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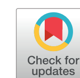
Holistic approach to chemical and microbiological quality of aquatic ecosystems impacted by wastewater effluent discharges



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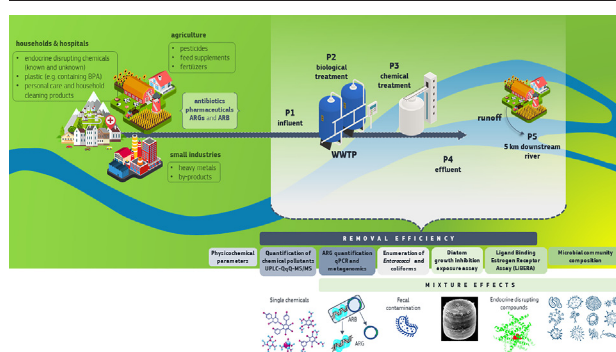
HIGHLIGHTS

- Combined methods used to assess WWTP removal efficiency of pollutants and ARGs
- Advantage of a holistic approach to better understand water contamination patterns
- Microbial community studied at each step of the WWTP and in the receiving river
- Data integration and link to land-use is required to target the unknown pollutants

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater treatment plants (WWTPs) collect wastewater from various sources and use different treatment processes to reduce the load of pollutants in the environment. Since the removal of many chemical pollutants and bacteria by WWTPs is incomplete, they constitute a potential source of contaminants. The continuous release of contaminants through WWTP effluents can compromise the health of the aquatic ecosystems, even if they occur at very low concentrations. The main objective of this work was to characterize, over a period of four months, the treatment steps starting from income to the effluent and 5 km downstream to the receiving river. In this context, the efficiency removal of chemical pollutants (e.g. hormones and pharmaceuticals, including antibiotics) and bacteria was assessed in a WWTP case study by using a holistic approach. It embraces different chemical and biological-based methods, such as pharmaceutical analysis by HPLC-MSMS, growth rate inhibition in algae, ligand binding estrogen receptor assay, microbial community study by 16S and shotgun sequencing along with relative quantification of resistance genes by quantitative polymerase chain reaction. Although both, chemical and biological-based methods showed a significant reduction of the pollutant burden in effluent and surface waters compared to the influent of the WWTP, no complete removal of pollutants, pathogens and antibiotic resistance genes was observed.

1. Introduction

The quality of aquatic ecosystem resources is constantly under threat due to chemical and biological pressures such as chemical mixtures,

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pathogens, antimicrobial resistance (AMR) and viruses. Conventional wastewater treatment plants (WWTPs) are designed to remove pathogens and coliforms and to reduce loads of carbon, nitrogen, and phosphorus. The removal of many expected and emerging (i.e. not yet regulated) contaminants, including pharmaceuticals and personal care products, hormones, and other industrial chemicals is, however, incomplete (Loos et al., 2013). Hence, WWTPs have been identified as a source of such pollutants in the aquatic environment. In particular, hospital wastewater has been identified as a problematic point source due to high concentrations of pharmaceuticals (Sörengård et al., 2019), including antibiotics. The complex mixtures of non-removed chemicals that are discharged to surface waters through WWTP effluents, even if they occur at low concentration when considered independently, may be potentially harmful to aquatic organisms. Among these micropollutants, antibiotics and hormones are of special concern since they act at very low concentrations (Gracia-Lor et al., 2012; Sanseverino et al., 2019; Fazolo et al., 2021; Khasawneh and Palaniandy, 2021).

The continuous release of antibiotics to surface waters may contribute to the emergence and spread of antibiotic resistant bacteria (ARB), compromising the effectiveness of antimicrobial therapy because these infectious organisms are becoming resistant to most antibiotics (O'Neill, 2014; Kraemer et al., 2019). The emergence and spread of ARB has been classified by the World Health Organization (WHO) as one of the biggest threats to public health in the 21st century (World Health, 2014). Thus, there is growing interest in exploring the occurrence of antibiotic resistance genes (ARGs) in the environment alongside the factors that contribute to their spread (Niegowska et al., 2021). Due to the continuous pollution linked to anthropogenic activities, aquatic ecosystems provide an ideal setting for the acquisition and diffusion of ARGs. Indeed, different scientific publications demonstrated a widespread occurrence of antibiotics and ARGs in urban and hospital wastewater and how effluents, even after treatment, contribute to the spread of these emerging pollutants in the aquatic environment (Rodriguez-Mozaz et al., 2015; Tang et al., 2016; Rodriguez-Mozaz et al., 2020).

WWTPs can also contribute to the discharge of endocrine disrupting compounds (EDC) to a river (Jálová et al., 2013). Municipal WWTPs are one of the main sources of estrogenic compounds. Some estrogenic chemicals, particularly steroid estrogens, are known to cause disruption of the endocrine system of fishes and abnormalities of the reproductive tract at ng/L concentrations, which commonly occur in the aquatic environment worldwide (Jarošová et al., 2014).

Chemical analyses of the aforementioned individual micropollutants cannot always identify total potential adverse effects due to molecular interactions that may occur. Several analytic-based techniques, such as high resolution mass spectrometry-based non-target screening (Aceña et al., 2015; Hollender et al., 2019; González-Gaya et al., 2021) allow the simultaneous detection of thousands of compounds in environmental samples. However, they do not cover the environmental risk posed by co-occurring pollutants (chemical mixtures) and their possible additive effects. Therefore, biological monitoring approaches are needed. In vitro bioassays and biomarkers can serve as a rapid, sensitive and relatively inexpensive integrative screening method to estimate total activity of all compounds in a mixture that act through the same mode of action (MoA). Indeed, the combination of chemical and bio-analytical protocols has been proposed as a holistic monitoring framework for the assessment of WWTP performance (Papa et al., 2016).

To our knowledge, this is the first study investigating in parallel the removal of chemical and biological contaminants, including ARGs, from a WWTP using complementary tools. Indeed, the novelty of this study is to advocate for the combination of several methods, such as chemical analysis with microbiological methods, in order to address emerging concerns for chemical mixtures. In this approach, the effects of critical chemicals are measured by implementing the use of bioassays, while the detection of AMR is performed by identifying target antibiotic resistances. It also aims to promote the data integration and correlations to land-use practices. The resulting overall picture, which also includes the same investigations performed in the downstream river, could contribute to improve the current understanding of the occurrence and removal of these contaminants in wastewater systems and their effects on the receiving river. To this respect, this study embraces a novel concept on how the water quality assessment should be implemented, bringing together chemical and microbiological analysis under water management and/or a regulatory framework (i.e. Urban Waste Water Treatment Directive (UWWTD), Water Framework Directive (WFD), Bathing Water Directive (BWD) and Drinking Water Directive (DWD)).

2. Material and methods

2.1. Wastewater sample collection and characterization

The selected WWTP is designed for 110,000 population equivalent. Wastewater samples were collected from an urban WWTP in Lombardy area (North Italy) from the influent (P1), after biological treatment (P2), after chemical treatment (P3), from the effluent (P4), and 5 km downstream the treatment plant (P5). Sample P1 was collected after the mechanical removal of suspended solids with a grid; P2 corresponds to the sedimentation tank downstream the biological treatments (nitrification followed by denitrification process), while the sample P3 was collected in the sedimentation tank downstream the chemical treatment (ferric chloride 40% which performs phosphorus removal). Water in the plant is then disinfected using sodium hypochlorite (14%, sample P4) and the remaining effluent is discharged into a river (sample P5). Flow-proportional 24 h composite samples were collected in P1 and P4, while grab samples were taken at P2 and P3. The water was collected during four different sampling campaigns in December 2018, January 2019, February 2019, and March 2019. The flow and the hydraulic retention time (HRT) in the four sampling campaigns were quite constant (24,640 ± 1351 m^3/d and 22.4 ± 1.2 h, respectively).

2.1.1. Physicochemical parameters

Physicochemical parameters of water samples, including dissolved oxygen (DO), pH and conductivity, were determined for each sample using Orion start A329 multi-meter (ThermoFisher Scientific).

2.1.2. Cell count and cell viability

For the microbiological assessment, total bacteria count in all samples was performed by fluorescence analysis. Eight mL of each sample were centrifuged at 10000 xg for 45 min. Supernatant was discarded and the pellet resuspended in 1.6 mL of phosphate buffered saline (PBS) (Sigma-Aldrich, Germany). The cell amount in each sample was determined using the regression equation generated by standard curve and the following equation (Martens-Habbena and Sass, 2006):

$$FI = \left({}^{Cell}/_{mL}\right)^a x\ 10^b \tag{1}$$

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

To generate the standard curve, a known concentration of *Escherichia coli* (*E. coli*) from an overnight culture was serial diluted (1:10) five times in order to obtain different concentrations.

To estimate bacterial cell viability, a suspension of live and 70% isopropyl-killed *E. coli* was prepared according to the Live/Dead BacLight Kit (Molecular Probes, Invitrogen), with small modifications (Feng et al., 2014). Live/dead cells were then mixed at various ratios (100:0; 75:25; 50:50; 25:75; 0:100).

The staining with SYBR Green I (for cell count) as well as with SYBR Green I together with propidium iodide (for cell viability) was done in untreated black 96-well microplates (ThermoFisher Scientific) using 200 μL aliquot of concentrated sample and 20 μL of staining mixture to a final concentration of 10×, and incubated at room temperature for 15 min under dark conditions. The fluorescence measurements were performed in

triplicate following Martens-Habbena and Sass indications (Martens-Habbena and Sass, 2006), using an Infinite F200 microplate reader (Tecan, Austria).

2.1.3. Enumeration of enterococci and coliforms

Fecal contamination of total coliforms, *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)/100 mL.

2.2. Solid phase extraction for chemical analysis and bioassay tests

One liter of water sample was stabilized by adding 10 mL of Na_2EDTA to avoid metal complexation (Opriş et al., 2013), homogenized and filtered on glass microfiber GF/F 0.7 μm nominal pore size (Whatman, Kent, UK). Each water sample was split in two aliquots (500 mL each): the pH of one aliquot was adjusted to 3 with hydrochloric acid (35%) while the other aliquot was not treated (pH > 7.5). Both aliquots were extracted with Oasis HLB cartridges (200 mg/6 mL, Waters, Milford, MA) and analyzed by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Detailed description of the procedure is reported in the Supplementary Material file 1.

Following the chemical analysis, the water sample extracts (pH >7.5 and pH 3) were mixed and tested in the bioassays (diatom exposure and ligand-binding estrogen receptor assay - LiBERA). The test concentrations of the water sample extracts were expressed as relative enrichment factor (REF) that incorporates the enrichment by solid phase extraction (SPE) and the dilution of the water extract in the bioassay. REF is a measure of how much a water sample would have to be enriched (REF > 1) or diluted (REF < 1) to achieve a given effect and is determined as follows (Escher et al., 2014):

$$REF = dilution\ factor\ bioassay \times enrichment\ factor\ SPE \quad (2)$$

where

$$enrichment\ factor\ SPE = \frac{Vwater}{Vextract} \quad (3)$$

and

$$dilution\ factor\ bioassay = \frac{Vextract\ added\ to\ the\ bioassay}{Total\ volume\ of\ the\ bioassay} \quad (4)$$

2.3. High performance liquid chromatography/tandem mass spectrometry

The studied substances belong to different categories: pharmaceuticals including numerous antibiotics and one transformation product (TP) derived from the degradation of an antiepileptic drug (gabapentin-lactam). All the analyzed substances were selected for their regulatory and environmental relevance. In particular, antibiotics were selected based on their presence in surface water and in WWTP effluents (Sanseverino et al., 2019). Meropenem and ertapenem were instead selected because they belong to carbapenems, which represent the major class of last-line treatments against multi resistant infections (WHO, 2022). The other pharmaceuticals were included in the analysis because they are refractory to removal in the treatment processes and occur at detectable concentrations in the outlet of urban WWTP in the Lombardy area (North Italy) (Palumbo et al., submitted to ET&C). The quantification of antibiotics, pharmaceuticals and the other compounds was carried out by ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UPLC-QqQ-MS/MS) (Thermo TSQ Quantum Access MAX) analysis.

The injection volume was 10 μL and the optimal chromatographic separation was obtained using a gradient of methanol and 0.1% HCOOH at 300 mL/min.

To confirm the compound identity, the mass spectrometer operated in ESI positive ionization mode and the quantification was performed using two SRM transitions. The calibration curves, freshly made before the analysis, were prepared with standards at concentrations ranging from 0 ng/L to 1000 ng/L and the labelled compounds were used to correct for losses during the extraction process and due to the matrix effect. Detailed description of the chromatographic and mass spectrometer settings is reported in the Supplementary Material file 1 (see Tables S1, S2, S3, S4 and S5 in Supplementary Material file 1).

2.3.1. Removal efficiency of pharmaceuticals

The removal efficiency of pharmaceuticals and antibiotics from the WWTP was estimated according to the following equation (de Jesus Gaffney et al., 2017):

$$Removal\ Efficiency\ (\%) = \frac{C_{inf} - C_{eff}}{C_{inf}} * 100 \quad (5)$$

where C_{inf} indicates average concentration calculated for the influent (P1) and C_{eff} stands for average concentration calculated for the effluent (P4).

The flow rate for influent and effluent was considered constant. The removal efficiency was estimated based on the average concentration calculated over all the sampling period, from December 2018 to March 2019. Limit of detection (LOD) was used in the calculation in case values were <LOD.

2.4. Diatom culture and exposure to water sample extracts

Thalassiosira pseudonana (*T. pseudonana*, strain CCMP 1335) was obtained as axenic culture from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbour, Maine, USA) and cultured in artificial seawater (ASW-f/2) at 16 °C with photoperiod 13/11-h light/dark. *T. pseudonana* cultures were synchronized according to Hildebrand et al. (2007) and exposed to the water extracts at cell density of 1×10^6 cells/mL in a total volume of 20 mL. A dose-dependent response of the water extracts ranging from 0.125 to 2.5 REF was investigated after 24, 48, and 72 h. A solvent control (1% methanol) and SPE control were also included in the study. Cell densities were determined and used to calculate growth rates and growth inhibition, as previously described by Bopp and Lettieri (2007). Data was fitted to a four-parameter non-linear regression curve (GraphPad Prism version 9.0.0, GraphPad Software, USA). EC_{10} and EC_{50} values (concentration of water sample required to induce growth rate inhibition of 10% and 50%, respectively) were calculated from the fit. The tests were conducted in two biological replicates and two technical replicates for each treatment.

2.5. Ligand binding estrogen receptor assay

The LiBERA assay was used to test the binding of substances present in the water sample extracts to the ligand-binding domain of the human estrogen receptor (ER) alpha ($ER\alpha^{LBD}$). This bioassay is a modified version of the PolarScreen™ ERα green assay developed by Life Technologies, according to Ferrero et al. (2014). It has been already used to test the effects of chemical mixtures by Carvalho et al. (2014), named $wtER\alpha^{LBD}$ binding assay and by Gómez et al. (2021). Dose-dependent responses ranging from 0.0025 to 2.5 REF were investigated. The data were fitted to a sigmoidal one-site competition four-parameter logistic curve using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, California USA). The fit provided IC_{10} and IC_{50} values (concentration of reference compound or water sample required to reduce the maximum % polarization to 90% and 50%, respectively). The natural hormone 17-β-estradiol (E2) was used as a reference compound to determine the estrogenic potential of the water samples, which was expressed as E2 equivalent concentration (EEQ, ng/L E2-Eq). The EEQ values of each water sample extract were derived by dividing the IC_{10} or IC_{50} value of the reference compound (E2) by IC_{10} or IC_{50} values of the water sample extracts (Escher et al., 2014; Kunz et al., 2017; Simon et al., 2019).

2.6. Next generation sequencing of water samples

2.6.1. Sample preparation and DNA extraction

Water aliquots of approximately 140 mL for P1, 250 mL for P2, P3, P4 and P5 were filtered in triplicate using MF-Millipore membrane filters, 0.22 μm pore size (Millipore). Water samples were filtered upon arrival at the laboratory, and all filters were stored at −20 °C until further analyses.

For DNA extraction, filters were incubated overnight in 50 mM KH_2PO_4 buffer and then sonicated for 15 min at 60 °C as described by Kisand et al. (2012), except for the lyticase incubation. Enzymatic digestion using lysozyme (100 mg/mL, Sigma) and β-mercaptoethanol (14 mM, Sigma) was performed prior to column-based DNA extraction with DNeasy Blood and Tissue Extraction Kit (Qiagen), according to supplier's instructions.

DNA concentration was checked at both Nanodrop (ThermoFisher Scientific) and Qubit (Invitrogen). Purified DNA samples were subjected to 16S, shotgun sequencing and quantitative polymerase chain reaction (qPCR).

2.6.2. 16S and shotgun sample preparation and sequencing

16S rDNA amplicons and total community genomic DNA were sequenced on the IonS5 Instrument (ThermoFisher Scientific) at the Joint Research Centre (JRC) in Ispra (Italy). Amplification of 16S rDNA (variable regions V3-V4) was performed using the primers S-D-Bact-0341-b-S-17 (5′ CCTACGGGNGGCWGCAG 3′) and S-D-Bact-0785-a-A-21 (5′ GACTACHVGGGTATCTAATCC 3′) (Klindworth et al., 2013). Polymerase chain reaction (PCR) was carried out using 25 ng of high-quality genomic DNA. The PCR conditions were: 1 cycle of 3 min at 95 °C followed by 25 cycles of 40 s at 95 °C, 2 min at 55 °C, 1 min at 72 °C and 7 min incubation at 72 °C. PCR amplicons were used to prepare amplicon libraries with the Ion Plus Fragment Library Kit (ThermoFisher Scientific) according to manufacturer's instructions.

For shotgun analysis, 100 ng of high-quality genomic DNA was used for library preparation. Libraries were constructed using the Ion Plus Fragment Library Kit (ThermoFisher Scientific). Input DNA was fragmented with the Ion Shear Plus Enzyme Mix (ThermoFisher Scientific) and purified with Agencourt AMPure XP Reagent (Beckman Coulter). The fragmentation was checked using the Bioanalyzer instrument (Agilent). Then, adaptors and a specific barcode were linked to the DNA fragments. Finally, the adapter-ligated and nick-repaired DNA was purified with Agencourt AMPure XP Reagent (Beckman Coulter) and the desired DNA library fragment length (400 bp) was size-selected using the *E*-Gel (ThermoFisher Scientific). Libraries were amplified using Platinum PCR SuperMix High Fidelity and Library Amplification Primer Mix (ThermoFisher Scientific).

2.6.3. Bioinformatics analysis

All 16S rDNA V3-V4 amplicon reads were initially converted into reads with the same (forward) sense by a custom Perl script and the quality filtered with TRIMMOMATIC v0.38 (Bolger et al., 2014). The data was then combined into one datafile and Operational Taxonomic Units (OTUs) were clustered at 97% identity with USEARCH (Edgar, 2010) and taxonomically classified by SINTAX (Edgar, 2016) using the GTDB r202 database (Parks et al., 2022). Clustering of OTU abundance was performed in R with heatmap.2 (complete agglomeration and Euclidean distance).

Shotgun reads were quality filtered with TRIMMOMATIC v0.38 as pairs and taxonomic banning was performed with KRAKEN2 (Wood and Salzberg, 2014) and GTDB r202. Details of taxonomic classification are available in the Supplementary Material file 2.

Assembly of shotgun read pairs was performed with MEGAHIT v1.2.9 (Li et al., 2015b) and mapped onto contigs from the combined dataset using BOWTIE2 (Langmead and Salzberg, 2012). Contigs were then binned with METABAT2 (Kang et al., 2019) and further analyzed by CHECKM (Parks et al., 2015) and GTDBtk (Chaumeil et al., 2019). Screening of Prodigal (Hyatt et al., 2010) predicted protein sequences against the UNIREF90 protein database was performed with DIAMOND (Buchfink et al., 2021). Virsorter2 (Guo et al., 2021) was used to detect potential phage genomes in the METABAT2 bins. Presence of ribosomal RNA (rRNA) genes in the assembled contigs was performed with CMSEARCH (Nawrocki et al., 2009) using the Rfam v14.7 (Kalvari et al., 2018) 16S small rRNA sequence profiles for bacteria (RF00177) and archaea (RF01959) and the 23S small ribosomal RNA for eukaryotes (RF01960). The resulting matches were then confronted with the RNA central database (Consortium, 2021) using BLASTN.

Detection of ARGs in assembled MEGAHIT contigs was performed with BLASTN using the CARD database (Alcock et al., 2020). BLASTN results were filtered accepting only hits with ≥99% sequence identity and spanning more than 90% of the CARD gene length. Relative abundance of single ARGs was determined, for each sample, as the ratio (counts for ARGs [gene i]/length(ARG[gene i])) divided by (counts for the 16S small ribosomal gene/average 16S gene length 1432 bp).

Detection of pathogens was performed with KRAKEN2 using a list of 538 pathogens (Li et al., 2015a), applying maximal stringency in KRAKEN2 through confidence = 1.0. Results were then validated with CONIFER (https://github.com/Ivarz/Conifer).

In all analysis using assembled contigs, the reported raw counts take into account the relative contig abundance (multi value) associated to each contig.

Principal component analysis (PCA) was performed on the antibiotics' concentration measurements and the sum of the ARG gene matches across each resistance class with the R language. Results were plotted using the ggbiplot function. All 16S amplicon and shotgun data is available in the NCBI SRA archive under accession number PRJNA793838 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA793838).

2.7. AMR genes detection by quantitative polymerase chain reaction

The extracted DNA was used for the quantification of four selected resistance genes: *sul1* (conferring resistance to sulfonamides), *qnrS1* (conferring resistance to quinolones), *mphE* (conferring resistance to macrolides), and bla_{OXA-58} (conferring resistance to β-lactams), using the primers listed in Table S6 (Supplementary Material file 1).

qPCR assays were performed in 25 μL reaction volume using 12.5 μL of Power SYBR Green I (ThermoFisher Scientific), 1.25 μL of both forward and reverse primers (10 μM) and 7.5 μL of water. Amplification was carried out using the Applied Biosystems 7900HP Real-Time PCR system (ThermoFisher Scientific) as follows: 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Calibration curves were built using a ten-fold dilution series, ranging from 10^0 to 10^5 of the synthetic plasmid pNORM constructed by Christophe Merlin (Rocha et al., 2020) which contains fragments encoding the target genes *sul1, qnrS1* and *16S* rDNA in a single plasmid. For the genes *mphE* and bla_{OXA-58}, a new plasmid (pAMR4U) was generated on a standard pUC57 vector (Twinhelix, Italy). A subset of samples was analyzed at five dilutions (1:10, 1:25, 1:50, 1:100, and 1:500) to determine the correct DNA concentration required to minimize inhibition. Dilutions varied depending on the samples: for the five target genes, 1:10 dilution was used in all samples, except for January P4, where 1:50 dilution was used.

2.8. Statistical analysis

The quantification of ARGs normalized with 16S were compared using RStudio Software (R version 3.6.1). Shapiro-Wilk test was used to evaluate the normality of the data. The values corresponding to concentrations of ARGs in the different treatments of the WWTP were compared with ANOVA test, followed by a Tukey's post hoc test. The *p* values <0.05 were considered statistically significant.

3. Results

3.1. Water characterization

3.1.1. Cell count and viability

During the wastewater treatment process, the number of bacteria/mL ranged from 6.90×10^5 (P4, January) to 3.16×10^8 (P1, March) being March the month with the highest cell count for all treatments, while the percentage of alive cells was in the range from 26% (P1, December) to

84.2% (P2, March) (see Table S7 in Supplementary Material file 1). Small seasonal variations in cell number and cell viability were observed among samples collected in the river (P5) during the sampling months (Table S7 in Supplementary Material file 1).

3.1.2. Fecal contamination

The MPN/100 mL for total coliforms, *E. coli* and *Enterococci* at each sampling point of the WWTP (P1, P2, P3, P4) and 5 km downstream the WWTP (P5) is shown in Table S7 (Supplementary Material file 1). The influent samples (P1) showed values from 10^5 to 10^7 orders of magnitude and recorded the highest total coliforms, *E. coli* and *Enterococci* counts among all samples. Total coliforms, *E. coli* and *Enterococci* detected in the influent (P1) were found to decrease in number during the treatment process. For total coliforms, the reduction was higher in January compared to December, February and March, whereas for *E. coli* and *Enterococci* the highest reduction was observed in March. *E. coli* concentrations in P4 were always in full compliance with the discharge limits issued by the Italian legislation (Italian Parliament, 2006). In March, the mean MPN/100 mL was higher in samples collected 5 km downstream the WWTP (P5) than in the final effluent (P4) with an increase of two-four orders of magnitude. All samples in P5 exceeded parametric values determined by the European Directive 2006/7/EC concerning the quality of bathing water (European Commission, 2006).

3.2. Chemical analysis

Concentrations of twenty selected antibiotics and nine pharmaceuticals were investigated in the selected WWTP during a sampling campaign performed from December 2018 to March 2019 (Table S8 and Table S9 in Supplementary Material file 1).

Ofloxacin, a broad-spectrum fluoroquinolone antibiotic used to treat different bacterial infections, was the most abundant antibiotic detected in P1 with the highest concentration in March 2019 (7179 ng/L), while azithromycin was detected in all wastewater treatments except P1 over four months of the study (Table S8 in Supplementary Material file 1). The concentration of amoxicillin was below LOD in December while, in the other sampling months, it was detected only in P1 (Table S8 in Supplementary Material file 1). Sulfamethoxazole appeared to accumulate along the wastewater treatment process, as also observed in December for clarithromycin, which however, in the other months, showed lower concentrations in P4 compared to P1 (Table S8 in Supplementary Material file 1). Most of the antibiotics were detected below the LOD (e.g. erythromycin, sulfamethazine, enrofloxacin) or at very low concentrations (e.g. chlorotetracycline, lincomycin, roxythromycin, tetracycline) (Table S8 in Supplementary Material file 1). Ciprofloxacin levels were always higher in P1 compared to the other treatment points (Table S8 in Supplementary Material file 1).

Pharmaceuticals were present in all treatment steps (Table S9 in Supplementary Material file 1). Irbesartan, a medication used to treat high blood pressure, showed higher concentrations in P4 and P5 compared to all the other pharmaceuticals (Table S9 in Supplementary Material file 1), while ketoprofen, a nonsteroidal anti-inflammatory drug, was the only pharmaceutical showing a progressive reduction during the wastewater treatment process when comparing P4 to P1 (Table S9 in Supplementary Material file 1).

The removal efficiency results suggest a variation for each pharmaceutical (Fig. 1 and Fig. 2). Only two antibiotics (amoxicillin and ciprofloxacin) showed a mean removal efficiency higher than 95% (Fig. 1A), while in the list of pharmaceuticals not including antibiotics, a positive mean removal efficiency in P4 compared to P1 was reported only for ketoprofen (Fig. 2A). All the remaining substances showed an increase of concentrations compared with measurements in the influent (Table S9 in Supplementary Material file 1).

Reduced levels of antibiotics and pharmaceuticals were detected in P5 (Table S8 and Table S9 in Supplementary Material file 1). Contrarily to the antibiotics, whose values in the river were generally below the LOD, pharmaceuticals were detected at *all sampling* locations for each sampling *month*.

3.3. Exposure of diatom cultures to water sample extracts

The removal efficiency of the WWTP was studied by comparing the toxicity of the water samples before and after the wastewater treatment process. Sample extracts of influent (P1), effluent (P4) and surface water (P5) were analyzed for their cytotoxic effect impacting the growth rate of the marine diatom *T. pseudonana*. The effect concentrations of the water sample extracts on the diatom cultures (EC_{10} and EC_{50}) are expressed as REF, a measure of how much the water sample is enriched (REF > 1) or diluted (REF < 1) (see Section 2.2). P1 exerted toxic effects to diatom cultures after 48 h exposure by strongly inhibiting the growth rate (Fig. 3) and the inhibitory effect was stronger after exposure to the wastewater sample collected in February (Fig. 3C) with an EC_{50} value of 0.40 REF (Table S10 in Supplementary Material file 1). The effects of P4 and P5 water extracts on

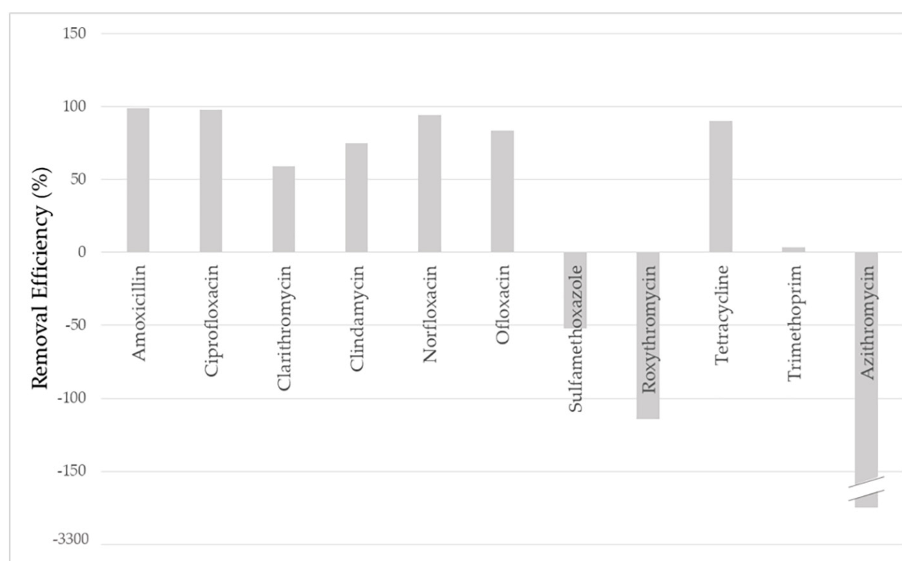

Fig. 1. Removal efficiency of antibiotics along the wastewater treatment plant (WWTP). The total removal efficiency of amoxicillin, ciprofloxacin, clarithromycin, clindamycin, norfloxacin, ofloxacin, sulfamethoxazole, roxythromycin, tetracycline, trimethoprim and azithromycin was estimated based on the mean concentration calculated over all the measurements performed from December 2018 to March 2019. The removal efficiency is reported as percentage of the antibiotic concentration detected in the WWTP effluent (P4) relative to the concentration in the influent (P1, see Section 2.3).

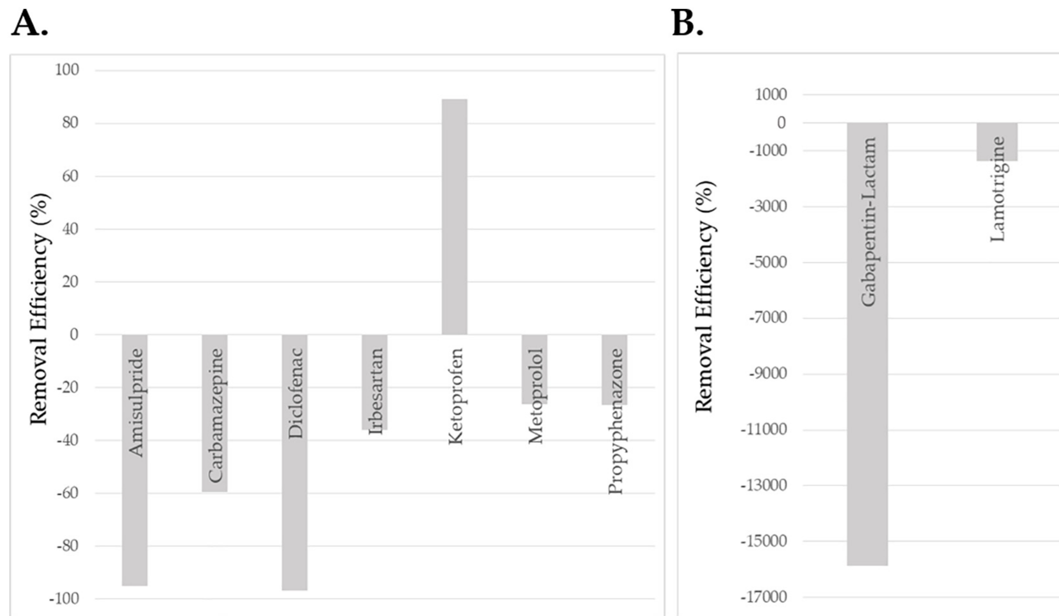

Fig. 2. Removal efficiency of pharmaceuticals (excluding antibiotics) along the wastewater treatment plant (WWTP). The total removal efficiency of amisulpride, carbamazepine, diclofenac, irbesartan, ketoprofen, metoprolol, propyphenazone (2A) gabapentin-lactam and lamotrigine (2B) was estimated based on the mean concentration calculated over all the measurements performed from December 2018 to March 2019. The removal efficiency is reported as percentage of the antibiotic concentration detected in the WWTP effluent (P4) relative to the concentration in the influent (P1, see Section 2.3).

the diatom's growth rate were lower compared to P1 in all months studied. Among the P4 sample extracts, the one from March exerted the strongest inhibitory effect (Fig. 3D) with an EC_{10} value of 0.58 REF (Table S10 in Supplementary Material file 1), while the effects of the P5 extract were higher in December (Fig. 3A) with an EC_{10} value of 2.11 REF (Table S10 in Supplementary Material file 1).

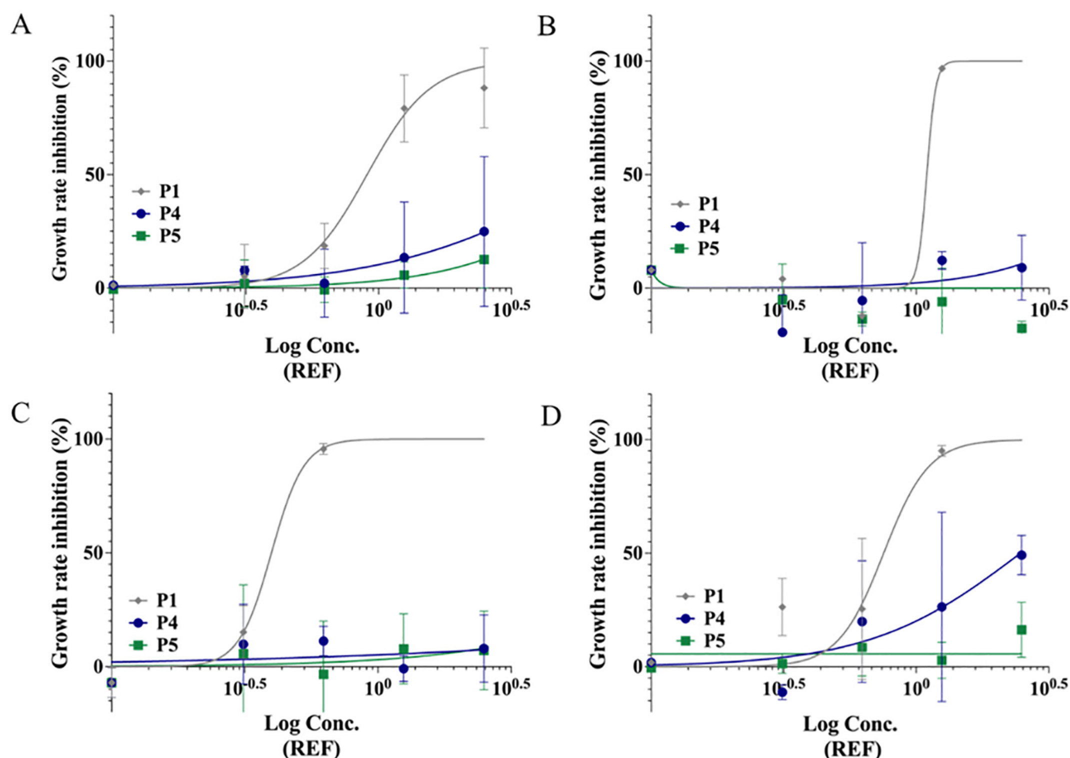

Fig. 3. Dose-response curves obtained after exposure of the diatoms (48 h) to the water sample extracts from three different points in the wastewater treatment plant (WWTP) (P1, P4, P5) and in different sampling months. A, December; B, January; C, February and D, March. The curves were prepared by fitting the data to a four-parameter non-linear regression curve. X-axis shows the relative enrichment factor (REF) expressed in logarithmic scale. Data are expressed as mean values, the vertical bars represent the mean standard error. Conc.: Concentration; P1: influent; P4: WWTP effluent; P5: 5 km downstream the WWTP.

3.4. Ligand binding estrogen receptor assay

The WWTP performance regarding the removal of estrogenic compounds was studied by testing the water samples collected from P1, P4 and P5 with the LiBERA assay. The natural hormone E2 was used as a reference compound to determine the estrogenic potential of the different water samples, which was expressed as E2 equivalent concentration (EEQ, ng/L E2-Eq) as described in Section 2.5. This bioassay covers exclusively the binding affinity of the compounds present in a sample (i.e., including both agonists and antagonists) to the $ER\alpha^{LBD}$. Fig. 4 shows the dose-response curves prepared with the different water sample extracts while the IC_{10}, IC_{50} and EEQ values derived from the curves are presented in Table S11 (Supplementary Material file 1). LiBERA was responsive to the water sample extracts from P1, P4 and P5 (Fig. 4). The effects of the tested extracts on the ER binding affinity were higher in the samples collected in March (Fig. 4D) in terms of IC_{10} and IC_{50}, being P1 the sample showing stronger effects (Table S11 in Supplementary Material file 1).

3.5. Microbial community

Variation of the bacterial community was first investigated through 16S amplicons metagenomics from water samples collected at sites P1, P2, P3, P4 and P5. At the phylum level, the microbial composition was relatively constant over the 4-month period at all sites, being however markedly different between P1, the three WWTP sites and P5 (Fig. S1 in Supplementary Material file 1). At P1, alpha diversity was markedly lower as evident from the rarefaction curve (Fig. S2 in Supplementary Material file 1). For P2, P3 and P4, clustering of OTU indicates a broad and highly diverse composition dominated mainly by Proteobacteria and Patescibacteria (Fig. S1 in Supplementary Material file 1). Instead, at the downstream site, the presence of Patescibacteria was less pronounced.

Rank level taxonomic classification of the OTU recapitulates these results with approximately 60% of Proteobacteria at P1 plus Bacteroidota, Firmicutes_A and Campylobacterota (Fig. 5). In WWTP samples P2, P3 and P4, Proteobacteria content was reduced to about 30% and Patescibacteria were present at about 30–40% (Fig. 5). At the downstream site P5, Proteobacteria content remained roughly constant, with some remaining Patescibacteria and, in addition to Bacteroidota, also Actinobacteria, Cyanobacteria and Verrucomicrobiota increased compared to P4 (Fig. 5). Archaea were almost absent with the exception of site P5 where a significant content of Asgardarchaeota was present, in particular in March, and a distinct OTU cluster unique to site P5 (Fig. 5) with a heterogeneous composition (Supplementary Material file 2).

While a nearly complete taxonomic classification was possible at the phylum level, the portion of unassigned OTU increased rapidly towards lower ranks, with the exception of site P1 (Supplementary Material file 2). At the genus level, only site P1 remained nearly completely taxonomically classified (around 90%), dominated by Acinetobacter, whereas all other sites, in particular sites P2, P3 and P4, had between 30% and 50% of OTU unassigned, with a near complete absence of genus level classification for Patescibacteria (Fig. S3 in Supplementary Material file 1). An explanation for this absence became evident when the OTU sequences were directly compared, using BLASTN, to the GTDB SSU database and the NCBI nt and envnt databases. In particular for Patescibacteria, the average percent sequence identity against GTDB SSU sequences, the basis for taxonomic classification, was distinctly lower compared to nearly all other phyla, even when considering the NCBI nt and envnt databases (Fig. S4 in Supplementary Material file 1). Closer inspection of the matches against nt and envnt, predominantly with sequence identities between 95 and 100%, revealed the existence of many taxonomically unassigned 16S sequences (taxonomy "Uncultured bacteria") apparently related to Patescibacteria and absent from the GTDB SSU database explaining why

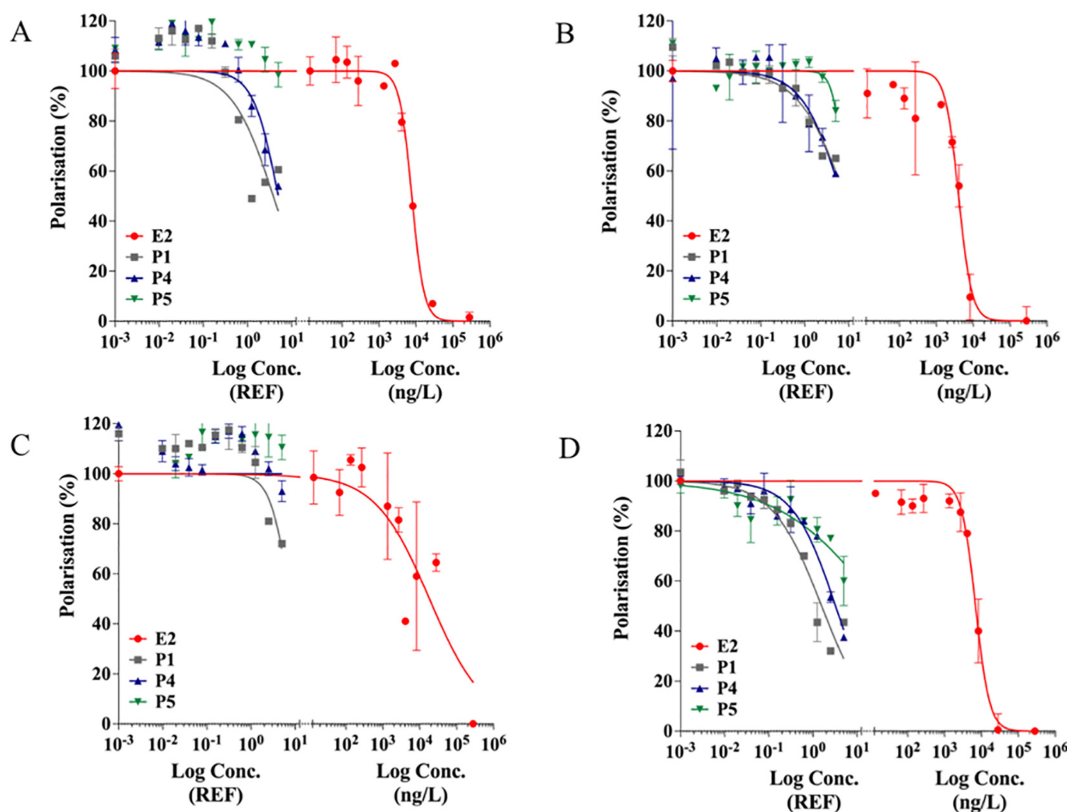

Fig. 4. Dose-response curves obtained using LiBERA after testing the water sample extracts from three different sampling points (P1, P4, P5) in four different months. A, December; B, January; C, February and D, March. The curves were prepared by fitting the data to a four-parameter non-linear regression curve. Data are expressed as polarization % respect to the positive control (E2). The vertical bars represent the mean standard error. X-axis shows the relative enrichment factor (REF) or ng/L expressed in logarithmic scale. Conc.: Concentration; P1: influent; P4: wastewater treatment plant (WWTP) effluent; P5: 5 km downstream the WWTP.

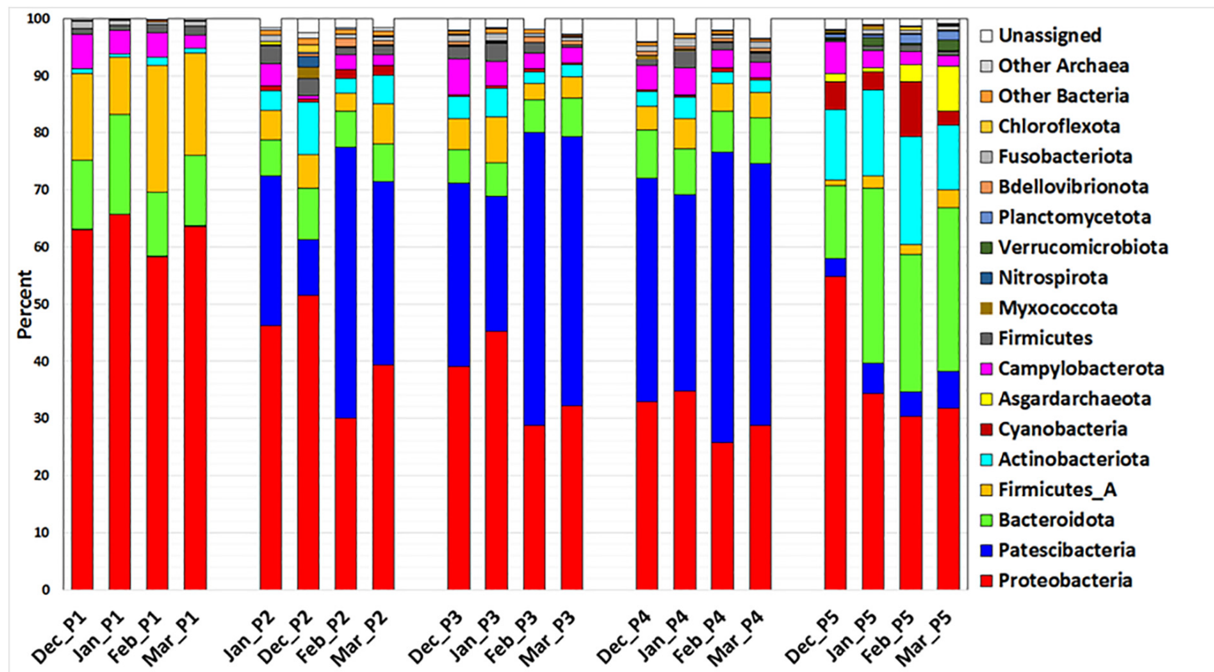

Fig. 5. Taxonomic composition at the phylum level as present in the 16S data. Only phyla with a relative abundance of at least 1% in one of the samples are listed. Phyla with less than 1% are represented as"Other Bacteria" or "Other Archaea". Blue and red dotted squares indicate high portions of Patescibacteria and Proteobacteria, respectively. P1: influent; P2: effluent of biological treatment; P3: effluent of chemical treatment; P4: effluent; P5: 5 km downstream the wastewater treatment plant (WWTP).

classification was only possible at the phylum level. An attempt to classify the OTU using the much larger SILVA database provided the same result (data not shown). Patescibacteria represent a large rapidly growing superphylum comprising previous phyla Parcubacteria, Microgenomates and Candidate Phylum Radiation (CPR) (Beam et al., 2020; Tian et al., 2020) with reduced genome size (~ 1 Mbp) and reduced genomic features (Beam et al., 2020). From the 16S analysis, it appeared that the Patescibacteria, frequently found in groundwater, sediments, lakes, and other aquifer environments, present in samples P2, P3 and P4 were taken up from the bacterial community existing inside the WWTP. At the species level, virtually all samples showed only very low successful classification with up to 85% of unassigned OTU, likely due to either the impossibility for the V3-V4 amplicon to discriminate between highly similar 16S sequences or the absence of 16S sequences with sufficiently high sequence identity (Supplementary Material file 2).

The results obtained from the 16S metagenomics analysis were further consolidated using the shotgun data available for sites P1, P4 and P5. Reads were classified with KRAKEN2 (Wood and Salzberg, 2014) using genomes available in GTDB release r202, the same database used in the 16S analysis. While data from site P1 could be taxonomically classified to a relatively high extend (around 80% of reads), values dropped to about 30% of classified reads for site P4 and around 40% of reads for site P5 (Fig. 6) (Supplementary Material file 2). Since KRAKEN2 classification relies on whole genome or genome assembly sequences, presence of only distantly related genomes in the GTDB database does not allow for a successful classification for P4 and P5. This was in particular the case for Patescibacteria in site P4, where even for the 16S V3-V4 amplicon sequences no close matches could be identified. Only for site P1 classification at lower ranks covered a relatively large portion of reads (around 65–70% at the species level) confirming the presence of various Acinetobacter species from the dominant Proteobacteria (Fig. 6 and Supplementary Material file 2). No relevant amounts of algae/plants/diatoms emerged from a KRAKEN2 analysis using instead the Genbank PLN database.

Assembly of shotgun reads into long(er) segments, so-called contigs, and binning of contigs into bins can in principle provide additional details about the microbial community, such as the length of genomes and possibly identity of species, and the co-existence of a phage community. METAHIT (Li et al., 2015b) assembly and subsequent METABAT2 (Kang et al., 2019) binning was however only partially successful providing, as expected, close to full-length genome assemblies only for site P1, in particular rather long (around 2 Mb) assemblies closely related to Acinetobacter species (Supplementary Material file 3). For sites P4 and P5, the most abundant METABAT2 bins (as estimated by the "depth" values associated with each bin) had a relatively short overall length suggesting a significant presence of phage genomes (Supplementary Material file 3). This was partially confirmed by submitting the METABAT2 bins to a VIROSORTER2 (Guo et al., 2021) analysis focusing on phage genomic markers (Supplementary Material file 3).

DNA-centric analysis of the shotgun reads is limited to cases where sequence identity is high enough to allow for unambiguous read assignment. In principle, protein segments encoded by the shotgun reads (or by the assembled contigs) might be able to reveal similarity to more distantly related organisms and provide further, even if only approximate, information about the composition of the community. Using DIAMOND (Buchfink et al., 2021) all shotgun reads were therefore confronted, applying a stringent threshold (80% of the shotgun read aligned, e-value cut-off 1.0e-10), against two different protein sequence databases, the UNIREF90 general protein database and a database comprising all PRODIGAL (Hyatt et al., 2010) predicted coding sequences in the GTDB r202 genome collection (Supplementary Material file 4). The analysis confirmed the high presence of Acinetobacter-related species in sample P1 and suggests a near complete absence of phages in this sample. Instead, for samples P4 and P5 across all four time points, uncultured Caudovirales phage emerged as the most frequently matched taxonomy. Like in the KRAKEN2 shotgun analysis, only very few taxonomies belonging to the Patescibacteria phylum were detected and no further details concerning the microbial composition at sites P4 and P5 could be identified. Overall, the number of protein sequences matched by P1 sample reads was considerably higher compared to sample P4 and P5, thus confirming again that these samples contain a relatively large number of microorganisms for which no sequence information is apparently yet available (Supplementary Material file 4), consistent with the METABAT2 results on the assembled bins.

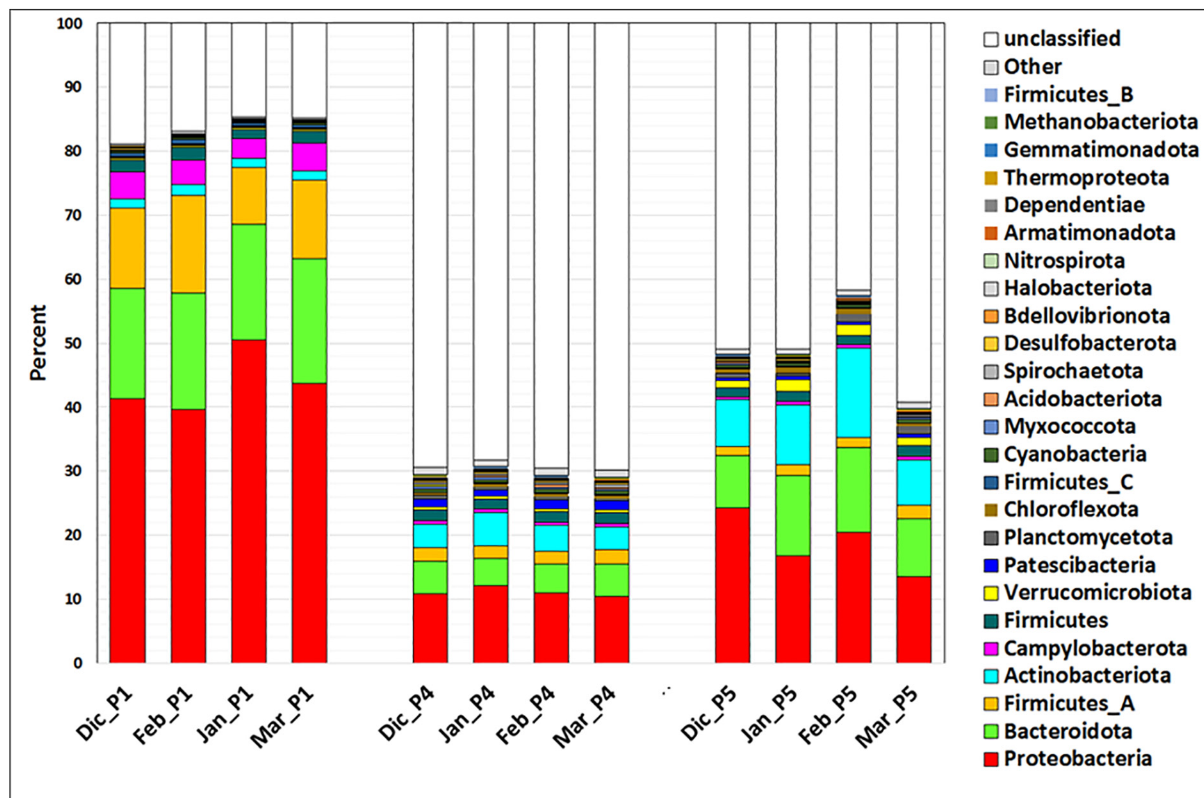

Fig. 6. Taxonomic composition at the phylum level as present in the shotgun data. Only phyla with a relative abundance of at least 0.1% in one of the samples are listed. Phyla with less than 0.1% are represented as"Other". P1: influent; P4: effluent; P5: 5 km downstream the wastewater treatment plant (WWTP).

A final analysis took advantage of the high level of sequence conservation of ribosomal RNA sequences. Using sequence profiles for bacterial, archaeal and eukaryotic small subunit ribosomal RNA available in Rfam (Kalvari et al., 2018), the MEGAHIT assemblies were examined with CMSEARCH from the INFERNAL package (Nawrocki et al., 2009). Different from the results obtained with KRAKEN2 for the Genbank plant database, the analysis revealed a significant level of eukaryotes at P4 and in particular for sample P5 (Table S12 in Supplementary Material file 1). Confirmation that these numbers represented true hits to the eukaryote RNA sequence profile was obtained with BLASTN searches against the RNAcentral database (Consortium, 2021). Most of the MEGAHIT assemblies reported by CMSEARCH to match the eukaryotic RNA profile showed relatively high sequence identity to eukaryotic sequences (often from unknown uncultured eukaryotes), although the level of identity frequently did not allow for a definite taxonomic assignment (Supplementary Material file 5).

3.6. In silico detection of potential pathogens community

To detect the presence of bacterial pathogens in the samples, we focused on a list of 538 potential pathogens from the literature (Li et al., 2015a). Since genome sequences or genome assemblies were available for most of these (Supplementary Material file 6), a custom KRAKEN2 pathogen genome database was generated and the shotgun data was analyzed applying the most stringent KRAKEN2 confidence parameter. Instead, count values for bacterial small ribosomal 16S RNA were determined with INFERNAL using sequence profiles (bacteria and archaea) from Rfam. After normalizing the counts for differences in cell/mL values of the samples (Table S7 in Supplementary Material file 1), it is evident that a large portion of pathogens have been removed from the water (Table S13 in Supplementary Material file 1). A complete list of results is available as Supplementary Material file 6.

3.7. Detection of ARGs using shotgun reads

For samples P1, P4 and P5, the assembled MEGAHIT contigs provided the best basis for the detection of resistance genes from the CARD database (Alcock et al., 2020) since they allowed, using BLASTN, to filter the results applying stringent criteria (≥99% sequence identity over at least 90% of the length of the CARD gene) (Fig. 7). Combining the results obtained for each ARG across the antibiotic class and normalizing them for 16S RNA count abundance showed that, at sites P4 and P5, the relative abundance generally diminished compared to site P1 (Table S14 in Supplementary Material file 1). Moreover, like for the pathogens, considering the differences in cell count per mL at the three sites and sampling dates, the total amount of detected ARGs at sites P4 and P5 was clearly much lower. More detailed results are available in the Supplementary Material file 6.

3.8. Detection of ARGs by qPCR

Four different ARGs (*bla*$_{\text{OXA-58}}$, *mphE, qnrS and sul1)* were quantified using a qPCR approach. These genes were selected because they were among the most frequently detected genes in *shotgun* metagenomic sequencing data (Fig. 7). They confer resistance to antibiotics which are critical for human health and were previously reported in WWTPs (Szczepanowski et al., 2009; Laht et al., 2014; Cacace et al., 2019). They also cover a wide range of resistance against antibiotics belonging to various classes: the *sul1* gene confers resistance to sulfonamides, *bla*$_{\text{OXA-58}}$ to β-lactams, *mphE* to macrolides and *qnrS* to quinolones.

To avoid inconsistencies among qPCR assays, we used 16S rRNA gene-normalized values. The gene *bla*$_{\text{OXA-58}}$ showed the highest relative abundance in all sampling months compared to the other genes (Fig. 8). A decreasing trend in relative abundance was observed for *bla*$_{\text{OXA-58}}$ from P1 to P5 in all sampling months. In February, the relative abundance of

Gene	Dic2018_P1	Jan2019_P1	Feb2019_P1	Mar2019_P1	Dic2018_P4	Jan2019_P4	Feb2019_P4	Mar2019_P4	Dic2018_P5	Jan2019_P5	Feb2019_P5	Mar2019_P5
mphE	9.1E-02	1.1E-01	2.2E-01	7.0E-02	2.2E-02	1.8E-02	7.6E-03	1.5E-02		1.1E-02	1.8E-02	5.1E-02
msrE	9.1E-02	1.1E-01	2.2E-01	3.3E-02	2.1E-02	1.8E-02	7.8E-03	1.5E-02		1.1E-02	1.8E-02	5.1E-02
tet(39)	6.3E-02	3.6E-02	3.8E-02	3.9E-02	5.8E-03	1.0E-02			5.6E-03		1.4E-02	1.5E-02
APH(3'')-Ib	1.9E-02	2.7E-02	3.9E-02	7.0E-02	8.3E-03		5.7E-03	1.2E-02				4.8E-03
APH(6)-Id	1.9E-02	2.7E-02	3.8E-02	7.0E-02	8.3E-03		5.7E-03					
OXA-164		9.8E-02	2.9E-02									6.8E-03
OXA-96		9.8E-02	2.9E-02									6.8E-03
OXA-397		9.8E-02	2.9E-02									6.8E-03
OXA-58		9.8E-02	2.9E-02									6.8E-03
OXA-97		9.8E-02	2.9E-02									6.8E-03
sul1	1.6E-02	1.5E-02	1.0E-02	2.7E-02	7.1E-03	1.0E-02		8.5E-03		6.3E-03	2.6E-03	8.3E-03
QnrS2	6.6E-03	1.7E-02	1.1E-02	3.1E-02	3.4E-03			6.0E-03			4.9E-03	
QnrS6	6.6E-03	1.7E-02	1.1E-02	3.1E-02	3.4E-03			6.0E-03			4.9E-03	
AAC(6')-Ib9	1.5E-02	1.3E-02	2.1E-03	2.4E-02	3.2E-03			3.9E-03		1.4E-03	3.9E-03	
aadA27	1.3E-02	1.5E-02	1.2E-02	8.1E-03								
aadA	6.6E-03	1.2E-02	5.3E-03	1.9E-02		9.9E-03		8.3E-03		4.6E-03		8.5E-03
qacEdelta1		1.4E-02		2.7E-02	7.1E-03		7.5E-03			6.4E-03		8.2E-03
tetW	8.3E-03	5.9E-03	8.7E-03	1.1E-02								

Fig. 7. Relative abundance (normalized against 16S) of the 20 most abundant CARD antibiotic resistance genes (ARGs). Both ARGs and 16S have been normalized for gene length. P1: influent; P4: effluent; P5: 5 km downstream the wastewater treatment plant (WWTP).

mphE, *qnrS* and *bla*$_{OXA-58}$ showed a reduction in P4 compared to P1, although the difference was statistically significant only for *qnrS* and *bla*$_{OXA-58}$. The relative abundance of all studied ARGs was significantly reduced in the outcome (P4) compared to the income (P1) of the WWTP in March (Fig. 8). On the other hand, in January, increased concentrations of *sul1, qnrS* and *mphE* were observed in P4 compared to the values reported for P1.

The relative abundance of the analyzed ARGs showed a reduction in all P5 samples compared to the income (P1). Statistical differences compared to the income (P1) are shown in Fig. 8.

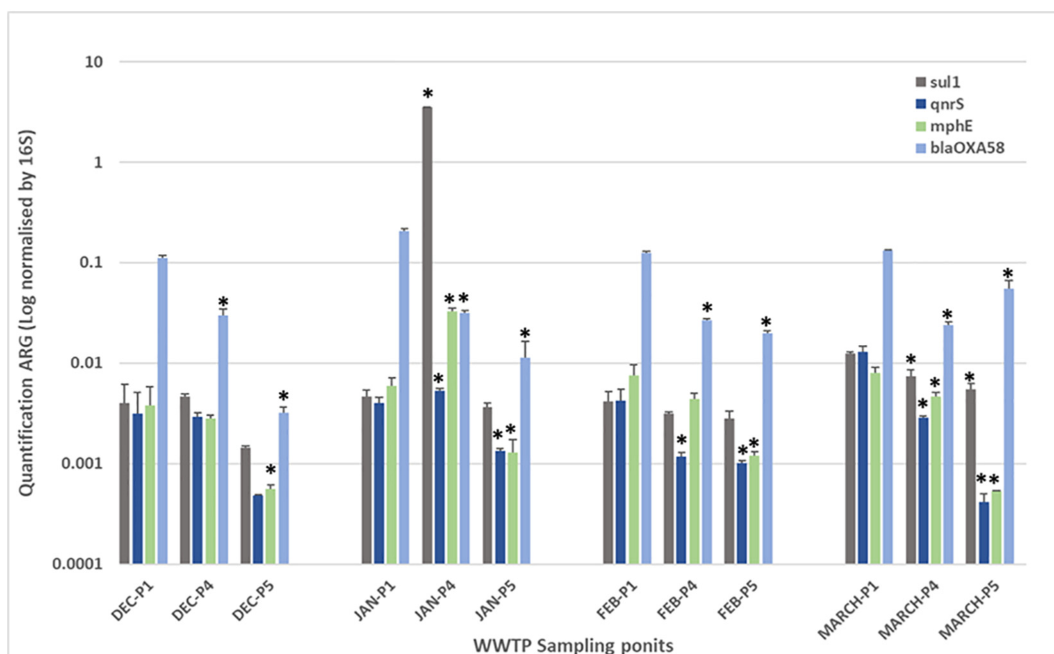

Fig. 8. Quantification of ARGs in the wastewater treatment plant (WWTP). Sul1 (grey), qnrS (dark blue), mphE (green), and bla$_{OXA-58}$ (light blue) quantification in different wastewater samples: P1, influent; P4, effluent; and P5, 5 km downstream the WWTP effluent. Data corresponds to the average value of ARG quantification normalized to the 16S rDNA copy number for each sample. The error bars represent the standard deviation from three qPCR replicates. Asterisks denote statistical significance for each sampling month compared to P1 (ANOVA, Tukey test $p < 0.05$).

3.9. Principal component analysis

Possible correlations between measured antibiotics' concentrations (Table S8) and detected ARG classes (Table S14) were examined by PCA and revealed, as expected, site P1 to dominate the analysis (Fig. S5). Strong correlations were observed between amoxicillin, clarithromycin and ofloxacin and their respective ARG classes at site P1 (Fig. S5). The remaining resistance classes showed only weak or inconsistent correlations with the detected ARGs. A strong correlation was also observed between amoxicillin and the non-corresponding ARGs conferring resistance to aminoglycosides. Site P4 correlated only with the presence of azithromycin and no correlation was detected for site P5.

4. Discussion

In this study, an integrated approach has been used for investigating at the same time pharmaceuticals occurrence (including antibiotics), levels of EDC, presence of herbicides/pesticides, bacterial indicators, microbial communities and ARGs in a selected WWTP and in the downstream river in order to assess their impact on the receiving river.

4.1. Load of fecal contamination along the WWTP and in the downstream river

We observed that total coliforms, *E. coli* and *Enterococci* decreased in number during the wastewater treatment process and the concentration of *E. coli* in the final effluent was always less than 5000 colony forming units (cfu)/100 mL, according to the Italian legislation (Italian Parliament, 2006). All samples in P5 exceeded parametric values concerning the quality of bathing water and included in the European Directive 2006/7/EC (European Commission, 2006). Considering that the guide values for "excellent" water quality in inland water are settled in the Directive to 200 cfu/100 mL for *Enterococci* and 500 cfu/100 mL for *E. coli*, the values detected in P5 (falling in the range of 6.44×10^2–2.65×10^3 MPN/100 mL for *Enterococci* and 1.10×10^3–1.43×10^4 MPN/100 mL for *E. coli*) are indicative of fecal contamination (Table S7 in Supplementary Material file 1). The slightly higher values observed in P5 compared to P4 in March (Table S7 in Supplementary Material file 1) can be explained by the presence of additional sources of contamination in the river (run-off), possibly represented by small farms or industries along the banks of the river or by the nearby lake. It has been indeed extensively reported that animal farming and the industrial sector have a negative impact on the microbial quality of aquatic ecosystems.

4.2. Chemical analysis for investigating the removal of pharmaceuticals and antibiotics

The chemical analysis revealed that most of the antibiotics were present in the WWTP at low concentrations (e.g. chlorotetracycline, lincomycin, roxythromycin, tetracycline) or at levels below the LOD (e.g. erythromycin, sulfamethazine, enrofloxacin) (Table S8 in Supplementary Material file 1). Azithromycin decreased along the plant (except in February) and, in P5, its values were always below the LOD. A negative removal efficiency in P4 compared to P1 was also observed for sulfamethoxazole while most of the other antibiotics showed an efficiency removal above 50%, with amoxicillin being the antibiotic most efficiently removed (98.8%).

Different authors highlight the relationship between the HRT of the WWTP and the removal efficiency of pollutants (Ejhed et al., 2018; Sabri et al., 2021). In our study, this parameter was rather constant during the four sampling campaigns (22.4 ± 1.2 h).

The antibiotics' concentration in the downstream river most likely revealed the effect of the dilution factor being very low and often below the LOD (Table S8 in Supplementary Material file 1). The analyzed antibiotics belong to different classes: macrolides (e.g. azithromycin), sulfonamides (e.g. sulfamethoxazole), quinolones (e.g. ofloxacin), carbapenems (e.g. meropenem, ertapenem), tetracyclines (e.g. tetracycline), beta-lactams (e.g. amoxicillin), lincosamides (e.g. lincomycin) and dihydrofolate reductase inhibitors (e.g. trimethoprim). Three of the selected antibiotics (azithromycin, clarithromycin, and erythromycin) were introduced in the 1st Watch List (WL) (Carvalho et al., 2015), a mechanism for obtaining high-quality monitoring data on emerging pollutants at European Union level that may pose a risk to the environment and human health. Two additional antibiotics (amoxicillin and ciprofloxacin) were added in the 2nd WL (Loos et al., 2018) while sulfamethoxazole and trimethoprim have been included in the 3rd WL (Gomez Cortes et al., 2020). In our study, the antibiotics azithromycin, clarithromycin, sulfamethoxazole and trimethoprim were found to exceed, in P4, the predicted no-effect concentration (PNEC) values, defined as the concentration below which no adverse effects are measured in an ecosystem. Clarithromycin was the only antibiotic showing levels above the PNEC also in P5, thus representing a potential concern for the water environment together with the other antibiotics found to be in excess in P4 (Table S15 in Supplementary Material file 1). However, so far there are no harmonized protocols and official guidelines for the risk assessment of antibiotics in waterbodies which would take into account their contribution to the spread of the antibiotic resistance, since the safety values are only based on ecotoxicological effects. Furthermore, the established safety values are defined for antibiotics as single substances without taking into account the cumulative effects caused by the simultaneous exposure to different antibiotics with the same MoA.

Among the pharmaceuticals analyzed, only ketoprofen, showed a positive removal efficiency. All the other substances were not completely removed during the wastewater treatment (e.g. amisulpride, carbamazepine, diclofenac, irbesartan, metoprolol, propyphenazone), and hence discharged to surface waters, as also reported by others (Bollmann et al., 2016; Hatoum et al., 2019; Angeles et al., 2020; Ferreiro et al., 2020). Among the analyzed pharmaceuticals, carbamazepine and diclofenac exceeded the safety limits provided in Table S16 in all WWTP steps, thus representing a potential risk to the aquatic environment (Supplementary Material file 1).

4.3. Use of bioassays to address the mixture effects

The main issue concerning the continuous discharge of pharmaceuticals into the aquatic compartment is that they could exert adverse effects on the aquatic organisms even if present at low concentrations (ranging from ng/L to μg/L) in water compartments (Khasawneh and Palaniandy, 2021). One limitation for assessing the risk of chemicals to ecosystems is the small number of substances taken into account in the regulation. For example, to assess the chemical status of European water bodies, the WFD focuses only on monitoring a small number of substances (45 priority substances, PS). Member States are also required to monitor other substances of national or local concern (River Basin Specific Pollutants, RBSP), which represent only a small percentage of all chemicals that occur in waterbodies. Several analytic-based techniques intended to overcome this limitation exist, such as high resolution mass spectrometry-based non-target screening (Aceña et al., 2015), which allows the simultaneous detection of thousands of compounds in a water sample. This method could be potentially used to support regulatory environmental monitoring and chemicals management (Sobus et al., 2018; Hollender et al., 2019; Lai et al., 2021). However, it does not cover the environmental risk posed by chemical mixtures and the additive effects of co-occurring chemicals acting through the same MoA. In this context, bioassays provide information on the overall effects of both known and unknown chemicals present in a water sample thus complementing chemical methods for evaluating the quality of a waterbody (Serra-Compte et al., 2021).

Bioassays allow to screen for total effects within a given biological pathway or MoA narrowing down the assessment to a few activated pathways and permit to assess cumulative toxicity of mixtures containing both known and unknown chemicals. In combination, bioassays can give an integrated measure of the toxicity of bioactive substances in environmental samples, especially for those compounds which cannot be fully explained by targeted analysis of known chemicals. Bioassays can be chosen based on chemical structure and common use of substances (e.g. herbicides, insecticides, polycyclic aromatic hydrocarbons) or according to common MoA

and toxicological endpoints (e.g. growth inhibition, endocrine disruption). In such a way, a "fingerprint" of each water body may be created by generating contamination profiles according to infrastructural changes in the surrounding area and adapting the selection of bioassays to newly introduced substances. On the other hand, the integrated data can help in the identification of unknown contaminants and sources of emission.

Here, we used diatoms growth rate inhibition and LiBERA as bioassays to estimate the overall removal of pesticides and EDC, respectively.

The marine diatom *T. pseudonana* growth rate inhibition assay was used as an indicator of the presence of pesticides, herbicides and other pollutants in influent and effluent wastewater. The toxicity tests were conducted to analyze the efficiency of the WWTP on removing potential hazard substances (complex mixtures of chemicals, e.g. herbicides, pesticides). The results of the toxicity tests indicate that the WWTP is not 100% efficient in removing substances affecting the diatom's growth rate. The removal was studied in a four-month period, resulting March the month showing a lower efficiency (Fig. 3). Nevertheless, when studied in surface water (along the river, downstream of the WWTP), such toxic effects were not detected (Fig. 3). Probably, the increase of toxicity in March could be explained by the application of pesticides, herbicides and fertilizers in agriculture concurring with the beginning of the springtime. However, to link the observed effects to the presence of a single chemical or group of chemicals, it would require the integration of additional information such as which pesticides are registered and used in that area and then to target them by analytical analysis.

A large variety of chemical compounds, collectively referred to as EDC, have been shown to be toxic to the aquatic organisms due to their ability to interfere with the normal function of the hormone receptors and hence, the endocrine system. Apart from natural and synthetic hormones, other substances such as bisphenol-A (BPA) and 4-nonylphenol (4NP) are able to bind to the ER, leading to potential adverse effects (e.g. reproductive and endocrine system malfunctions, cancer and feminization in some fish and amphibians). Monitoring the presence of a vast number of different EDC in water and studying their biochemical effect on ER could help ensuring healthy ecosystems (Bilal et al., 2021).

The study by Ferrero et al. (2014) provides a method for detecting compounds that interact with the ER using LiBERA. This method was also applied in an interlaboratory study by Gómez et al. (2021), aimed to determine the estrogenic effects of chemical mixtures by using a panel of estrogenicity bioassays. In the present study, data generated by LiBERA indicate that the removal of estrogenic compounds was not complete in the studied WWTP and that even the samples collected downstream (P5) elicited estrogenic potential (Fig. 4). Although, our results do not allow us linking the observed effects to the presence of any single chemical, they highlight the importance of addressing the overall effects of the water samples by using bioassays covering specific MoAs and mixture effects in addition to the concentration analysis of single compounds. The next step would be then to derive a trigger value for this kind of bioassays or to determine a safety value as relative potency value.

4.4. Changes in the microbial community and in the pathogen prevalence revealed by metagenomics analysis

The 16S profile along the WWTP showed a profound modulation of the microbial community composition. Bacteria belonging to the phylum Proteobacteria were dominant at all the treatment steps and, together with Bacteroidota, are probably responsible for the removal of nutrients and carbon (Weissbrodt et al., 2014). Firmicutes, a phylum representing the 30% of human gut microbiota, decreased in relative abundance, as expected, in all treatment steps compared to the income (P1). Patescibacteria appeared after the biological treatment (P2) up to site P4 being less prominent at P5 in the river. This taxon includes the superphyla *Parcubacteria* (OD1) and *Microgenomates* (OP11) and is characterized by a ultra-small cell size (Luef et al., 2015; Chaudhari et al., 2021) and a small genome, retaining only simple metabolic functions related to glucose and pyruvate (Brown et al., 2015; Beam et al., 2020; Tian et al., 2020). Patescibacteria have been found in freshwater, groundwater, lakes and in WWTP (Proctor et al., 2018) (Tian et al., 2020) but their biological functions are not completely understood due to the difficulty in culturing them in the laboratory. In a livestock wastewater, Patescibacteria together with Proteobacteria and Chloroflexi decreased in concomitance with the degradation of ammonia nitrogen and were negatively correlated with total N, COD, and total P (Yan et al., 2021). Since measurements of the operational and environmental parameters were available only for P1 and P4, intermediate points would have allowed to better understand the reduction of Bacteroidota and Firmicutes and the concomitant increase of Patescibacteria observed after P1. However, the denitrification process upstream the P2 collection site, and the chemical treatment with ferric chloride are known to induce a reduction of total N and total P, respectively. Additionally, our data clearly show a reduction of total N, COD and total P at the end of the treatment processes (P4) (Table S17 in Supplementary Material file 1). Therefore, Patescibacteria may be involved in the nitrogen and phosphorus removal along the WWTP although a recent paper would suggest that the metabolic capacities predicted for Patescibacteria lack nitrogen marker genes (Suguru, 2021). The taxonomic composition in the river (P5) showed a different pattern compared to all the other sampling points and was characterized by an increased presence of phyla typically found in freshwater environments such as Cyanobacteria and Verrucomicrobiota (Sanseverino et al., 2021; Orellana et al., 2022). Actinobacteria increased in P5 compared to the other sampling points and this trend was probably due to the presence of Nanopelagicales in the water environment, as suggested by their detection in Lake Varese (located upstream the P5 site) (Sanseverino et al., 2021), and to the contribution of Actinobacteria like Acidimicrobiales involved in wastewater treatment processes and discharged into the receiving river (Rossetti et al., 2005).

Concerning the shotgun analysis, the data reproduced relatively well the profile obtained by the 16S. However, only 30% and 40% of reads for site P4 and P5 were taxonomically classified already at the phylum level, compared to about 80% at site P1. This likely indicates the presence of many bacteria from human sources at P1, thus justifying their accurate annotation in the databases. Instead, at sites P4 and P5 there was a significant increase, relative to P1, of hits against eukaryotic 18S and 23S genes strongly suggesting a relevant presence of eukaryotes. It is therefore plausible that this presence, even if representing only a small percentage of the cells in the sample, strongly reduces the fraction of bacterial shotgun reads due to a much larger eukaryotic genome size (generally more than 10- or 20-fold larger). A routine screening of the shotgun data for the presence of 18S and 23S genes might therefore help to more completely understand, at least qualitatively, the range of organisms present in the samples. An additional confounding factor might be the presence, at sites P4 and P5, of a relatively large population of phages.

Shotgun analysis revealed that most of the pathogens were reduced in the WWTP. The genus *Acinetobacter* dominated the P1 samples followed by the genera *Bacteroides* and *Pseudomonas.* At the species level, among the detected human pathogenic bacteria, *Phocaeicola vulgatus* was the most abundant species in P1 (Table S13 in Supplementary Material file 1) (Tang et al., 2016). Considering that it can cause intra-abdominal infections and other infections that originate from the gut flora, its persistence at P4 should deserve attention. Additional pathogenic bacteria which were higher in P1 compared to P4 and P5 are: *Acinetobacter johnsonii CIP 64.6*, *Escherichia coli*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Table S13 in Supplementary Material file 1). *A. johnsonii* is usually found in the aquatic environment and it can cause septicemia, endocarditis, abscess and urinary tract infections in humans (Turton et al., 2010; Feng et al., 2016). *Acinetobacter baumannii,* belonging to the same genus Acinetobacter, is a hospital pathogen representing a leading cause of nosocomial infections worldwide (Dexter et al., 2015). In the last decades, *Acinetobacter baumannii* has been frequently detected in hospital WWTP and its emission to the environment represents a serious concern (Ferreira et al., 2011; Seruga Music et al., 2017; Higgins et al., 2018; Dekic et al., 2019). Although, according to the sequencing data, the analyzed WWTP seems highly efficient in reducing the *Acinetobacter*

baumannii, we do not have information on which specific operational step was mainly involved in its reduction. However, the release of *Acinetobacter baumannii* into the natural environment via WWTP effluents, even if at very low concentration, could concur to the spread of AMR. Isolates of *Acinetobacter baumannii* have been found to contain resistance genes against carbapenems, macrolides, aminoglycosides, tetracycline, and chloramphenicol (Higgins et al., 2018). This is in line with the inclusion of *A. baumanii* in the list of the ESKAPE pathogens which are characterized by exhibiting multiple antibiotic resistances. Among the ESKAPE pathogens, *Klebsiella pneumoniae* is a nosocomial pathogen causing infections with high mortality rates (up to 50%) (Bassetti et al., 2018). Although its removal, in the analyzed WWTP, is high, the release of this pathogen in the river could contribute to the release of ARGs in the environment. This data underlines that it is of great concern to investigate the pathogenic bacteria discharged from WWTPs to the receiving water not only because detected microorganisms are harmful for human health, animals and the environment, but also because the horizontal transfer of ARGs could occur into human bacterial pathogens along the wastewater treatment process and increase the risk of these genes to be disseminated through the waterbodies.

4.5. Contribution of the wastewater treatments in the removal of ARGs

Our metagenomic analysis on ARGs showed that their relative abundance generally decreased along the treatment plant, with only few resistance genes having an enrichment in the freshwater sampling site (P5) compared to the WWTP effluent (P4) (Fig. 7). The qPCR generally confirmed the reduction trend in December, February and March, while in January an increase of *sul1*, *qnrS* and *mphE* was observed in P4 compared to P1 (Fig. 8). An increase of ARGs abundance in the effluent has also been reported in other studies (Laht et al., 2014), and further investigations are needed to better understand this trend. Discrepancies in the relative abundances of the analyzed ARGs between qPCR and the shotgun metagenomic analysis highlight the complexity of the resistome dynamics in the aquatic environments and may derive from the different detection sensitivity and potential inhibition associated with either of the methods. Main ARGs found in the WWTP belong to the macrolides (e.g. *mphE* and *msrE*), β-lactams (e.g. bla_{OXA-58}), sulfonamides (e.g. *sul1*) and quinolones (e.g. *qnrS*). In Europe, macrolide antibiotics are approved for both human and veterinary use. In humans, they are used for treating respiratory, skin and gastrointestinal infections, and as a common alternative for patients with a penicillin and cephalosporin allergy. In animals, and in particular in cattle, macrolides are extremely useful in the treatment of respiratory tract infections. The European Medicines Agency has included them, together with other antibiotic classes like quinolones, sulfonamides and β-lactams, in the "Categorization of antibiotics for use in animals for prudent and responsible use" (https://www.ema.europa.eu/en/news/categorisation-antibiotics-used-animals-promotes-responsible-use-protect-public-animal-health).

Finally, we performed a PCA analysis to gain a first insight into the correlation between the relative abundance of ARG classes and the detected concentrations of antibiotics at the different sampling sites (P1, P4 and P5). Our data showed a strong relationship between antibiotics and their corresponding or non-corresponding ARGs in P1, but not in the effluent and in the downstream river. The observed correlations in P1 not necessarily reflect the induction or promotion of resistances triggered by treatment with the measured antibiotics due to factors arising from an existing intrinsic natural resistance (Reygaert, 2018), and therefore should be considered with the necessary caution. Reasons that could contribute to the missing correlation between antibiotics and ARGs in P4 and P5 may include their different environmental fate. Furthermore, considering that our testing method includes only selected substances, possible correlations between ARGs and not analyzed antibiotics may have been excluded.

Although further investigations are necessary to establish such relationships, our study showed that the selected WWTP can reduce but not completely eliminate the ARGs. Therefore, the downstream river water still contains these genes which potentially constitute health risks for humans and animals via pathogenic and non-pathogenic bacteria. For this reason, ARGs will be proposed as AMR indicators along the European One Health Action Plan (European Commission, 2017) and the strategy on Pharmaceutical in the Environment (PiE) (European Commission, 2019) which among their actions include the reduction of the emergence and spread of AMR and the development and availability of new effective antimicrobials.

5. Conclusions

Although the contribution from the WWTP to the water pollution is widely documented, in this pilot study, we envisage a holistic approach by combining different methodologies and data integration to generate a contamination profile in a given waterbody. Particularly, we emphasize the urgent need of risk assessment methods for emerging threats/concerns as AMR and chemical mixtures.

For the latter, we show that bioassays provide a useful screening approach to determine the removal efficiency of WWTPs and presence/effects of pollutants in a receiving river. Two in vitro bioassays, LiBERA and algal growth rate inhibition assay, were chosen to target selected groups of substances, i.e. endocrine disruptors and pesticides, respectively. However, bioassays covering other MoAs (e.g. carcinogenicity) can be applied for detecting the presence of known and unknown pollutants depending on the source of contamination. This could, then, help to narrow the chemical analysis to specific chemicals or class of chemicals to be monitored for the emission source by integrating data on anthropogenic activities in the area.

For AMR, we identified the most enriched ARGs in freshwater suggesting their potential for monitoring as indicators of contamination by resistance genes in the environment. However, the natural resistance should be investigated to establish the abundance of ARGs in anthropogenically-impacted areas. Waterbodies can act as reservoirs of resistance–carrying genes and favour the continuous ARGs transfer from water environment to human pathogens, compromising the ability of microorganisms to withstand the effects of antibiotics. Overall, this novel data-integration approach is the way to pursue for better understanding of the contamination patterns in a specific waterbody required for water management. At the same time, it will contribute to achieve the goals of European initiatives such as Zero Pollution Action Plan and Farm to Fork Strategy under the Green Deal Agenda.

CRediT authorship contribution statement

Teresa Lettieri: Conceptualization; **Isabella Sanseverino**, **Livia Gómez**, **Francesca Cappelli, Anna Navarro Armin Lahm, Rosalba Pedraccini, Simone Crosta, Maurizio Barbiere:** Investigation and Formal analysis; **Armin Lahm, Isabella Sanseverino, Livia Gómez, Francesca Cappelli, Sara Valsecchi, Anna Navarro**: Data Curation; **Isabella Sanseverino, Livia Gómez**, **Armin Lahm, Magdalena Niegowska, Elena Porcel-Rodríguez**: Visualization; **Isabella Sanseverino, Livia Gómez, Armin Lahm, Magdalena Niegowska, Anna Navarro, Teresa Lettieri:** Writing - original draft;, **All co-authors**: Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

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