Investigation of pharmaceuticals in a conventional wastewater treatment plant: removal efficiency, seasonal variation and impact of a nearby hospital

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Abstract

Discharges from the wastewater treatment plants (WWTPs) are among the main sources of contamination to receiving surface water, therefore the quality of treated wastewater needs to be properly monitored. However, not only the effluents of larger WWTPs employing advanced treatment processes have been considered, but also those from more conventional WWTPs. In this study, the occurrence and behavior of pharmaceuticals have been investigated in a conventional WWTP which receives wastewater from an urban area and a near-by hospital. 24-h composite samples were collected during one week before (influent wastewater, IWW) and after (effluent wastewater, EWW) treatment along three monitoring campaigns distributed over one year. Moreover, seven daily IWW samples discharged from a hospital were also collected. A preliminary wide-scope screening using liquid chromatography (LC) coupled to high resolution mass spectrometry allowed to identify a wide number of pharmaceuticals in the samples. Based on the screening findings, a list of 40 compounds was established for subsequent target quantitative analyses by LC-tandem mass spectrometry. Up to 75% of the compounds investigated were present in all wastewater samples. Analyte concentrations in hospital discharge samples were significantly higher, evidencing an important contribution in terms of pharmaceuticals content. Antibiotics showed the highest concentrations during the winter season, which could be related to the increase in the prescription of these compounds to treat respiratory infections. Data from this work show that the biological treatment applied was able to eliminate nearly half of the compounds under study, although still 12 pharmaceuticals were not or poorly removed.

Keywords: Pharmaceuticals; Antibiotics; Wastewater treatment; Hospital discharge; WWTP removal efficiency

1. INTRODUCTION

The investigation on the occurrence of contaminants of emerging concern (CECs), specifically pharmaceuticals, in the aquatic environment has gained much interest due to their widely use and frequent detection in the water cycle at concentrations even higher than classical persistent and/or priority substances (Corada-Fernández et al., 2017; Afonso-Olivares et al., 2017; Bellver-Domingo et al., 2019). CECs are normally not included in the routine analysis due to the lack on regulation and high analytical cost, but their presence may have a negative impact on the environment and shows on human public health (Gracia-Lor et al., 2012; Agüera et al., 2013; Galindo-Miranda et al., 2019; Hernández et al., 2019a). Environmental regulations have barely included the control of pharmaceuticals in water bodies. However, due to the growing concern about this subject, policy makers have become aware of this potential environmental and public health problem. Hence, the European Commission updated the Watch List of the Water Framework Directive (Commission Implementing Decision 2018/840) to obtain more EU-wide monitoring data, with the final goal to better regulate priority pollutants in the aquatic environment (Directive 2000/60/EC). Five antibiotics have been already included in the Watch List i.e. the penicillin amoxicillin, the fluoroquinolone ciprofloxacin and three macrolides erythromycin, clarithromycin and azithromycin. Yet in the near future, the requirements of water quality will be probably modified and become stricter, especially in relation to pharmaceutical discharges from the wastewater treatment plants (WWTPs), since the quality of wastewater effluent is of great relevance as it is one of the main sources of contamination to receiving surface water (Delgado et al., 2012).

64 Conventional treatments applied by WWTPs do not commonly remove these
65 compounds efficiently, and they can thus end-up in effluent wastewater (EWW) at
66 relatively high concentrations, frequently exceeding 1 μg/L (Gros et al., 2010; Alidina et
67 al., 2014; Montes-Grajales et al., 2017). Consequently, it is not surprising that
68 pharmaceuticals are found in receiving surface water (Dai et al., 2015; Vione et al.,
69 2018; Celic et al., 2019) and even in drinking water (Reis et al., 2019; Carmona et al.,
70 2014; Luján-Facundo et al., 2019). A number of papers have highlighted the need for
71 improving the treatment applied in the WWTPs, employing additional tertiary
72 treatment processes (Sousa et al., 2018). Although additional advanced oxidation
73 processes (AOPs) are recommended to improve the elimination of pollutants, they will
74 imply additional costs that may be difficult to bear for relatively small WWTPs.

75 The efficiency of treatment and thus the extent of pharmaceutical removal by a WWTP
76 can be restricted depending on the compounds concentration, chemical structure,
77 solubility, charge and the existence of viable bacteria in the WWTP with degradative
78 capabilities (Comber et al., 2019). Previous studies have demonstrated that the removal
79 efficiency (RE) for pharmaceuticals can vary among different and even in the same
80 treatment processes (Lee et al., 2019; Spataro et al., 2019; Papageorgiou et al., 2019).
81 Therefore, regular monitoring campaigns are required to obtain information about the
82 actual functioning of the WWTP and to evaluate the potential impact of treated water
83 on the aquatic environment. Detection, reliable identification and accurate
84 quantification of CECs is a challenge in modern analytical chemistry. Liquid
85 chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most widely
86 applied technique for the determination of pharmaceuticals in wastewater, focusing on
87 a limited list of target compounds (Serna-Galvis et al., 2019; Lee et al., 2019; de Oliveira

et al., 2020). However, the use of pharmaceuticals between regions varies spatially and temporally due to different regulations, prescription practices, etc., so the application of target methods may not be sufficient as many compounds other than analytes remain ignored in the analysis. Therefore, wide-scope screening methodologies making use of high-resolution mass spectrometry (HRMS) become necessary in order to detect and identify a high number of contaminants, allowing to select the most relevant compounds for subsequent quantitative target analysis (Hernández et al., 2015a; Hernández et al., 2015b; Wielens Becker et al., 2020; Gago-Ferrero et al., 2020).

The objectives of this work were: 1) Investigate the contribution of a continuous discharge from a hospital located in the nearby area to a small WWTP in the north of Spain; 2) Estimate the removal efficiency of the WWTP for a selected group of pharmaceuticals after application of a conventional treatment; 3) Evaluate the seasonal variation of pharmaceuticals detected in the WWTP. For this purpose, a preliminary screening by LC coupled to quadruple time of flight (QTOF) MS was carried out in order to detect and identify the most abundant pharmaceuticals in wastewater. Then, a list of 40 target pharmaceuticals was established for subsequent quantitative analysis based on LC-MS/MS with triple quadrupole (QqQ). A total of 42 samples, 21 IWW (influent wastewater) and 21 EWW (effluent wastewater), from the WWTP were quantitatively analyzed in three sampling campaigns distributed over a year. Additionally, 7 wastewater samples from the hospital were also analyzed during the first monitoring. The comparison of daily loads (g/day) in influent and effluent water allowed the estimation of RE for the selected pharmaceuticals.

2. MATERIALS AND METHODS

2.1. Pharmaceutical standards and reagents

40 pharmaceuticals (**Table 1**) from different groups and physicochemical characteristics were selected for target quantitative analysis. More details are included in the Supplementary Material (SM).

2.2. Description of the wastewater treatment plant

The WWTP from Ricao, located in Asturias (Northern Spain), treats urban wastewater of different municipalities belonging to the public sanitation system of the Güeña, Sella and Piloña rivers. The WWTP also receives different authorized industrial discharges, mainly related to the chemical, pharmaceutical, food and services sectors, so the characteristics of their discharges are usually heterogenous.

The WWTP Ricao is designed to treat discharges from an equivalent population of 54,000 inhabitants. Its maximum pre-treatment flow rate is 41,208 m^3/day and a maximum of 20,640 m^3/day when an A20 type biological process with anaerobic, anoxic chambers and aerated carousel channels is applied. The biological process is designed for organic matter, nitrogen and phosphorus removal. This treatment is a conventional treatment of active sludge, which incorporates at the reactor inlet an anaerobic zone that receives the influent residual water and the recirculated sludge, producing the fermentation reaction and phosphate elimination. The biological reactor has a capacity of 16,076 m^3 and the biologically treated effluent ends in two circular decanters (28 meters in diameter and 3,50 meters of useful height). The treated water from the WWTP is discharged to the Sella River.

The quality parameters of the effluent of the WWTP must be in accordance with the discharge authorization nº V/33/01838 of 21 April 2015 (see **Table S1** in SM), which mainly includes the parameters defined in the Water Framework Directive 2000/60/EC, such as biochemical or chemical oxygen demand (BOD5 or COD), organic matter, suspended solids and nutrients (nitrogen and phosphorus).

2.3. Sample collection

A preliminary sampling and HRMS screening were carried out before performing the three campaigns of quantitative analysis. To this aim, a 24-h composite IWW and a 24-h composite EWW sample from the WWTP Ricao were collected in June 2018. In addition, a 24-h composite sample from a continuous discharge of a hospital located in the surrounding area was also collected. 24-h composite wastewater samples were collected using a time-proportional sampling mode (100 mL, every 15 min). All these samples were screened by LC-QTOF MS. For quantitative LC-MS/MS analyses, IWW and EWW samples (24-h composite) were collected over seven consecutive days along three campaigns: 1st (September 2018), 2nd (January 2019) and 3rd (April 2019). Additionally, in the 1st campaign, seven 24-h composite samples reaching the WWTP from the hospital were also collected. **Table S2** in SM shows sampling dates and the corresponding wastewater flow rates.

All samples were collected in high-density polyethylene bottles, stored at <-20 °C, and transported to the laboratory after the last sample of the week was collected. Upon reception in the laboratory, samples were stored in the dark at -20 °C until analysis (i.e. within 2 weeks).

2.4. Sample treatment

155 A generic solid-phase extraction (SPE) procedure based on Gracia et al., 2012 was
156 applied for the screening analysis. In order to reduce matrix complexity, IWW and
157 hospital discharge samples were previously diluted x4 with Milli-Q water.

158 The procedure for quantitative determination of pharmaceuticals was based on those
159 previously developed by our research group (Boix et al., 2015; Botero-Coy et al., 2018),
160 employing direct injection of the (diluted) samples. In this work, a simple dilution x5
161 (IWW and hospital discharge) or x2 (EWW) with Milli-Q water was made in order to
162 reduce matrix complexity.

163 More details are included in SM, section 2.4.

164 ***2.5. Instrumentation***

165 Qualitative screening was performed using a Waters Acquity UPLC (Waters Corp.)
166 interfaced to a hybrid quadrupole-TOF mass spectrometer (Xevo G2 QTOF, Waters
167 Corp.), using a Z-spray electrospray (ESI) was used. Two acquisition functions with
168 different collision energies were used for MS^E experiments: the low energy (LE),
169 selecting a collision energy of 4 eV in order to obtain information about the protonated
170 molecule and adducts (if present), and the high energy (HE) function, with a collision
171 energy ramp ranging from 15 to 40 eV, in order to obtain a greater range of fragment
172 ions. The LE and HE functions settings were for both a scan time of 0.3 s.

173 Quantitative analyses were performed using a Waters Acquity H-Class UPLC (Waters
174 Corp.), equipped with a binary pump system, was interfaced to a triple quadrupole
175 (Xevo TQ-STM, Waters Corp.) mass spectrometer (Waters Corp.) with an ESI source. See
176 SM for more details.

177 ***2.6. Analysis***

2.6.1. Qualitative screening: QTOF data processing

Accurate-mass data provided by QTOF, generated at low and high collision energy (MS^E mode) during the same run, were processed using ChromaLynx XS software (within MassLynx) in combination with a homemade database containing around 1.000 pharmaceuticals and 250 metabolites (Hernández et al., 2015a; Ibañez et al., 2017). The data were automatically processed and the chromatograms obtained (Extracted Ion Chromatogram, EIC) with a narrow mass window (nw-EIC) of 20 mDa for each *m/z* ion selected. The different approaches of data processing are described in SM.

2.6.2. Target quantitative analysis

On the basis of screening results, 40 pharmaceuticals were selected in order to perform quantitative target analysis by LC-MS/MS. The experimental conditions are shown in **Table 1**. In order to facilitate accurate quantification, up to fourteen isotopically labelled internal standard (ILIS) were used for matrix effects correction. All compounds, including ILIS, were measured in positive ionization mode, with only 4 exceptions as shown in **Table 1**.

Quality controls (QC) consisted of two samples of different type, each fortified at two levels: 0.5 and 5 μg/L (IWW), and 0.2 and 2 μg/L (EWW). QCs recoveries between 60 and 140 % were considered satisfactory (SANTE/12682/2019).

For fourteen pharmaceuticals (see **Table 1**), quantification was performed using internal standard method with their corresponding ILIS. In the case of levamisole, cocaethylene-d8 was used as ILIS based on our previous experience (Boix et al., 2015). The rest of compounds were quantified by external standards with calibration curves prepared in solvent. The limit of quantification was estimated from the lowest calibration level (LCL)

taking into account the sample dilution: LCLx5 (for IWW and hospital discharge) and LCLx2 (for EWW).Positive samples will be considered as “detected” when the concentration was below LCL and at least one q/Q ratio was accomplished. For the constructions of graphs, the detected positives were given the value of half of their LCL.

3. RESULTS AND DISCUSSION

3.1. Preliminary QTOF screening

With the objective to identify a large number of pharmaceuticals in wastewaters, three different sample types (hospital discharge, IWW and EWW) were subjected to wide-scope screening by LC-QTOF MS after SPE pre-concentration to enable the detection of analytes at the low concentrations normally present.

A large number of pharmaceuticals and relevant metabolites from different therapeutic groups were investigated. Several compounds could be confirmed by comparison of retention time and experimental fragments because the reference standard was available at the laboratory. However, other compounds could only be tentatively identified (suspect screening) due to the lack of analytical standard (**Table 2)**. In such cases, the presence of the protonated molecule and fragment ions was evaluated in the low energy (LE) and high energy (HE) functions, respectively, as well as the characteristic isotope pattern when Cl or Br were present. Tentative identification was based on the information obtained by LC-QTOF MS (i.e. accurate mass of the protonated molecule and fragment ions), which was compared with online databases, such as MassBank or MetLin, or previously reported fragments in the literature. In total, 40 pharmaceuticals and/or their metabolites were identified in the three samples studied. 17 out of 40 compounds could be confirmed with their corresponding reference standard, while the remaining were tentatively identified on the basis of the accurate mass information provided by QTOF MS. The compounds confirmed with standards corresponded to pharmaceuticals and/or metabolites commonly found in wastewaters (Boix et al., 2015; Ibáñez et al., 2017; Botero-Coy et al., 2018; Rivera-

Jaimes et al., 2018; Celic, et al., 2019; Hernández et al., 2019b; Picó et al., 2019). As expected, the greatest number of pharmaceuticals was found in the hospital wastewater, while the EWW presented the lowest number of positives.

As an example, **Figure 1** shows a finding of the analgesic acetaminophen in hospital wastewater (chromatographic peak at 2.00 min). It can be observed the presence of the protonated molecule and several fragment ions, all with mass errors <5 ppm, in the HE spectrum (**Figure 1a**, top) and the LE spectrum (**Figure 1a**, bottom) of this peak. The nw-XICs for five *m/z* fragment ions are also depicted and were perfectly aligned, demonstrated that they all come from the same compound **(Figure 1b**).

Figure 2 illustrates the process followed for a tentative identification, taking as example an angiotensin II receptor antagonist found in the hospital discharge water sample. The LE spectrum in ESI positive of the chromatographic peak detected at 7.41 min, showed an abundant signal at *m/z* 425.1542 (**Figure 2a**, bottom). This would correspond to the protonated molecule of eprosartan ($C_{23}H_{25}N_2O_4S^+$, with a mass error of 1.6 ppm in relation with its theoretical exact mass). The HE spectrum showed four fragment ions at *m/z* 295.1447 ($C_{18}H_{19}N_2O_2^+$, 0 ppm), 273.1059 ($C_{15}H_{17}N_2OS^+$, -1.1 ppm), 207.1131 ($C_{11}H_{15}N_2O_2^+$, -1.4 ppm) and 135.0445 ($C_8H_7O_2^+$, -0.7 ppm) (**Figure 2a**, top). As it can be seen, the structure of these fragment ions was justified on the basis of their measured accurate masses, and all were compatible with the structure of the candidate compound. In addition, the four fragment ions were in accordance with the scientific literature (MassBank). All these data strongly support the tentative identification of the compound as eprosartan.

From the results of the wide-scope screening, a list of pharmaceuticals was established to perform target quantitative analysis in the next monitoring campaigns. In total, 40 compounds were selected including the 17 pharmaceuticals that were identified and confirmed by QTOF MS. Those compounds tentatively identified that could not be confirmed due to the absence of reference standards in our laboratory, remained as priority compounds for subsequent works in the area, because of the working calendar did not allow us to wait the acquisition of such new standards. The rest of the pharmaceuticals until completing the list of target compounds were added based on our previous experience on wastewater analysis from different WWTPs, and on their occurrence in such type of samples (their reference standards were also available in our laboratory).

3.2. Quantitative analysis by LC-MS/MS

3.2.1. Quality control analysis

The analytical methodology applied for the quantitative determination of the 40 pharmaceuticals has been previously developed and validated in our laboratory (Boix et al., 2015; Botero-Coy et al., 2018), where particular attention was paid to the evaluation of the matrix effects. Due to the high complexity and variability of the sample matrices studied in the present work, special emphasis was made on the analysis of representative quality control sample in order to support the reliability of quantitative data reported (see section 2.7). **Table 3** summarizes the average QCs recoveries for IWW and EWW, which were in general, satisfactory with values between 60 and 140 % (SANTE/12682/2019). (See **Tables S3, S4** and **S5** in SM for detailed information).

The complexity of the matrix samples analysed in combination with the low analyte concentrations make this type of analysis complicated, being quite difficult finding a compromise to get fully satisfactory data for all compounds. Thus, some exceptions, among the 40 pharmaceuticals investigated, were observed. The most remarkable were the antibiotics ciprofloxacin and norfloxacin, for which poor reproducibility and average recoveries out of the established range 60-140 % were obtained. Three more compounds, all analysed in negative ESI (gemfibrozil, ketoprofen and naproxen), could not be properly evaluated due to the lack of sensitivity in negative ionization mode at the fortified levels tested. The antibiotics clarithromycin, roxithromycin and trimethoprim presented average recoveries near the acceptable range, but slightly greater than 140 %, especially at the high fortification levels. A possible explanation could be that these antibiotics are more prone to matrix enhancement resulting in apparent higher recoveries, which could not be corrected due to the lack of analyte ILIS. For the antibiotic azithromycin, the average recovery was also near the acceptable range, but slightly below 50 % .

Regarding the impact of the above mentioned exceptions in data reported, it was limited to only those cases where positive detections were found. The most noticeable corresponded to the antibiotics ciprofloxacin and norfloxacin, which could not be quantified with the required accuracy, despite being found in all samples at relatively high concentrations. For these two compounds, guidance data are presented, which should be considered as approximate concentration range.

3.2.2. Occurrence of pharmaceuticals in wastewaters

A total of 21 IWW 24-h composite samples were collected from the WWTP along the three sampling campaigns (see “Materials and methods”). During the same period, 21 EWW 24-h composite samples were also collected in order to evaluate the removal efficiency of the WWTP. **Table 4** summarizes the average weekly concentrations of pharmaceuticals in IWW and EWW samples in the three sampling campaigns. For more details see **Tables S6-S11** in SM. As it can be seen, 34 out of 40 pharmaceuticals were found, illustrating the wide presence of these emerging contaminants in wastewater, even after the treatment applied in the WWTP based on a combined biological process (anaerobic-anoxic-aerobic). Among them, the four antibiotics included in the European Watch List (Commission Implementing Decision 2018/840) - the fluoroquinolone ciprofloxacin and three macrolides, erythromycin, clarithromycin and azithromycin – were also found. Only six compounds from the target list were not detected in any of the samples analysed: three antibiotics (furaltadone, lincomycin and roxithromycin), two hypolipidemic agents (bezafibrate and gemfibrozil) and one anti-inflammatory (ketoprofen).

In IWW samples, the highest concentrations corresponded to the analgesic acetaminophen (5.4 μg/L), the anti-inflammatory naproxen (2.4 μg/L) and the antiepileptic gabapentin (3.2 μg/L). The majority of the pharmaceuticals showed markedly lower average concentrations in treated waters, which indicates that most of them were eliminated/retained in the WWTP, at least partially. Thus, in EWW most concentrations did not exceeded the average weekly value of 0.1 μg/L, with a few exceptions such as gabapentin (1.1 μg/L), irbesartan (0.13 μg/L) and tramadol (0.37 μg/L). Several compounds, such as clindamycin, levamisole, lorazepam, oxolinic acid, pantoprazole, tramadol and venlafaxine, were found at similar concentration levels in

IWW and EWW, or even at higher concentrations in EWW, which suggests the non-removal of these compounds using the primary treatment applied in the WWTP. Pharmaceutical elimination in WWTPs is probably a complex process as many plants are equipped with the main objective of removing biodegradable carbon, nitrogen and phosphorus compounds and microbiological organisms (Pereira et al., 2020) and not equipped to remove complex contaminants. The finding of higher concentrations in the treated water has been reported several times in the scientific literature (Botero-Coy et al., 2018; Jelic et al., 2011; Gros et al., 2010). The low removal efficiency of the WWTP for these compounds together with the possible release of conjugates (usually glucuronides and sulphates) during the treatment of wastewater might be possible causes of the increase in concentrations (Lacey et al., 2008; Vieno et al., 2007). In addition, matrix effects (commonly ionization suppression) are much higher in IWW than in EWW which may hamper the detection/quantification of some compounds in IWW, particularly when they are present at very low concentrations (Bijlsma et al., 2012).

Special attention should be paid to the presence of antibiotics in wastewater, especially EWW, due to their potential hazardous to the aquatic environment. Recent investigations show that WWTPs constitute hotspots for antibiotic emissions, contributing to the enrichment of resistance genes in surface water ecosystems (Buelow et al., 2020). In Spain, several macrolide antibiotics were determined (Gusamaroli et al., 2019), being azithromycin the compound detected at the highest concentration level, both in IWW and EWW. Moreover, Rodriguez-Mozaz et al. performed a comprehensive monitoring of antibiotics in wastewater samples of WWTPs from 7 European countries, where Spain presented the highest concentrations for azithromycin, ciprofloxacin,

343 clindamycin, clarithromycin, metronidazole and sulfamethoxazole (Rodríguez-Mozaz et
344 al., 2020). The results obtained in these works were in agreement with the present
345 study, where thirteen of the 16 antibiotics investigated were detected in both IWW and
346 EWW and of which azithromycin, ciprofloxacin, trimethoprim, clarithromycin and
347 norfloxacin showed in general the highest concentrations.

348 The comparison of average concentrations for the several pharmaceutical families
349 studied allows to obtain interesting conclusions (see **Figure 3**). The season with lowest
350 total concentrations for nearly all families of compounds was winter (green bars, 2nd
351 campaign, January 2019), in both IWW and EWW, but there was an evident exception
352 with the group of antibiotics, which concentrations in wastewater were notably higher
353 in winter. A fact that is not surprising due to the expected increase in the prescription of
354 antibiotics to fight respiratory infections typically during winter. It is also illustrative, by
355 comparing the top and bottom graphics, the notable decrease in concentrations for all
356 families in the EWW (bottom). This evidences a certain removal efficiency in the WWTP
357 as will be discussed in section 3.2.4.

358 Although the results obtained in this study correspond to the dissolved phase of
359 wastewater samples, in every campaign a preliminary analysis of a sludge sample was
360 also performed. Compared to the wastewater analyzed at the same period, much less
361 pharmaceuticals could be quantified in the particulate material, surely due to their
362 absence or their very low concentrations. This could be explained by the medium-high
363 polarity of the compounds under study, making them more soluble in the aqueous
364 phase and being hardly adsorbed on the sludge. This suggests that analysis of the
365 particulate phase should not significantly modify the results presented in this work.

3.2.3. Contribution of the hospital discharge

In the first campaign (September 2018), in addition to the seven IWW samples from the WWTP, another seven 24-h composite samples were collected from a continuous discharge of an hospital in the nearby area. The results of quantitative analysis by LC-MS/MS for IWW and hospital discharge samples are included in **Tables S6** and **S12**, respectively, in SM.

In the hospital samples, 28 out of the 40 pharmaceuticals investigated were detected. In general, pharmaceutical concentrations were similar along the sampling week. Some exceptions were erythromycin, losartan, pantoprazole, phenazone, sulfamethoxazole, trimethoprim and valsartan, which presented greater variations (RSD above 50%). The highest concentrations in hospital samples corresponded to the widely consumed analgesic acetaminophen (159 μg/L), the antiepileptic gabapentin (23 μg/L) and the anti-inflammatory naproxen (2.9 μg/L).

Similarly, 28 out the 40 compounds were also detected in IWW collected during the same days, of which 24 coincided with those found in hospital water. In general, the concentration levels in IWW were rather consistent throughout the whole week, with the exception of phenazone, which presented greater variation (RSD greater than 50 %). Similarly to the hospital discharge, the highest concentrations corresponded to the analgesic acetaminophen (8.7 μg/L), the antiepileptic gabapentin (4.7 μg/L) and the anti-inflammatory naproxen (2.4 μg/L), whose concentrations were significantly lower than in the hospital wastewater, and in agreement with data reported in the literature (e.g. Santos et al., 2013; de Oliveira et al., 2020; Niemi et al., 2020).

Figure 4 shows the average weekly concentrations of pharmaceuticals in the hospital discharge and in IWW during the first campaign. In order to represent the compounds detected (but not quantified), a concentration value equal to half of their LCL was estimated. In general, concentrations in the hospital samples were clearly higher than in IWW of the WWTP, except for five compounds – clarithromycin, irbesartan, levamisole, primidone and tetracycline – which showed mean concentrations slightly higher in the IWW. The results suggest that a large part of the pharmaceuticals studied reached the WWTP mainly through the discharge from the hospital. This was expected, and it is in agreement with Bellver-Domingo et al. (2019), who reported hospitals as one of the main facilities that discharge anti-inflammatories into Valencian urban wastewater.

3.2.4. Estimation of removal efficiencies of pharmaceuticals in the WWTP

The efficiency of pharmaceuticals removal in a WWTP can be estimated from the compound concentrations and/or from pharmaceutical daily loads in IWW and in EWW. Most estimations are based on analyte concentrations (Postigo et al., 2010; Gracia-Lor et al., 2012; Bijlsma et al., 2014; Botero-Coy et al., 2018; Villar-Navarro et al., 2018), however in this study we have used daily loads (g/day), which were calculated taking into account the concentrations in wastewater and the daily flows of IWW and EWW. Although the use of concentrations is a useful approach, the estimation based on total loads seems more realistic as it takes into account the total amount of pharmaceuticals entering into the WWTP and the total loads in the treated water, and therefore it takes into account the influence of the amount of water in each case (see **Table S2** in SM). Thus, we compared the daily loads at the entrance and the exit of the next day, assuming a residence time at the WWTP of 24h. From the seven daily loads, we are able

411 to calculate the average daily loads for the whole week (g/day), which were finally used
412 for RE estimation. Data on daily and average weekly loads are shown in **Tables S13-S18**
413 and **Table S19**, respectively, in SM, for the three sampling campaigns. From these data,
414 the daily and average RE (%) were calculated for each campaign, as shown in **Tables**
415 **S20-S22** of SM.

416 **Figure 5** shows the average RE of pharmaceuticals in each monitoring campaign (the
417 two antibiotics with estimated concentrations, ciprofloxacin and norfloxacin, are not
418 included). Different behaviours were observed, with a first group including 34 % of the
419 compounds which were removed almost completely, with average RE above 75 %
420 (acetaminophen, atorvastatin, azithromycin, enalapril, losartan, metronidazole,
421 naproxen, salbutamol, tetracycline, trimethoprim and valsartan). A second group
422 included pharmaceuticals for which the elimination was not total, but greater than 50 %
423 (diclofenac, gabapentin and phenazone). Another six compounds presented slightly
424 variable RE along the three campaigns, with a tendency to poor removal (RE ≤ 40 %)
425 (irbesartan, levamisole, lorazepam, primidone, tramadol and venlafaxine). A fourth
426 group corresponded to 18 % of compounds detected which did not seem to be
427 eliminated, with RE near 0 % or even negative RE (alprazolam, clindamycin, metoprolol,
428 nalidixic acid, pantoprazole and sulfadiazine). The remaining analytes showed highly
429 variable elimination data along the three sampling campaigns, with no clear tendency
430 (carbamazepine, clarithromycin, erythromycin, omeprazole sulphide 4-OH, oxolonic acid
431 and sulfamethoxazole).

432 In summary, 14 out of 32 pharmaceuticals detected, which account for 44 % of the
433 compounds, were removed (more than 50 %) in the WWTP using a conventional

treatment based on a combined biological process (anaerobic-anoxic-aerobic). The fact that RE were calculated based on three weekly sampling campaigns in different periods of the year (i.e. different climatic conditions) makes data reported more robust, especially for those pharmaceuticals that showed consistent behavior. The results obtained are mostly in agreement with those reported elsewhere (Gros et al., 2010; Jelic et al., 2011; Gracia-Lor et al., 2012; Botero-Coy et al., 2019; Reis et al., 2019; Lee et al., 2019; Serna-Garvis et al., 2019).

It is important to remark the potential impact on the aquatic environment of emerging contaminants present in treated wastewater. Although around half of the pharmaceuticals investigated in this work were partially or totally removed in the WWTP, the use of a secondary and an optional tertiary treatment process seems necessary in order to improve the removal of these compounds and to protect the environment, although those additional treatments are always associated with a higher cost (Pereira et al., 2020). Yet in the near future, the requirements of water quality will be surely modified and become stricter, especially in relation to pharmaceutical discharges from WWTPs, since the quality of wastewater effluent is of great relevance as it is one of the main sources of contamination to receiving surface water (Delgado et al., 2012). Frequent monitoring campaigns are needed to determine the quality of treated water in terms of emerging contaminants, but risk assessment studies also are required to establish the potential harmful effects on these compounds on the aquatic environment. Conducting monitoring campaigns making use of advanced analytical techniques will be necessary to update European regulations particularly in relation to the quality of wastewater effluents.

4. CONCLUSIONS

The occurrence of pharmaceuticals in wastewater from a conventional WWTP has been investigated, as well as their possible elimination as a result of the treatment applied. IWW and EWW samples from the WWTP were collected in three seasonal campaigns, as well as raw wastewater samples from an hospital discharge nearby the plant to evaluate the impact in terms of pharmaceuticals content. Due to the high number of pharmaceuticals that may be present in this type of samples, a preliminary wide-scope screening using LC-HRMS with QTOF MS was applied to identify the most relevant/abundant compounds in the samples. Based on data from the screening, 40 compounds were selected for subsequent target quantitative analysis by LC-MS/MS with QqQ.

Most of pharmaceuticals detected in IWW from the WWTP were identified in hospital discharge samples at concentrations significantly higher, which seems to indicate that a large part of pharmaceuticals reach the WWTP mainly through the discharge from the hospital.

The removal efficiency of pharmaceuticals was estimated from daily loads in the IWW and in EWW, which were calculated for the three one-week campaigns. From the 32 compounds detected in the water samples, the wide majority presented lower concentrations in treated water compared to raw wastewater. Thus, around 50 % of the compounds were totally (> 80 %) or partially (RE > 50%) removed using the conventional biological treatment, but still a large number of compounds could not be efficiently eliminated. Most of concentrations in EWW did not exceed the average weekly value of 0.1 µg/L, with a few exceptions such as gabapentin (1.1 µg/L), irbesartan (0.13 µg/L) and

tramadol (0.37 μg/L). Other compounds, such as clindamycin, levamisole, lorazepam, oxolinic acid, pantoprazole and venlafaxine, were found at similar concentrations in IWW and EWW, which suggests the non-removal of these compounds in the WWTP. The fact that some pharmaceuticals still remain in the treated wastewater may suppose a risk for the aquatic environment. Therefore, additional treatments are required to improve the removal of these emerging contaminants, as well as conducting periodically ambitious monitoring campaigns to evaluate the performance of the WWTP and the potential impact of treated water on the aquatic environment.

Finally, the study of seasonal variation demonstrated that concentration levels of antibiotics were notably higher in winter due to typical infections of that period of the year.

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Table 1. LC-MS/MS conditions (cone value 10V) for pharmaceuticals selected. All compounds were measured in positive mode, with the exception of 4 compounds that were measured in negative mode (marked as (-)). Quantification (Q) and confirmation (q) transitions. Collision energy (CE). Lowest calibration level (LCL, * x5 for raw and x2 for treated samples), estimated as the limit of quantification. In italic, ILIS used for quantification of their corresponded analyte. [a] Compounds included in the Watch List of the Commission Decision 2018/840

Family	Compounds	Transition (Q)	CE (eV)	Transition (q)	CE (eV)	LCL* (ng/L)
Analgesic	Acetaminophen	152.0 > 110.0	15	152.0 > 93.0	20	5
				152.0 > 65.0	25	
	Acetaminophen-d_4	*156.0 > 114.0*	*10*	-	-	-
Benzodiazepine	Alprazolam	309.0 > 281.0	25	309.0 > 205.0	25	5
				309.0 > 274.0	25	
Hypolipidemic agent	Atorvastatin	559.0 > 440.0	20	559.0 > 466.0	15	5
				559.0 > 292.0	25	
	Atorvastatin-d_5	*564.0 > 445.0*	*20*	-	-	-
Antibiotic	Azithromycin[a]	749.4 > 591.4	25	749.4 > 82.9	45	50
				749.4 > 116.1	45	
	Azithromycin-d_3	*752.2 > 594.2*	*25*	-	-	-
Hypolipidemic agent	Bezafibrate (-)	360.0 > 274.0	20	360.0 > 154.0	25	1000
				360.0 > 85.0	15	
Antiepileptic	Carbamazepine	237.0 > 194.0	20	237.0 > 179.0	25	5
				237.0 > 192.0	10	
	Carbamazepine 10,11-epoxide-d_{10}	*263.0 > 190.0*	*25*	-	-	-
Antibiotic	Ciprofloxacin[a]	332.0 > 231.0	25	332.0 > 288.0	15	50
				332.0 > 314.0	20	
	Ciprofloxacin-d_8	*340.1 > 322.1*	*20*	-	-	-
Antibiotic	Clarithromycin[a]	590.0 > 158.0	20	590.0 > 116.0	25	5
				590.0 > 98.0	25	
Antibiotic	Clindamycin	425.1 > 126.0	20	425.1 > 337.0	20	5
				425.1 > 389.0	15	
Nonsteroidal anti-inflammatory	Diclofenac	296.2 > 214.2	30	296.2 > 250.0	10	5
				296.2 > 278.0	5	
	Diclofenac-d_4	*300.1 > 219.2*	*20*	-	-	-
Antihypertensive	Enalapril	377.0 > 234.0	15	377.0 > 117.0	25	5
				377.0 > 303.0	15	
Antibiotic	Erythromycin[a]	734.0 > 158.0	25	734.0 > 576.0	15	10
				734.0 > 558.0	15	
	Erythromycin-^{13}C-d_3	*738.1 > 161.9*	*35*	-	-	-
Antibiotic	Furaltadone	325.0 > 100.0	20	325.0 > 252.0	15	5
				325.0 > 281.0	10	
Antiepileptic	Gabapentin	172.0 > 137.0	15	172.0 > 154.2	15	5
				172.0 > 95.0	20	
Hypolipidemic agent	Gemfibrozil (-)	249.0 > 113.0	10	249.0 > 121.0	20	1000
				249.0 > 127.0	10	
Antihypertensive	Irbesartan	429.0 > 207.0	25	429.0 > 195.0	20	5
				429.0 > 180.0	25	
	Irbesartan-d_6	*435.1 > 213.3*	*25*	-	-	-
Nonsteroidal anti-inflammatory	Ketoprofen (-)	253.0 > 79.0	10	253.0 > 92.0	20	1000
				253.0 > 209.0	10	
Anthelmintic agent	Levamisole	205.0 > 178.0	20	205.0 > 91.0	25	5
				205.0 > 123.0	25	
	Cocaethylene-d_8	*326.0 > 204.0*	*20*	-	-	-
Antibiotic	Lincomycin	407.0 > 126.0	20	407.0 > 359.0	15	5
				407.0 > 389.0	15	

Family	Compounds	Transition (Q)	CE (eV)	Transition (q)	CE (eV)	LCL* (ng/L)
Benzodiazepine	Lorazepam	321.0 > 275.0	20	321.0 > 303.0	15	10
				321.0 > 229.0	25	
Antihypertensive	Losartan	423.1 > 207.1	15	423.1 > 377.1	15	5
				423.1 > 405.1	10	
Beta-blocker agent	Metropolol	268.2 > 116.0	15	268.2 > 74.0	20	5
				268.2 > 191.0	15	
Antibiotic	Metronidazole	172.0 > 127.9	15	172.0 > 82.1	20	5
				172.0 > 55.9	20	
Antibiotic	Nalidixic acid	233.0 > 187.0	25	233.0 > 215.0	10	5
				233.0 > 159.0	25	
Nonsteroidal anti-inflammatory	Naproxen (-)	229.0 > 170.	20	229.0 > 185.0	12	1000
				185.0 > 169.0	20	
Antibiotic	Norfloxacin	320.0 > 233.0	25	320.0 > 276.0	15	50
				320.0 > 302.0	20	
	Norfloxacin-d_5	*325.0 > 238.0*	*20*	-	-	-
Antiulcer drug	Omeprazole sulfide, 4-hydroxy[a]	316.0 > 168.0	20	316.0 > 149.0	20	5
				316.0 > 283.0	15	
	Omeprazole-d_3	*349.0 > 198.0*	*10*	-	-	-
Antibiotic	Oxolinic acid	262.0 > 216.0	25	262.0 > 244.0	15	5
				262.0 > 158.0	25	
Antiulcer drug	Pantoprazole	384.0 > 200.0	10	384.0 > 138.0	25	5
				384.0 > 153.0	15	
Nonsteroidal anti-inflammatory	Phenazone	189.3 > 131.1	20	189.3 > 104.1	20	10
				189.3 > 58.1	20	
Antiepileptic	Primidone	219.2 > 162.0	10	219.2 > 91.0	20	5
				219.2 > 119.2	15	
Antibiotic	Roxithromycin	679.0 > 158.0	25	679.0 > 116.0	25	5
				679.0 > 98.0	25	
Beta-blocker agent	Salbutamol	240.0 > 148.0	15	240.0 > 222.1	10	5
				240.0 > 166.1	10	
Antibiotic	Sulfadiazine	251.0 > 156.0	15	251.0 > 92.0	25	5
				251.0 > 108.0	20	
Antibiotic	Sulfamethoxazole	254.0 > 92.0	25	254.0 > 156.0	15	5
				254.0 > 108.0	20	
	Sulfamethoxazole-$^{13}C_6$	*260.0 > 162.0*	*15*	-	-	-
Antibiotic	Tetracycline	445.0 > 154.0	25	445.0 > 410.0	15	5
				445.0 > 427.0	10	
Analgesic	Tramadol	264.0 > 58.0	10	264.0 > 121.0	25	5
				264.0 > 246.0	10	
Antibiotic	Trimetroprim	291.0 > 123.0	25	291.0 > 230.0	20	5
				291.0 > 261.0	25	
Antihypertensive	Valsartan	436.0 > 207.0	25	436.0 > 235.0	15	5
				436.0 > 261.0	15	
	Valsartan-d_8	*444.0 > 207.0*	*25*	-	-	-
Antidepressant	Venlafaxine	278.0 > 58.0	15	278.0 > 260.0	10	5
				278.0 > 121.0	25	
	Venlafaxin-d_6	*284.3 > 64.1*	*25*	-	-	-

Table 2. Pharmaceuticals identified in wastewater samples from the WWTP after UHPLC-QTOF MS screening

IWW	**EWW**	**Hospital discharge**	
4-FAA	*4-FAA*	*4-AA*	Levofloxacin*
4-AAA	*4-AAA*	*4-FAA*	Lidocaine*
Acetaminophen	Amperozide*	*4-MAA*	**Losartan**
Amperozide*	**Carbamazepine**	*4-AAA*	*Meclofenamic acid**
Diclofenac	*Clopidogrel carboxylic acid*	**Acetaminophen**	**Metronidazole**
Fenofibric acid	**Diclofenac**	*Acethyl-sulfamethoxazole**	**Naproxen**
Gabapentin	**Gabapentin**	Amoxicilline*	Memantine*
Gemfibrozil	**Irbesartan**	Amperozide*	*o-Desmethyl venlafaxine*
Irbesartan	Lamotrigine*	Atenolol*	Ofloxacin*
Ketoprofen	*Meclofenamic acid*	**Atorvastatin**	***Omeprazole sulfide 4-OH***
Naproxen	Narasin*	**Ciprofloxacin**	Oxcarbazepine*
Narasin*	*o-Desmethyl venlafaxine*	*Clopidogrel carboxylic acid*	Pregabalin*
o-Desmethyl venlafaxine	Oxcarbazepine*	**Diclofenac**	Propanolol*
Oxcarbazepine*		Eprosartan*	Quetiapine*
Venlafaxine		Esomeprazole*	Rimantadine*
		Fenofibric acid	**Sulfamethoxazole**
		Gabapentin	Sulfapyridine*
		Gemfibrozil	**Trimethoprim**
		Irbesartan	**Valsartan**
		Ketoprofen	**Venlafaxine**

4-AA: 4-aminoantipyrine
4-AAA: 4-acethylaminoantipyrine
4-FAA: 4-formylaminoantipyrine
4-MAA: 4-methylaminoantipyrine
Metabolites are shown in italic
In bold, pharmaceuticals included in the subsequent quantitative analysis by UHPLC-MS/MS
** Suspect compound, tentative identification*

Table 3. Average recoveries (%) of QCs analyzed in the three sampling campaigns for wastewaters (IWW and EWW) from the WWTP

		IWW		EWW	
		0.5	**5**	**0.2**	**2**
Compounds	**ILIS**	**µg/L**	**µg/L**	**µg/L**	**µg/L**
Acetaminophen	Acetaminophen-d_4	92	100	83	100
Alprazolam	-	87	107	84	94
Atorvastatin	Atorvastatin-d_5	106	112	90	88
Azitromycin	Azitromycin-d_3	***34***[a]	***48***[a]	***30***[a]	65[a]
Bezafibrate	-	102[b]	78[a]	136[b]	82[a]
Carbamazepine	Carbamazepine 10,11-epoxide-d_{10}	113	[c]	109	[c]
Clarithromycin	-	127[a]	***161***[a]	122	***145***[b]
Clindamycin	-	105	119	117	121
Diclofenac	Diclofenac-d_4	94	109	102	110
Enalapril	-	83	87	85	87
Erythromycin	Erythromycin-^{13}Cd3	83	94	82	107[a]
Furaltadone	-	107	106	106	105
Gabapentin	-	114	115	133	113
Gemfibrozil	-	-	112[b]	-	101[b]
Irbesartan	Irbesartan-d_6	85	121	117	107
Ketoprofen	-	-	109[b]	-	84[b]
Levamisole	Cocaethylene-d_8	95	127	107	136
Lincomycin	-	120	124	110	101
Lorazepam	-	105	80	77	94
Losartan	-	88	90	89	91
Metoprolol	-	102[b]	119[b]	104[b]	127[b]
Metronidazole	-	98	102	100	106
Nalidixic acid	-	84	84	81	78
Naproxen	-	-	67[a]	-	90
Omeprazole sulfide, 4-OH	Omeprazole-d_3	100	87	97	89
Oxolinic acid	-	74	70	79	70
Pantoprazole	-	104	99	103	82
Phenazone	-	101	113	87	98
Primidone	-	98	99	91	97
Roxithromycin	-	114[a]	127[b]	139	***145***[b]
Salbutamol	-	102	118	131	126
Sulfadiazine	Sulfamethoxazole-$^{13}C_6$	95	100	80	90
Sulfamethoxazole	Sulfamethoxazole-$^{13}C_6$	107	109	103	109
Tetracycline	-	80	81	89	69
Tramadol	-	104[b]	112[b]	107[b]	116[b]
Trimetroprim	-	123[b]	***149***[b]	125[b]	***156***[b]
Valsartan	Valsartan-d_8	78	91	90	91
Venlafaxine	Venlafaxin-d_6	86	111	84	110

*In **bold** and **italic**, recoveries out of accepted range (60-140 %) are shown*

[a] Average of two available values

[b] Only one available value

[c] Not calculated due to lack of linearity at high concentration levels

- Value not available due to the lack of sensitivity, which prevents reaching the lowest concentrations tested

Table 4. Average weekly concentrations (ng/L) of pharmaceuticals in influent and effluent wastewater samples from the WWTP in the three sampling campaigns

	IWW				EWW			
Compounds	**1st**	**2nd**	**3rd**	**Average**	**1st**	**2nd**	**3rd**	**Average**
Acetaminophen	6490	4564	5030	**5361**	-	d	-	**d**
Alprazolam	-	d	-	**d**	d	d	d	**d**
Atorvastatin	87	d	88	**88**[a]	d	-	-	**d**
Azitromycin	186	328	-	**257**[a]	-	d	-	**d**
Bezafibrate	-	-	-	**-**	-	-	-	**-**
Carbamazepine	d	-	-	**d**	d	-	d	**d**
Ciprofloxacin	149700	8191	1270	**53054**	3640	2242	700	**2194**
Clarithromycin	97	192	-	**145**[a]	48	107	37	**64**
Clindamycin	-	-	-	**-**	d	-	d	**d**
Diclofenac	232	56	223	**170**	143	26	126	**98**
Enalapril	50	-	29	**40**[a]	-	-	-	**-**
Erythromycin	25	64	d	**45**[a]	28	d	13	**21**[a]
Furaltadone	-	-	-	**-**	-	-	-	**-**
Gabapentin	4013	1836	3775	**3208**	1555	528	1125	**1069**
Gemfibrozil	-	-	-	**-**	-	-	-	**-**
Irbesartan	223	63	181	**156**	175	57	159	**130**
Ketoprofen	-	-	-	**-**	-	-	-	**-**
Levamisole	29	-	-	**29**[b]	28	d	13	**21**[a]
Lincomycin	-	-	-	**-**	-	-	-	**-**
Lorazepam	34	-	25	**30**[a]	44	d	21	**33**[a]
Losartan	168	27	67	**87**	12	10	15	**12**
Metoprolol	d	d	d	**d**	d	d	d	**d**
Metronidazole	d	37	54	**46**[a]	d	d	d	**d**
Nalidixic acid	-	-	-	**-**	d	-	-	**d**
Naproxen	2365	-	-	**2365**[b]	-	-	-	**-**
Norfloxacin	880	10386	530	**3932**	800	2455	350	**1202**
Omeprazole sulfide. 4-OH	66	d	50	**58**[a]	38	d	54	**46**[a]
Oxolinic acid	-	d	-	**d**	-	-	15	**15**[b]
Pantoprazole	-	d	d	**d**	19	d	d	**19**[a]
Phenazone	32	42	-	**37**[a]	d	-	-	**d**
Primidone	76	-	50	**63**[a]	72	11	40	**41**
Roxithromycin	-	-	-	**-**	-	-	-	**-**
Salbutamol	d	d	d	**d**	d	-	d	**d**
Sulfadiazine	-	-	d	**d**	-	-	d	**d**
Sulfamethoxazole	74	d	34	**54**[a]	33	13	14	**20**
Tetracycline	44	103	55	**67**	19	-	16	**18**
Tramadol	625	119	471	**405**	594	112	398	**368**
Trimetroprim	137	96	231	**155**	15	21	37	**24**
Valsartan	507	136	446	**363**	26	31	37	**31**
Venlafaxine	162	43	123	**109**	172	35	119	**109**

d: detected, not quantified. Concentration below LCL and at least one q/Q ratio was accomplished

Underlined: estimated concentration

[a] *Average data from two samplings*

[b] *Data from only one sampling*

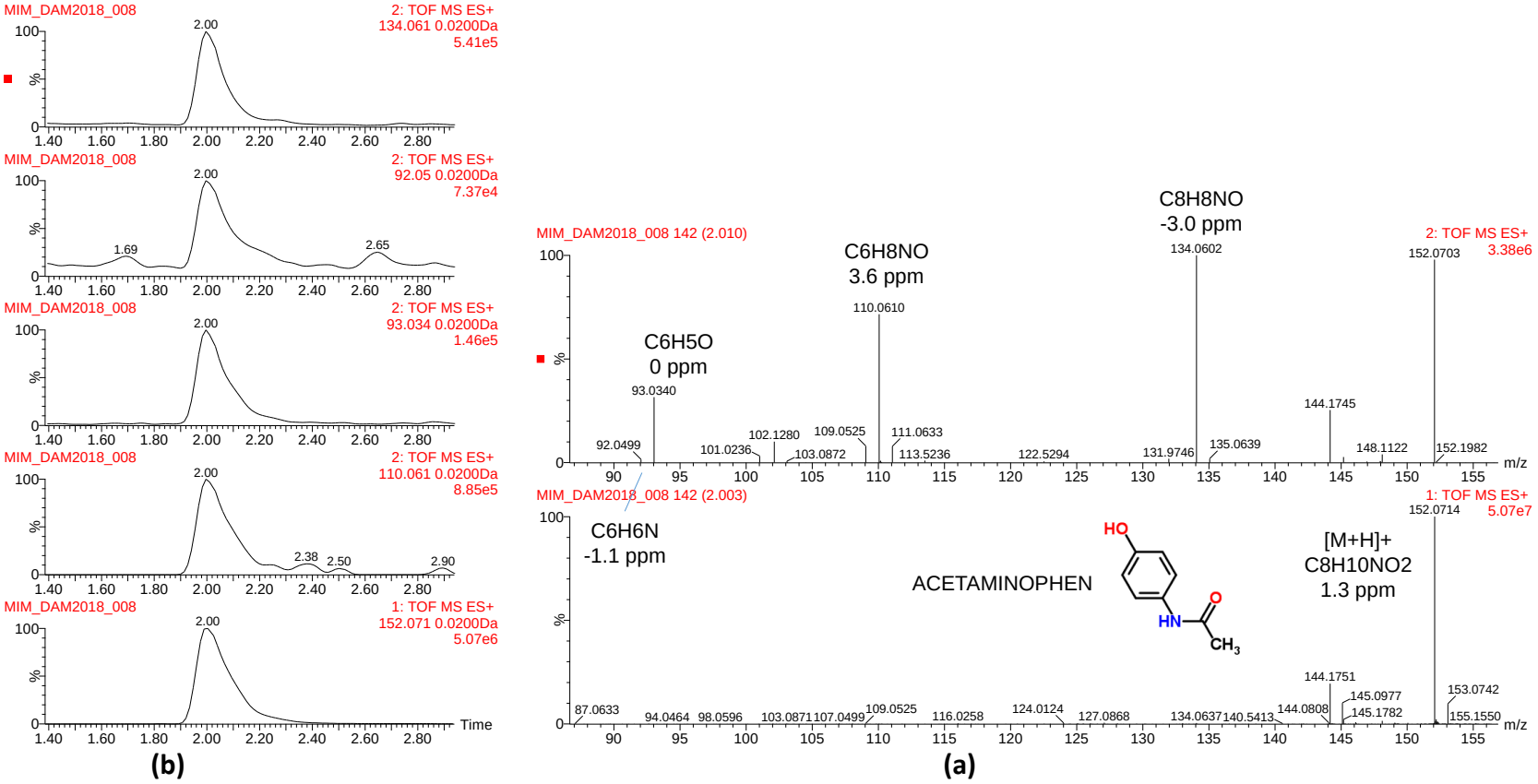


Figure 1. Detection and identification of acetaminophen in the analysis by LC-QTOF MS of the sample corresponded to the hospital discharge. (a) LE (bottom) and HE (top) mass spectra of the chromatographic peak at retention time 2.00 min. (b) XICs with 0.02 Da mass window for the protonated molecule in LE and different ions observed in HE

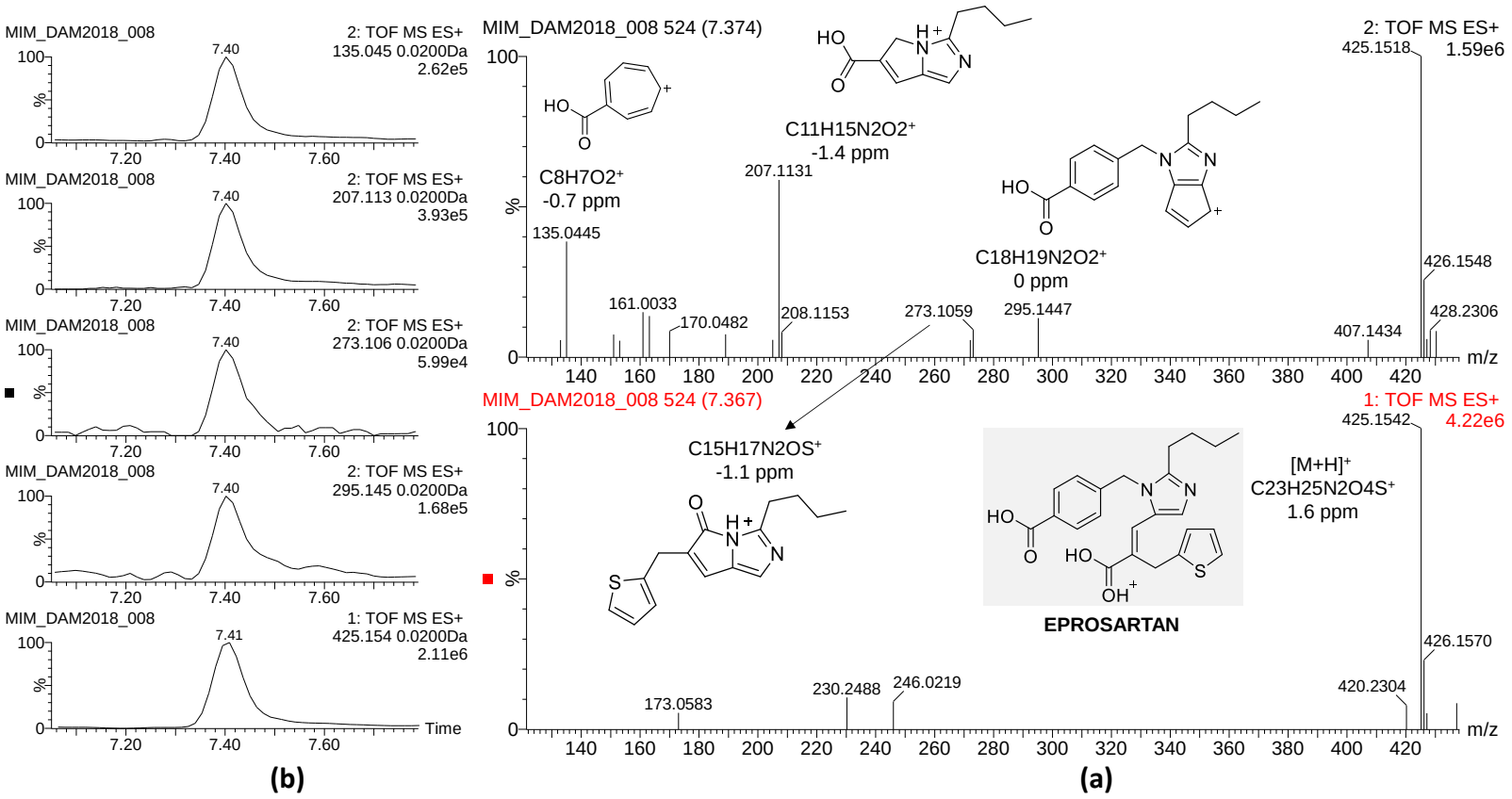
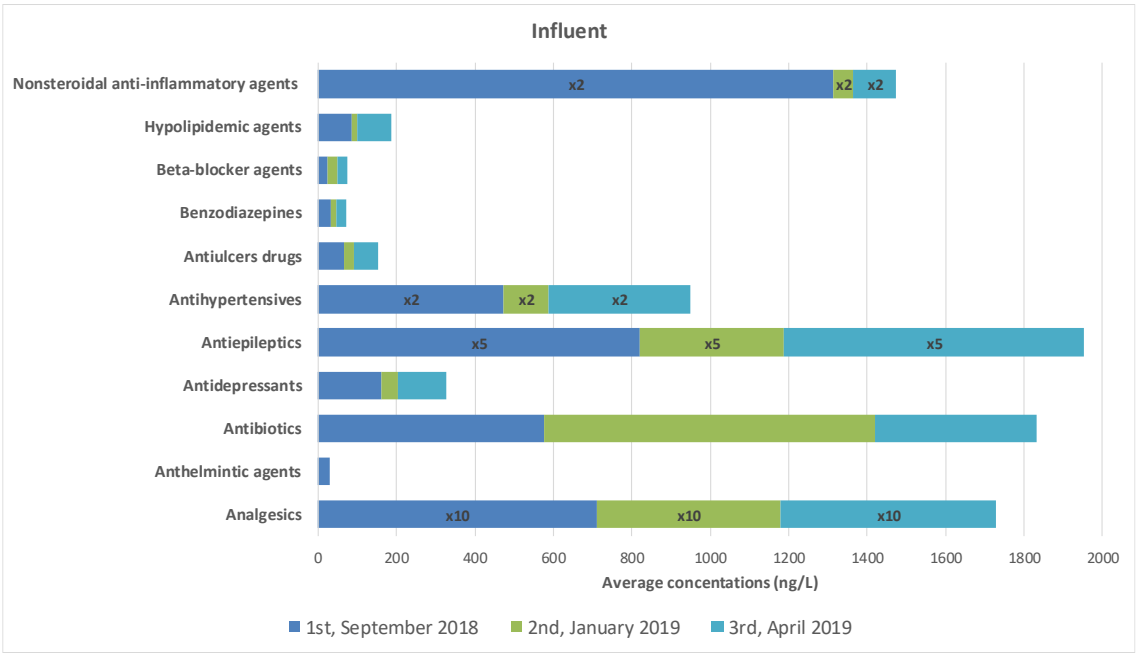
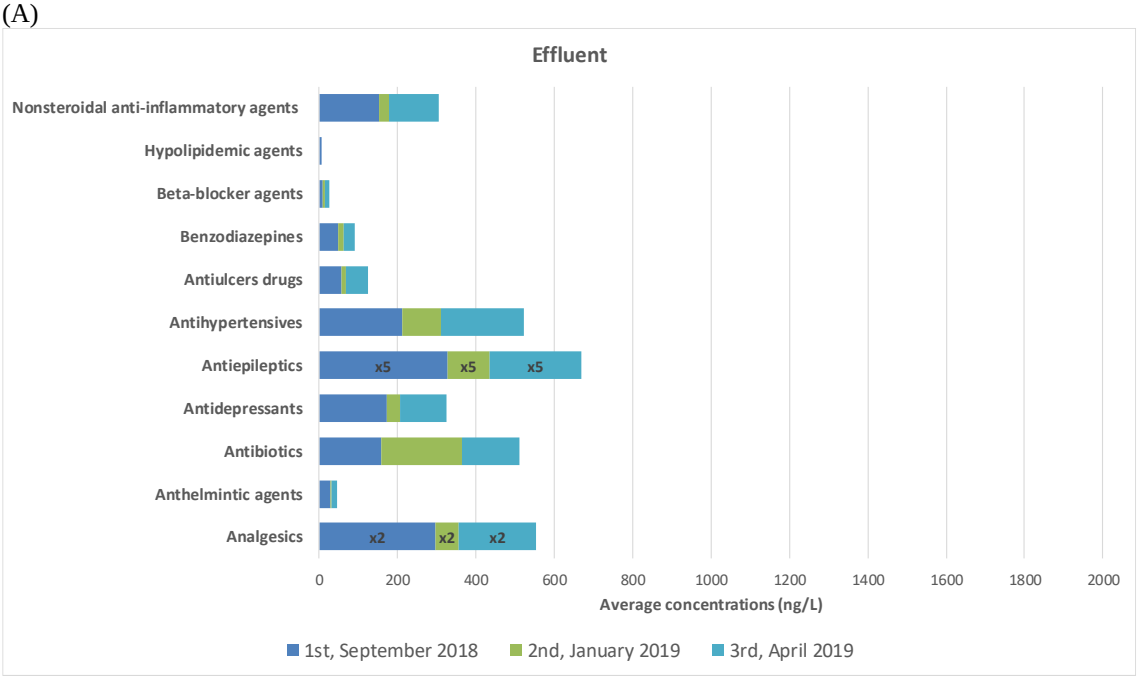


Figure 2. Detection and tentative identification of eprosartan in the analysis by LC-QTOF MS of the sample corresponded to the hospital discharge. (a) LE (bottom) and HE (top) mass spectra of the chromatographic peak at retention time 7.41 min. (b) XICs with 0.02 Da mass window for the protonated molecule in LE and different ions observed in HE



(A)



(B)

Figure 3. Average concentrations of different pharmaceutical groups in the influent (A) and the effluent (B) of the WWTP Ricao along three sampling campaigns. To build the graphs, data reported as "d" (detected) have been assigned a value equal to half of the LCL. Ciprofloxacin and norfloxacin have not been included in the total of antibiotics as their concentrations were indicative. The annotation (x2, x5 and x10) into the bars indicates that concentration level is 2, 5 or 10 times higher than the level presented in the graphic.

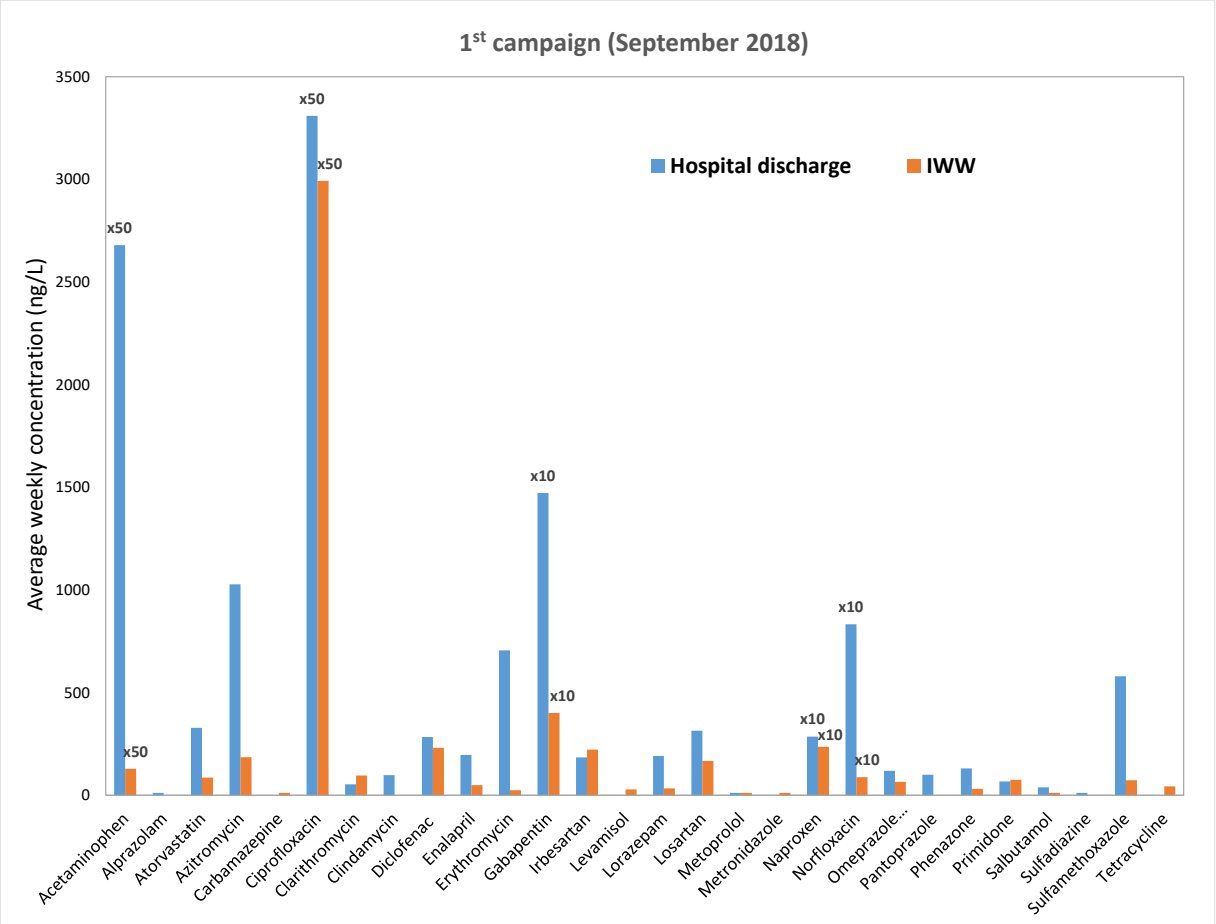


Figure 4. Concentrations of pharmaceuticals (ng/L) calculated as the average of the seven days from the September campaign in hospital discharge and in IWW from the WWTP. To build the graph, data reported as "d" (detected) have been assigned a value equal to half of the LCL. Ciprofloxacin and norfloxacin data are indicative. The annotation (x10 and x50) on the bars indicates that concentration level is 10 or 50 times higher than the level presented in the graphic.

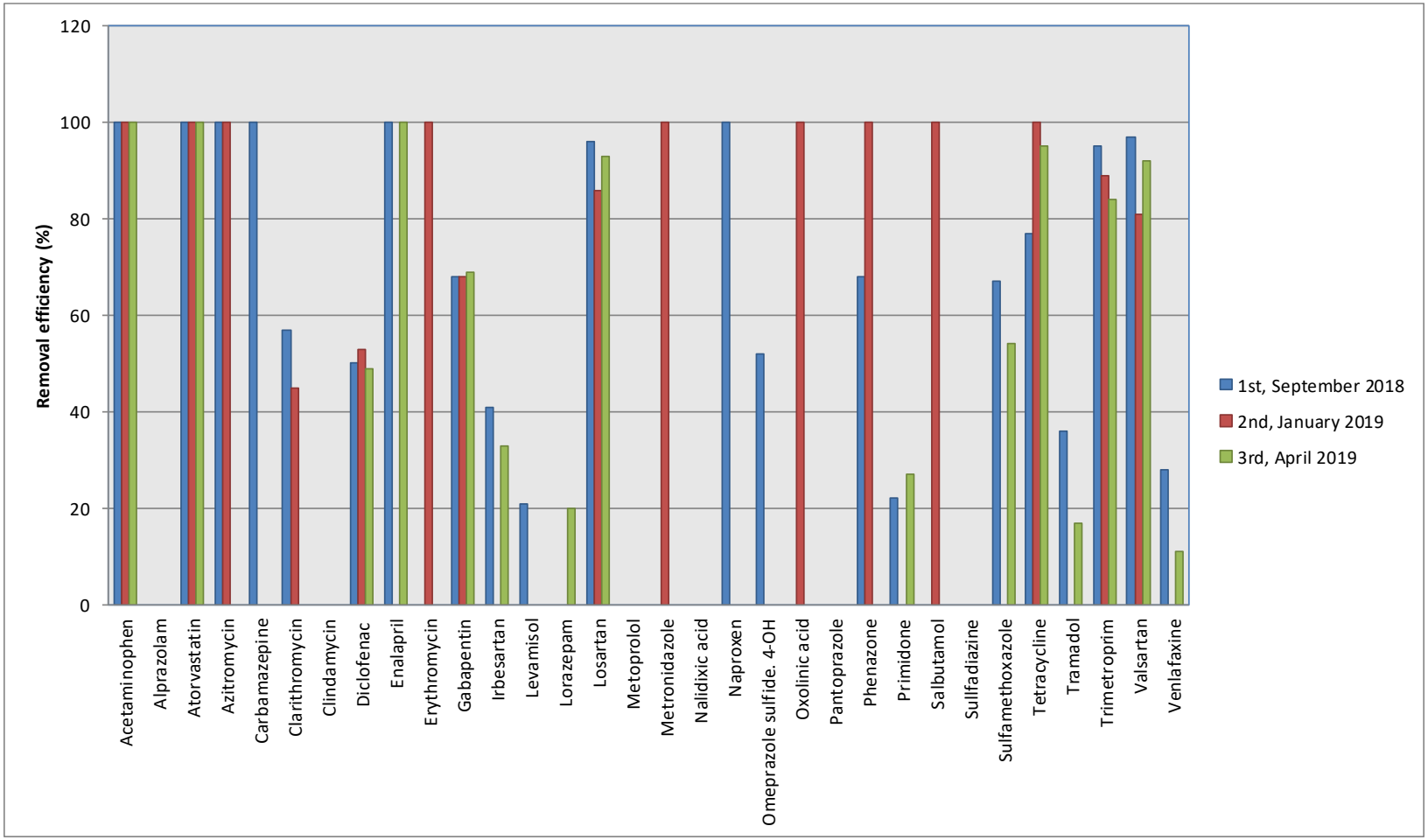


Figure 5. Average removal efficiency (%) for pharmaceuticals in the WWTP estimated for the three monitoring campaigns (the absence of a bar indicates RE is near or below 0 %)