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Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings

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ABSTRACT

There is increasing public concern regarding the fate of antibiotic resistance genes (ARGs) during wastewater treatment, their persistence during the treatment process and their potential impacts on the receiving water bodies. In this study, we used quantitative PCR (qPCR) to determine the abundance of nine ARGs and a class 1 integron associated integrase gene in 16 wastewater treatment plant (WWTP) effluents from ten different European countries. In order to assess the impact on the receiving water bodies, gene abundances in the latter were also analysed.

Six out of the nine ARGs analysed were detected in all effluent and river water samples. Among the quantified genes, *intI1* and *sul*1 were the most abundant. Our results demonstrate that European WWTP contribute to the enrichment of the resistome in the receiving water bodies with the particular impact being dependent on the effluent load and local hydrological conditions. The ARGs concentrations in WWTP effluents were found to be inversely correlated to the number of implemented biological treatment steps, indicating a possible option for WWTP management.

Furthermore, this study has identified bla_{OXA-58} as a possible resistance gene for future studies investigating the impact of WWTPs on their receiving water.

1. Introduction

The evolution of antibiotic resistance has historically been viewed as a strictly clinical issue and considered to be exclusively related to the use and misuse of antibiotics (Ainsa, 2002). In recent years, the fate of antibiotic resistance genes (ARGs) emitted from

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urban wastewater treatment plants (WWTP) has received increasing attention (Berendonk et al., 2015; Jia et al., 2017; Lorenzo et al., 2018; Sabri et al., 2018; Stoll et al., 2012). There is a worldwide consensus that raw municipal wastewater, treated effluent wastewater, and wastewater sludge, where environmental bacteria with genetically different "composition" interact with each other, are reservoirs of ARGs (Auerbach et al., 2007; Ghosh et al., 2009; Zhang et al., 2009) and hotspots for the evolution and spread of antibiotic resistance (Caucci et al., 2016; Guo et al., 2017; Rizzo et al., 2013).

Even though treated wastewater contains significantly lower amounts of ARGs than raw wastewater, various researchers have demonstrated that the discharge of treated municipal wastewater can increase the quantities of ARGs in the receiving water bodies (Amos et al., 2018; Jäger et al., 2018; LaPara et al., 2011; Pruden et al., 2012). Aquatic environments downstream of WWTPs can eventually be enriched also with mobile genetic elements, such as conjugative plasmids, integrative and conjugative elements (ICEs), transposons and integrons (Di Cesare et al., 2016; Marano and Cytryn, 2017; van Hoek et al., 2011), which represent effective carriers of ARGs (including multi-resistances). Currently, little is known about the mechanisms controlling transport, transfer and accumulation of ARGs in these aquatic systems. According to the scientific literature, we hypothesize that WWTP effluents may serve as a source of ARGs and mobile elements carrying resistance for downstream aquatic environments, independent from the treatment processes in a specific WWTP and the country of origin (Freeman et al., 2018; Karkman et al., 2018; Liu et al., 2018). In addition, among the screened ARGs and genetic elements, we hypothesize that *intI1* is the most abundant genetic component in both effluents and water environments, relative to other antibiotic resistance elements that are being screened, since it is a genetic platform enabling the capture of over a hundred known resistance gene cassettes (Gillings et al., 2015; Khan et al., 2013). Nevertheless, studies on a larger geographic scale supporting these findings are still largely lacking. This is unfortunate, because it is generally accepted that ARGs, as environmental contaminants, have to be monitored in order to generate information as a base for risk assessment (Berendonk et al., 2015). A large-scale geographic analysis of the levels of ARGs discharged by WWTPs, comprising different treatment technologies and geographical locations, and their effect on the receiving waters is still missing. To obtain such data "a method that can achieve high specificity, based on targeted detection, rendering reliable and reproducible quantifications and therefore quantifiable ranges of variation, is the best choice. Real-time quantitative PCR (qPCR) is currently the best candidate for such an application" (Rocha et al., 2018).

Accordingly, the objective was to quantify the abundance of selected ARGs and *intI1* in WWTPs effluents and their receiving waters across Europe and to document the possible impact of WWTPs on their receiving water.

Ten antibiotic resistance determinants, considered to be relevant indicators of the resistome of different environments (Berendonk et al., 2015; Rocha et al., 2018), were investigated in this study. The ARGs conferring resistance to antibiotics of different classes such as beta-lactams, sulfonamides and tetracycline were chosen, as recognised to be widely occurring in urban wastewater and water environments (Narciso-da-Rocha and Manaia, 2016; Szczepanowski et al., 2009). The ARGs encoding for broad (bla_{KPC-3}) and extended-spectrum (bla_{OXA}, bla_{CTX-M} *and* bla_{TEM}) β-lactamases (ESBL) were chosen in relation to their ability to confer resistance against an essential class of antibiotics for the treatment of infectious disease (Graham et al., 2016). The gene *mcr-1* encodes colistin resistance and its occurrence and prevalence in WWTPs and surrounding environments is still largely unknown (Poirel et al., 2016). This gene is of particular interest because, to the best of our knowledge, it has only been detected in treated wastewater by two other groups (Hembach et al., 2017; Lekunberri et al., 2017). The *intI1* gene, encoding the class 1 integron integrases, is highly abundant in both wastewater and freshwater environments and therefore, is commonly used as an indicator for anthropogenic pollution because of linkage to gene cassettes containing genes conferring resistance to antibiotics (Gatica et al., 2016; Power et al., 2013).

2. Materials and methods

2.1. Sample collection and DNA extraction

Two different sampling campaigns were performed. In the first campaign 1 L of a 24 h composite treated wastewater samples were collected during three consecutive days (14th, 15th and 16th of October 2014) from 16 urban WWTPs in 10 different European countries (France, Italy, Norway, Portugal, Germany, Netherlands, Cyprus, Turkey, Austria and United Kingdom) (Fig. S1, Table 1). All participants provided metadata (e.g. weekly discharge averages, WWTP characteristics, weather conditions etc.) by filling in a common survey (Table 1, Table S2).

The second sampling campaign took place 11 months later (8th, 9th and 10th September 2015). In addition to the WWTP effluent samples (same procedure as in the first campaign), the corresponding receiving water bodies were sampled as well. Four WWTPs (IT, NO1, CY and DE6) had to be excluded from this extended sampling because the receiving waters could not be accessed with reasonable logistic effort.

Grab samples were collected both upstream and downstream of the discharge point of the WWTP. Volumes of 500 mL of water were collected from each of the two sides of the water body and subsequently mixed to obtain 1 L integrated grab sample. In order to harmonize and standardise the sampling amongst the participating countries, the downstream samples were taken at a specified distance from the WWTP equalling 10 times the width of the river.

After sampling, the samples were transferred in sterile glass bottles to local laboratories, where three 150 mL aliquots were filtered through polycarbonate membrane filters (0.22 μm) and stored at −20 °C prior to DNA extraction. DNA was extracted from the three filters using the PowerWater DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. Total DNA was recovered in 100 μL of elution buffer and replicates were pooled to reduce variations in extraction efficiencies and sampling. The concentration of extracted DNA was measured using a NanoDrop® Spectrophotometer ND-1000 (Thermo Scientific, Waldham, USA). The extracted DNA was stored at −20 °C pending further analysis.

2.2. Quantification of antibiotic resistance genes

A total of nine resistance genes: bla_{TEM}, bla_{OXA-48}, bla_{OXA-58}, $bla_{CTX-M-15}$, $bla_{CTX-M-32}$, bla_{KPC-3}, *sul1, tetM, mcr*-1, the 16S rRNA encoding genes and the class 1 integron integrase *intI1* gene were quantified.

qPCRs were performed in 20 μl reaction volumes in 96-well plates on a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) containing 10 μl 1× Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, USA), 0.5 μL of the respective primers (stock concentration 10 μM), 4 μl of Nuclease-free water and 5 μL of template DNA (2 ng/μL) (Heβ et al., 2018). The qPCR cycling conditions for all reactions were as follows: 95 °C for 10 min for DNA polymerase activation followed by 40 cycles of 15 s at 95 °C and 1 min at respective temperature for primer annealing and elongation (Table S1). Melting curves were obtained to confirm

Table 1
WWTPs sampled in this study.

WWTP code	Country	Size (Population Equivalent)	Building year	Hospitals in catchment	Nr. Biological stages
FR	France	500,000	2002	7	1
IT	Italy	700,000	1988	1	1
NO1	Norway	700,000	2008	5	2
NO2	Norway	150,000	2010	2	2
PT	Portugal	160,000	1986	0	1
DE1	Germany	140,000	2001	2	2
DE2	Germany	740,000	2006	8	1
DE3	Germany	550,000	2007	10	1
DE4	Germany	4,500	2013	1	2
DE5	Germany	440,000	2015	3	1
DE6	Germany	50,000	2012	–	1
NL	Netherlands	500,000	1991	3	2
CY	Cyprus	100,000	2012	0	2
UK	United Kingdom	900,000	1950	1	1
TR	Turkey	–	2008	5	1
AT	Austria	42,000	2007	1	1

amplification specificity. Both samples and standards were analyzed in technical duplicates. All the analyses were performed in the same laboratory. Data analysis was performed by using the *Realplex* software (Eppendorf AG, Hamburg, Germany) and according to the procedures described in Rocha et al. (2018).

Abundance of the above-listed ARGs was quantified via qPCR targeting different fragments of ARGs that were cloned into plasmid vectors and used as standards. The fragments of 16S rRNA, *intI1*, *sul1* and $bla_{\text{CTX-M-32}}$ were embedded into a single plasmid "pNORM" designed by Christophe Merlin for the NORMAN project (www.norman-network.net; Hembach et al., 2017; Rocha et al., 2018; Stalder et al., 2014). The rest of the ARGs were quantified by using pUC19 plasmids including the corresponding gene fragment (Heβ et al., 2018). Plasmid-DNA was extracted by a QIAprep Spin Miniprep Kit (Hilden, Germany) and used after linearization to generate a standard curve (between 10^8 and 10^1 copy numbers) in duplicate for each qPCR run.

2.3. Data analysis and statistics

The impact of a WWTP on the receiving river is best reflected by the ratio of ARG concentrations measured downstream and upstream of the discharge site (C_d/C_u). In practice, it is difficult to define and uniformize the Cd and Cu sampling sites and therefore assess how representative those values are for the respective cross-sections. Moreover, even though composed water samples from both river banks were used in this study, the quality of the downstream measurements (C_d) remains particularly sensitive to incomplete mixing. While the downstream sample could have been taken very far from the WWTP so as to guarantee complete mixing of effluent and river water under all circumstances, this option has not been adopted for two reasons. On the one hand, the measurements might easily be affected by further unknown sources of ARG located between the effluent pipe and the sampling point. On the other hand, the effects of in-stream processes such as settling and bacteria attachment would gain in importance as the travel time between the effluent and the downstream sampling site increases. In view of these difficulties, and also as an exercise to test whether the use of available data can be used to predict the dissemination of ARGs in surface water, it was decided to complement measured values of C_d with independent estimates derived from the mass balance (Eqn. (1)) where C_e and Q_e denote the concentration and discharge of the WWTP effluent, respectively and Q_u represents stream flow upstream of the plant.

$$C_d = (C_u{}^*Q_u + C_e{}^*Q_e)/(Q_u + Q_e)\ . \qquad 1$$

As compared to measured values of C_d, the estimates computed with Eqn. (1) are unaffected by incomplete mixing or in-stream processes, nevertheless, they are sensitive to inaccuracies in the flow rates Q_e and Q_u.

All statistical analyses and visualizations were performed in R version 3.4.4. The Welch test was used to check for significant shifts in location and input data were log-transformed as necessary. Where appropriate, p-values underwent conventional adjustment by Holm's method to minimize the chance of false positive outcomes in multiple test problems (Holm, 1979). Data associated with the individual days of a sampling campaign were not aggregated into mean values but rather processed in original form so as to retain as much of the variance as possible.

3. Results

3.1. Spatial overview of the abundance of ARGs abundance in wastewater effluents in Europe

Averaged bacterial abundance (measured as 16S *rRNA* genes) was quantified in 16 different European effluents of WWTPs over the two sampling campaigns. Also the abundance of nine ARGs, conferring resistance to beta-lactams (bla_{TEM}, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-58}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-32}}$, $bla_{\text{KPC-3}}$), sulfonamides (*sul1*), tetracycline (*tetM*) and polymixin (*mcr-1*) and of the class 1 integrase gene (*intI1*) were determined. Effluent samples from the two different sampling campaigns showed no considerable variation for each of the screened ARGs. In 50% of the cases the ARG concentrations between the two campaigns did not differ by more than a factor of 2. Inter-campaign variations exceeding one order of magnitude were rarely observed (8% of the cases, mostly attributed to a single plant).

In general, the variation of the abundance of the analysed genetic determinants was consistent over all WWTPs and therefore the absolute abundance (ARGs/100 ml) of the analyzed genes could be ranked along the following order: *intI1* > *sul1* > *tetM* > $bla_{\text{OXA-58}}$ > bla_{TEM} > $bla_{\text{OXA-48}}$ > $bla_{\text{CTX-M-32}}$ > *mcr-1* > $bla_{\text{CTX-M-15}}$ > $bla_{\text{KPC-3}}$ (Fig. 1). The limit of quantification (LOQ) of the screened genetic components ranged from 10^4/100 ml (16S *rRNA and intI1*) to 10^3/100 ml (*sul1, tetM,* $bla_{\text{OXA-58}}$, bla_{TEM}, $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-32}}$, *mcr-1*, $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-3}}$). In addition, it is important to note that *mcr-1*, $bla_{\text{OXA-48}}$ and $bla_{\text{KPC-3}}$ were not detected in all effluents. The overall absolute abundance of ARGs and *intI1* in the WWTP effluent samples did not follow a clear geographical pattern (Fig. S2). The samples from the WWTP in Cyprus contained always the lowest abundance of ARGs in effluents, except in the case of $bla_{\text{CTX-M-32}}$.

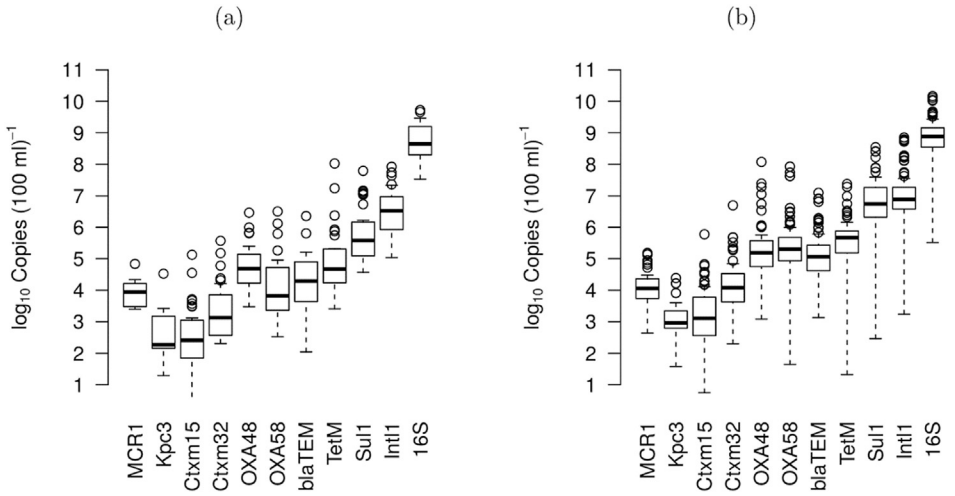

Fig. 1. Absolute abundance of the monitored ARGs in the receiving rivers upstream (a) and in the effluents of European WWTPs (b) for both sampling campaigns. Boxes indicate quartiles and the median. Points represent outliers, the latter being defined as data points being more than 1.5 times the interquartile range from the box. Whiskers extend to the most extreme data points not being classified as outliers.

Fig. 1 illustrates the median of the measured absolute abundance. Essentially, based on their absolute abundance, three groups of genes can be distinguished: *16S, intI1, sul1* and *tetM,* forming the group with the highest concentration; resistance genes coding for beta-lactamases bla_{TEM}, bla_{OXA-58}, bla_{OXA-48}, $bla_{CTX-M-32}$, $bla_{CTX-M-15}$ and bla_{KPC-3} cluster forming a group with intermediate/low concentrations. The gene *mcr-1* was detected and quantified in all of the investigated effluents, except for NO2 and CYP (Fig. S2).

3.2. Absolute abundance of ARGs in relation to WWTP characteristics

In order to identify which parameter might affect the absolute abundance of ARGs in each of the effluents of WWTPs, the collected data were separated into two main groups for each of the metadata parameters assessed for the WWTPs (nr. of hospital beds in the catchment, nr. of biological stages, COD effluent concentration, nr. of hospitals in the catchment, geographical latitude, plant size). The results of the analysis are represented in Fig. 2. The only factor that shows a significant correlation with the absolute abundance of ARGs is the number of biological steps. Both *intI1* and bla_{TEM} ($p < 0.001$), together with *16S* and *sul1* ($p < 0.01$), are significantly reduced in WWTPs combining a high loaded first step (C-removal) with a subsequent low loaded second biological step (N removal). The same is noticeable for bla_{OXA-58} and *tetM* ($p < 0.05$). For all the other investigated parameters, no clear trend or pattern correlating with ARGs concentrations was observed.

3.3. Absolute abundance of ARGs upstream of the WWTPs

During the second sampling campaign, besides the WWTP effluents, samples from the receiving river upstream and downstream of the discharge points were also collected. The results of the measured absolute ARGs abundance are also shown in Fig. 2. Interestingly, we can observe a similar ranking for the ARGs abundance as in the WWTP effluent samples, with some minor variation within the above-formulated groups.

The observed pattern (Fig. 3) shows that for most of the quantified genetic elements, the absolute abundance is higher in WWTP effluents compared to upstream corresponding samples. A more detailed analysis of Fig. 4 shows, that with the exception of Norway (NO2), TR, AT and DE5, all the investigated locations show the same relation of the two sampling points (Effluent > Upstream) for the most abundant analysed components (bla_{OXA-58}, *sul1*, *tetM* and *intI1*). While DE5 and NO2 do not show any considerable difference between upstream and effluent, TR and AT display a higher absolute abundance of ARGs upstream than in the effluent, suggesting that the receiving water body has eventually been exposed to a considerable source of ARGs further upstream to the sampling point e.g. by combined sewer overflows (Garner et al., 2017) or surface runoff (Almakki et al., 2019). The absolute abundance of *intI1* was always highest in all the locations and in both sampling points, the only exceptions were the effluent of TR and NL. In the rivers, the *mcr-1* gene has been detected in four cases upstream the WWTP (TR, PT, DE5, NL). It was not possible to reliably quantify the gene, as its number of copies per 100 ml was rarely above its LOQ.

3.4. Impact of WWTP effluents on the receiving river

As outlined in Sec. 2.3, the impact of treatment plant effluents on the receiving rivers has been quantified in terms of the ratio C_d/C_u relating the ARG concentration downstream of the effluent site to the corresponding upstream value. In accordance with expectation, the ratio C_d/C_u exceeded the value of 1 for the majority of WWTPs and resistance genes, indicating a rise in the in-stream level of ARG due to the plant effluent (Fig. 4). However, Fig. 4 also indicates that the estimated ratios are not strong predictors of resistance dissemination. The use of measured ARG concentrations at the downstream sampling site (C_d) generally results in larger estimates of C_d/C_u as compared to a situation where C_d is approximated by Eqn. (1).

Since reliable hydrological information was unavailable for some canals and highly regulated rivers, Eqn. (1) was not applicable everywhere and the analysis depicted in Fig. 4 was thus restricted to a total of nine locations.

For the genes *mcr-1*, $bla_{CTX-M-15}$, $bla_{CTX-M-32}$ and bla_{KPC-3} reliable estimates of C_d/C_u could not be computed because the measured in-river concentrations upstream (C_u) of the plant were mostly close to/below the LOQ of about 10^3 copies $(100\,mL)^{-1}$. However, downstream of the plants these ARGs were quantifiable in many cases, suggesting an impact of the effluent on the receiving water body. Advanced methods such as droplet PCR might be able to

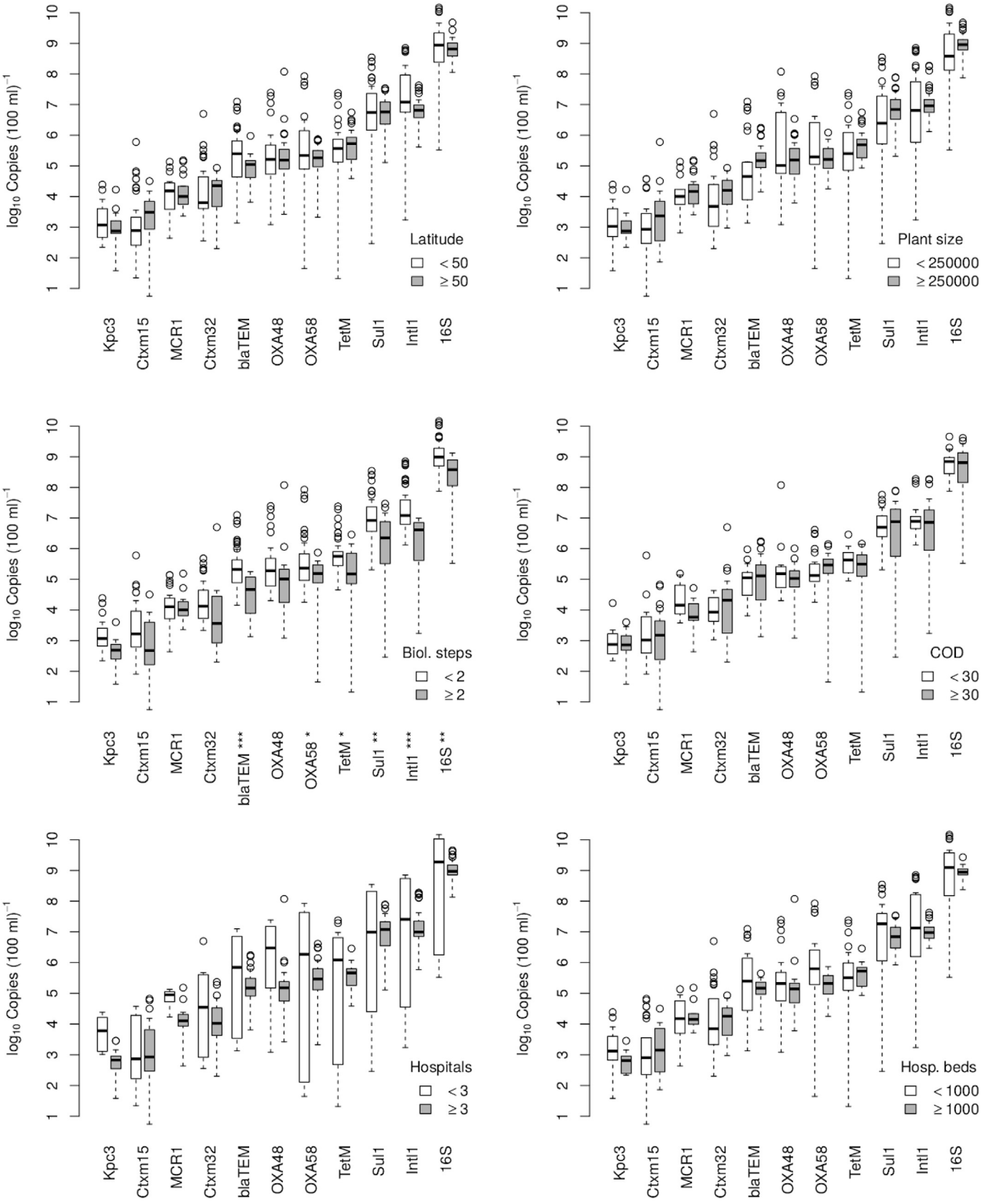

Fig. 2. Absolute abundance of the quantified genes in WWTP effluents for both sampling campaigns. Boxes indicate quartiles and the median; points represent outliers according to the conventional 1.5 × IQR rule. Whiskers extend to the most extreme data points not being classified as outliers. A two-sample t.test was carried out on the log-transformed data to test for shifts in location due to the respective factor (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; p-values were adjusted for each of the sub-figures accounting for the 11 tested genes).

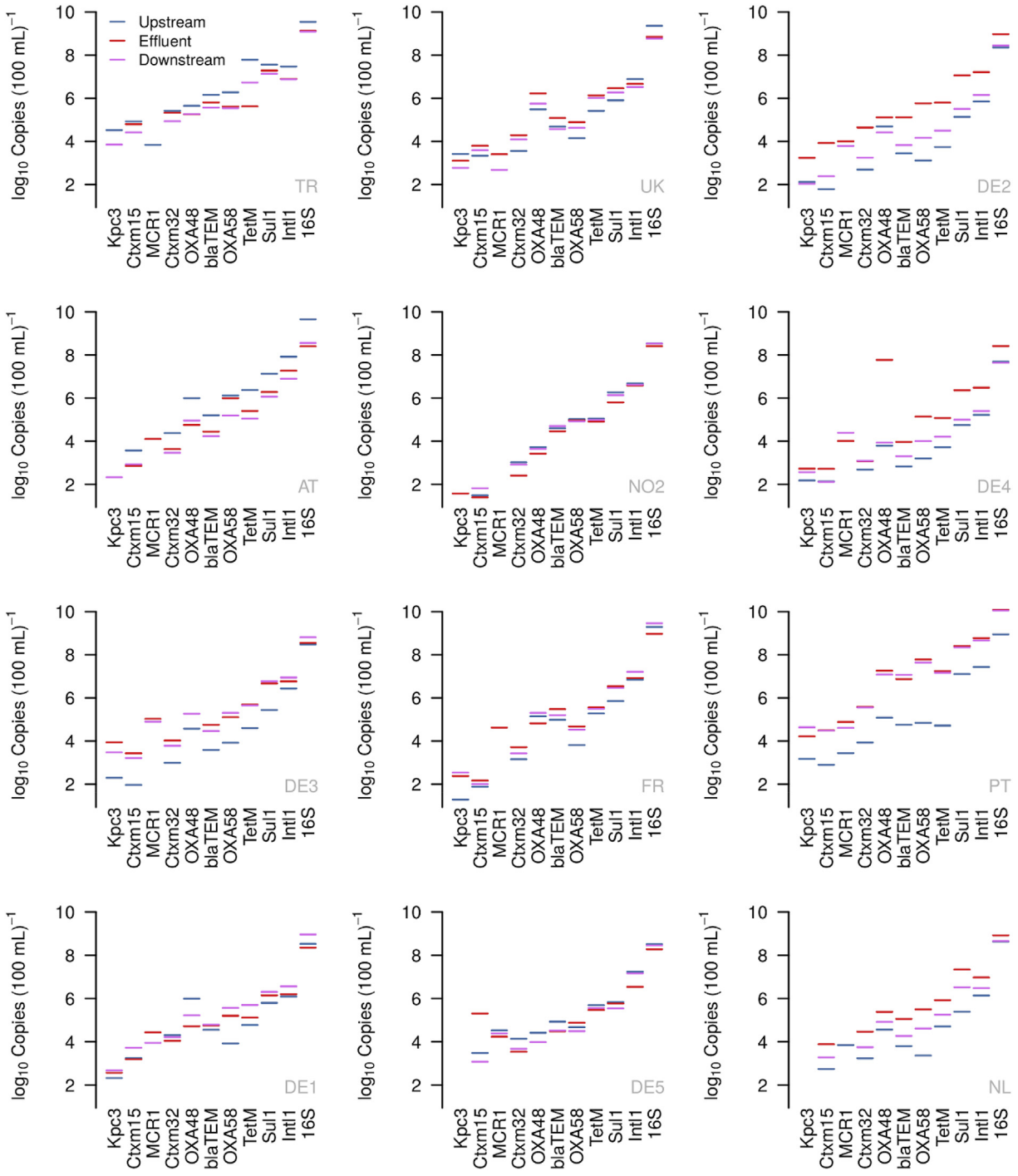

Fig. 3. Absolute abundance of screened ARGs upstream (blue), downstream (pink) and in the effluent (red) of each of the investigated WWTP. ARGs are ranked from left to right in ascending order.

allow to precisely quantify low abundant ARGs, and could be used to investigate the impact of the effluents of WWTP on the receiving environments.

4. Discussion

This study has achieved its first objective and produced a comprehensive qPCR dataset on quantified selected ARGs in WWTP effluents and the receiving rivers across various European countries. The data provided by this study give a first environmental standard for qPCR based quantifications of these selected ARGs. Due to the uniform distribution pattern of absolute abundance of ARGs throughout Europe, future studies can compare their quantifications to this study and conclude if they have detected comparatively high or low concentrations of ARGs. Furthermore, this investigation verifies previous case studies results that the analysed component with the highest concentration is *intl1* followed by the *sul1* gene, which is often associated to class 1

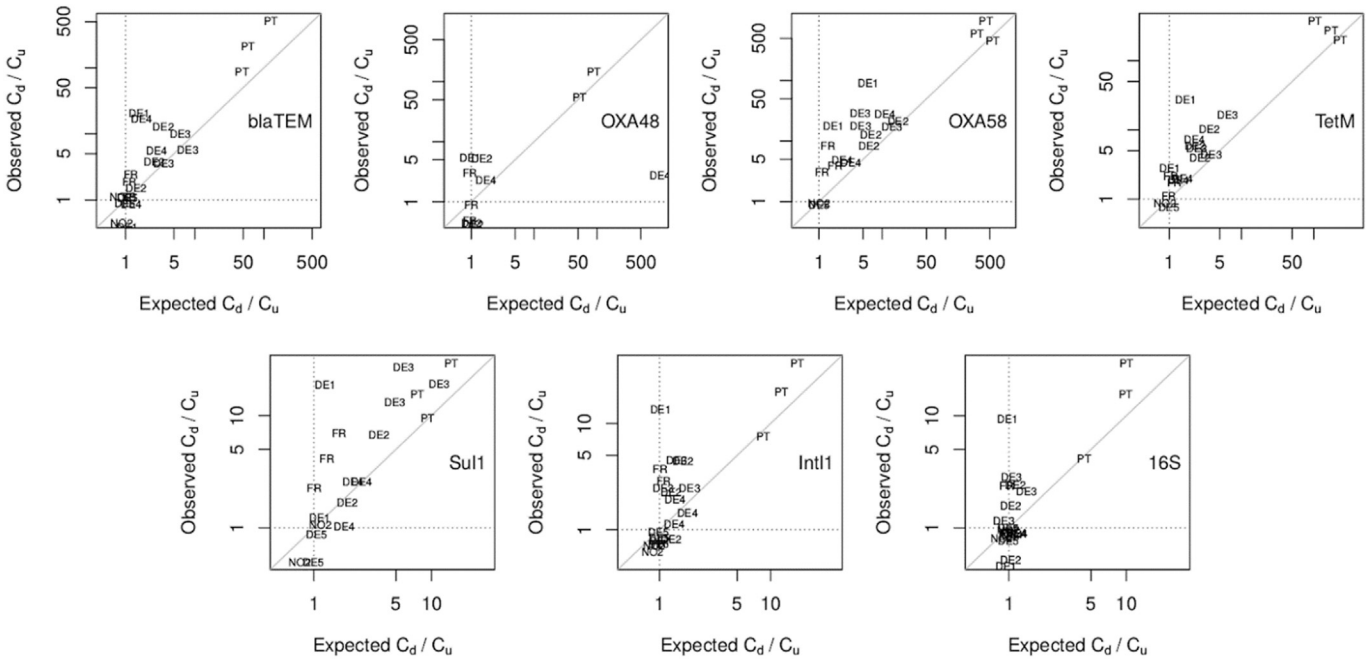

Fig. 4. Impact of WWTP effluents on the receiving rivers expressed as the ratio of ARG concentrations downstream (C_d) and upstream (C_u) of the discharge site. Each label represents a particular plant and sampling date. The observed ratio (vertical axis) was inferred from measured ARG concentrations alone while the expected ratio (lower axis) is based on values of C_d obtained from Eqn. (1). Labels close to the diagonal indicate a perfect match between the two estimates of C_d/C_u. The vertical and horizontal line separate cases with $C_d > C_u$ (apparent pollution) from cases where $C_d < C_u$ (apparent dilution). Axes are log scaled due to different rations between investigated genetic elements and sampling points.

integrons (Gillings et al., 2015). Furthermore, the results demonstrated that the abundance of the analysed resistance genes could be clustered in three groups, and these results were surprisingly consistent throughout Europe. The first group of abundant ARGs consisted of the above mentioned *intI1* and *sul1* genes, the second group of intermediate abundance, with all values above the LOQ, were *tetM*, bla_{TEM}, bla_{OXA-48} and bla_{OXA-58}. The third group *mcr-1*, $bla_{CTX-M-15}$ and bla_{KPC-3} could be viewed as emerging ARGs in the environment as they are detected at comparatively low abundance, and sometimes the values did not pass the LOQ threshold. However, if scientists perform future studies and endeavour to compare their results to this study, they should follow the guidelines given in Rocha et al. (2018) and Heβ et al. (2018) and they should be aware of the fact that, an interlaboratory error margin or variation of up to 30% needs to be considered (Rocha et al., 2018). If taken these considerations into account the values of the concentrations differ strongly to the here presented ones, it can be concluded that the values might indicate unique local or regional processes.

4.1. Biogeography of ARGs in European WWTP effluents

This study is one of the first studies on a pan-continental scale investigating the abundance and dissemination of ARGs in WWTPs and inland freshwater bodies. Absolute abundance of ARGs did not exhibit biogeographical patterns in WWTP effluents. This suggests that it is unlikely that environmental factors, such as climate or latitude, are driving the pattern of ARGs in European WWTP effluents. Similar results have been documented by Zhu et al. (2017) for a continent-scale study in China.

All across the sampled WWTPs, the most abundant genes were the class 1 integron integrase gene *intI1* and the *sul1* gene coding for sulfonamide resistance. Both have been extensively reported to be identified as genetic markers associated with anthropogenic influenced environments such as WWTPs (Gillings et al., 2015; Nardelli et al., 2012) and surface waters receiving their treated effluents.

The samples originating from the Cypriot WWTP effluents showed the lowest absolute abundance of ARGs among the investigated plants. Possibly this result is due to the wastewater treatment technology used. As a matter of fact, unlike the other investigated WWTPs, the Cyprus facility implements membrane (MBR) technology for the separation of treated water and activated sludge, which has been proven to be an efficient technology to reduce the absolute concentration of both antibiotic resistant bacteria (Schwermer et al., 2018) and subsequently ARGs (Kappell et al., 2018) due to particle retention.

Our findings show that even WWTPs that are equipped with a secondary clarifier are responsible for a significant reduction of four ARGs (*sul1, tetM,* bla_{OXA-58}, bla_{TEM}) and *intI1*. Considering the simultaneous reduction of 16S rRNA, this observation can be attributed to sedimentation of activated sludge particles in the clarifier. The finding is in agreement with the results of previous studies (Li et al., 2017; McConnell et al., 2018), that investigated the fate of the same genetic markers.

Hospitals are generally considered a direct source of ARGs (Hocquet et al., 2016; Szekeres et al., 2017; Varela et al., 2013). According to our data, however, the number of hospitals and the number of hospitalized patients do not have a significant impact on the amount of ARGs released from the WWTPs. The results rather confirm that hospital wastewater - in terms of ARGs load, have a minor contribution (0.2–2%) to the total amount of wastewater collected from an urban area (Buelow et al., 2018; Carraro et al., 2016). This observation, however, does not preclude the possibility that locally, hospitals may have an impact on the WWTP effluents. Furthermore, it should be noted that neither effluent COD nor the size and geographical location (latitude) of the WWTP correlate to the concentration of ARGs copies released from the treatment facilities.

4.2. Estimated effects of WWTPs on downstream freshwater environments

WWTPs and their effluents represent a situation where a high diversity of bacteria, antibiotics and other pollutants from the urban area mix together with environmental bacteria, turning them into hotspots for the evolution and spread of antibiotic resistance (Karkman et al., 2018; Pruden and Arabi, 2011; Rizzo et al., 2013; Wu et al., 2019). ARGs have been extensively reported to be ubiquitous in both wastewater and in downstream environments across all Europe (Freeman et al., 2018; Moura et al., 2007; Oberlé et al., 2012; Szczepanowski et al., 2009). Similarly, our findings indicate that European WWTPs are releasing treated effluent containing concentrations of ARGs that are higher in absolute abundance than those measured in the receiving environment.

However, the impact on the receiving water body cannot be deduced from the effluent concentration data alone but the discharge from the plant in relation to stream flow must be taken into account (Fig. 4). Our data suggest that the impact of a particular WWTP on the receiving river is determined by both, the gene concentration in the effluent water and the local hydrological situation. Six out of ten measured genes were always detected (bla_{TEM}, bla_{OXA-58} $bla_{CTX-M-32}$, *sul1, tetM*, *intI1*) in all sampling points (Upstream, Effluent and Downstream) for each of the screened locations (Fig. 3).

In this investigation, special attention has been given to *mcr-1*. This ARG, located on highly mobile plasmids, has been detected for the first time by Liu et al. (2016) in *E. coli* isolated from raw meat and Chinese inpatients. It has been afterwards detected in bacterial isolates originated from pig farms or slaughterhouses of various countries such as Mexico (Garza-Ramos et al., 2018), Japan (Kusumoto et al., 2016), USA (McGann et al., 2016), Vietnam (Nakayama et al., 2018), Korea (Yoon et al., 2018) and throughout Europe (El Garch et al., 2018). On the other hand, the occurrence of *mcr-1* in freshwater environments is confined to a limited number of studies (Hembach et al., 2017; Lekunberri et al., 2017; Yang et al., 2017). The most likely explanation for the numerous cases of reported presence of *mcr-1* in this study, in both WWTP effluents and surrounding environments, is that this ARG is widespread within European WWTPs and that, in accordance with Hembach et al. (2017), it is able to withstand the treatment processes and eventually to be persistent in downstream environments.

Also, in the case of the receiving rivers, *intI1* and *sul1* were the most predominant genes,and they are often used as proxies of anthropogenic pollution (Djordjevic et al., 2013; Domingues et al., 2012; Eckert et al., 2018) in both WWTP and surrounding environments. Our results question the sole use of *sul1* and *intI1* as proxies, at least in an urban environment, as their concentrations in the WWTPs and the receiving rivers are similar (Figs. 1 and 3), making it difficult to assess the impact of a WWTP on the receiving system. The reason may be that the receiving water is already enriched with genes such as *intI1* or *sul1*, due to e.g. combined sewer overflows, intensive agriculture, or WWTP effluents located upstream of the sampling points. Note that the similar pattern of *intI1* and *sul1* is not surprising, as they are suggested to be physically linked in many cases (Uyaguari-Díaz et al., 2018). Our data suggest that gene bla_{OXA-58} would be a better proxy, since it often occurs at lower concentrations upstream, but at higher concentrations in the WWTP effluent and downstream of the effluent (Figs. 3 and 4 and supplement Fig. S3 and Fig. S4). In addition, the concentrations of bla_{OXA-58} were always above the LOQ. Therefore, this work encourages further studies on the prevalence of bla_{OXA-58}. This ARG has been considered to be almost exclusively associated with *Acinetobacter baumannii*, an opportunistic pathogen known to cause nosocomial infections (Heritier et al., 2005). But recent studies have found bla_{OXA-58} also to be linked to several environmental bacteria genera, suggesting that this ARG is prevalent in more bacterial groups than originally thought (Hultman et al., 2018; Xin et al., 2019).

The results of this study indicate that many European surface waters are enriched with ARGs due to the discharge of treated municipal wastewater (see, e.g., the cases of DE2, DE3, NL, and PT in Fig. 3). Nevertheless, some unexpected local exceptions were also encountered as illustrated, for example, by the data of the Austrian and Turkish WWTP and their receiving rivers. Here, future studies are warranted to find an explanation of these unusual exceptions (Fig. 3, cases AT and TR).

For several ARGs and sampling points, considerable mismatches between the observed and computed concentrations downstream of the WWTP were identified (Fig. 4). These deviations possible reflect incomplete mixing between effluent and river water, uncertainty in the hydrological information processed through Eqn. (1), or unknown sources and sinks of ARG within the studied river sections. Interestingly, the observed concentrations downstream of the WWTP were typically equal or even higher than anticipated from the mass balance (Eqn. (1)) suggesting that the emitted ARGs do not undergo instant elimination from water phase. Generally, the collection of consistent data in a multinational context turned out to be challenging. Specifically, this study has identified two major critical points, which should be considered for future studies: a) To obtain sound and complete information on the river catchment and the local hydrological situation, the collection of reliable metadata must receive particular attention. This should also include detailed attention towards potential pollution sources upstream the target impact point. b) To define an accurate sampling strategy aimed to minimize the risk of biased measurements, resulting e.g. from incomplete mixing, accordingly the downstream sampling site must be chosen with special care.

In spite of these challenges, the study also demonstrated an unexpected consistency: namely the ranking of ARG abundances ($intI1 > sul1 > tetM > bla_{OXA-58} > bla_{TEM} > bla_{OXA-48} > bla_{CTX-M-32} > mcr\text{-}1 > bla_{CTX-M-15} > bla_{KPC-3}$) was almost identical for all WWTPs effluents and the receiving waters within Europe and therefore these results may well be of general significance.

5. Conclusions

The challenges connected to the study of antibiotic resistance in environmental reservoirs are mainly related to the definition and understanding of the processes behind the transport of contaminants in the different environmental matrices (soil, water, sludge, etc.). Thus, the identification of strategies to limit the spread of clinically relevant ARGs is the driving force behind this work.

In this study, we monitored WWTP effluents across Europe and estimated their loadings of ARGs in downstream water environments, in order to provide basic data on the proliferation of ARGs in the environment.

5.1. Specific implications include the following

- WWTP are responsible for the discharge of considerable amounts of ARGs in the downstream water bodies. Abundance of the monitored genetic markers could be ranked according to the following order: $intI1 > sul1 > tetM > bla_{OXA-58} > bla_{TEM} > bla_{OXA-48} > bla_{CTX-M-32} > mcr\text{-}1 > bla_{CTX-M-15} > bla_{KPC-3}$
- The calculation of mass balances, in order to evaluate the impact of WWTPs on the receiving waters, needs a carefully designed sampling scheme, with repeated spatial and

temporal sampling in the receiving waters along with detailed metadata on the catchment.

- Further research is needed to understand the dynamics that occur downstream of the WWTPs. In order to be able to reliably study and understand the fate and behaviour of ARGs in natural environments, this study has identified *bla*$_{OXA-58}$ as a possibly suitable candidate resistance gene for such studies.

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Declaration of interests

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.06.039.

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