Science of the Total Environment 857 (2023) 159378

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Study of antibiotic resistance in freshwater ecosystems with low anthropogenic impact

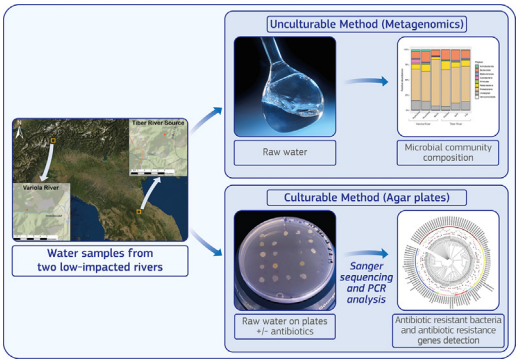
Anna Navarro [a,1], Isabella Sanseverino [a,1], Francesca Cappelli [b,c], Armin Lahm [d], Magdalena Niegowska [a], Marco Fabbri [a], Valentina Paracchini [a], Mauro Petrillo [e], Helle Skejo [a], Sara Valsecchi [c], Rosalba Pedraccini [f], Stefano Guglielmetti [f], Stefano Frattini [f], Gabriella Villani [g], Teresa Lettieri [a,*]

[a] European Commission Joint Research Centre, Via E. Fermi 2749, 21027 Ispra, VA, Italy
[b] Department of Science and High Technology, University of Insubria, Via Valleggio 11, 22100 Como, Italy
[c] Water Research Institute IRSA-CNR, Via del Mulino 19, Brugherio 20861, MB, Italy
[d] Bioinformatic project support, P.za S.M. Liberatrice 18, 00153 Roma, Italy
[e] Seidor Italy srl, Milano, Italy
[f] Alfa S.r.l., via Carrobbio, 3, 21100 Varese, Italy
[g] Energy and Sustainable Economic Development (ENEA), Via E. Fermi 2749, 21027 Ispra, VA, Italy

HIGHLIGHTS

- Bacterial isolates from two low-impacted rivers show resistance to two or more antibiotics.
- ARGs were detected in river water samples not experimentally exposed to antibiotics.
- bla_{TEM} was found in ~58 % of total isolates upon exposure to antibiotics.
- Most isolates belonged to Enterobacterales, Micrococcales and Pseudomonadales orders.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
Freshwater
Antibiotics
Antibiotic resistance
Natural bacterial
Antibiotic resistance genes (ARG)

ABSTRACT

This study aimed to investigate the bacterial diversity and the background level of antibiotic resistance in two freshwater ecosystems with low anthropogenic impact in order to evaluate the presence of natural antimicrobial resistance in these areas and its potential to spread downstream. Water samples from a pre-Alpine and an Apennine river (Variola and Tiber, respectively) were collected in three different sampling campaigns and bacterial diversity was assessed by 16S sequencing, while the presence of bacteria resistant to five antibiotics was screened using a culturable approach. Overall bacterial load was higher in the Tiber River compared with the Variola River. Furthermore, the study revealed the presence of resistant bacteria, especially the Tiber River showed, for each sampling, the presence of resistance to all antibiotics tested, while for the Variola River, the detected resistance was variable, comprising two or more antibiotics. Screening of two resistance genes on a total of one hundred eighteen bacterial isolates from the two rivers showed that bla_{TEM}, conferring resistance to β-lactam antibiotics, was dominant and present in ~58 % of isolates compared to only ~9 % for *mefA/E* conferring resistance to macrolides. Moreover, β-lactam resistance was detected in various isolates showing also resistance to additional antibiotics such as macrolides, aminoglycosides and tetracyclines. These

* Corresponding author at: Via E. Fermi 2749, 21027 Ispra, VA, Italy.
E-mail address: teresa.lettieri@ec.europa.eu (T. Lettieri).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.scitotenv.2022.159378
Received 13 July 2022; Received in revised form 28 September 2022; Accepted 7 October 2022
Available online 20 October 2022

observations would suggest the presence of co-resistant bacteria even in non-anthropogenic environments and this resistance may spread from the environment to humans and/or animals.

1. Introduction

Antimicrobial resistance (AMR) is one of the global concerns for public health, which affects the effectiveness of antibiotics to treat life-threatening infections and the healthcare system (World Health, 2015). Given its clinical relevance, studies on AMR have mainly been focused on the analysis of pathogens from patients or contaminated environments such as wastewater treatment plants (WWTPs) (Czekalski et al., 2014; Pärnänen et al., 2019; Sanseverino et al., 2022a; Szczepanowski et al., 2009). Recently, it has become evident that AMR is common not only among commensal bacteria of humans and animals, but also among environmental bacteria in different ecosystems (Pal et al., 2016), from sediments, including ancient permafrost sediments (30,000 years old) (D'Costa et al., 2011), to surface water samples (Jardine et al., 2019; Lima-Bittencourt et al., 2007). Misuse and overuse of antibiotics with their consequent discharge to the environment have been claimed as the main drivers in the development of antibiotic resistant bacteria (ARB) (WHO, 2021). Besides, chemical pollutants such as heavy metals (e.g. silver) (Durán et al., 2016; Larimer et al., 2014; Li et al., 2017) or other biocides (e.g. triclosan) (Schweizer, 2001) present in environmental matrices constitute an additional selective pressure leading to the occurrence of AMR (Komijani et al., 2021; Lu et al., 2020).

Antibiotics are compounds produced naturally by microorganisms (Ben et al., 2019), and antibiotic resistance is an ancient and natural phenomenon (D'Costa et al., 2011). Indeed, bacteria develop resistance naturally via spontaneous mutations or/and through the acquisition of genetic determinants from other bacteria as a survival mechanism in the presence of environmental stressors (Sanseverino et al., 2018). The occurrence of resistance plasmids in pristine environments without any record of the presence of antibiotics was described in the first field studies investigating antibiotic resistance (Gardner et al., 1969). Other studies have demonstrated that resistance can also be maintained without antibiotic selective pressures (Pontes et al., 2009a), however the extent to which environmental factors may affect the resistance is not fully understood. Additionally, it has been observed that the excessive use of antibiotics in medicine and agriculture stimulates the proliferation and selection of resistance in bacterial communities (Prestinaci et al., 2015).

AMR is mainly transmitted among humans, animals and in natural environments (Iossa and White, 2018) through the acquisition of antibiotic resistance genes (ARG) via mobile genetic elements (MGEs) such as integrons, transposons and plasmids (van Hoek et al., 2011). Recent evidence would suggest that many ARGs found in pathogens may have an environmental origin (Bhullar et al., 2012; D'Costa et al., 2011). Indeed, in the last years, the environment, in particular water, has been identified as a potential source and transmission route for ARGs, posing therefore a potential risk for human health (Ashbolt et al., 2013). However, antibiotic resistance from pristine environments with no or low anthropogenic impact have been much less studied. Consequently, there is a need to investigate in more detail the role of environmental bacteria and their capacity to maintain and to spread AMR.

The aim of this work was to investigate the natural presence of antibiotic resistance in environmental bacterial communities. Water samples were collected at or close to the river source, at three different time points between September 2018 and July 2019 in two freshwater ecosystems with low anthropogenic impact: the Variola River and the Tiber River, Italy. Freshwater ecosystem was considered of having low anthropogenic impact when no agricultural activity, industrial sites, or WWTP effluents were located in the nearby area. We assessed the microbial community composition by 16S ribosomal RNA (rRNA) amplicon sequencing and the presence of bacteria resistant to five antibiotics (two β-lactams, macrolide, aminoglycoside and tetracycline) using a culturable method. In addition, the presence of two ARGs, bla_{TEM} and *mefA/E*, encoding resistance to β-lactams and macrolide antibiotics, respectively, was tested by Polymerase Chain Reaction (PCR) as resistance to these antibiotics was the most abundant in the isolates of both rivers.

2. Materials and methods

2.1. Sampling points

To study the natural background of antibiotic resistance in bacterial population, two different locations were selected as pilot cases, the Variola River and the source of the Tiber River (Fig. 1). The Variola River is located in Monte Ossolano, an alpine area of the Dagliano Valley in Piedmont (North of Italy) with the sampling point located 7 km from the river source, in a rocky riverbed and surrounded by grassland with few trees. The Tiber River is the third longest river in Italy arising in Monte Fumaiolo, in the Northern Apennine in Emilia-Romagna (Central Italy). Sampling was performed at the source, located in a beech forest at 1268 m above the sea level. The sampling point was surrounded by a forest and presented numerous leaves on the riverbed. Sampling campaigns were conducted in September/November 2018 and March 2019 for the Variola River; and in October 2018, April and July 2019 for the Tiber River. The surroundings of the sampling points do not present farms, industries, or effluents of WWTPs. The area of both rivers is frequented by tourists and hikers.

Water for microbiological analysis was collected using 2 acid-washed 10 L bottles, which were rinsed twice before collection. All water samples were transported to the laboratory on ice and processed immediately.

A separate one-liter aliquot of water was collected in a high-density polyethylene (HDPE) dark bottle for antibiotic analysis in each sampling site. The water was immediately stabilized adding 10 mL of ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA{\cdot}2H_2O$) to prevent metal complexation (Opriş et al., 2013). Samples were then transported to the laboratory and stored at 4 °C until extraction, which was performed within 2 days.

2.2. Physicochemical and microbiological parameters

Physicochemical parameters of water samples, including temperature (°C), dissolved oxygen (DO, %), pH and conductivity (μg mL^{-1}) were determined on site for each campaign using an Orion start A329 multi-meter (ThermoFisher Scientific).

Immediately upon arrival to the laboratory, 500 mL and 1 L of each water sample were filtered using GF/C membranes (Whatman™, Kent, UK) for Chlorophyll a (Chl-*a*) analysis. Filter membranes were folded and placed in a 15 mL falcon tube, protected from light with aluminum foil and stored at −20 °C for a maximum of one day. The samples were analyzed by spectrophotometric analysis using a methanol-based extraction (HMSO, 1983). Briefly, 14 mL of 100 % methanol were added to each filter. The samples were then boiled at 70 °C for about 5 min and centrifuged for 7 min at 3500 rpm. The supernatant was carefully poured into a quartz cuvette and optical density (OD) was measured at 665 nm and 750 nm. Chl-*a* content was determined applying the following equation (HMSO, 1983):

$$\text{Chl-}a\left(\mu\text{g L}^{-1}\right) = [13.9 * (\text{OD665nm} - \text{OD750nm}) * \text{v} * \text{d}]/\text{P} * \text{V}$$

where: v - added volume of methanol, d - dilution factor (when applicable), P - cuvette path in cm, V - filtered water sample in liters.

Total suspended solids (TSS) were determined on samples filtered through 0.45 μm pore size filter with the standard method APAT CNR

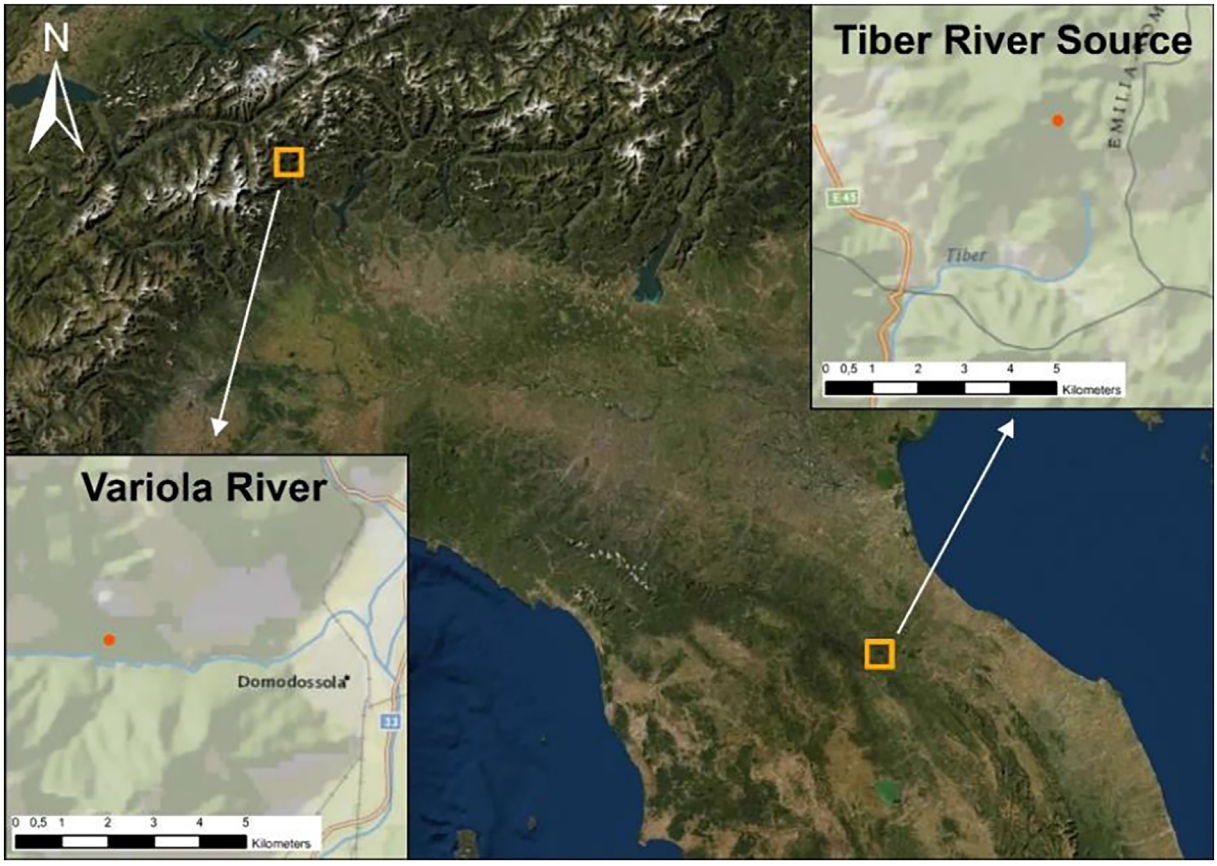


Fig. 1. Geographical location of the sampling points (National Geographic Cartography Basemap). Water samples from the Variola River (46°07′31.2″N - 8°13′29.3″E) were collected in September, November 2018, and March 2019; for the Tiber River source (43°47′10.6″N - 12°04′44.4″E), sampling campaigns took place in October 2018, April and July 2019.

IRSA 2090B Man 29 2003. Anions (NO^-_3 and NO^-_2), total nitrogen (TN) and total organic carbon (TOC) were determined in water samples filtered through 0.22 μm pore size filter. Anions were analyzed by ion chromatography using the standard method APAT CNR IRSA 4020. Total nitrogen was determined using the standard method UNI 11759:2019, while TOC was analyzed using the kit LCK 385 (Hach, Germany) following manufacturer's instructions.

For each sampling time and location, total coliforms, *Escherichia coli (E. coli)* and *Enterococcus* spp. concentrations were determined using Colilert and Enterolert defined substrate assay (IDEXX Laboratories, Westbrook, ME) following the manufacturer's instructions. The values are reported as the most probable number (MPN) per 100 mL^{-1}.

Total bacterial count of all samples was performed by fluorescence analysis. Eight milliliters of each sample were centrifuged at 10,000 $\times g$ for 45 min. The supernatant was discarded, and the pellet resuspended in 1.6 mL of phosphate buffered saline (PBS) (Sigma-Aldrich, Germany) in order to have a sufficient concentration of bacteria during measurements. The staining was done in untreated black 96-well microplates (ThermoFisher Scientific) using 200 μL aliquot of a concentrated sample and 20 μL of SYBR Green I to a final concentration of 10× and incubated at room temperature for 15 min in the dark. Cell amount in each sample was determined using a regression equation generated by a standard curve with a known concentration of a serial diluted (1:10) overnight culture of *E. coli,* with a limit of detection (LOD) of 10^2 cells mL^{-1}. The fluorescence measurements (FI) were performed in triplicate according to Martens-Habbena and Sass (2006) instructions, using an Infinite F200 microplate reader (Tecan, Austria), and the following formula:

$$FI = (Cell/mL)^a \times 10^b$$

where FI is the value of fluorescence, while a and b are the values obtained in the regression equation.

2.3. Antibiotic analysis

Water samples were analyzed for the presence of twenty-two antibiotic compounds belonging to several classes (Table S1). The choice of the antibiotics to be measured was based on their wide presence in the aquatic environment (Sanseverino et al., 2018). All the standards and solvents were purchased from Sigma-Aldrich (Sigma-Aldrich, Germany) at analytical reagent grade or LC-MS grade purity. The samples were filtered on glass micro-fiber GF/F 0.7 μm nominal pore size (Whatman, Kent, UK) and split in two aliquots of 500 mL. The pH of one aliquot was adjusted to 3 with hydrochloric acid (35 %) and used to measure tetracycline antibiotics, ofloxacin, levofloxacin, ciprofloxacin, norfloxacin and amoxicillin. The other aliquot was used to quantify the remnant analytes. Fifty microliters of each internal standard solution (1 μg mL^{-1}) were added to the sample aliquots.

The sample aliquots were extracted on preconditioned Oasis HLB cartridges (200 mg 6 mL^{-1}, Waters, Milford, MA). Elution was performed on cartridges air-dried under vacuum using 6 mL of methanol and then the eluate was concentrated to 1 mL under a stream of nitrogen. Two hundred microliters of extracts were completely dried and then reconstituted with 200 μL of ultrapure water (UW)/methanol (90:10, v/v) to be analyzed by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS). Generally, recoveries were above 75 % with relative standard deviations (RSD) below 15 %; LOQs ranged from 0.2 to 126 ng L^{-1}. The detailed extraction protocol, liquid chromatography parameters and the mass spectrometer settings are reported in the Supplementary material (Paragraph S1 and Tables S1, S2, S3, S4).

2.4. DNA extraction and next generation sequencing (NGS)

For DNA extraction, three biological replicates of 5 L and 1 L from the Variola River and the Tiber River source, respectively, were filtered using a 0.22 μm pore size filter and stored at −20 °C until further processing.

DNA extraction was performed according to the protocol described in Sanseverino et al. (2022b). DNA extracted from the replicates was pooled to avoid internal variations and was stored at −20 °C until further analysis.

2.4.1. Library preparation and DNA sequencing

Amplification of 16S ribosomal RNA (rRNA, variable regions V3-V4) was performed using primer pairs shown in Table S5. Libraries were prepared following the method described in Sanseverino et al. (2021). Sequencing was performed using the IonS5 Instrument (ThermoFisher Scientific) at the Joint Research Centre (JRC) in Ispra (VA, Italy). All 16S rRNA samples were sequenced as 400 bp reads, using Ion 520 Chip Kits (ThermoFisher Scientific).

2.4.2. 16S rRNA sequencing data analysis

Raw read data was first filtered retaining only reads of at least 300 nucleotides in length. Reads that contained amplicons in the reverse sense were reverted to sense strand sequences. To allow for an unbiased comparison between samples, 295,000 reads were then randomly selected from each sample using USEARCH (Edgar, 2010) followed by IU clustering at 97 % sequence identity and taxonomic binning was performed with the SINTAX algorithm (Edgar, 2016) against the GTDB 16S sequence database (Parks et al., 2018). All read data has been deposited at ENA under project ID PRJEB52263 (https://www.ebi.ac.uk/ena/browser/view/PRJEB52263).

2.5. Antibiotic stock solution

Five different antibiotics (erythromycin, ampicillin, amoxicillin, kanamycin, and tetracycline) were purchased from Sigma-Aldrich (Germany). Antibiotic stocks were prepared under aseptic conditions by dissolving the powder in sterile distilled water for ampicillin (10 mg mL^{-1}), amoxicillin (3 mg mL^{-1}) and kanamycin (10 mg mL^{-1}), and ethanol 100 % or 70 % for erythromycin (10 mg mL^{-1}) and tetracycline (10 mg mL^{-1}), respectively. The stocks were sterilized through filtration and aliquots stored at −20 °C. Working stock solutions of antibiotics were freshly prepared the day of agar plate preparation using sterile distilled water.

2.6. Antibiotic resistance test

For the antibiotic resistance test, 10 mL of each water sample were concentrated by centrifugation 5 times at 10,000 ×*g* for 45 min, and resuspended with PBS. An aliquot of 100 μL of the resuspended buffered solution was plated on Luria-Bertani (LB) agar plate containing erythromycin (16 μg mL^{-1}), amoxicillin (10 μg mL^{-1}), ampicillin (32 μg mL^{-1}), kanamycin (35 μg mL^{-1}) or tetracycline (16 μg mL^{-1}). Aliquots were also plated on agar plates without antibiotics as control. These concentrations were used previously in other studies (Garcia-Bermejo et al., 1998; Tacão et al., 2012; Watkinson et al., 2007). To monitor any possible bacterial contamination by handling and plating during the study, a bottle of MilliQ water was carried to the field. It was opened during the water collection, transported, concentrated, and plated as negative control following the protocol for the river water samples. No bacterial growth was observed on the agar plates.

Erythromycin and amoxicillin were selected as they are included on the European Commission's Water Framework Directive priority hazardous substances Watch List (WL) (Carvalho et al., 2015; Loos et al., 2018). Among the β-lactam antibiotics, we also selected ampicillin, while kanamycin and tetracycline were included in the analysis as bacteria resistant to these antibiotics had already been detected in pristine environments (Chen et al., 2017; Leff et al., 1993).

The experiment was performed in triplicate. The agar plates were incubated at room temperature for 6 days to allow slow-growing bacteria to be included in the study with isolates being counted daily. It must be considered that not all natural bacteria are culturable in laboratory, and the agar medium used can also affect bacterial species in obtained colonies.

A representation of five isolates from each treatment (with or without antibiotics), or all isolates in case the number of colonies was lower, were randomly picked, preserved in glycerol and stored at −80 °C for further characterization and determination of resistance.

2.7. DNA extraction and 16 S sequencing from bacterial isolates

DNA from all collected isolates was extracted using the DNeasy Blood and Tissue extraction kit (Qiagen, Germany) according to a slightly modified protocol designed for DNA extraction of Gram-positive bacteria. Briefly, the lysis step was performed using an enzymatic lysis buffer prepared with 37 mg mL^{-1} of lysozyme and an incubation of 3 h. Incubation time for the proteinase was increased to 1 h. The concentration and quality of DNA extracted was determined by spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific) and stored at −20 °C for further analysis.

Twenty-five nanogram of genomic DNA was used for PCR with Klindworth primers (Klindworth et al., 2013) (Table S5). The PCR cycling conditions were: hot start 95 °C for 5 min, 25 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 2 min and elongation at 72 °C for 1 min, followed by a final elongation step at 72 °C for 7 min. Amplicon size was determined using Bioanalyzer (Agilent Technologies). The resulting PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified products were sequenced on Applied Biosystems 3730 (Applied Biosystems) DNA Sequencer with the Big Dye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) in accordance with the producer's indications. Identical primers for PCR and DNA sequencing were used. Sequences obtained were then taxonomically classified using SILVA (Pruesse et al., 2012), aligned and subjected to a phylogenetic analysis with MEGA X (Kumar et al., 2018) and the resulting tree was displayed with iTOL (Letunic and Bork, 2021).

2.8. Detection of antibiotic resistance genes (ARGs) in bacterial isolates

The isolates collected from treated and untreated plates were tested for the presence/absence of two different ARGs: $bla_{TEM,}$ which confers resistance to β-lactam antibiotics and *mefA/E* conferring macrolide resistance. The target genes were amplified by PCR in a SureCycler 8800 thermocycler (Agilent Technologies) using primers shown in Table S5 and the following program: 95 °C for 10 min, then 35 cycles of 95 °C for 5 min, specific annealing temperatures listed in Table S5 for 1 min, and 72 °C for 30 s, with a final extension step of 5 min at 72 °C. Amplification mixtures were prepared in a total volume of 25 μL, including 1× final concentration of PCR buffer, 1.5 mM dNTP, 2.5 mM $MgCl_2$ for bla_{TEM} and 4 mM for *mefA/E*, 0.3 μM of each primer, 1 U Taq DNA polymerase (Qiagen, Germany), and 25 ng of bacterial genomic DNA (gDNA). A negative control was added in all PCR assays to monitor contamination and random amplifications. The amplicons were analyzed on Bioanalyzer (Agilent Technologies) in order to verify the size of the amplification products. Sanger sequencing of one amplified PCR product of each ARG was confirmed by DNA sequencing in an Applied Biosystems 3730 (Applied Biosystems) DNA Sequencer. The amplified sequences were compared to the corresponding reference genes by BLAST algorithm at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.9. Statistical analysis

Statistical analyses to compare culturable and resistant bacteria were performed using R® Studio Software (R version 3.6.1). Shapiro test was used to assess if the data follows a normal distribution. The range mean and standard error of colony forming unit (CFU) mL^{-1} were calculated for isolates and data were compared using Kruskal-Wallis (Dunn test) or ANOVA (Tukey test), depending on the normalization test of the data. p-Values <0.1 for isolates from the Variola River and <0.05 for the Tiber

River were established as threshold for statistical significance. Statistical differences are indicated by small letters labelled within each group (months or antibiotic treatments); different letters indicate significant differences at $p < 0.05$ and $p < 0.1$.

Pearson's correlation analysis was applied to evaluate the relationship between the different antibiotics measured in the sampling campaigns and the presence of ARB isolates and ARGs (*mefA/E* and bla_{TEM}).

A canonical corresponding analysis (CCA) was performed using the vegan package in R (Oksanen et al., 2019). The parameters analyzed were: i) the antibiotic treatments, ii) the bacterial order identified from the isolates collected on the agar plates, iii) the presence or absence of two ARGs in each isolate and iv) the sampling month.

3. Results

3.1. Water characterization

The physicochemical and microbiological parameters of the water samples collected in both rivers (Variola and Tiber) are shown in Table S6. Temperature variations were detected among samples due to the seasonality, with higher temperatures in summer (September and July) as expected. Differences between the two rivers were observed in the conductivity, Chl-*a*, and bacterial abundance while similar values were registered for DO and pH. The Tiber River showed high conductivity values ranging from 200.8 to 331.2 μs cm^{-1} compared with the values observed in the Variola River which were found in a range from 30 to 64 μs cm^{-1}. The highest concentrations of CO_2 fixation organisms were detected in the Tiber River, as confirmed by Chl-*a* measurements (Table S6). A problem during the Chl-*a* measurement occurred for the Variola River sampling in September 2018 and no value could be determined for that month. For both rivers, total organic carbon, nitrite, nitrates, total nitrogen, and total suspended solids were low (Table S6). The measurements of nitrates ranged from 0.3 to 0.4 mg L^{-1} in the Variola River and 0.8 mg L^{-1} in the Tiber River, values below the European limit of 50 mg L^{-1} as mentioned in the Nitrates Directive (91/676/EC). The values of the total nitrogen ranged from <0.1 to 0.3 mg L^{-1} in the Variola River and from 0.8 to 0.9 mg L^{-1} in the Tiber River. Total organic carbon measurements were below LOD (<3 mg L^{-1}) in all sampling campaigns except for March in the Variola River that showed a value of 3.6 mg L^{-1} (Table S6).

Measurements of total coliforms, *E. coli* and *Enterococcus* spp., were carried out as indication of fecal contamination. As displayed in Table S6, the Tiber River showed the highest number of total coliforms during the entire sampling period compared to the lower levels detected in the Variola River. Despite these differences, both rivers are classified as excellent water quality under the European Bathing Water Directive (2006/7/EC) (European Commission, 2006).

To investigate the anthropogenic contamination in the rivers, twenty-two antibiotics (Table S1), belonging to nine different classes, were analyzed in the water samples. Among them, only three antibiotics showed values above the LOQ in at least one sampling month. Clarithromycin was identified in both rivers, at concentrations ranging from of 1 to 2.6 ng L^{-1} while ofloxacin and chlortetracycline were only detected in the Tiber River, at concentrations of 2 and 1.6 ng L^{-1}, respectively (Table S7). Clarithromycin and ofloxacin are semi-synthetic antibiotics, whereas chlortetracycline is a natural antibiotic isolated from *Streptomyces aureofaciens*. In the sampling campaign carried out in April in the Tiber River, three additional antibiotics, ciprofloxacin, norfloxacin and oxytetracycline, were analyzed but their values were below the LOQ (Table S1).

3.2. Bacterial community analysis

Seasonal and site variations in the bacterial community were investigated using 16S amplicon sequencing. As shown in Fig. 2, at the phylum level, the microbial composition was overall similar across all water samples from both rivers with the exception of Cyanobacteria that were predominantly identified only in samples from the Variola River collected in September 2018

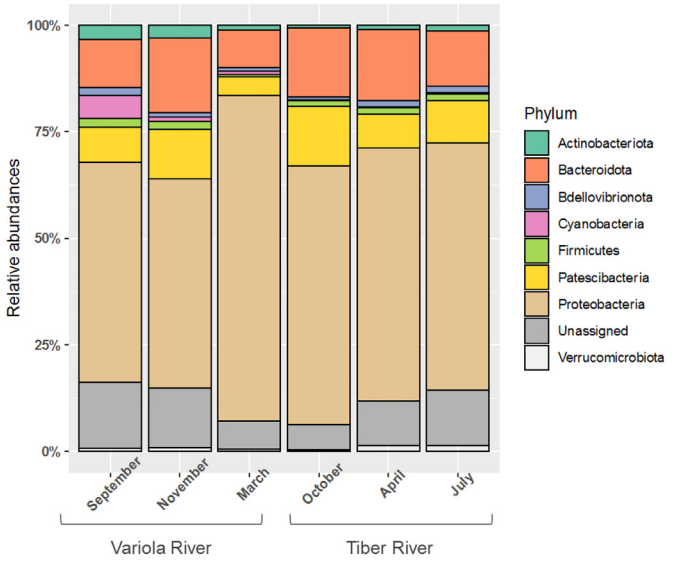


Fig. 2. Taxonomic bacterial composition at the phylum level. Relative abundance of the microbial community composition in each sampling month, for each river (x-axis) as determined by 16S V3-V4 amplicon sequencing. Values represent the percent fraction of all 16S V3-V4 amplicons in the sample (relative abundance, y-axis). Only dominant phyla (values ≥ 1 %) are shown.

(5.4 % relative abundance). However, no detection of Chl-*a* was possible during this sampling month due to a problem with the measurement (Table S6). Thirty different phyla were identified, the most abundant being Proteobacteria followed by Bacteroidota and Patescibacteria (Fig. 2 and Table S8). These three phyla represented over 75 % of the relative abundance in most of the analyzed samples, except for the one collected in September (69 %). Differences among seasonal samplings were observed mainly for the Variola River (Table S8), where Proteobacteria accounted for ~75 % of the relative abundance in March 2019 comparing to the other samplings. Furthermore, the phyla Firmicutes and Actinobacteria showed a higher relative abundance in September and November 2018 compared to March 2019, while values recorded for the Tiber River were constant across the sampling campaigns, with percentages of relative abundance of 1.4 % and 0.6–1.3 %, respectively (Table S8 and Fig. 2).

Among the orders identified, Enterobacterales, Burkholderiales and Sphingomonadales (phylum Proteobacteria) were found as the most representative taxa, followed by the order UBA9983_A (phylum Patescibacteria), Flavobacteriales and Cytophagales (phylum Bacteroidota) (Fig. S1 and Table S9). A remarkable seasonal difference in the Variola River samples and between the two rivers was evident for the order Enterobacterales which showed a higher relative abundance (47.9 %) in March 2019 (Variola) compared to the 13 % observed in the Tiber River (April 2019 sample). The relative abundance of the orders Sphingomonadales, UBA9983_A, Cytophagales and Pseudomonadales were always higher in the fall season respect to the other sampling periods, particularly in the Tiber River and except for Cytophagales (Fig. S1).

3.3. Antibiotic resistant bacteria (ARB) isolates

The presence of ARB was tested on agar plates with and without antibiotics (Fig. 3 and Table S10). The number of isolates obtained from the control (no antibiotics) varied significantly across sampling sites and months (Kruskal-Wallis, $p < 0.001$), with values ranging from 1.3 ± 1.3 to 10 ± 2.3 CFU mL^{-1} in the Variola River and 28.7 ± 2.9 to 95.3 ± 2.7 CFU mL^{-1} in the Tiber River (Fig. 3, Tables S11 and S12). Results of the Variola River should be considered cautiously as they may be compromised by the low number of culturable bacteria which is in line with the data on total cell count whose values were below the LOD (<10^2 cell mL^{-1}). Instead, for samples collected in the Tiber River, the total cell count did not correlate

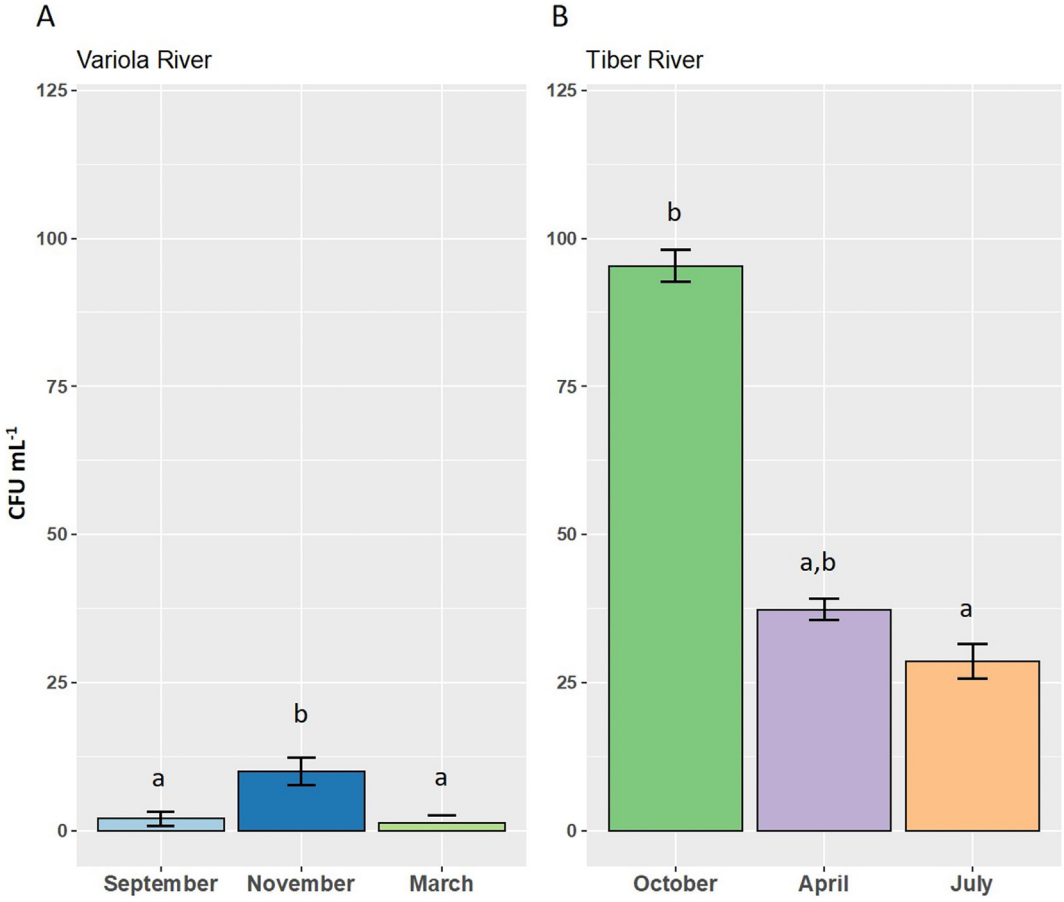


Fig. 3. Average number of bacterial isolates grown on agar plates without antibiotics. The graph shows the average number of culturable bacteria for the Variola River (A) and the Tiber River (B) across different months expressed as colony forming units (CFU) mL^{-1}. Generally, the highest number of culturable bacteria was observed in both rivers during the fall season. The vertical bars represent standard errors. Different letters indicate statistical differences among samples from each river, while an identical letter indicates a statistically not significant difference (Variola: Tukey test, $p < 0.1$, Tiber: Dunn test, $p < 0.05$).

with the number of culturable bacteria, indeed the highest cell count observed in July 2019 (1.7×10^4 CFU mL^{-1}, Table S6) corresponded to the lowest number of culturable bacteria (28.7 ± 4.1 CFU mL^{-1}) as shown in Fig. 3.

Samples collected in autumn (November and October 2018) showed a significantly higher average number of culturable bacteria, with 10 ± 2.31 CFU mL^{-1} (Tukey, $p < 0.1$) and 95.33 ± 2.67 CFU mL^{-1} (Dunn test, $p < 0.05$) in the Variola and the Tiber River, respectively (Fig. 3, Tables S10, S11 and S12). However, for the Tiber River, a statistically significant difference was observed only between values obtained in October and July.

Isolates were also observed on agar plates supplemented with antibiotics (erythromycin, amoxicillin, ampicillin, kanamycin and tetracycline), indicating the presence of ARB in the sampling sites (Fig. 4).

For the Variola River, no statistically significant differences were found between the number of isolates observed on the antibiotic-supplemented plates for any sampled month (Table S13). Water samples collected in November showed the highest number of resistant bacteria compared to samples collected in September and March (Fig. 4). Only resistance to kanamycin was constantly detected in the Variola River, while no isolate showed resistance to tetracycline (Fig. 4). Contrarily, in the Tiber River, isolates were observed in all antibiotic treatments and their number showed statistical significance among treatments performed in October and April (Tukey, $p < 0.05$, Table S14). The highest numbers of ARB were counted on plates supplemented with β-lactams (amoxicillin and ampicillin) and erythromycin followed by kanamycin (Fig. 4 and Table S10). Tetracycline-resistant isolates were observed in all samples collected in the Tiber River, even though at lower levels (2–3 isolates) compared to those isolated upon exposure to the other antibiotics (erythromycin, ampicillin, amoxicillin and kanamycin) (Fig. 4).

3.4. Identification of bacterial isolates and antibiotic resistance genes (ARGs)

A total of 118 bacterial isolates, 36 from the Variola River and 82 from the Tiber River, were collected from agar plates. Twenty-six isolates were collected from the control plates without antibiotics (11 from the Variola River and 15 from the Tiber River). Subsequently, the isolates, upon DNA extraction, were characterized by 16S Sanger sequencing showing that most of the amplicons were present also in the NGS data (Table S15), and by PCR analysis for the detection of two ARGs (bla_{TEM}, and *mefA/E*). Taxonomic analysis revealed 27 different bacterial genera from 17 families, distributed across eleven orders and belonging to two phyla of Gram-negative bacteria (Bacteroidota and Proteobacteria) and two phyla of Gram-positive bacteria (Actinobacteriota and Firmicutes) (Table S16). At first glance, the distribution at the phylum level showed higher variability between the treatments in samples from the Variola River compared to the Tiber River (Fig. 5). Firmicutes were detected only in the Variola River upon treatment with erythromycin and interestingly neither of the two ARGs was present (Fig. 5A and Table S17). In the Variola River, the phylum Bacteroidetes was detected in the presence of erythromycin, ampicillin and amoxicillin (Fig. 5A). In the Tiber River, Bacteroidota was detected on control plates and those treated with ampicillin and kanamycin (Fig. 5B). The observed resistance in Bacteroidota was attributed to bla_{TEM} except the isolate from November, identified as genera *Pedobacter* carrying the *mefA/E* gene (Table S17).

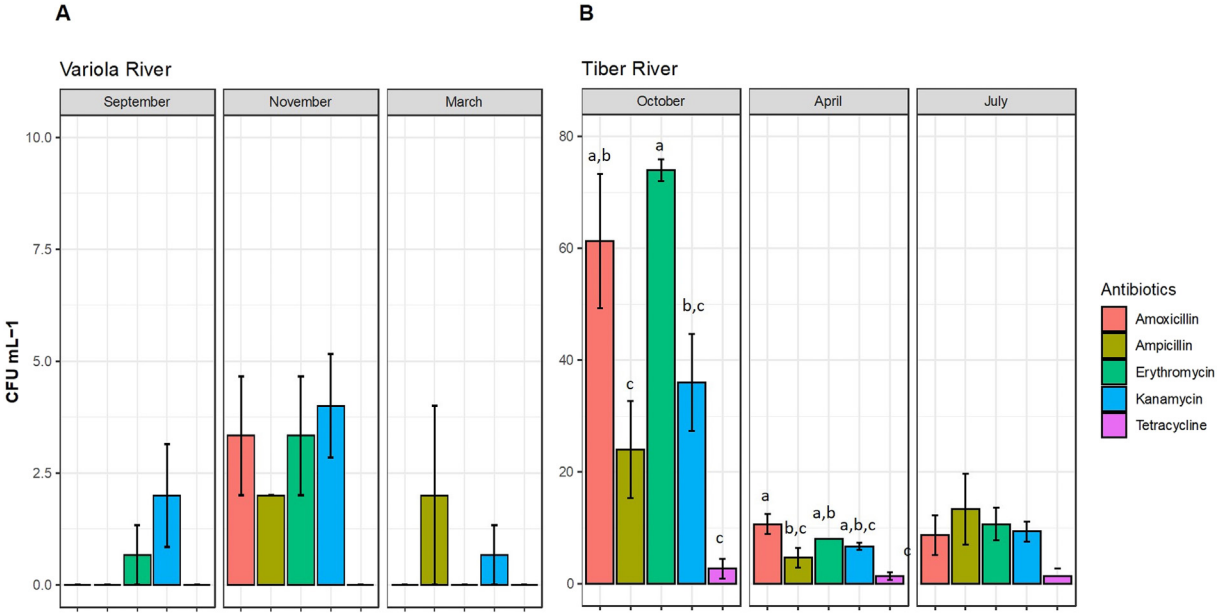


Fig. 4. Bacterial isolates grown on agar plates supplemented with antibiotics. The graphs show the number of isolates for the Variola River (A) and the Tiber River (B) expressed as colony forming units (CFU) mL^{-1} (y-axis). The average of triplicates is shown on the x-axis and the vertical bars represent standard errors. Different letters indicate statistical differences between samples from each river while an identical letter indicates a statistically not significant difference (Tukey $p < 0.05$).

Also isolates obtained from the control treatment already showed the presence of the bla_{TEM} gene. In the Variola River, one isolate was additionally positive to *mefA/E* (Fig. 5 and Table S18). The order Pseudomonadales, highly represented in the Tiber River (35 isolates), was mainly detected in amoxicillin and erythromycin treatment agar plates, while for the Variola River the most frequently detected order was Micrococcales (16 isolates), predominantly in kanamycin treatment followed by the control treatment (Table S17).

For the resistance genes, bla_{TEM} was present in ~58 % (69 colonies) of the total isolates, while *mefA/E* was detected only in ~9 % (11 isolates) of the isolates. Moreover, bla_{TEM} was detected in isolates from all antibiotic treatments, most frequently on plates supplemented with ampicillin (15 isolates) and amoxicillin (13 isolates), as expected (Table S18). Instead *mefA/E* was detected primarily on the erythromycin and tetracycline treatment plates (4 isolates of 21 and 2 isolates of 7, respectively) (Table S18). Overall, 42 isolates were PCR negative for both genes, suggesting that other ARGs were responsible for resistance.

Aligned V3-V4 16S amplicon sequences from all isolates were then displayed as a phylogenetic tree (neighbor-joining consensus tree) together with their associated data: presence/absence of *mefA/E* and bla_{TEM}, treatment and taxonomic classification at the genus and order rank (Fig. 6). Three orders, Enterobacterales, Micrococcales and Pseudomonadales dominate the tree. For Pseudomonadales, only genera *Pseudomonas* and *Acinetobacter* are present. All *Pseudomonas* isolates showed at least one form of resistance, most frequently upon amoxicillin or erythromycin exposure, some displayed ampicillin resistance, only one isolate showed kanamycin resistance and no tetracycline resistance. The gene bla_{TEM} was detected in many but not all of these isolates. For the genus *Acinetobacter*, one isolate was tetracycline resistant. Overall, very few isolates spread among different genera and orders were resistant to tetracycline treatment. On the other hand, resistance to kanamycin exposure was observed mainly for a subset of isolates from the order *Micrococcales*, with three isolates (out of five overall) having both bla_{TEM} and *mefA/E*. Isolates from the control (no treatment) carried mainly the bla_{TEM} gene.

3.5. Canonical corresponding analysis (CCA)

A canonical corresponding analysis (CCA) was performed to investigate a possible correlation between the bacterial orders of the isolates, the antibiotic treatments (exposure or no exposure, i.e. control), and the presence/absence of bla_{TEM} and *mefA/E*. The CCA analysis for the Variola River (Fig. 7A) showed a significant positive correlation between the ampicillin treatment, the presence of the *mefA/E* gene ($F = 2.11$, $p < 0.1$, Table S19) and the orders Bacillales and Propionibacteriales, as indicated by the length of the arrows. Conversely, bla_{TEM} and orders Pseudomonadales and Chitinophagales were positively correlated with the control condition (no antibiotics). Sphingobacteriales and Micrococcales were correlated with erythromycin, amoxicillin and kanamycin treatments at all time-points (September, November and March).

For the Tiber River (Fig. 7B), a different pattern was observed, and the presence of bla_{TEM} was strongly correlated with the ampicillin treatment and with the orders Pseudomonadales and Enterobacterales. The detection of *mefA/E* weakly correlated with the amoxicillin and kanamycin treatment, and a positive correlation was observed between the erythromycin and amoxicillin treatments (Fig. 7B). Erythromycin treatment showed a positive correlation with the presence of Solirubrobacterales and Sphingobacteriales bacteria, while kanamycin with Aeromonadales and Xanthomonadales bacteria.

4. Discussion

In this study, we investigated the presence of ARB in two rivers with low anthropogenic impact in order to assess the presence of antibiotic resistance in natural environments. A pre-Alpine River in Northern Italy (Variola River) and the Tiber River source in the Apennine Mountains, were selected to cover two different freshwater ecosystems. The analysis of the physicochemical parameters showed that the two rivers were of good water quality, with high levels of DO and pH values at optimal range (6–9) (Grochowska, 2020). For conductivity, the normal range found in freshwater streams is from 0 to 1500 μs cm^{-1}. The Tiber River presented values in the range considered adequate to support aquatic biodiversity (values between 150 and 500 μs cm^{-1}), while the Variola River showed lower values (<100 μs cm^{-1}) (Behar et al., 1996). The levels of temperature, pH, conductivity and DO observed in the Tiber River were in the range of the values obtained in another study at the same sampling point (Saccà et al., 2019). The indicators of fecal contamination (*E.coli* and *Enterococci*) also showed an excellent water quality level in both rivers (European Commission, 2006). The main difference observed between the two rivers was the

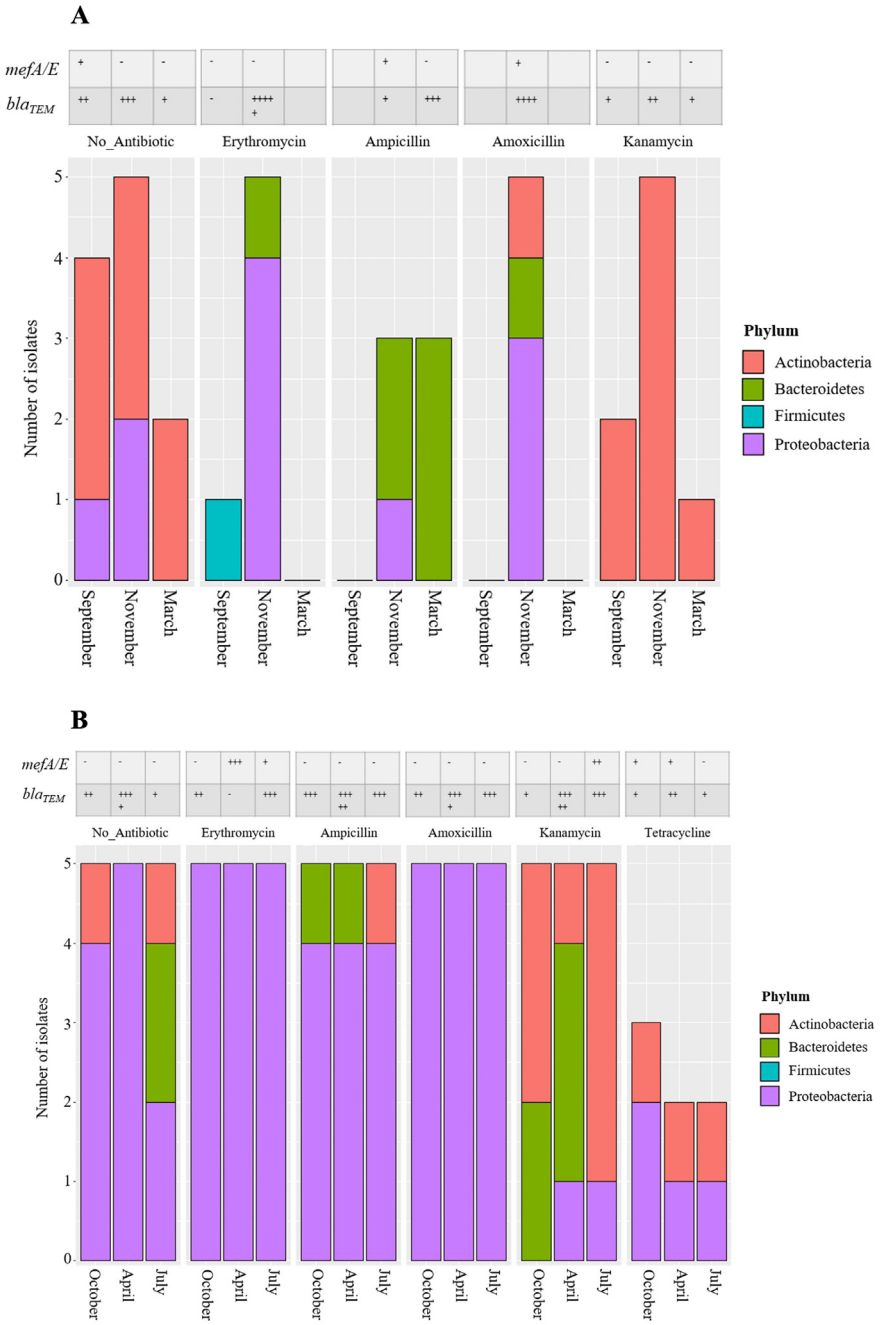


Fig. 5. Phylum-level taxonomic identification of bacterial isolates from agar plates with and without antibiotics and antibiotic resistance genes (ARGs) detection from water samples collected in the Variola River (A) and in the Tiber River (B). Graphics show the microbial composition in each sampling month (x-axis) determined by 16S Sanger sequencing. The results are expressed as the number of isolates identified at phylum level. Detection of the two resistance genes, *mefA/E* and bla_{TEM}, is shown on the top of each graph. The number of + symbols corresponds to the number of isolates which were positive in the PCR.

bacterial load, being considerably lower for the Variola River (isolates and cell count) compared to the Tiber River (Table S6, Fig. 3). This difference is consistent with low levels of conductivity observed in the Variola River; indeed, a high conductivity in water is associated with a high concentration of ions, which could be advantageous to bacterial growth (Wang et al., 2021). Measurements of nitrate, nitrite, total nitrogen, total organic carbon and total suspended solids were low as expected in pristine waters (Table S6).

From a list of 22 antibiotics analyzed during the sampling campaigns, only three were detected in the rivers: two synthetic antibiotics (clarithromycin and ofloxacin), and one natural antibiotic (chlortetracycline) which is extracted from *Streptomyces aureofaciens*. Clarithromycin (macrolide) and ofloxacin (fluoroquinolone) are used to treat a variety of bacterial infections including pneumonia, bronchitis and urinary infections, and an increase in their use is usually expected during cold months to treat cold-related illnesses. Indeed, ofloxacin and clarithromycin have been detected in waters collected from September to November, but their concentrations were always below the environmental safety limits (Alliance, 2018; European Commission, 2018) in both rivers. The detection of pharmaceuticals at the source of the rivers has also been observed in other studies, and their presence was attributed to the infiltration of untreated wastewaters (Paíga et al., 2016), or to the tourism in the area (Mandaric et al., 2017). In our case, further investigations should be conducted to identify the source of the two synthetic antibiotics.

To gain insight into the bacterial community of the two rivers, a 16S amplicon sequencing analysis was performed to investigate the entire microbial community, considering that only a small fraction of bacteria

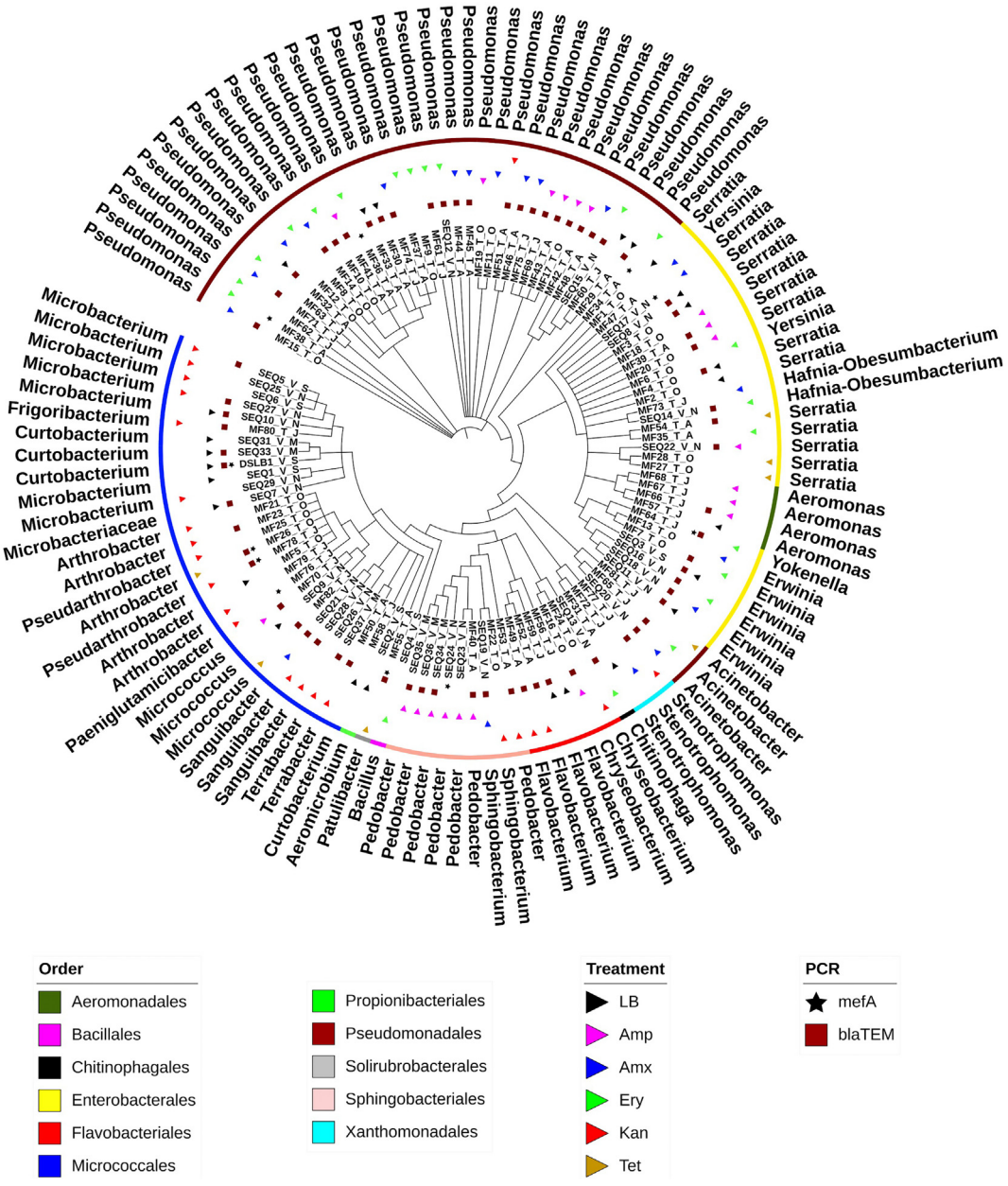


Fig. 6. Phylogenetic tree of isolates. Bacterial order for each genus (outer ring) is represented by the colored circular line according to the scheme at the bottom. Starting from the center, the following information is shown: presence of the antibiotic resistance genes (ARGs) *mefA/E* (black star) and bla_{TEM} (brown square) followed by the treatments represented as triangles: LB as no antibiotic treatment (black), ampicillin (pink), amoxicillin (blue), erythromycin (green), kanamycin (red) and tetracycline (brown). Isolate sequences are labelled including their source (T - Tiber, V - Variola) and the month of sample collection. No antibiotic (LB), erythromycin (Ery), amoxicillin (Amx), ampicillin (Amp), kanamycin (Kan) and tetracycline (Tet).

can be cultured in the laboratory (Schloss and Handelsman, 2004; Stewart, 2012). The most abundant phyla observed in the two sites were consistent with the bacteria usually detected in freshwater ecosystems, in particular Proteobacteria and Bacteroidota together with Actinobacteriota, Cyanobacteria, Firmicutes and Verrucomicrobiota (Lemke et al., 2009; Savio et al., 2015; Tierno de Figueroa et al., 2013; Zwart et al., 2002). Patescibacteria, a phylum widely observed in subsurface habitats and in ground water due to a mobilization process from soil (Herrmann et al., 2019), were present at higher relative abundance in water samples collected in autumn from both rivers compared to the other sampling months. The increase in relative abundance of Patescibacteria could be explained by the precipitation events occurred during October and November that might have resuspended the sediments and mixed the soil bacteria in water. The metagenomic analysis also demonstrated that despite the distance between the two studied areas, the bacterial community did not show big variations. However, in all analyzed samples, >20 % of reads were taxonomically unclassified at the order level, indicating the presence of bacteria not yet annotated in the databases.

The classical approach, based on culturable method, was implemented to study the presence of ARB. Sanger sequencing was then used to identify the individual isolates to better understand which bacteria were resistant to the treatment with selected antibiotics. Despite the differences in bacterial load, an increased number of culturable bacteria were observed on agar plates from samples collected in November from the Variola River and in October from the Tiber River. This finding is in line with the 16S sequencing results showing an increase in the relative abundance of Patescibacteria in these two months. For the same months, the highest number of isolates was observed on agar plates treated with amoxicillin, ampicillin, erythromycin

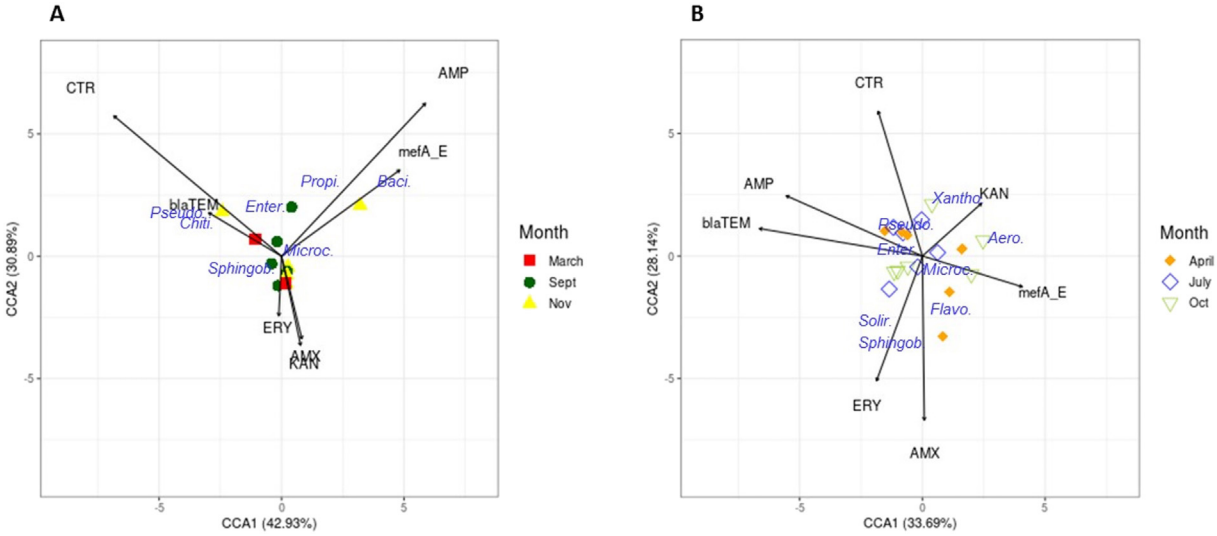


Fig. 7. Canonical corresponding analysis (CCA) of the correlation among the bacterial isolates at order level, treatments in agar plates and antibiotic resistance genes (ARGs) detected. The CCA analysis was performed separately for the Variola River (A) and the Tiber River (B). The arrows indicate the direction and magnitude of the correlation between antibiotic treatment on agar plates where 118 isolates (36 for the Variola River and 82 for the Tiber River) were collected, and the presence/absence of two ARGs associated with the bacterial orders (blue). The antibiotic treatments on agar plates were: no antibiotic (CTR), erythromycin (ERY), amoxicillin (AMX), ampicillin (AMP), kanamycin (KAN) and tetracycline (TET). The ARGs analyzed were: bla_{TEM} (conferring resistance to β-lactams) and *mefA/E* (conferring resistance to macrolides). The symbols represent the different sampling months studied: solid green symbol September (Sept), solid yellow November (Nov) and solid red March for the Variola River; and open symbol green October (Oct), solid orange April and open blue July for the Tiber River. Bacterial orders: Aeromonadales (Aero.), Bacillales (Baci.), Chitinophagales (Chiti.), Enterobacterales (Enter.), Flavobacteriales (Flavo.), Micrococcales (Microc.), Propionibacteriales (Propi.), Pseudomonadales (Pseudo.), Solirubrobacterales (Solir.), Sphingobacteriales (Sphingob.) and Xanthomonadales (Xantho.).

and kanamycin, suggesting a non-specific increase of resistance potentially influenced by soil bacteria (Araya et al., 2003; Di Cesare et al., 2017).

The 16S V3-V4 amplicon sequencing analysis on a total of 118 isolates from agar treatment plates (with and without antibiotics) showed *Pseudomonadaceae* as the most frequently identified family followed by *Yersiniaceae* (both Proteobacteria phylum) in the Tiber River, and *Microbacteriaceae* followed by *Sphingobacteriaceae* (Actinobacteriota and Bacteroidota phylum, respectively) in the Variola River.

Compared to the culture-based method employed in this study, the NGS 16S metagenomic analysis showed, as expected, a more complex microbiome. Differences in results obtained with these two methods are mainly due to the ability of NGS to detect both culturable and unculturable bacteria and also to a different taxonomic classification among databases. However, for 104 out of the 118 isolates, the Sanger amplicon matched a NGS amplicon with sequence identity higher or equal to 99 % and 71 sequences at 100 %.

We selected two resistance genes, bla_{TEM} and *mefA/E* to be monitored in the isolates as they were associated with a higher number of isolates during the study. In our study, bla_{TEM} and *mefA/E* were detected together only in five different isolates, three Actinobacteriota and one Proteobacteria from the Tiber River samples, and one Actinobacteriota from the Variola River. The gene bla_{TEM} confers resistance to penicillins, 1st and 2nd generation of cephalosporins, and the extended-spectrum β-lactamases (ESBLs). It is frequently present on plasmids and transposons (Bradford, 2001; Hooban et al., 2020; Singh et al., 2018) allowing resistance to be transferred to other bacteria by horizontal gene transfer (HGT), using for example the conjugation pathway (Del Grosso et al., 2002). Importantly, bla_{TEM} has been detected worldwide in different freshwater ecosystems (Alves et al., 2020), including those considered at low anthropogenic impact (Lima-Bittencourt et al., 2007; Scott et al., 2020) and in drinking water (Alpay-Karaoglu et al., 2007; Hsu et al., 2014). Sub-inhibitory or environmentally relevant concentrations of anthropogenic contaminants in aquatic ecosystems usually promote HGT (Jiang et al., 2022; Niegowska et al., 2021; Wang et al., 2019). At the same time, water ecosystems have been considered ideal habitats for the spreading of antibiotic resistance (Zhu et al., 2017). In the present study, bla_{TEM} was mainly detected in isolates from agar plates treated with amoxicillin, while *mefA/E,* which confers resistance to macrolides, was mostly observed after treatment with erythromycin, as expected, but also following tetracycline treatments. These results show that macrolide resistance is co-selected after exposure to tetracycline, for example, by the same resistance mechanism such as expression of efflux pumps (Speer et al., 1992; Sutcliffe et al., 1996), and co-selective effects have also been detected following exposure to other antibiotics. This implies that the observed co-selection, which is an important mechanism to induce/enhance ARB in water, was probably due to a natural resistance or to the presence of antibiotics in water which, even at very low concentrations, may contribute to this phenomenon. Indeed, it is known that low levels of these substances in the environment can enhance the development of resistance (Esiobu et al., 2002). Moreover, the detection of ARGs in the selected low-impacted sampling sites is not unexpected, considering that the antibiotic resistance is a naturally occurring phenomenon.

As for bla_{TEM}, the *mef* gene is located on MGEs, in transposons. *Mef*-carrying bacteria can transfer this element to other bacteria by the conjugation pathway and facilitate the spread of resistance (Del Grosso et al., 2002).

Looking at the analyses performed at the order/genus level, within the order Pseudomonadales, one of the most diverse and ecologically significant groups of bacteria, the most frequently detected genus was *Pseudomonas*, bearing bla_{TEM} in 23 out of 35 isolates. Due to its versatility and adaptability, it can be found in the major natural aquatic and terrestrial environments (Palleroni, 2015; Spiers et al., 2000). The genus *Pseudomonas* may endanger human health due to species considered opportunistic pathogens infecting humans, such as *P. aeruginosa* (Mena and Gerba, 2009), which is on the WHO list of priority pathogens posing threat to human health (WHO, 2017). For the order Enterobacterales, *Serratia* was the most frequently identified genus with the detection of bla_{TEM} in 7 out of 14 isolates, followed by *Erwinia* with bla_{TEM} in 5 out of 6 isolates (Table S17). The genus *Serratia* can be found in different environments, including pristine freshwater ecosystems (Lima-Bittencourt et al., 2007; Pontes et al., 2009b), soil (Rusznyák et al., 2012) and plants (Grimont et al., 1981). Although most of the species belonging to this genus are non-pathogenic, *S. marcescens* is the main pathogenic species for either humans or animals. The genus *Erwinia* contains mostly plant pathogenic species, although occasionally it can cause

infection in humans, becoming an opportunistic pathogen (Bottone and Schneierson, 1972). We also identified resistance genes in *Pedobacter* (Bacteroidota phylum) which also has been detected in pristine environments (Markúsdóttir et al., 2013), and in *Arthrobacter*, known to include environmental superbugs and opportunistic pathogens, respectively (Huang et al., 2005; Viana et al., 2018).

In the CCA analysis, which was performed to identify possible correlations between bacterial orders, antibiotic treatments, and resistance genes, a correlation between bla_{TEM} and the control plates was observed for samples collected in the Variola River. However, this result should be interpreted with caution due to the low number of bacterial isolates obtained. For the Tiber River, bla_{TEM} detection was correlated with ampicillin treatment, as expected. The lack of correlation between *mefA/E* and erythromycin treatment may be due to unanalyzed genes conferring resistance to macrolides such as *erm* or *mph* (Varaldo et al., 2009), and suggests a co-selection of resistance.

The positive correlation between clarithromycin concentrations and the amount of isolates resistant to erythromycin suggests that antibiotic concentrations below the proposed environmental safety limits (Bengtsson-Palme and Larsson, 2016) influence the functional resistance of environmental bacteria, an effect not easily detectable by the presence of the ARGs. Further studies need be carried out to assess a possible correlation between low concentrations of antibiotics and the presence of ARGs, as well as the effect of these concentrations on the environmental resistome.

Overall, this study allowed characterizing the background level of resistance in two low-impacted areas, which will be useful in a future comparison to downstream sites subjected to different anthropogenic pressures. In particular, our results show the presence of resistant and co-resistant bacteria in the selected sampling sites and further analysis will be necessary to better understand if the observed resistance developed following exposure to particular stimuli (e.g. metals) or if it represents a naturally-occurring phenomenon. Additionally, the detection of isolates bearing ARGs which are frequently detected in MGEs may represent a factor concurring to the spread of resistance thorough HGT. Considering that some of the detected bacteria belong to genera including opportunistic human pathogens, their spread in the environment could have serious health implications on both humans and animals. Surveillance is therefore required to monitor ARB and ARG prevalence in the environment to better understand the potential threat they may pose to public health.

5. Conclusions

Although the AMR impact on water environments has been extensively investigated in areas with high anthropogenic activities, less is known on the selective pressure promoted by antibiotics in low-impacted sites. In our study, we demonstrate the presence of bacterial resistance to at least two antibiotics in a pre-Alpine river and to all antibiotics tested in an Apennine river with low anthropogenic impact. The difference of resistance found in the rivers suggests that low concentration of antibiotics could affect the resistome in the river. We also identified genera of culturable bacteria, which have recently been recognized or suggested to include opportunistic pathogens as carriers of ARGs. Our results showed the presence of cross-resistance and the presence of resistance to macrolides and β-lactams simultaneously in some genera which could contribute to the spread of AMR in the ecosystem. On the other hand, metagenomics enabled us to characterize the overall microbial community, including unculturable bacteria. In this case, further studies would be needed to link the ARGs to species carrying the resistance. Although additional investigations need to be performed to better define the development of resistance and the impact of the antibiotic contamination in water ecosystems, our findings show which bacteria can be source of AMR from the environments to humans and animals. At the same time, our study contributes to the generation of ARG profiles in areas with low anthropogenic activities. Such profiles, once integrated with studies performed on impacted sites, could help in the identification of selected ARB and ARGs as indicators of AMR spread and in the implementation of strategic monitoring programs to mitigate the AMR and its effects on humans and environmental health.

CRediT authorship contribution statement

Anna Navarro: Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Isabella Sanseverino:** Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Francesca Cappelli:** Investigation, Formal analysis, Writing – review & editing. **Armin Lahm:** Data curation, Visualization, Writing – original draft, Writing – review & editing. **Magdalena Niegowska:** Visualization, Writing – original draft, Writing – review & editing. **Marco Fabbri:** Data curation, Writing – review & editing. **Valentina Paracchini:** Investigation, Formal analysis, Writing – review & editing. **Mauro Petrillo:** Data curation, Writing – review & editing. **Helle Skejo:** Investigation, Formal analysis, Writing – review & editing. **Sara Valsecchi:** Investigation, Formal analysis, Writing – review & editing. **Rosalba Pedraccini:** Investigation, Formal analysis, Writing – review & editing. **Stefano Guglielmetti:** Investigation, Formal analysis, Writing – review & editing. **Stefano Frattini:** Investigation, Formal analysis, Writing – review & editing. **Gabriella Villani:** Visualization, Writing – review & editing. **Teresa Lettieri:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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.

Acknowledgments

These studies have been performed within the Project Antibiotic4you, “Antibiotics in aquatic environment: mechanisms and key factors to understand the environmental contribution to the antimicrobial resistance” funded by the European Commission Joint Research Centre (JRC) as an exploratory project. The authors wish to express their gratitude to the Exploratory Research community for funding the project. A special thanks to Simona Tavazzi for her support with the chemical analysis and Robert Loos (JRC) for having organized and participated in the water sampling campaign; to Dimitar Marinov for validating the results in the statistical analysis, and to Stefano Polesello (Water Research Institute - CNR) for his support. Antibiotic analysis have been carried out in the framework of PhD of Francesca Cappelli (Ph.D. Program in Chemical and Environmental Sciences, University of Insubria, Italy) who thanks Roberta Bettinetti (Department of Human Sciences and Innovation for the Territory, University of Insubria, Como, Italy) for tutoring. We also thank Dr. Desdemona Oliva and Dr. Silvia Longhi for their help on the nutrient's measurements in the water samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159378.

References

Alliance, A.I., 2018. AMR Industry Alliance ANtibiotic Discharge Targets. list of Predicted No-Effect COncentrations (PNECs).

Alpay-Karaoglu, S., Ozgumus, O.B., Sevim, E., Kolayli, F., Sevim, A., Yesilgil, P., 2007. Investigation of antibiotic resistance profile and TEM-type β-lactamase gene carriage of ampicillin-resistantEscherichia coli strains isolated from drinking water. Ann. Microbiol. 57, 281.

Alves, J., Dias, L., Mateus, J., Marques, J., Graças, D., Ramos, R., Seldin, L., Henriques, I., Silva, A., Folador, A., 2020. Resistome in Lake bolonha, brazilian Amazon: identification of genes related to resistance to broad-Spectrum antibiotics. Front. Microbiol. 11.

Araya, R., Tani, K., Takagi, T., Yamaguchi, N., Nasu, M., 2003. Bacterial activity and community composition in stream water and biofilm from an urban river determined by fluorescent in situ hybridization and DGGE analysis. FEMS Microbiol. Ecol. 43, 111–119.

Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., et al., 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ. Health Perspect. 121, 993–1001.

Behar, S., Byrne, J., Dates, G., 1996. Testing the Waters: Chemical and Physical Vital Signs of a River (River Watch Network).

Ben, Y., Fu, C., Hu, M., Liu, L., Wong, M.H., Zheng, C., 2019. Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: a review. Environ. Res. 169, 483–493.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ. Int. 86, 140–149.

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A., Wright, G.D., 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE 7, e34953.

Bottone, E., Schneierson, S.S., 1972. Erwinia species: an emerging human pathogen. Am. J. Clin. Pathol. 57, 400–405.

Bradford, P.A., 2001. Extended-spectrum & #x3b2;-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14, 933–951.

Carvalho, R.N., Ceriani, L., Ippolito, A., Lettieri, T., 2015. Development of the First Wtach List Under the Environmental Quality Standards. (Publication Office of the European Union).

Chen, Z., Yu, D., He, S., Ye, H., Zhang, L., Wen, Y., Zhang, W., Shu, L., Chen, S., 2017. Prevalence of antibiotic-resistant *Escherichia coli* in drinking water sources in Hangzhou City. Front. Microbiol. 8.

Czekalski, N., Díez, E.G., Bürgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 8, 1381.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., 2011. Antibiotic resistance is ancient. Nature 477, 457.

Del Grosso, M., Iannelli, F., Messina, C., Santagati, M., Petrosillo, N., Stefani, S., Pozzi, G., Pantosti, A., 2002. Macrolide efflux genes mef(A) and mef(E) are carried by different genetic elements in Streptococcus pneumoniae. J. Clin. Microbiol. 40, 774–778.

Di Cesare, A., Eckert, E.M., Rogora, M., Corno, G., 2017. Rainfall increases the abundance of antibiotic resistance genes within a riverine microbial community. Environ. Pollut. 226, 473–478.

Durán, N., Durán, M., de Jesus, M.B., Seabra, A.B., Fávaro, W.J., Nakazato, G., 2016. Silver nanoparticles: a new view on mechanistic aspects on antimicrobial activity. Nanomed. Nanotechnol. Biol. Med. 12, 789–799.

Edgar, 2016 SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. bioRxiv.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.

Esiobu, N., Armenta, L., Ike, J., 2002. Antibiotic resistance in soil and water environments. Int. J. Environ. Health Res. 12, 133–144.

European Commission, E., 2006. Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing Water Quality and Repealing Directive 76/160/EEC. (Off. J. Eur. Union.), pp. 37–51.

European Commission, E., 2018. COMMISSION IMPLEMENTING DECISION (EU) 2018/840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495.

Garcia-Bermejo, I., Cacho, J., Orden, B., Alós, J.I., Gómez-Garcés, J.L., 1998. Emergence of erythromycin-resistant, clindamycin-susceptible Streptococcus pyogenes isolates in Madrid, Spain. Antimicrob. Agents Chemother. 42, 989–990.

Gardner, P., Smith, D., Beer, H., Moellering, R., 1969. Recovery of resistance (R) factors from a drug-free community. Lancet 294, 774–776.

Grimont, P.A.D., Grimont, F., Starr, M.P., 1981. Serratia species isolated from plants. Curr. Microbiol. 5, 317–322.

Grochowska, J., 2020. Assessment of Water Buffer Capacity of Two Morphometrically Different, Degraded. Urban Lakes. Water 12, 1512.

Herrmann, M., Wegner, C.-E., Taubert, M., Geesink, P., Lehmann, K., Yan, L., Lehmann, R., Totsche, K.U., Küsel, K., 2019. Predominance of Cand. Patescibacteria in Groundwater Is Caused by Their Preferential Mobilization From Soils and Flourishing Under Oligotrophic Conditions. Front. Microbiol. 10.

HMSO, 1983 The Determination of chlorophyll a in aquatic environments 1980. in: Methods for the extraction of water and associated materials.

Hooban, B., Joyce, A., Fitzhenry, K., Chique, C., Morris, D., 2020. The role of the natural aquatic environment in the dissemination of extended spectrum beta-lactamase and carbapenemase encoding genes: a scoping review. Water Res. 180, 115880.

Hsu, C.-Y., Hsu, B.-M., Ji, W.-T., Chang, T.-Y., Kao, P.-M., Tseng, S.-F., Shen, T.-Y., Shih, F.-C., Fan, C.-W., Liu, J.-H., 2014. A potential association between antibiotic abuse and existence of related resistance genes in different aquatic environments. Water Air Soil Pollut. 226, 2235.

Huang, Y., Zhao, N., He, L., Wang, L., Liu, Z., You, M., Guan, F., 2005. Arthrobacter scleromae sp. nov. isolated from human clinical specimens. J. Clin. Microbiol. 43, 1451–1455.

Iossa, G., White, P.C., 2018. The natural environment: a critical missing link in national action plans on antimicrobial resistance. Bull. World Health Organ. 96, 858–860.

Jardine, J., Mavumengwana, V., Ubomba-Jaswa, E., 2019. Antibiotic resistance and heavy metal tolerance in cultured bacteria from hot springs as indicators of environmental intrinsic resistance and tolerance levels. Environ. Pollut. 249, 696–702.

Jiang, Q., Feng, M., Ye, C., Yu, X., 2022. Effects and relevant mechanisms of non-antibiotic factors on the horizontal transfer of antibiotic resistance genes in water environments: a review. Sci. Total Environ. 806, 150568.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1.

Komijani, M., Shamabadi, N.S., Shahin, K., Eghbalpour, F., Tahsili, M.R., Bahram, M., 2021. Heavy metal pollution promotes antibiotic resistance potential in the aquatic environment. Environ. Pollut. 274, 116569.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.

Larimer, C., Islam, M.S., Ojha, A., Nettleship, I., 2014. Mutation of environmental mycobacteria to resist silver nanoparticles also confers resistance to a common antibiotic. Biometals 27, 695–702.

Leff, L.G., Dana, J.R., McArthur, J.V., Shimkets, L.J., 1993. Detection of Tn5-like sequences in kanamycin-resistant stream bacteria and environmental DNA. Appl. Environ. Microbiol. 59, 417–421.

Lemke, M.J., Lienau, E.K., Rothe, J., Pagioro, T.A., Rosenfeld, J., DeSalle, R., 2009. Description of freshwater bacterial assemblages from the Upper Paraná River Floodpulse System, Brazil. Microb. Ecol. 57, 94–103.

Letunic, I., Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49, W293–W296.

Li, L.-G., Xia, Y., Zhang, T., 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. ISME J. 11, 651–662.

Lima-Bittencourt, C.I., Cursino, L., Gonçalves-Dornelas, H., Pontes, D.S., Nardi, R.M., Callisto, M., Chartone-Souza, E., Nascimento, A.M., 2007. Multiple antimicrobial resistance in Enterobacteriaceae isolates from pristine freshwater. Genet. Mol. Res. 6, 510–521.

Loos, R., Marinov, D., Sanseverino, I., Napierska, D., Lettieri, T., 2018. Review of the 1st Watch List Under the Water Framework Directive and Recommendations for the 2nd Watch List. (Publications Office of the European Union.).

Lu, J., Wang, Y., Zhang, S., Bond, P., Yuan, Z., Guo, J., 2020. Triclosan at environmental concentrations can enhance the spread of extracellular antibiotic resistance genes through transformation. Sci. Total Environ. 713, 136621.

Mandaric, L., Diamantini, E., Stella, E., Cano-Paoli, K., Valle-Sistac, J., Molins-Delgado, D., Bellin, A., Chiogna, G., Majone, B., Diaz-Cruz, M.S., et al., 2017. Contamination sources and distribution patterns of pharmaceuticals and personal care products in Alpine rivers strongly affected by tourism. Sci. Total Environ. 590–591, 484–494.

Markúsdóttir, M., Heiðmarsson, S., Eyþórsdóttir, A., Magnússon, K.P., Vilhelmsson, O., 2013. The natural and anthropogenic microbiota of Glerá, a sub-arctic river in northeastern Iceland. Int. Biodeterior. Biodegradation 84, 192–203.

Martens-Habbena, W., Sass, H., 2006. Sensitive determination of microbial growth by nucleic acid staining in aqueous suspension. Appl. Environ. Microbiol. 72, 87–95.

Mena, K.D., Gerba, C.P., 2009. Risk Assessment of Pseudomonas aeruginosa in Water. In: Whitacre, D.M. (Ed.)Reviews of Environmental Contamination and Toxicology. Vol 201. Springer, US), (Boston, MA, pp. 71–115.

Niegowska, M., Sanseverino, I., Navarro, A., Lettieri, T., 2021. Knowledge gaps in the assessment of antimicrobial resistance in surface waters. FEMS Microbiol. Ecol. 97.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., 2019. Vegan: Community Ecology Package. R Package Version 2.5-6.

Opriş, O., Soran, M.-L., Coman, V., Copaciu, F., Ristoiu, D., 2013. Determination of some frequently used antibiotics in waste waters using solid phase extraction followed by high performance liquid chromatography with diode array and mass spectrometry detection. Cent. Eur. J. Chem. 11, 1343–1351.

Paíga, P., Santos, L.H.M.L.M., Ramos, S., Jorge, S., Silva, J.G., Delerue-Matos, C., 2016. Presence of pharmaceuticals in the Lis river (Portugal): sources, fate and seasonal variation. Sci. Total Environ. 573, 164–177.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2016. The structure and diversity of human, animal and environmental resistomes. Microbiome 4, 54.

Palleroni, N.J., 2015. Pseudomonas. In Bergey's Manual of Systematics of Archaea and Bacteria 1 1.

Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.-A., Hugenholtz, P., 2018. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat. Biotechnol. 36, 996–1004.

Pärnänen, K.M.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., et al., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5, eaau9124.

Pontes, D.S., Pinheiro, F.A., Lima-Bittencourt, C.I., Guedes, R.L., Cursino, L., Barbosa, F., Santos, F.R., Chartone-Souza, E., Nascimento, A.M., 2009a. Multiple antimicrobial resistance of gram-negative bacteria from natural oligotrophic lakes under distinct anthropogenic influence in a tropical region. Microb. Ecol. 58, 762–772.

Pontes, D.S., Pinheiro, F.A., Lima-Bittencourt, C.I., Guedes, R.L.M., Cursino, L., Barbosa, F., Santos, F.R., Chartone-Souza, E., Nascimento, A.M.A., 2009b. Multiple antimicrobial resistance of Gram-negative bacteria from natural oligotrophic lakes under distinct anthropogenic influence in a tropical region. Microb. Ecol. 58, 762–772.

Prestinaci, F., Pezzotti, P., Pantosti, A., 2015. Antimicrobial resistance: a global multifaceted phenomenon. Pathog. Glob. Health 109, 309–318.

Pruesse, E., Peplies, J., Glöckner, F.O., 2012. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28, 1823–1829.

Rusznyák, A., Akob, D.M., Nietzsche, S., Eusterhues, K., Totsche, K.U., Neu, T.R., Frosch, T., Popp, J., Keiner, R., Geleteny, J., et al., 2012. Calcite biomineralization by bacterial isolates from the recently discovered pristine karstic herrenberg cave. Appl. Environ. Microbiol. 78, 1157–1167.

Saccà, M.L., Ferrero, V.E.V., Loos, R., Di Lenola, M., Tavazzi, S., Grenni, P., Ademollo, N., Patrolecco, L., Huggett, J., Caracciolo, A.B., et al., 2019. Chemical mixtures and fluorescence in situ hybridization analysis of natural microbial community in the Tiber river. Sci. Total Environ. 673, 7–19.

Sanseverino, I., Navarro Cuenca, A., Loos, R., Marinov, D., Lettieri, T., 2018. State of the Art on the Contribution of Water to Antimicrobial Resistance. European Union, Brussels.
Sanseverino, I., Navarro Cuenca, A., Lahm, A., Petrillo, M., Fabbri, M., Gomez Cortes, L., Farnleitner, A., Kirschner, A., Zarfel, G., Zaccara, S., et al., 2021. Chapter 21 Microbe: Microbial community and environmental DNA analysis. oint Danube Survey 4 Scientific Report: A shared analysisof the Danube River. ICPDR – International Commission for the Protection of the Danube River, pp. 199–212 (Vienna, Austria).
Sanseverino, I., Gómez, L., Navarro, A., Cappelli, F., Niegowska, M., Lahm, A., Barbiere, M., Porcel-Rodríguez, E., Valsecchi, S., Pedraccini, R., et al., 2022a. Holistic approach to chemical and microbiological quality of aquatic ecosystems impacted by wastewater effluent discharges. Sci. Total Environ. 835, 155388.
Sanseverino, I., Pretto, P., António, D.C., Lahm, A., Facca, C., Loos, R., Skejo, H., Beghi, A., Pandolfi, F., Genoni, P., et al., 2022b. Metagenomics analysis to investigate the microbial communities and their functional profile during cyanobacterial blooms in Lake Varese. Microb. Ecol. 83, 850–868.
Savio, D., Sinclair, L., Ijaz, U.Z., Parajka, J., Reischer, G.H., Stadler, P., Blaschke, A.P., Blöschl, G., Mach, R.L., Kirschner, A.K., et al., 2015. Bacterial diversity along a 2600 km river continuum. Environ. Microbiol. 17, 4994–5007.
Schloss, P.D., Handelsman, J., 2004. Status of the microbial census. Microbiol. Mol. Biol. Rev. 68, 686–691.
Schweizer, H.P., 2001. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiol. Lett. 202, 1–7.
Scott, L.C., Lee, N., Aw, T.G., 2020. Antibiotic resistance in minimally human-impacted environments. Int. J. Environ. Res. Public Health 17, 3939.
Singh, N.S., Singhal, N., Virdi, J.S., 2018. Genetic environment of blaTEM-1, blaCTX-M-15, blaCMY-42 and characterization of integrons of *Escherichia coli* isolated from an indian urban aquatic environment. Front. Microbiol. 9.
Speer, B.S., Shoemaker, N.B., Salyers, A.A., 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin. Microbiol. Rev. 5, 387–399.
Spiers, A.J., Buckling, A., Rainey, P.B., 2000. The causes of Pseudomonas diversity. Microbiology 146, 2345–2350.
Stewart, E.J., 2012. Growing unculturable bacteria. J. Bacteriol. 194, 4151–4160.
Sutcliffe, J., Grebe, T., Tait-Kamradt, A., Wondrack, L., 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. 40, 2562–2566.
Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.-H., Gutzkow, T., Eichler, W., Puhler, A., Schluter, A., 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. (Author abstract)(Report). Microbiology 155, 2306.
Tacão, M., Correia, A., Henriques, I., 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of bla(CTX-M)-like genes. Appl. Environ. Microbiol. 78, 4134–4140.
Tierno de Figueroa, J.M., López-Rodríguez, M.J., Fenoglio, S., Sánchez-Castillo, P., Fochetti, R., 2013. Freshwater biodiversity in the rivers of the Mediterranean Basin. Hydrobiologia 719, 137–186.
van Hoek, A.H., Mevius, D., Guerra, B., Mullany, P., Roberts, A.P., Aarts, H.J., 2011. Acquired antibiotic resistance genes: an overview. Front. Microbiol. 2, 203.
Varaldo, P.E., Montanari, M.P., Giovanetti, E., 2009. Genetic elements responsible for erythromycin resistance in streptococci. Antimicrob. Agents Chemother. 53, 343–353.
Viana, A.T., Caetano, T., Covas, C., Santos, T., Mendo, S., 2018. Environmental superbugs: the case study of Pedobacter spp. Environ. Pollut. 241, 1048–1055.
Wang, J., Fan, H., He, X., Zhang, F., Xiao, J., Yan, Z., Feng, J., Li, R., 2021. Response of bacterial communities to variation in water quality and physicochemical conditions in a river-reservoir system. Glob. Ecol. Conserv. 27, e01541.
Wang, Y., Lu, J., Mao, L., Li, J., Yuan, Z., Bond, P.L., Guo, J., 2019. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. ISME J. 13, 509–522.
Watkinson, A.J., Micalizzi, G.R., Bates, J.R., Costanzo, S.D., 2007. Novel method for rapid assessment of antibiotic resistance in Escherichia coli isolates from environmental waters by use of a modified chromogenic agar. Appl. Environ. Microbiol. 73, 2224–2229.
WHO, 2017. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis.
WHO, 2021 Antimicrobial Resistance.
World Health, O., 2015. Global action plan on antimicrobial resistance. World Health Organization, Geneva.
Zhu, Y.-G., Zhao, Y., Li, B., Huang, C.-L., Zhang, S.-Y., Yu, S., Chen, Y.-S., Zhang, T., Gillings, M.R., Su, J.-Q., 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. Nat. Microbiol. 2, 16270.
Zwart, G., Kamst-van Agterveld, M., Hagen, F., Han, S.-K., Byron, C., 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat. Microb. Ecol. 28, 141–155.