotic-specific: whereas high levels of ofloxacin were measured at the end of the experiment, this was not the case for ciprofloxacin. The higher lipophilicity of ciprofloxacin compared to ofloxacin [31] may have favored ciprofloxacin adsorption onto particulate matter in river water. Ciprofloxacin lipophilicity reaches its maximum at a neutral pH [32], whereas the Rhône river has a slightly basic pH (between 7.5 and 8). The occupation of primary sorption sites by ciprofloxacin may explain the faster loss of ciprofloxacin followed by a stabilization at low doses, as well as the faster ofloxacin loss in samples that have been exposed to lower doses of ciprofloxacin. Regarding metals, copper showed a higher persistence over time than zinc.

However, the main driver of changes at the community and resistome level was not the pollutant that showed the highest levels after 27 days, demonstrating the complexity of dose-response relationships in aquatic ecosystems and the need for quantitative studies to determine non-effect doses for major aquatic pollutants. Whereas fluoroquinolones did not correlate to the abundance of fluoroquinolone resistance genes and ARG-containing bacteria, copper showed the strongest impact on overall community composition and on the abundance of fluoroquinolone and metal resistance genes. Our results are consistent with previous research suggesting that copper is a pollutant that induces mechanisms related to the emergence and dissemination of antibiotic resistance, such as oxidative stress, cell membrane permeability, the SOS response and the conjugative transfer of ARGs at sub-inhibitory doses in *E. coli* [11, 33] and the increase of conjugative transfer from *E. coli* to freshwater bacteria at doses similar to the one used in this study [34]. On the other hand, in our study, zinc did not show a strong impact on the selection of antibiotic resistance in river water. Zinc effects on antibiotic resistance selection are controversial: some evidence points to an increase of mutations involved in quinolone resistance and to the expression of multidrug efflux pumps in *E. coli* in the presence of sublethal levels of zinc [35]. However, other studies have shown a limited impact of zinc on oxidative stress and SOS response induction, which was suggested to be linked to its antioxidant activity [11], and even a reduced growth rate and final density of a ciprofloxacin-resistant *E. coli* strain [36]. Zinc is involved in bacterial metabolism and is thus essential to bacterial life [37]. This double-edged role of zinc on bacterial physiology may explain the contrary results found in different experiments and highlights the need for further research to understand the impact of zinc on complex bacterial

communities and antibiotic resistance selection in environmental settings. Finally, regarding the limited impact of fluoroquinolones on antibiotic resistance selection in this study, previous research points to metals as a potentially stronger driver of antibiotic resistance selection in aquatic ecosystems than antibiotics [38]. However, fluoroquinolone-induced mutations in fluoroquinolone targets, which are a major fluoroquinolone resistance mechanism [39], were not addressed in this study.

Our research shows a transient selection for antibiotic resistance in aquatic ecosystems mediated mainly by copper and strongly linked to the short-term selection of some *Pseudomonas* subpopulations that harbor efflux pumps involved in fluoroquinolone resistance as well as copper resistance genes. Strong correlations were found between copper levels, the abundance of *Pseudomonas* (MAG and ASVs) and fluoroquinolone resistance genes (including the *mexAB-OprM* efflux pump found in the *Pseudomonas* MAG) that point to the selection of this member of the community as the main driver of the increase in antibiotic resistance abundance in Rhône river microcosms. *Pseudomonas* is commonly found in this ecosystem and is involved in the response to aquatic pollutants [9, 40]. Some *Pseudomonas* species found in the environment are opportunistic pathogens [41, 42] and the selection of *Pseudomonas* containing genes that encode for multidrug resistance in aquatic ecosystems could increase human health risk, although MAG reconstruction in this study did not provide species-level resolution. In addition, *Prosthecobacter* and *Aquisediminimonas* were also selected under metal long-term sub-inhibitory pressure, although no fluoroquinolone and metal resistance genes were identified in *Aquisediminimonas.* Since MAGs in this study were not 100% complete, the possibility that ARGs/MRGs contained by *Aquisediminimonas* were missed using metagenomics cannot be overlooked. Finally, the short-term increase in the relative proportion of fluoroquinolone resistance genes under metal pollution can be partially explained by the toxic, inhibitory impact of this pollution on some members of the community as illustrated by the reduced diversity in metal-polluted samples at day 3 and the selection against several MAGs that were not associated to ARGs, such as *Limnohabitans*, *MAG-120802* and *SYFN01*. These results illustrate the notion that different individual sensitivities to metals may generate a gradient of responses to overall sub-inhibitory metal levels, with some members being inhibited [43].

The fluoroquinolone resistance genes selected by metals in this study were mainly associated with antibiotic efflux. Although cross-resistance to antibiotics and metals mediated by the same efflux pumps is assumed to be a major mechanism for metal-mediated antibiotic resistance co-selection [7], recent findings suggest that antibiotic and metal efflux pumps are substrate-specific and that the ambiguous annotation in databases may lead to the overestimation of cross-resistance in metagenomic studies [44]. Another major mechanism of antibiotic resistance gene co-selection by metals, co-resistance, is associated with the simultaneous presence of antibiotic and metal resistance genes in the same genetic element [7], usually plasmids [45], insertion sequences [46] and integrons [47]. Plasmid-associated fluoroquinolone resistance genes were not found in our results, and plasmids assembled from the metagenomes contained no ARGs (results not shown). Plasmid-encoded fluoroquinolone resistance genes have been detected at a low abundance (or were under the detection limit) in previous research on the Rhône river [40]. Low-impacted environmental settings such as the Rhône river may have a lower abundance of plasmid-encoded ARGs than highly-polluted ecosystems such as the human microbiome [48], although this lack of detection could also be associated with technical biases in DNA extraction and sequencing. At the sequencing depths obtained in this study, it is likely that plasmid-associated genes, as well as other ARGs that are not amongst the most abundant in the system, remain undetected. Further studies focusing on the plasmidome should determine the relevance of plasmid-mediated resistance in low-impacted environments and the role that metal pollution plays on its abundance and spread. Although several studies have correlated the abundance of ARGs, MRGs and MGE under metal exposure and extrapolated relationships between these [49–51], the metal-induced selection of particular taxa that contain both the metal resistance gene needed to resist metal pressure and one or several ARGs, even if not in the same genetic element, also leads to the co-selection of antibiotic resistance [52]. The alternative mechanism proposed by Gillieatt and Coleman [12], where changes in bacterial dynamics induced by heavy metal exposure in complex communities may promote species that incidentally harbor ARGs, seems more appropriate to describe our results than traditional co-resistance definitions that imply co-occurrence of ARGs and MRGs in a mobile genetic element.

Conclusions

This study addressed a critical gap concerning dose-response relationships in low-impacted freshwater ecosystems and the medium-term impact of individual and combined anthropogenic selective pressures at overall sub-inhibitory levels on bacterial communities and the antibiotic and metal resistomes in river water at both an overall and an individual scale. Our research showed that pollutant persistence in freshwater ecosystems was strongly compound-dependent and that metals should not be assumed to be present in the dissolved phase

longer than antibiotics. In addition, this study demonstrates the short and medium-term impacts of heavy metal pollution at overall sub-inhibitory levels on bacterial communities in freshwater ecosystems with the selection of ARG-harboring members of the community from which *Pseudomonas* stands out. Our results highlight the complexity of dose-response relationships in the environment and the need to establish non-effect levels in a set of environments for different individual pollutants. Finally, this research reflects major differences between the MGE-mediated co-dissemination of antibiotic and metal resistance widely reported in highly polluted ecosystems and the metal-driven antibiotic resistance maintenance in low-impacted freshwater environments, which seems to be mediated by the selection of specific taxa under metal pollution that incidentally harbor ARGs. Further studies should determine the impact of heavy metal pollution on the antibiotic mobilome in low-impacted environments to avoid extrapolating conclusions from highly-polluted scenarios.

Supplementary Information

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Supplementary Material 1

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Author contributions
Conceptualization: CS. Methodology: ED, SV, CS. Formal analysis: ED, CS. Investigation: ED, SV, CS. Data curation: CS. Writing– original draft: ED, CS. Writing– review & editing– SV, AM, SFB, SW, AD, TMV. Visualization: ED, CS. Supervision: CS. Project administration: TMV, CS. Funding acquisition: TMV.

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Data availability
The datasets generated and analyzed in this study are available at the DDBJ repository, BioProject PRJDB19965, DRA accession DRR631545-DRR631663. All codes used in this study is available at: https://github.com/concscid/Dehon-et-al-2025.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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