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Characterisation of microbial communities and quantification of antibiotic resistance genes in Italian wastewater treatment plants using 16S rRNA sequencing and digital PCR

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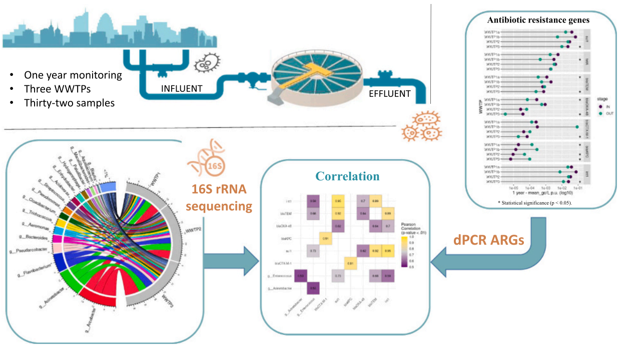
HIGHLIGHTS

- A one-year study of antimicrobial resistance in WWTPs influent and effluent.
- Microbial communities and ARGs analyzed using 16S rRNA sequencing and dPCR.
- The effluents were found to have greater microbial diversity than the influents.
- The sul1 gene was the one that was present in the higher concentrations.
- An increase in ARGs in effluents was observed in some instances

GRAPHICAL ABSTRACT



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ABSTRACT

The spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in humans, animals and environment is a growing threat to public health. Wastewater treatment plants (WWTPs) are crucial in mitigating the risk of environmental contamination by effectively removing contaminants before discharge. However, the persistence of ARB and ARGs even after treatment is a challenge for the management of water system.

To comprehensively assess antimicrobial resistance dynamics, we conducted a one-year monitoring study in three WWTPs in central Italy, both influents and effluents. We used seasonal sampling to analyze microbial communities by 16S rRNA, as well as to determine the prevalence and behaviour of major ARGs (*sul*1, *tet*A, bla_{TEM}, $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-1 group}}$, bla_{KPC}) and the class 1 Integron (*int*1). Predominant genera included in order: *Arcobacter*, *Acinetobacter*, *Flavobacterium*, *Pseudarcobacter*, *Bacteroides*, *Aeromonas*, *Trichococcus*, *Cloacibacterium*,

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Pseudomonas and *Streptococcus*. A higher diversity of bacterial communities was observed in the effluents compared to the influents. Within these communities, we also identified bacteria that may be associated with antibiotic resistance and pose a significant threat to human health. The mean concentrations (in gene copies per liter, gc/L) of ARGs and *int*1 in untreated wastewater (absolute abundance) were as follows: *sul*1 (4.1×10^9), *tet*A (5.2×10^8), bla_{TEM} (1.1×10^8), $bla_{\mathrm{OXA\text{-}48}}$ (2.1×10^7), $bla_{\mathrm{CTX\text{-}M\text{-}1\ group}}$ (1.1×10^7), bla_{KPC} (9.4×10^5), and *int*1 (5.5×10^9). The mean values in treated effluents showed reductions ranging from one to three log. However, after normalizing to the 16S rRNA gene (relative abundance), it was observed that in 37.5 % (42/112) of measurements, the relative abundance of ARGs increased in effluents compared to influents. Furthermore, correlations were identified between ARGs and bacterial genera including priority pathogens. This study improves our understanding of the dynamics of ARGs and provides insights to develop more effective strategies to reduce their spread, protecting public health and preserving the future efficacy of antibiotics.

1. Introduction

Antibiotic resistance is a threat to human and animal health worldwide, and key measures are required to reduce the risks posed by antibiotic bacteria (ARB) or antibiotic resistance genes (ARGs) that occur in the environment (Berendonk et al., 2015). Although the complete picture is still unclear, there is a growing body of evidence on the mass flows of both selective agents and resistant bacteria that reach the environment from different sources and through distinctive pathways (Larsson and Flach, 2022). In order to comprehensively assess the impact of different sources of antibiotics and ARBs on the environment, it is crucial to understand the abundance, characteristics and both natural and anthropogenic variability of ARGs and mobile genetic elements (MGEs). In addition, determining their mobilisation and transfer in the environment is essential for a thorough assessment. All these elements will be necessary in defining containment measures, including the identification of critical control points, the development of reliable surveillance and risk assessment procedures, and the implementation of technological solutions that can prevent environmental contamination with ARB and ARGs.

Recognizing AMR as a major One Health challenge, the WHO proposed a global action plan to extend surveillance to all human interfaces, including the environment (WHO, 2021). In June 2017, the EU adopted an action plan focusing on the role of the environment in the development of AMR (EU, 2017). More recently, the Council underlined the urgent need to monitor AMR in the environment to assess the associated risks to human health (Council of the European Union, 2023).

The European Commission has proposed monitoring obligations for the presence of AMR in urban wastewaters (EU, 2022).

Among all environmental habitats, urban and hospital wastewater are recognized as the primary point sources of environmental contamination by AMR determinants (Berendonk et al., 2015). Although wastewater treatment plants (WWTPs) are designed to remove pollutants, there is significant evidence that treatments are not very effective in eliminating ARB and ARGs (Rizzo et al., 2013; Singh et al., 2019). The presence of antimicrobial residues in the effluent entering wastewater treatment plants, even at sub-inhibitory concentrations, can promote the proliferation of ARBs and the eventual transfer of antibiotic resistance genes through horizontal gene transfer (HGT) (Berendonk et al., 2015; Krzeminski et al., 2019). This is facilitated by suitable conditions such as the abundance of nutrients and the proximity of cells, which enhance cell-to-cell interactions. Sewage provides a continuous input of ARGs, ARB, and highly diverse commensal and pathogenic bacteria from human and animal microbiomes. Wastewater-based epidemiology offers a promising approach to monitor and analyze the prevalence and dissemination of antibiotic resistance in community populations through sewage analysis (Chau et al., 2022). ARGs often assemble near one another on MGEs generating complex resistance regions (CRRs) (Nguyen et al., 2021). Thus, conditions found in WWTPs can create an ideal environment for the evolution of new and more complex CRRs as well as their HGT to new hosts. In addition, after disinfection, extracellular DNA produced by the destruction of resistant bacterial cells is released into the receiving water bodies. The acquisition and integration of ARGs from extracellular DNA by competent bacteria in aquatic environments represent a rout for the antibiotic resistance transmission to human pathogens, hence posing a risk to human health (Soucy et al., 2015; Moura et al., 2007; Stanton et al., 2022). It is also important to note that the use of conventional disinfection processes may be only partially effective in the inactivation of ARBs and could also lead to the selection of ARBs among the bacteria that survive the treatment (Fiorentino et al., 2015; Di Cesare et al., 2016b).

Assessing the risk to human health associated with the spread of ARBs and ARGs in the environment is currently challenging and represents a significant knowledge gap, as no alert levels have been established with a view to prevention (Manaia, 2017). Therefore, conducting environmental monitoring is crucial for understanding the fate of ARBs and ARGs during wastewater treatment. This is a complex issue due to various external factors and the treatment system's complexity. In recent years, numerous research groups worldwide have embarked on initiatives to examine antibiotic-resistant bacteria and resistance genes in the environment (Cacace et al., 2019; Gatica et al., 2016; Hendriksen et al., 2019; Miłobedzka et al., 2022; Pärnänen et al., 2019; Rocha et al., 2020).

In Italy, several studies have investigated the occurrence of ARB and ARGs in untreated and/or treated wastewater. Some of these studies have focused on specific targets, such as *Klebsiella pneumoniae*, *Escherichia coli*, or *Enterococcus* sp. (Ferro et al., 2016; Fiorentino et al., 2015, 2019; Piccirilli et al., 2019; Pellegrini et al., 2011; Rizzo et al., 2013; Turolla et al., 2018; Zanotto et al., 2016). Some studies have focused on genotypic evaluation alone, while others have combined genotypic and phenotypic assesment (Bonetta et al., 2023; Corno et al., 2019; Di Cesare et al., 2016a, 2016b; Fiorentino et al., 2019; Piccirilli et al., 2019; Pellegrini et al., 2011; Subirats et al., 2019; Turolla et al., 2018; Zanotto et al., 2016). In addition, a few of these studies investigated the effectiveness of wastewater disinfection treatments in removing ARB and ARGs (Bonetta et al., 2023; Fiorentino et al., 2015; Rizzo et al., 2013). Although a significant reduction in ARBs and ARGs was observed in wastewater treatment plants after treatment in these studies, they were not completely removed.

The present study undertaken in three WWTPs located in central Italy was carried out with different objectives: i) to investigate the diversity of the microbial community in both the influent and effluent of the WWTPs, while assessing the potential presence of genera that may include pathogens of public health significance ("priority pathogens") for AMR, as outlined by WHO (WHO, 2017); ii) to identify and quantify some selected key antibiotic resistance genes between the most diffused and transferable, which are critical for understanding the landscape of antibiotic resistance dissemination; iii) to evaluate the impact of wastewater treatment processes in the occurrence of ARB and ARGs. iv) to attempt to correlate the levels of ARGs with the genera of bacteria including priority pathogens.

2. Materials and methods

2.1. Sampling and DNA isolation

Composite (24 h) influent and effluent samples were collected from three WWTPs (WWTP1–3) located in central Italy. In particular, WWTP1 consists of two separate lines originating from two different areas of the same city, namely WWTP1-a and WWTP1-b, processed independently. Supplementary Table 1 provides geographical, demographic, and technical characteristics for each of these WWTPs. Raw influent (50 mL) and treated effluent (500 mL) samples were collected. Sampling was performed every three months (seasonal sampling) over the course of one year, from both the inlet and outlet of the plants. In addition, pre-disinfection samples were taken during the summer season when an additional disinfection step is carried out to reduce the risk associated to bathing activities, giving a total of 36 samples (16 influent, 16 effluent, 4 pre-disinfection). These samples were transported to the laboratory under refrigerated conditions (+4 °C) and processed immediately.

Samples were filtered through sterile polycarbonate membranes with a porosity of 0.22 μm (Whatman, UK). For each sampling campain a blank samples was filtered. Total DNA was extracted using the DNeasy PowerWater Kit (QIAGEN) according to the manufacturer's instructions. Each sample yielded 100 μL of final extract, was aliquoted and stored at −80 °C for subsequent analysis. DNA quality and concentration were determined using both the Qubit dsDNA HS fluorometric assay kit (Ref. Q32851, Invitrogen, Carlsbad, CA 92008, USA) and the Agilent® High Sensitivity DNA kit (Ref. 5067–4626).

2.2. Amplicon sequencing of 16S rRNA

The 16S rRNA V3-V4 variable region (~460 bp) (Klindworth et al., 2013) was amplified according to the MiSeq rRNA amplicon sequencing protocol (Illumina, San Diego, CA) with some modifications. The PCR was performed in a final volume of 25 μL, containing 5 μL of genomic DNA (10 ng/μL in H2O), 1× PCRBIO HiFi Buffer (PCR BIOSYSTEMS, USA) consisting of 1 mM dNTPs and 3 mM MgCl2, 0.5 units of PCRBIO HiFi Polymerase and 0.2 μM of each primer. Cycling conditions were as follows: initial denaturation at 95 °C for 3 min, 25 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (30 s), final extension at 72 °C for 5 min, hold at 4 °C. DNA amplicons were purified using MagSi-NGSPREP Plus beads (Euroclone, Milan, IT) and Illumina Nextera adaptor primers were used to barcode each sample by PCR as follows: 5 μL of cleaned up DNA amplicon, 1 × PCRBIO HiFi and 0.2 μM of each primer. Cycling conditions were as follows: initial denaturation at 95 °C for 3 min, 8 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (30 s), final extension at 72 °C for 5 min, hold at 4 °C. DNA amplicons were purified using MagSi-NGSPREP Plus beads (Euroclone, Milan, IT) and the final library was quantified using the QuDye dsDNA HS Kit (Lumiprobe, Hannover, DE). Samples were pooled and sequenced on an Illumina MiSeq™ platform according to the manufacturer's specifications to generate 300 base-length paired-end reads.

2.3. Bioinformatic analysis and diversity indexes

Bioinformatic analysis was performed using the Quantitative Insights Into Microbial Ecology v2022.2 (QIIME2) software suite and R packages. Environmental microbiome data were analyzed using DADA2 algorithm and an in-house trained classifier for taxonomic assignment following QIIME2 protocol. The Silva database, release 138.1, served as the reference database for the identification of bacteria present in each sample. Amplicon sequence variant (ASVs) clustering was performed using a 97 % sequence similarity cut-off, coupled with a taxonomic assignment confidence level > 0.75. Dominant bacterial genera were defined as those representing >1.0 % of the total community, consistent with criteria established in previous studies (Numberger et al., 2019).

Alpha and beta diversity analyses were performed using the *vegan v.2.6–4* package for R, using default parameters. Alpha diversity was calculated using the Shannon-Weaver index (0 to infinite) to measure differences within each community in a single sample. Beta diversity was calculated using the Bray-Curtis index (0 to 1) to assess differences between microbial communities in the influent and effluent of the same WWTP during the same season. Both indexes were calculated on the entire community without applying any abundance cut-off.

2.4. Digital PCR (dPCR)

Digital PCR (dPCR) was employed to measure the abundance of the 16S rRNA gene, the class 1 Integron (*int*1), and a panel of genes associated with antibiotic resistance, including sulfonamides (*sul*1), tetracyclines (*tet*A), and β-lactams (bla_{TEM}, bla_{OXA48}, $bla_{\mathrm{CTX\text{-}M\text{-}1\ group}}$, bla_{KPC}). Quantification was performed using the QIAcuity digital PCR System (Qiagen) five-plex device, and Nanoplate with a 26 K configuration. Quantitative results were generated using the Quicuity Softwere Suite (Version 2.2.0.26) (Qiagen, Hilden, Germany).

The dPCR Microbial DNA Detection Assays (Qiagen) were used to quantify the genes *sul*1, *tet*A, bla_{TEM}, bla_{OXA48}, $bla_{\mathrm{CTX\text{-}M\text{-}1\ group}}$. The *int*1 gene was quantified using a published assay (Barraud et al., 2010). The QIAcuity UCP Probe PCR Kit (Cat. No. / ID: 250121) was used to prepare all prereactions, following the manufacturer's instructions.

The QIAcuity Eva Green Kit (Cat. No. / ID: 250111) was used to perform the bla_{KPC} assay (home-designed using Primer3Plus, https://www.primer3plus.com/primer3plusPackage.html) and the 16S rRNA assay (Muyzer et al., 1993) following the manufacturer's instructions. Details of the assays used in this study are provided in supplementary Table 2. As positive control a *Salmonella enterica* subsp. *enterica,* serovar Choleraesuis (ATCC™ 10708) strain was used for 16S rRNA, a ceftazidime/avibactam-resistant KPC-producing *K. pneumoniae* CZA-R (Accession: PRJNA866305) strain CZ5 (Bongiorno et al., 2023) for *sul*1, *tet*A, bla_{TEM}, $bla_{\mathrm{CTX\text{-}M1\ group}}$, $bla_{\mathrm{OXA\text{-}48,}}$ bla_{KPC} and *int*1. A negative control was systematically included in each run.

To setup the assay and work within the dPCR instrument's dynamic range, 10-fold serial dilutions, (ranging from 10^{-1} to 10^{-8}) were prepared for both target genes and their relative positive controls. Subsequently, based on the results obtained, we determined whether singleplex or duplex use was appropriate for the target genes. Each assay was performed in duplicate, using 5 μL of template. The target concentration (copies/μl) in each sample was calculated by utilizing the instrument outcome and applying the formula:

$$\text{Sample concentration (copies}/\mu\text{l}) = \text{dPCR outcome (copies}/\mu\text{l})\\ \times (\text{Reaction vol.})/\text{DNA vol.}$$

The total number of genome copies in the initial sample volume was then obtained by multiplying by the appropriate dilution factors, and the concentration was finally expressed as copies/L.

The abundance of ARGs and *int*1 was quantified in terms of both absolute abundance (gene copies/L) and relative abundance (gene copies normalized to the 16S rRNA gene).

Potential inhibition was assessed by measuring the linearity of target gene concentrations in 10-fold serial dilutions. The *intra-assay coefficient* of *variation* (CV) for each gene was calculated as follow: mean (SD of replicate mean / replicate mean × 100).

The entire workflow was performed in accordance to the Digital MIQE Guidelines (dMIQE Group and Huggett JF, 2020) (Table S5).

2.5. Statistical analysis

The assessment of statistical significance ($p < 0.05$) on the absolute abundance variation of ARGs between influent and effluent stages was conducted using a two-sided paired *t*-test. For each ARG, two groups were constructed based on all sampling points within one year per WWTP (2 replicates × 4 seasons = 8) and per influent or effluent stage.

This analysis was carried out using *stats v.4.3.1* package for R.

To assess potential associations between the relative abundance of ARGs and genera that include priority pathogens, we calculated the Pearson's correlation coefficient and its statistical significance (p-value <0.01) with a two-sided unpaired t-test for all sampling points in the combined distribution of WWTPs. This includes 4 seasons, 3 WWTPs, and 2 stages (influent and effluents), resulting in a total of 24 data points. This analysis was carried out using *Hmisc v.5.1–2* package for R.

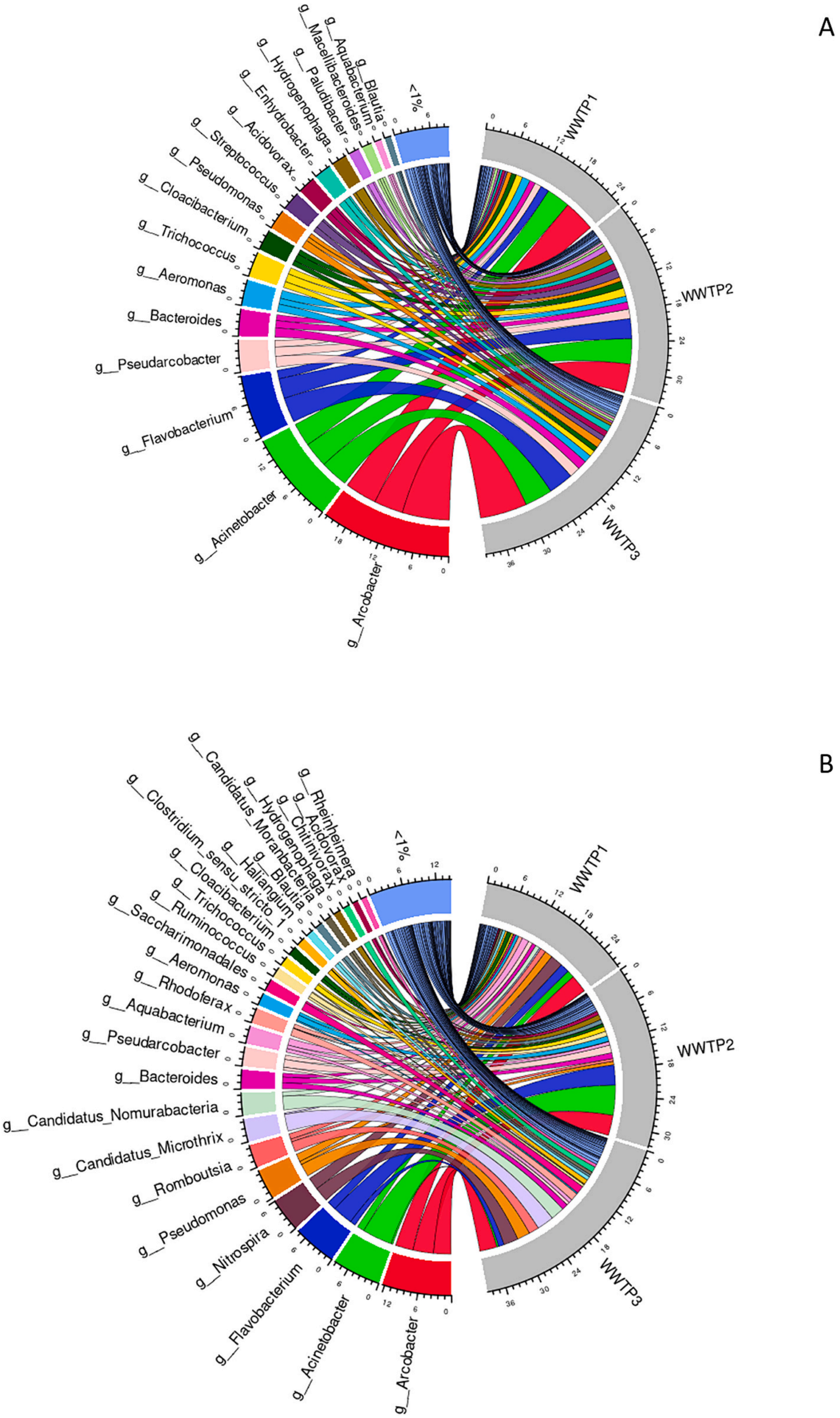

Fig. 1. Proportion of bacterial genera in influent samples (A) and effluent samples (B) for each WWTP throughout the entire sampling period, aggregated over all four seasons.

3. Results

3.1. Amplicon sequencing of 16S rRNA, bacterial community composition and diversity

A total of 27 samples were sequenced instead of 36, since samples from WWTP1 lines a and b were combined for sequencing purposes. The Illumina 300 bp paired-end sequencing generated 2,051,425 sequence reads (mean length of 416 bp). The number of reads per sample ranged from 45,365 to 143,777. After quality filtering, 1,405,887 reads passed the filter. Subsequent merging and filtering (ASVs confidence level > 0.75) resulted in 464,404 non-chimeric reads, with a frequency per sample ranging from 9935 to 39,758. A total of 7210 ASVs were identified describing the complete community diversity for all samples as showed by rarefaction curves plateau (Fig. S1). Subsequently, ASVs were aggregated at genus level as the minimum discriminant taxonomic unit.

The community composition of influent and effluent samples for each WWTP, divided by season, is listed in the Supplementary material (Table S3). In the influent, the predominant phyla included: *Proteobacteria* (37 %), *Bacteroidota* (23.4 %), *Firmicutes* (11.2 %), *Campylobacterota* (27.4 %), *Fusobacteriota* (0.5 %), *Actinobacteriota* (0.4 %) and *Nitrospirota* (0.1 %).

The effluent contained five additional dominant phyla: *Proteobacteria* (32.1 %), *Firmicutes* (13 %), *Bacteroidota* (15.9 %), *Campylobacterota* (15.8 %), *Patescibacteria* (8.9 %), *Actinobacteriota* (5.7 %), *Nitrospirota* (6 %), *Myxococcota* (1.4 %), *Bdellovibrionota* (0.6 %), *Cyanobacteria* (0.2 %), *Fusobacteriota* (0.1 %) and *Verrucomicrobiota* (0.1 %). The dominant bacterial groups identified in the samples taken before disinfection were largely similar to those found in the corresponding effluents. However, there were instances where a slight decrease (up to 4 %) in abundance was observed, while in other cases, there was an increase (up to 10 %).

To describe the microbial communities in the wastewater at the genus level, we classified the taxonomic units into two groups: dominant genera (with a relative abundance >1 %) and genera that may include priority pathogens (regardless of their abundance). Based on the above criteria, 43 genera from 26 families were characterised as dominant in the influent, while 71 genera from 53 families were detected in the effluent. The chord diagrams in Fig. 1 illustrate the proportion of genera in influent and effluent samples for each WWTP throughout the whole sampling period, aggregated across all four seasons. In the influents, approximately half of the microbial community, in terms of relative abundance, consisted of only three genera: *Arcobacter* (22 %), *Acinetobacter* (15.5 %) and *Flavobacterium* (10.9 %). This pattern was consistent across all WWTPs. In the effluents, the same genera were dominating but with lower percentages: *Arcobacter* (12.5 %), *Acinetobacter* (8.7 %) and *Flavobacterium* (7.8 %). It's worth mentioning the presence of *Aeromonas* and *Pseudomonas* genera in both influent and effluent, accounting for about 2–5 % of the microbial community. Four genera were found to be consistently present in influents: *Acinetobacter*, *Aeromonas*, *Arcobacter*, and *Pseudarcobacter*; five genera were almost always present: *Acidovorax*, *Bacteroides*, *Cloacibacterium*, *Flavobacterium*, *Trichococcus*. On the other hand, in the effluents, the *Romboutsia* genera was present in all sampling points and three genera were almost always present: *Flavobacterium*, *Arcobacter* and *Pseudoarcobacter* (Supplementary Table 3).

The relative abundance of genera between influents and effluents across seasons is plotted in Fig. 2. Different genera were present in different seasons: *Arcobacter* was dominant in winter in all WWTPs, while *Acinetobacter* prevailed in spring in all WWTPs. *Flavobacterium* showed a maximum abundance in spring and summer, a decrease in autumn and a minimal presence in winter. *Pseudomonas* also followed this trend, but with a lower relative abundance.

In addition to characterising the predominant microbial communities, our investigation focused on identifying potential pathogens of public health concern. For this purpose, we referred to the WHO global priority pathogen list for antimicrobial resistance (WHO, 2017), which catalogues 12 bacteria that pose the greatest threat to human health and are categorised as critical, high or medium priority based on the urgency of the need for new antibiotics. Due to the limitations of our method (16S RNA sequencing) in species-level characterisation, we identified the genera that potentially include the priority pathogens. Seven of these were detected in influent and effluent: *Acinetobacter* (15.5 % and 8.7 %

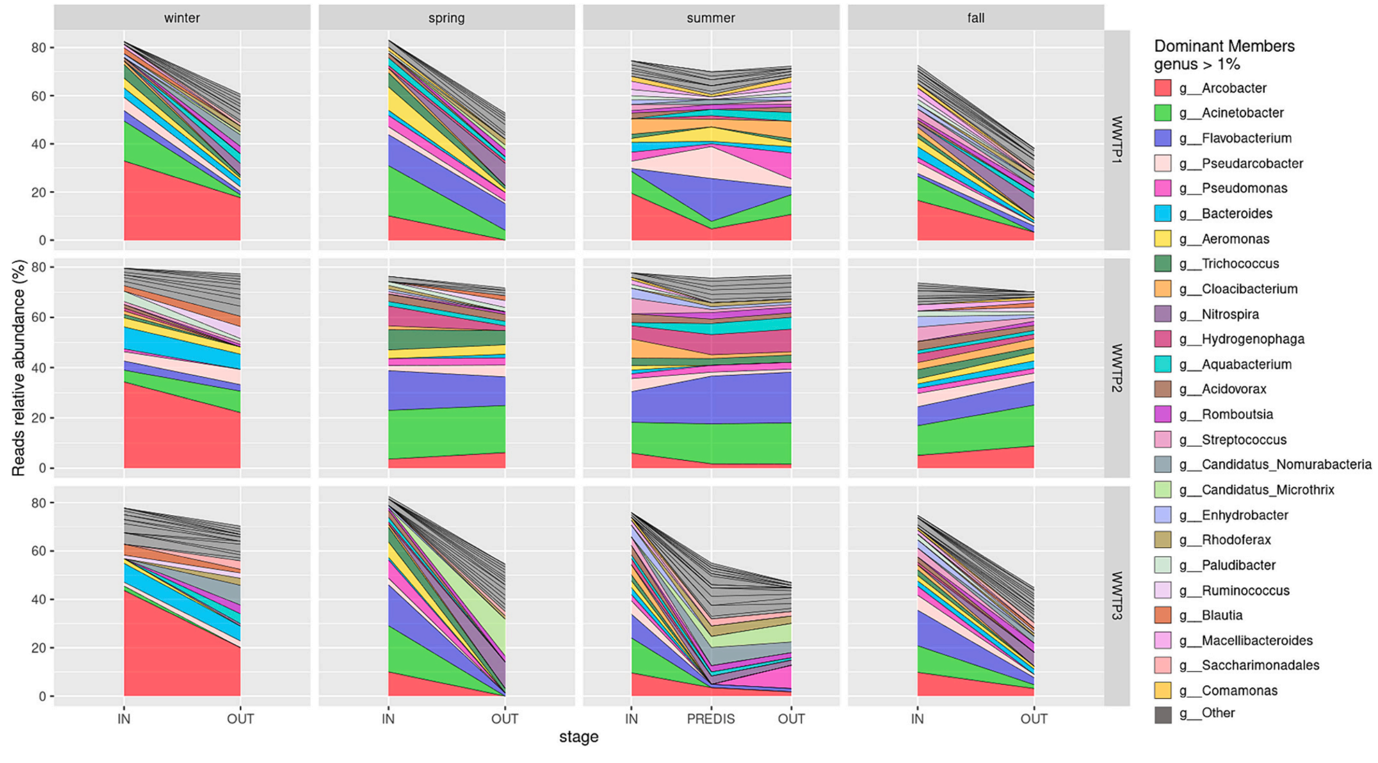

Fig. 2. Stacked area plot comparing the relative abundance of genera between influents, pre-disinfection (only during summer season) and effluents.

respectively), *Enterococcus* (0.2 %; 0.1 %), *Escherichia-Shigella* (0.1 %; 0.1 %), *Mycobacterium* (0.2 %; 0.6 %), *Pseudomonas* (3.3 %; 5.4 %), and *Streptococcus* (2.5 %; 0.6 %). *Klebsiella* was found exclusively in effluents (0.1 %) (Fig. 3). The relative abundance of *Enterococcus* and *Escherichia-Shigella* in both influent and effluent was consistently <1 %. *Mycobacterium* was present in all seasons, mainly in effluents.

Overall, alpha diversity varied across different stages of WWTPs. Effluents generally displayed the highest alpha diversity values with the exception of WWTP2 which showed a much lower increase. Additionally, the diversity index values were similar between the pre-disinfection stage and the effluents for all WWTPs. Beta dissimilarity highlighted significant differences in microbial communities between influent and effluent, with variations observed across seasons and WWTPs. WWTP3 showed the greatest dissimilarity between influent and effluent microbial communities across all seasons, followed by WWTP1 and WWTP2. The alpha and beta diversity of the microbial communities is shown in Supplementary Fig. 2. The rarefaction curves (Fig. S1) demonstrate that the plateau was consistently reached, indicating that sequencing depth did not impact the alpha or beta indexes.

3.2. 16S rRNA and ARGs quantification

The absolute abundance (gene copies/L) and the relative abundance (gene copies/16S rRNA gene copy) were calculated for ARGs and *int*1 (Table 1). The results obtained from the 10-fold serial dilutions demonstrated linearity, thus indicating that no inhibition was observed in the samples. Examples of the graphical output for the positive and negative controls for each gene are reported in Supplementary Material

Table 1

Range of concentrations for absolute abundance (g.c./L) and relative abundance (g.c./16S rRNA g.c.) in Influents (IN) and Effluents (OUT).

Gene	Absolute abundance (g.c./L)		Relative abundance (g.c./16S rRNA g.c.)	
	IN	OUT	IN	OUT
16S rRNA	9.9×10^{9}–6.0×10^{11}	2.4×10^{7}–2.6×10^{11}	–	–
sul1	4.7×10^{8}–1.2×10^{10}	10.0×10^{4}–9×10^{8}	2.3×10^{-3}–2.0×10^{-1}	7.8×10^{-4}–5.9×10^{-2}
tetA	4.4×10^{7}–1.8×10^{9}	7.7×10^{3}–8.0×10^{7}	2.0×10^{-4}–1.4×10^{-2}	3.9×10^{-5}–1.0×10^{-2}
$\mathit{bla}_{\mathrm{TEM}}$	1.1×10^{7}–2.5×10^{8}	4.7×10^{4}–2.0×10^{7}	3.2×10^{-4}–6.6×10^{-3}	9.5×10^{-7}–2.4×10^{-3}
$\mathit{bla}_{\mathrm{OXA\text{-}48}}$	5.9×10^{5}–1.2×10^{8}	1.4×10^{3}–5.0×10^{7}	8.2×10^{-6}–3.1×10^{-3}	2.5×10^{-8}–2.0×10^{-3}
$\mathit{bla}_{\mathrm{CTX\text{-}M\text{-}1}}$ group	2.1×10^{5}–4.5×10^{7}	1.1×10^{4}–4.0×10^{6}	1.9×10^{-6}–9.9×10^{-4}	1.4×10^{-7}–3.5×10^{-1}
$\mathit{bla}_{\mathrm{KPC}}$	1.8×10^{5}–2.2×10^{6}	7.4×10^{3}–2.2×10^{6}	1.4×10^{-6}–6.6×10^{-5}	3.0×10^{-6}–9.8×10^{-4}
int1	4.3×10^{8}–1.5×10^{10}	1.5×10^{5}–1.3×10^{9}	1.8×10^{-2}–2.3×10^{-1}	9.8×10^{-4}–5.8×10^{-2}

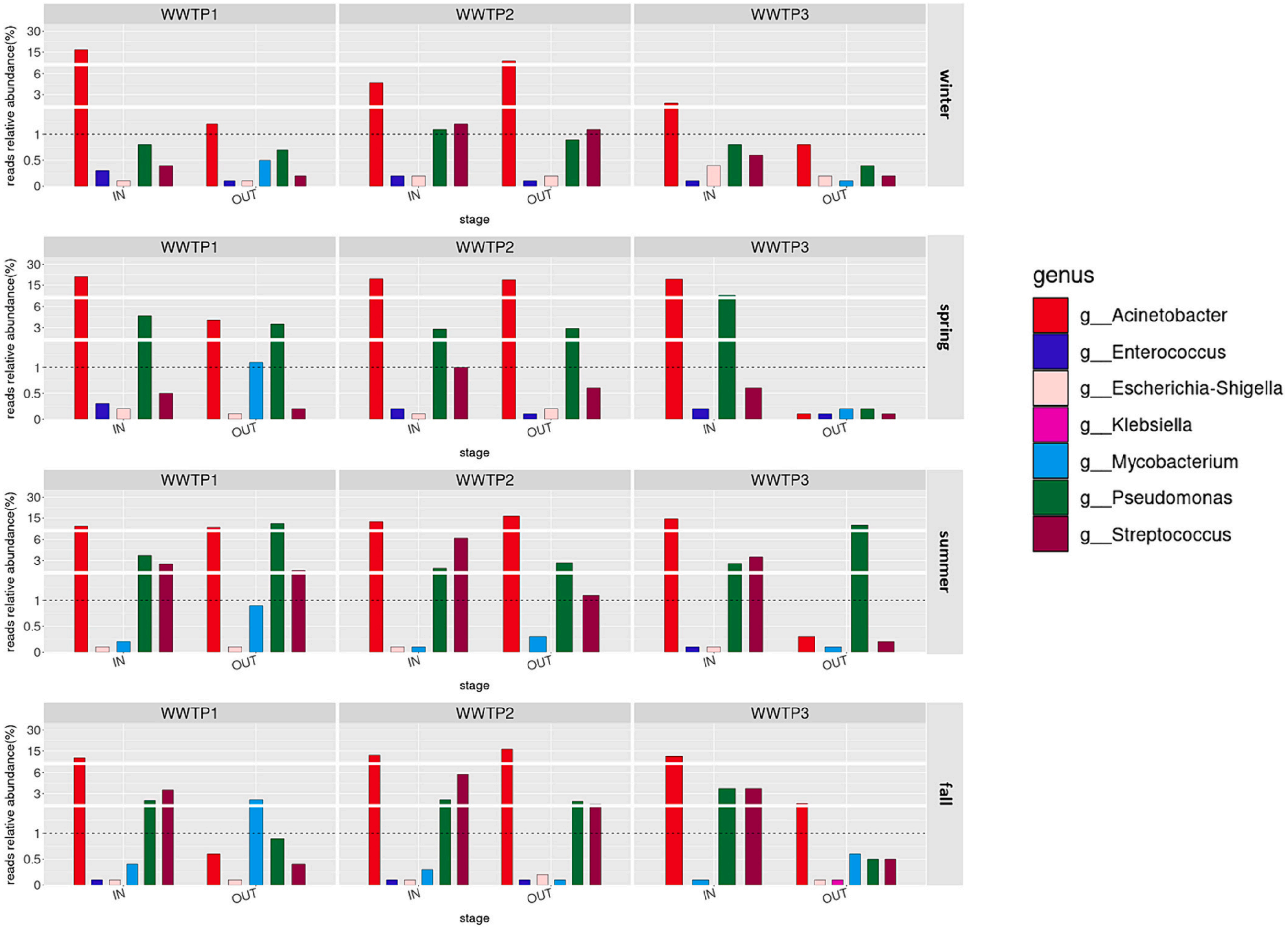

Fig. 3. Relative abundance of genera including pathogens with significant public health implications (according to the WHO Global Priority Pathogens) in WWTPs.

(Fig. S4). Coefficient variation (CV%) data for each gene intra-assay are reported in Table S6.

All samples tested positive for each target gene. Based on the average concentration, the ARGs in the influents were ranked as follows: *sul*1 > *tet*A > *bla*$_{\text{TEM}}$ > *bla*$_{\text{OXA-48}}$ > *bla*$_{\text{CTX-M-1 group}}$ > *bla*$_{\text{KPC}}$.

In general, the absolute abundance of all genes was reduced by one to three logs in the effluent compared to the influent (Table S4). However, a higher reduction was observed for *sul*1 and *tet*A (4 log), and *bla*$_{\text{OXA-48}}$ (5 log) during winter at WWTP1-b. Notably, the *sul*1 gene had the highest concentration in both influent and effluent, while *bla*$_{\text{KPC}}$ displayed the lowest concentration in both. In few instances, two genes, *tet*A and *bla*$_{\text{KPC}}$, had higher concentrations in the effluent compared to the influent, albeit remaining within the same log.

In terms of seasonal trends (Supplementary Fig. 3), an increase in the absolute concentration was observed for *sul*1, *tet*A, *bla*$_{\text{TEM}}$, *bla*$_{\text{KPC}}$ and *int*1 during the fall in influents. In contrast, *bla*$_{\text{CTX-M-1}}$ group, *bla*$_{\text{OXA-48}}$, did not display significant variations throughout the year.

The differences in relative gene concentrations between influent and effluent from the 3 WWTPs over the course of a year, together with their respective statistical significance ($p < 0.05$), are shown in Fig. 4. As can be seen, the differences are not always statistically significant, with some exceptions. For example, the relative abundance of the *bla*$_{\text{KPC}}$ gene in the effluents was consistently higher than in the influents in all seasons and in all WWTPs, and this was statistically significant. In WWTP3, the differences between IN and OUT were significant for all genes, except for *tet*A.

To assess the impact of wastewater treatment processes in reducing the prevalence of ARGs, the relative abundance results were grouped into four categories: high reduction or high increase (more than one log), reduction and increase (within one log). Overall, we observed a general trend towards reduction/high reduction in 62.5 % of the 112 data points (4 seasons × 4 sampling points × 7 targets), although increases in effluents ARGs concentrations were also observed. Specifically, we observed that 15.2 % ($n = 17$) exhibited a high decrease, 47.3 % ($n = 53$) a decrease, 29.5 % ($n = 33$) an increase, and 8 % ($n = 9$) a high increase. In addition, we observed a seasonal pattern, such as a constant increase for all genes in WWTP1a in the summer or a general decrease in WWTP3 in the spring (Fig. 5).

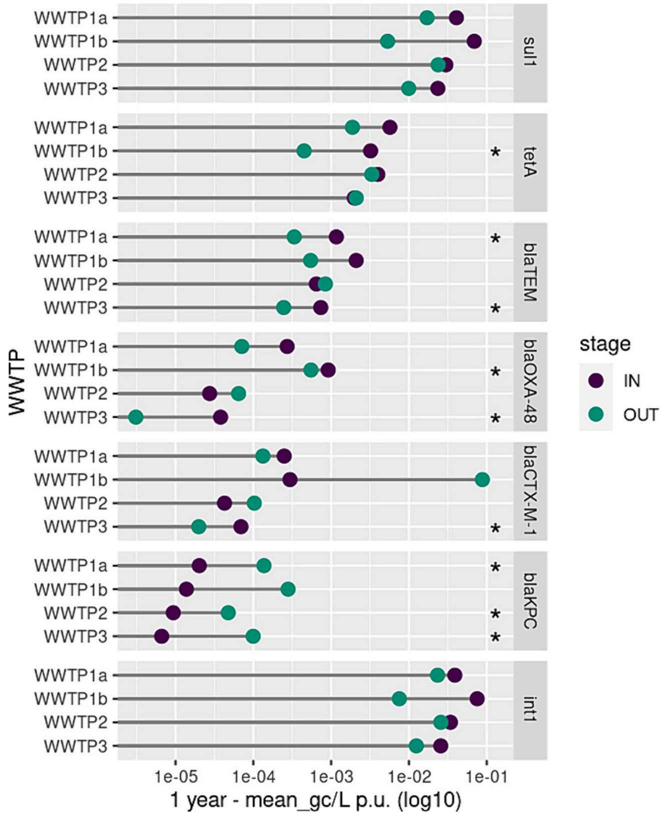

Fig. 4. Relative abundance - gene copies normalized per unit (p.u.) 16S rRNA gene copy - in influent and effluent of all 3 WWTPs (4 sampling points, as WWTP1 include lines *a* and *b*) (during one year monitoring). * Statistical significance ($p < 0.05$).

3.3. Correlations between ARGs and genera including priority pathogens

We investigated whether there was a correlation between the genes and the genera including priority pathogens, or among different genes or different genera, to determine whether an increase in one corresponds to an increase in the other or vice versa. Fig. 6 shows the correlation heatmap, which represents the strength of the relationships between the variables. By specifically identifying statistically significant strong positive correlations (correlation coefficient $r > 0.5$, p-value <0.01), we observed correlations between *int*1, *bla*$_{\text{TEM}}$, and *sul*1 with *Enterococcus.* We also found a strong correlation between *bla*$_{\text{KPC}}$ and *bla*$_{\text{CTX-M-1 group}}$, as well as between *int*1, *bla*$_{\text{TEM}}$, *bla*$_{\text{OXA-48}}$, and *sul*1.

4. Discussion

Antimicrobial resistance is a critical global public health challenge that threatens human and animal well-being on a large scale. Adopting the One Health approach, which emphasises the interconnectedness of human, animal and environmental health, is essential to effectively address this multifaceted problem. Environmental monitoring plays a key role in understanding the spread of resistant bacteria and genes between environmental compartments and their potential transmission

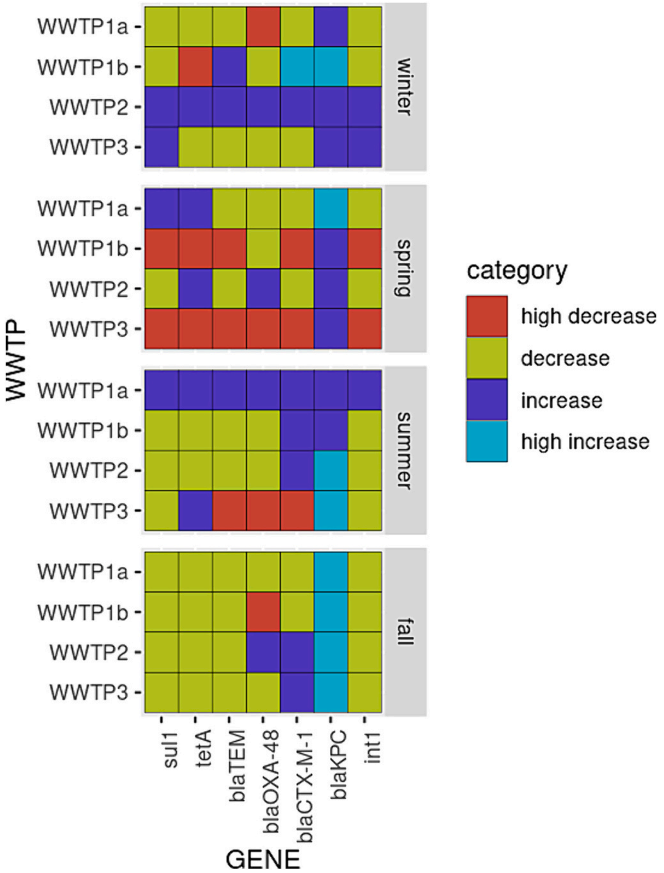

Fig. 5. WWTPs effectiveness for ARGs: high decrease (>1 log), decrease (within 1 log).

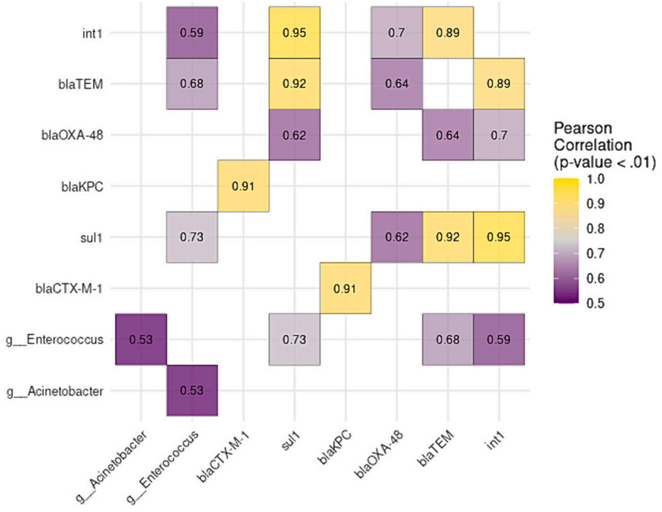

Fig. 6. Pearson correlation of ARGs vs bacterial genera including priority pathogens. Only strong and significant correlation ($r > 0.5$; p-value <0.01) are shown.

to humans and, in this contest, the role of WWTPs as hotspots for the spread of ARBs and ARGs, due to the selective pressure induced by sub-inhibitory concentrations of antibiotics, has been widely recognized by the scientific community (Rizzo et al., 2013; Vaz-Moreira et al., 2014). Although wastewater treatment processes reduce the release of ARBs and ARGs to the environment, they do not eliminate them, resulting in increased levels of HGTs among bacteria within the plants. As a result, affected receiving waters, such as surface waters, have the potential to act as reservoirs of AMR (Manoharan et al., 2022).

Here, we aimed to describe the microbial community diversity, assess the occurrence and abundance of relevant ARGs, and evaluate the impact of the wastewater treatment process in removing ARGs. Initially, we described the microbial community in the wastewater, using 16S rRNA sequencing, focusing on bacteria with a relative abundance >1 %. *Arcobacter*, *Acinetobacter* and *Flavobacterium* were consistently the most abundant genera in all WWTPs, observed in both influents and effluents. These genera are commonly found in WWTPs worldwide (Cai et al., 2014; Limayem et al., 2019; Marti et al., 2013; Numberger et al., 2019; Ye and Zhang, 2013) and include bacterial opportunistic species, that readily adapt to environmental conditions. Among the most represented genera, *Arcobacter*, *Acinetobacter*, *Aeromonas* and *Pseudomonas* are known for their propensity to acquire multidrug resistance (Gabucci et al., 2023; Ibrahim et al., 2021; Carusi et al., 2024; Yasuda et al., 2023) contributing to the global spread of the antimicrobial resistance issue. All of these genera showed a decrease in abundance after treatment, but remained detectable in the treated effluents, suggesting their potential dissemination in the environment, consistent with findings from previous studies (Numberger et al., 2019; Do et al., 2019). Among them, *Acinetobacter* and *Pseudomonas* include species of particular concern due to their impact on human health such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: both microrganisms are opportunistic pathogens, causing infections in immunocompromised patients with high mortality. These bacteria have significant antimicrobial resistant profiles putting them at the top of the list of MDR or pan drug resistant (PDR) organisms (Cavallo et al., 2023).

We also investigated the presence of genera including priority pathogens as a benchmark to identify bacteria that pose the greatest potential threat to human health. In addition to *Acinetobacter* and *Pseudomonas* (Ayoub Moubareck and Hammoudi, 2020; Qin et al., 2022), others were identified, including *Enterococcus*, *Streptococcus*, *Escherichia-Shigella*, *Mycobacterium* and *Klebsiella* (Alsayed and Gunosewoyo, 2023; Brouwer et al., 2023; Dong et al., 2022; Kaper et al., 2004; Zhou et al., 2020). These pathogens pose a significant threat in healthcare settings such as hospitals and nursing homes. They are notorious for causing serious and often fatal infections such as bloodstream infections and pneumonia. Alarmingly, they have developed resistance to a wide range of antibiotics, including carbapenems and third generation cephalosporins, considered the most effective treatments against MDR bacteria. In addition, some of them, such as *Streptococcus* spp., are versatile and opportunistic bacteria, adapting to different environmental conditions and surviving in organic-rich habitats. Their ability to transfer genetic material via mobile genetic elements is well known, facilitating the spread of antibiotic resistance among different bacterial populations. About *Mycobacterium* spp., the large group of non-tuberculous mycobacteria are normal inhabitants of aquatic environments and soils and include opportunistic pathogens of humans and animals. Some species have been suggested to potentially play a beneficial role in certain biological treatment applications in wastewater treatment systems (Guo et al., 2019). Occasional reports have highlighted the presence of pathogenic mycobacteria in sewage treatment plants (Pickup et al., 2006).

The sequencing approach used in this study, which relies on short reads of 16S rRNA, has limitations. Short reads may not provide the optimal resolution for identifying species within certain bacterial genera. Additionally, some bacteria within the same family may share high sequence similarity, even in variable regions of their 16S rRNA sequences. For instance, members of the Enterobacteriaceae family that are closely related, such as *Escherichia coli* and *Shigella* spp., are difficult to differentiate and are identified together as *Escherichia-Shigella*.

Our study found significant variability in alpha diversity values across different stages of wastewater treatment, with effluents generally having the highest diversity. Beta dissimilarity analysis revealed differences between influent and effluent microbial communities across seasons and between WWTPs, as previously reported in other studies (Numberger et al., 2019; Do et al., 2019). Several factors may have contributed to an increase in the variability of bacterial species in wastewater effluents after treatment, despite an overall reduction in bacterial load. For example, some wastewater treatment processes may create favourable conditions for the growth of some species that were previously present at lower levels. The microbial content showed significant seasonal variations in terms of genera present in WWTPs, with *Arcobacter* prevalent in winter, *Acinetobacter* in spring, and *Flavobacterium* and *Pseudomonas* showing higher abundance in spring and summer, decreasing in autumn and almost absent in winter. These findings highlight the importance of considering seasonal dynamics in order to maintain optimal WWTP treatment efficiency. The second aim of our study was to detect and quantify ARGs in both influent and effluent, in order to assess their prevalence and potential dissemination following the wastewater treatment process. Some studies have addressed this issue by using a metagenomic approach (Hendriksen et al., 2019; Karkman et al., 2020) which allows for the detection of a wide range of resistance genes present in wastewater. However, it is worth noting that metagenomic analysis produces complex and extensive data that require significant computational resources for interpretation. Additionally, it may be less effective and sensitive in detecting resistance genes present at low levels, and it may be more expensive and time-consuming to perform as part of routine environmental monitoring. Therefore, digital PCR may be more cost-effective and practical compared to the metagenomic approach, reducing costs and simplifying environmental monitoring routines. The technique's ability to perform absolute quantification enables precise determination of ARGs abundance, which is crucial for assessing the efficacy of wastewater treatment processes and understanding the dynamics of resistance dissemination. Moreover, reaction volume partitioning bypasses PCR inhibitors commonly found in wastewater, ensuring accurate detection even in complex matrices (Sidstedt et al., 2020). By providing robust quantification unaffected by inhibition, dPCR enhances our ability to track ARGs, thereby facilitating proactive strategies to mitigate the spread of antibiotic resistance in environmental reservoirs. The selection of target genes, used as a proxy, was based on their known prevalence in wastewater associated with their importance in the clinical setting as reported in the literature (Pazda et al., 2019). Indeed, *bla*$_{TEM}$, *bla*$_{OXA-48}$, *bla*$_{CTX-M-1\ group}$, and *bla*$_{KPC}$ genes encode different types of beta-lactamases that confer resistance to penicillins, cephalosporins, and carbapenems, which are important classes of antibiotics used in clinical settings (Bush and Bradford, 2020). *tet*A gene is one of the most represented genes encoding for tetracycline efflux pumps, that confer resistance to tetracycline antibiotics, which are widely used in both human and veterinary medicine (Shutter and Akhondi, 2023). The *sul*1 gene is associated with resistance to sulfonamides, a class of antibiotics commonly used in both human and veterinary medicine. Finally, the class 1 integron-integrase gene, *int*1, is found in a wide variety of pathogenic and non-pathogenic bacteria and is commonly linked to genes conferring resistance to antibiotics, disinfectants, and heavy metals (Gatica et al., 2016). Among all the analyzed genes, *sul*1 had the highest concentration, while *bla*$_{KPC}$ had the lowest. Overall, gene concentrations increased in both influent and effluent samples, especially in the fall. This phenomenon could be related to the seasonal increase in illness during autumn (especially respiratory diseases) and the subsequent increase in antibiotic use. However, it's important to note that the presence of antibiotics was not evaluated in this study, and we cannot confirm whether there was an increase in their residues, as this was not one of the specific objectives of this study. The significantly higher concentration of *sul*1 is not surprising, given its association with sulfonamide resistance (Sköld, 2001). In fact, the sulfonamide resistance genes *sul*1 and *sul*2 have been proposed as indicator genes for anthropogenic activities, due to their widespread presence in environmental samples. Similarly, *int*1, which was found at elevated levels in this study, is also used as an indicator of the level of antimicrobial contamination in the environment (Haenelt et al., 2023). Furthermore, we observed a clear correlation between *sul*1 and *int*1, consistent with previous findings (Cacace et al., 2019; Uyaguari-Díaz et al., 2018). The presence and stable prevalence of the *tet*A gene in all samples collected from all WWTPs suggests its high abundance in bacterial population in wastewater, as previously documented (Xu et al., 2023; Liao et al., 2021). Indeed, this gene has already been pointed out as a potential indicator of the abundance of tetracycline resistance genes (Marti et al., 2014). The *bla*$_{KPC}$ gene, associated with carbapenem resistance, was consistently detected at lower concentrations in both influents and effluents, compared to other genes. However, despite the low concentrations, the *bla*$_{KPC}$ gene consistently showed an increase in treated wastewater, compared to raw wastewater. Overall, wastewater treatment showed different results in reducing ARGs in different seasons. It is noteworthy that 37.5 % of the observations reported an increase in ARGs in the effluents compared to the influents. The increase in gene concentrations in the effluents compared to the influents has already been documented in the literature (Raza et al., 2022) and could be attributed to several factors. From one side, it may be due to the selective proliferation of bacteria carrying these genes during the wastewater treatment process, in particular, in activated sludge where ARB may be exposed to antibiotics or their metabolites. Additionally, ARGs can be transferred from one bacterium to another through the process of horizontal gene transfer, which can occur within a wastewater treatment plant. In this process, bacteria exchange genetic material containing resistance genes, thereby contributing to the increased concentration of these genes in the effluents. It is important to note that one of the limitations of our study was related to the lack of specific association of any ARG with its hosts. Therefore, the analysis cannot differentiate whether the genes are present in living bacteria, non-living bacteria, bacteriophage, or as free DNA fragments. Future investigations could focus on using advanced metagenomic techniques to elucidate the host specificity of ARGs within microbial communities, or conducting complementary experimental studies, such as culturing approaches, to determine the functional relevance of these genes in living bacteria. Despite these challenges, we attempted to determine if there was a correlation between genes and genera including priority pathogens. This refers to whether an increase in one would result in a corresponding increase in the other. In our study we found both expected correlations, such as for *int*1 and *sul*1 with *Enterococcus*, and unexpected results, as the correlation of *bla*$_{TEM}$ with *Enterococcus*. Although it is well known that beta-lactamases usually provide resistance specific to Gram-negative bacteria, *bla*$_{TEM}$ was previously detected in two gram-positive resistant strains, *E. avium* and *E. faecium*, isolated from hospital sewage (Farkas et al., 2022) and also in *E. faecalis* (Chouchani et al., 2012). These findings suggest that enterococci may carry both plasmid-based and chromosomal resistance genes, making them reservoirs for drug resistance traits and potential vectors for the transfer of these genes to other Gram-positive pathogens. Additionally, wastewater is a complex matrix, which represents a faecal pool, in which multiple factors (e.g., temperature changes, fluctuations in nutrient availability, and interactions among different microbial species) may contribute to the simultaneous increase in the abundance of different elements, even if they are not directly correlated.

5. Conclusions

Our study identified significant microbial diversity at all stages of wastewater treatment, with higher diversity in the effluent. Seasonal variations in the abundance of bacterial genera highlight the need to consider seasonal dynamics when optimising the efficiency of treatment plants. In addition, we identified several genera potentially carrying priority pathogens, highlighting health risks in untreated or poorly treated wastewater. The persistent presence of genera such as *Acinetobacter* and *Pseudomonas* in treated wastewater highlights the challenges of effectively eliminating antibiotic-resistant bacteria. Despite the limitations of our sequencing approach, our results provide valuable insights into the dynamics of antibiotic resistance in wastewater. Future research could explore advanced metagenomic techniques to understand the host specificity of ARGs and conduct complementary studies to assess the functional relevance of these genes in living bacteria.

In conclusion, this study improves our understanding of the microbial community structure and the spread of antimicrobial resistance in wastewater treatment plants and shows that conventional wastewater treatment processes including both physicochemical and biological approaches are not effective to reduce ARGs, showing a limited capacity to address antibiotic-resistant pollutants.

By shedding light on the diffusion dynamics of ARGs, this investigation provides valuable insights into the need to optimise operating conditions and develop novel process designs at WWTPs, enhancing their role as a key barrier to prevent the spread of AMR from wastewater to the aquatic environment.

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CRediT authorship contribution statement

Giusy Bonanno Ferraro: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Carmelo Bonomo:** Data curation, Investigation, Methodology, Writing – review & editing. **David Brandtner:** Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Pamela Mancini:** Investigation, Methodology, Writing – review & editing. **Carolina Veneri:** Investigation, Methodology, Writing – review & editing. **Rossella Briancesco:** Investigation, Methodology, Writing – review & editing.

Anna Maria Coccia: Investigation, Methodology, Writing – review & editing. **Luca Lucentini:** Resources, Supervision, Writing – review & editing. **Elisabetta Suffredini:** Data curation, Methodology, Validation, Writing – review & editing. **Dafne Bongiorno:** Data curation, Investigation, Methodology, Writing – review & editing. **Nicolò Musso:** Investigation, Methodology, Writing – review & editing. **Stefania Stefani:** Conceptualization, Data curation, Supervision, Writing – review & editing. **Giuseppina La Rosa:** Conceptualization, Data curation, Supervision, Writing – original draft, 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.

Data availability

Data will be made available on request.

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