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Monitoring the exposure and emissions of antibiotic resistance: Co-occurrence of antibiotics and resistance genes in wastewater treatment plants

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ABSTRACT

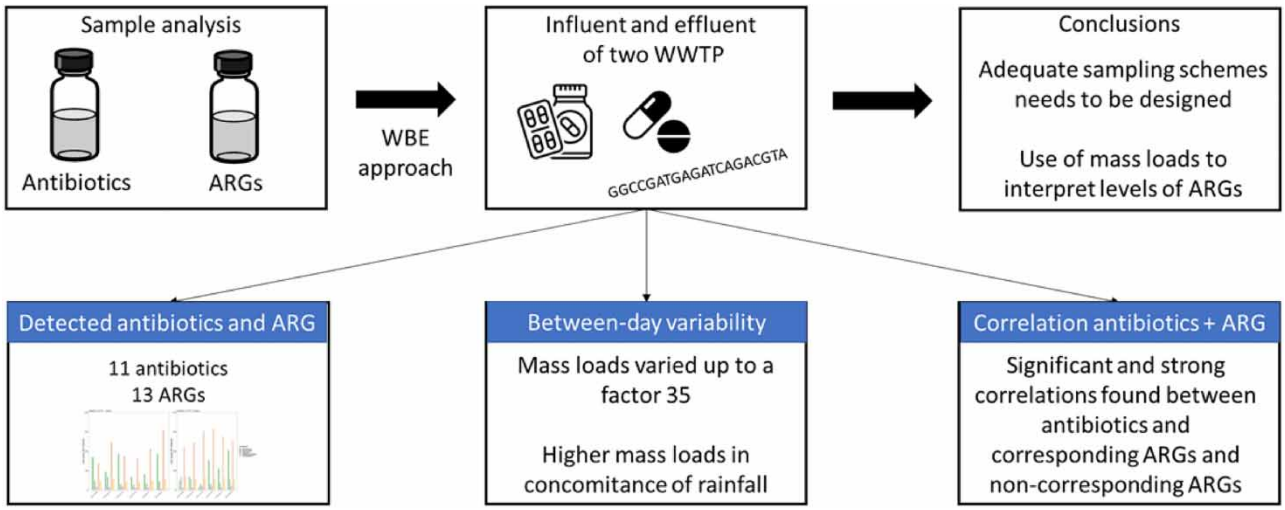
The COVID-19 pandemic has brought new momentum to 'wastewater-based epidemiology' (WBE). This approach can be applied to monitor the levels of antibiotic-resistant genes (ARGs), which in terms are used to make inferences about the burden of antimicrobial resistance (AMR) in human settlements. However, there is still little information about temporal variability in ARG levels measured in wastewater streams and how these influence the inferences made about the occurrence of AMR in communities. The goal of this study was hence to gain insights into the variability in ARG levels measured in the influent and effluent of two wastewater treatment plants in The Netherlands and link these to levels of antibiotic residues measured in the same samples. Eleven antibiotics were detected, together with all selected ARGs, except for *VanB*. Among the measured antibiotics, significant positive correlations ($p>0.70$) with the corresponding resistance genes and some non-corresponding ARGs were found. Mass loads varied up to a factor of 35 between days and in concomitance with rainfall. Adequate sampling schemes need to be designed to ensure that conclusions are drawn from valid and representative data. Additionally, we advocate for the use of mass loads to interpret levels of AMR measured in wastewater.

Key words: antibiotic-resistant genes, antibiotics, between-day variability, human exposure and emission, wastewater-based epidemiology

HIGHLIGHTS

- Monitoring of antibiotics and antibiotic-resistant genes (ARGs) by wastewater-based epidemiology (WBE).
- Able to detect 11 antibiotics and 13 corresponding ARGs.
- Correlations found between antibiotics and ARGs in effluent.
- Important variability in ARG mass loads observed between sampling days.
- WBE needs to be implemented to design adequate sampling schemes to avoid misinterpreting the burden of antibiotic resistance.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The COVID-19 pandemic, which has been affecting people globally since the early 2020, has brought new momentum to a discipline known as 'wastewater-based epidemiology' (WBE) or 'sewage surveillance'. This approach has been used to monitor the circulation of infectious disease agents found in urine and feces since the 1980 s (Asghar *et al.* 2014) and mid-2000 s, to monitor (illicit) drug use and exposure to contaminants (Gracia-Lor *et al.* 2017). Additionally, this approach relies on the measurement of relevant parameters (e.g., RNA, DNA, chemicals and human metabolites) in wastewater samples collected at the influent of wastewater treatment plants (WWTPs). Levels of these parameters, measured at different locations and times, are then used as proxy to derive information relevant for public health, as is currently being done with SARS-CoV-2 (Medema *et al.* 2020a). Prior to the COVID-19 outbreak, one of the most important and promising applications of WBE has been the monitoring of antimicrobial resistance (AMR), and more specifically, antibiotic resistance (Aarestrup & Woolhouse 2020). The interest around sewage surveillance to monitor AMR raised from the acknowledgement that WWTPs are a major player in the spread of antibiotic resistance genes (ARGs) to the environment (Schlüter *et al.* 2007; Bouki *et al.* 2013; Gatica & Cytryn 2013; Rizzo *et al.* 2013). While these studies mainly focused on emissions and the risk that ARGs reaching surface waters pose for human health and the environment (Rizzo *et al.* 2013; Vaz-Moreira *et al.* 2014), researchers recently recognized that influents of WWTPs can be used to monitor the burden of AMR at the community level. In fact, there has been an increasing number of global studies which have monitored resistance at the population level by analysis of samples collected at the inlet of WWTPs (Hendriksen *et al.* 2019; Hutinel *et al.* 2019; Pärnänen *et al.* 2019). Following a WBE approach, these studies aim to provide valuable information about resistance prevalence, in a similar manner to traditional clinical surveillance. However, in the WBE approach, the information provided is at the community level and at different geographical and temporal resolutions (Huijbers *et al.* 2019). The available environmental data can then be linked to other relevant epidemiological and/or socio-economic factors and potentially identify significant covariates such as levels of sanitation and antibiotic use (Hendriksen *et al.* 2019; Hutinel *et al.* 2019; Pärnänen *et al.* 2019). Furthermore, the implementation of metagenomic sequencing provides the opportunity to characterize entire communities (i.e., the general population), which would otherwise not be monitored (Aarestrup & Woolhouse 2020). Similarly to ARGs, antibiotic residues have also been largely measured in WWTP influents and effluents (Wang *et al.* 2020). While most of these studies focused on assessing removal and emissions to the environment, recent reports focusing on estimating antibiotic use through wastewater analysis have also been reported (Castrignanò *et al.* 2020). Studies evaluating levels of both antibiotics and resistance genes in wastewater samples have also been reported, although to a lesser extent, limiting the strength of established relationships (Duarte *et al.* 2019). The combined analysis of antibiotic residues and ARGs in WWTPs could complement existing conventional surveillance systems in humans and animals which in developing countries, are affected by limited data and resources.

Additionally, these studies can help to better understand the occurrence of AMR in non-pathogenic bacteria in large (healthy) communities, which is currently largely unknown (Manaia *et al.* 2018; Muloi *et al.* 2018).

However, before this approach can be routinely and reliably applied to monitor the occurrence of AMR and potentially assess the resistance burden in large communities across the world, additional research is necessary. Studies carried out so far, especially those whose goal was to map resistance in various countries through the wastewater analysis, focused less on short-temporal variability of gene counts, its impact on sampling strategies, and the derivation of (gene) mass loads (used to compare results across different catchments) (Hendriksen *et al.* 2019; Hutinel *et al.* 2019; Pärnänen *et al.* 2019). For example, some extensive studies reported on the short-term and long-term fluctuations of antibiotic mass loads at the influent of WWTPs and the impact on samplings and removal strategies (Coutu *et al.* 2013), but less is known about fluctuations in ARG counts and how these affect inferences made from wastewater analysis about the occurrence of AMR in investigated communities. The goal of this study was hence to gain insights into the between-day variability of ARG levels measured at the influent and effluent of two WWTPs in The Netherlands and link these to levels of antibiotic residues measured in the same samples. Between-day variability in levels of ARGs and antibiotics, and their relationships were then investigated to determine the impact that these have on sampling strategies and inferences drawn about occurrence of AMR in communities.

2. MATERIALS AND METHODS

2.1. Sampling

Samples were taken from WWTP 1 (52.09083 N°, 5.12222 E°, 300,000 inhabitants served, mean flow rate 20,700 m^3/day) and WWTP 2 (52.02 N°, 5.04306 E°, 96,000 inhabitants served, mean flow rate 54,400 m^3/day). A map with the sampling locations are shown in Supplementary Figure S1 and the distance between the two WWTPs is 12.3 km. One liter of influent and effluent at the beginning of the WWTPs was sampled in six sampling rounds (the second and the last week of each month) in May, July and September 2019 to have three seasons covered in the sampling campaign. At the WWTPs, 24-h volume proportional composite samples were taken. In total, 12 influent and 12 effluent samples were collected in a large bucket made of high-density polyethylene (HDPE). Samples were immediately transported to the laboratory and separated in two aliquots. The first was immediately stored at −20 °C (for chemical analysis, see the following section), while the second was immediately processed according to the protocol described below (for biological analysis). Samples were collected with an iron spoon in HDPE bottles that were previously cleaned with methanol and ultrapure water. All glassware used for chemical and biological analyses was precleaned with acetone and petroleum ether.

2.2. Sample preparation

2.2.1. Microbiological sample preparation

Samples were transported directly to the laboratory and vacuum filtered through 0.22-μm pore-size polycarbonate track-etch filter membranes (Sartorius, Göttingen, Germany). The filter membranes were pre-treated with ammonium oxalate to remove iron, which diminish the quantitative polymerase chain reactions (qPCRs). Additionally, an internal control plasmid was added prior to DNA extraction (2.5×10^4 gene copies/μL) to allow quantification of the DNA loss caused by the extraction process, as done previously (Wullings *et al.* 2007). Samples were stored at −20 °C until DNA was extracted. DNA was subsequently extracted from the filters using DNeasy PowerSoil Kit (QIAGEN Benelux B.V., Venlo, the Netherlands). DNA extraction was performed according to the DNeasy PowerSoil Kit instructions. DNA extracts were stored at −20 °C until further analysis.

2.2.2. Chemical sample preparation

All reference standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). The analyzed antibiotics are listed in Supplementary Table S1 with their corresponding limit of quantification (LOQ). These antibiotics were chosen because they correspond to the ARGs targeted in this study and they are prescribed to humans in the Netherlands. The analytical procedures is based on previous research (Thomas *et al.* 2012; Hernández *et al.* 2018; Paulus *et al.* 2019) and changes are made to optimize the sample preparation and analysis for the analyzed antibiotics. Individual stock solutions of the reference standards, including mass labeled ones, were prepared at a concentration of 100 mg/L in acetonitrile with 5% dimethyl sulfoxide and 5% ultrapure water (18.2 MΩ/cm, ELGA LabWater, Lane End, UK). Individual stock solutions were stored at −20 °C. Work solutions containing all individual stocks were freshly prepared in ultrapure water (500 μg/L) each time a

new set of samples was processed and analyzed. Samples for analysis by direct injection were prepared as follows: a 50-mL flask was rinsed three times with a small volume of the sample. Samples were subsequently transferred to the flasks and spiked with 100 μL internal standard. Samples were then filtered through a 0.20-μm pore-size filter and transferred to a 1.8-mL amber vial for analysis. For solid-phase extraction, 100 mL of each sample was transferred in a precleaned HDPE bottle. 100 μL of internal standard work solution was added to each sample, 0.1% EDTA (v/v) was added, and then adjusted to pH=2.5 with hydrochloric acid. The samples were horizontally shaken for 5 min at 120 rpm. Samples were extracted with Strata-X SPE columns (6 mL, 200 mg, Phenomenex, USA). The columns were conditioned with two column volumes of methanol, followed by two column volumes of acidified ultrapure water (pH=2.5). Samples were then gently loaded onto the cartridges. Subsequently, the columns were washed with two column volumes of acidified ultrapure water (pH=2.5) and dried under vacuum for 1 h. Thereafter, the columns were eluted with 3×2 mL of MeOH. Eluates were collected in glass tubes and evaporated to dryness under a gentle stream of nitrogen at 40 °C. Eluates were then reconstituted in 500 μL of ultrapure water with 5% of MeOH and vortexed for 5 s. The extract was transferred in 1.8 mL vials with inserts for analysis.

2.3. Analyses

2.3.1. Microbiological analysis

Extracted DNA was used for qPCR analysis of total bacterial counts and ARGs. For the quantification of total bacterial counts, 16S rRNA gene counts were obtained using a SYBR Green qPCR assay. For quantification of specific ARGs (see Supplementary Table S2), multiplex qPCR assays were performed. The analysis included standards and positive and a negative control to confirm multiplex qPCR quality in every assay. The standards were made up with five subsequent dilutions (2.5×10^4–2.5×10^0 gene copies/μL) and the multiplex qPCR assays were performed using a iQ™ Multiplex Powermix (Bio Rad, München, Germany) and qPCR reactions were performed using a CFX96™ Real-Time PCR Detection System (Bio Rad, München, Germany) as described by Paulus *et al.* (2019). Quantified genes were reported as copies/L by CFX Manager v.3.1.1517.0823 (Bio Rad, München, Germany). Data were reported if (a) the amplification efficiency was 85–105%, (b) $R^2>0.9$, (c) the efficiency of the internal control was >20%, and (d) the number of gene copies was higher than the LOQ. If the number of gene copies was between the limit of detection (LOD) and the LOQ, the data were qualitatively reported as 'indicative'. A $R^2>0.9$ and high efficiencies (from 81.7 to 104.8%) confirmed the linearity and the sensitivity of the qPCR assays. The absolute concentrations of the ARGs are shown in Supplementary Table S3.

2.3.2. Chemical analysis

Analysis of selected antibiotics (Supplementary Table S1) in the water samples for both direct injection and SPE was performed by liquid chromatography tandem mass spectrometer (LC-MS/MS). Chromatographic separation was achieved using a Nexera X2 LC-30 AD (Shimadzu, Tokyo, Japan) equipped with a Luna Omega Polar C18 (2.1 mm×100 mm, 1.6 μm) column (Phenomenex, CA, USA). The injection volume was 100 μL and the column compartment was set to 30 °C. Mobile phases consisted of ultrapure water with 0.1% formic acid (A) and MeOH with 0.1% formic acid (B). A linear gradient from 95% A to 0% over 10 min was used. This was held for 3 min and then starting conditions were reset after 0.5 min and held for equilibration for another 2.5 min, for a total run time of 16 min. The flow rate was kept at 300 μL/min through the whole analysis for both direct injection and SPE. The mass analyser used was a Sciex Triple Quad 6500+ (SCIEX, MA, USA) equipped with an IonSpray source operated in positive and negative mode. Analyses were performed in multiple reaction monitoring mode (MRM) using an H-ESI interface. The spray voltage was set to 5,000 and −4,500 V for positive and negative ionization, respectively. Electrospray temperature (TEM) was set to 400 °C, the curtain gas (CUR) was set to 30 psi, collision gas (CAD) was 8 psi and the ion source gas pressures (GS1 and GS2) were set to 40 psi. Declustering potential (DP), collision energy (CE), collision cell (CXP), and resolution (Q1 and Q3) were optimized for each compound (Supplementary Table S4). The dwell time varied between 3 and 50 ms, depending on the target antibiotic.

The validation was based on the guidelines developed by Peters *et al.* (2007) and the guidelines for bioanalytical method validation by the European Medicines Agency (EMA) (van Amsterdam *et al.* 2013). During the validation, performance parameters such as precision, LOD, LOQ, linearity, recovery, selectivity and carry-over were evaluated and met the criteria for the 25 antibiotics (Supplementary Table S1b).

2.3.3. Data analysis

Influent and effluent qPCR data were normalized to the measured 16S rRNA gene copy number (genes/L), the flow (m^3/day) and the number of inhabitants connected to the WWTP (i.e., copies/16S gene/day/1,000 inhabitants). All antibiotic concentrations were also normalized to the number of citizens connected to the WWTP and the flow (i.e., mg/day/1,000 inhabitants). The latter was done to facilitate comparisons between the two locations which have a different number of inhabitants served.

A Shapiro–Wilk test (if $n \geq 3$) was used to test for normality of the data. Significant differences between location, time, and/or measurements were assessed by paired or unpaired *T*-tests with Bonferroni correction to counteract the problem of multiple comparisons. If the data did not meet the criteria for normality, a Wilcoxon signed-ranked test with Bonferroni correction or a Kruskal–Wallis one-way analysis of variance (ANOVA) was used instead. If the data did not meet the criteria for normality, a was used for both ARGs and antibiotics.

Correlations between antibiotics and ARG mass loads were calculated using Pearson's Rank correlation coefficient (if the data were linear) or a Spearmen's correlation coefficient (for non-parametric data). Data were correlated if the *p*-value < 0.05 and if there were four or more data points available. Correlation was determined at $R^2 > 0.5$, with a moderate correlation if $0.5 > R^2 > 0.7$, and high correlation if $R^2 > 0.7$. All data were collected and analyzed with R version 3.6.1 (R, Vienna, Austria).

Redundancy analysis was conducted to find correlations between antibiotics and ARGs. If data were lower than the detection limit, ARG data were set to 0, only if the efficiency of the internal control was higher than 20%. If the efficiency was lower than 20%, the missing data were set to 'NA'. Antibiotic concentrations were set to half the LOQ if the target compound was detected in at least 70% of the samples at concentrations <LOQ.

3. RESULTS AND DISCUSSION

3.1. Antibiotic-resistant genes

Figure 1 shows the normalized ARG loads of 14 ARGs in WWTPs 1 and 2. These ranged from 0.011 to 30,734 copies/16S/day/1,000 inhabitants for WWTP 1, with *sul1* as the most prevalent ARG. No significant differences were found neither

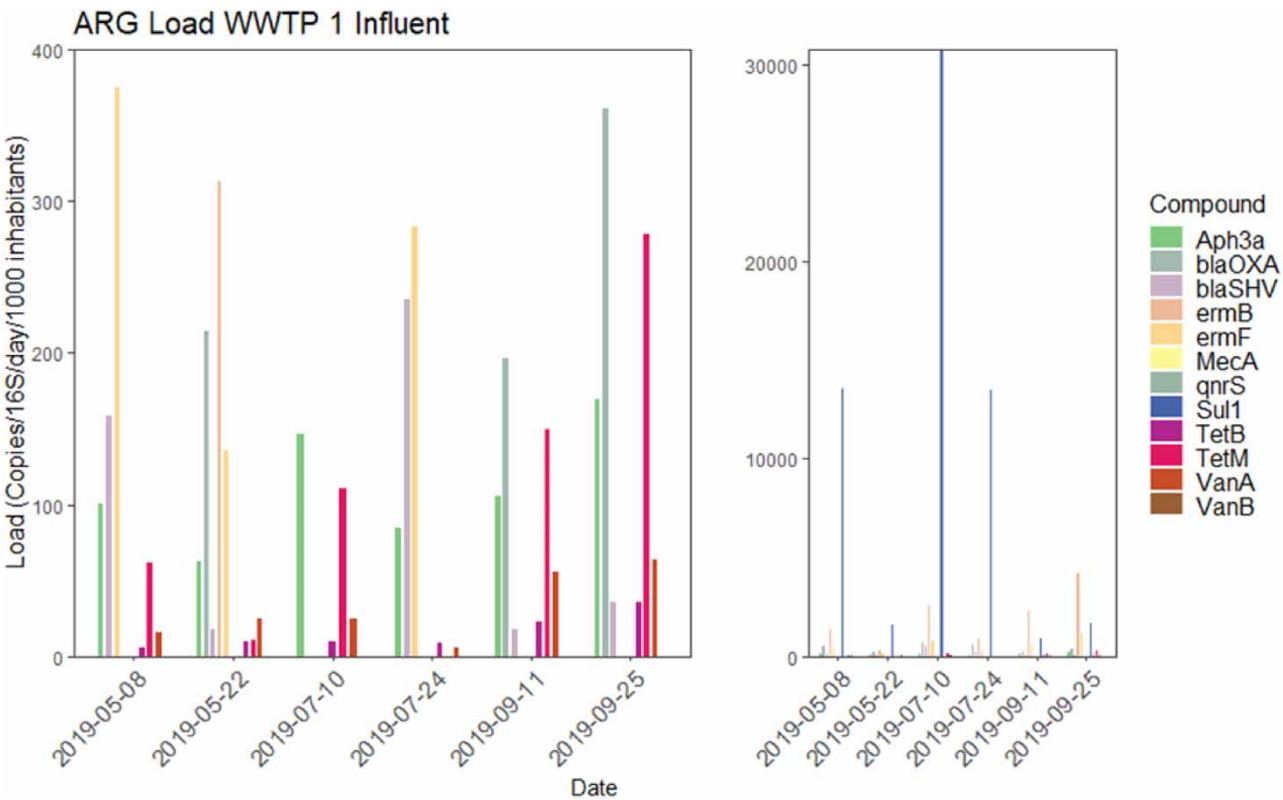


Figure 1 | ARG loads of the detected genes in influent wastewater of WWTP 1. In the left graph, the less prevalent ARGs are shown and on the right all the detected ARGs are presented.

between days nor months of sampling when considering the sum of all genes detected (one-way ANOVA or Kruskal–Wallis, p-value <0.05). Higher mass loads were seen for most of the detected ARGs on the 25th of September, a day with 5 mm of rainfall (KNMI n.d.). Overall, a substantial variability in mass loads between sampling days was observed for all considered ARGs as shown in Figure 1. For instance, *ermB* loads in influent and effluents varied up to one order of magnitude between July and September in WWTP 1. Substantial variations were also visible within the same month, with *blaOXA* for instance, decreasing by a factor of two within the month of May in the influent of WWTP 2. *Sul1* loads in the influent were 35 times higher on 10th of July (also a day with 5 mm of rainfall) compared to the 11th of September. Similarly, *ermF* loads in the influent were seven times higher on the 25th of September compared to the 22nd of May. For WWTP 2, normalized mass loads of ARGs ranged from 0.014 to 13,537 copies/16S/day/1,000 inhabitants, while *sul1* ranged between 0.044 and 23,280 copies/16S/day/1,000 inhabitants, making it again the most prevalent ARG (see Figure 2). As for WWTP 1, no significant differences between days nor months of sampling were found when considering the sum of all detected genes (one-way ANOVA or Kruskal–Wallis, p-value<0.05). All detected genes showed higher mass loads on the 25th of September, especially in the effluent. Similar to WWTP 1, a high variability between the measuring dates was clearly visible with highest loads in September, but for instance *sul1* mass loads in the influent were 18 times higher on the 10th of July compared to the 11th of September (i.e., 23,280 vs. 1,267 copies/16S/day/1,000 inhabitants). No significant differences were found between ARG mass loads in the influent nor effluent of WWTPs 1 and 2. With regards to ARGs concentrations, figures reported here were in the same range reported by Paulus *et al.* (2019), who measured ARGs in samples collected from the Netherlands. Additionally, a study from Finland and Estonia, reported similar *blaOXA* concentration in influents compared to here (mean concentration 1×10^{11} vs. 2.5×10^{11} genes/L) (Laht *et al.* 2014). *Sul1* and *tetM* concentrations were substantially higher compared to here, namely 3×10^{11} and 10^{12} genes/L compared to 4×10^{10} and 2.5×10^{9} genes/L. In a study from Michigan (USA), *sul1* concentrations in raw wastewater was approximately 10 times higher compared to findings from this study (Gao *et al.* 2012).

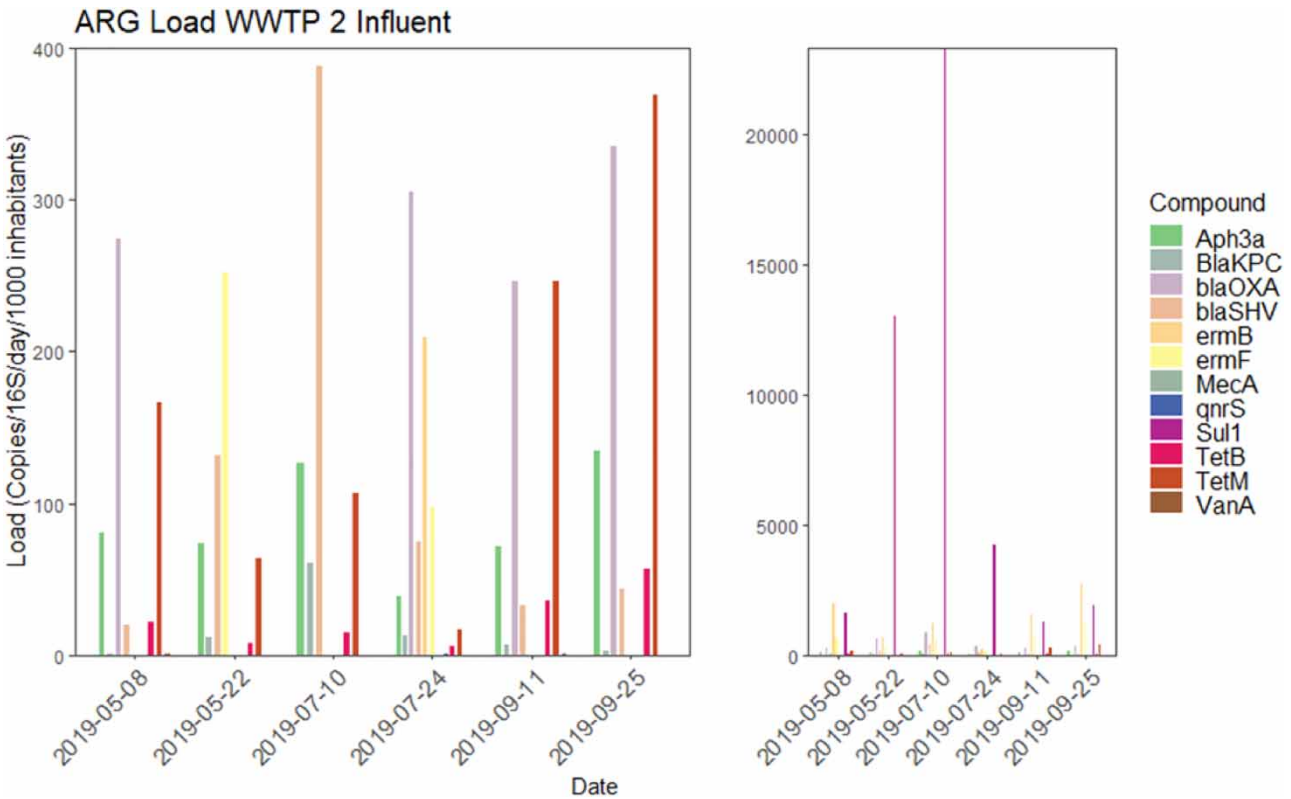


Figure 2 | ARG loads of the detected genes in influent wastewater of WWTP 2. In the left graph, the less prevalent ARGs are shown and on the right all the detected ARGs are presented.

3.2. Antibiotics

In total, 11 antibiotics were found in the influent and effluent wastewater of the WWTP. The absolute concentrations (μg/L) of the detected antibiotics can be found in Supplementary Table S5. The variability in measured mass loads antibiotics between sampling days can be seen in Figure 3. For instance, ceftazidime concentration on 25th of July in influent of WWTP 1 was 10 times higher than the 10th of July (0.08–0.80 μg/L). Ceftazidime concentrations in effluent of WWTP 2 were almost three times as high on the 10th of July compared to the 25th of July. Also, trimethoprim concentration in effluent of WWTP 2 was six times higher on the 8th of May compared to the 25th of July. No significant differences could be found between sampled months. Significant differences were found between influent and effluent for sulfamethoxazole in both WWTPs and for trimethoprim and doxycycline in WWTP 1 (*t*-test, p-value<0.05). The concentration range of the detected antibiotics and their detection frequencies are listed in Table 1. Antibiotics measured in this context have also been reported in other studies. For instance, Rodriguez-Mozaz *et al.* (2015) reported maximum sulfamethoxazole concentrations of 0.38 μg/L in influents and 0.065 μg/L in effluents. As for the study by Gracia-Lor *et al.* (2014), these results are in the same range as those reported here. However, mass loads of ciprofloxacin measured by Castrignanò *et al.* (2020) in influents in Utrecht (i.e., 58.7 mg/1,000 people/day) were roughly three times lower compared to mass loads reported in this study (i.e., 176.5 mg/1,000 people/day). Similarly, ofloxacin mass loads reported here (i.e., 13.4 mg/1,000 people/day) were approximately three times lower compared to results reported by Castrignanò *et al.* (2020) (i.e., 4.3 mg/1,000 people/day). Influent concentrations for sulfamethoxazole reported here (i.e., 1.24 μg/L) were comparable to findings by Yang *et al.* (2005) (i.e. 1.09 μg/L), who analyzed samples collected from WWTPs in Fort Collins, Colorado, USA. The authors also reported lower effluent concentrations compared to findings from this study (i.e., 0.10 vs. 0.22 μg/L in this study).

3.3. Correlation between ARGs and antibiotics

Redundancy analysis was conducted to explore correlations between measured ARGs and antibiotics. When all samples were considered together (i.e., influents and effluents), significant correlations ($p>0.50$) were found (Table 2). Significant

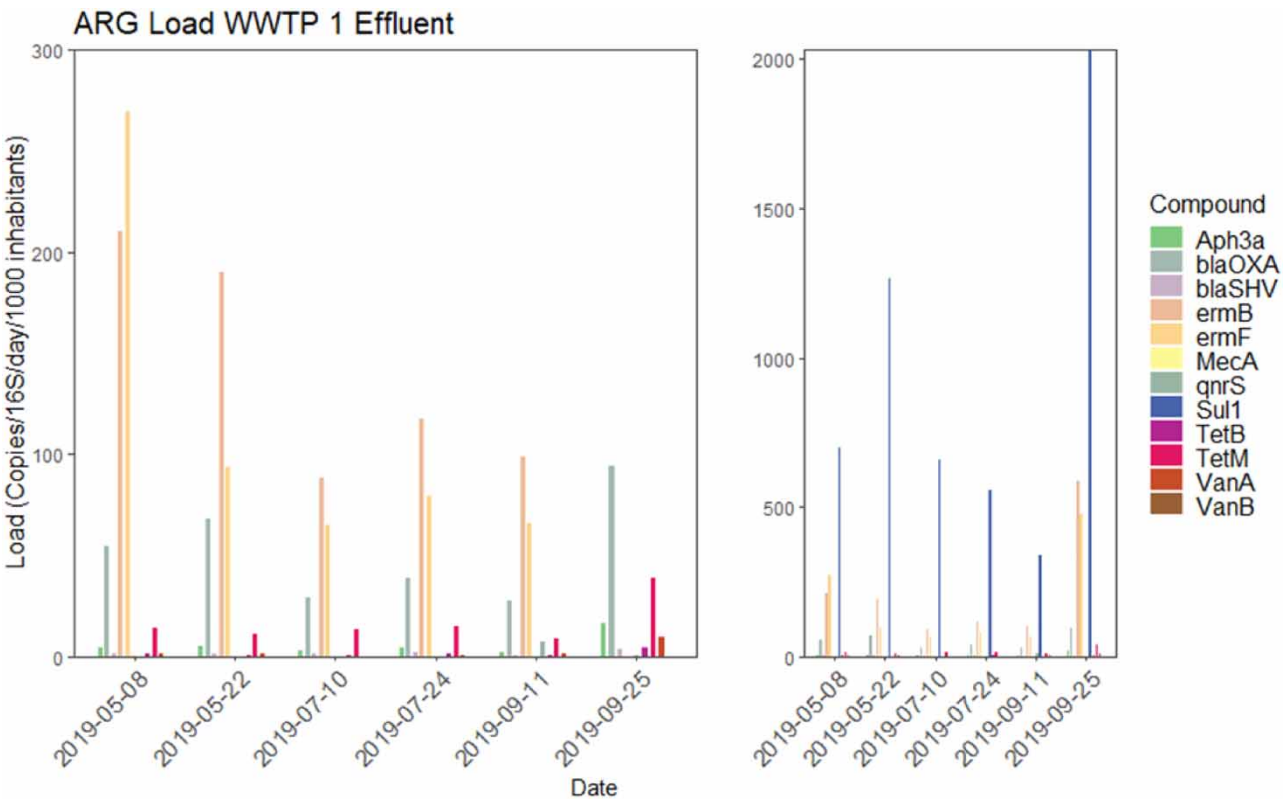


Figure 3 | ARG loads of the detected genes in effluent wastewater of WWTP 1. In the left graph, the less prevalent ARGs are shown and on the right all the detected ARGs are presented.

Table 1 | Mean values of detected antibiotics and detection frequency

	WWTP 1 [μg/L]		WWTP 2 [μg/L]	
Antibiotics	**Influent**	**Effluent**	**Influent**	**Effluent**
Ceftazidime	0.73 (100%)	0.59 (100%)	0.45 (100%)	0.32 (100%)
Trimethoprim	0.28 (100%)	0.08 (100%)	0.29 (100%)	0.09 (100%)
Sulfamethoxazole	1.16 (100%)	0.18 (100%)	1.33 (100%)	0.26 (100%)
Ofloxacin	0.08 (100%)	<LOQ	0.08 (83.3%)	0.02 (66.7%)
Ciprofloxacin	1.08 (33.3%)	<LOQ	1.47 (16.7%)	0.16 (83.3%)
Doxycycline	0.24 (100%)	0.01 (33.3%)	0.19 (100%)	0.02 (83.3%)
Piperacillin	<LOQ	0.01 (16.7%)	<LOQ	0.01 (33.3%)
Ceftriaxon	<LOQ	0.10–0.20 (83.3%)	<LOQ	0.20 (16.7%)
Flucloxacillin	<LOQ	0.04 (33.3%)	<LOQ	0.03 (100%)
Amoxicillin	<LOQ	0.02 (16.7%)	<LOQ	<LOQ
Sulfadiazine	0.06 (33.3%)	0.01 (16.7%)	0.07 (16.7%)	<LOQ

<LOQ, results were below the limit of quantification.

Table 2 | Significant correlation coefficients of all samples together

All	**Intl1**	**qnrS**	**Sul1**	**TetB**	**blaSHV**	**MecA**	**blaOXA**	**Aph3a**	**VanA**	**VanB**	**TetM**	**ermB**	**ermF**	**blaKPC**
Sulfamethoxazole	–	–	0.79	0.88	0.85	–	0.85	0.86	–	–	0.78	0.80	0.85	–
Ofloxacin	0.83	–	0.83	0.78	0.85	–	0.78	0.78	–	–	0.70	0.75	0.78	–
Ceftazidime	0	–	0	0	0	–	0	0	0.43	–	0	0	0	–
Trimethoprim	0.79	–	0.79	0.83	0.74	–	0.86	0.81	0	–	0.79	0.84	0.85	–
Ciprofloxacin	0	–	0	0	0	–	0	0	0	–	0	0	0	–
Doxycycline	0.76	–	0.79	0.84	0.75	–	0.86	0.84	–	–	0.78	0.88	0.85	–
Flucloxacillin	0.73	–	0.73	0.72	0.70	–	0.73	0.71	0.57	–	0.60	0.74	0.71	–
Ceftriaxon	–	–	–	–	–	–	–	–	–	–	–	–	–	–

correlations were also found between ARGs and antibiotics measured in effluents of both WWTPs as shown in Table 3. For instance, correlations were found between sulfomethoxazole and *sul1*, trimethoprim and *sul1* and between flucloxacillin and *blaSHV*. These correlations were found when considering all samples together (influents and effluents) as well as when considering only WWTP effluents. These findings might indicate that although WWTPs reduce total ARG and antibiotic

Table 3 | Significant correlation coefficients of effluent samples together

Effluent	**Intl1**	**qnrS**	**Sul1**	**TetB**	**blaSHV**	**MecA**	**blaOXA**	**Aph3a**	**VanA**	**VanB**	**TetM**	**ermB**	**ermF**	**blaKPC**
Sulfamethoxazole	0.6	–	0.59	0.71	0.80	–	0.59	0.71	–	–	–	–	0.64	–
Ofloxacin	–	–	–	–	–	–	–	–	0.64	–	–	–	–	–
Ceftazidime	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trimethoprim	0.61	–	0.61	–	–	–	0.64	–	–	–	0.59	0.80	0.69	–
Ciprofloxacin	–	–		–	0.86	–	–	–	–	–	–	–	–	–
Doxycycline	–	–	0.64	–	0.63	–	0.70	–	–	–	0.69	0.72	0.58	–
Flucloxacillin	0.55	0.22	–	0.66	0.78	–	0.58	0.69	–	–	0.50	–	0.69	–
Ceftriaxon	–	–	–	–	–	–	–	–	–	–	–	–	–	–

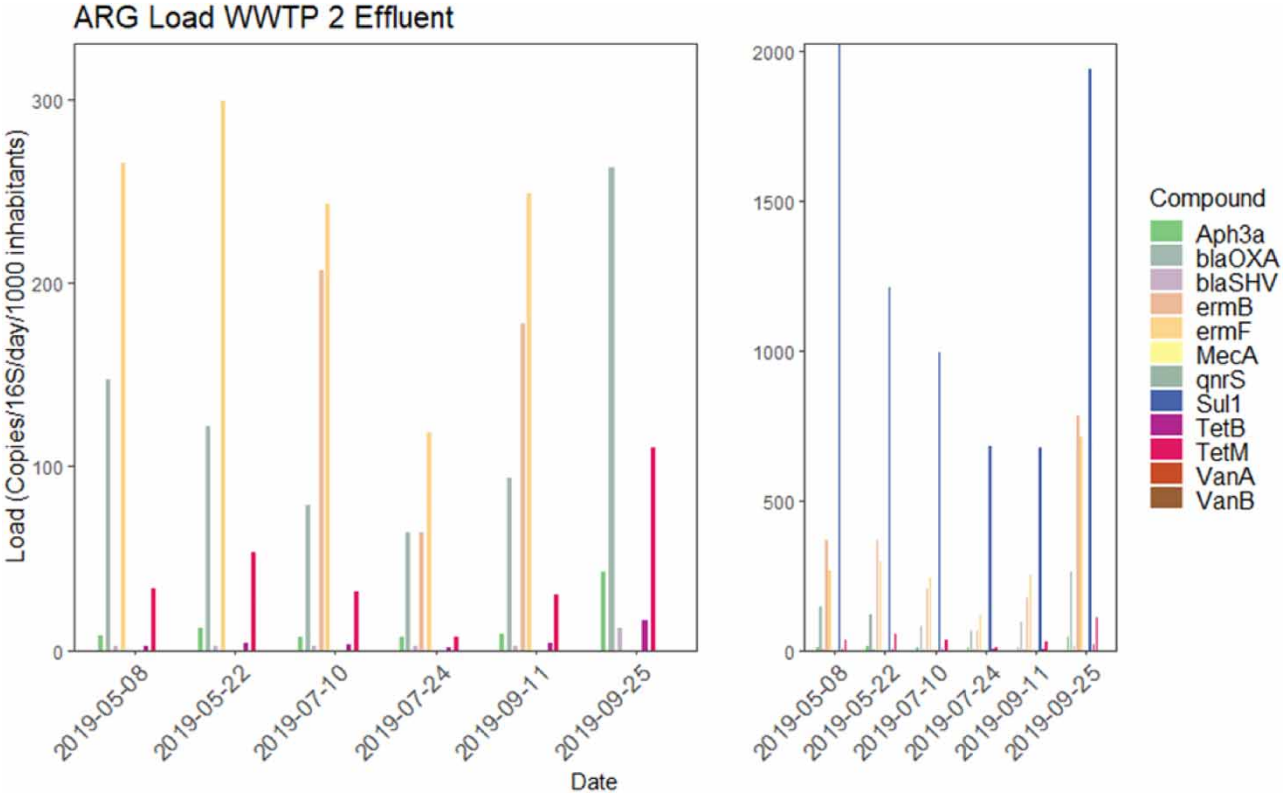


Figure 4 | ARG loads of the detected genes in effluent wastewater of WWTP 2. In the left graph, the less prevalent ARGs are shown and on the right all the detected ARGs are presented.

concentrations, selective pressure for some of these ARGs might still be exerted in WWTPs. However, a larger sampling campaign needs to be carried out before these results can be confirmed. When considering the influents of both WWTPs, no significant correlations were found. This was also the case when influent and effluent samples of both WWTPs were investigated individually. The correlation found between sulfamethoxazole and total sulfonamide concentration with *sul1* was similar with findings from previous studies (Luo *et al.* 2010; Gao *et al.* 2012; Rodriguez-Mozaz *et al.* 2015; Xu *et al.* 2016). In all cases, higher antibiotic concentrations corresponded to higher ARG concentrations, which is in line with the hypothesis that exposure to antibiotics leads to selective pressure for ARGs (Li *et al.* 2012; Rodriguez-Mozaz *et al.* 2015).

Correlations between antibiotics and non-corresponding ARGs were also found (Tables 2 and 3). Although there is currently a lack of knowledge about the significance of such relationships, lateral gene transfer of plasmids containing multidrug resistance is plausible. In fact, it has been reported that *sul1* genes are probably carried on mobile genetic elements, explaining the frequently observed co-occurrence of *sul1* and the class 1 integron gene *Int1* (Luo *et al.* 2010; Xu *et al.* 2016; Subirats *et al.* 2018; Zhang *et al.* 2018). Class 1 integrons play an important role in antibiotic resistance dissemination in many multidrug resistant gram-negative bacteria (Arabi *et al.* 2015). A positive correlation was also found between sulfamethoxazole and trimethoprim, which is not surprising as these antibiotics are often used in combination, both for human and veterinary purposes (Greeff & Mouton 2017). Results presented here show a strong correlation between *Int1* and *Sul1*, but also between *Int1* and other ARGs, such as *blaSHV, blaOXA* and *qnrS* (Spearman correlation$>$0.8, $p<0.05$). This suggests that these other ARGs might also be carried on mobile generic elements, which appears to be the case for most ARGs detected in WWTPs (Che *et al.* 2019). Furthermore, it has been postulated that human multidrug use aggravates co-selective pressure for antibiotic resistance (Xu *et al.* 2016). The occurrence of ARGs also correlates to heavy metals, implying that ARG proliferation is not only dependent on the presence of antibiotics, but also on the presence of heavy metals (Ohore *et al.* 2019).

Redundancy analysis was conducted to explore correlations between measured ARGs and antibiotics. When considering influents of both WWTPs and separately, no significant correlations were found.

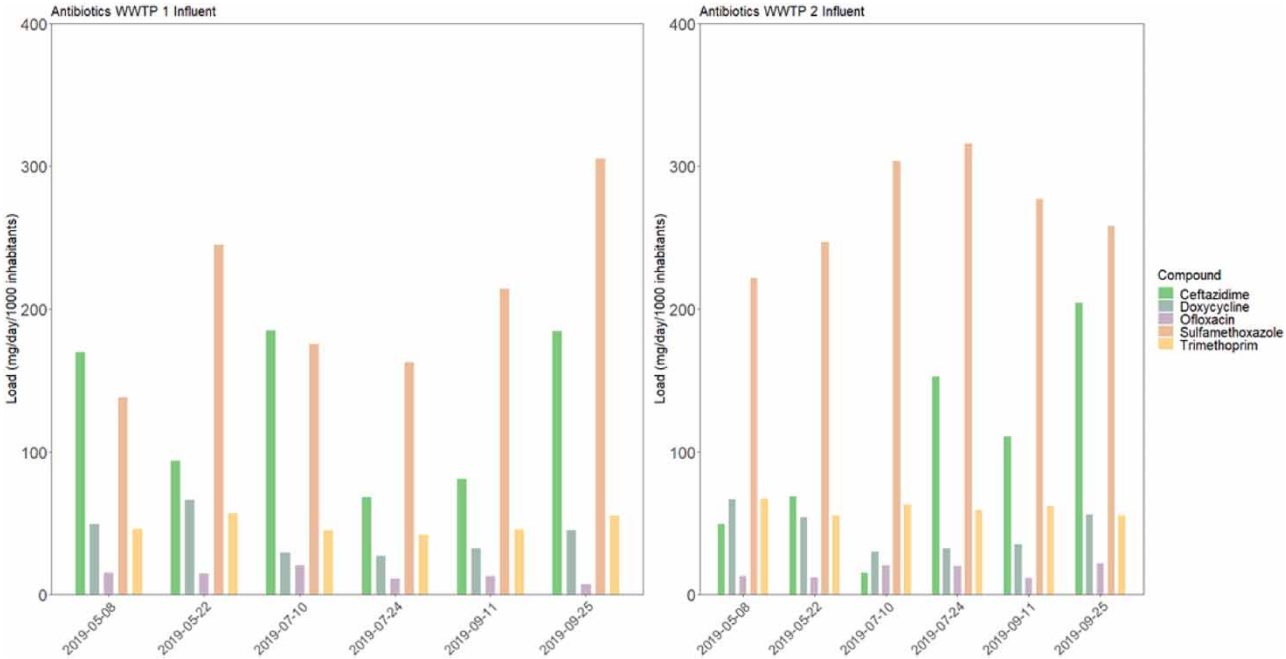


Figure 5 | Detected antibiotics in influent wastewater of WWTP 1 and WWTP 2.

3.4. Between-day variability

Particularly high mass loads were observed in concomitance of rain events. Potential dilution effects due to rain would be accounted for when considering mass loads, as concentrations are multiplied by wastewater flows. However, in this case, particularly high mass loads were still observed for some ARGs. This can be explained by the fact that rainfall increases the typical hydraulic load of the WWTP, which significantly reduce the efficiency of wastewater treatment removal of genes

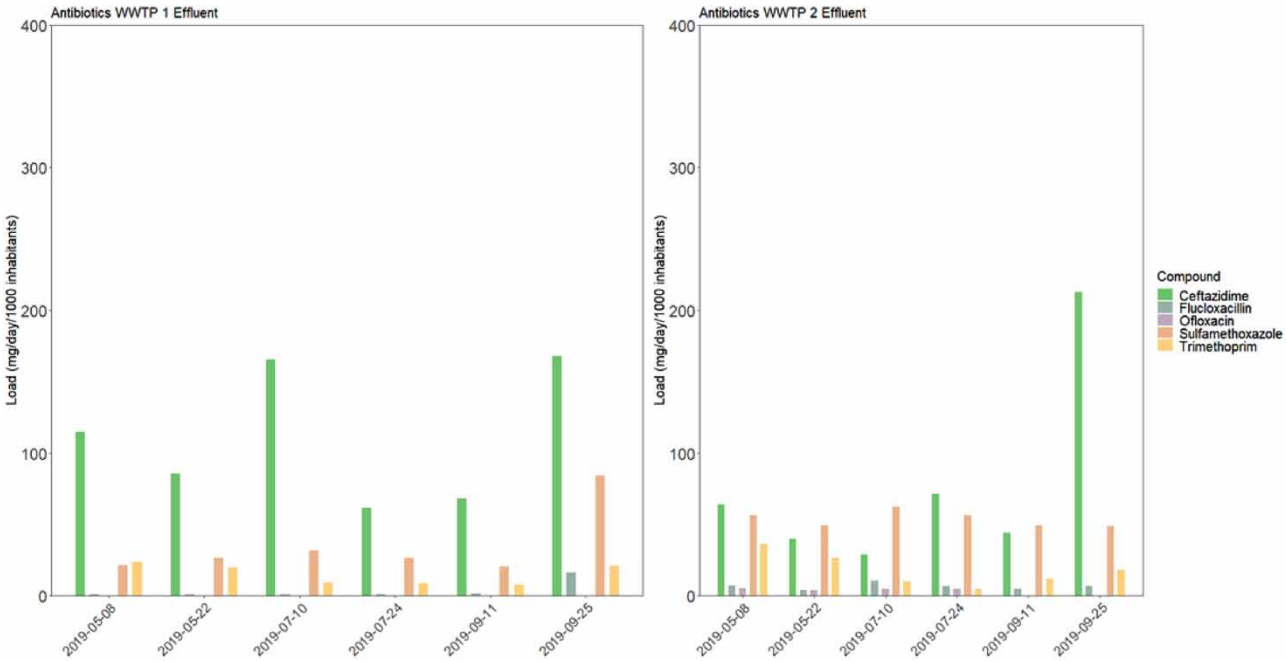


Figure 6 | Detected antibiotics in influent wastewater of WWTP 1 and WWTP 2.

(Pallares-Vega *et al.* 2019). Previous studies have shown that rainfall can increase ARGs abundance in surface waters (Amos *et al.* 2014; Zhang *et al.* 2016; Di Cesare *et al.* 2017). In the specific case of antibiotic residues, previous studies have, however, shown that these hardly fluctuate on a day-to-day basis in wastewater influents (Coutu *et al.* 2013). Increased ARG loads in wastewater during rainfall could be due to various factors, such as flushing of biofilms from sewer walls due to sudden and dramatic increases in flows in sewers. In fact, microorganisms attach to surfaces to protect themselves from environmental hazards (Costerton *et al.* 1978) and studies that screened biofilms have shown that the latter are reservoirs of ARGs (Balcázar *et al.* 2015). For instance, in a study by Schwartz *et al.* (2003), *VanA* and *ampC* genes were detected in wastewater biofilms. Similarly, sediment resuspension during high flows in rivers might also contribute to the increase observed in ARG mass loads (He *et al.* 2018). A similar phenomenon could take place in sewers, as solids are resuspended due to higher flows (Bailey *et al.* 2020). Finally, surface runoffs (in combined sewers) during rainfall could also be involved and would thus suggest that urban runoff might contribute to ARG loads and spreading of AMR (Almakki *et al.* 2019). Regardless of the reason behind the increase in ARG mass loads observed during rain events, the substantial variability in mass loads observed between days, even when no rainfall was involved, has important implications for sampling schemes. In fact, as sewage surveillance (or WBE) is increasingly recognized as an important addition to existing methods used to monitor the burden of AMR (Hendriksen *et al.* 2019; Pärnänen *et al.* 2019) or to monitor the circulation of pathogens at the community level (Medema *et al.* 2020b), it is important to develop sampling schemes which can capture the overall variability of target markers (e.g., resistance genes or RNA). From the results obtained here, collection of samples on single days, even if 24-h composites are collected within the same month (thus precluding any seasonal effects), does not appear sufficient to capture the total variability of ARG loads. Hence, individual 24-h composite samples are not adequate to obtain a representative image of the AMR burden in a community. This becomes even more important when the goal is to compare different communities and assess differences in AMR burden. Incorrect conclusion might be drawn due to the variability in ARG loads at the influent of WWTPs. Although to a lesser extent, important variations were also observed in effluents suggesting, when monitoring AMR emissions to the environment, adequate sampling schemes need to be designed. Future studies should thus involve the collection of multiple (consecutive) samples rather than individual 24-h composites. Similarly, to what has been done for antibiotics, additional studies should be carried out to determine the short-term (e.g., day-to-day) fluctuations in ARG loads measured at the influent of WWTPs.

4. CONCLUSION

Among the measured antibiotics, trimethoprim, sulfamethoxazole and flucloxacillin showed a significant positive correlation with the corresponding resistance genes *sul1* and *blaSHV*. Significant and strong correlations were also found between the integron Int1 and other ARGs, as well as between antibiotics and non-corresponding ARGs. These correlations were observed in effluents only, not in influents of the tested WWTPs. Mass loads varied substantially over time, especially for ARGs, which varied up to a factor of 35 between July and September. Moreover, higher mass loads were observed in concomitance with rainfall. Biofilm release, resuspension of particulate matter and urban runoff are among the considered explanations for these observations. However, an important variability in mass loads was observed between days, in particular for ARGs and regardless of rainfall. This suggests that higher sampling frequencies (using 24-h composite samples) are needed to adequately capture the fluctuations throughout the year and obtain representative estimates of ARG mass loads. This has important implications for studies which use sewage surveillance to assess AMR burden, or monitor the circulation of pathogens, at the community level. Although being highly useful to complement conventional surveillance methods in assessing community-wide exposure to AMR, adequate sampling schemes need to be designed to ensure that conclusions are drawn from valid and representative data. Additionally, following current practice for monitoring trends in chemicals measured in wastewater, including antibiotics, we advocate for the use of mass loads to interpret levels of AMR measured in wastewater.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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