Comprehensive study on the potential environmental risk of temporal antibiotic usage through wastewater discharges

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CONFLICT OF INTEREST

The authors declare they have nothing to disclose.

ABSTRACT

Antibiotic residues can reach aquatic ecosystems through urban wastewater discharges, posing an ecotoxicological risk for aquatic organisms and favoring the development of bacterial resistance. To assess the emission rate and hazardousness of these compounds, it is important to carry out periodic chemical monitoring campaigns that provide information regarding the actual performance of wastewater treatment plants (WWTPs) and the potential impact of the treated wastewater in the aquatic environment. In this study, 18 of the most widely consumed antibiotics in Spain were determined by liquid chromatography-tandem mass spectrometry in both influent (IWW) and effluent wastewater (EWW) samples collected over four seasons along 2021-2022. Eleven antibiotics were detected in EWW with azithromycin, ciprofloxacin and levofloxacin showing the highest concentration levels (around 2 µg L^{-1} of azithromycin and 0.4 µg L^{-1} of quinolone compounds).Data showed that only 4 out of the 11 compounds were removed by more than 50 % in the WWTP, with sulfamethoxazole standing out with an average removal efficiency > 80 %. The risk that treated water could pose to the aquatic environment was also assessed, with 6 compounds indicating a potential environmental risk by exceeding established ecotoxicological and resistance thresholds. Based on the risk assessment, the WWTP removal efficiency required to reduce such risk for antibiotics was estimated. In addition, pooled wastewater samples were screened by LC coupled to high resolution mass spectrometry with ion mobility separation, searching for metabolites and transformation products of the antibiotics investigated to widen future research. Studies like this are crucial to map the impact of antibiotic pollution and to provide the basis for designing water quality and risk prevention monitoring programs.

Keywords: Antibiotics**;** metabolites;sewage;removal efficiency;environmental impact; risk assessment

1. INTRODUCTION

Undoubtedly, the use of antibiotics has improved human life expectancy during the last century, as well as decreased mortality from diseases caused by pathogenic bacteria (Aminov, 2010; Elder et al., 2021; Kumar et al., 2019). However, their inappropriate and increasing usage in human and veterinary medicine have resulted in increasing environmental emissions and contributed to the antimicrobial resistance burden. The spread of antibiotic resistant (ABR) bacteria in the human population reduces the success to treat common infectious diseases and, consequently, can increase mortality and economic costs. The World Health Organization (WHO) has identified antibiotic resistance as one of the greatest threats to human health and highlighted the urgency to advance towards a more comprehensive and accurate assessment and surveillance (WHO, 2023). It is now recognized that the environment plays a key role in the development and spread of ABR (Elder et al., 2021), being necessary to improve our knowledge regarding the presence and behaviour of antimicrobials in environmental compartments (European Commission, 2017).

Antibiotics enter the sewage system after consumption and excretion (including their metabolites) or due to direct disposal. Subsequently, due to incomplete removal by wastewater treatment plants (WWTPs), antibiotic residues may enter the aquatic environment through wastewater discharges. Many studies have reported the presence of antibiotics in different aquatic environments such as surface water (Van Hoi et al., 2021) and reclaimed water (Campos-Mañas et al., 2017; Martínez-Piernas et al., 2021). Some papers have also highlighted that antibiotic removal by WWTPs can vary among different locations, even when using the same treatment processes (Kovalakova et al., 2020; McCorquodale-Bauer et al., 2023). Hence, advanced treatment processes are required to reduce the negative impact of antibiotics in aquatic ecosystems (Lien et al., 2016a). However, novel and economic solutions are currently limited available or not accessible. Therefore, performing regular monitoring

campaigns are pivotal to understand the current status and environmental risks posed by these compounds. In fact, the European Commission included four antibiotics (sulfamethoxazole, trimethoprim, clindamycin and ofloxacin) in the last Watch List of substances in the field of water policy (European Commission, 2022), demonstrating the concern about the entry of these compounds into the environment and their potential consequences for aquatic ecosystems and human health.

Monitoring antibiotics requires the use of highly selective and sensitive analytical techniques, being liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) one of the most applied to obtain reliable quantitative data. As indicated above, antibiotics can be excreted unaltered or as metabolites. It should be noted that metabolites could be found at higher concentrations than the parent antibiotic, and they may have the same or higher level of toxicity to the environment than unaltered compound. However, only data on concentrations of parent compounds are usually reported, but the information on the presence of metabolites in the aquatic media is crucial to obtain a comprehensive overview of the current situation (Fabregat-Safont et al., 2023a; Ibáñez et al., 2017, 2021; Löffler et al., 2023; Wielens Becker et al., 2020). Yet, reference standards of metabolites are not always available. Under this situation, the complementary quantitative target analysis, usually focused on parent antibiotics, and the wide-scope screening based on high-resolution mass spectrometry (HRMS) can provide relevant information on the presence of both antibiotics and their metabolites (Fabregat-Safont et al., 2023a; Fabregat-Safont et al., 2021).

Besides the promotion of antibiotic resistance, antibiotics may pose toxicological effects for organisms, principally bacteria and primary producers, thus affecting the structure of aquatic ecosystems and important ecosystem functions such as organic matter decomposition or nitrification (Le Page et al., 2017; Roose-Amsaleg&Laverman, 2016). Thus, the risk assessment of antibiotics should combine several protection goals. Few studies have

developed ecotoxicological and resistance thresholds for largely used antibiotics based on laboratory toxicity data for aquatic standard test species and minimum inhibitory concentrations for pathogenic bacteria, respectively (Bengtsson-Palme & Larsson, 2016; Rico et al., 2017; Tell et al., 2019). The comparison of ecotoxicological and resistance thresholds shows that neither is always protective of the other, so both should be preferably used together to make a holistic risk assessment of antibiotic pollution in areas affected by WWTP emissions (Le Page et al., 2017). Few studies have demonstrated that concentrations of some antibiotics measured in aquatic ecosystems impacted by urban or industrial wastewaters can exceed such thresholds (Fonseca et al., 2020; Hanna et al., 2023; Kelly & Brooks, 2018), however their use to set effective wastewater treatment methods and processes is to be further developed.

In this work the occurrence of 18 highly consumed antibiotics was investigated in wastewater samples collected between April 2021 to January 2022 from the WWTP of Castelló (Spain). The objectives of this study were: 1) to evaluate seasonal variations of concentrations and removal efficiencies of the antibiotics studied, after conventional wastewater treatment; 2) to apply a complementary screening of relevant metabolites to better characterize environmental exposure, using advanced analytical methodology based on HRMS with ion mobility separation (IMS); 3) to assess risks regarding their potential to contribute to ecotoxicological effects and antibiotic resistance in the environment, determining the required wastewater treatment efficiencies that should be achieved to reduce such risks.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The selection of compounds included in this study was based on antibiotic prescription data in collaboration with theHealth Department of Castelló(**Table S1**), and the annual sales data provided by suppliers and the Pharmacy Services of Castelló (Spain) (**Table S2**). Such information is summarized in **Figure 1**, where it is observed that β-lactams and macrolides were the main families of antibiotics prescribed during 2020.

Finally, 18 antibiotics were chosen to be part of this study: amoxicillin, ampicillin, azithromycin, cefditoren (purchased as cefditoren pivoxil), cefuroxime, ciprofloxacin, clarithromycin, clindamycin, cloxacillin, doxycycline, erythromycin, levofloxacin, metronidazole, moxifloxacin, norfloxacin, roxithromycin, sulfamethoxazole and trimethoprim. Isotopically-labelled analogues (**Table S3**) were used as internal standards (ILIS) for each selected antibiotic, with the exception of cefditoren. All the analytical reference standards were purchased from LGC (Teddington, UK) and Merck (Darmstadt, Germany). Methanol, acetonitrile and formic acid (LC-MS grade) and ammonium acetate (> 98 %) were acquired from Scharlab (Scharlab, Barcelona, Spain). LC-MS grade water was obtained using an Ultramatic Plus GR from Wasserlab (Navarra, Spain).

2.2. Description of the WWTP

The selected WWTP (39°59'09.2"N 0°0'21.8"W) treats urban wastewater from Castelló de la Plana and Borriol (Spain) and serves a population of 179,661 inhabitants (based on census data of 2020 (Instituto Nacional de Estadistica, 2023)). The WWTP applies a conventional treatment consisting of a basic biological process and has a treatment capacity of 45,000 m^3 / day. The water line includes a pretreatment (roughing filtration, desanding and degreasing), a primary treatment (primary sedimentation), a conventional activated sludge biological

treatment, followed by a tertiary treatment (operated with sand filtration and ultraviolet oxidation). Finally, the treated water is discharged into the Mediterranean Sea or used to irrigate parks and gardens after tertiary treatment with an additional chlorination step.

2.3. Wastewater samples

24-h composite samples of influent wastewater (IWW) and effluent wastewater (EWW) were collected from the WWTP, from April to October 2021 and during January 2022, covering the four seasons. Wastewater sampling was carried out two weeks per month (only in one week in August) collecting IWW and EWW samples of two days each week. A total of 30 IWW and 30 EWW samples were analysed. **Table S4** shows the sampling dates, and the flow rates of the WWTP on these days.

All samples were collected in high-density polyethylene bottles, stored at -20 ^{o}C, and transported to the laboratory when the last sample of the week was collected. After reception in the laboratory, samples were stored in the dark at -20 ^{o}C until analysis.

2.4. Instrumentation

2.4.1. LC-MS/MS

An Acquity UPLC^{TM} H-Class liquid chromatography system (Waters Corp., Milford, MA, USA) interfaced to a triple quadrupole mass spectrometer Xevo TQ-S^{TM} (Waters Corp., Manchester, UK) and equipped with an orthogonal Z-Spray electrospray ionization interface (ESI) (Waters Corp, Manchester, UK) was used for quantitative sample analysis. MS/MS conditions are shown in **Table S3**. Further information regarding antibiotic determination, analytical method and validation can be found in the literature (Fabregat-Safont et al., 2023b).

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167 *2.4.2. LC-IMS-HRMS*

168 Metabolite screening was performed using an Acquity UPLC^{TM} I-Class system (Waters
169 Corp., Milford, MA, USA) coupled to a Vion IMS QTOF mass spectrometer (Waters Corp.,
170 Wilmslow, Manchester, UK), using an ESI interface operating in both positive and negative
171 ionization modes. Further information regarding instrumentation, data treatment, results
172 evaluation and compound identification is described in the literature (Celma et al., 2020;
173 Fabregat-Safont et al., 2021; Lopez et al., 2022).

174 **2.5. Sample analysis**

175 For quantification, samples were analysed by direct injection (DI)-LC-MS/MS based on a
176 previously developed methodology (Fabregat-Safont et al., 2023b). Briefly, a volume of 2
177 mL of centrifuged wastewater was 5-fold (IWW) or 2-fold (EWW) diluted with ultrapure
178 water, taking 200 µL IWW or 500 µL EWW, adding 40 µL of ILIS mixture 5 µg L^{-1} and
179 adjusting the volume to 1 mL with ultrapure water. Finally, 100 µL of the diluted samples
180 were injected into the LC-MS/MS system.

181 For metabolite screening analyses by LC-IMS-HRMS, two IWW and two EWW pooled
182 samples were prepared by mixing individual samples as follows: the first IWW pooled
183 sample was prepared using two randomly selected samples, collected one in April and the
184 other one in May 2021; and the second IWW pooled sample using samples collected in June
185 and July 2021. The same strategy was used for EWW, mixing the corresponding EWW
186 samples. Sample treatment was performed using 100 mL of sample (2-fold diluted with
187 ultrapure water to avoid clogging) and passed by gravity through an Oasis HLB 200 mg
188 cartridge (Waters Corp.). Cartridges were eluted with 10 mL of methanol, evaporated to

dryness at 40 ºC under gentle nitrogen stream, and redissolved in 500 µL of water:methanol (90:10, v:v). Finally, 10 µL of sample extract was injected in the LC-IMS-HRMS system.

For compound identification in the screening, an in-house database containing information about the major metabolites reported for these antibiotics was used, following the analytical strategy described in literature (Fabregat-Safont et al., 2023a; Fabregat-Safont et al., 2021; Lopez et al., 2022). More details about the confidence levels of the metabolite identification can be found in identification Celma et al. (2020) and in section 2.5 of the Supporting Information.

2.6. Estimation of removal efficiencies

The removal efficiency (RE) of the WWTP was estimated by comparing the daily mass loads of antibiotics in IWW and EWW, estimated from the daily antibiotic concentrations and the WWTP flow rates (m^3/24 h).RE was calculated using **Eq. 1**, where q_I is the daily mass load (g/24h) of IWW at day x and q_E is the mass load of EWW at day x + 1, assuming a residence time at the WWTP of approximately 24h.

$$RE\ (\%) = \frac{q_I - q_E}{q_I} \times 100 \quad \textbf{Eq. 1}$$

A concentration equivalent to half the quantification value (or the daily load corresponding to this concentration value) was considered for the calculation of RE of an antibiotic detected i.e., above its limit of detection (LOD) but below its limit of quantification (LOQ).

2.7. Risk assessment

Antibiotic concentrations in WWTP effluents were compared with Predicted No Effect Concentrations for ecotoxicological effects ($PNEC_{ecotox}$) and the promotion of antibiotic resistance ($PNEC_{resistance}$). $PNEC_{ecotox}$ values were obtained from (Tell et al., 2019), which had been derived from laboratory toxicity data for cyanobacteria (NOEC; growth inhibition)

212 divided by an assessment factor of 10 following the recommendations established by the
213 European Chemicals Agency (ECHA) (2008) and the European Commission (2018). For
214 some compounds, the $PNEC_{ecotox}$was not available (e.g. levofloxacin, moxifloxacin). For
215 these, the $PNEC_{ecotox}$was derived from published toxicity data for *Microcystis sp.* following
216 the same approach (**Table 1**).

217 $PNEC_{resistance}$ values were obtained from Bengtsson-Palme & Larsson (2016, which were
218 derived from Minimum Inhibitory Concentrations (MICs) obtained from the European
219 Committee on Antimicrobial Susceptibility Testing (EUCAST) database (EUCAST, 2022).
220 The method applied (Bengtsson-Palme & Larsson, 2016) uses the 1% lowest observed MICs
221 rounded down to the lowest concentration in the EUCAST testing scale after application of
222 an assessment factor of 10. Besides the $PNEC_{resistance}$ proposed by Bengtsson-Palme &
223 Larsson (2016), we also implemented the method proposed by Rico et al. (2017) to derive
224 $PNEC_{resistance}$ values, which is based on the calculation of the Hazardous Concentration for
225 the 5% (HC5) of the estimated minimum selective concentrations for bacteria. Similarly to
226 Bengtsson-Palme & Larsson (2016), the minimum selective concentrations (MSC) for each
227 bacterial taxon is extrapolated from the MIC data available in the EUCAST database
228 applying an assessment factor of 10. However, a log-normal distribution is then fitted to the
229 MSCs extrapolated for the bacteria included in the EUCAST database. To implement this
230 method, the MIC data for the antibiotics found in the WWTP effluents was downloaded from
231 the EUCAST database (EUCAST, 2022; **Table S5**). The taxa for which there were less than
232 30 MIC observations for each antibiotic were removed. Then the lowest MIC for each taxon-
233 antibiotic combination was derived. The lowest MIC was defined as the lowest MIC from the
234 available MICs reported in the different studies that contained at least 10 observations. Such
235 approach was implemented to reduce the risk of including individual, low MIC values that
236 may be considered outliers or flawed too much the MIC distribution. Next, the MSC was

derived by dividing the lowest MIC for each taxon-antibiotic combination by an extrapolation factor of 10. Finally, a log-normal distribution was fitted to the MSC data available for antibiotic to calculate the HC5 and their lower (5%) and upper (95%) confidence limits using the ETX2.3 software (Van Vlaardingen et al., 2004) and the methods described by Aldenberg & Jaworska 2000). The lower confidence limit of the calculated HC5 interval was chosen as the $PNEC_{resistance}$, assuming that this is the maximum concentration that prevents the development of antibiotic resistance in environmental bacteria.

The empirical cumulative distribution functions for the measured antibiotic concentrations in the WWTP effluents were compared with the ecotoxicological and resistance PNECs. We calculated the percentage of samples that exceeded both threshold concentrations. Furthermore, we estimated the antibiotic removal efficiencies that should be implemented at the studied WWTP to produce effluent concentrations below the lowest antibiotic threshold, considering both ecotoxicological and antimicrobial resistance effects.

3. RESULTS AND DISCUSION

3.1. Determination of antibiotics in IWW and EWW

Analytical quality assurance

In this work, special attention was paid to the quality of the analysis to support the reliability of the results (Hernández et al., 2023). To this aim, quality control (QC) samples were prepared from four real "blank" wastewater samples of different type (IWW and EWW), each spiked at three different concentration levels, 100, 500 and 5000 ng L^{-1}. All samples, including the "blank" samples for preparing the QCs, were analysed in 4 different sequences.

QCs recoveries in both IWW and EWW were mainlybetween 60 and 140%, which is the acceptability range for individual recoveries of control samples according to the SANTE guideline (SANTE, 2021)(**Table S6**). In some cases, the calculation of QCs recovery at the low concentration (100 ng L^{-1}) was problematic due to the presence of the analyte in the "blank" sample used for the QC preparation at concentration similar or higher than the spiked level (*e.g.*,azithromycin, clarithromycin, erythromycin, roxithromycin and cefditoren) (Hernández et al., 2023). As an example, the antibiotics clarithromycin and azithromycin showed recoveries slightly below 60% (between 51 to 56%) in QCs prepared at the low and medium concentrations. These two compounds were present at high concentrations in the samples, except for some EWW where clarithromycin was foundat lower concentration levels, butstill above 100 ng L^{-1}, and therefore no correction factor was applied for its quantification. Furthermore,Cefuroxime showed anomalous QCs at 100 ng L^{-1} in EWW QC samples, therefore no average value has been reported. Relative standard deviations (RSDs) (see **Table S6**) were mostly below 20-25%, although greater variations could be observed for some antibiotics, especially in IWW samples (*e.g.*,clindamycin and metronidazole). It is worth noting that data presented in Table S6 do not correspond to replicates of the same

sample (*i.e.*, repeatability), but to individual data from different samples analysed throughout this study.

Occurrence of antibiotics in IWW and EWW samples

The 18 antibiotics were analyzedin IWW and EWW samples collected in the different campaigns. From their concentrations, the daily mass loads were calculated by multiplying them by the daily flow rate (m^3/day) entering the WWTP. This is typically applied to correct for dilution factors related to the sewage system and weather conditions (i.e., rainwater). Concentrations and mass loads of antibiotics can be found in **Tables S7** to **S10**. As it is described in section 2.5, the samples were centrifuged before analysis, so the results shown correspond to the dissolved phase of wastewater samples. Although analysis of both liquid and solid phases surely providesa more accurate estimation of removal efficiency, the medium-high polarity of the antibiotics selected imply that they are more soluble in the aqueous phase, and hardly absorbed to the suspended particles. This suggests that analysis of the particulate phase should not significantly modify the results presented in this work.

Antibiotics belonging to the β-lactam family were not detected in any of the samples analyzed, including amoxicillin, one of the most consumed antibiotics in Spain. This fact could be related to the poor stability of these compounds in aqueous samples (Fabregat-Safontet al., 2023b), which might be explained by the limited stability of the β-lactam ring, common to all antibiotics belonging to this family of antibiotics (Lien et al., 2016b; Zuccato et al., 2005). Furthermore, the tetracycline doxycycline and the macrolide roxithromycin were not found in any sample, and the quinolone moxifloxacin was only found in EWW.

In IWW samples, five antibiotics (azithromycin, ciprofloxacin, clarithromycin, levofloxacin/ofloxacin and sulfamethoxazole) were detected in all the samples analyzed and exceeded the concentration level of 1 μg L^{-1} in at least one sample. The compounds with

highest concentrations in IWW, also showed the highest levels in the corresponding EWW samples, generally below 1 μg L^{-1} (except for azithromycin, up to 4 μg L^{-1}), which revealed low elimination rates in the WWTP. Our results are in accordance with a study from Italy (Zuccato et al., 2010) where clarithromycin, sulfamethoxazole, and the fluoroquinolones ciprofloxacin and levofloxacin/ofloxacin were the most abundant antibiotics in the four WWTPs investigated. Similarly, in another European research (Rodriguez-Mozaz et al., 2020), fluoroquinolones were observed at the highest concentrations in Portugal and Cyprus, while the macrolides azithromycin and clarithromycin were found in all the seven studied countries, Spain among them.

As can be observed in **Tables S9** and **S10**, the sum of the daily mass loads (g/day) of the detected antibiotics varies depending on the sampling day. In the case of the IWW samples, the values ranged from 89 to 453 g/day, highlighting the samples of May (I-007, I-010 and I-011) and June (I-013 and I-014) with the highest sum of daily loads, in all cases higher than 400 g/day. As regards to the EWW samples, the values were lower, between 46 to 250 g/day, with the highest total daily loads (≥ 200 g/day) in May (E-011 and E-012) and June (E-014 and E-015) as well.**Figure 2** shows the annual evolution of the daily mass load (as sum of antibiotics and antibiotic families) in both IWW and EWW samples (β-lactams are not included in this figure because they were not found in any of the samples). Macrolides were the antibiotics found at the highest mass loads, followed by the fluoroquinolones. The evolution profile of both families was similar to the sum of antibiotics in both water types, observing a decrease in mass loads at the end of summer (i.e., September) and reaching higher levels again in January. This data is consistent with Spanish data on antibiotic resistances (Plan Nacional Resistencia Antibioticos (PRAN), 2023) and described by (Solaun et al., 2022), where a decline in antibiotic prescription is observed annually with the approach of summer and a considerable increase is observed in January. The rest of antibiotics showed

a more regular pattern, although the highest daily mass loads were also reached in January, especially in IWW samples.

The antibiotics concentrations found in wastewater samples are in the line of other recent studies performed around the world (**Table S11**)and illustrate the anthropogenic impact of the use ofpharmaceuticals on urban wastewaters. Considering this widely reported issue, only the efficient removal efficiency in the WWTPs would allow to minimize the potential negative impact on the aquatic environment.

Removal efficiencies

RE of the WWTP for the selected antibiotics was estimated as described in section 2.6 (see **Tables S9** and **S10** for daily mass loads). The obtained results for the WWTP RE of the selected antibiotics are shown in **Figure 3**. In order to facilitate the visualization, RE was considered equal to 0% when a compound was undetected in IWW, but it could be quantified in EWW (e.g. a common situation for clindamycin, metronidazole and moxifloxacin). The RE estimated in the different campaigns were rather variable, particularly for some compounds (e.g trimethoprim), which could be due to some factors that affect the WWTP removal, such as temperature and hydraulic retention time (Subedi et al., 2014; Vieno et al., 2007). Highly variable elimination was also observed in another WWTP for some compounds with no clear tendency along three sampling campaigns (Bijlsma et al., 2021). The highest variability in the RE estimated in the different monitoring campaigns occurred for trimethoprim and specially for metronidazole as shown in **Figure 3**.

The average RE was above 50% for five antibiotics (azithromycin, clarithromycin, ciprofloxacin, norfloxacin, sulfamethoxazole), with sulfamethoxazole being efficiently eliminated (RE around 80%). On the contrary, erythromycin, levofloxacin/ofloxacin and trimethoprim were poorly removed with mean RE below 30%. These data and the variability

observed in RE are in line with other data reported in the literature (Behera et al., 2011; Pereira et al., 2020; Bijlsma et al., 2021; Karthikeyan & Meyer, 2006; Seifrtová et al., 2010; Zuccato et al., 2010;Lopez et al., 2022). In the case of moxifloxacin, its non-elimination observed in the present study does not agree with studies performed in US and China (He et al., 2015; Yan et al., 2014), where an elimination around 50% was reported.

Three compounds (moxifloxacin, clindamycin, and metronidazole) showed no elimination (**Figure 3**). RE=0 or even negative RE may be explained by the fact that removal in a WWTP is not only related to the treatment applied but also to the physic-chemical properties of the compounds (such as pKa, log K_{ow} and biodegradability) (Desbiolles et al., 2018; Rodriguez-Mozaz et al., 2020). It is challenging to link the antibiotics' physicochemical characteristics to the RE attained in an activated sludge system since many variables are involved (Verlicchi et al., 2012). Although more polar compounds (log K_{ow}< 2.5) usually have low sorption potential (Rogers, 1996), fluoroquinolones (log K_{ow}< 1; see **Table S3**) could bind to the sludge due to their zwiterionic character (Golet et al., 2003), causing low or even negative elimination from the WWTP (Golovko et al., 2021; Sabri et al., 2020a; Zuccato et al., 2010), as it has been observed for moxifloxacin in the present study. Negative efficiencies obviously imply that no removal occurs in the WWTP. The fact that EWW present higher pharmaceutical concentrations than IWW may be due to the cleavage of phase II metabolites, such as glucuronides and sulphates (Lacey et al., 2008; Vieno et al., 2007), during wastewater treatment, releasing thus parent compound and increasing their concentrations after treatment (Yan et al., 2014). The low removal found for metronidazole may be justified by its high solubility in water (log K_{ow} -0.02) and its low biodegradability. This compound is considered a difficult pollutant to be eliminated by using only conventional treatments (Lien et al., 2016a).

3.2. Metabolite screening

In this work, four antibiotic metabolites were tentatively identified in the screening based on the exact mass information provided by HRMS, interpretation of the fragmentation observed and agreement with ion fragments reported in the literature.

3-desmethyl trimethoprim

Trimethoprim undergoes oxidative metabolism, with the demethylated 3'- and 4'- metabolites accounting for approximately 65% and 25% of the total metabolite formation, respectively (Goldman et al., 2015). After oral administration, 50% to 60% of trimethoprim is excreted in urine within 24 hours, approximately 80% of which is unchanged parent drug (FDA, 2016). The identification of this metabolite was based on the presence of two common fragment ions shared with trimethoprim, at *m/z* 261 and 123, stablishing thus the position of the demethylation (**Table 2**, **Figure 4A**). Nevertheless, it cannot be assured which is the demethylated methoxy group, as the three moieties can be metabolized and will produce the same fragmentation (compound identified at Level 3).

Clindamycin sulfoxide

Clindamycin is manly metabolized in the liver CYP3A4 and CYP3A5 (FDA, 2019), producing two inactive metabolites: clindamycin sulfoxide and *N*-desmethyl clindamycin (FDA, 2019). Approximately 10% of unchanged clindamycin is excreted in the urine, 3.6% in the feces, and the remaining as inactive metabolites (FDA, 2019). The identification of this metabolite was based on the presence of two common fragment ions shared with clindamycin at *m/z* 126 and 377. The diagnostic ion *m/z* 377 stablishes the position of the oxide group, a sulfoxide in this case (**Table 2**, **Figure 4B**). Additionally, the observed isotope pattern fits with the presence of Cl and S atoms in the compound structure, similarly to clindamycin (compound identified at Level 2b).

N-acetyl ciprofloxacin and oxociprofloxacin

Ciprofloxacin is primarily metabolized by CYP1A2 (FDA, 2021), producing oxociprofloxacin and sulociprofloxacin (3-8% of the total dose each) (FDA, 2021). Ciprofloxacin is also metabolized to desethylene ciprofloxacin and formylciprofloxacin (both minor) (FDA, 2021), being together with the previously mentioned metabolites the 15% of a total oral dose (FDA, 2021). Unchanged ciprofloxacin resulted in 45% recovery in urine and 62% recovery in feces(LeBel, 1988). In this work, the metabolite *N*-acetyl ciprofloxacin was identified based on one shared fragment ion with the parent ciprofloxacin at *m/z* 231, stablishing thus the position of the biotransformation (compound identified at Level 2b) (**Table 2**, **Figure S1A**). For oxociprofloxacin, no common shared fragment ions were observed. Nevertheless, the three observed ion fragments were justified based on its proposed structure, although the oxo group could be located in different parts of the piperazine ring (compound identified at Level 3) (**Table 2**, **Figure S1B**).

3.3. Antibiotic risk assessment

The comparison of measured antibiotic concentrations with environmental thresholds shows that 6 out of the 11 antibiotics detectedin the WWTP effluents exceeded either the ecotoxicological or the resistance thresholds. The antibiotics with the highest percentage of exceedances were azithromycin and clarithromycin (exceedance in 100% of samples), followed by ciprofloxacin (97%), norfloxacin (77%), metronidazole (70%) and levofloxacin/ofloxacin (63%; **Table 1**). Azithromycin and clarithromycin exceeded both the $PNEC_{ecotox}$ and the $PNEC_{resistance}$ in all cases, while ciprofloxacin exceeded the $PNEC_{resistance}$ in 97% of cases and the $PNEC_{ecotox}$ in 7% of them (**Figure 5**). The calculated risks for the rest of compounds were driven by the exceedance of the resistance thresholds. The magnitude of exceedances ranged from about 3 for metronidazole and levofloxacin, to 209 for azithromycin.

Except for metronidazole, which is often used to treat bacterial vaginosis, the compounds showing the highest potential risk belong to the macrolide and quinolone groups, which are classified as antibiotics of critical importance for human health (WHO, 2019). Other studies have also pointed to these compounds as major contributors to resistance development in aquatic ecosystems. For example, Fonseca et al. (2020) identified azithromycin, ciprofloxacin and norfloxacin as the most hazardous compounds in surface waters of the Mijares River (Spain) based on a similar approach. Other studies assessing the environmental risks of ciprofloxacin at a global scale based on a literature review showed that 58% of municipal effluents exceeded the established resistance threshold, while 16% the ecotoxicity one (Kelly and Brooks 2018). A more recent study on the environmental occurrence of antibiotics and other pharmaceuticals in surface waters of 104 countries showed that 70% of the monitored antibiotics exceeded resistance thresholds in at least one location, and pointed at ciprofloxacin, clarithromycin, enrofloxacin (a quinolone mostly used in veterinary medicine) and metronidazole as the compounds showing the largest potential contribution to antibiotic resistance in European surface waters (Wilkinson et al., 2022).

Differences between the resistance thresholds calculated by Bengtsson-Palme & Larsson (2016) and those derived based on the HC5 of the MSC distribution according to Rico et al. (2017) varied for the different compounds and were, in most cases, within a factor of 2, suggesting that both approaches yield similar results and that none of the two is consistently lower or higher than the other. To date, the number of experimentally-derived MSCs that can be used to validate the theoretical approaches used by these two methods to establish environmental thresholds is very limited (but see (Gullberg et al., 2011; Liu et al., 2011)). Therefore, further experimental approaches are needed to generate MSC and to calculate MIC-MSC extrapolation ratios to refine risk calculations for the antibiotics that show a

higher contribution to the environmental resistance burden, such as ciprofloxacin, azithromycin, or clarithromycin.

To protect environmental and public health it is important to assess the degree of selection pressure by antibiotic pollution in different scenarios (Pruden et al., 2013). The outcomes of this study suggest that the emission point of these WWTP effluents constitute a marine hotspot for ecotoxicological impacts and antibiotic resistance development, where cumulative impacts may be expected due to co-exposures and the continuous nature of the WWTP effluent emission. The extension and magnitude of such impact at the discharge point will also depend on factors such as water depth, currents, or sediment characteristics. By applying a precautionary approach that considers minimal dilution in the aquatic environment, our study shows that efforts are needed to eliminate antibiotic residues during the wastewater treatment process. Based on the lowest PNEC as benchmark, we estimated target REs of approximately 60% for levofloxacin/ofloxacin and metronidazole, 80% for clarithromycin, and above 90% for azithromycin and ciprofloxacin. These elimination targets should be added to the elimination percentages already achieved by the conventional wastewater treatment methods implemented at the WWTP. In this work, most antibiotics were partially removed in the WWTP, therefore additional treatments would be required to reach the targets RE estimated in this study. Thus, conventional treatments may need optimization or advanced treatments should be implemented to improve RE (Sabri et al., 2020b), such as microfiltration and reverse osmosis (Golovko et al., 2021; Watkinson et al., 2007), phytoremediation (McCorquodale-Bauer et al., 2023) or advanced oxidation processes, ultraviolet radiation, or ozonation (Gao et al., 2012; Luo et al., 2014).

4. CONCLUSIONS

A comprehensive monitoring study has been carried to assess the input and environmental emission of antibiotics in a conventional WWTP. Azithromycin, ciprofloxacin, clarithromycin, levofloxacin/ofloxacin and sulfamethoxazole were the antibiotics found at the highest concentrations. The measured antibiotic concentrations were relatively constant throughout the year, with a decline at the end of the summer season, which highlights the potential of these kind of analyses to reflect antibiotic consumption patterns. The estimation of the WWTP removal efficiency revealed that only 5 antibiotics (sulfamethoxazole, norfloxacin, clarithromycin, ciprofloxacin and azithromycin) were eliminated above 50%, being sulfamethoxazole the only compound that could be considered completely eliminated (RE approximately 80%). About half of the detected compounds exceeded ecotoxicological and/or resistance thresholds, being azithromycin, clarithromycin, and ciprofloxacin the compounds that showed the largest number of exceedances. In total, 18 compounds were monitored, 11 detected and 6 exceeded PNEC. Despite the RE for these compounds was notable (> 40%), this study recommends the application of advanced treatment technologies to meet the proposed ecotoxicological and resistance standards. An additional screening of metabolites reported in the literature allowed the identification of four compounds derived from the antibiotics trimethoprim, clindamycin, and ciprofloxacin, illustrating the interest of including metabolites and transformation products as well in monitoring studies and to derive ecotoxicological and resistance thresholds for these compounds.

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CRediT authorship contribution statement

Elisa Gracia-Marín: Writing – original draft, Methodology, Formal analysis, Data curation, Visualization; **Andreu Rico:** Writing – original draft; Investigation, Formal analysis, Data Curation; **David Fabregat-Safont**: Writing – original draft, Investigation, Formal analysis, Data Curation; **Francisco J. López**: Writing – original draft, Investigation, Formal analysis, Data Curation; **Félix Hernández**: Resources, Funding acquisition, Writing – review & editing; **Elena Pitarch**: Project administration, Funding acquisition, Conceptualization, Supervision, Writing – review & editing; **Lubertus Bijlsma:** Funding acquisition, Conceptualization, Supervision, Data curation, Writing – review & editing.

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