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

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Environmental Science and Pollution Research

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

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Abstract:	<p>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 intl 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.</p> <p>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</p>

	represents a potential route of transmission to human.
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Università degli Studi di Torino
**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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1 **TITLE PAGE**

2 **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., *tetA*, *bla_{TEM}* and *sulII*) and *intI1* 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

61 **Introduction**

62 Antimicrobial resistance is of primary concern for the public health (WHO, 2014). Globally, it is estimated that
63 700,000 people each year could die because of antimicrobial resistant bacterial infections (Carvalho et al., 2016).
64 If effective interventions are not carried out to overcome infections attributable to microorganisms resistant to
65 antimicrobials, there could be an increase in deaths estimated up to 10 million people in the world by 2050, each
66 year (O'Neil 2016). The main driver for the spread and the persistence of antibiotic resistance is the overuse and
67 misuse of antibiotics in human and animal medicine (Sanganyado et al., 2019). Antibiotic resistant bacteria and
68 resistance genes (ARB and ARGs) together with residues of antibiotics are released in sewer, that constitutes a
69 reservoir of ARB and ARGs (Rizzo et al., 2013).

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

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

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

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

91 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
92 per inhabitant equivalent; of these at least 10^7 - 10^{10} showed some form of antibiotic resistance (Rizzo et al.,
93 2013). Moreover, a recently pan-European survey on treated wastewater demonstrates that WWTPs are
94 responsible for the discharge of considerable amounts of ARGs in the downstream water bodies (Cacace et al.,
95 2019). These data underline the main role of WWTPs in the accumulation and spreading of ARB and ARGs into
96 open waters.

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

101 The investigation of antibiotic resistance in the urban water cycle represents a relevant issue considering the risk
102 that ARB and ARGs in the "unclean" phases of the cycle can reach the final consumer of the water (Manaia et
103 al., 2016). Although the direct impact on human health by ARB and ARGs in drinking water is not well
104 established, antibiotic resistance threatens human health by two main different mechanisms: first, pathogenic and
105 opportunistic ARB that can survive in drinking water may also enter the human microbiome following water
106 consumption (Vaz-Moreira et al., 2014); second, ARGs in drinking water can occasionally be transferred by
107 horizontal gene transfer (HGT) to human pathogenic bacteria (Manaia 2017). Therefore, drinking water can be
108 considered as a potential transmission route of antibiotic resistance to humans (Chang et al., 2015; Manaia et al.,
109 2016).

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

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

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

121 *sulII*; against tetracycline, β -lactams, and sulphonamides, respectively) as well as those of the integrase gene of
122 the class 1 integrons (*intI1*), used as proxy of the anthropogenic pollution and of the antibiotic resistance in the
123 environment (Gillings et al., 2015; Ma et al., 2017) were measured using quantitative Real-Time PCR (qPCR).

124

125 **Materials and methods**

126 **Sampling**

127 **WWTPs**

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

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

138

139 **DWTPs**

140 Drinking water source (raw surface water from a large river) and finished water (drinking water) samples were
141 collected in two DWTPs (plants D and E) in the same geographical area of WWTPs (~20 km² in North-West of
142 Italy). Plants D and E treat 130.000 m³/day and 86.400 m³/day of drinking water, respectively, sourced from the
143 same drinking water source.

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

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

150

151 **Sample processing and ARB quantification**

152 ARB were isolated in a basic culture medium for the count of heterotrophic bacteria (R₂Agar) supplemented
153 with ampicillin, tetracycline and sulfamethoxazole at a concentration of 32 mg/L, 16 mg/L and 50.4 mg/L,
154 respectively.

155 The antibiotic concentrations tested were chosen considering the highest dose used in standard methods to
156 establish resistance to antibiotics with clinical strains (breakpoint) (CLSI, 2018) or the concentration reported in
157 previous study (Gao et al., 2012). Total heterotrophic count (HPC) was determined on media without antibiotics.
158 Serial dilutions of wastewater samples (influent and effluent) were plated in duplicate on media with and without
159 antibiotics. Serial dilution of surface water or different volumes of drinking water (0.5-1L for HPC and 2 L for
160 ARB) were filtered (0.22 um pore nitrocellulose size filter membrane, Millipore) and then the membranes were
161 placed on R₂Agar plates with and without antibiotics.

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

165

166 **Sample processing and DNA extraction**

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

173

174 **Real-time qPCR of ARGs**

175 The abundance of the selected ARGs (*tetA*, *bla*_{TEM} and *suIII*) of *intI1* and of the 16 rRNA gene was measured by
176 qPCR using a RT-thermocycler (CFX Connect, Bio-Rad). The protocol used in qPCR assays was previously
177 described by Di Cesare et al. (2015). The qPCR program was 95 °C for 2 min, 35 cycles of 95 °C for 15 s,
178 annealing temperature reported in Supplementary Table S1 for 30 s and 72 °C for 15 s. Melt curve analysis was
179 performed from 60 °C to 95 °C with increments of 0.5 °C/5 s. Standard calibration curves were carried out using
180 the purified, quantified and ten-fold diluted amplicon of each gene as described in Di Cesare et al. (2013). Each

181 reaction was carried out in duplicate for each sample. The limits of quantification (LOQ) per each quantified
182 gene was determined as described in Bustin et al. (2009). They were 1.55×10^3 , 1.22×10^2 , 3.57×10^1 , $4.88 \times$
183 10^2 and 1.12×10^1 gene copy/ μ L for 16 rRNA, *tetA*, *sulIII*, *bla*_{TEM} and *intI1* genes respectively. The mean value \pm
184 standard deviation of the reaction efficiencies was $98.32 \pm 8.23\%$ and the R^2 was always more than 0.97. The
185 potential inhibition of the qPCRs due to the type of analysed matrix was calculated by dilution method (Di
186 Cesare et al. 2013) and no inhibition was obtained. The ARG and *intI1* abundances were expressed as absolute
187 abundance (log gene copies/ml) and relative abundance (gene copies/16S rRNA gene copy) and a mean value of
188 the abundance for each gene was calculated. The interpretation of the results in case of abundance values lower
189 than the LOQ and in case of discordance between the two replicates was made as previously reported (Di Cesare
190 et al., 2015).

191

192 **Statistical Analyses**

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

203

204 **Results**

205 **Dynamics of ARB and ARGs in urban water cycle**

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

210 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}, *tetA*, *suII* and *intI1* 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 *tetA*, *suII* and *intI1* respect to 16S rRNA gene, when quantifiable ranged from 3.3×10^{-4} to 9.1×10^{-4} , 1.1×10^{-3} to
237 3.7×10^{-3} , 2.0×10^{-1} to 8.7×10^{-1} and 1.6×10^{-3} to 3.7×10^{-3} copies/16 rRNA gene copy, respectively. *suII* showed
238 for both, absolute and relative abundance, the highest mean values (mean: 9.4 log gene copies/mL and 3.8×10^{-1}
239 copies/16 rRNA gene copy) (Fig 2).

240 The absolute abundance of *bla*_{TEM}, *tetA*, *suII* and *intI1* when quantifiable ranged in the effluents from 7.3 to 8.2,

241 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
242 quantifiable of *bla*_{TEM} was 1.9×10^{-4} copies/16 rRNA gene copy, the relative abundance of *tetA*, *suII* and *intI1*
243 ranged from 2.7×10^{-5} to 1.5×10^{-3} , 1.3×10^{-1} to 9.1×10^{-1} and 5.6×10^{-4} to 3.1×10^{-3} copies/16 rRNA gene copy.
244 As observed already in the influents, the highest mean value was obtained for *suII* (mean: 9.5 log gene
245 copies/mL and 3.6×10^{-1} copies/16 rRNA gene copy) (Fig 2).

246 Although the absolute abundance of ARGs generally did not show differences between the influents and
247 effluents of WWTPs, a significant reduction of the relative abundance of *tetA*, *bla*_{TEM} and *intI1* was observed in
248 the effluents.

249 Significant differences among the WWTPs were reported in effluents only for the relative abundance of *suII*
250 (WWTP2>WWTP1 and WWTP3), *tetA* (WWTP1>WWTP2) and *intI1* (WWTP1>WWTP2).

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

252 A correlation between relative abundance of *intI1* vs *tetA* ($p<0.001$), *intI1* vs *bla*_{TEM} ($p<0.05$) and *bla*_{TEM} vs *tetA*
253 ($p<0.001$) was observed ($p<0.05$). No relationship was reported for ARGs and ARB, except a correlation
254 between TRB and *tetA* ($p<0.05$) and TRB and *intI1* ($p<0.05$)

255

256 **Dynamics of ARB and ARGs in DWTPs**

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

259 ARB were observed in all analyzed surface water samples with concentrations ranging between 4.7 to 6.3 for
260 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
261 concentration was 6.1 Log CFU/mL for SRB, and the lowest 4.9 Log CFU/mL for TRB. The highest ratio is
262 36% for SRB and the lowest 5% for TRB.

263 Considering the drinking water, SRB were observed in all analyzed samples with concentrations ranging from
264 1.0 to 2.1 Log CFU/mL. On the contrary, TRB and AmRB were found in the 50% and 25% of the analyzed
265 samples and the concentration when quantifiable ranged from 1.2 to 1.9 Log CFU/mL for AmRB and from 0.4 to
266 1.5 Log CFU/mL for TRB. Moreover, the highest mean concentration was 1.5 Log CFU/mL for SRB, and the
267 lowest 0.4 Log CFU/mL for AmRB. According to the ARB concentration, the highest resistance rate was
268 observed for SRB (mean value: 27%).

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

271 treatment and sampling period did not impact abundances and rates of TRB, SRB and AmRB.
272 In all samples of the surface water *intI1*, *tetA* and *suII* were observed, while *bla_{TEM}* was never detected. The
273 absolute abundance of *tetA*, *suII* and *intI1* in these samples ranged from 8.2 to 9.6, 8.6 to 10.0, 8.4 to 10.3 log
274 gene copies/mL, respectively (Fig 4a). The relative abundances of *tetA*, *suII* and *intI1* ranged from 5.9×10^{-5} to
275 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. *intI1* showed the
276 highest mean values for both, absolute and relative abundance (mean: 9.6 log gene copies/mL and 7.5×10^{-4}
277 copies/16 rRNA gene copy) (Fig 4b).
278 *bla_{TEM}* and *tetA* were not detected in drinking waters, except for three samples (1 for DWTP D, 2 for DWTP E)
279 that presented only one replicate positive but not quantifiable for *tetA*, while all drinking water samples were
280 positive for *suII*, although this gene was not quantifiable. The absolute and relative abundance of *intI1* was 7.5
281 Log gene copies/mL and 1.3×10^{-3} copies/16 rRNA gene copy, respectively.
282 The relative and absolute abundance of *tetA* and *suII* were reduced by each water treatment, moreover for
283 DWTP E also the absolute abundance of *intI1* was lower in the drinking water (Fig 4a). The relative abundance
284 of *intI1* in the drinking water was the sole parameter evidencing a difference between the DWTPs (DWTP
285 E>DWTP D; Fig 4b). The absolute and relative ARG abundances were not influenced by the sampling period
286 (Table 3).
287 Moreover, both relative and absolute abundances of *tetA* showed a correlation with the absolute abundance of
288 *suII* ($p < 0.001$). No relationship was detected between the values of ARGs and ARB, except for a correlation
289 between SRB and *suII* gene ($p < 0.001$).

290

291 **Discussion**

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

297 The overall ARB and ARG dynamics in the WWTPs and DWTPs sampled in this study, highlighted, as
298 expected, higher concentrations of antibiotic resistances in the WWTPs than in DWTPs. This is in agreement
299 with several studies that identified the WWTP as hot spot for the spread of antibiotic resistances (Rizzo et al.,
300 2013; Pazda et al., 2019). The concentration of ARB in the influent of WWTPs observed in our study were

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

306 Moreover, the stability of the antibiotic resistant rates in the influents and effluents, (proportion of ARB in the
307 HPC for each sample), underlined that the different treatments did not promote the selection of ARB. This was
308 also confirmed by the relative abundance of the ARGs that showed a reduction for *bla*_{TEM}, *tetA* and *intI1* with
309 values significantly lower in the effluent. The results reported in literature that considered the effect of the
310 treatment on the variation of the ARG relative abundances are discordant. Indeed, in two studies, performed in
311 Canada and in China, no significant differences were observed in the relative abundance of ARGs between
312 influent and effluent of two WWTPs (Mc Connell et al., 2018; Ma et al., 2015). On the contrary Di Cesare et al.
313 (2016) showed an increase of some ARGs (e.g. *suII*) and of *intI1* in the effluents of three Italian WWTPs.

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

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

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

331 ARGs and ARB (3-4 orders of magnitude) respect to the data reported for other rivers in China and Poland
332 influenced by anthropogenic activities (e.g., sewage discharges, agricultural runoff, swine farm) (Bondarczuk et
333 al., 2019; Yang et al., 2020; Hu et al., 2019) underlining its role in the spreading of antibiotic resistance. The
334 presence of ARB and ARGs in surface water used as drinking water source highlights the importance to
335 investigate all the phases composing the urban water cycle and to deep the effect of the drinking water treatment.
336 None of the detected ARB or ARGs were completely removed in drinking water by the applied water treatments,
337 according to Hu (Hu et al, 2019) and Siedlecka (Siedlecka et al., 2021) who investigated ARGs in a DWTP in
338 China and Poland. As previously reported in other studies, the presence and the total concentration of ARGs in
339 drinking water significantly decreased in comparison to corresponding water source (Sanganyado et al., 2019;
340 Stange et al., 2019). Moreover, the limited variations in antibiotic resistance rates between surface water and
341 drinking water associated with the reduction of relative ARG abundances showed, as observed in WWTPs, that
342 the treatment did not apply any selective pressure. These results highlight the key role played by the drinking
343 water treatment in reducing ARGs and controlling ARB from the water source. As observed for WWTPs, the
344 different drinking water treatments seemed to display a limited impact on the spread of SRB and ARGs,
345 although DWTP D was equipped with an ozonation step that resulted the best treatment to reduce ARB and
346 ARG in some studies (Stange et al., 2019; Yang et al., 2020). It is important to highlight that the effectiveness of
347 the disinfection step can be influenced by numerous factors such as the bacterial community composition in the
348 raw water used as drinking water source, the effectiveness of other treatment steps, and the co-selection of
349 antibiotic resistance enhanced by disinfection by-products (Sanganyado and Gwenzi, 2019).

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

353 Considering that some genes can be utilized as proxy for the overall abundance of ARGs in waters (Su et al.,
354 2018), Pearson correlation was carried out among the different measured ARGs in WWTPs and DWTPs to
355 evaluate the suitability of the monitored target as indicator. The results highlighted that a positive correlation was
356 observed only for *bla*_{TEM} vs *tetA* in WWTPs and *sulIII* vs *tetA* in DWTPs underlining that none of the selected
357 genes could be used to predict the fate and the potential contamination by determinants of antibiotic resistance in
358 the urban water cycle. A similar result was also observed for *intI1* that was correlated only with *bla*_{TEM} and *tetA*
359 in WWTPs. These results claim for the need to perform more studies by using untargeted based quantification
360 approach, *i.e.*, shotgun metagenomics, to characterize the overall antibiotic resistome (total content of ARGs) of

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

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

382

383 **Author contribution**

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

390

391 **Declarations**

392 **Ethics approval**

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

394 **Consent to participate**

395 No consent of participation is to be claimed.

396 **Consent for publication**

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

399 **Competing interests**

400 The authors declare no competing interests.

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403 **Availability of data and materials**

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

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600 Fig. 1: (a) Log-transformed concentrations of ampicillin, tetracycline, sulfonamide resistant bacteria and HPC in
601 influent and effluent of the WWTPs investigated. Box plots represent median and range values. (b) Antibiotic
602 resistance rate of ampicillin, tetracycline and sulfonamide resistant bacteria in WWTPs. All values are
603 normalized to HPC abundances. Box plots represent median and range values.
604

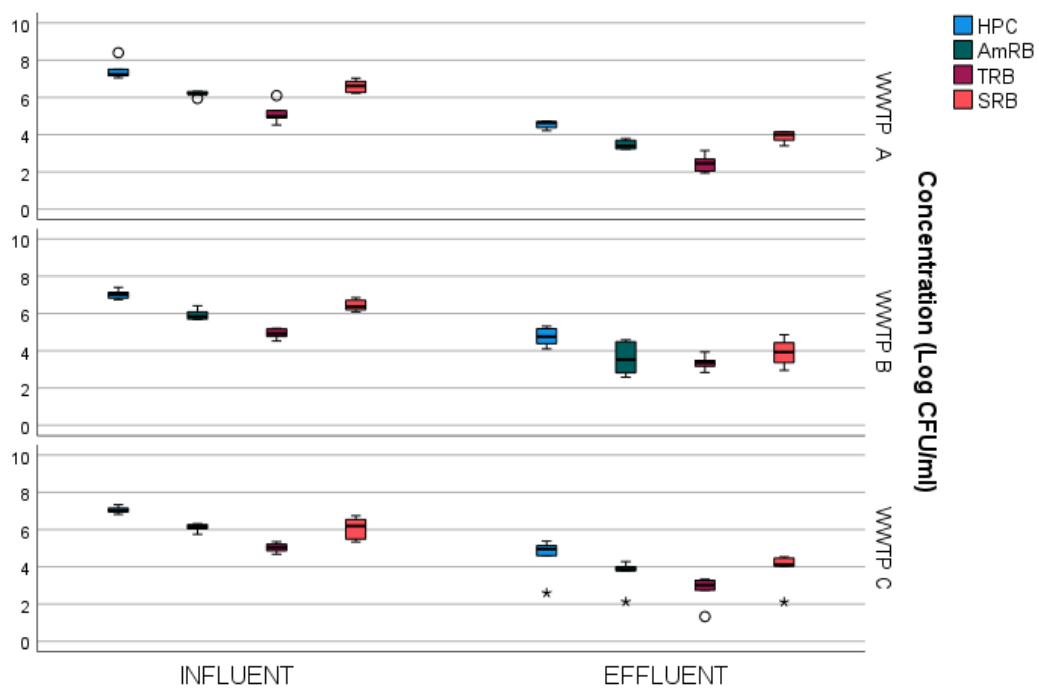
605 Fig. 2: (a) Absolute abundance of tetA, sulII, blaTEM and intI1 gene in influent and effluent of the WWTPs
606 investigated. Box plots represent median and range values. (b) Relative abundance of of tetA, blaTEM, intI1 (1)
607 and sulII (2) gene in WWTPs. All values are normalized to 16S rRNA gene copy. Box plots represent median
608 and range values.
609

610 Fig. 3: (a) Log-transformed concentrations of ampicillin, tetracycline, sulfonamide resistant bacteria and HPC in
611 influent and effluent of the DWTPs investigated. Box plots represent median and range values. (b) Antibiotic
612 resistance rate of ampicillin, tetracycline and sulfonamide resistant bacteria in DWTPs. All values are
613 normalized to HPC abundances. Box plots represent median and range values.
614

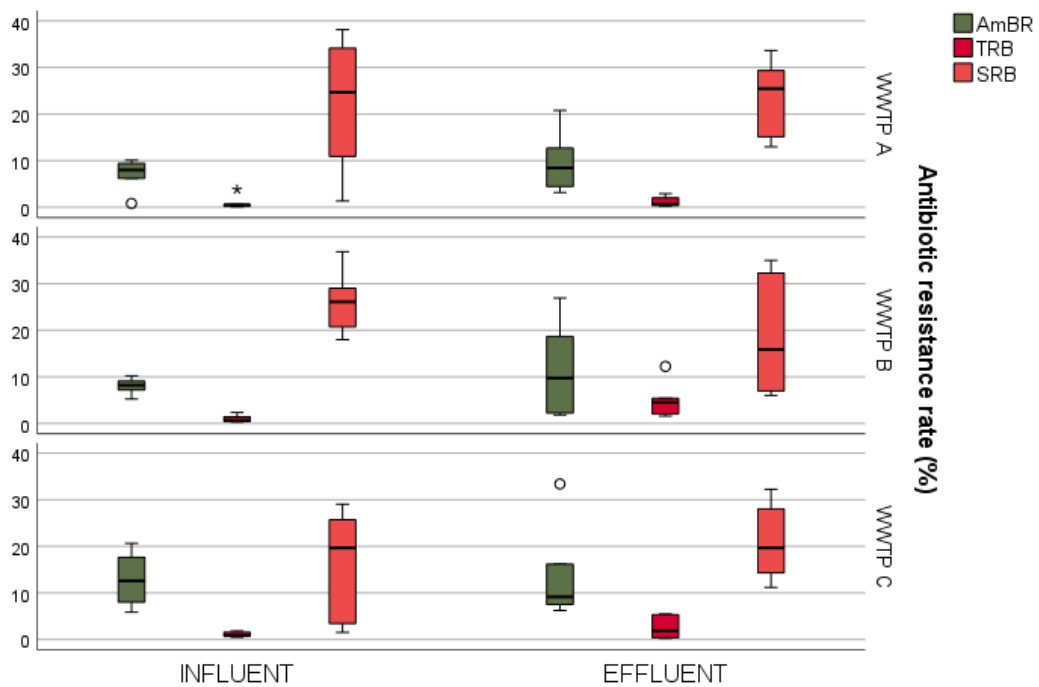
615 Fig. 4: (a) Absolute abundance of tetA, sulII, blaTEM and intI1 gene in influent and effluent of the DWTPs
616 investigated. Box plots represent median and range values. (b) Relative abundance of of tetA, blaTEM, sulII and
617 intI1 gene in DWTPs. All values are normalized to 16S rRNA gene copy.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

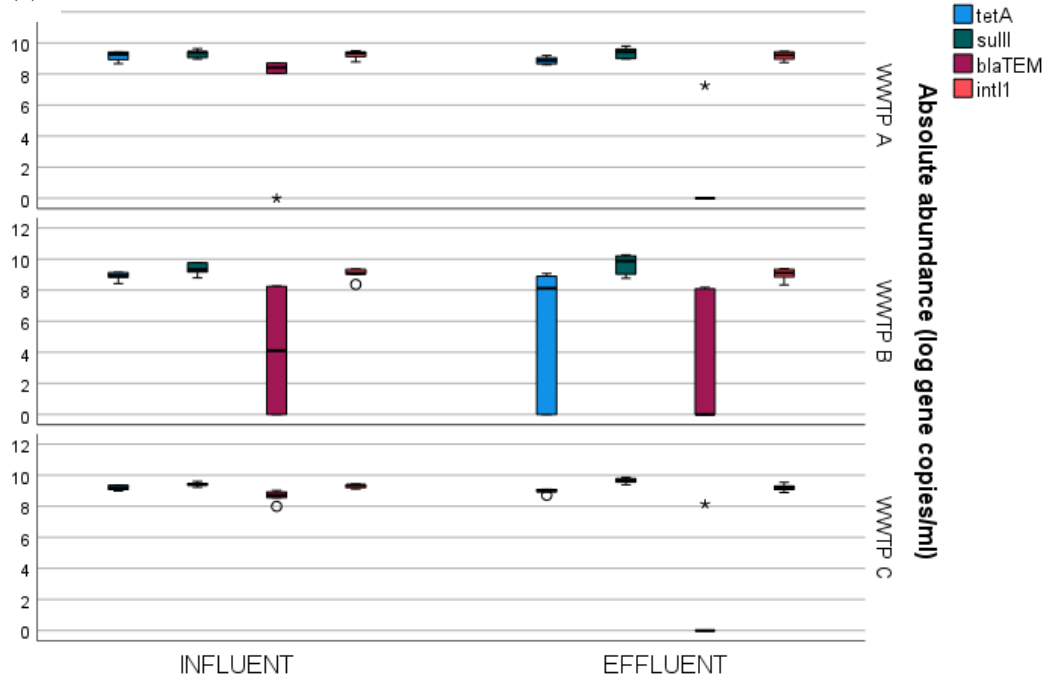
(a)



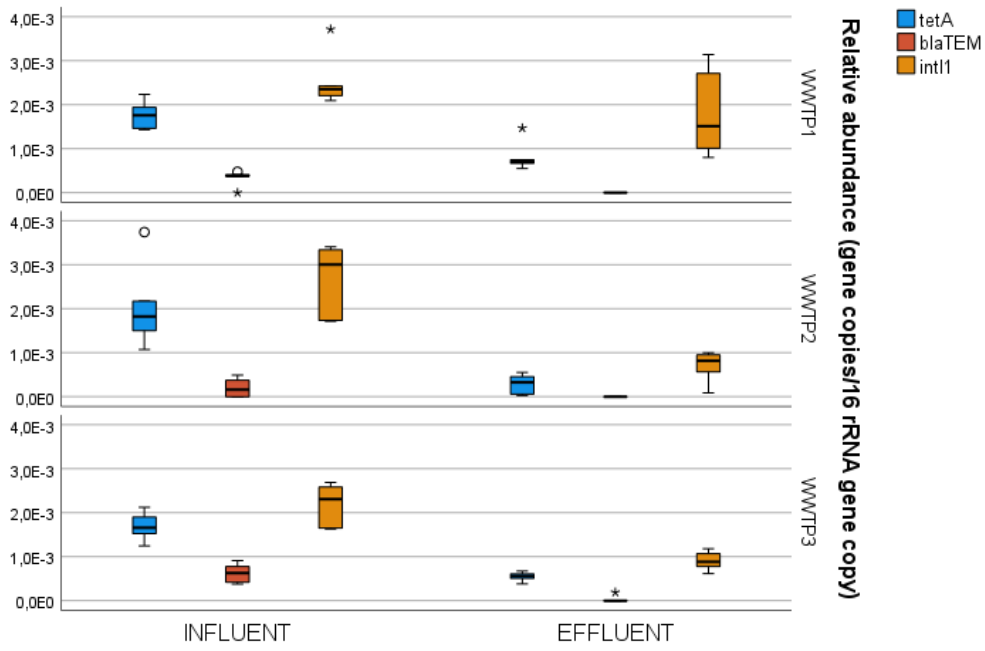
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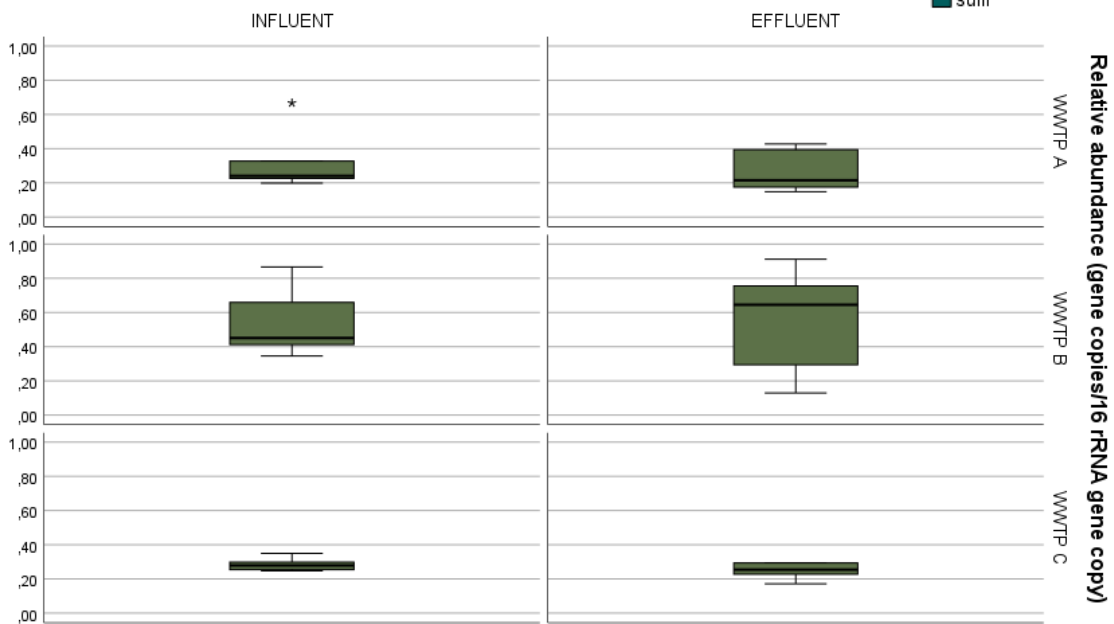
(a)



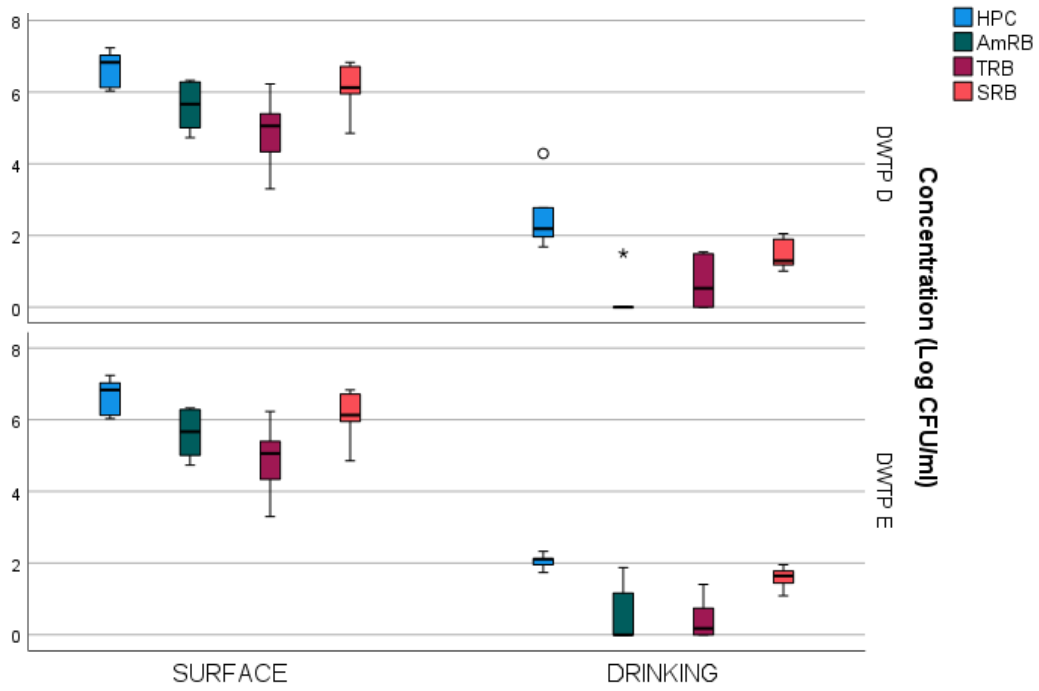
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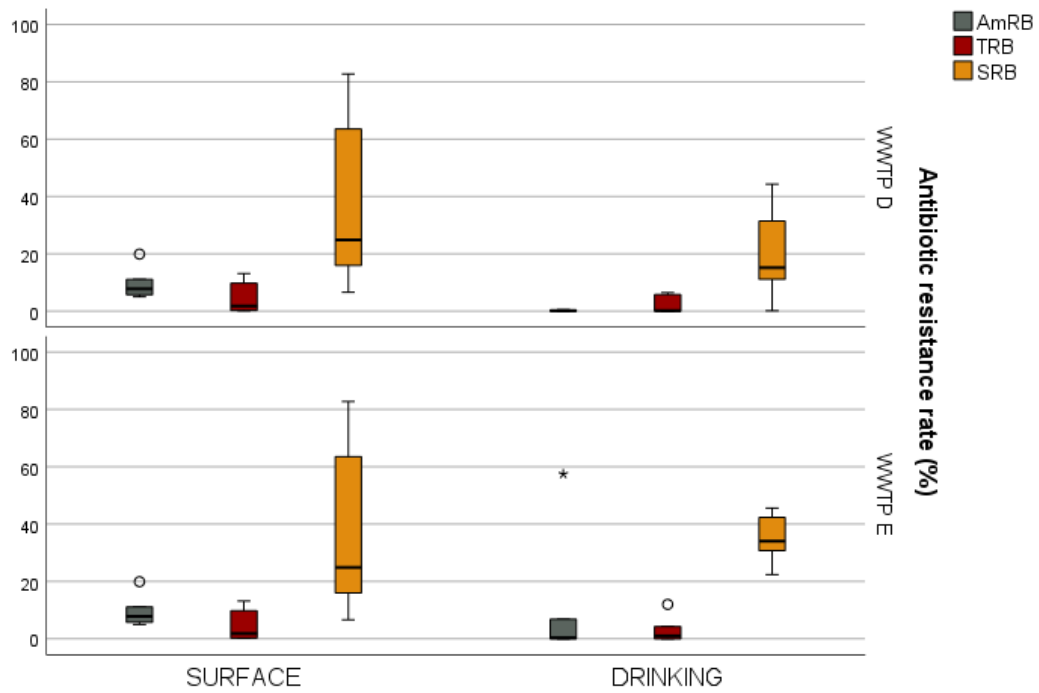
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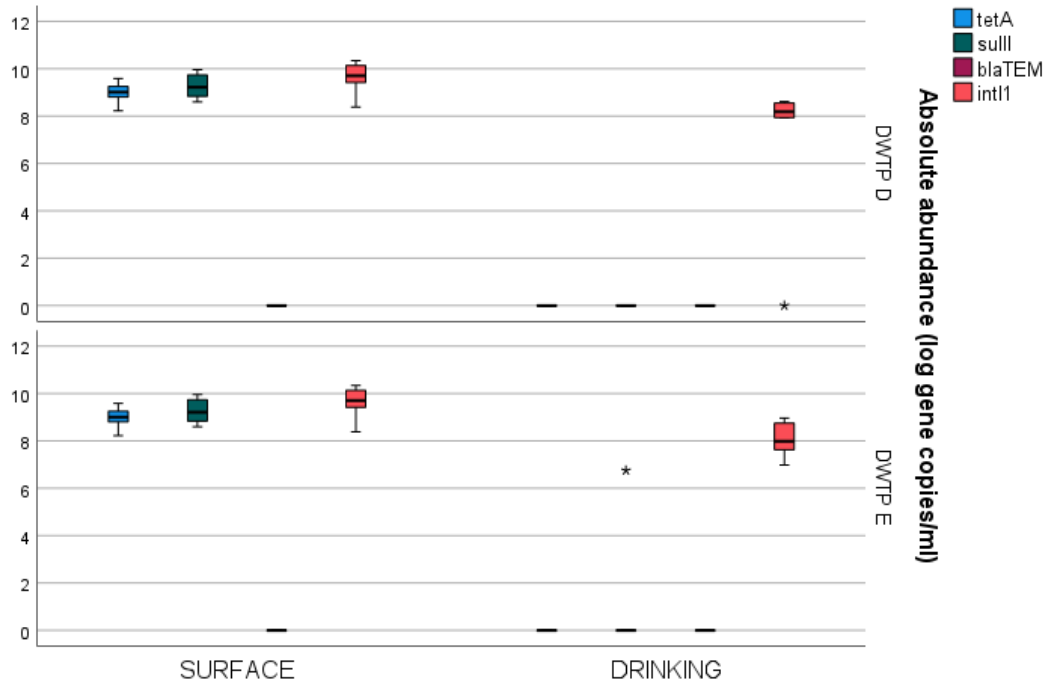
(a)



(b)



(a)



(b)

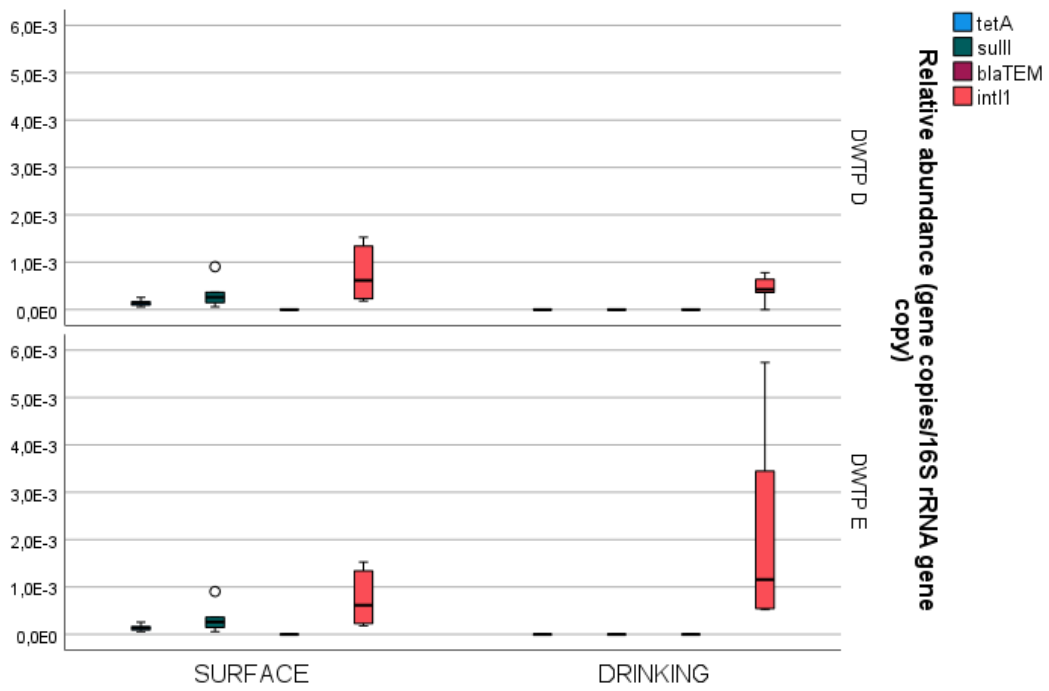


Table 1. Results of the Student's T-test and the one-way ANOVA test considering the ARB and ARGs in urban water cycle

	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
<i>bla</i> _{TEM}	p<0.001	p<0.001	p<0.001	p<0.001	n.s.	n.s.
<i>tetA</i>	p<0.001	p<0.001	p<0.001	p<0.001	n.s.	n.s.
<i>suII</i>	p<0.001	p<0.001	p<0.001	p<0.001	n.s.	n.s.
<i>intI1</i>	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
<i>bla_{TEM}</i>	n.s.	p<0.001	n.s.	n.s.	n.s.	n.s.
<i>tetA</i>	n.s.	p<0.001	n.s.	p<0.05 ¹	n.s.	n.s.
<i>suII</i>	n.s.	n.s.	n.s.	p<0.05 ¹	n.s.	n.s.
<i>intI1</i>	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
<i>bla</i> _{TEM}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>tetA</i>	p<0.001	p<0.001	n.s.	n.s.	n.s.	n.s.
<i>suII</i>	p<0.001	p<0.001	n.s.	n.s.	n.s.	n.s.
<i>intI1</i>	p<0.05	n.s.	n.s.	p<0.05	n.s.	n.s.



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