



Understanding associations between antimicrobial agents usage and antimicrobial resistance genes prevalence at the community level using wastewater-based epidemiology: A Spanish pilot study

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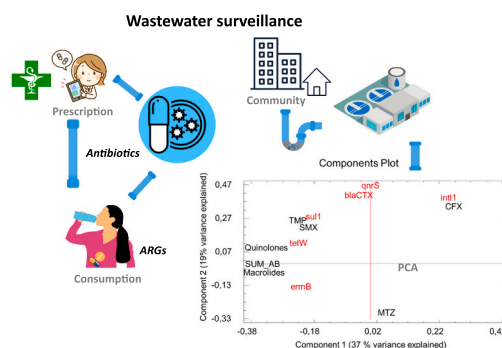
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HIGHLIGHTS

- Application of WBE to assess community usage of antibiotics and spread of ARGs
- Normalized WBE data of antibiotics partly coincided with prescription data
- A steady decrease in antibiotic use, independent of the season, was observed
- Fluctuations in antibiotic mass loads were not directly reflected in ARGs loads
- ARGs correlate with 16S rRNA, linking microorganism abundance to population size

GRAPHICAL ABSTRACT



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ABSTRACT

Understanding the development and spread of antimicrobial resistance (AMR) is important for combating this global threat for public health. Wastewater-based epidemiology (WBE) is a complementary approach to current surveillance programs that minimizes some of the existing limitations. The aim of the present study is to explore WBE for monitoring antibiotics and antibiotic resistance genes (ARGs) in wastewater samples collected during 2021/2022 from the city of Castellon (Spain). Eighteen commonly prescribed antibiotics have been selected and measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), with triple quadrupole mass analysers. Moreover, qPCR for specific ARGs has been performed to obtain information of these genes in co-presence with antibiotics. All selected ARGs, along with a total of 11 antibiotics, were identified. The highest population-normalized daily loads were observed for the macrolide azithromycin, followed by the quinolones ciprofloxacin and levofloxacin. Subsequently, daily consumption estimates based on wastewater data were compared with prescription data of antibiotics. Statistical analyses were conducted to explore if there is correlation between antibiotics and ARGs. While no correlations were found between antibiotics and their corresponding ARGs, certain correlations ($p < 0.05$) were identified among non-corresponding ARGs. In addition, a strong positive correlation was found between the sum of all antibiotics and the *int1* gene. Moreover, population-

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normalized ARG loads significantly correlate with the 16S rRNA-normalized ARG loads, serving as an indicator for population size. Results provide a baseline for future work and a proof-of-concept emphasising the need for future work and long-term surveillance, and highlight the need of similar programs at a regional and global levels worldwide.

1. Introduction

Antibiotic usage and the resulting antimicrobial resistance (AMR) pose one of the most significant threats to global public health. This crisis raises the alarming possibility that some of our essential medicines may cease to be effective, complicating the treatment of common infectious diseases. Current surveillance initiatives are mainly focused on a clinical level and have proven difficult to coordinate and harmonize both sampling and testing results (Aarestrup and Woolhouse, 2020). Furthermore, the assessment of antibiotic usage and spread of AMR in a wider community can be challenging. Hence, improvements are urgently required to obtain more epidemiological information on a population scale and ensure more accurate assessment of the threat (World Health Organization, 2022).

Wastewater-based epidemiology (WBE), also known as wastewater surveillance, is an exploratory and complementary approach to current clinical surveillance programs that minimizes some of the existing limitations such as sampling biases, long realization time, ethical approval and high economic costs. WBE relies on the determination of specific chemical and biological markers in wastewater produced by a population as a proxy to derive information about its health-related aspects (Daughton, 2012). It is relatively cheap, straightforward to implement and there are less ethical considerations, as the anonymity of individuals is maintained (Prichard et al., 2014). The WBE approach has primarily been applied to monitor lifestyle habits (González-Mariño et al., 2020; Gracia-Lor et al., 2017). However, it also found application in virus surveillance (Hellmér et al., 2014; Iaconelli et al., 2020). Yet, it entered a new era when providing fast response to SARS-CoV-2 transmission, demonstrating its unique features (Bivins et al., 2020; Medema et al., 2020). Accordingly, the European Commission recently recognized WBE by proposing a new directive concerning urban wastewater treatment where several public health parameters are recommended for monitoring in wastewater (European Commission, 2022).

Surveillance of AMR through wastewater analysis is one of the most important and promising applications for the future. It can provide data that is otherwise hard to obtain and may sometimes be the easiest route to providing any information at all, especially in low- and middle-income countries, where prescription and sales data are limited, or antimicrobial medications can be bought over-the-counter with ease (Aarestrup and Woolhouse, 2020; Hendriksen et al., 2019; Perry et al., 2018; Sims et al., 2023a; Zieliński et al., 2021). Since one of the major drivers of AMR is the use (and misuse) of antibiotics, the combined analysis of antibiotic residues and antibiotic resistance genes (ARGs) in the same influent wastewater is complementary to better interpret the dynamic nature and burden of AMR. Thus, even a single sample can be representative of a large urban community, allowing for a complete profile (occurrence and abundance) of antibiotic medication and ARGs within that population.

Some promising results have been published, which allowed spatiotemporal profiling of antibiotics and ARGs in WWTPs and evaluate the potential exposure and emission of AMR (Cacace et al., 2019; Holton et al., 2022b; Munk et al., 2022; Osińska et al., 2020; Pärnänen et al., 2019; Steenbeek et al., 2022). Moreover, WBE data and prescription data were triangulated to provide more comprehensive information and insight in antibiotic use at the community level (Holton et al., 2022b; Sims et al., 2023a). Although these outcomes are encouraging, more research is needed before WBE can be implemented routinely and ensure valid, reliable, and comparable spatial and temporal data from wastewater catchment areas across the world.

The aim of the present study is to explore the potential of WBE by combining chemical and biological analysis for the monitoring of antibiotics and ARGs in the same wastewater samples collected from the city of Castellón (Spain). Variability in levels of antibiotics and ARGs, and their relationships are investigated. Moreover, the data are compared to other studies to evaluate if similar occurrence and correlations are observed. To the best of our knowledge, this is the first pilot study in Spain that can provide a baseline for future and ongoing surveillance allowing the evaluation of trends and effects of interventions and highlight the need of similar programs at a regional level worldwide.

2. Materials and methods

2.1. Chemicals and reagents

Eighteen antibiotics belonging to different classes: β -lactams (amoxicillin - AMX, ampicillin - AMP, cloxacillin - CLX, cefditoren - CFT, cefuroxime - CFX), macrolides (azithromycin - AZM, clarithromycin - CLR, erythromycin - ERY, roxithromycin - ROX), quinolones (ciprofloxacin - CIP, levofloxacin - LEV, moxifloxacin - MXF, norfloxacin - NFX), lincosamide (clindamycin - CLI), tetracycline (doxycycline - DOX), nitroimidazole (metronidazole - MTZ), sulfonamides (sulfamethoxazole - SMX) and diaminopyridine (trimethoprim - TMP) were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in influent wastewater. These compounds were selected based on information on antibiotic consumption provided by suppliers, the General University Hospital and the Pharmacy Service of the Health Department of Castellón (Gracia-Marín et al., 2024) and on the ARGs targeted in this study. Quantitative PCR (qPCR) analysis of six specific ARGs have been performed: β -lactams (*bla_{CTX-M}*), macrolides (*ermB*), quinolones (*qnrS*), tetracyclines (*tetW*), sulfonamides (*sul 1*), class 1 integron-integrase gene (*intI 1*) and bacterial 16S rRNA. The general selection criteria of the target ARGs was based on: 1) their presence in wastewater; 2) the antibiotic to which they confer resistance; and 3) the mechanism of resistance. All of the selected ARGs, except for *tetW*, have been suggested as possible indicators to assess the antibiotic resistance status in environmental settings (Berendonk et al., 2015). Reference standards of antibiotics and their isotopic labelled internal standard (ILIS) used for quantification were purchased from LGC (Teddington, UK) and Merck (Darmstadt, Germany). Sterile Cellulose Nitrate Membranes (0.2 μ m; Whatman, UK) and LC-MS grade methanol, acetonitrile, formic acid, and ammonium acetate (> 98 %) were acquired from Scharlab (Scharlab, Barcelona, Spain). LC-MS grade water was obtained by purifying demineralized water using an Ultramatic Plus GR from Wasserlab (Navarra, Spain). Table 1 gives an overview of the antibiotics and ARGs studied.

2.2. Sample collection

In total, 23 untreated wastewater samples were collected at the inlet of the wastewater treatment plant (WWTP) of Castellón, Spain, that serves approximately 178,141 inhabitants (inh., based on census data of 2021). Composite samples were collected during 24 h (time-proportional every 10 min, starting and finishing time at 8:00 am) using a temperature-controlled (4 °C) automatic sampler device to limit potential degradation (Holton et al., 2022b). After each sampling day, the sample was immediately transported to the laboratory, and stored in the dark at -20 °C until analysis. Moreover, the flow rate (m³/day) entering the WWTP was recorded and used to calculate daily loads. Samples were collected between April 2021 and May 2022. More details on sampling

Table 1

Antibiotic class, abbreviations and corresponding ARGs of the antibiotics studied.

| Antibiotic class | Antibiotic | Abbreviation | ARG |
|------------------|------------------|--------------|---|
| β-Lactams | Amoxicillin | AMX | <i>bla_{CTX-M}</i> |
| | Ampicillin | AMP | |
| | Cloxacillin | CLX | |
| | Cefditoren | CFT | |
| Macrolides | Cefuroxima | CFX | <i>ermB</i> |
| | Azithromycin | AZM | |
| | Clarithromycin | CLR | |
| | Clindamycin | CLI | |
| | Erythromycin | ERY | |
| | Roxithromycin | ROX | |
| Quinolones | Ciprofloxacin | CIP | <i>qnrS</i> |
| | Levofloxacin | LEV | |
| | Moxifloxacin | MXF | |
| | Norfloxacin | NFX | |
| Tetracycline | Doxycycline | DOX | <i>tetW</i> |
| Sulphonamide | Sulfamethoxazole | SMX | <i>sul 1</i> |
| Diaminopyridine | Trimethoprim | TMP | <i>intl 1^a</i> <i>16S^b</i> |
| Nitroimidazole | Metronidazole | MTZ | |

^a Class 1 integron-integrase gene.

^b Bacterial 16S rRNA.

date and flow rate can be found in Table S1 of the Supporting information (SI). When available, water quality parameters *i.e.*, biological oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (N) total phosphorus (P), and pH measured by the WWTP were also included.

2.3. Chemical analysis

The determination of antibiotics in wastewater was performed by employing a validated methodology based on a direct injection approach previously developed by our research group (Fabregat-Safont et al., 2023). Briefly, 2 mL untreated influent wastewater was centrifuged for 10 min at 12,500 rpm and the supernatant was 5-fold diluted with ultrapure water (final volume 1 mL). Subsequently, 40 µL of the ILIS mix was added to the diluted samples to reach a final ILIS concentration of 200 ng L⁻¹. The samples were injected (100 µL) and analysed using a Waters Acquity UPLC™ H-Class liquid chromatography system (Waters Corp, Milford, MA, USA) with an Atlantis T3 3.0 × 150 mm, 3 µm analytical column (Waters Corp, Wexford, Ireland). The column temperature was set at 40 °C and the mobile phases consisted of water (solvent A) and methanol (solvent B), both with 2 mM ammonium acetate and 0.1 % formic acid, at a flow rate of 0.4 mL min⁻¹. The gradient elution was: 0 min 10 % B, 6.0 min 99 % B, 8.0 min 99 % B, and 8.1 min 10 % B maintained to 10 min for re-equilibration of the column. The LC system was interfaced to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp, Manchester, UK) equipped with a Z-Spray electrospray ionization interface (ESI) (Waters Corp, Manchester, UK). LC-MS/MS data were acquired and processed using MassLynx 4.1 software and TargetLynx application (Waters Corp, Manchester, UK).

2.4. Microbiological analysis

ARGs in wastewater were determined by applying a previously developed methodology based on qPCR (Xu et al., 2019). First, each sample was homogenized thoroughly just before filtration and DNA extraction was carried out under identical conditions for all samples. Approximately 60 mL of untreated influent wastewater were filtered using a vacuum filtration apparatus through 0.2 µm Cellulose Nitrate Membranes and stored at -20 °C until DNA extraction. Genomic DNA extraction was performed according to the manufacturer's instructions using a FastDNA SPIN Kit for Soil (MP Bio, UK). DNA yield was

measured by Qubit 4.0 Fluorometer (Thermo Fischer Scientific, Germany) and kept at -20 °C for downstream qPCR analysis. More information about plasmids and primers are included in Supporting Information (Table S2).

Six-point standard curves with copy numbers ranging from 10³ to 10⁸ for qPCR were generated using 10-fold serial dilutions of the plasmid DNA carrying target ARGs. Detailed information on standard preparation and primer sets used in this study can be found in a previous study (Xu et al., 2019). A final volume of 20 µL reaction mixture was used for qPCR, consisting of 10 µL of Luna Universal qPCR Master Mix (New England Biolabs, UK), 1 µL of each primer (10 µM), 1 µL DNA template, and 7 µL of PCR grade water. The qPCR protocol was as follows: 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and then a final melt curve stage with temperature ramping from 60 to 95 °C. Each reaction run in triplicate and a non-template control was included. All the qPCR assays were performed, as per the instructions of the manufacturer, in an AriaMx Real-Time PCR system (Agilent Technologies, UK). qPCR data were acquired and processed using AriaMx software.

2.5. Quality assurance

Antibiotics chemical analyses were performed by employing a fully validated methodology using LC-MS/MS. Quality control samples (QCs), *i.e.*, spiked wastewater samples with known concentrations of the antibiotics, were also measured in each sequence of analysis. The analyses were considered satisfactory, when QC recoveries were in the range of 60–140 % for each analyte (European Commission, 2021). More information about method validation and QCs is presented in Fabregat-Safont et al. (2023).

The detection and quantification of the six ARGs was carried out using qPCR assays. The qPCR assays were considered satisfactory if the standard curves demonstrated good linearity R² > 0.99, and high efficiency between 90 and 110 %. Furthermore, samples were measured in triplicate and the relative standard deviation was, in general, ≤ 20 %, illustrating a good precision.

2.6. Data analysis

The derivation (back-calculation) of antibiotic and ARG mass loads were used to compare results across different days. Daily mass loads and population normalized daily mass loads of antibiotics (mg day⁻¹ 1000 inh⁻¹) and ARGs (copies day⁻¹ 1000 inh⁻¹) were calculated using concentrations (C in ng L⁻¹ or copies L⁻¹), flowrate of wastewater (F in m³ day⁻¹) and the population contributing to the sample (P based on census data):

$$\text{Population normalized daily loads} = \frac{C \times F}{P}$$

In addition, qPCR data were normalized to the measured 16S rRNA gene copies as a proxy of the abundance of ARGs per total bacteria and to investigate possible selection occurrence. Its ratio can be referred to as the ARG prevalence (Pärnänen et al., 2019).

Statistical analysis was performed as follows: a Shapiro-Wilk test was employed to test for a normal distribution of the data. If the data did not meet the normality criteria, data were transformed using the formula Log(x + 1). Correlation between the variables were calculated using Pearson's coefficient. A correlation between variables was considered when *p* < 0.05. Differences between summer and winter loads of antibiotics and ARGs were studied by means of ANOVA I (*p* < 0.05). Finally, a Principal Component Analysis (PCA) was performed to obtain the maximum relevant information on the relationships between the variables considered in this study.

All calculations were carried out using MS EXCEL and STAT-GRAPHICS Centurion XV.

3. Results and discussion

3.1. Occurrence of antibiotics

In total, 11 antibiotics were detected in the untreated wastewater samples analysed. Table S3 and Fig. S1 show the absolute concentrations and corresponding boxplots, respectively. When an antibiotic was detected (*i.e.*, above limit of detection (LOD) but below limit of quantification (LOQ)), a concentration corresponding to half the LOQ was taken for further calculations and statistical analysis. Quantified concentrations ranged from 53 ng L⁻¹ for MTZ to 6565 ng L⁻¹ for AZM, with population normalized daily mass loads from 9.6 mg day⁻¹ 1000 inh⁻¹ for MTZ to 1343 mg day⁻¹ 1000 inh⁻¹ for AZM (Figs. 1, S2 and Table S4). In general, population normalized daily mass loads found in wastewater were highest for AZM followed by CIP > LEV > CLR > SMX > NFX > TMP > ERY and MTZ. This did not entirely coincide with the estimated normalized daily consumption of antibiotics in 2021 in Castellón based on prescription data (Fig. 2 and the non-continuous arrows in Fig. 1). Where these determined antibiotics can be arranged in the following order: CIP > MTZ > SMX > AZM > LEV > CLR > TMP > NFX > ERY. Moreover, several antibiotics monitored were only detected occasionally (CFX and CLI) or not detected at all *i.e.*, AMX, CLX, DOX, CFT, AMP, MXF and ROX. The latter three were also sold, prescribed and presumably consumed at lower quantities. However, estimated daily consumption of AMX was 9.4 g day⁻¹ 1000 inh⁻¹, whereas no AMX was detected in any of the wastewater samples collected. The analysis of AMX has shown to be challenging, due to its polar nature. Moreover, the stability of this antibiotic in wastewater, affecting sampling procedure and storage, was questioned (Fabregat-Safont et al., 2023). Several of the antibiotics included in this study (AZM, SMX, TMP, CLR and CIP) are stable in wastewater under different temperature conditions. Other compounds, such as MTZ degrade under refrigerated conditions, whereas concentrations of ofloxacin (not included in this study) can increase possibly due to biotransformation of metabolites (Holton et al., 2022b). In this study all samples were stored in the dark at -20 °C in order to limit possible degradation. Nevertheless, more research related to the stability of antibiotics in wastewater is required, especially for those compounds for which limited information is available. Other reasons for lower detection rates of some of the target antibiotics could be related to inefficient ionization in the LC-MS/MS source, matrix composition (*i.e.*, some compounds are more prone to matrix effects), losses due to sorption to solids or sampling containers, and/or metabolic and excretion pathways. For example, estimated total consumption of CLI and CLR in the year 2021 was about 14 kg per antibiotic. However, CLR was quantified in most samples (up to 1005 ng L⁻¹), whereas CLI could only be detected in some of the samples, this could be due to the different excretion rates. CLI is excreted in its original form (unchanged) approximately 11 % in urine and faeces after human consumption, whereas CLR is excreted around 34 % (Drugbank; Holton et al., 2022b). The determination of appropriate metabolites could be an alternative (if reference standards are available) to obtain concentration data in wastewater that can be used to estimate consumption by a population. However, in both CLI and CLR, the unaltered form was excreted at a higher proportion than the corresponding desmethyl metabolites. Yet, these metabolites should be considered as viable options in future monitoring campaigns as they may estimate consumption better (Holton et al., 2022b).

The back-calculation to population normalized daily mass loads is typically applied to correct for dilution factors related to the size of the population, the sewage system and weather conditions (*i.e.*, rainwater). It allows a more accurate comparison of the results across different days and locations. Population normalized daily mass loads of the encountered antibiotics can be found in Figs. 1, S2 and Table S4. Overall, variations in mass loads between sampling days are in the same order of magnitude. However, it is interesting to observe that several antibiotics were only detected towards the end of our study. For instance, in May

2022, the macrolides AZM and CLR were mainly detected (not quantified *i.e.*, < LOQ) as well as the quinolone NFX indicating a decrease in consumption. On the contrary, the β -lactam CFX, which was not previously found, could be detected in these samples. A clear explanation cannot be given, stability does not seem to be a major issue since most samples were stored over similar periods of time (max. 3 months) until analysis. Besides, as previously mentioned, macrolides are stable in wastewater, and would affect the β -lactam CFX more. A possible hypothesis is related to the higher number of COVID-19 cases and subsequent treatment with antibiotics in 2021, which declined over the studied period due to changes in the vaccination status of the population, among others (Diaz-Höberg et al., 2021).

In the literature, population normalized data is not always available thus, in those cases, concentrations were used for comparison of the results with those reported in other studies. However, it is noteworthy that higher concentrations do not necessarily mean higher antibiotic usage as samples could simply be less diluted by for example rainfall. In general, the mean population normalized data obtained in this study are in the same range as observed elsewhere. For example, mean daily loads for SMX were 220 mg day⁻¹ 1000 inh⁻¹ (the Netherlands (Steenbeek et al., 2022)), 131 mg day⁻¹ 1000 inh⁻¹ and 45 mg day⁻¹ 1000 inh⁻¹ (UK, Bath and Keynsham, respectively (Holton et al., 2022b; Sims et al., 2023a)) and in this study 93 mg day⁻¹ 1000 inh⁻¹. In China, however, there was more variation with daily loads ranging from 3 to 1715 mg day⁻¹ 1000 inh⁻¹ (Gao et al., 2022) and in South Africa mean daily loads of 481 mg day⁻¹ 1000 inh⁻¹ were found (Holton et al., 2022a). In Spain, the concentrations reported over the years were in the same range as reported in this study *i.e.*, 426 ng L⁻¹ (Carballa et al., 2008; Escolà Casas et al., 2021; Rodríguez-Mozaz et al., 2015). Another example, TMP was found at a mean daily mass load of 30 mg day⁻¹ 1000 inh⁻¹ (concentration 136 ng L⁻¹) in this study, comparable to the mass loads and concentrations found in the previously mentioned European studies. Yet, a higher mean daily mass load of 102 mg day⁻¹ 1000 inh⁻¹ (concentration 1173 ng L⁻¹) was observed in South Africa (Holton et al., 2022a), whereas a lower mean daily mass load 10 mg day⁻¹ 1000 inh⁻¹ (concentration 34 ng L⁻¹) was reported in China, where the consumption of TMP was considered lowest among all antibiotics studied (Gao et al., 2022). Finally, concentrations of the studied antibiotics within the Spanish studies were generally within the same range over the years, except for AZM where a high variation could be observed *i.e.* 135 ng L⁻¹ in 2015 (Rodríguez-Mozaz et al., 2015), 1370 ng L⁻¹ in 2019 (Escolà Casas et al., 2021) and 3231 ng L⁻¹ in 2021/2022, this study. A possible explanation could be related to the COVID-19 pandemic, where specifically the use of AZM increased up to 400 % (Gonzalez-Zorn, 2021). However, this is not necessarily the only explanation as the catchment areas covered in each study should also be considered *e.g.*, number of hospitals connected to the sewer network.

3.2. Abundance on ARGs

Gene copies of the six ARGs and bacterial 16S rRNA measured in the influent wastewater samples collected were normalized to the population served within the catchment area (*i.e.*, the entire city of Castellón). Population normalized mass loads (copies day⁻¹ 1000 inh⁻¹) are shown in Table S5. Subsequently, Fig. 1 and Table S6 shows the data normalized to the measured 16S rRNA gene copies (copies 16S gene⁻¹ day⁻¹ 1000 inh⁻¹). Loads ranged from 0.2 to 22,546 copies 16S gene⁻¹ day⁻¹ 1000 inh⁻¹, with *ermB* as the most prevalent ARG. Although β -lactams are the antibiotic class mostly prescribed (approximately 75 % of all antibiotics, Fig. 2), *bla*_{CTX-M} was the less abundant gene present in the wastewater samples analysed. Moreover, only β -lactam CFX could be detected in the wastewater samples. For future studies other bla indicator genes *e.g.*, *bla*_{SHV}, *bla*_{OXA}, *bla*_{TEM} should be considered to better assess the β -lactam resistance status in wastewater (Berendonk et al., 2015). Possible correlations between antibiotics and ARGs are further discussed in Section 3.3.

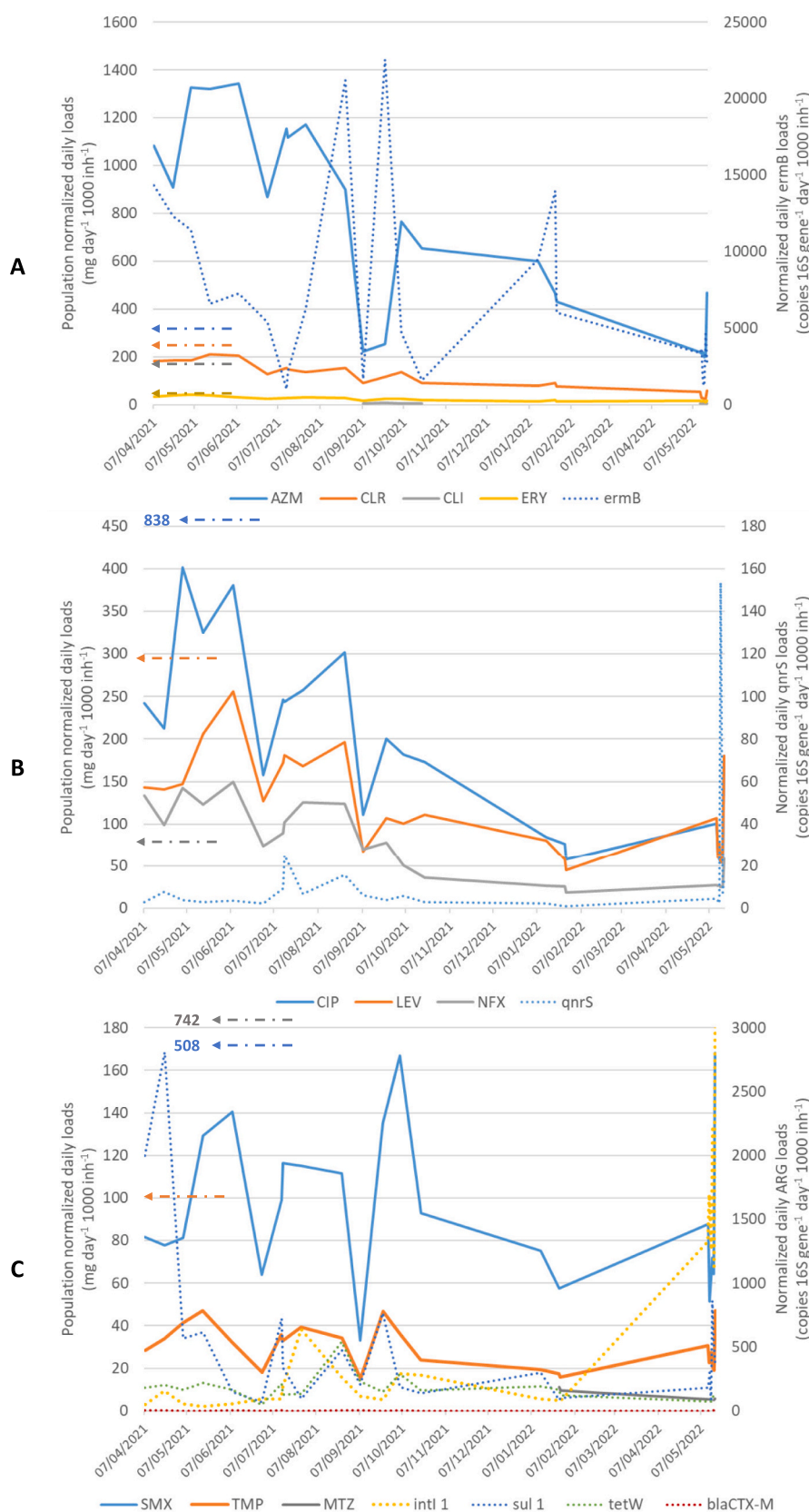


Fig. 1. Normalized daily mass loads of antibiotics ($\text{mg day}^{-1} 1000 \text{ inh}^{-1}$) and ARGs (copies $16\text{S gene}^{-1} \text{ day}^{-1} 1000 \text{ inh}^{-1}$) in the influent wastewater samples collected. Daily loads corresponding to (A) macrolides AZM, CLR, CLI, ERY and the *ermB* gene; (B) quinolones CIP, LEV, NFX and the *qnrS* gene and; (C) other antibiotic classes SMX, TMP, MTZ and the genes *intl 1*, *sul 1*, *tetW* and *blaCTX-M*. The non-continuous arrows indicate the estimated normalized daily consumption ($\text{mg day}^{-1} 1000 \text{ inh}^{-1}$) based on prescription data of Castellón in 2021 (the colour corresponds to the antibiotic).

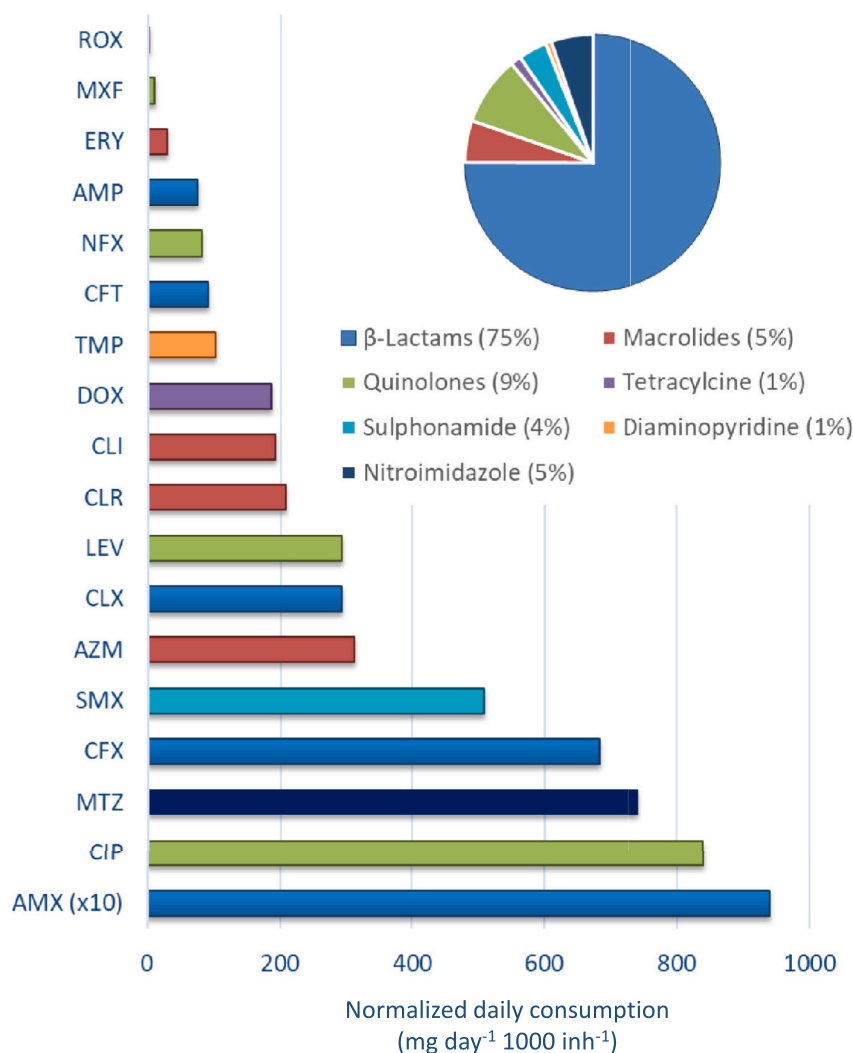


Fig. 2. Estimated normalized daily consumption (mg day⁻¹ 1000 inh⁻¹) of antibiotics in Castellón in 2021 based on information provided by suppliers, the General University Hospital and the Pharmacy Service of the Health Department of Castellón (insert percentage of antibiotic class consumed in 2021).

In general, high variability in mass loads between sampling days can be observed for all measured ARGs. This could be associated with the inherent sampling biases or other phenomena that influence the samples. More research and data are needed to understand better all variables affecting environmental AMR. Yet, some noteworthy observations are, for example, the *ermB* loads of August 25th and September 23rd 2021 with >20,000 copies 16S gene⁻¹ day⁻¹ 1000 inh⁻¹, whereas the sampling day in between September 7th showed loads by a factor of 10-fold lower (i.e., 1689 copies 16S gene⁻¹ day⁻¹ 1000 inh⁻¹). Similarly, the high *qnrS* loads on May 15th 2022, and the much lower loads on May 14th and 16th 2022 (Table S6) or the high loads of *sul1* in April 2021 compared to the other sampling dates. Furthermore, substantial increase by one order of magnitude could be observed for *int1* in May 2022 compared to January 2022 and earlier measurements in 2021. This increase of *int1* coordinates with the prevalence in May 2022, where more antibiotics were detected. Significant daily variability in mass loads in influent wastewater was also seen in other studies (Pärnänen et al., 2019; Steenbeek et al., 2022). For instance, *sul1* and *ermB* loads varied up to one order of magnitude between July and September 2019 in the Netherlands (Steenbeek et al., 2022). However, variability of ARGs in the UK was less prone, whereas seasonal fluctuations of several antibiotics were observed, indicating that antibiotic usage might not directly affect most ARG levels but rather spans over a longer period (Sims et al.,

2023b).

For further comparison with the literature, Tables S7 and S8 shows ARGs concentrations (gene copies L⁻¹) and absolute ARG concentrations [Log (gene copies mL⁻¹)], respectively. In the UK, concentrations of *sul1*, *ermB*, *qnrS* and *int1* were all within the same range as reported here (Sims et al., 2023b). However, in Finland and Estonia, average concentrations of ARGs *bla*_{CTX-M}, *sul1* and *tetW* in influent wastewater were significantly higher than in this study, 5 × 10⁹ vs 2 × 10⁶ *bla*_{CTX-M} copies L⁻¹, 3 × 10¹¹ vs 7 × 10⁸ *sul1* copies L⁻¹, 1 × 10¹² vs 2 × 10⁸ *tetW* copies L⁻¹, respectively (Laht et al., 2014). Moreover, higher average concentrations i.e., by a factor 100, were also observed for *sul1* and *int1* in the Netherlands compared to the current study. Whereas average concentrations of *ermB* and *qnrS* were very similar 1 × 10¹⁰ and 1 × 10⁷ copies L⁻¹, respectively (Paulus et al., 2019). In Spain, mean absolute ARG concentrations in influent wastewater were reported by Rodriguez-Mozaz et al. in 2015. Compared to this study, they observed slightly higher concentrations for *qnrS* (log ~5 copies mL⁻¹) and *tetW* (log ~6 copies mL⁻¹), and similar concentrations for *ermB* (log ~6 copies mL⁻¹) and *sul1* (log ~5 copies mL⁻¹) (Rodriguez-Mozaz et al., 2015). Although no effluent wastewater was monitored in this work, incomplete removal of antibiotics and ARGs by WWTPs has been demonstrated (Amarasiri et al., 2020; Rodriguez-Mozaz et al., 2015). Consequently, releasing these effluents into the aquatic environment will contribute to the

spread AMR with potential impact on associated environmental and human health risks (Amarasiri et al., 2020).

3.3. Correlation between antibiotics and ARGs

A Shapiro-Wilk test ($p < 0.05$) was used to check the normality of the data. In order to comply with normality, data were transformed using the formula $\text{Log}(x + 1)$. Correlations between 16S rRNA normalized loads of ARG vs. population normalized loads of antibiotics, and between 16S rRNA normalized loads vs. population normalized ARG loads, were calculated using the Pearson's correlation coefficient (Tables 2 and 3, respectively). When analysing the correlations between loads of individual antibiotics and ARGs (Table 2) only CFX showed significant correlations with *intI1*, *ermB* and *tetW*. However, no correlation was observed for *bla_{CTX-M}*, the ARG target gene for β -lactams such as CFX. Yet it should be taken into account that CFX was only detected in some of the samples therefore more research and quantitative data is needed to confirm these relationships. Furthermore, results also illustrate that the antibiotic classes macrolides and quinolones correlate with *intI1* and *tetW* (but not with *ermB*, an ARG more specific to macrolides), whereas the sum of all antibiotics only correlate with *intI1* loads. This is in accordance to other studies, where strong correlations were found between the antibiotics and non-corresponding ARGs (Paulus et al., 2019; Steenbeek et al., 2022). Despite the limited understanding of the significance of these relationships, it is plausible that horizontal gene transfer of plasmids contains multidrug resistance (Wang et al., 2019). Moreover, it has been reported that ARGs are more stable than fluctuations in antibiotic use (Sims et al., 2023b). The results obtained in our study would support this fact. Hence, changes in antibiotic consumption would not be immediately reflected in ARGs in wastewater. Higher sampling frequencies were therefor suggested to capture fluctuations and obtain representative estimates (Steenbeek et al., 2022).

Table 3 shows significant correlations between ARGs loads normalized to the measured 16S rRNA and ARGs loads normalized to the population served within the catchment area. As can be observed, correlation coefficients were statistically significant in all cases except for *tetW* ($p > 0.05$). Based on the results, it can be deduced that the normalization of gene loads to the measured 16S rRNA can be used as a proxy for the number of inhabitants within the localities studied. This correlation has been also found by Sims et al. in a one-year study in two cities (Sims et al., 2023b). Hence, this indicates a clear relationship between population size and the abundance of microorganisms in the western Mediterranean area, similar to Southern UK.

Possible differences in population normalized loads of antibiotics and ARGs over the studied period were also explored employing ANOVA I. Table 4 shows significant differences (ANOVA I, p -value = 0.0088)

Table 2

Statistically significant Pearson's correlation coefficients between pairs of variables (ARGs data normalized to the measured 16S rRNA gene copies (copies 16S gene⁻¹ day⁻¹ 1000 inh⁻¹) and population normalized loads of antibiotics (mg day⁻¹ 1000 inh⁻¹)).

| | CFX ^a | Macrolides | Quinolones | Sum_AB ^b | <i>intI1</i> | <i>ermB</i> | <i>tetW</i> |
|--------------|-------------------|-------------------|-------------------|---------------------|-------------------|-------------|-------------|
| CFX | | | | | | | |
| Macrolides | -0.7160 0.0001 | | | | | | |
| Quinolones | -0.5309 0.0091 | 0.8572 0.0001 | | | | | |
| Sum_AB | -0.7019 0.0002 | 0.9857 0.0001 | 0.9131 0.0001 | | | | |
| <i>intI1</i> | 0.8731 0.0001 | -0.6364 0.0011 | -0.4878 0.0185 | -0.6080 0.0021 | | | |
| <i>ermB</i> | -0.4320 0.0395 | ns | ns | ns | -0.4719 0.0230 | | |
| <i>tetW</i> | -0.5528 0.0062 | 0.4441 0.0337 | 0.4528 0.0300 | ns | ns | ns | |

Correlation coefficient and p value (in italics). ns: not significant.

^a CFX was only detected.

^b Sum of the loads of all antibiotics.

Table 3

Pearson's correlation coefficients between ARGs loads normalized to the measured 16S rRNA and population normalized ARG loads.

| | <i>intI1</i> | <i>sul1</i> | <i>tetW</i> | <i>ermB</i> | <i>qnrS</i> | <i>bla_{CTX-M}</i> |
|-----------------|--------------|-------------|-------------|-------------|-------------|----------------------------|
| Pearson's coef. | 0.9493 | 0.9598 | 0.1916 | 0.7476 | 0.9893 | 0.6324 |
| p -Value | 0.0001 | 0.0001 | 0.3810 | 0.0001 | 0.0001 | 0.0012 |

Table 4

Results of ANOVA I (LSD "a posteriori test") applied to the population normalized loads of antibiotics over time.

| Sampling period | Mean (in population normalized loads of antibiotics) | Homogeneous groups |
|--------------------|--|--------------------|
| Spring/summer 2021 | 0.0802 (0.0690–0.0960) | X |
| Autumn/winter 2021 | 0.0477 (0.0264–0.0690) | X X |
| Spring/summer 2022 | 0.0225 (0.0012–0.0438) | X |

(Confidence limits.)

along the different seasons presenting decreasing loads of antibiotics over time, while ARGs loads do not present differences (data not shown). This is in accordance with the hypothesis of steadier ARGs loads over time with respect to fluctuations in antibiotic use (Sims et al., 2023b). Yet, Steenbeek et al. observed substantial variance in ARG loads between days and over the studied period (Steenbeek et al., 2022). As for the population normalized antibiotic loads, a decrease in the use of antibiotics in summer would be expected, however the results obtained illustrate a continuous decrease in the antibiotic loads from the summer of 2021 to the summer of 2022. Considering that in 2021 there was still a strong pressure of COVID-19 cases, which declined over the studied period, this may explain this continuous drop in antibiotic use, masking the typical summer-winter fluctuations (Xu et al., 2022).

Principal Components Analysis (PCA) was applied to annotate the different components and obtain all relevant information from the acquired data. As can be observed in Fig. 3, *intI1* and CFX are positioned on the right-hand side of component 1 i.e., opposite to a set of variables that include population normalized loads of antibiotics and ARGs. The variance explained in component 1 would be based on the opposite behaviour of CFX and *intI1* vs. most of the other variables, without any apparent cause. Considering that, as discussed above, fluctuations in antibiotic use are not reflected in ARGs. Hence, the PCA results are consistent with that fact. Similarly, component 2 interprets opposite behaviour between MTZ loads and those of the *qnrS* and *bla_{CTX-M}* genes. It is interesting to note that the plot components show antibiotics or

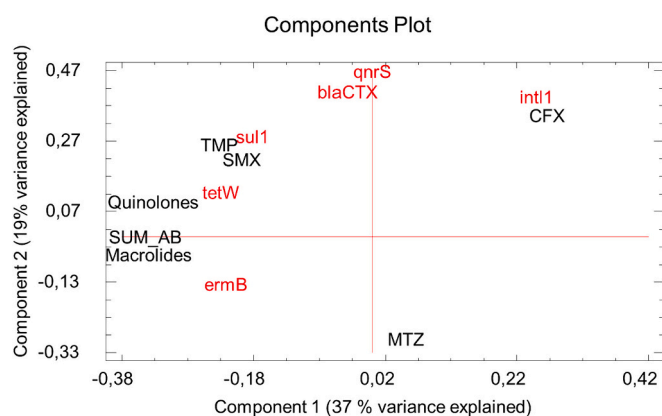


Fig. 3. Principal component analysis (PCA) scores plot for the two components at different sampling time in antibiotics (black) and ARGs (red).

families of antibiotics that are very close, although the correlation coefficients were not significant, such as TMP and SMX with *sul1* (ARG target gene for sulfonamides) and macrolides with *ermB* (ARG target gene for macrolides). Larger studies have described significant correlations between the last two (Sims et al., 2023b). However, other studies did not find direct one to one correlations between antibiotics and ARGs, which would indicate more complex interactions between antibiotics and ARGs and the need for further research (Cai et al., 2022; Huang et al., 2015; Zhao et al., 2021).

Fig. 4 shows the scatter plot of objects (days of sampling). As can be observed the samples are approximately ordered along component 1, coinciding with the chronological order of sampling. This component could be interpreted as the decreasing tendency in antibiotic use from 2021 to 2022, as previously discussed above. Prior to and during the COVID-19 pandemic, a decrease in community antibiotic consumption has been observed in the European Union (EU), especially between 2019 and 2020 (Diaz-Höberg et al., 2021). However, interpretation could not be extrapolated to the hospital sector and an increase in use and overuse by the community have also been reported during the pandemic (Langford et al., 2021).

This study illustrates that WBE can provide complementary insight in current AMR surveillance. However, changes in antibiotic consumption and possible implications to antibiotic resistance need to be further evaluated, and long-term monitoring programs are required worldwide. These advancements are essential and urgently needed as highlighted by the World Health Organization (WHO). Moreover, the study aligns with the One Health concept, where the aquatic environment is considered an important compartment for the evolution and spread of AMR (Milo-bedzka et al., 2022). Finally, within the EU there is a proposal for a harmonised and integrated action, between health and wastewater competent authorities, for surveying key public health parameters in wastewater in the near future (European Commission, 2022).

4. Conclusions

Tracking changes in AMR and understanding consumption habits in communities is critical for appropriate policy making to this growing concern. This study explored wastewater analysis as a complementary approach to reach an understanding of antibiotic usage and AMR within the city of Castellón (Spain). In total, 11 antibiotics were determined in the untreated wastewater samples collected during 2021/2022. Highest population normalized daily loads were measured for the macrolide AZM followed by the quinolones CIP and LEV. This did not entirely coincide with the estimated normalized daily consumption of antibiotics in 2021 in Castellón based on prescription data. Variability in mass loads between sampling days can be observed for all six ARGs measured. However, fluctuations in ARGs were not directly reflected in antibiotic

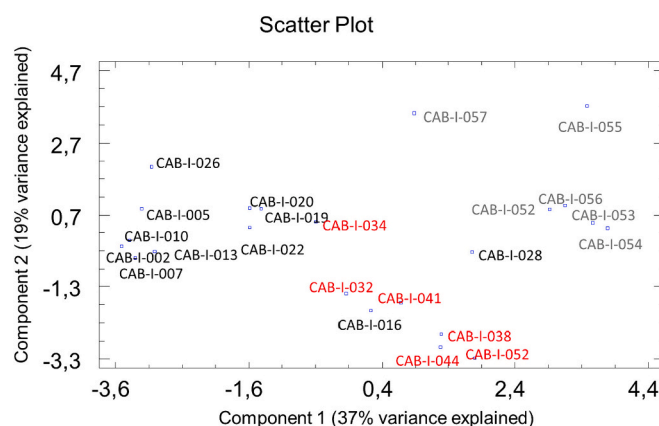


Fig. 4. Scatter Plot obtained by PCA analysis of samples described by antibiotics and ARGs (black: summer samples 2021; red: winter samples 2021/2022; grey: summer samples 2022).

loads. Certain correlations were identified among non-corresponding ARGs i.e., macrolides and quinolones correlated with *int1* and *tetW*, suggesting a potential cross-selection mechanism of bacterial resistance. Besides a correlation was found between the sum of all antibiotics and *int1*. Moreover, population normalized ARG loads correlated significantly with the ARGs loads normalized to the 16S rRNA, indicating that the abundance of this gene is proportional to population. Furthermore, the analysis over the studies period suggests a steady decrease in antibiotic use, independent of the season. This might be related to the treatment during the COVID-19 pandemic i.e., a steady decline of cases and change in vaccination status over time. Finally, results obtained in this study provide functional information and a baseline to further develop and validate this tool to predict the burden and potential risk of communities to AMR based on the analysis of wastewater.

CRedit authorship contribution statement

Lubertus Bijlsma: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Like Xu:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Elisa Gracia-Marín:** Writing – review & editing, Methodology, Formal analysis. **Elena Pitarch:** Writing – review & editing, Resources, Project administration, Conceptualization. **Roque Serrano:** Writing – review & editing, Visualization, Investigation, Data curation. **Barbara Kasprzyk-Hordern:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.171996>.

References

- Aarestrup, F.M., Woolhouse, M.E.J., 2020. Using sewage for surveillance of antimicrobial resistance. *Science* (80-.) 367, 630–632. <https://doi.org/10.1126/science.aba3432>.
- Amarasiri, M., Sano, D., Suzuki, S., 2020. Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: current knowledge and questions to be answered. *Crit. Rev. Environ. Sci. Technol.* 50, 2016–2059. <https://doi.org/10.1080/10643389.2019.1692611>.
- Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martínez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol.* 13, 310–317. <https://doi.org/10.1038/nrmicro3439>.
- Bivins, A., North, D., Ahmad, A., Ahmed, W., Alm, E., Been, F., Bhattacharya, P., Bijlsma, L., Boehm, A.B., Brown, J., Buttiglieri, G., Calabro, V., Carducci, A., Castiglioni, S., Cetecioglu, G., Chakraborty, S., Costa, F., Curcio, S., De Los Reyes, F.L., Delgado Vela, J., Farkas, K., Fernandez-Casi, X., Gerba, C., Gerrity, D., Girones, R., Gonzalez, R., Haramoto, E., Harris, A., Holden, P.A., Islam, M.T., Jones, D.L., Kasprzyk-Hordern, B., Kitajima, M., Kotlarz, N., Kumar, M., Kuroda, K., La Rosa, G., Malpei, F., Mautus, M., McLellan, C.B., Medema, G., Meschke, J.S., Mueller, J., Newton, R.J., Nilsson, D., Noble, R.T., Van Nuijs, A., Peccia, J., Perkins, T.A., Pickering, A.J., Rose, J., Sanchez, G., Smith, A., Stadler, L., Stauber, C., Thomas, K., Van Der Voorn, T., Wigginton, K., Zhu, K., Bibby, K., 2020. Wastewater-based epidemiology: global collaborative to maximize contributions in the fight against COVID-19. *Environ. Sci. Technol.* 54, 7754–7757. <https://doi.org/10.1021/acs.est.0c02388>.
- Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: a pan-European survey of urban settings. *Water Res.* 162, 320–330. <https://doi.org/10.1016/j.watres.2019.06.039>.
- Cai, M., Wang, Z., Gu, H., Dong, H., Zhang, X., Cui, N., Zhou, L., Chen, G., Zou, G., 2022. Occurrence and temporal variation of antibiotics and antibiotic resistance genes in hospital inpatient department wastewater: impacts of daily schedule of inpatients and wastewater treatment process. *Chemosphere* 292, 133405. <https://doi.org/10.1016/j.chemosphere.2021.133405>.
- Carballa, M., Omil, F., Lema, J.M., 2008. Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage. *Chemosphere* 72, 1118–1123. <https://doi.org/10.1016/j.chemosphere.2008.04.034>.
- Daughton, C.G., 2012. Using biomarkers in sewage to monitor community-wide human health: isoprostanates as conceptual prototype. *Sci. Total Environ.* 424, 16–38. <https://doi.org/10.1016/j.scitotenv.2012.02.038>.
- Diaz-Höberg, L., Vlahović-Palčevski, V., Pereira, C., Weist, K., Monnet, D.L., Group, E.-N. study, 2021. Decrease in community antibiotic consumption during the COVID-19 pandemic, EU/EEA, 2020. *Eurosurveillance* 26, 1–5. <https://doi.org/10.2807/1560-7917.ES.2021.26.46.2101020>.
- Drugbank online. URL: <https://go.drugbank.com/> (WWW Document, n.d.).
- Escolà Casas, M., Schröter, N.S., Zammit, I., Castaño-Trias, M., Rodríguez-Mozaz, S., Gago-Ferrero, P., Corominas, L., 2021. Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: comparison against prescription data collected at fine spatial resolution. *Environ. Int.* 150, 106404. <https://doi.org/10.1016/j.envint.2021.106404>.
- European Commission, 2021. Guidance Document on Analytical Quality Control and Method Validation for Pesticide Residues Analysis in Food and Feed SANTE 11312/2021. *Sante/11312/2021* 1–57.
- European Commission, 2022. Directive of the European Parliament and of the Council Concerning Urban Wastewater Treatment, vol. 541, pp. 1–68.
- Fabregat-Safont, D., Gracia-Marín, E., Ibáñez, M., Pitarch, E., Hernández, F., 2023. Analytical key issues and challenges in the LC-MS/MS determination of antibiotics in wastewater. *Anal. Chim. Acta* 1239. <https://doi.org/10.1016/j.jaca.2022.340739>.
- Gao, J., Li, L., Duan, L., Yang, M., Zhou, X., Zheng, Q., Ou, Y., Li, Z., Lai, F.Y., 2022. Exploring antibiotic consumption between urban and sub-urban catchments using both parent drugs and related metabolites in wastewater-based epidemiology. *Sci. Total Environ.* 827, 154171. <https://doi.org/10.1016/j.scitotenv.2022.154171>.
- González-Mariño, I., Baz-Lomba, J.A., Alygizakis, N.A., Andrés-Costa, M.J., Bade, R., Bannwarth, A., Barron, L.P., Been, F., Benaglia, L., Berset, J.D., Bijlsma, L., Bodík, I., Brenner, A., Brock, A.L., Burgard, D.A., Castrignanò, E., Celma, A., Christophoridis, C.E., Covaci, A., Delémont, O., de Voogt, P., Devault, D.A., Dias, M. J., Emke, E., Esseiva, P., Fatta-Kassinos, D., Fedorova, G., Fytianos, K., Gerber, C., Grabic, R., Gracia-Lor, E., Grüner, S., Gunnar, T., Hapeshi, E., Heath, E., Helm, B., Hernández, F., Kankaanpää, A., Karolák, S., Kasprzyk-Hordern, B., Krizman-Matic, I., Lai, F.Y., Lechowicz, W., Lopes, A., López de Alda, M., López-García, E., Löve, A.S.C., Mastroianni, N., McEneff, G.L., Montes, R., Munro, K., Nefau, T., Oberacher, H., O'Brien, J.W., Oertel, R., Olafsdottir, K., Picó, Y., Plósz, B.G., Polesel, F., Postigo, C., Quintana, J.B., Ramin, P., Reid, M.J., Rice, J., Rodil, R., Salgueiro-González, N., Schubert, S., Senta, I., Simões, S.M., Sremacki, M.M., Styszko, K., Terzic, S., Thomaidis, N.S., Thomas, K.V., Tschärke, B.J., Udrisard, R., van Nuijs, A.L.N., Yargeau, V., Zuccato, E., Castiglioni, S., Ort, C., 2020. Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring. *Addiction* 115, 109–120. <https://doi.org/10.1111/add.14767>.
- Gonzalez-Zorn, B., 2021. Antibiotic use in the COVID-19 crisis in Spain. *Clin. Microbiol. Infect.* 27, 646–647. <https://doi.org/10.1016/j.cmi.2020.09.055>.
- Gracia-Lor, E., Castiglioni, S., Bade, R., Been, F., Castrignanò, E., Covaci, A., González-Mariño, I., Hapeshi, E., Kasprzyk-Hordern, B., Kinyua, J., Lai, F.Y., Letzel, T., Lopardo, L., Meyer, M.R., O'Brien, J., Ramin, P., Rousis, N.I., Rydevik, A., Ryu, Y., Santos, M.M., Senta, I., Thomaidis, N.S., Veloutsos, S., Yang, Z., Zuccato, E., Bijlsma, L., 2017. Measuring biomarkers in wastewater as a new source of epidemiological information: current state and future perspectives. *Environ. Int.* 99, 131–150. <https://doi.org/10.1016/j.envint.2016.12.016>.
- Gracia-Marín, E., Rico, A., Fabregat-Safont, D., López, F.J., Hernández, F., Pitarch, E., Bijlsma, L., 2024. Comprehensive study on the potential environmental risk of temporal antibiotic usage through wastewater discharges. *Chemosphere* 346. <https://doi.org/10.1016/j.chemosphere.2023.140587>.
- Hellmér, M., Paxéus, N., Magnus, L., Enache, L., Arnholm, B., Johansson, A., Bergström, T., Norder, H., 2014. Detection of pathogenic viruses in sewage provided early warnings of hepatitis A virus and norovirus outbreaks. *Appl. Environ. Microbiol.* 80, 6771–6781. <https://doi.org/10.1128/AEM.01981-14>.
- Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijs, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T. L.C., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Bego, A., Rees, C., Cassar, S., Coventry, K., Collignon, P., Allerberger, F., Rahube, T.O., Oliveira, G., Ivanov, I., Vuthy, Y., Sopheak, T., Yost, C.K., Ke, C., Zheng, H., Baisheng, L., Jiao, X., Donado-Godoy, P., Coulibaly, K.J., Jergović, M., Hrenovic, J., Karpisková, R., Villacs, J.E., Legesse, M., Eguale, T., Heikinheimo, A., Malania, L., Nitsche, A., Brinkmann, A., Saba, C.K.S., Kocsis, B., Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Alebouyeh, M., Morris, D., Cormican, M., O'Connor, L., Moran-Gilad, J., Alba, P., Battisti, A., Shakenova, Z., Kiyukia, C., Ng'eno, E., Raka, L., Avsejkenko, J., Bērziņš, A., Bartkevics, V., Penny, C., Rajandas, H., Parimannan, S., Haber, M.V., Pal, P., Jeunen, G.J., Gemmell, N., Fashae, K., Holmstad, R., Hasan, R., Shakoor, S., Rojas, M.L.Z., Wasyl, D., Bosevka, G., Kochubovskii, M., Radu, C., Gassama, A., Radosavljevic, V., Wuertz, S., Zuniga-Montanez, R., Tay, M.Y.F., Gavačová, D., Pastuchova, K., Truska, P., Trkov, M., Esterhuysen, K., Keddy, K., Cerdà-Cuellar, M., Pathirage, S., Norrgren, L., Örn, S., Larsson, D.G.J., Heijden, T. Van der, Kumburu, H. H., Sannhe, B., Bidjaja, P., Njanpop-Lafourcade, B.M., Nikiema-Pessinaba, S.C., Levent, B., Meschke, J.S., Beck, N.K., Van, C.D., Phuc, N. Do, Tran, D.M.N., Kwenda, G., Tabo, D. adjim, Wester, A.L., Cuadros-Orellana, S., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* 10. <https://doi.org/10.1038/s41467-019-08853-3>.
- Holton, E., Archer, E., Fidal, J., Kjeldsen, T., Wolfaardt, G., Kasprzyk-Hordern, B., 2022a. Spatiotemporal urban water profiling for the assessment of environmental and public exposure to antimicrobials (antibiotics, antifungals, and antivirals) in the Eerste River Catchment. *South Africa. Environ. Int.* 164, 107227. <https://doi.org/10.1016/j.envint.2022.107227>.
- Holton, E., Sims, N., Jagadeesan, K., Standerwick, R., Kasprzyk-Hordern, B., 2022b. Quantifying community-wide antimicrobials usage via wastewater-based epidemiology. *J. Hazard. Mater.* 436, 129001. <https://doi.org/10.1016/j.jhazmat.2022.129001>.
- Huang, R.J., Zhang, Y., Bozzetti, C., Ho, K.F., Cao, J.J., Han, Y., Daellenbach, K.R., Slowik, J.G., Platt, S.M., Canonaco, F., Zotter, P., Wolf, R., Pieber, S.M., Bruns, E.A., Crippa, M., Ciarelli, G., Piazzalunga, A., Schwikowski, M., Abbaszade, G., Schnelle-Kreis, J., Zimmermann, R., An, Z., Szidat, S., Baltensperger, U., El Haddad, I., Prévôt, A.S.H., 2015. High secondary aerosol contribution to particulate pollution during haze events in China. *Nature* 514, 218–222. <https://doi.org/10.1038/nature13774>.
- Iaconelli, M., Ferraro, G.B., Mancini, P., Suffredini, E., Veneri, C., Ciccaglione, A.R., Bruni, R., Libera, S., Della, Bignami, F., Brambilla, M., De Medici, D., Brandtner, D., Schembri, P., D'amato, S., La Rosa, G., 2020. Nine-year nationwide environmental surveillance of hepatitis E virus in urban wastewaters in Italy (2011–2019). *Int. J. Environ. Res. Public Health* 17, 1–18. <https://doi.org/10.3390/ijerph17062059>.
- Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., Kisand, V., 2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS One* 9, 1–8. <https://doi.org/10.1371/journal.pone.0103705>.
- Langford, B.J., So, M., Raybardhan, S., Leung, V., Soucy, J.P.R., Westwood, D., Daneman, N., MacFadden, D.R., 2021. Antibiotic prescribing in patients with COVID-19: rapid review and meta-analysis. *Clin. Microbiol. Infect.* 27, 520–531. <https://doi.org/10.1016/j.cmi.2020.12.018>.
- Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environ. Sci. Technol. Lett.* 7, 511–516. <https://doi.org/10.1021/acs.estlett.0c00357>.
- Milobedzka, A., Ferreira, C., Vaz-Moreira, I., Calderón-Franco, D., Gorecki, A., Purkrtova, S., Bartacek, Jan, Dziewit, L., Singleton, C.M., Nielsen, P.H.,

- Weissbrodt, D.G., Manaia, C.M., 2022. Monitoring antibiotic resistance genes in wastewater environments: the challenges of filling a gap in the One-Health cycle. *J. Hazard. Mater.* 424 <https://doi.org/10.1016/j.jhazmat.2021.127407>.
- Munk, P., Brinch, C., Møller, F.D., Petersen, T.N., Hendriksen, R.S., Seyfarth, A.M., Kjeldgaard, J.S., Svendsen, C.A., van Bunnik, B., Berglund, F., Bego, A., Power, P., Rees, C., Lambrinidis, D., Neilson, E.H.J., Gibb, K., Coventry, K., Collignon, P., Cassar, S., Allerberger, F., Begum, A., Hossain, Z.Z., Worrell, C., Vandenberg, O., Pieters, I., Victorien, D.T., Gutierrez, A.D.S., Soria, F., Grujić, V.R., Mazalica, N., Rahube, T.O., Tagliati, C.A., Rodrigues, D., Oliveira, G., de Souza, L.C.R., Ivanov, I., Juste, B.I., Oumar, T., Sopheak, T., Vuthy, Y., Ngandijo, A., Nzouankeu, A., Olivier, Z.A.A.J., Yost, C.K., Kumar, P., Brar, S.K., Tabo, D.A., Adell, A.D., Paredes-Osses, E., Martinez, M.C., Cuadros-Orellana, S., Ke, C., Zheng, H., Baisheng, L., Lau, L.T., Chung, T., Jiao, X., Yu, Y., JiaYong, Z., Morales, J.F.B., Valencia, M.F., Donado-Godoy, P., Coulbaly, K.J., Hrenovic, J., Jergović, M., Karpíšková, R., Deogratias, Z.N., Elsborg, B., Hansen, L.T., Jensen, P.E., Abouelnaga, M., Salem, M. F., Koolmeister, M., Legesse, M., Eguale, T., Heikkinheimo, A., Le Guyader, S., Schaeffer, J., Villacis, J.E., Sanneh, B., Malania, L., Nitsche, A., Brinkmann, A., Schubert, S., Hesse, S., Berendonk, T.U., Saba, C.K.S., Mohammed, J., Feglo, P.K., Banu, R.A., Kotzamanidis, C., Lytras, E., Lickes, S.A., Kocsis, B., Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Ballal, M., Banger, S.R., Fani, F., Alebouyeh, M., Morris, D., O'Connor, L., Cormican, M., Moran-Gilad, J., Battisti, A., Diaconu, E.L., Corno, G., Di Cesare, A., Alba, P., Hisatsune, J., Yu, L., Kuroda, M., Sugai, M., Kayama, S., Shakenova, Z., Kiiyukia, C., Ng'eno, E., Raka, L., Jamil, K., Fakhruldeen, S.A., Alaati, T., Bērziņš, A., Avsejkeno, J., Kokina, K., Streikisa, M., Bartkevics, V., Matar, G.M., Daoud, Z., Pereckienė, A., Butrimaite-Ambrozeviene, C., Penny, C., Bastaraut, A., Rasolofoaarison, T., Collard, J.M., Samison, L.H., Andrianarivelo, M.R., Banda, D.L., Amin, A., Rajandas, H., Parimannan, S., Spiteri, D., Haber, M.V., Santchurn, S.J., Vujacic, A., Djurovic, D., Bouchrif, B., Karraouan, B., Vubil, D.C., Pal, P., Schmitt, H., van Passel, M., Jeunen, G.J., Gemmell, N., Chambers, S.T., Mendoza, F.P., Huete-Perez, J., Vilchez, S., Ahmed, A.O., Adisa, I.R., Odetokun, I.A., Fashae, K., Sørgaard, A.M., Wester, A.L., Ryrfors, P., Holmstad, R., Mohsin, M., Hasan, R., Shakoor, S., Gustafson, N.W., Schill, C.H., Rojas, M.L.Z., Velasquez, J.E., Magtibay, B.B., Catangcatang, K., Sibulo, R., Yauce, F.C., Wasyl, D., Manaia, C., Rocha, J., Martins, J., Alvaro, P., Di Yoong Wen, D., Shin, H., Hur, H.G., Yoon, S., Bosevska, G., Kochubovskii, M., Cojocar, R., Burdunuc, O., Hong, P.Y., Perry, M.R., Gassama, A., Radosavljevic, V., Tay, M.Y.F., Zuniga-Montanez, R., Wuertz, S., Gavačová, D., Pastuchová, K., Truska, P., Trkov, M., Keddy, K., Esterhuysen, K., Song, M.J., Quintela-Balaja, M., Lopez, M.G., Cerdá-Cuellar, M., Perera, R.R.D.P., Bandara, N.K. B.K.R.G.W., Premasiri, H.I., Pathirage, S., Charlemagne, K., Rutgersson, C., Norrgren, L., Örn, S., Boss, R., Van der Heijden, T., Hong, Y.P., Kumburu, H.H., Mdegela, R.H., Hounmanou, Y.M.G., Chonsin, K., Suthienkul, O., Thamlikitkul, V., de Roda Husman, A.M., Bidjaja, B., Njanpop-Lafourcade, B.M., Nikiema-Pessinaba, S.C., Levent, B., Kurekci, C., Ejobi, F., Kalule, J.B., Thomsen, J., Obaidi, O., Jassim, L.M., Moore, A., Leonard, A., Graham, D.W., Bunce, J.T., Zhang, L., Gaze, W.H., Lefor, B., Capone, D., Sozzi, E., Brown, J., Meschke, J.S., Sobsey, M.D., Davis, M., Beck, N.K., Sukapantharam, P., Truong, P., Lilienthal, R., Kang, S., Wittum, T.E., Rigamonti, N., Baklayan, P., Van, C.D., Tran, D.M.N., Do Phuc, N., Kwenda, G., Larsson, D.G.J., Koopmans, M., Woolhouse, M., Aarestrup, F. M., 2022. Genomic analysis of sewage from 101 countries reveals global landscape of antimicrobial resistance. *Nat. Commun.* 13, 1–16. <https://doi.org/10.1038/s41467-022-34312-7>.
- Osińska, A., Korzeniewska, E., Harnisz, M., Felis, E., Bajkacz, S., Jachimowicz, P., Niestepski, S., Konopka, I., 2020. Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. *J. Hazard. Mater.* 381, 121221 <https://doi.org/10.1016/j.jhazmat.2019.121221>.
- Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T. T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J. L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Sci. Adv.* 5 <https://doi.org/10.1126/sciadv.aau9124>.
- Paulus, G.K., Hornstra, L.M., Alygizakis, N., Slobodnik, J., Thomaidis, N., Medema, G., 2019. The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. *Int. J. Hyg. Environ. Health* 222, 635–644. <https://doi.org/10.1016/j.ijheh.2019.01.004>.
- Perry, M., McClean, D., Simonet, C., Woolhouse, M., McNally, L., 2018. Focussing on resistance to front-line drugs is the most effective way to combat the antimicrobial resistance crisis. *bioRxiv*, 498329. <https://doi.org/10.1101/498329>.
- Prichard, J., Hall, W., de Voogt, P., Zuccato, E., 2014. Sewage epidemiology and illicit drug research: the development of ethical research guidelines. *Sci. Total Environ.* 472, 550–555. <https://doi.org/10.1016/j.scitotenv.2013.11.039>.
- Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sánchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. <https://doi.org/10.1016/j.watres.2014.11.021>.
- Sims, N., Holtan, E., Jagadeesan, K., Standerwick, R., Barden, R., Kasprzyk-Hordern, B., 2023a. Community infectious disease treatment with antimicrobial agents – a longitudinal one year study of antimicrobials in two cities via wastewater-based epidemiology. *J. Hazard. Mater.* 454, 131461 <https://doi.org/10.1016/j.jhazmat.2023.131461>.
- Sims, N., Kannan, A., Holtan, E., Jagadeesan, K., Mageiros, L., Standerwick, R., Craft, T., Barden, R., Feil, E.J., Kasprzyk-Hordern, B., 2023b. Community and Hospital Derived Antimicrobials and Resistance Genes in a Two-city One-year Longitudinal Study. <https://doi.org/10.2139/ssrn.4235052> (pre-print SSRN).
- Steenbeek, R., Timmers, P.H.A., van der Linde, D., Hup, K., Hornstra, L., Been, F., 2022. Monitoring the exposure and emissions of antibiotic resistance: co-occurrence of antibiotics and resistance genes in wastewater treatment plants. *J. Water Health* 20, 1157–1170. <https://doi.org/10.2166/wh.2022.021>.
- Wang, Y., Lu, J., Mao, L., Li, J., Yuan, Z., Bond, P.L., Guo, J., 2019. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. *ISME J.* 13, 509–522. <https://doi.org/10.1038/s41396-018-0275-x>.
- WHO. WHO: Global Antimicrobial Resistance and Use Surveillance System (GLASS). URL: <https://www.who.int/initiatives/glass>. (Accessed 26 March 2024) (WWW Document, n.d.).
- World Health Organization, 2022. *Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2022*. Switzerland, Geneva.
- Xu, L., Chen, H., Canales, M., Ciric, L., 2019. Use of synthesized double-stranded gene fragments as qPCR standards for the quantification of antibiotic resistance genes. *J. Microbiol. Methods* 164, 105670. <https://doi.org/10.1016/j.mimet.2019.105670>.
- Xu, L., Zang, J., Cong, W., Holton, E., Jiang, L., Sheppard, S.K., Wang, Y., Wang, N., Weeks, J., Fu, C., Jiang, Q., Lambert, H., Kasprzyk-Hordern, B., 2022. Assessment of community-wide antimicrobials usage in Eastern China using wastewater-based epidemiology. *Water Res.* 222, 118942 <https://doi.org/10.1016/j.watres.2022.118942>.
- Zhao, B., Xu, J., Zhang, G., Lu, S., Liu, X., Li, L., Li, M., 2021. Occurrence of antibiotics and antibiotic resistance genes in the Fuxian Lake and antibiotic source analysis based on principal component analysis-multiple linear regression model. *Chemosphere* 262, 127741. <https://doi.org/10.1016/j.chemosphere.2020.127741>.
- Zieliński, W., Korzeniewska, E., Harnisz, M., Drzymała, J., Felis, E., Bajkacz, S., 2021. Wastewater treatment plants as a reservoir of integrase and antibiotic resistance genes – an epidemiological threat to workers and environment. *Environ. Int.* 156 <https://doi.org/10.1016/j.envint.2021.106641>.