Occurrence of antibiotic-resistant bacteria and resistance genes in the urban water cycle

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	represents a potential route of transmission to human.
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DIPARTIMENTO di SCIENZE DELLA VITA E BIOLOGIA DEI SISTEMI

Torino, September 1th, 2022

Dear Editor,

We are pleased to submit our paper entitled, “Occurrence of antibiotic resistant bacteria and resistance genes in the urban water cycle”.

Antimicrobial resistance is of primary concern for the public health. The urban water cycle that includes the collection, treatment, and discharge of treated wastewater into the environment as well as the abstraction, disinfection and distribution of water for drinking purposes represents an interesting model for tracing the fate of antibiotic resistance in the environment and for assessing the risk of transmission to humans. In this context, wastewater treatment plants (WWTPs) are among the most important receptors and hot-spots for the release of Antibiotic resistant bacteria and resistance genes (ARB and ARGs) into the environment. Moreover, a relevant issue is represented by the risk that ARB and ARGs in the "unclean" phases of the cycle can reach the final consumer of the water. Although drinking water treatment drastically abates the overall bacterial numbers, the standard Drinking Water Treatment Plants (DWTPs) are not specifically designed to reduce ARB and ARGs. This study investigates the antibiotic resistance fate in the urban water cycle, evaluating the dynamics of ARB and ARGs in three different full-scale WWTPs and two DWTPs located in the same geographical area (North-west of Italy). In our opinion, the issues investigated in this study reflect the Aims and Scope of the “Environmental Science and Pollution Research”. The paper has not been submitted to a preprint server prior to submission on “Environmental Science and Pollution Research”.

We hope that this manuscript is suitable for publication in your journal.

Yours sincerely,

Dr Silvia Bonetta, on behalf of all the authors

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TITLE PAGE

Occurrence of antibiotic resistant bacteria and resistance genes in the urban water cycle

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ABSTRACT

This study investigates the antibiotic resistance fate in the urban water cycle, evaluating the dynamics of ARB and ARGs in three different full-scale WWTPs and two DWTPs located in the same geographical area (North-west of Italy). ARB (tetracycline, ampicillin, and sulfonamide resistant bacteria) were quantified by plate counting and the abundances of selected ARGs (i.e., *tet*A, bla_{TEM} and *sul*II) and *intI*1 gene were measured using quantitative Real-Time PCR (qPCR). Higher concentrations of ARB and ARGs were observed in the WWTPs respect to the DWTPs identifying the WWTP as hot spot for the spread of antibiotic resistances. Although a significant reduction of ARB and ARGs was observed in WWTPs and DWTPs after the treatment, none of the detected ARB or ARGs were completely removed in drinking water. The stability of the antibiotic resistant rates between inlet and outlet associated with the reduction of relative ARGs abundances underlined that both the treatments (WWTs and DWTs) did not apply any selective pressure.

The overall results highlighted the importance to investigate the antibiotic resistance dynamics in aquatic ecosystems involved in urban water cycle integrating the information obtained by culture-dependent method with the culture independent one and the need to monitor the presence of ARB and ARGs mainly in drinking water that represents a potential route of transmission to human.

Keywords: urban water cycle, antibiotic resistance, antibiotic resistance bacteria, antibiotic resistance genes, wastewater treatment, drinking water treatment

Introduction

Antimicrobial resistance is of primary concern for the public health (WHO, 2014). Globally, it is estimated that 700,000 people each year could die because of antimicrobial resistant bacterial infections (Carvalho et al., 2016). If effective interventions are not carried out to overcome infections attributable to microorganisms resistant to antimicrobials, there could be an increase in deaths estimated up to 10 million people in the world by 2050, each year (O'Neil 2016). The main driver for the spread and the persistence of antibiotic resistance is the overuse and misuse of antibiotics in human and animal medicine (Sanganyado et al., 2019). Antibiotic resistant bacteria and resistance genes (ARB and ARGs) together with residues of antibiotics are released in sewer, that constitutes a reservoir of ARB and ARGs (Rizzo et al., 2013).

In this context, waters, favouring dispersion and smoothing physical factors (e.g. temperature, UV radiation) reducing the survival rates of allochthonous bacteria in the environment, allow the spread of human and animal derived bacteria; therefore it plays an important role in the release of antibiotic resistances into the environment. In particular, the urban water cycle that includes the collection, treatment, and discharge of treated wastewater into the environment as well as the abstraction, disinfection and distribution of water for drinking purposes represents an interesting model for tracing the fate of antibiotic resistance in the environment and for assessing the risk of transmission to humans (Manaia et al., 2016; Almakki et al., 2019).

In the last two decades, several studies reported the presence of ARB and ARGs in different concentrations in aquatic environments all over the world (Baquero et al., 2008; Li et al., 2015; Yang et al., 2019). In particular, urban wastewater treatment plants (WWTPs) are among the most important receptors and hot-spots for the release of antibiotic resistance into the environment (Rizzo et al., 2013). A recent overview on ARGs occurrence in WWTPs highlights that the absolute abundance of the most frequently detected ARGs in influent worldwide ranged from 4.5 to 7 Log copies/mL (Wang et al., 2020). Also antibiotic resistant feacal indicators (*E.coli*, coliforms) or ARB isolated from heterotrophic flora were frequently reported in WWTP influents, with a mean concentration of 4 and 6 Log of Colony Forming Units (CFU)/ml detected in different studies for tetracycline and sulphonamide resistant bacteria, respectively (Gao et al., 2012; Munir et al., 2011).

Despite the wastewater treatments generally lead to a reduction of ARG and ARB abundance (~2-3 Logs), some studies observed higher resistance rates in the effluents with respect to the influent, in relation to the different treatment processes investigated (Pazda et al., 2019; Wang et al., 2020; Stachurova et al., 2021).

Thus, it is important to underline that even a well-functioning WWTP equipped with secondary and even tertiary treatment will be able to release high concentrations of ARBs and ARGs into the environment. Some studies

reported that, in a final effluent of a WWTP, it is possible to detect about 10^9-10^{12} CFU of total bacteria per day per inhabitant equivalent; of these at least 10^7-10^{10} showed some form of antibiotic resistance (Rizzo et al., 2013). Moreover, a recently pan-European survey on treated wastewater demonstrates that WWTPs are responsible for the discharge of considerable amounts of ARGs in the downstream water bodies (Cacace et al., 2019). These data underline the main role of WWTPs in the accumulation and spreading of ARB and ARGs into open waters.

Several studies evaluated the antibiotic resistance in WWTPs worldwide observing highly variable abundances of ARB and ARGs; these results suggested the influence of local environmental and anthropogenic factors (e.g. antimicrobial residue levels, bacterial taxonomic composition, local use of antimicrobials) and they highlighted the need to specifically evaluate antibiotic resistance in different geographical areas (Hendriksen et al., 2019).

The investigation of antibiotic resistance in the urban water cycle represents a relevant issue considering the risk that ARB and ARGs in the "unclean" phases of the cycle can reach the final consumer of the water (Manaia et al., 2016). Although the direct impact on human health by ARB and ARGs in drinking water is not well established, antibiotic resistance threats human health by two main different mechanisms: first, pathogenic and opportunistic ARB that can survive in drinking water may also enter the human microbiome following water consumption (Vaz-Moreira et al., 2014); second, ARGs in drinking water can occasionally be transferred by horizontal gene transfer (HGT) to human pathogenic bacteria (Manaia 2017). Therefore, drinking water can be considered as a potential transmission route of antibiotic resistance to humans (Chang et al., 2015; Manaia et al., 2016).

Although drinking water treatment drastically abates the overall bacterial numbers, including the number of ARB and ARGs, still a small but quantifiable number of cells and genes have been detected in several drinking water systems. Indeed, the standard Drinking Water Treatment Plants (DWTPs) are not specifically designed to reduce ARB and ARGs (Huang et al., 2021). Moreover, biofilm formation (Zhang et al., 2019), presence of chorine residues (Bai et al., 2015) and heavy metals allow the persistence of antibiotic resistances in drinking water systems (Seiler et al., 2012).

This study investigates the antibiotic resistance fate in the urban water cycle, evaluating the dynamics of ARB and ARGs in three different full-scale WWTPs and two DWTPs located in the North-west of Italy.

The combination of culture-dependent and independent approach was used. In particular, tetracycline, ampicillin, and sulfonamide resistant bacteria in the influent and in the final effluent of each WWTPs and DWTPs were quantified by plate counting. Moreover, the abundances of selected ARGs (i.e., *tet*A, bla_{TEM} and

*sul*II; against tetracycline, β-lactams, and sulphonamides, respectively) as well as those of the integrase gene of the class 1 integrons (*intI*1), used as proxy of the anthropogenic pollution and of the antibiotic resistance in the environment (Gillings et al., 2015; Ma et al., 2017) were measured using quantitative Real-Time PCR (qPCR).

Materials and methods

Sampling

WWTPs

Influent and effluent samples were collected in three WWTPs (plants A, B and C) located in North-West of Italy. The WWTP A (population equivalent of 3,800,000) employs preliminary treatment (screening and aerated grit removal), primary sedimentation, denitrification step, biological oxidation/nitrification process, secondary settling, then phosphorus removal and filtration steps. The WWTPs B and C (60.000 and 276.000 population equivalent, respectively) after screening and grit removal, employs a denitrification step, the biological treatment, and the secondary settling. Only the WWTP B has a primary sedimentation tank, before the biological treatment. Finally, a tertiary treatment is carried out with ultrafiltration in WWTP B and with a chlorination step in WWTP C.

Six wastewater sampling were performed during one year (March 2019 - January 2020). Samples collected in sterile plastic bottles were transported on ice to the laboratory and analyzed within 24 h.

DWTPs

Drinking water source (raw surface water from a large river) and finished water (drinking water) samples were collected in two DWTPs (plants D and E) in the same geographical area of WWTPs (~20 km^2 in North-West of Italy). Plants D and E treat 130.000 m^3/day and 86.400 m^3/day of drinking water, respectively, sourced from the same drinking water source.

The DWTP D involves pre-decantation, addition of powder activated carbon (PAC), ozonation followed by clarification/flocculation, two granular activated carbon (GAC) filtrations and final disinfection using chlorine dioxide. The DWTP E employs chlorination, clarification, granular activated carbon (GAC) filtration and final disinfection using chlorine dioxide.

Six wastewater sampling were performed during one year (March 2019 - January 2020). Samples collected in sterile plastic bottles were transported on ice to the laboratory and analyzed within 24 h.

Sample processing and ARB quantification

ARB were isolated in a basic culture medium for the count of heterotrophic bacteria (R_2Agar) supplemented with ampicillin, tetracycline and sulfamethoxazole at a concentration of 32 mg/L, 16 mg/L and 50.4 mg/L, respectively.

The antibiotic concentrations tested were chosen considering the highest dose used in standard methods to establish resistance to antibiotics with clinical strains (breakpoint) (CLSI, 2018) or the concentration reported in previous study (Gao et al., 2012). Total heterotrophic count (HPC) was determined on media without antibiotics. Serial dilutions of wastewater samples (influent and effluent) were plated in duplicate on media with and without antibiotics. Serial dilution of surface water or different volumes of drinking water (0.5-1L for HPC and 2 L for ARB) were filtered (0.22 um pore nitrocellulose size filter membrane, Millipore) and then the membranes were placed on R_2Agar plates with and without antibiotics.

All plates were incubated at 30°C for seven days and the results are expressed as log CFU/mL. The antibiotic resistance rate for each antibiotic was calculated as the ratio between the CFU/mL of each ARB and the CFU/mL of HPC.

Sample processing and DNA extraction

Samples of wastewater influent (20 ml), wastewater effluent (250 ml), drinking water source (700 ml) and finished water (3 L) were filtered in triplicate on 0.22 um pore size polycarbonate filter membrane. The filters were stored at -20 °C until DNA extraction. Subsequently, the filters were used for DNA extraction using the DNeasy PowerWater kit (Qiagen) according to the manufacturer's instructions. Concentration of the extracted DNA of each sample was quantified by spectrophotometry (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE).

Real-time qPCR of ARGs

The abundance of the selected ARGs (*tet*A, bla_{TEM} and *sul*II) of *intI*1 and of the 16 rRNA gene was measured by qPCR using a RT-thermocycler (CFX Connect, Bio-Rad). The protocol used in qPCR assays was previously described by Di Cesare et al. (2015). The qPCR program was 95 °C for 2 min, 35 cycles of 95 °C for 15 s, annealing temperature reported in Supplementary Table S1 for 30 s and 72 °C for 15 s. Melt curve analysis was performed from 60 °C to 95 °C with increments of 0.5 °C/5 s. Standard calibration curves were carried out using the purified, quantified and ten-fold diluted amplicon of each gene as described in Di Cesare et al. (2013). Each

reaction was carried out in duplicate for each sample. The limits of quantification (LOQ) per each quantified gene was determined as described in Bustin et al. (2009). They were 1.55 x 10^3, 1.22 x 10^2, 3.57 x 10^1, 4.88 x 10^2 and 1.12 x 10^1 gene copy/µL for 16 rRNA, *tet*A, *sul*II, bla_{TEM} and *intI*1 genes respectively. The mean value ± standard deviation of the reaction efficiencies was 98.32 ± 8.23% and the R^2 was always more than 0.97. The potential inhibition of the qPCRs due to the type of analysed matrix was calculated by dilution method (Di Cesare et al. 2013) and no inhibition was obtained. The ARG and *intI*1 abundances were expressed as absolute abundance (log gene copies/ml) and relative abundance (gene copies/16S rRNA gene copy) and a mean value of the abundance for each gene was calculated. The interpretation of the results in case of abundance values lower than the LOQ and in case of discordance between the two replicates was made as previously reported (Di Cesare et al., 2015).

Statistical Analyses

The statistical analysis of the data was carried out using the SPSS package (Version 25.0) for Windows. The bacterial concentrations and ARG abundances were converted to log10 (Log CFU/mL or Log copies/mL), whereas relative ARG abundances were transformed in the arcsine of their square root (Arcsine of square root of gene copy/16 rRNA gene copy) (Crawley, 2012). For the comparison of ARB concentration or antibiotic resistance rate or ARG abundance between influent *vs* effluent in the WWTPs or raw surface water *vs* drinking water in the DWTPs the Student's T-test was used. This test was also applied to evaluate the differences between DWTP D and DWTP E. The one-way ANOVA test, followed by Tukey's post-hoc analysis, was used to study the differences among the sampling and the WWTPs. The relationship among ARGs (relative abundance) and between ARGs (relative abundance) and ARB (antibiotic resistance rate) was analysed with Pearson correlation. test.

Results

Dynamics of ARB and ARGs in urban water cycle

The dynamics of antibiotic resistance in the urban water cycle, that includes WWTPs and DWTPs, highlighted a statistically significant difference (Table 1) of the ARB concentrations and ARGs (relative and absolute abundance) between WWTPs and DWTPs, with higher values in the WWTPs (Fig. 1-4). On the contrary, no difference was revealed for the antibiotic resistance rates (%) with respect to the plant (WWTPs *vs* DWTPs).

A generally higher ARB concentration and ARGs relative and absolute abundance were observed in the inlet of

211 the treatment plants (influent of WWTPs and surface raw water treated in the DWTPs) with respect to the outlet
212 (effluent and drinking water), although no differences were detected when considering the antibiotic resistance
213 rates (%).
214 The sampling period seemed to not affect the dynamics of antibiotic resistance.
215

216 **Dynamics of ARB and ARGs in WWTPs**

217 Total HPCs, ampicillin-resistant bacteria (AmRB), tetracycline-resistant bacteria (TRB), and sulfonamide-
218 resistant bacteria (SRB) detected in the influents and in the effluents of the three WWTPs investigated are
219 reported in Figure 1. In the influents, the concentrations ranged from 5.7 to 6.4 for AmRB, from 4.5 to 6.1 for
220 TRB and from 5.4 to 7.0 Log CFU/mL for SRB. The highest mean concentration reported was 6.4 Log CFU/mL
221 for SRB, and the lowest 6.1 Log CFU/mL for AmRB. The mean resistance rates of AmRB, TRB and SRB were
222 9%, 1% and 22%, respectively and their highest ratio reached up to 21%, 4% and 38%.
223 The concentrations in the effluents ranged from 2.1 to 4.5 for AmRB, from 1.3 to 3.9 for TRB and from 2.1 to
224 4.9 Log CFU/mL for SRB. Moreover, the highest mean concentration was 3.9 Log CFU/mL for SRB, and the
225 lowest 2.8 Log CFU/mL for TRB. As observed in the influent, the decreasing trend of the mean resistance rates
226 of ARBs were SRB>AmRB>TRB (21%> 12%> 3%) and their highest ratio reached up to 35%, 33% and 12%,
227 respectively.
228 A statistically significant reduction of heterotrophic bacteria and ARB was observed in WWTPs after the
229 treatment (Table 2; Fig1a). On the contrary, for all ARB monitored, the antibiotic resistance rate did not show a
230 significant trend (influent *vs* effluent, Table 2), except for TRB (higher value in the effluent of WWTPs) (Fig1b).
231 Moreover, no difference was observed among the different WWTPs both, considering the results expressed as
232 Log CFU/ml and as antibiotic resistance rates. Also, the sampling period seemed not to affect the abundances
233 and the rates of TRB, SRB and AmRB.
234 The absolute abundance of bla_{TEM}, *tet*A, *sul*II and *intI*1 in influents, when quantifiable ranged from 8.0 to 9.0,
235 8.7 to 9.4, 8.8 to 9.8 and 8.4 to 9.5 log gene copies/mL, respectively (Fig 2a). The relative abundances of bla_{TEM},
236 *tet*A, *sul*II and *intI*1 respect to 16S rRNA gene, when quantifiable ranged from 3.3 10^{-4} to 9.1 $\times 10^{-4}$, 1.1 $\times 10^{-3}$ to
237 3.7 $\times 10^{-3}$, 2.0 $\times 10^{-1}$ to 8.7 $\times 10^{-1}$ and 1.6 $\times 10^{-3}$ to 3.7 $\times 10^{-3}$ copies/16 rRNA gene copy, respectively. *sul*II showed
238 for both, absolute and relative abundance, the highest mean values (mean: 9.4 log gene copies/mL and 3.8 $\times 10^{-1}$
239 copies/16 rRNA gene copy) (Fig 2).
240 The absolute abundance of bla_{TEM}, *tet*A, *sul*II and *intI*1 when quantifiable ranged in the effluents from 7.3 to 8.2,

7.6 to 9.2, 8.8 to 10.3 and 8.4 to 9.6 log gene copies/mL, respectively (Fig 2a). The relative abundances when quantifiable of bla_{TEM} was 1.9 $\times 10^{-4}$ copies/16 rRNA gene copy, the relative abundance of *tet*A, *sul*II and *intI*1 ranged from 2.7 $\times 10^{-5}$ to 1.5 $\times 10^{-3}$, 1.3 $\times 10^{-1}$ to 9.1 $\times 10^{-1}$ and 5.6 $\times 10^{-4}$ to 3.1 $\times 10^{-3}$ copies/16 rRNA gene copy. As observed already in the influents, the highest mean value was obtained for *sul*II (mean: 9.5 log gene copies/mL and 3.6 $\times 10^{-1}$ copies/16 rRNA gene copy) (Fig 2).

Although the absolute abundance of ARGs generally did not show differences between the influents and effluents of WWTPs, a significant reduction of the relative abundance of *tet*A, bla_{TEM} and *intI*1 was observed in the effluents.

Significant differences among the WWTPs were reported in effluents only for the relative abundance of *sul*II (WWTP2>WWTP1 and WWTP3), *tet*A (WWTP1>WWTP2) and *intI*1 (WWTP1>WWTP2).

The absolute and relative abundances were not influenced by the sampling period.

A correlation between relative abundance of *intI*1 vs *tet*A ($p<0.001$), *intI*1 vs bla_{TEM} ($p<0.05$) and bla_{TEM} vs *tet*A ($p<0.001$) was observed ($p<0.05$). No relationship was reported for ARGs and ARB, except a correlation between TRB and *tet*A ($p<0.05$) and TRB and *intI*1 ($p<0.05$)

Dynamics of ARB and ARGs in DWTPs

ARB concentration and antibiotic resistance rate (%) detected in surface and drinking water of the two DWTPs were reported in Figure 3.

ARB were observed in all analyzed surface water samples with concentrations ranging between 4.7 to 6.3 for AmRB, 3.3 to 6.2 for TRB and 4.9 to 6.9 Log CFU/mL for SRB (Fig. 3a). Moreover, the highest mean concentration was 6.1 Log CFU/mL for SRB, and the lowest 4.9 Log CFU/mL for TRB. The highest ratio is 36% for SRB and the lowest 5% for TRB.

Considering the drinking water, SRB were observed in all analyzed samples with concentrations ranging from 1.0 to 2.1 Log CFU/mL. On the contrary, TRB and AmRB were found in the 50% and 25% of the analyzed samples and the concentration when quantifiable ranged from 1.2 to 1.9 Log CFU/mL for AmRB and from 0.4 to 1.5 Log CFU/mL for TRB. Moreover, the highest mean concentration was 1.5 Log CFU/mL for SRB, and the lowest 0.4 Log CFU/mL for AmRB. According to the ARB concentration, the highest resistance rate was observed for SRB (mean value: 27%).

A significant reduction (Table 3) was detected after the treatments for both heterotrophic bacteria and ARB (Log CFU/mL) in DWTP (Fig 3a), while no abatement was observed for antibiotic resistance rates (Fig 3b). Water

treatment and sampling period did not impact abundances and rates of TRB, SRB and AmRB.

In all samples of the surface water *intI*1, *tet*A and *sul*II were observed, while bla_{TEM} was never detected. The absolute abundance of *tet*A, *sul*II and *intI*1 in these samples ranged from 8.2 to 9.6, 8.6 to 10.0, 8.4 to 10.3 log gene copies/mL, respectively (Fig 4a). The relative abundances of *tet*A, *sul*II and *intI*1 ranged from 5.9×10^{-5} to 2.6×10^{-4}, 5.8×10^{-5} to 9.1×10^{-4}, 1.8×10^{-4} to 1.5×10^{-3} copies/16 rRNA gene copy, respectively. *intI*1 showed the highest mean values for both, absolute and relative abundance (mean: 9.6 log gene copies/mL and 7.5×10^{-4} copies/16 rRNA gene copy) (Fig 4b).

bla_{TEM} and *tet*A were not detected in drinking waters, except for three samples (1 for DWTP D, 2 for DWTP E) that presented only one replicate positive but not quantifiable for *tet*A, while all drinking water samples were positive for *sul*II, although this gene was not quantifiable. The absolute and relative abundance of *intI*1 was 7.5 Log gene copies/mL and 1.3×10^{-3} copies/16 rRNA gene copy, respectively.

The relative and absolute abundance of *tet*A and *sul*II were reduced by each water treatment, moreover for DWTP E also the absolute abundance of *intI*1 was lower in the drinking water (Fig 4a). The relative abundance of *intI*1 in the drinking water was the sole parameter evidencing a difference between the DWTPs (DWTP E>DWTP D; Fig 4b). The absolute and relative ARG abundances were not influenced by the sampling period (Table 3).

Moreover, both relative and absolute abundances of *tet*A showed a correlation with the absolute abundance of *sul*II ($p<0.001$). No relationship was detected between the values of ARGs and ARB, except for a correlation between SRB and *sul*II gene ($p<0.001$).

Discussion

The urban water cycle that comprises both wastewater and drinking water treatment represents a possible route for spreading for ARB and ARGs into the environment with a subsequently potential return to humans with a direct impact on the human health (Manaia et al., 2017). The treatment plants (WWTPs and DWTPs) investigated in our study, being in the same geographical area, are particularly interesting to track the fate of ARB and ARGs in the urban water cycle.

The overall ARB and ARG dynamics in the WWTPs and DWTPs sampled in this study, highlighted, as expected, higher concentrations of antibiotic resistances in the WWTPs than in DWTPs. This is in agreement with several studies that identified the WWTP as hot spot for the spread of antibiotic resistances (Rizzo et al., 2013; Pazda et al., 2019). The concentration of ARB in the influent of WWTPs observed in our study were

similar or higher than those reported in other studies for TRB and SRB (Gao et al., 2012; Munir et al., 2011) and, generally, the monitored ARGs showed absolute abundances substantially higher than those reported by other authors (Fiorentino et al. 2019, Wang et al. 2020 and Narciso-da-Rocha et al. 2018). The reduction of ARB concentrations between the influent and the effluent of the studied WWTPs was comparable with similar studies (Wang et al., 2020).

Moreover, the stability of the antibiotic resistant rates in the influents and effluents, (proportion of ARB in the HPC for each sample), underlined that the different treatments did not promote the selection of ARB. This was also confirmed by the relative abundance of the ARGs that showed a reduction for bla_{TEM}, *tet*A and *intI*1 with values significantly lower in the effluent. The results reported in literature that considered the effect of the treatment on the variation of the ARG relative abundances are discordant. Indeed, in two studies, performed in Canada and in China, no significant differences were observed in the relative abundance of ARGs between influent and effluent of two WWTPs (Mc Connell et al., 2018; Ma et al., 2015). On the contrary Di Cesare et al. (2016) showed an increase of some ARGs (e.g. *sul*II) and of *intI*1 in the effluents of three Italian WWTPs.

Although the treatment process, the dimension, and the quality of the treated effluent varied in the investigated WWTPs, no significant difference in presence and abundance of ARB and ARGs was observed among the three WWTPs, highlighting that these factors had a limited impact on the fate of the resistances released by the plants. This is in agreement with the literature that hypothesizes a role for the biological process, present in all the investigated WWTPs, in the establishment of a bacterial community exerting specific ecological factors (e.g. enhanced competition, predation, cooperation) determining the presence of ARB and ARGs (Manaia et al., 2016; Rizzo et al., 2013).

As previously observed among ARB and ARGs investigated SRB and *sul*II were the most abundant (Munir et al., 2011; Ferro et al., 2016; Ben et al., 2017).

In order to evaluate the dynamics of antibiotic resistance in the urban water cycle, it is also important to consider the spread of ARB and ARGs in surface waters utilized for drinking purposes that are influenced by the discharge of wastewater and by other anthropogenic activities. The role of surface water is confirmed in our study by the presence of all ARB and most of the quantified genes in all samples of raw surface water analyzed used as drinking water source, with high abundances of SRB and *sul*II, according to the results obtained in the three WWTPs that are in the same geographical area. The high abundance of antibiotic resistances against sulfonamides in surface water, was observed also in other rivers impacted by human activities (Yang et al., 2020; Hu et al., 2019). The results obtained in the investigated surface water revealed a higher absolute abundance of

ARGs and ARB (3-4 orders of magnitude) respect to the data reported for other rivers in China and Poland influenced by anthropogenic activities (e.g., sewage discharges, agricultural runoff, swine farm) (Bondarczuk et al., 2019; Yang et al., 2020; Hu et al., 2019) underlining its role in the spreading of antibiotic resistance. The presence of ARB and ARGs in surface water used as drinking water source highlights the importance to investigate all the phases composing the urban water cycle and to deep the effect of the drinking water treatment.

None of the detected ARB or ARGs were completely removed in drinking water by the applied water treatments, according to Hu (Hu et al, 2019) and Siedlecka (Siedlecka et al., 2021) who investigated ARGs in a DWTP in China and Poland. As previously reported in other studies, the presence and the total concentration of ARGs in drinking water significantly decreased in comparison to corresponding water source (Sanganyado et al., 2019; Stange et al., 2019). Moreover, the limited variations in antibiotic resistance rates between surface water and drinking water associated with the reduction of relative ARG abundances showed, as observed in WWTPs, that the treatment did not apply any selective pressure. These results highlight the key role played by the drinking water treatment in reducing ARGs and controlling ARB from the water source. As observed for WWTPs, the different drinking water treatments seemed to display a limited impact on the spread of SRB and ARGs, although DWTP D was equipped with an ozonation step that resulted the best treatment to reduce ARB and ARG in some studies (Stange et al., 2019; Yang et al., 2020). It is important to highlight that the effectiveness of the disinfection step can be influenced by numerous factors such as the bacterial community composition in the raw water used as drinking water source, the effectiveness of other treatment steps, and the co-selection of antibiotic resistance enhanced by disinfection by-products (Sanganyado and Gwenzi, 2019).

Despite the observed reduction after drinking water treatment, the presence of ARB and ARGs in drinking water should be kept under observation, taking into account that the amount of ARB could increase in tap water, due to biofilm detachment in the distribution system (Zhang et al., 2018).

Considering that some genes can be utilized as proxy for the overall abundance of ARGs in waters (Su et al., 2018), Pearson correlation was carried out among the different measured ARGs in WWTPs and DWTPs to evaluate the suitability of the monitored target as indicator. The results highlighted that a positive correlation was observed only for bla_{TEM} vs *tet*A in WWTPs and *sul*II vs *tet*A in DWTPs underlining that none of the selected genes could be used to predict the fate and the potential contamination by determinants of antibiotic resistance in the urban water cycle. A similar result was also observed for *intI*1 that was correlated only with bla_{TEM} and *tet*A in WWTPs. These results claim for the need to perform more studies by using untargeted based quantification approach, *i.e.*, shotgun metagenomics, to characterize the overall antibiotic resistome (total content of ARGs) of

a microbial community aiming to find the best targets as proxy of the antibiotic resistome in aquatic ecosystems. Another important topic is related to the meaning of the results obtained with the two approaches (cultivation dependent and independent) utilized in this study for characterizing the antibiotic resistance. Molecular results highlighted a trend (influent > effluent; raw surface water > drinking water) that was not shown with cultivation dependent methods, moreover no clear relationship between ARB and the corresponding ARGs was observed. This is probably related to the different information that the two approaches provide. The cultivation dependent method provides a general overview of resistant bacteria but considers only a part of the viable bacteria because no information about VBNC (Viable but not culturable bacteria) can be provided. Moreover, other drawbacks associated to this approach are present, for example the results obtained can be influenced by the antibiotic tested and the concentration used. On the contrary the cultivation independent method has high specificity and sensitivity and the detection is not influenced by the physiological status of bacteria; these characteristics make this method a useful tool to evaluate the potential of antibiotic resistance spreading in environment. However, as highlighted before, qPCR is a target-based quantification method that cannot provide a complete overview of the antibiotic resistome of a microbial community.

In conclusion the results obtained underline the importance to investigate the antibiotic resistance dynamics in aquatic ecosystems involved in urban water cycle integrating the information obtained by culture-dependent method with the culture independent one. The WWTPs were confirmed to be a hot-spot of antibiotic resistance. Although the processes applied for treating wastewater and for drinking water production allowed to reduce the concentration of ARB and ARGs and did not apply selective pressures, the results highlighted the need to monitor the presence of ARB and ARGs mainly in drinking water that represents a potential route of transmission to human with a direct impact on human health.

Author contribution

All authors contributed to the study conception and design. Experiments were performed by Cristina Pignata, Marco Panizzolo, Manuela Macrì and Raffaella Sabatino, while data collection and analysis was done by Sara Bonetta, Silvia Bonetta, Andrea Dicesare, Raffaella Sabatino. The first draft of the manuscript was written by Silvia Bonetta, Sara Bonetta, Andrea Dicesare and all authors commented on previous versions of the manuscript. Gianluca Corno and Elisabetta Carraro were in charge of supervision and greatly contributed to final review and editing of the paper. All authors read and approved the final manuscript.

Declarations

Ethics approval

This is an original article that did not use other information that requires ethical approval.

Consent to participate

No consent of participation is to be claimed.

Consent for publication

All of the authors have read and approved the paper for publication. We confirmed that it has not been published previously nor is it being considered by any other peer-reviewed journal.

Competing interests

The authors declare no competing interests.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article

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Fig. 1: (a) Log-transformed concentrations of ampicillin, tetracycline, sulfonamide resistant bacteria and HPC in influent and effluent of the WWTPs investigated. Box plots represent median and range values. (b) Antibiotic resistance rate of ampicillin, tetracycline and sulfonamide resistant bacteria in WWTPs. All values are normalized to HPC abundances. Box plots represent median and range values.

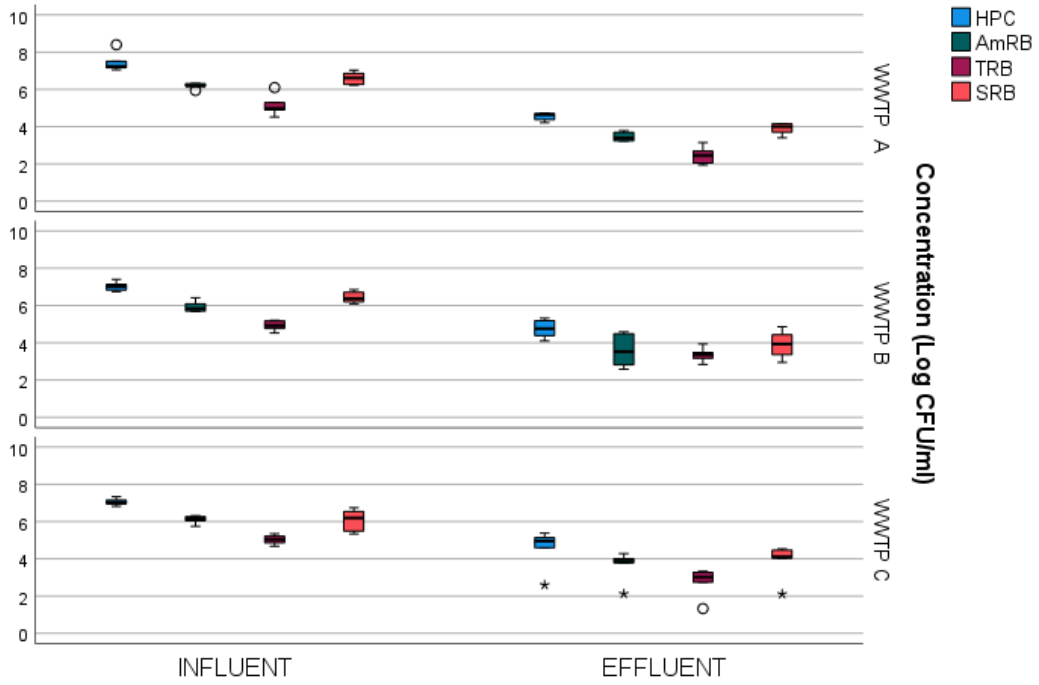
Fig. 2: (a) Absolute abundance of tetA, sulII, blaTEM and intI1 gene in influent and effluent of the WWTPs investigated. Box plots represent median and range values. (b) Relative abundance of of tetA, blaTEM, intI1 (1) and sulII (2) gene in WWTPs. All values are normalized to 16S rRNA gene copy. Box plots represent median and range values.

Fig. 3: (a) Log-transformed concentrations of ampicillin, tetracycline, sulfonamide resistant bacteria and HPC in influent and effluent of the DWTPs investigated. Box plots represent median and range values. (b) Antibiotic resistance rate of ampicillin, tetracycline and sulfonamide resistant bacteria in DWTPs. All values are normalized to HPC abundances. Box plots represent median and range values.

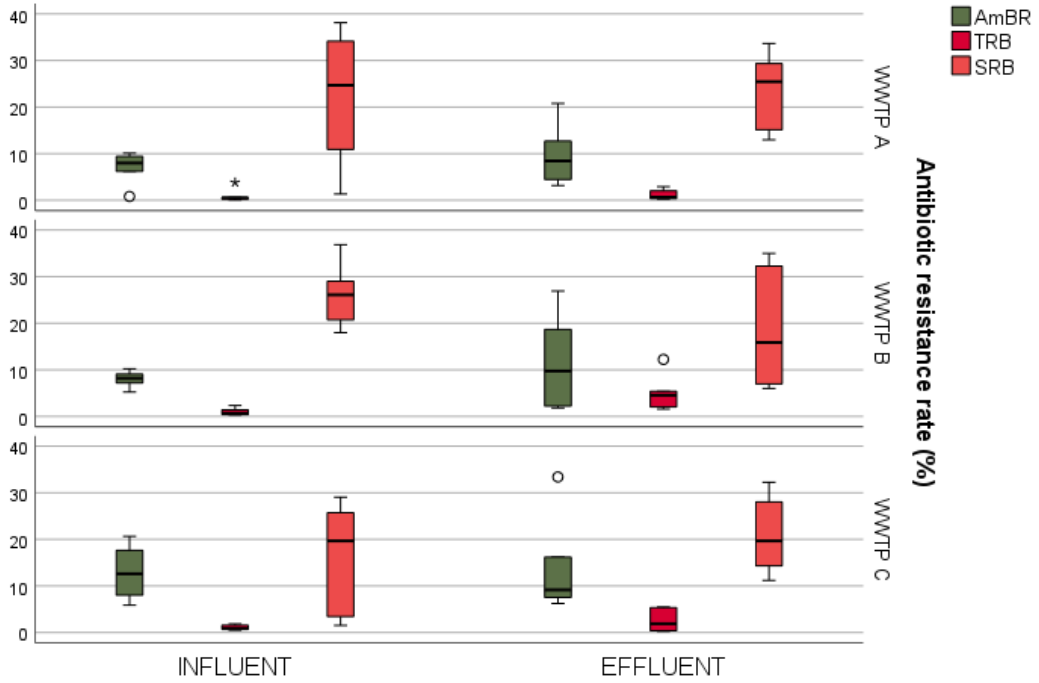
Fig. 4: (a) Absolute abundance of tetA, sulII, blaTEM and intI1 gene in influent and effluent of the DWTPs investigated. Box plots represent median and range values. (b) Relative abundance of of tetA, blaTEM, sulII and intI1 gene in DWTPs. All values are normalized to 16S rRNA gene copy. Box plots represent median and range values.

Figure

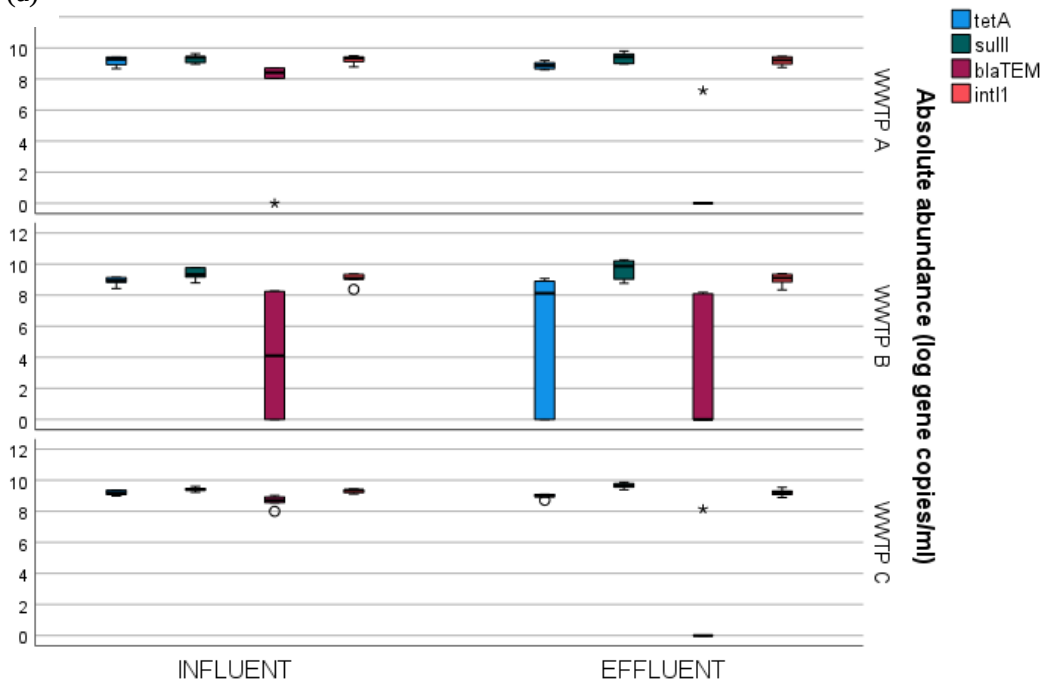
(a)


HPC
AmRB
TRB
SRB
WWTP A
WWTP B
WWTP C
Concentration (Log CFU/ml)
INFLUENT
EFFLUENT

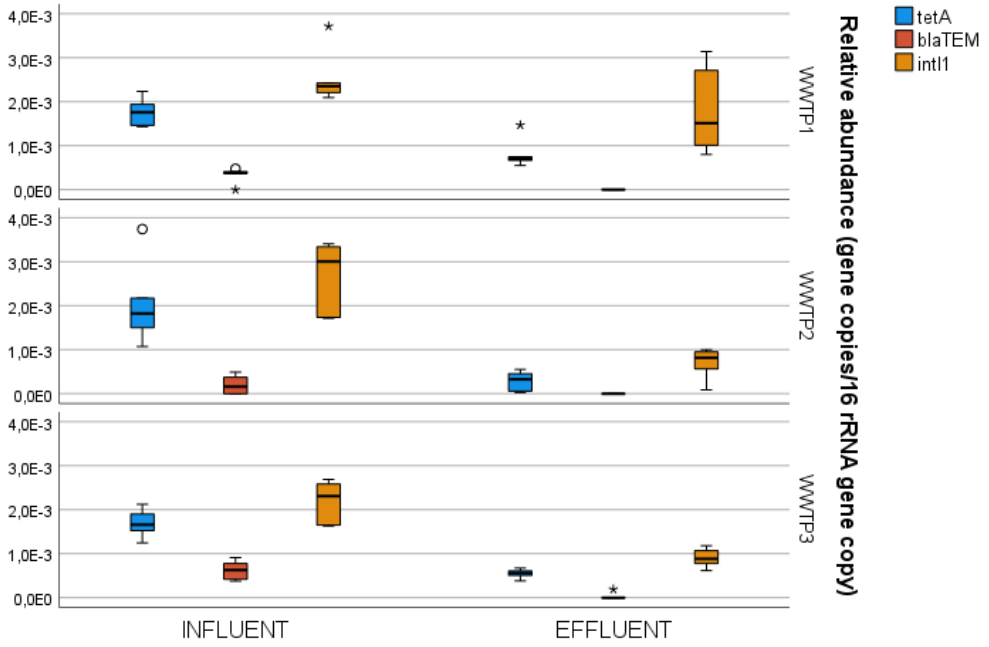
(b)


AmBR
TRB
SRB
WWTP A
WWTP B
WWTP C
Antibiotic resistance rate (%)
INFLUENT
EFFLUENT

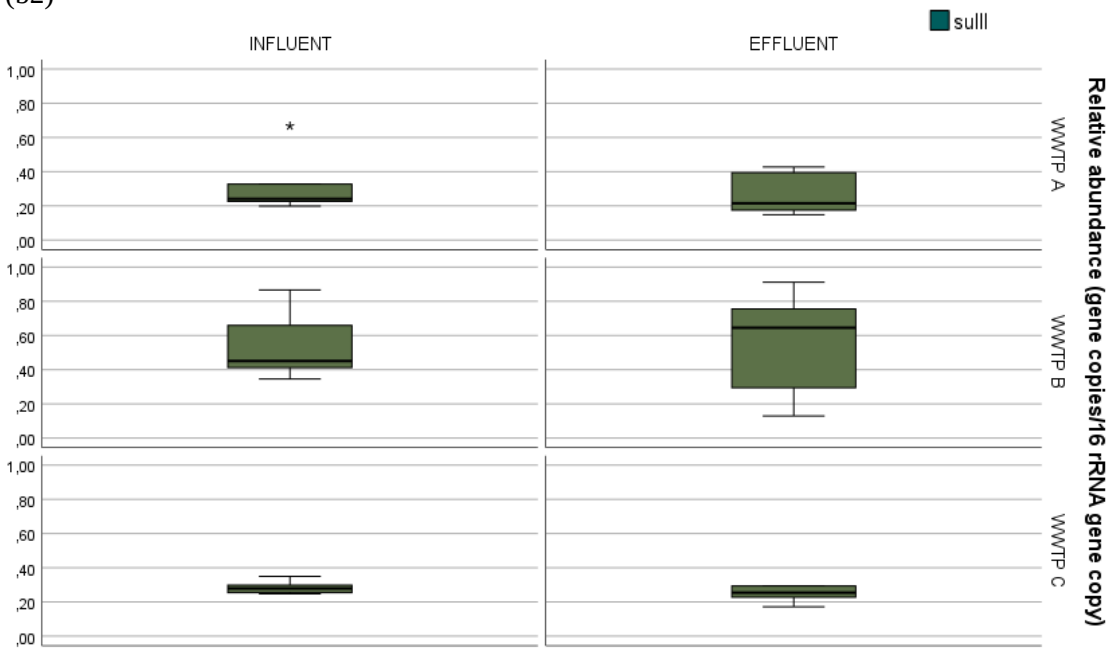
(a)


tetA
sulII
blaTEM
intI1
Absolute abundance (log gene copies/ml)
WWTP A
WWTP B
WWTP C
INFLUENT
EFFLUENT

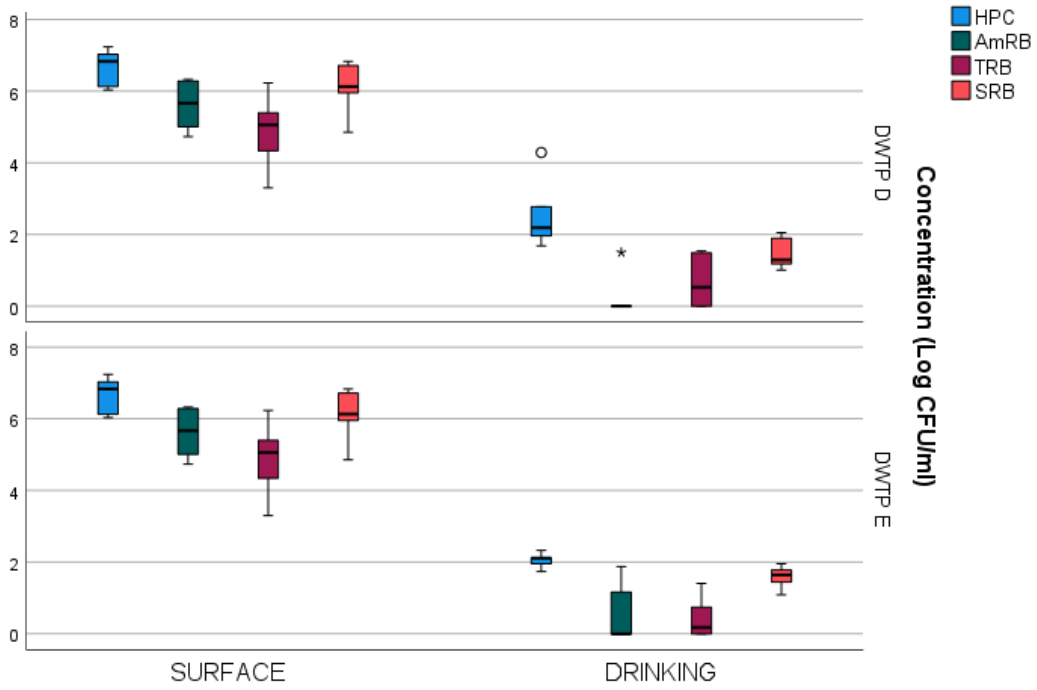
(b1)


tetA
blaTEM
intI1
Relative abundance (gene copies/16 rRNA gene copy)
WWTP1
WWTP2
WWTP3
4,0E-3
3,0E-3
2,0E-3
1,0E-3
0,0E0
INFLUENT
EFFLUENT

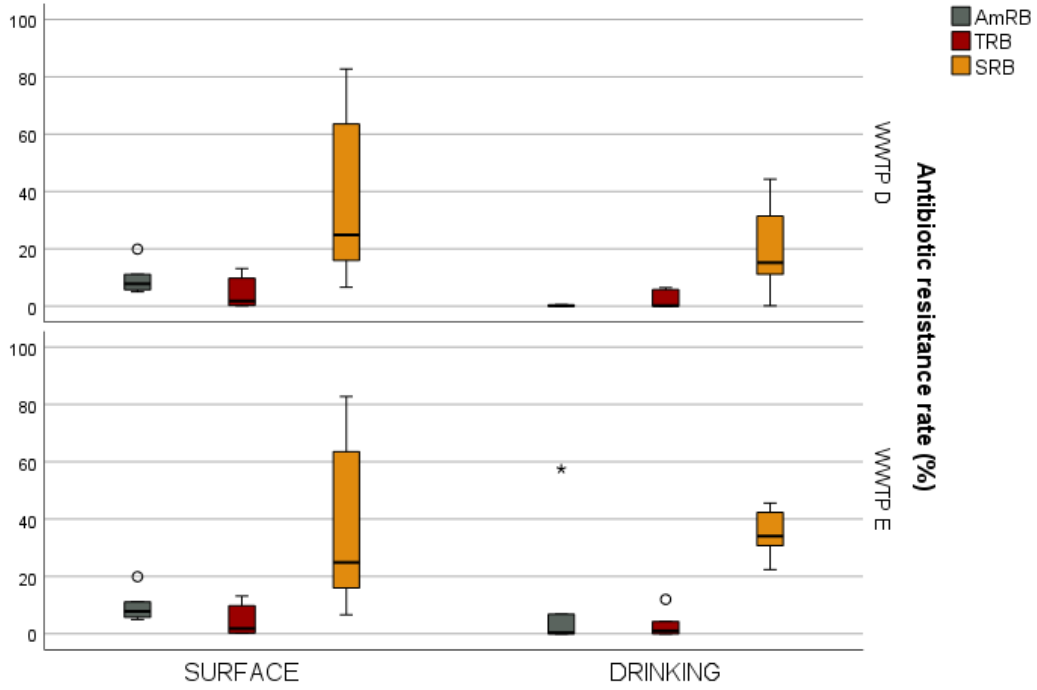
(b2)


sulII
INFLUENT
EFFLUENT
Relative abundance (gene copies/16 rRNA gene copy)
WWTP A
WWTP B
WWTP C
1,00
,80
,60
,40
,20
,00

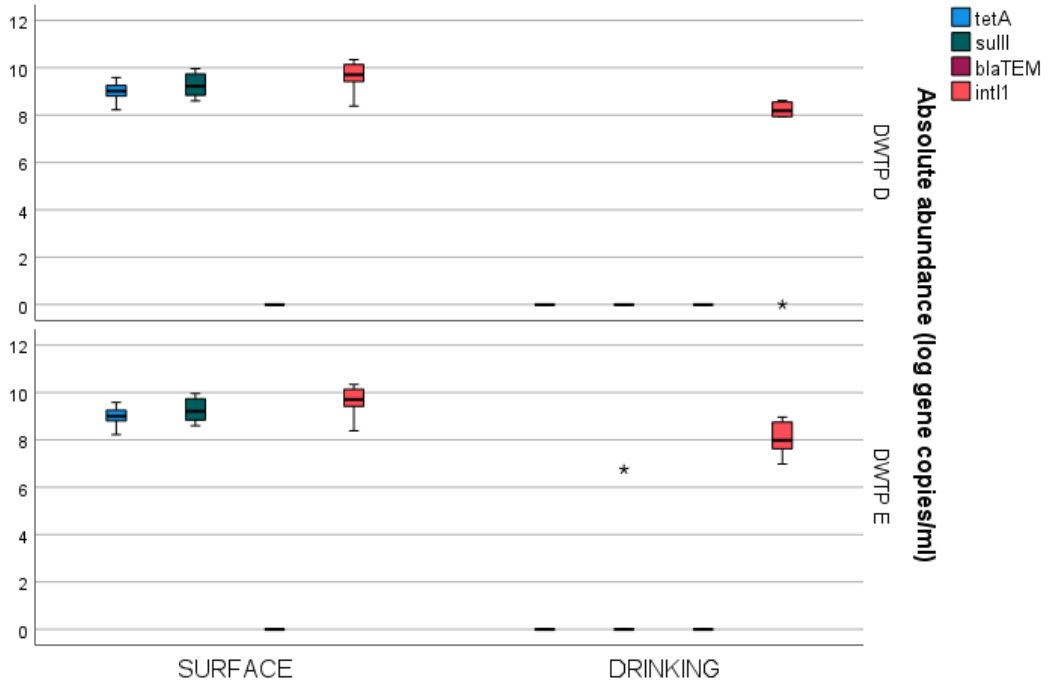
(a)


HPC
AmRB
TRB
SRB
Concentration (Log CFU/ml)
DWTP D
DWTP E
0
2
4
6
8
SURFACE
DRINKING

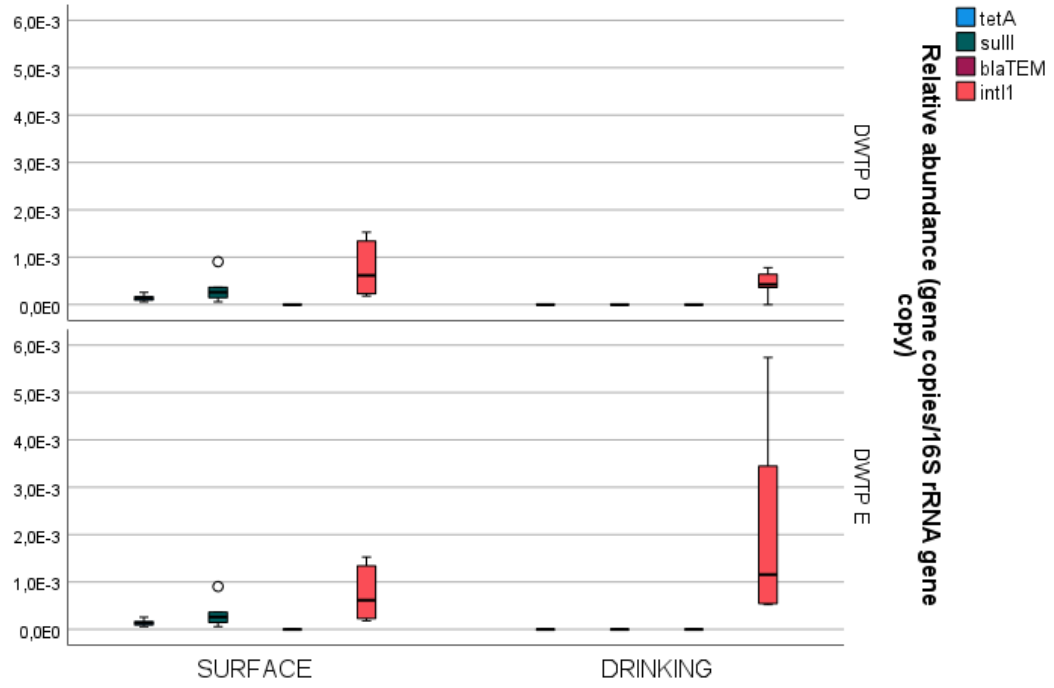
(b)


AmRB
TRB
SRB
Antibiotic resistance rate (%)
WWTP D
WWTP E
0
20
40
60
80
100
SURFACE
DRINKING

(a)


tetA
sulII
blaTEM
intI1
Absolute abundance (log gene copies/ml)
DWTP D
DWTP E
12
10
8
6
4
2
0
SURFACE
DRINKING

(b)


tetA
sulII
blaTEM
intI1
Relative abundance (gene copies/16S rRNA gene copy)
DWTP D
DWTP E
6,0E-3
5,0E-3
4,0E-3
3,0E-3
2,0E-3
1,0E-3
0,0E0
SURFACE
DRINKING

Table

Table 1. Results of the Student's T-test and the one-way ANOVA test considering the ARB and ARGs in urban water cycle

	Inlet [1] vs Outlet [2]		**WWTPs vs DWTPs**		**Sampling period**	
	Concentration	**Antibiotic resistance rate**	**Concentration**	**Antibiotic resistance rate**	**Concentration**	**Antibiotic resistance rate**
HPC	p<0.001	n.s.	p<0.05	n.s.	n.s.	n.s.
AmRB	p<0.001	n.s.	p<0.05	n.s.	n.s.	n.s.
TRB	p<0.001	n.s.	p<0.05	n.s.	n.s.	n.s.
SRB	p<0.001	n.s.	p<0.05	n.s.	n.s.	n.s.
	Absolute abundance	**Relative abundance**	**Absolute abundance**	**Relative abundance**	**Absolute abundance**	**Relative abundance**
***bla*TEM**	p<0.001	p<0.001	p<0.001	p<0.001	n.s.	n.s.
***tet*A**	p<0.001	p<0.001	p<0.001	p<0.001	n.s.	n.s.
***sul*II**	p<0.001	p<0.001	p<0.001	p<0.001	n.s.	n.s.
***intI*1**	p<0.05	p<0.05	n.s.	p<0.001	n.s.	n.s.

[1]: influents of WWTPs+drinking water source; [2]: effluents of WWTPs+drinking water

Table 2. Results of the Student's T-test and the one-way ANOVA test considering the ARB and ARGs in WWTPs

	Influent vs Effluent		**Wastewater treatment**		**Sampling period**	
	Concentration	**Antibiotic resistance rate**	**Concentration**	**Antibiotic resistance rate**	**Concentration**	**Antibiotic resistance rate**
HPC	p<0.001	n.s.	n.s.	n.s.	n.s.	n.s.
AmRB	p<0.001	n.s.	n.s.	n.s.	n.s.	n.s.
TRB	p<0.001	p<0.05	n.s.	n.s.	n.s.	n.s.
SRB	p<0.001	n.s.	n.s.	n.s.	n.s.	n.s.
	Absolute abundance	**Relative abundance**	**Absolute abundance**	**Relative abundance**	**Absolute abundance**	**Relative abundance**
***bla*TEM**	n.s.	p<0.001	n.s.	n.s.	n.s.	n.s.
***tet*A**	n.s.	p<0.001	n.s.	p<0.05[1]	n.s.	n.s.
***sul*II**	n.s.	n.s.	n.s.	p<0.05 [1]	n.s.	n.s.
***intI*1**	n.s.	p<0.001	n.s.	p<0.05 [1]	n.s.	n.s.

[1]: only for the effluent

Table 3. Results of the Student's T-test and the one-way ANOVA test considering the ARB and ARGs in DWTPs

	Surface water vs Drinking water		**Water treatment**		**Sampling period**	
	Concentration	**Antibiotic resistance rate**	**Concentration**	**Antibiotic resistance rate**	**Concentration**	**Antibiotic resistance rate**
HPC	p<0.001	n.s.	n.s.	n.s.	n.s.	n.s.
AmRB	p<0.001	n.s.	n.s.	n.s.	n.s.	n.s.
TRB	p<0.001	n.s.	n.s.	n.s.	n.s.	n.s.
SRB	p<0.001	n.s.	n.s.	n.s.	n.s.	n.s.
	Absolute abundance	**Relative abundance**	**Absolute abundance**	**Relative abundance**	**Absolute abundance**	**Relative abundance**
***bla*$_{\mathbf{TEM}}$**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
***tet*A**	p<0.001	p<0.001	n.s.	n.s.	n.s.	n.s.
***sul*II**	p<0.001	p<0.001	n.s.	n.s.	n.s.	n.s.
***intI*1**	p<0.05	n.s.	n.s.	p<0.05	n.s.	n.s.