Investigation of Drugs in Wastewater:

Removal Efficiency in a Treatment Plant and Environmental Impact Assessment

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Contents

Summary

This study assessed the presence of 41 pharmaceuticals in wastewater samples from the north of Spain. The samples under study were collected during a week, before the treatment (influent) and after treatment (effluent) in a wastewater treatment plant (WWTP). Additionally, samples reaching the inlet of the WWTP from hospital discharge were also collected. The wastewater samples were analysed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry using triple quadrupole analyzer (UHPLC-MS/MS QqQ) due to its great selectivity and sensitivity. Analyses were performed by direct injection (DI) of the samples; so, pre-concentration treatments were avoided. Moreover, in order to correct matrix effects, Isotopically-Labelled Internal Standards (ILIS) available for some pharmaceuticals were added to the samples. *Acetaminophen* and *Gabapentin* were the most frequently detected compounds in both influent wastewater (IWW) and Hospital discharge. In EWW, it was observed a decrease in the concentrations of pharmaceuticals in comparison to the influent samples. The removal efficiency (RE) (%) was calculated by comparing the mass load of the influent and the effluent samples. From the 30 compounds detected in wastewater, only three pharmaceuticals (*Acetaminophen*, *Atorvastatin* and *Enalapril*) presented a 100% RE, which means that they were completely eliminated after passing through the WWTP. The results of this work indicate that most of pharmaceuticals under study are present in treated wastewater, and consequently they reach the aquatic environment. Hazard quotients (HQ) were calculated to assess the potential environmental risks of these pharmaceuticals. The results showed that generally there are not significant risks associated to the presence of pharmaceuticals in the aquatic environment although *Ciprofloxacin*, *Diclofenac*, *Norfloxacin* and *Venlafaxine* presented an HQ > 1 implying that the amount of these compounds released into the environment is above the non-effect concentration. The results from this study contribute to the better understanding of potential risks associated to the presence of pharmaceuticals in urban wastewater and their impact on the aquatic environment.

1. Introduction

Water is essential for life. It is a fundamental component of the nature and it is necessary for the living beings, who are composed by around 70% of water. Water acts as a transporter of nutrients, among other functions for the living beings, and it is also used to cultivate lands, breeding of cattle… The aquatic living beings spend most of their lives in rivers, oceans and lakes. For that many aforementioned reasons, it is why that the water should be kept free from waste and micropollutants. Water is considered as polluted if it contains some substances or if it exists a specific condition that does not allow the water to be used for other purposes (Owa, 2014).

"In the European Union (EU) around 3000 different Pharmaceutically Active Compounds (PhACs) are used in human medicine belonging to different medicinal classes" (Gros et al., 2010). These pharmaceuticals, after being consumed, are excreted via urine and faeces to the aquatic environment (Gaso-Sokac et al., 2017) so it can imply a potential environmental threat (Boix et al., 2015). Therefore, the scientific interest to know the presence and the effects of PhACs and other compounds of emerging concern in the aquatic environment has increased in the last few years (Gracia-Lor et al., 2011; Martínez-Morcillo et al., 2020).

In order to avoid the presence of the pharmaceuticals and other compounds, such as pesticides and illicit drugs in the aquatic environment, most cities have at least one wastewater treatment plant (WWTP). With physical, chemical and biological processes herein, the WWTP should be able to eliminate most of the emergent contaminants. However, the wide majority of conventional WWTPs do not completely eliminate these compounds, and commonly only a partial or even null elimination is reached. Some studies (Radjenović et al., 2009) suggest an improvement and implementation of technologies of advanced treatment in order to manage high quality treated effluents.

"Among pharmaceuticals, the presence of antibiotics in water causes more concern because they can induce bacterial resistance, even at low concentration, through their continuous exposure" (Gracia-Lor et al., 2011). So, different studies (Li et al., 2019; Na et al., 2019) have opted to evaluate the risk that the emerging contaminants generate with a parameter called Hazard Quotient (HQ), which in case of being higher or equal to one, the studied pharmaceutical is considered dangerous for aquatic environment.

The main objectives of this work are 1) to investigate the presence of 41 pharmaceuticals in the wastewater, both in the influent wastewater (IWW) and effluent wastewater (EWW) of a WWTP as well as an hospital discharge in a location from the north Spain; 2) to calculate the removal efficiency (RE) for pharmaceuticals of the WWTP; 3) to evaluate the potential environmental impact that these pharmaceuticals might produce. In order to carry out this study, the highly sensitive technique resulting from the combination of ultra-high performance liquid chromatography (UHPLC) with MS/MS using triple quadrupole (QqQ) has been used. In order to minimise issues associated with the manipulation of samples, direct injection (DI) has been used for analysis. Moreover, in order to correct the matrix effect, different isotope-labelled internal standards (ILIS) have been added to the standards used for preparation of the calibration curves as well as to the samples.

1.1. Analytical techniques

The analytical technique used in this study was ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) with triple quadrupole (QqQ). It was selected by different reasons: excellent sensitivity and selectivity, robustness at low concentration levels and fully compatible with aqueous samples without the need to make solvent exchange. Moreover, recent advances in MS instrumentation have made possible the direct injection of samples, reaching concentrations at the sub-ppb levels without any type of sample treatment, and minimizing analytical errors associated to sample manipulation.

1.1.1. Ultra-high performance liquid chromatography (UHPLC)

Chromatography may be defined as a physical method of separation in which the mixture to resolve is introduced in a system formed by a fluid (mobile phase) that moves around in a close contact with a solid or liquid phase, which is immobile during the process (stationary phase). According to the characteristics of the mobile phase, the chromatography may be divided into three types: liquid, gaseous and supercritical fluids. This section focuses on the liquid chromatography. "UHPLC separations represent separations performed with columns packed with sub-2µm fully porous particles or sub-3µm core-shell particles, respectively, in optimized low-dispersion high-pressure LC systems with the aim to achieve fast and high-resolution analyses" (Nováková et al., 2017).

The chromatographic technique has been modified and improved through the years. Since 15 years ago, important advances have taken place in conventional High Performance Liquid Chromatography (HPLC). These advances include improvements in the stationary phase and in the instrumentation (Nováková et al., 2017). The decrease of the particle size improves the chromatographic separation, as demonstrated in the Van Deemter's theory **(Equation 1)** because the efficiency that corresponds to the height of the plate (H) is in proportion to the particle size (d_p).

$$H = A + \frac{B}{u} + Cu = 2\lambda d_p + \frac{2\gamma D_M}{u} + \frac{f(k) d_p^2 u}{D_M} \qquad \textit{(Equation 1)}$$

The sample is introduced in the column together with mobile phase thanks to a pump. According to the polarity of the compounds that are in the sample in the stationary phase and mobile phase, these compounds will be retained and eluted at different times. As a result, different chromatograms will be observed at different retention times. A detector is needed in order to transform these chromatograms into more comprehensible information. Although there are a lot of detectors, the most reliable for this type of analysis is the mass spectrometry.

1.1.2. Mass spectrometry. Triple quadrupole instruments (QqQ)

Mass spectrometry (MS), is an analytical technique that produces ions and separates them. MS classifies the ions in relation to time and space in the gaseous phase according to the relation *m/z*. The result is a plot than represents relative intensity vs *m/z*. It is a very sensitive technique and it is commonly used in different fields. It is important its use in environmental sciences with the purpose of studying the presence of organic pollutants (Hernández et al., 2012).

The sample is in liquid condition in LC but as it has been previously mentioned, the MS works in the gaseous phase, so it is needed a phase change that is produced thanks to the electrospray ionization (ESI). "ESI is considered as the most important ionization technique for coupling between LC and MS" (Nováková et al., 2017). A voltage and high temperature are applied to the sample that evaporates the solvent of the droplets formed and ends up generating ionic species.

The essential part of the MS is the analyser. The resolution (capacity of differentiating between similar masses), system's sensitivity, the capacity of measuring the exact mass and kinetics depend on the analyser. Moreover, its function is to separate the ions based on their *m/z* ratio. In this study, a triple quadrupole (QqQ) analyser has been used for analysis. The QqQ consists of four circular parallel bars. The ions get separated because of their stability in the electric fields applied to bars. The QqQ is based on the control of the transition among the molecular ions (or precursor ions) and the ions of the fragments (or product ions). To this end, specific amount of m/z are allowed throughout the first quadrupole (*Q1*). Afterwards, in the collision cell (second quadrupole *q2*), a collision energy is applied in order to fragment the precursor ion (generated in *Q1*) and to generate product ions. Finally, the third quadrupole (*Q3*) is designed to isolate the specific *m/z*. Accordingly, after the selection of the molecular ions in the *Q1*, they get fragmented in the collision cell so at the end, only some ions in *Q3* are traced, which results in the monitoring of the transitions among the molecular ions and the fragments. Only one ion with the correct *m/z* will have the correct trajectory and will get to the detector (Agilent Technologies, 2016; Heeren, 2006).

Analysis have been made with LC-MS/MS QqQ working under the Selected Reaction Monitoring (SRM) mode, i.e. a precursor ion in selected, typically the $[M+H]^+$, in the first quadrupole, and specific product ins are measured in the third quadrupole. In other words, several transitions "precursor ion > product ion" are measured, one of then used for quantification (known as Quantification transition), which commonly is the most abundant, and the remaining ones, used for confirmation of the identify (known as qualitative transitions).

1.2. Importance of Isotopically-labelled internal standards

Although UHPLC-MS/MS is widely accepted for pharmaceuticals analysis, there is an issue of major concern when applied to complex-matrix sample. The presence of coeluting compounds with the analytes of interest might alter the ionisation process by default (ionization suppression) or by excess (ionization enhancement) (Gracia-Lor et al., 2011). This is the so called, matrix effect, and if it is not properly corrected or eliminated, may affect both the identification

and the quantification of the analyte (Bijlsma et al., 2014). Matrix effect can be very high in complex matrix samples, such as wastewater, and therefore is necessary its appropriate correction to ensure the reliability of data reported.

The ILIS are compounds isotopically marked that are used as surrogates in order to compensate the matrix effect and the possible losses during sample treatment (Bijlsma et al., 2014; Botero-Coy et al., 2018). The high cost and low commercial availability of ILIS for some compounds is a limitation to apply this approach, which however is the recommended when only a few pharmaceuticals are under study (Botero-Coy et al., 2018). More specifically, in this study 15 ILIS have been used and they have contributed to the satisfactory correction of matrix effects for around half of the compounds investigated.

1.3. Removal efficiency of Wastewater treatment plant

In order to maintain aquatic systems, such as rivers, sea or lakes, free from pollution, it is necessary the application of appropriate treatments for removal of organic contaminants in the WWTPs. The treatments are commonly based on chemical, physical and biological processes that allow the elimination of the majority of organic matter. However, many organic micro-pollutants are not efficiently removed using the conventional treatments applied. Therefore, EWW usually contain an amount of pollutants such as pharmaceuticals, pesticides or drugs, among others.

In order to calculate the removal efficiency (RE), it is convenient to calculate the daily mass loads because in this way the calculation is more realistic than just comparing concentrations in IWW and EWW. The mass load (**Equation 2**) is calculated (e.g. ng/day), considering the daily IWW flow (F), shown in **Table 3**, and the concentration found in the sample extract (C). In order to estimate the efficiency of the elimination of the plant, the mass loads of the studied pharmaceuticals in IWW and EWW are compared. RE is calculated by applying **Equation 3**, where L_{IWW} is the load of the influent at day x and L_{EWW} is the load of the effluent at day x + 1, assuming in this way a residence time at the plant of approximately 24h. The result is obtained in percentage (%).

$$Mass\ load\ \left(\frac{ng}{day}\right) = C \cdot F \cdot\ 10^{-3} \qquad \textit{(Equation 2)}$$

$$RE(\%) = \left(1 - \frac{L_{EWW}(x+1)}{L_{IWW}(x)}\right) \cdot 100 \qquad \textit{(Equation 3)}$$

1.4. Environmental risk assessment

The increasing use of pharmaceuticals and personal care products (PPCPs) entails a continuous increase of their presence in wastewater (Li et al., 2019). For this reason, scientific attention on the presence and effects of PhACs and other compounds of emerging concern in aquatic systems has experienced a notable increase over the last decades (Martínez-Morcillo et al., 2020).

These organic micro-pollutants may generate a consequent impact on the environment, also affecting the growth and reproduction of microorganisms (Li et al., 2019). Therefore, several studies in different research fields have been carried out. Liu et al. (X. Liu et al., 2020) evaluated the danger of the bioaccumulation of the PPCPs in different food such as cucumber, eggplant, wheat and long bean. Moreover, they also analysed the risk to human health associated with the consumption of these crops. Some studies (Prosser & Sibley, 2015) examined the manure among others in order to check if the plant tissue was free of PPCPs. A study carried out in Galicia (Martínez-Morcillo et al., 2020) evaluated the risk for the human health when consuming seafood species that contain pharmaceutical compounds.

The WWTPs are considered as one of the main sources of pharmaceutical wastes in the aquatic system. However, the problem of WWTPs is that they mainly work to eliminate the already known macro-pollutants (solids, nutrients and total organic matter) and they do not focus on the emerging pollutants such as pharmaceuticals (Sim et al., 2010). Furthermore, although in the north of Europe many WWTPs include tertiary wastewater treatment, in other Countries, Spain included, only two treatments are usually applied (Gros et al., 2010). For this reason, the high concentration of the pharmaceuticals in the wastewater might be attributed to a poor removal in the WWTP (X. Liu et al., 2020).

The parent pharmaceuticals reach wastewater through urine and/or faeces (Gaso-Sokac et al., 2017), but a great part of the compound is eliminated as a variety of free metabolites (transformation products) (Gracia-Lor et al., 2014; Han & Lee, 2017) or as conjugates of glucuronic and sulphuric acid (Gros et al., 2010). Some data reported (Han & Lee, 2017; X. Liu et al., 2020) show that the potential risk caused by some metabolites may exceed their predecessors.

Due to the potential problems derived from their presence in the aquatic environment, it is necessary to evaluate the environmental risk that PPCPs and their metabolites may produce. A possible way to evaluate the risks is through the use of the Hazard Quotients HQ. The HQ was determined in this work based on several studies (Li et al., 2019; Na et al., 2019) by dividing the measured environmental concentration (MEC) by the predicted non-effect concentration (PNEC). When HQ is equal or higher than one, it suggest that the substance may cause potential adverse ecological effects (Gros et al., 2010).

2. Objectives

The three main objectives of this work were:

- To detect and quantify 41 pharmaceuticals in different wastewater of north of Spain, through the use of UHPLC-MS/MS with QqQ. These wastewaters are IWW, EWW and Hospital discharge.
- To estimate WWTP removal efficiency for pharmaceuticals by comparison of IWW and EWW samples.
- To evaluate the possible environmental impact that drugs can generate in the aquatic environment with the data obtained from the EWW samples

3. Materials and methods

3.1. Pharmaceutical standards and reagents

Pharmaceutical reference standards were purchased from different companies: Sigma-Aldrich (St Louis, MO, USA), LGC Promochem (London, UK), Toronto Research Chemicals (Ontario, Canada), Across Organics (Geel, Belgium), Bayer Hispania (Barcelona, Spain), Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid, Spain) and Aventis Pharma (Madrid, Spain). Moreover, the ILIS were from CDN Isotopes (Quebec, Canada), Toronto Research Chemicals, Cambridge Isotope Laboratories (Andover, MA, USA), Sigma-Aldrich (St Louis, MO, USA) and Cerilliant (Texas, USA). All the reference standards obtained from the aforementioned sources presented high purity levels of more than 93%.

Stock standard solutions of each compound were prepared at 500 mg/L in methanol or acetonitrile and were stored at -20ºC. The individual stock solutions were diluted ten times with methanol in order to prepare 50 mg/L intermediate solutions. Finally, mixed working solutions were prepared by dissolving intermediate solutions in water. These working solutions contained all the analytes, so they were used to prepare the calibration standards and for spiking samples.

A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to purify water through filtration, osmosis and other processes so as to obtain LC-MS grade water. Besides, LC-MS grade methanol (MeOH), LC-MS grade acetonitrile (ACN), formic acid (HCOOH, content > 98%) and ammonium acetate (NH_4AC, reagent grade), were acquired from Scharlab (Barcelona, Spain).

3.2. Description of the wastewater treatment plant

The wastewater treatment plant (WWTP) included in this study treats urban wastewater from the public sanitation system of different towns. The WWTP is located in the north of Spain and it also receives different authorized industrial discharges, mainly related to the chemical, pharmaceutical, food and services sectors.

The WWTP applies a pre-treatment with a maximum flow rate of 41,208 m^3/day and A20 type biological process with a maximum of 20,640 m^3/day. This biological process removes organic matter, nitrogen and phosphorus with anaerobic, anoxic chambers and aerated carousel channels. Moreover, it is a variant of the conventional treatment of active sludge, which incorporates an anaerobic zone at the reactor inlet. In this reactor, which has a capacity of 16,076 m^3, the influent wastewater and recirculated sludge are received, producing the fermentation reaction and phosphate elimination. The biologically treated effluent ends in two circular decanters of 28 meters in diameter, so the treated water from WWTP is returned to the river.

The quality parameters of the WWTP's effluent must be in accordance with the requirements of the Water Framework Directive 2000/60/EC (Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a Framework for Community Action in the Field of Water Policy., 2000) (e.g. organic matter, suspended solids and nutrients: nitrogen and phosphorus) as shown in **Table 1**.

Table 1. *Water Quality Parameters of EWW from WWTP.*

Parameter	Emission limit
pH	Between 6-9
Suspended solids	< 15 mg/L
BOD_5	< 12 mg O_2/L
COD	< 60 mg O_2/L
Total ammonium	< 3 mg NH_4/L
Nitrates	< 35 mg NO_3/L
Kjeldahl nitrogen	< 5 mg N/L
Total nitrogen	< 15 mg N/L
Total phosphorus	< 2 mg P/L

3.3. Selected pharmaceuticals

In preliminary stages of the study, a high resolution mass spectrometry screening was carried out and the list of pharmaceuticals to be included in this particular study was based on the results obtained in the screening. A total of 41 pharmaceuticals were selected including 17 compounds previously identified and confirmed in the screening (*data not published)* and 24 additional compounds of interest based on the prevalence of use and availability of reference standard at the laboratory. The complete list of pharmaceuticals to be studied is shown in **Table 2.**

3.4. Experimental

3.4.1. Sample collection

IWW and EWW 24-h composite samples at the inlet and outlet of the WWTP, respectively, were collected during seven consecutive days in September. Additionally, daily composite samples reaching the inlet of the WWTP from hospital discharge were also collected. All the samples were collected in high-density polyethylene bottles, stored at < -20ºC, and transported to the laboratory once the last sample of the week was collected. After reception in the laboratory, samples were stored in the dark at -20ºC and analysed within 2 weeks. Water flow rates of the IWW, EWW and Hospital WW are shown in **Table 3**.

Table 2. *List of pharmaceuticals to be studied.*

Compound	Family
Acetaminophen	Analgesic
Alprazolam	Benzodiazepine
Atorvastatin	Hypolipidemic agent
Azithromycin	Antibiotic
Bezafibrate	Hypolipidemic agent
Carbamazepine	Antiepileptic
Ciprofloxacin	Antibiotic
Clarithromycin	Antibiotic
Clindamycin	Antibiotic
Diclofenac	Nonsteroidal anti-inflammatory
Enalapril	Antihypertensive
Erythromycin	Antibiotic
Furaltadone	Antibiotic
Gabapentin	Antiepileptic
Gemfibrozil	Hypolipidemic agent
Irbesartan	Antihypertensive
Ketoprofen	Nonsteroidal anti-inflammatory
Levamisol	Anthelmintic agent
Lincomycin	Antibiotic
Lorazepam	Benzodiazepine
Losartan	Antihypertensive
Metoprolol	Beta-blocker agent
Metronidazole	Antibiotic
Nalidixic acid	Antibiotic
Naproxen	Nonsteroidal anti-inflammatory
Norfloxacin	Antibiotic
Omeprazole sulfide, 4-OH	Antiulcer drug
Oxolinic acid	Antibiotic
Pantoprazole	Antiulcer drug
Phenazone	Nonsteroidal anti-inflammatory
Primidone	Antiepileptic
Roxithromycin	Antibiotic
Salbutamol (Albuterol)	Beta-blocker agent
Simvastatin	Hypolipemiant
Sulfadiazine	Antibiotic
Sulfamethoxazole	Antibiotic
Tetracycline	Antibiotic
Tramadol	Analgesic
Trimethoprim	Antibiotic
Valsartan	Antihypertensive
Venlafaxine	Antidepressant

3.4.2. Analytical procedure

Sample analysis was performed according to previously developed, validated and published methodologies (Boix et al., 2015; Botero-Coy et al., 2018). Briefly, depending on the origin of the samples (i.e. IWW, hospital discharge, EWW), two different sample treatments were applied. Firstly, 1ml of raw WW sample was centrifuged at 12.000 rpm for 10 min. For IWW and Hospital discharge samples, a x5 dilution was performed with Milli-Q water in order to reduce the matrix complexity. For this purpose, a 200 µL-aliquot of the centrifuged sample was diluted with 750 µL of Milli-Q water and 50 µL of mix ILIS solution (2 µg/L). The EWW samples were diluted x2, so a 500 µL- aliquot of the centrifuged EWW sample was diluted with 450 µL Milli-Q water and 50 µL of mix ILIS solution (2 µg/L). ILIS were at a final concentration of 0.1 µg/L in all samples. Finally, 50 µL of diluted samples were injected in the UHPLC-MS/MS system in order to perform the analyses. **Figure 1** shows the schematic analytical procedure followed for the extraction of IWW and Hospital samples (**Figure 1a**) and EWW samples (**Figure 1b**).

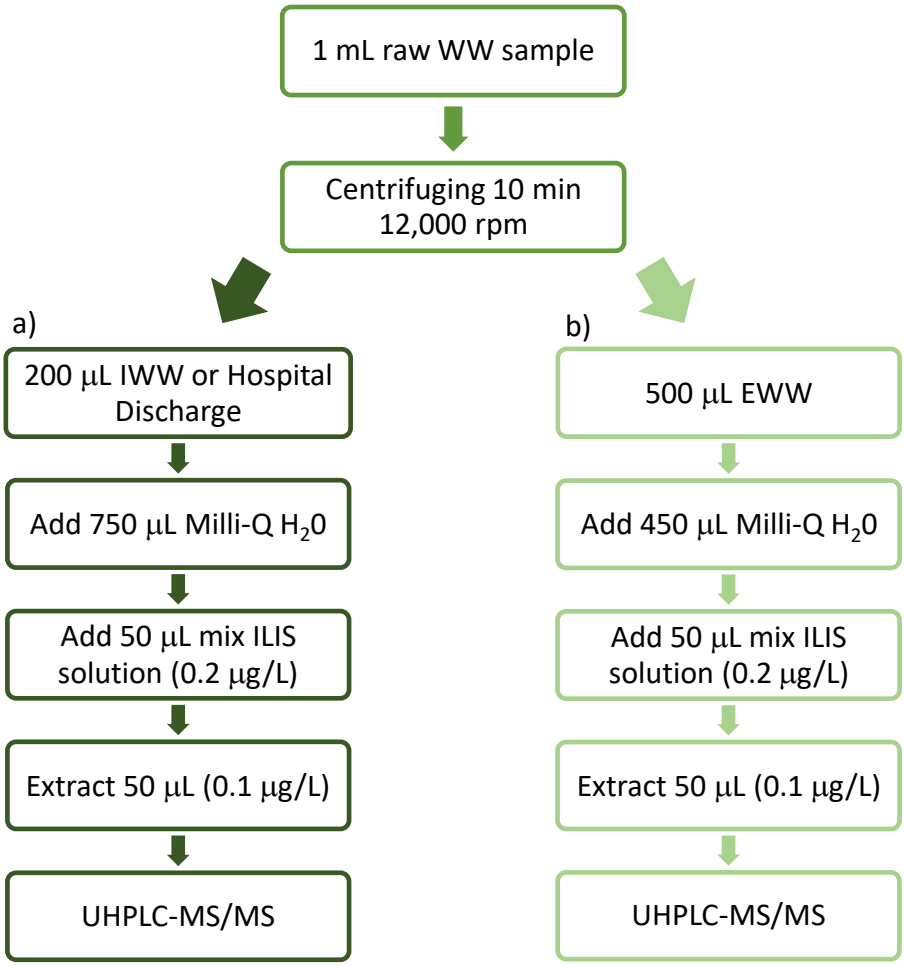

Figure 1. *Flow chart for the analysis of (a) IWW and Hospital and (b) EWW samples.*

Table 3. *Flow date for Hospital, IWW and EWW water streams samples over a period of 7 consecutive days*

Day	Flow (m^3/day)		
	Hospital	IWW	EWW
Monday	na	22.120	16.980
Tuesday	na	20.828	16.830
Wednesday	na	17.184	18.110
Thursday	na	17.992	16.680
Friday	na	22.208	16.590
Saturday	na	20.796	15.860
Sunday	na	18.996	18.680

na: data not available.

3.4.3. Instrumentation

UHPLC analysis was carried out with a Waters ACQUITY ultra performance liquid chromatography (UPLC™) system (Waters Corp., Milford, MA, USA). A CORTECS C_{18} column (100 x 2.1 mm i.d., particle size 2.7 μm) (Waters) with a constant flow rate of 0.4 mL/min was used for chromatographic separation. The mobile phases were A= methanol LC-MS and B= water LC-MS, both with 0.01% HCOOH and 1 mM ammonium acetate. Finally, the column was kept at 40°C and the percentage of solvent A was changed as follows: 0 min, 5% A; 7 min, 95% A; 8 min, 95% A; 8.1 min, 5% A for the re-equilibration of the column with a total run time of 9.5 min.

The UPLC system was interfaced to a triple quadrupole mass spectrometer Xevo TQ-S™ (Waters Micromass, Manchester, UK), equipped with an orthogonal Z-spray electrospray ionization interface (ESI) and operated in positive and negative ion mode. Furthermore, a capillary voltage was fixed at 3.5 kV (ESI+) and 2 kV (ESI-) with a cone voltage of 10 V. Then, nitrogen (Praixar, Valencia, Spain) was optimized at 250 L/h to be used as a cone gas and optimized at 1200 L/h to be used as a desolvation gas. Regarding the operation in MS/MS mode, the collision gas was Argon 99.995% (Praixar, Valencia, Spain) with a flow of 0.15 mL/min in the collision cell. Moreover, the desolvation temperature was set to 650°C and the source temperature was set to 150°C.

All the data were acquired using MassLynx Software v 4.1 (Waters Corporation) and the concentrations levels of the target analytes were quantified with TargetLynx application.

3.5. MS conditions for target compounds

Out of 41 compounds, 36 were determined with ESI operating in positive ionization mode and using the protonate molecule $[M+H]^+$ as precursor ion. The remaining 5 compounds were determined in negative ionization using $[M-H]^-$ as precursor ion, as shown in **Table 4.**

The acquisition of three SRM transitions for every compound allowed the simultaneous quantification and identification of the positive findings. In addition, 15 isotopically labelled internal standards were used for matrix effects correction. Regarding the reliable identification of the compounds found in the sample, the calculation of the ion ratios (peak area) between the confirmation (q_1 and q_2) and quantification (Q) transitions (*q1/Q* and *q2/Q* enabled the confirmation of the identity. The finding was considered as positive when at least one ion-ratio and the retention time of the compound in sample were within the tolerance ranges of the deviation compared standards used for calibration (maximum deviation ± 30% for ion ratio, ± 0.1 min for the retention time) (Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed (SANTE/12682/2019)., 2019).

The selected transitions (quantification -*Q*- and confirmation -*q*-), Collision Energies (CE), Limits of Quantification (LOQ), ILIS used for matrix effect correction, and the retention times are shown in **Table 4** for each compound. Cone voltage was 10 V for all compounds.

3.6. Method performance

The quantification transition (*Q*) and external calibration with standards in solvent were used in order to quantify the analytes. In those cases that the analyte-ILIS was available, relative areas were used for quantification. At least eight-point calibration curves (0-20.000 ng/L) were included at the beginning and the end of each sequence of analysis. Linearity was assumed when regression coefficient (R) was > 0.99 with residuals lower than 30%. Regarding the LOQ, it was considered as the lowest calibration level (LCL) taking into account the sample dilution, i.e. LOQs were LCLx5 for IWW and hospital discharge and LCLx2 for EWW.

Quality control (QC) samples consisted of real-word wastewaters, which were fortified at two concentration levels: 0.5 and 5 μg/L (for Hospital and IWW) and 0.2 and 2 μg/L (for EWW). QCs and the samples were treated in the same way and therefore injected in every batch of analysed samples. QCs recoveries were considered as satisfactory when they were between 60% and 140% (Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed (SANTE/12682/2019)., 2019).

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Table 4. *Conditions of analysis by UHPLC-MS/MS.*

Compound	ESI	ILIS used	Transition (Q)	CE (eV)	Transition (q)	CE (eV)	LOQ (ng/L) *	Rt (min)
Acetaminophen	+	Acetaminophen-d_4	152 > 110	15	152 > 93 152 > 65	20 25	5	1.5
Alprazolam	+	(-)	309 > 281	25	309 > 205 309 > 274	25 25	**	5.36
Atorvastatin	+	Atorvastatin-d_5	559 > 440	20	559 > 466 559 > 292	15 25	**	6.4
Azithromycin	+	Azitromycin-d_3	749.4 > 591.4	25	749.4 > 82.9 749.4 > 116.1	45 45	50	4.27
Bezafibrate	-	(-)	360 > 274	20	360 > 154 360 > 85	25 15	1000	5.97
Carbamazepine	+	Carbamazepine 10, 11-epoxide-d_{10}	237 > 194	20	237 > 179 237 > 192	25 10	5	4.92
Ciprofloxacin	+	Ciprofloxacin-d_8	332 > 231	25	332 > 288 332 > 314	15 20	***	2.75
Clarithromycin	+	(-)	590 > 158	20	590 > 116 590 > 98	25 25	5	5.68
Clindamycin	+	(-)	425.1 > 126	20	425.1 > 377 425.1 > 389	20 15	5	4.42
Diclofenac	+	Diclofenac-d_4	296.2 > 214.2	30	296.2 > 250 296.2 > 278	10 5	5	6.53
Enalapril	+	(-)	377 > 234	15	377 > 117 377 > 303	25 15	5	4.61
Erythromycin	+	Erythromycin-$^{13}C_3$	734 > 158	25	734 > 576 734 > 558	15 15	5	5.12
Furaltadone	+	(-)	325 > 100	20	325 > 252 325 > 281	15 10	5	2.06
Gabapentin	+	(-)	172 > 137	15	172 > 154.2 172 > 95	15 20	5	1.89
Gemfibrozil	-	(-)	249 > 113	10	249 > 121 249 > 127	20 10	**	7.52
Irbesartan	+	Irbesartan-d_6	429 > 207	25	429 > 195 429 > 180	20 25	5	5.96
Ketoprofen	-	(-)	253 > 79	10	253 > 92 253 > 209	20 10	1000	5.39
Levamisol	+	Cocaethylene-d_8	205 > 178	20	205 > 91 205 > 123	25 25	5	1.99
Lincomycin	+	(-)	407 > 126	20	407 > 359 407 > 389	15 15	5	2.36
Lorazepam	+	(-)	321 > 275	20	321 > 303 321 > 229	15 25	5	5.36
Losartan	+	(-)	423.1 > 207.1	15	423.1 > 377.1 423.1 > 405.1	15 10	5	5.48

**LOQ was considered as LQLX5 for the Hospital and IWW samples, and LQLx2 for EWW samples. **LOQs for Alprazolam and Atorvastatin were 25 for Hospital and IWW, 5 for EWW. LOQs for Gemfibrozil were 25000 for Hospital and IWW, and 2000 for EWW. ***Data not available.*

Table 4 (cont).

Compounds	ESI	ILIS used	Transition (Q)	CE (eV)	Transition (q)	CE (eV)	LOQ (ng/L) *	RT (min)
Metoprolol	+	(-)	268.2 > 116	15	268.2 > 74	20	5	3.2
					268.2 > 191	15		
Metronidazole	+	(-)	172 > 127.9	15	172 > 82.1	20	5	1.68
					172 > 55.9	20		
Nalidixic acid	+	(-)	233 > 215	10	233 > 187	25	5	4.53
					233 > 159	25		
Naproxen	-	(-)	229 > 170	20	229 > 185	10	1000	2.65
					185 > 169	20		
Norfloxacin	+	Norfloxacin-d_5	320 > 233	25	320 > 276	15	***	
					320 > 302	20		
Omeprazole sulfide, 4-OH	+	Omeprazole-d_3	316 > 168	20	316 > 149	20	5	4.12
					316 > 283	15		
Oxolinic acid	+	(-)	262 > 216	25	262 > 244	15	5	3.83
					262 > 158	25		
Pantoprazole	+	(-)	384 > 200	10	384 > 138	25	5	4.83
					384 > 153	15		
Phenazone	+	(-)	189.3 > 131.1	20	189.3 > 104.1	20	5	3.01
					189.3 > 58.1	20		
Primidone	+	(-)	219.2 > 162	10	219.2 > 91	20	5	3.36
					219.2 > 119.2	15		
Roxithromycin	+	(-)	679 > 158	25	679 > 116	25	5	5.78
					679 > 98	25		
Salbutamol (Albuterol)	+	(-)	240 > 148	15	240 > 222.1	10	5	1.5
					240 > 166.1	10		
Simvastatin	+	Simvastatin-d_6	419.1 > 199	10	419.1 > 285	5	***	7.51
					419.1 > 225.1	20		
Sulfadiazine	+	Sulfamethoxazole-$^{13}C_6$	251 > 156	15	251 > 92	25	5	1.68
					251 > 108	20		
Sulfamethoxazole	+	Sulfamethoxazole-$^{13}C_6$	254 > 92	25	254 > 156	15	5	2.86
					254 > 108	20		
Tetracycline	+	(-)	445 > 154	25	445 > 410	15	5	2.87
					445 > 427	10		
Tramadol	+	(-)	264 > 58	10	264 > 121	25	5	3.12
					264 > 246	10		
Trimethoprim	+	(-)	291 > 123	25	291 > 230	20	5	2.39
					291 > 261	25		
Valsartan	+	Valsartan-d_8	436 > 207	25	436 > 235	15	5	5.96
					436 > 261	15		
Venlafaxine	+	Venlafaxin-d_6	278 > 58	15	278 > 260	10	5	4
					278 > 121	25		

**LOQ was considered as LQLX5 for the Hospital and IWW samples, and LQLx2 for EWW samples. **LOQs for Alprazolam and Atorvastatin were 25 for Hospital and IWW, 5 for EWW. LOQs for Gemfibrozil were 25000 for Hospital and IWW, and 2000 for EWW. ***Data not available due.*

Table 4 (cont).

ILIS	ESI	Transition (Q)	CE (eV)
Acetaminophen-d_4	+	156 > 114	10
Atorvastatin-d_5	+	564 > 445	20
Azitromycin-d_3	+	752.2 > 594.2	25
Carbamazepine 10, 11- epoxide-d_{10}	+	263 > 190	25
Ciprofloxacin-d_8	+	340.1 > 322.1	20
Diclofenac-d_4	+	300.1 > 219.2	20
Erythromycin-$^{13}C_3$	+	738.1 > 161.9	35
Irbesartan-d_6	+	435.1 > 231.3	25
Cocaethylene-d_8	+	326 > 204	20
Norfloxacin-d_5	+	325 > 238	20
Omeprazole-d_3	+	349 > 198	10
Simvastatin-d_6	+	425.2 > 199.1	10
Sulfamethoxazole-$^{13}C_6$	+	260 > 162	15
Valsartan-d_8	+	444 > 207	25
Venlafaxin-d_6	+	284.3 > 64.1	25

**LOQ was considered as LQLX5 for the Hospital and IWW samples, and LQLx2 for EWW samples. **LOQs for Alprazolam and Atorvastatin were 25 for Hospital and IWW, 5 for EWW. LOQs for Gemfibrozil were 25000 for Hospital and IWW, and 2000 for EWW. ***Data not available due.*

4. Results and discussion

4.1. Quality Control

The quality control of the methodology used for the determination of drugs by UHPLC-MS/MS was carried out through the analysis of Quality Control samples (QCs) in order to support the robustness, effectiveness and reliability of the method applied and support the quantitative data reported. Each batch analysed included at least two QCs for every type of samples (i.e. IWW, EWW, hospital discharge). The average recoveries (%) for the QCs prepared for IWW, EWW and hospital discharges (n=2) are shown in **Table 5**.

In general, the recoveries were satisfactory with values between 60 and 140%, acceptable range for individual QCs values in the field of residues analysis (Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed (SANTE/12682/2019)., 2019), which gives reliability to the results obtained. The achievement of satisfactory results for most QCs was undoubtedly facilitated by the use of a high number of ILIS and the absence of complex sample treatment in the analytical process. However, both the complexity of the sample matrix and the low analyte concentrations, make this type of analysis problematic. Therefore, it was necessary to find a right compromise for application of the analytical multiclass method to a high number of compounds.

The most relevant exceptions to satisfactory QCs recoveries among the 41 investigated pharmaceuticals were *Ciprofloxacin*, *Norfloxacin* and *Simvastatin* for which average recovery values greater than 200% and poor reproducibility was observed, surely due to inefficient correction of ILIS. Other 4 compounds (i.e. *Bezafibrate*, *Gemfibrozil*, *Ketoprofen* and *Naproxen*) analysed in negative ESI could not be properly evaluated due to the lack of instrumental sensitivity at the fortified level tested. Therefore, no recovery values were included for these pharmaceuticals. In addition, the antibiotics *Clarithromycin* and *Roxithromycin* presented unsatisfactory recoveries (over 200%), especially at the high fortification levels because the analyte ILIS was not available for matrix effects correction. Finally, the average recovery of the antibiotic *Azithromycin* in the IWW and EWW samples was also near the acceptable range but slightly below 50%.

Regarding the sample analysis, the failure to obtain fully satisfactory QC recoveries only affected to a few cases, in which positive detections were found. The most noticeable were the antibiotics *Ciprofloxacin* and *Norfloxacin*. Despite these two substances were found in all samples at concentrations above the limit of quantification, they could not be accurately quantified. Accordingly, for these two compounds, the presented data should be considered as an approximate concentration range.

Table 5. *Average recoveries (%) of the QCs for wastewater analysis, at two levels of fortification.*

	Hospital		IWW		EWW	
Drugs	**0.5 µg/L**	**5 µg/L**	**0.5 µg/L**	**5 µg/L**	**0.2 µg/L**	**2 µg/L**
Acetaminophen	*a*	*a*	132	125	97	107
Alprazolam	82	87	110	106	101	125
Atorvastatin	103	104	107	103	106	106
Azithromycin	81	59	*	*	**15**	69
Bezafibrate	-	**49**	-	63	-	81
Carbamazepine	89	*b*	91	*b*	74	*b*
Ciprofloxacin	*	*	*	*	*	*
Clarithromycin	118	*	139	*	124	*
Clindamycin	135	130	77	81	108	121
Diclofenac	116	108	98	96	96	94
Enalapril	111	104	102	107	104	106
Erythromycin	95	108	78	103	90	108
Furaltadone	84	100	82	74	115	95
Gabapentin	101	122	105	109	**149**	117
Gemfibrozil	-	-	-	-	-	-
Irbesartan	100	108	92	100	*a*	106
Ketoprofen	-	66	-	-	-	-
Levamisole	106	125	99	121	138	**176**
Lincomycin	116	89	97	110	124	108
Lorazepam	112	98	111	93	77	100
Losartan	115	93	106	103	105	104
Metoprolol	103	110	110	121	112	126
Metronidazole	72	86	98	99	103	116
Nalidixic acid	81	95	90	94	97	96
Naproxen	-	-	-	-	-	78
Norfloxacin	*	*	*	*	*	*
Omeprazole sulfide, 4-OH	73	**57**	96	80	67	64
Oxolinic acid	99	83	94	79	107	85

Recoveries outside the accepted range 60%-140% are shown in ***bold****. a. Presence of analyte in the blank at high concentration, which prevents recovery calculation. b. Value not calculated due to lack of linearity at high concentration levels. -. Value not available due to lack of sensitivity, which prevents reaching the lowest tested concentration levels. *. Anomalous recovery value, not acceptable value.*

Table 5 (cont.)

	Hospital		IWW		EWW	
Drugs	**0.5 µg/L**	**5 µg/L**	**0.5 µg/L**	**5 µg/L**	**0.2 µg/L**	**2 µg/L**
Pantoprazole	129	124	119	121	118	69
Phenazone	138	**148**	114	134	81	96
Primidone	87	88	102	105	94	106
Roxithromycin	*	*	*	*	*	*
Salbutamol (Albuterol)	100	96	111	104	143	139
Simvastatin	*	*	*	*	*	*
Sulfadiazine	88	96	110	120	73	87
Sulfamethoxazole	117	110	114	116	109	111
Tetracycline	86	**57**	95	79	83	**37**
Tramadol	84	93	104	103	108	115
Trimethoprim	84	98	116	124	118	132
Valsartan	87	126	68	70	86	103
Venlafaxine	96	105	96	114	107	118

*Recoveries outside the accepted range 60%-140% are shown in **bold**. a. Presence of analyte in the blank at high concentration, which prevents recovery calculation. b. Value not calculated due to lack of linearity at high concentration levels. -. Value not available due to lack of sensitivity, which prevents reaching the lowest tested concentration levels. *. Anomalous recovery value, not acceptable value.*

4.2. Occurrence of pharmaceuticals in wastewater samples

In this work three types of wastewater (i.e. IWW, EWW and Hospital discharge) were analysed by UHPLC-MS/MS. In order to study the efficiency of the elimination of the WWTP, data obtained from IWW and EWW were compared. Moreover, with the results of EWW it was possible to know which pharmaceuticals and at which concentration end up in the environment, so as to evaluate the environmental impact based on the use of the HQ. In addition, Hospital water samples were analysed and compared with the IWW samples.

It is important to highlight that most QCs of the *Ciprofloxacin* and *Norfloxacin* did not obtain any successful recovery so they could not be quantified with the required accuracy. However, these antibiotics were found in most of the water samples, presenting higher concentrations by far above the limit of quantification. For these two pharmaceuticals, approximate data are given, which must be understood as an approximate range of concentration.

4.2.1. IWW samples

A total of 30 out of 41 pharmaceuticals were observed in the IWW (**Table 6**). In general, pharmaceutical concentrations for a given compound were similar throughout the week, with the exception for *Phenazone*, which could only be quantified on Monday and Sunday at quite different concentrations.

Acetaminophen (6490 ng/L) and *Gabapentin* (4014 ng/L) presented the highest levels of concentration. Moreover, total daily mass load of each pharmaceutical (**Table 7**) was calculated in ng/day, from the **Equation 2**. **Figure 2** and **Figure 3** show the chromatograms obtained for positive samples containing *Acetaminophen* (Monday) and *Gabapentin* (Thursday), respectively.

4.2.2. EWW samples

The results of the analysis are shown in **Table 8** with the concentrations expressed in ng/L. The total daily mass loads (mg/day) are shown in **Table 9**, considering the EWW flow rates indicated in **Table 3**. A total of 29 out of 41 pharmaceuticals were detected in the EWW. The concentrations throughout the week were constant and lower than 100 ng/L. However, *Gabapentin*, *Tramadol*, *Irbesartan* and *Diclofenac* presented an average concentration higher than 100 ng/L.

After the application of the treatment in the WWTP, the concentrations of most pharmaceuticals decreased, presenting levels in the EWW lower than in IWW, which reveal that these compounds were removed or retained in the WWTP, at least partially. However, *Alprazolam*, *Clindamycin*, *Lorazepam*, *Metoprolol*, *Metronidazole*, *Nalidixic Acid*, *Pantoprazole* and *Salbutamol* presented the same or even higher concentrations than in IWW, which illustrates that the treatment applied in the WWTP does not remove these pharmaceuticals. A similar behaviour has been observed in other studies performed around the world (Botero-Coy et al., 2018; Gros et al., 2010).

4.2.3. Hospital discharge samples

In the Hospital discharge samples, 28 out of 41 studied pharmaceuticals were detected. Their concentration throughout the week of sampling were also rather constant, with the exception of *Erythromycin*, *Losartan*, *Pantoprazole*, *Phenazone*, *Sulfamethoxazole*, *Trimetroprim*, *Valsartan* and *Ciprofloxacin*.

Acetaminophen and *Gabapentin* presented the highest levels of concentration throughout the week. More specifically, on Saturday, *Acetaminophen* presented its peak with a concentration of 158840 ng/L and on Friday, *Gabapentin* presented its peak with a concentration of 22947 ng/L (shown in **Table 10**). For the Hospital samples, it has not been possible to calculate the total daily mass load of the pharmaceuticals because the flow rate data was unknown for the analyst.

For more information, see the Annex, where for each sample two types of graphs have been made to observe the trends for quantitative data. (**Figures 6-11**).

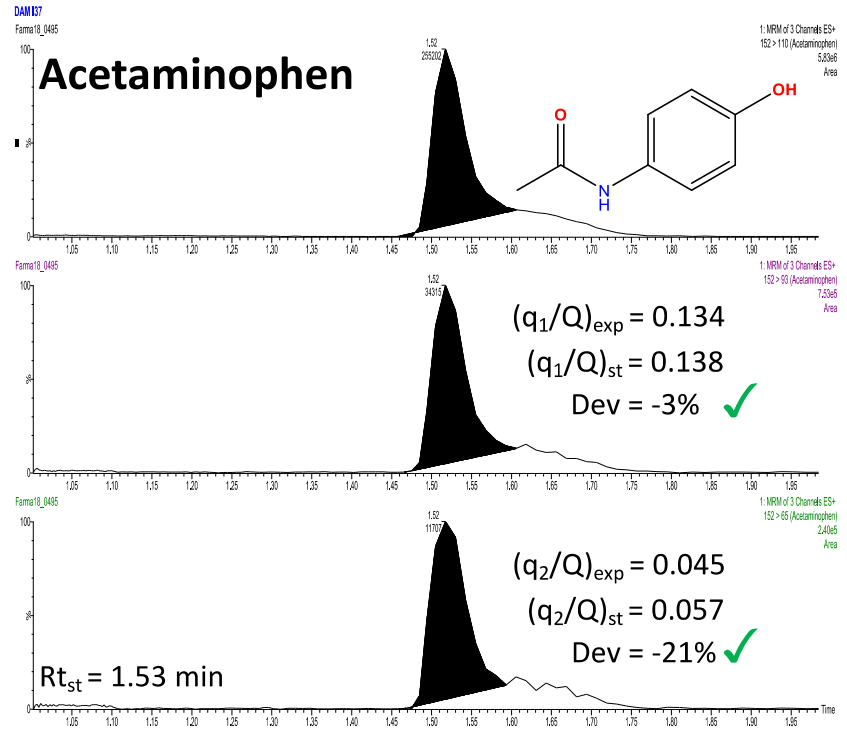

Figure 2. *LC-MS/MS chromatogram for Acetaminophen. IWW sample collected on Monday.*

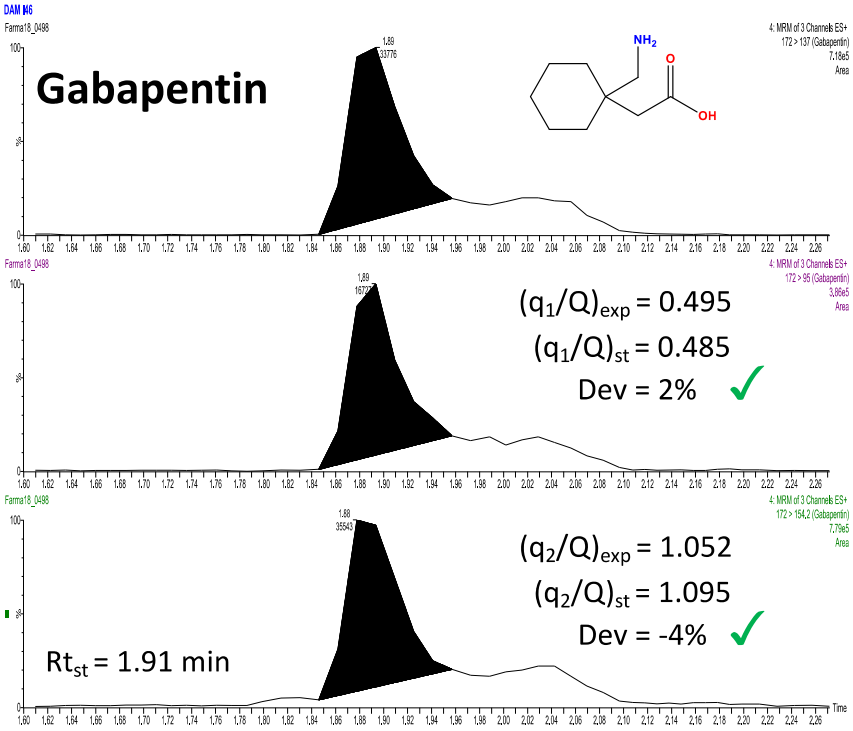

Figure 3. *LC-MS/MS chromatogram for Gabapentin. IWW sample collected on Thursday.*

Table 6. *Concentrations (ng/L) of pharmaceuticals in IWW samples.*

Drugs	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Acetaminophen	5185	8713	7336	6649	7171	7733	2643
Alprazolam	-	-	-	-	-	-	d
Atorvastatin	86	90	72	81	84	113	80
Azithromycin	d	d	d	d	d	d	d
Bezafibrate	-	-	-	-	-	-	-
Carbamazepine	d	d	d	d	d	d	d
Ciprofloxacin	99439	460160	88450	48479	-	-	51776
Clarithromycin	117	96	127	76	70	101	95
Clindamycin	-	-	-	-	-	d	-
Diclofenac	407	247	199	197	165	252	157
Enalapril	45	66	51	49	49	58	34
Erythromycin	-	35	30	32	d	33	d
Furaltadone	-	-	-	-	-	-	-
Gabapentin	3561	3875	4311	3641	4087	4678	3942
Gemfibrozil	-	-	-	-	-	-	-
Irbesartan	215	240	189	248	224	264	185
Ketoprofen	-	-	-	-	-	-	-
Levamisol	26	-	43	29	27	32	27
Lincomycin	-	-	-	-	-	-	-
Lorazepam	34	29	41	35	-	-	-
Losartan	156	181	156	178	165	221	120
Metoprolol	d	d	d	-	d	d	d
Metronidazole	d	d	d	-	-	d	-
Nalidixic acid	d	-	-	-	-	-	-
Naproxen	-	d	d	d	-	d	-
Norfloxacin	-	1269	1966	1030	551	178	-
Omeprazole sulfide, 4-OH	64	65	65	52	66	90	63
Oxolinic acid	-	-	-	-	-	-	-
Pantoprazole	-	-	-	-	-	-	-
Phenazone	93	-	d	d	-	d	56
Primidone	80	71	69	84	63	82	88
Roxithromycin	-	-	-	-	-	-	-
Salbutamol (Albuterol)	-	d	d	d	d	d	d
Simvastatin	-	-	-	-	-	-	-
Sulfadiazine	-	-	-	-	-	-	-
Sulfamethoxazole	70	129	70	56	57	101	36
Tetracycline	49	53	-	-	-	-	-
Tramadol	574	570	649	593	660	730	603
Trimethoprim	180	199	126	109	93	146	109
Valsartan	457	552	390	602	444	651	456
Venlafaxine	146	151	154	153	180	191	161

-. Not detected.

d. Detected but not quantified. Concentration below LOQ.

Underlined. Indicative data of concentration.

Table 7. *Daily loads (ng/day) of pharmaceuticals in IWW samples.*

Drugs	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Acetaminophen	115	181	126	120	159	161	50
Alprazolam	-	-	-	-	-	-	d
Atorvastatin	1.9	1.9	1.24	1.5	1.9	2	1.5
Azithromycin	d	d	d	d	d	d	d
Bezafibrate	-	-	-	-	-	-	-
Carbamazepine	d	d	d	d	d	d	d
Ciprofloxacin	2200	9584	1520	872	-	-	984
Clarithromycin	3	2	2	1.4	1.6	2	1.8
Clindamycin	-	-	-	-	-	d	-
Diclofenac	9	5	3	4	4	5	3
Enalapril	1	1.4	-	-	1.09	1..21	0.65
Erythromycin	-	0.73	0.52	0.58	d	0.69	d
Furaltadone	-	-	-	-	-	-	-
Gabapentin	79	81	74	66	91	97	75
Gemfibrozil	-	-	-	-	-	-	-
Irbesartan	5	5	3	5	5	6	4
Ketoprofen	-	-	-	-	-	-	-
Levamisol	0.58	-	0.74	0.52	0.60	0.67	0.51
Lincomycin	-	-	-	-	-	-	-
Lorazepam	0.75	0.60	0.70	0.63	-	-	-
Losartan	4	4	3	3	4	5	2
Metoprolol	d	d	d	-	d	d	d
Metronidazole	d	d	d	-	-	d	-
Nalidixic acid	d	-	-	-	-	-	-
Naproxen	-	d	d	d	-	d	-
Norfloxacin	-	26	34	19	12	4	-
Omeprazole sulfide, 4-OH	1.4	1.4	1.12	0.93	1.5	1.9	1.20
Oxolinic acid	-	-	-	-	-	-	-
Pantoprazole	-	-	-	-	-	-	-
Phenazone	2	-	d	d	-	d	1.06
Primidone	1.8	1.5	1.19	1.5	1.4	1.7	1.7
Roxithromycin	-	-	-	-	-	-	-
Salbutamol (Albuterol)	-	d	d	d	d	d	d
Simvastatin	-	-	-	-	-	-	-
Sulfadiazine	-	-	-	-	-	-	-
Sulfamethoxazole	1.6	3	1.20	1	1.3	2	0.68
Tetracycline	1.08	1.10	-	-	-	-	-
Tramadol	13	12	11	11	15	15	12
Trimethoprim	4	4	2	2	2	3	2
Valsartan	10	12	7	11	10	14	9
Venlafaxine	3	3	3	3	4	4	3

-. Not detected.

d. Detected but not quantified. Concentration below LOQ.

Underlined. Indicative data of concentration.

Table 8. *Concentrations (ng/L) of pharmaceuticals in EWW samples.*

Drugs	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Acetaminophen	-	-	-	-	-	-	-
Alprazolam	d	d	d	d	d	6	d
Atorvastatin	-	d	-	-	d	-	-
Azithromycin	-	-	-	-	-	-	-
Bezafibrate	-	-	-	-	-	-	-
Carbamazepine	-	-	-	-	d	d	-
Ciprofloxacin	1163	-	610	1558	11082	3769	-
Clarithromycin	53	44	57	34	58	47	43
Clindamycin	d	d	-	d	d	d	10
Diclofenac	182	151	141	148	126	135	119
Enalapril	-	-	-	-	-	-	-
Erythromycin	36	24	26	41	23	23	22
Furaltadone	-	-	-	-	-	-	-
Gabapentin	1669	1638	1599	1550	1391	1535	1506
Gemfibrozil	-	-	-	-	-	-	-
Irbesartan	150	134	167	194	191	192	195
Ketoprofen	-	-	-	-	-	-	-
Levamisol	37	30	27	28	25	24	27
Lincomycin	-	-	-	-	-	-	-
Lorazepam	44	44	41	31	57	41	47
Losartan	12	13	10	d	11	15	d
Metoprolol	d	d	d	-	10	14	d
Metronidazole	d	d	-	d	-	d	-
Nalidixic acid	d	d	d	d	-	-	d
Naproxen	-	-	-	-	-	-	-
Norfloxacin	-	-	-	2078	531	515	-
Omeprazole sulfide, 4-OH	39	40	39	37	34	36	40
Oxolinic acid	-	18	-	-	-	-	-
Pantoprazole	21	20	19	19	17	18	23
Phenazone	d	d	-	d	d	d	d
Primidone	66	59	69	74	77	74	84
Roxithromycin	-	-	-	-	-	-	-
Salbutamol (Albuterol)	-	d	d	d	d	d	d
Simvastatin	-	-	-	-	-	-	-
Sulfadiazine	-	-	-	-	-	-	-
Sulfamethoxazole	44	46	36	31	19	18	38
Tetracycline	-	30	18	-	-	-	d
Tramadol	-	529	503	530	-	910	271
Trimethoprim	12	15	10	-	24	-	d
Valsartan	-	31	-	-	35	19	18
Venlafaxine	184	180	162	175	155	160	191

-. Not detected.

d. Detected but not quantified. Concentration below LOQ.

Underlined. Indicative data of concentration.

Table 9. *Daily loads (ng/day) of pharmaceuticals in EWW samples.*

Drugs	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Acetaminophen	-	-	-	-	-	-	-
Alprazolam	d	d	d	d	d	0.10	d
Atorvastatin	-	d	-	-	d	-	-
Azithromycin	-	-	-	-	-	-	-
Bezafibrate	-	-	-	-	-	-	-
Carbamazepine	-	-	-	-	d	d	-
Ciprofloxacin	20	-	11	26	184	60	-
Clarithromycin	0.90	0.74	1.03	0.57	0.96	0.75	0.80
Clindamycin	d	d	-	d	d	d	0.19
Diclofenac	3	3	3	3	2	2	2
Enalapril	-	-	-	-	-	-	-
Erythromycin	0.61	0.40	0.47	0.68	0.38	0.36	0.41
Furaltadone	-	-	-	-	-	-	-
Gabapentin	28	28	29	26	23	24	28
Gemfibrozil	-	-	-	-	-	-	-
Irbesartan	3	2	3	3	3	3	4
Ketoprofen	-	-	-	-	-	-	-
Levamisol	0.63	0.50	0.49	0.47	0.41	0.38	0.50
Lincomycin	-	-	-	-	-	-	-
Lorazepam	0.75	0.74	0.74	0.52	0.95	0.65	0.88
Losartan	0.20	0.22	0.18	d	0.18	0.24	d
Metoprolol	d	d	d	-	0.17	0.22	d
Metronidazole	d	d	-	d	-	d	-
Nalidixic acid	d	d	d	d	-	-	d
Naproxen	-	-	-	-	-	-	-
Norfloxacin	-	-	-	35	9	8	-
Omeprazole sulfide, 4-OH	0.66	0.67	0.71	0.62	0.56	0.57	0.74
Oxolinic acid	-	0.30	-	-	-	-	-
Pantoprazole	0.36	0.34	0.34	0.32	0.28	0.29	0.43
Phenazone	d	d	-	d	d	d	d
Primidone	1.12	0.99	1.3	1.23	1.3	1.17	1.6
Roxithromycin	-	-	-	-	-	-	-
Salbutamol (Albuterol)	-	d	d	d	d	d	d
Simvastatin	-	-	-	-	-	-	-
Sulfadiazine	-	-	-	-	-	-	-
Sulfamethoxazole	0.75	0.77	0.65	0.52	0.32	0.29	0.71
Tetracycline	-	0.50	0.33	-	-	-	d
Tramadol	-	9	9	9	-	14	5
Trimethoprim	0.20	0.25	0.18	-	0.40	-	d
Valsartan	-	0.52	-	-	0.58	0.30	0.33
Venlafaxine	3	3	3	3	3	3	4

-. Not detected.

d. Detected but not quantified. Concentration below LOQ.

Underlined. Indicative data of concentration.

Table 10. *Concentrations (ng/L) of pharmaceuticals in Hospital discharge samples.*

Drugs	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Acetaminophen	144160	145630	117290	144600	115150	158840	112340
Alprazolam	d	d	d	d	-	-	-
Atorvastatin	294	277	745	206	221	266	294
Azithromycin	1140	1387	998	1134	1349	1059	130
Bezafibrate	-	-	-	-	-	-	-
Carbamazepine	-	-	-	-	-	-	-
Ciprofloxacin	1180	4197	138890	197580	23212	112847	680508
Clarithromycin	73	-	-	-	35	-	-
Clindamycin	86	167	88	83	-	-	-
Diclofenac	385	237	189	221	236	331	392
Enalapril	253	212	59	233	189	171	263
Erythromycin	3132	296	303	525	61	436	190
Furaltadone	-	-	-	-	-	-	-
Gabapentin	13995	18114	2311	13377	22947	18943	13439
Gemfibrozil	-	-	-	-	-	-	-
Irbesartan	240	292	163	114	117	185	184
Ketoprofen	-	-	-	-	-	-	-
Levamisol	-	-	-	-	-	-	-
Lincomycin	-	-	-	-	-	-	-
Lorazepam	235	199	96	189	-	264	169
Losartan	105	451	96	181	146	778	450
Metoprolol	d	d	25	34	-	d	d
Metronidazole	-	-	-	-	-	-	-
Nalidixic acid	-	-	-	-	-	-	-
Naproxen	d	-	d	-	-	-	-
Norfloxacin	2154	9605	9200	14776	7264	10657	4703
Omeprazole sulfide, 4-OH	159	118	80	105	117	127	127
Oxolinic acid	-	-	-		-	-	-
Pantoprazole	101	48	25	85	204	71	174
Phenazone	40	-	367	-	72	-	47
Primidone	-	-	-	-	68	-	-
Roxithromycin	-	-	-	-	-	-	-
Salbutamol (Albuterol)	58	43	d	33	35	42	42
Simvastatin	-	-	-	-	-	-	-
Sulfadiazine	d	d	d	d	d	d	d
Sulfamethoxazole	-	-	-	d	-	144	1016
Tetracycline	-	-	-	-	-	-	43
Tramadol	1528	1388	1787	1490	1564	1740	1061
Trimethoprim	31	d	d	29	40	348	1299
Valsartan	3404	3152	1949	5925	2737	63	2924
Venlafaxine	1119	865	764	917	1161	1477	946

-. Not detected.

d. Detected but not quantified. Concentration below LOQ.

Underlined. Indicative data of concentration.

4.3. Estimation of the pharmaceuticals removal efficiency at the wastewater treatment plant

In order to estimate the removal efficiency of the plant, **Equation 3** was used, taking into account the values included in **Table 7** and **Table 9**.

According to the removal efficiency (RE) (%) data shown in **Table 11**, *Acetaminophen*, *Atorvastatin* and *Enalapril*, were totally eliminated, with an average efficiency of 100%. Furthermore, *Losartan*, *Trimetroprim* and *Valsartan*, also presented a great efficiency of elimination but lower than 100%. For other compounds RE was around or higher than 50%, such as *Clarithromycin*, *Diclofenac*, *Gabapentin*, *Omeprazole sulfide 4-OH*, *Sulfamethoxazole* and *Tetracycline*. Moreover, *Irbesartan*, *Levamisol*, *Primidone* and *Venlafaxine* present an average efficiency of less 35%, which means that the treatments applied in this WWTP in order to eliminate the compounds does not efficiently eliminate these pharmaceuticals. **Figure 4** shows in a more visual way the average RE for one-week data expressed in %.

Table 11 also includes pharmaceuticals (e.g. *Alprazolam*, *Azithromycin*, *Clindamycin*, *Metoprolol*) with concentrations lower than the limit of the quantification (marked with different characters). For these compounds, a removal efficiency could not be calculated, because it was not possible to obtain a numerical value, being the present compound as d.

Erythromycin, *Lorazepam*, *Primidone* and *Venlafaxine* present higher levels of concentration in the water of the exit than in the water of the entrance, so they present negative efficiencies. As it has been mentioned before, the fact of founding pharmaceuticals with higher concentrations in the treated water has been already analysed in the scientific research. Moreover, the uncertainty associated to the calculations need to be considered because the water from the effluent of the following day was compared with the water from the influent of the previous day, so 24h is the estimated time of residence in the WWTP.

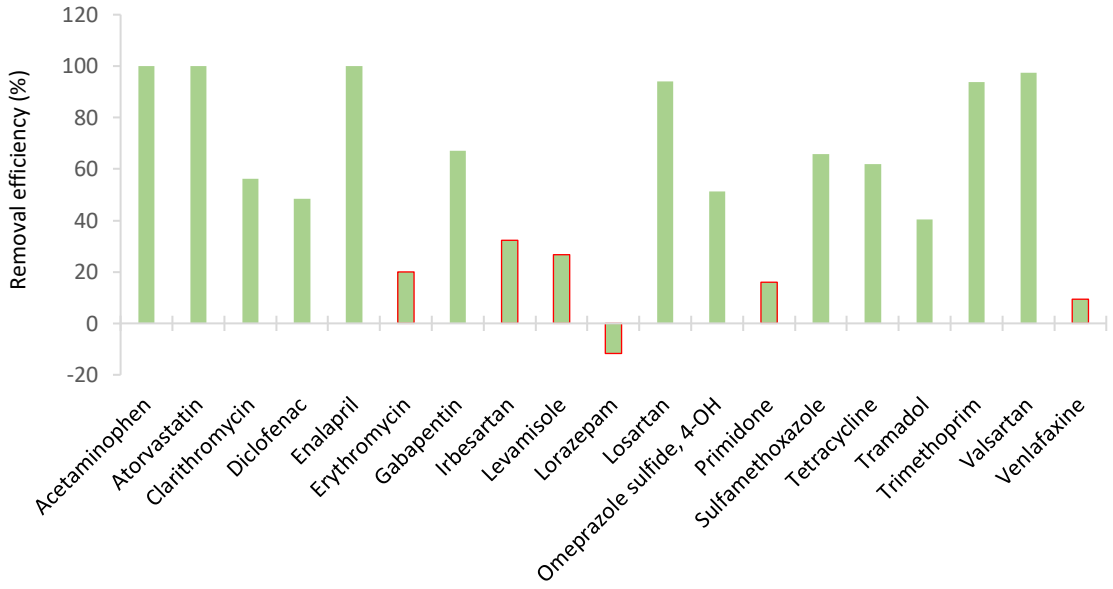

Figure 4. *Average one-week removal efficiency (%) for quantified drugs in the WWTP.*

Table 11. *Removal efficiency (%) of pharmaceuticals, w*here the weekday specified refers to the day of collection of EWW.

Drugs	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	Average
Acetaminophen	100	100	100	100	100	100	100
Alprazolam	*a*	*a*	*a*	*a*	*e*	*c*	*a*
Atorvastatin	c	100	100	*c*	100	100	100
Azithromycin	*b*	*b*	*b*	*b*	*b*	*b*	*b*
Bezafibrate	-	-	-	-	-	-	-
Carbamazepine	*b*	*b*	*b*	0	0	*b*	*b*
Ciprofloxacin	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Clarithromycin	71.4	48.5	73.9	29.9	51.6	61.9	56.2
Clindamycin	*A*	-	*a*	*a*	*a*	*d*	*a*
Diclofenac	71.8	50.4	27.8	41	41.5	57.6	48.4
Enalapril	100	100	-	-	100	100	100
Erythromycin	*e*	35.6	-30.8	34.5	*d*	40.6	20
Furaltadone	-	-	-	-	-	-	-
Gabapentin	65	64.1	65.1	64.8	73.2	71.1	67
Gemfibrozil	-	-	-	-	-	-	-
Irbesartan	52.5	39.6	0.3	28.9	38.6	33.7	32.3
Ketoprofen	-	-	-	-	-	-	-
Levamisol	13.8	*e*	36.5	21.2	36.7	25.4	26.7
Lincomycin	-	-	-	-	-	-	-
Lorazepam	1.3	-23.3	25.7	-50.8	*e*	*e*	-11.7
Losartan	93.6	95.2	*c*	94.4	93.4	*c*	94.1
Metoprolol	0	0	*b*	*e*	*d*	0	0
Metronidazole	0	*b*	0	-	*a*	*b*	0
Nalidixic acid	0	*a*	*a*	-	-	*a*	*a*
Naproxen	-	*b*	*b*	*b*	-	*b*	*b*
Norfloxacin	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Omeprazole sulfide, 4-OH	52.8	47.4	44.6	39.8	61.2	60.4	51.3
Oxolinic acid	*e*	-	-	-	-	-	-
Pantoprazole	*e*	*e*	*e*	*e*	*e*	*e*	*e*
Phenazone	*c*	-	0	0	*a*	0	0
Primidone	44.1	15.5	-3.4	15.2	16.4	8.2	16
Roxithromycin	-	-	*b*	-	-	-	-
Salbutamol (Albuterol)	*a*	0	0	0	0	0	0
Simvastatin	-	-	-	-	-	-	-
Sulfadiazine	-	-	-	-	-	-	-
Sulfamethoxazole	50.3	75.8	56.7	68	77.2	66.2	65.7
Tetracycline	53.7	70	-	-	-	*a*	61.9
Tramadol	30.5	23.3	20.7	100	1.6	66.7	40.5
Trimethoprim	93.7	95.7	100	79.6	100	*c*	93.8
Valsartan	94.9	100	100	94.6	97	97.6	97.4
Venlafaxine	6.2	7	-10.2	6.6	36.5	10.1	9.4

-. Not detected. a. When an undetected compound appears after treatment but cannot be quantified. b. When a compound detected but not quantified is no longer detected in the EWW sample. c. When a compound is detected after treatment, but quantification is not possible. d. When a compound is detected before treatment and can be quantified afterwards. e. When compound is not detected is quantified after passing through the WWTP. n.c. Not calculated as being indicative data. 0. When compound is detected in both influent and effluent samples.

4.4. Comparison of pharmaceuticals prevalence in IWW and Hospital water

When comparing **Table 6** and **Table 10**, it can be observed that most of pharmaceuticals from the Hospital samples presented average concentrations significantly higher than in urban IWW.

With the purpose of visually illustrate the differences between the streams that enter in the WWTP, **Figure 5** compares the average weekly concentration of pharmaceuticals in both Hospital water and IWW. Only *Clarithromycin*, *Irbesartan*, *Levamisol*, *Primidone* and *Tetracycline* showed a slightly higher average concentration in the water of the entrance into the WWTP than the Hospital sewage. *Levamisol* and *Primidone* were not detected in the water of the Hospital, but they presented an average concentration in the IWW waters of 29 ng/L and 76 ng/L respectively.

Acetaminophen and *Gabapentin* presented the highest average concentration in both the Hospital WW and the IWW samples. *Acetaminophen* (i.e. the active principle of Paracetamol) is a daily used pharmaceutical that is first in the ranking of the consumption of analgesics' active principles (Sanitarios, 2019). Moreover, it is second in the ranking of the first twenty active principles with the highest consumption in containers (Ministerio de Sanidad, 2019). Therefore, it is understandable that it presents high concentrations in wastewater. On the other hand, the high levels of *Gabapentin* seems surprising because it is not in the ranking of the twenty active principles with the highest consumption in containers (Ministerio de Sanidad, 2019). However, it must be taken into account the metabolism of the pharmaceutical, because it highly influences the levels of the parent compound excreted. Thus, it may occur that a compound reaches high levels in WW not only due to its high consumption but also to its low metabolism in humans, i.e. cases when the parent compound is the main product excreted in urine. This seems to be the situation for *Gabapentin*. Other studies (Goodman, C. W., & Brett, 2017; Mondón, S., Nogué, S., Urbano, D., & Rovira, 2010) have shown an increasing Gabapentin's prescription and consumption, although there are concerns about its possible misuse and abuse.

4.5. Environmental risk assessment

The continued introduction of PPCPs into the aquatic environment as well as their bioactivity and known modes of action, convert them to emerging contaminants, which may have ecotoxicological effects on aquatic and terrestrial organisms and public health (X. Liu et al., 2020), and may produce antibiotic resistance and endocrine disruptions on humans (M. Liu et al., 2019). Therefore, the assessment of their potential effect in the environmental compartment is of utmost importance for a sustainable aquatic system.

For this purpose, the parameter known as HQ has been estimated in order to evaluate the impact of the pharmaceuticals in the aquatic environment. The HQ has been calculated for treated water dividing the concentration of each identified pharmaceutical in the EWW with by its PNEC (Predicted No Effect Concentration) value, as shown in **Equation 4**.

$$HQ = \frac{C_{EWW}}{PNEC} \qquad \textbf{(Equation 4)}$$

C_{EWW} corresponds to the weekly average concentration of the pharmaceutical (ng/L) in the effluent sample (shown in **Table 8**). Furthermore, the PNEC value has been taken from the literature, and is shown in **Table 12** together with the calculated value of the HQ.

Among the 22 HQ calculated values, only 4 showed a HQ higher than 1, the value taken as a reference in risk assessment: *Ciprofloxacin* (40.85), *Diclofenac* (2.86), *Norfloxacin* (1.33) and *Venlafaxine* (4.53). As previously mentioned, the concentrations of the pharmaceuticals *Ciprofloxacin* and *Norfloxacin* must be taken as an estimated value, so the HQ values are also approximate values, but they are sufficiently high values to be taken into account, particularly for *Ciprofloxacin*. Moreover, *Diclofenac* and *Venlafaxine* are considered to have higher impact in the aquatic environment as the HQ is above 1.

In general, the obtained results are satisfactory from an environmental point of view because almost all the pharmaceuticals presented HQ lower than 1. However, it must be considered that these estimations have been made considering the parent pharmaceuticals only, regardless their metabolites. Previous research (Han & Lee, 2017) has reported that pharmaceuticals' metabolites may produce similar, even higher risk in the aquatic environment than the pharmaceuticals themselves. For this reason, it would be interesting in future works to include, at least some relevant metabolites, in both studies on occurrence in wastewater and environmental risk assessment.

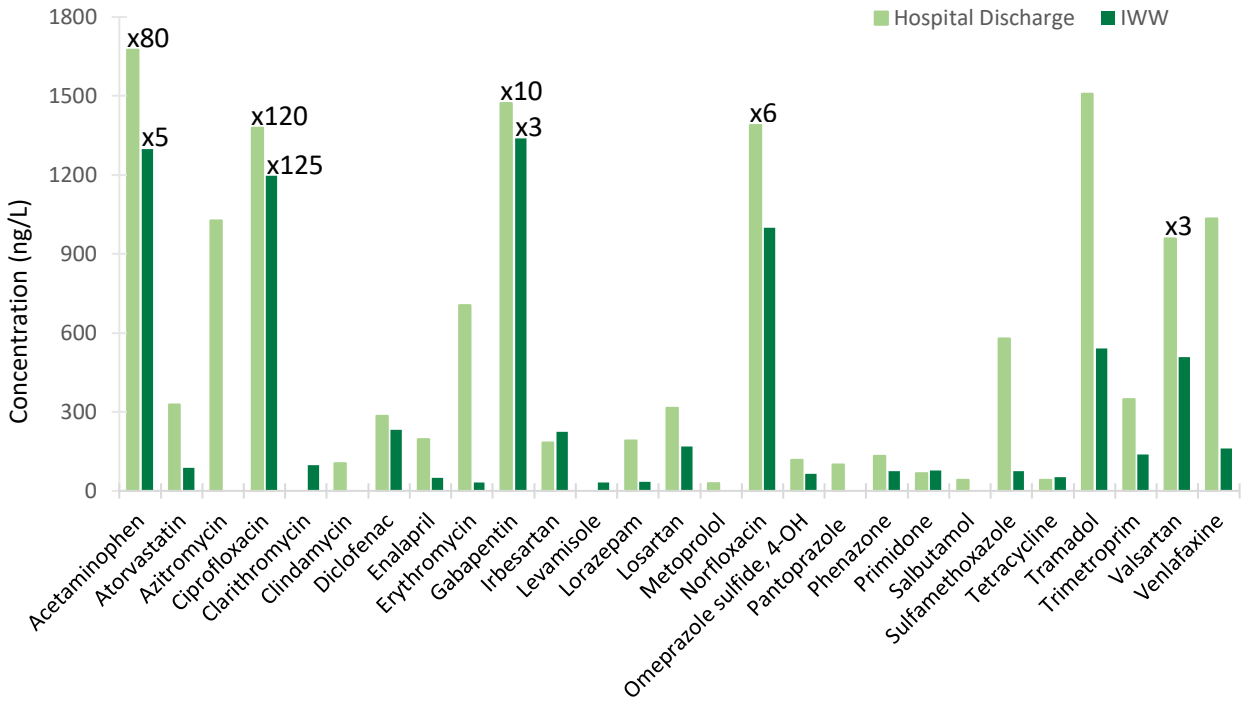

Figure 5. *Comparison of average weekly concentrations of drugs detected in IWW and Hospital wastewaters.*

Table 12. *PNEC data and calculated HQ values.*

Compound	CAS No.	PNEC (µg/L)	HQ	Reference
Acetaminophen	103-90-2	134	-	(Network, 2020)
Alprazolam	28981-97-7	0.077	0.08	(Network, 2020)
Atorvastatin	134523-00-5	0.01	-	(Network, 2020)
Azithromycin	83905-01-5	0.019	-	(Network, 2020)
Bezafibrate	41859-67-0	2.3	-	(Network, 2020)
Carbamazepine	298-46-4	0.05	-	(Network, 2020)
Ciprofloxacin	85721-33-1	0.089	40.85	(Network, 2020)
Clarithromycin	81103-11-9	0.12	0.40	(Network, 2020)
Clindamycin	18323-44-9	1.7	-	(Network, 2020)
Diclofenac	15307-86-5	0.05	2.86	(Network, 2020)
Enalapril	75847-73-3	1.58	-	(Network, 2020)
Erythromycin	114-07-8	0.2	0.14	(Network, 2020)
Furaltadone	139-91-3	19.2	-	(Network, 2020)
Gabapentin	60142-96-3	10	0.16	(Network, 2020)
Gemfibrozil	25812-30-0	0.5	-	(Network, 2020)
Irbesartan	138402-11-6	704	0	(Network, 2020)
Ketoprofen	22071-15-4	2.1	-	(Network, 2020)
Levamisole	14769-73-4	1.81	0.02	(Network, 2020)
Lincomycin	154-21-2	3.95	-	(Network, 2020)
Lorazepam	846-49-1	0.096	0.46	(Network, 2020)
Losartan	114798-26-4	78	0	(Network, 2020)
Metoprolol	37350-58-6	8.6	0	(Network, 2020)
Metronidazole	443-48-1	6.76	-	(Wielens Becker et al., 2020)
Nalidixic acid	389-08-2	8.98	-	(Network, 2020)
Naproxen	22204-53-1	1570	-	(Wielens Becker et al., 2020)
Norfloxacin	70458-96-7	0.78	1.33	(Network, 2020)
Omeprazole sulfide, 4-OH	103876-98-8	0.26	0.15	(Network, 2020)
Oxolinic acid	14698-29-4	1.07	0.02	(Network, 2020)
Pantoprazole	102625-70-7	0.68	0.03	(Network, 2020)
Phenazone	60-80-0	21.1	-	(Network, 2020)
Primidone	125-33-7	9.11	0.01	(Network, 2020)
Roxithromycin	80214-83-1	0.083	-	(Network, 2020)
Salbutamol (Albuterol)	18559-94-9	17.1	-	(Network, 2020)
Simvastatin	79902-63-9	2.63	-	(Network, 2020)
Sulfadiazine	68-35-9	1	-	(Network, 2020)
Sulfamethoxazole	723-46-6	0.6	0.06	(Network, 2020)
Tetracycline	60-54-8	0.5	0.05	(Network, 2020)
Tramadol	27203-92-5	8.65	0.06	(Network, 2020)
Trimethoprim	738-70-5	120	0	(Network, 2020)
Valsartan	137862-53-4	560	0	(Network, 2020)
Venlafaxine	93413-69-5	0.038	4.53	(Network, 2020)

Underlined. Indicative data.

5. Conclusions

A total of 41 pharmaceuticals have been determined (identification and quantification) by UHPLC-MS/MS with QqQ in raw influent and effluent wastewater from a WWTP in the north Spain. In this way, the occurrence of these compounds in both types of water have been evaluated, as well as the removal efficiency at the wastewater treatment plant for the selected pharmaceuticals. In addition, on the basis of concentrations found in treated water (effluent) the potential hazardous effect of these contaminants in the environment has been assessed. In order to support the reliability of quantitative data reported, several QCs were prepared and analysed in every batch of samples, showing recovery values between 60% and 140% for the great majority of compounds under study.

Most of the compounds were found in the IWW entering into the WWTP and in the Hospital discharge, illustrating the consumption of the pharmaceuticals in the population. The high concentration of *Acetaminophen* and *Gabapentin* stood out in comparison to the other pharmaceuticals. The removal efficiency of the plant was studied by comparing the mass loads in IWW and EWW samples. In general, the wastewater treatment plant was able to remove, at least partially, a major part of the pharmaceuticals. However, only *Acetaminophen*, *Atorvastatin* and *Enalapril* were totally eliminated. Hazard quotients were calculated from the concentration data obtained from the EWW samples to assess the potential hazard posed by their presence in the aquatic system. HQ was calculated for each pharmaceutical as the ratio between the concentration found in EWW and the PNEC value, which was based on data reported by the scientific literature. PNEC refers to the concentration that will probably not have any toxic effect. Only 4 compounds presented HQ > 1, the value taken as reference to consider a risk in the aquatic environment. *Ciprofloxacin*, with a HQ of 40.85 is remarkable because suggesting high aquatic environmental risk. Furthermore, *Diclofenac*, *Norfloxacin* and *Venlafaxine* also shown a hazard quotient higher than 1.

This work shows that although most of pharmaceuticals are partially removed in the WWTP, the treatments applied should be improved for a more efficient elimination. At present, with the conventional treatments applied in the WWTP, most of the pharmaceuticals released into the environmental do not pose a significant risk to the aquatic environment, but still four compounds are found at levels that may suppose an hazard that would notably decrease by adding tertiary treatments (e.g. advanced oxidation processes, among others) in the WWTP.

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Annex

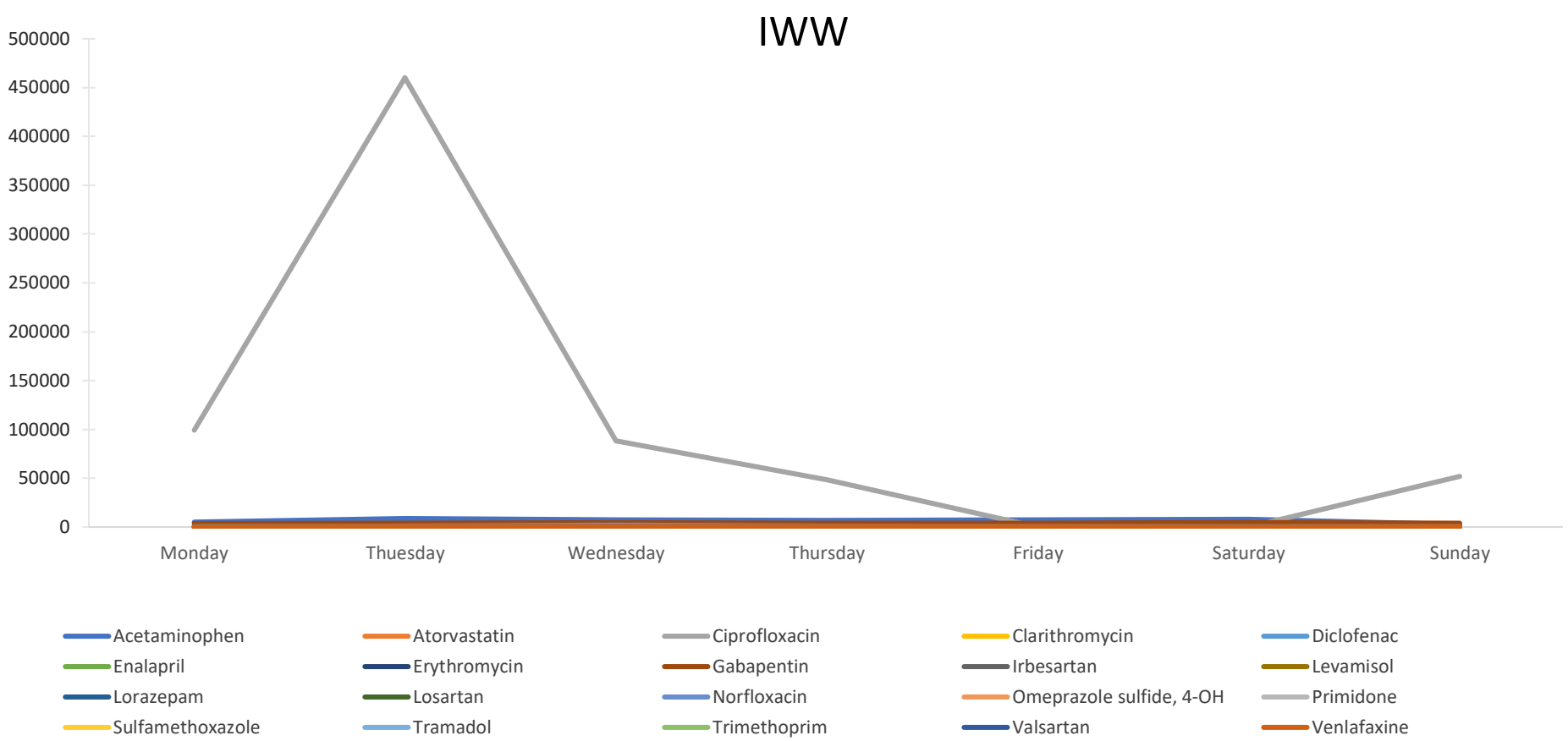

Figure 6. *Quantification trend graph of all drugs in the one-week IWW samples.*

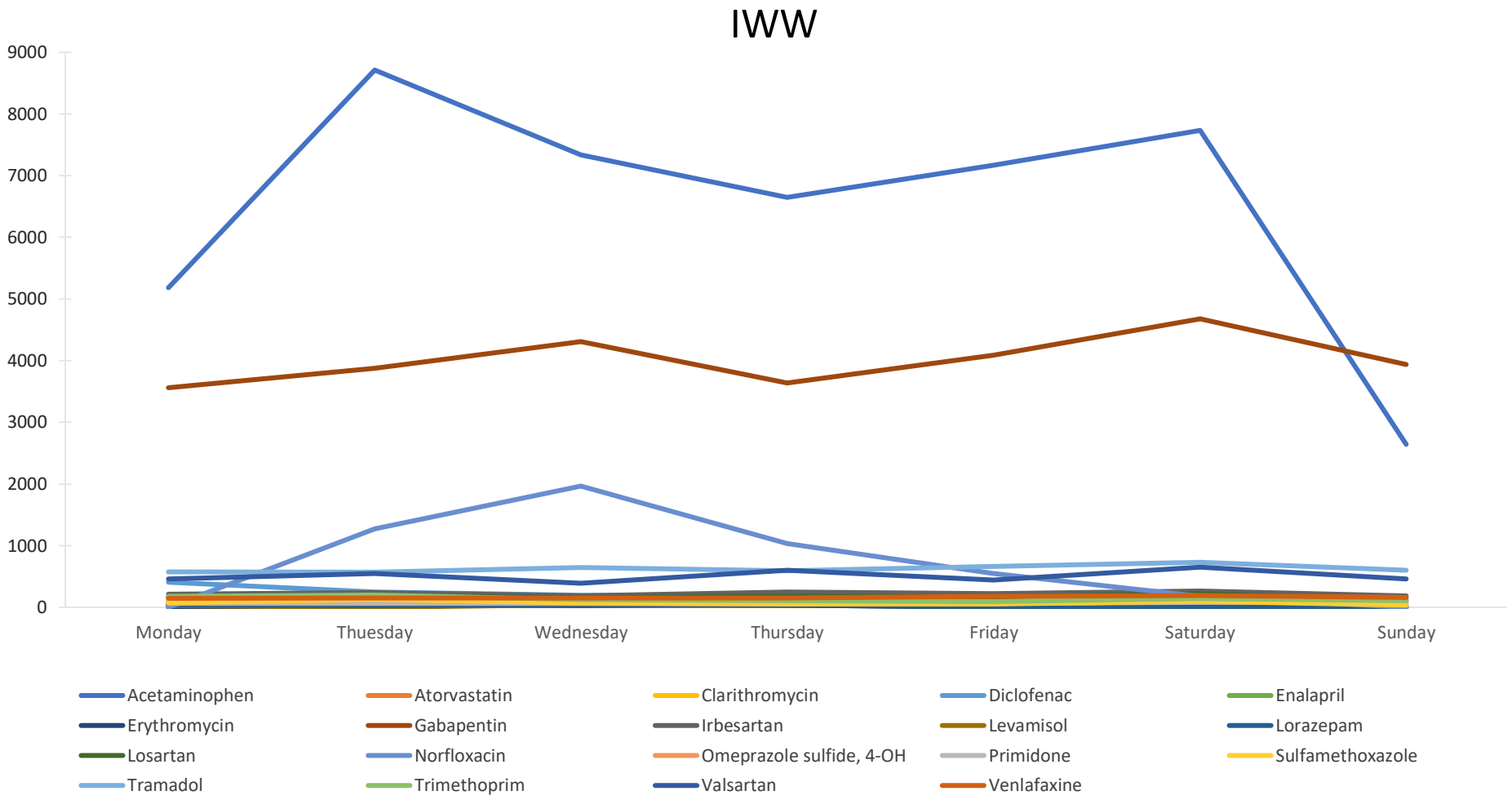

Figure 7. *Quantification trend graph of all drugs in the one-week IWW samples except Ciprofloxacin.*

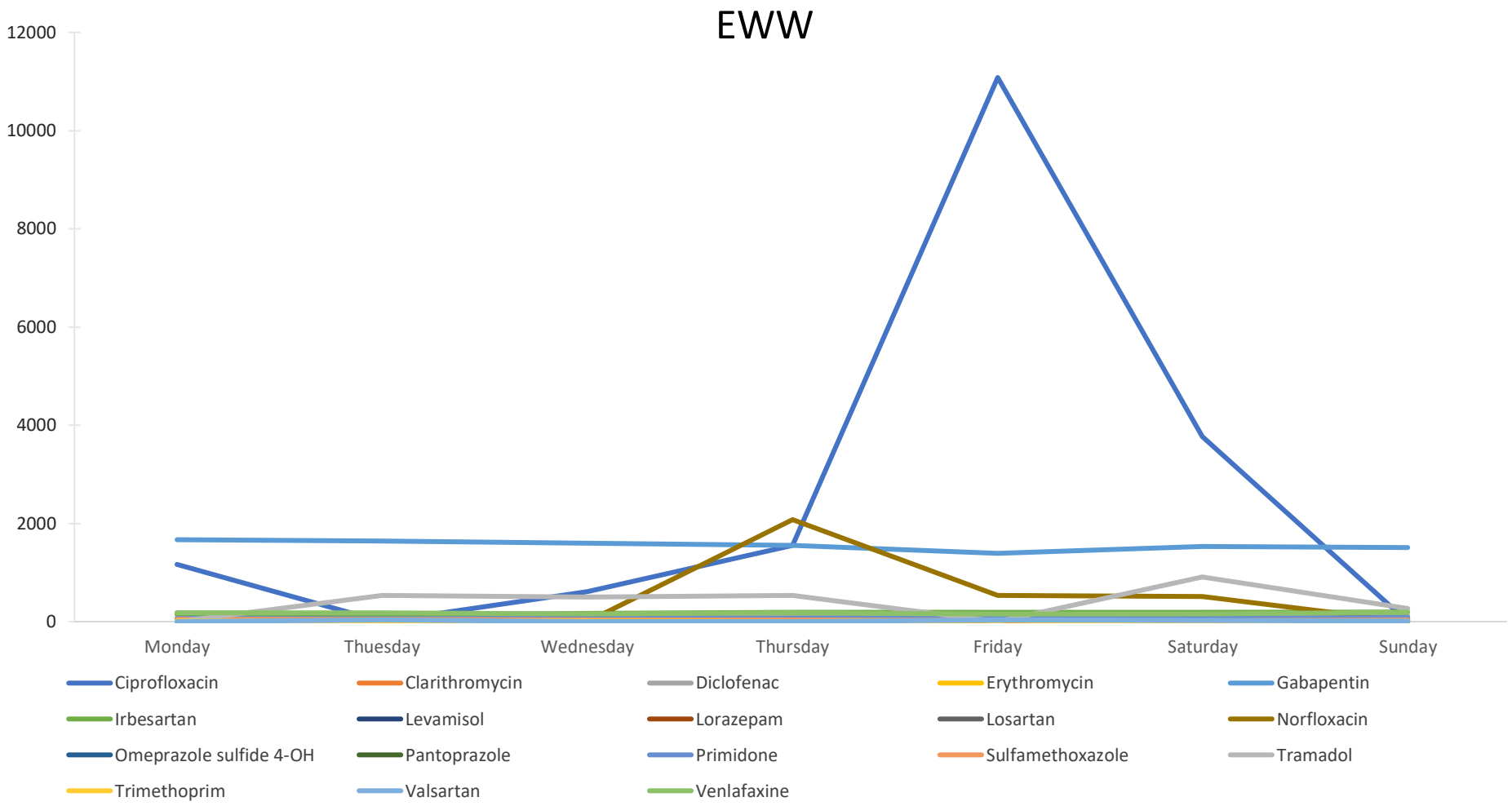

Figure 8. *Quantification trend graph of all drugs in the one-week EWW samples.*

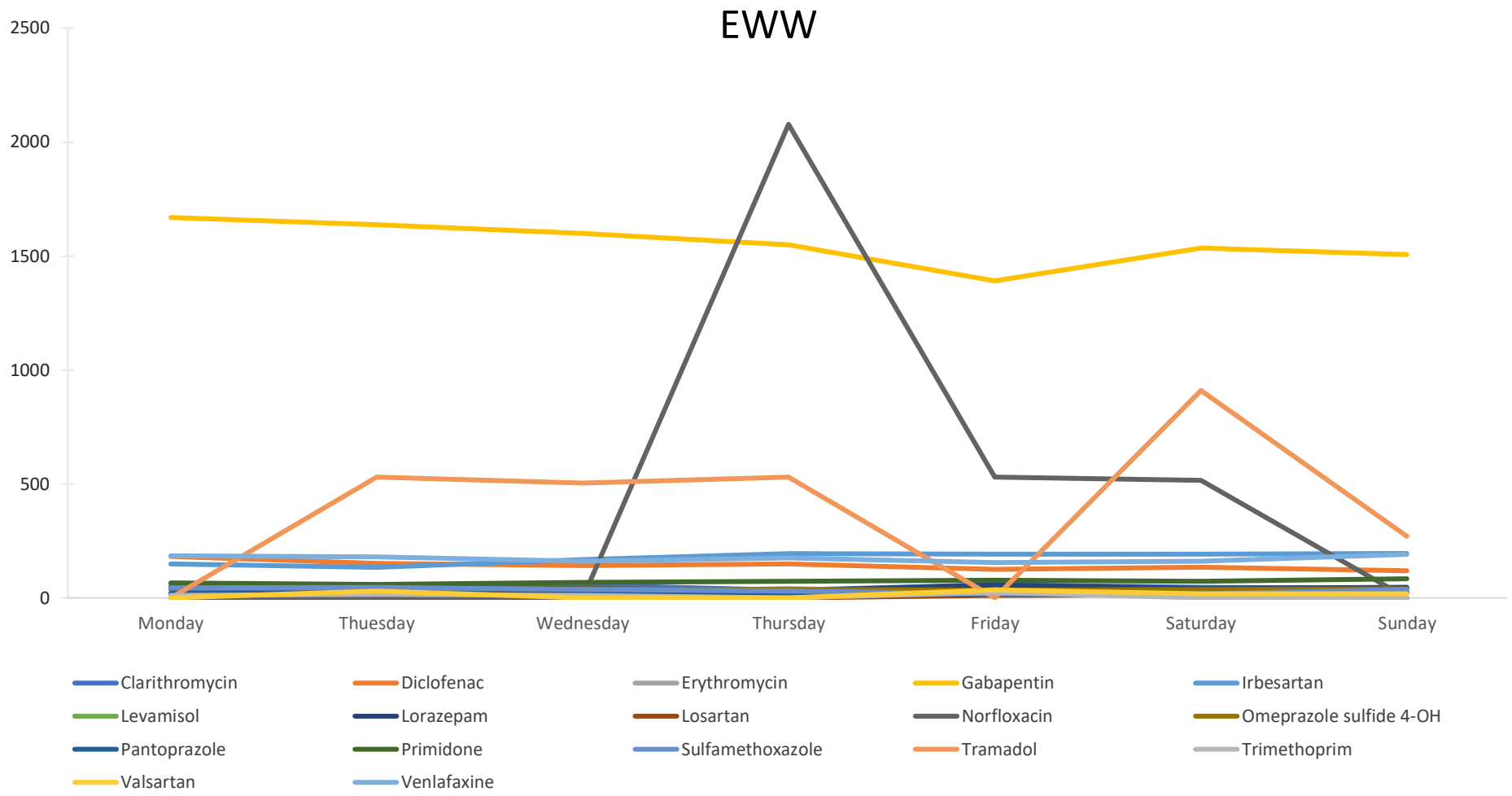

Figure 9. *Quantification trend graph of all drugs in the one-week EWW samples except Ciprofloxacin.*

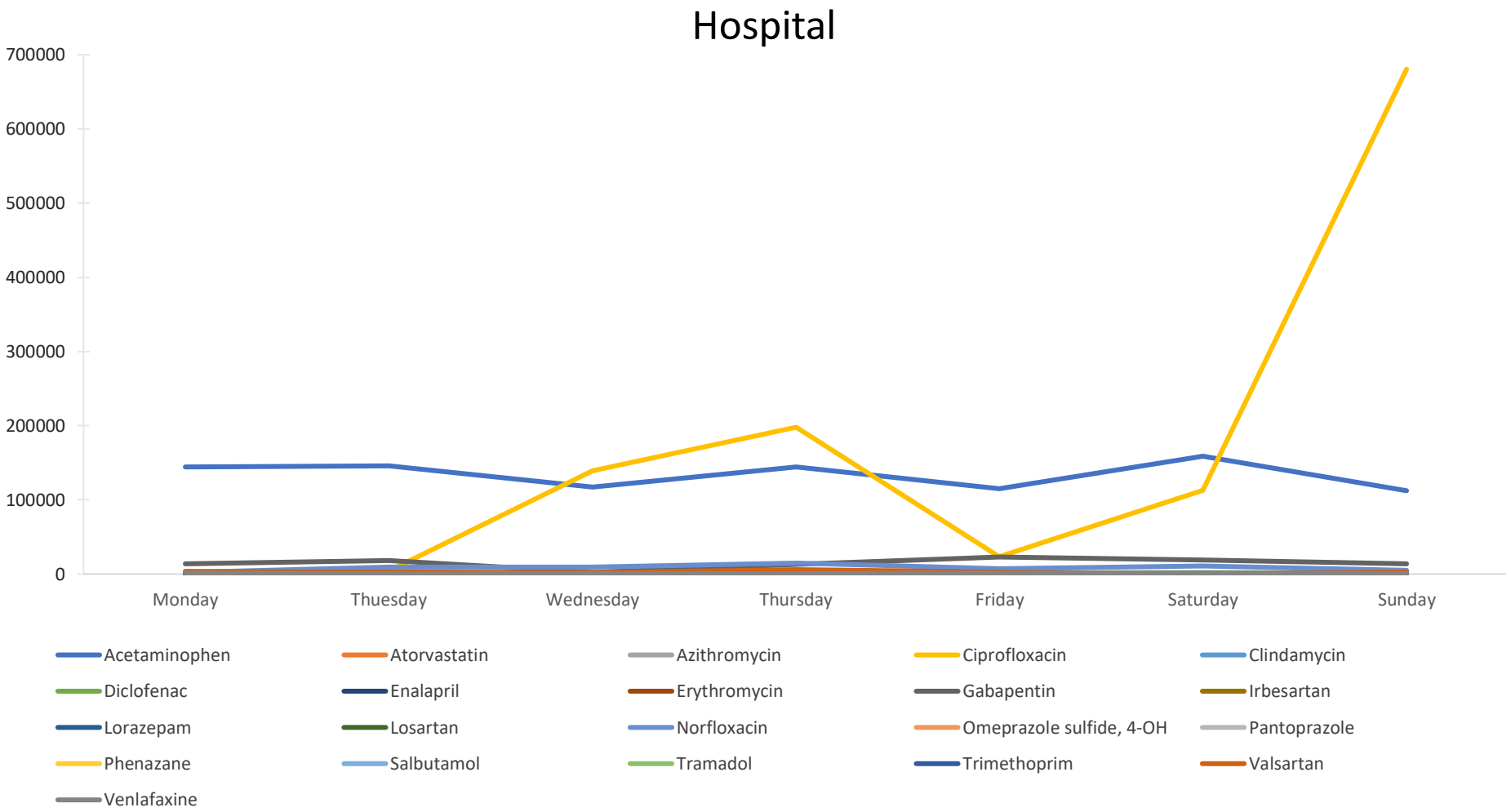

Figure 10. *Quantification trend graph of all drugs in the one-week Hospital samples.*

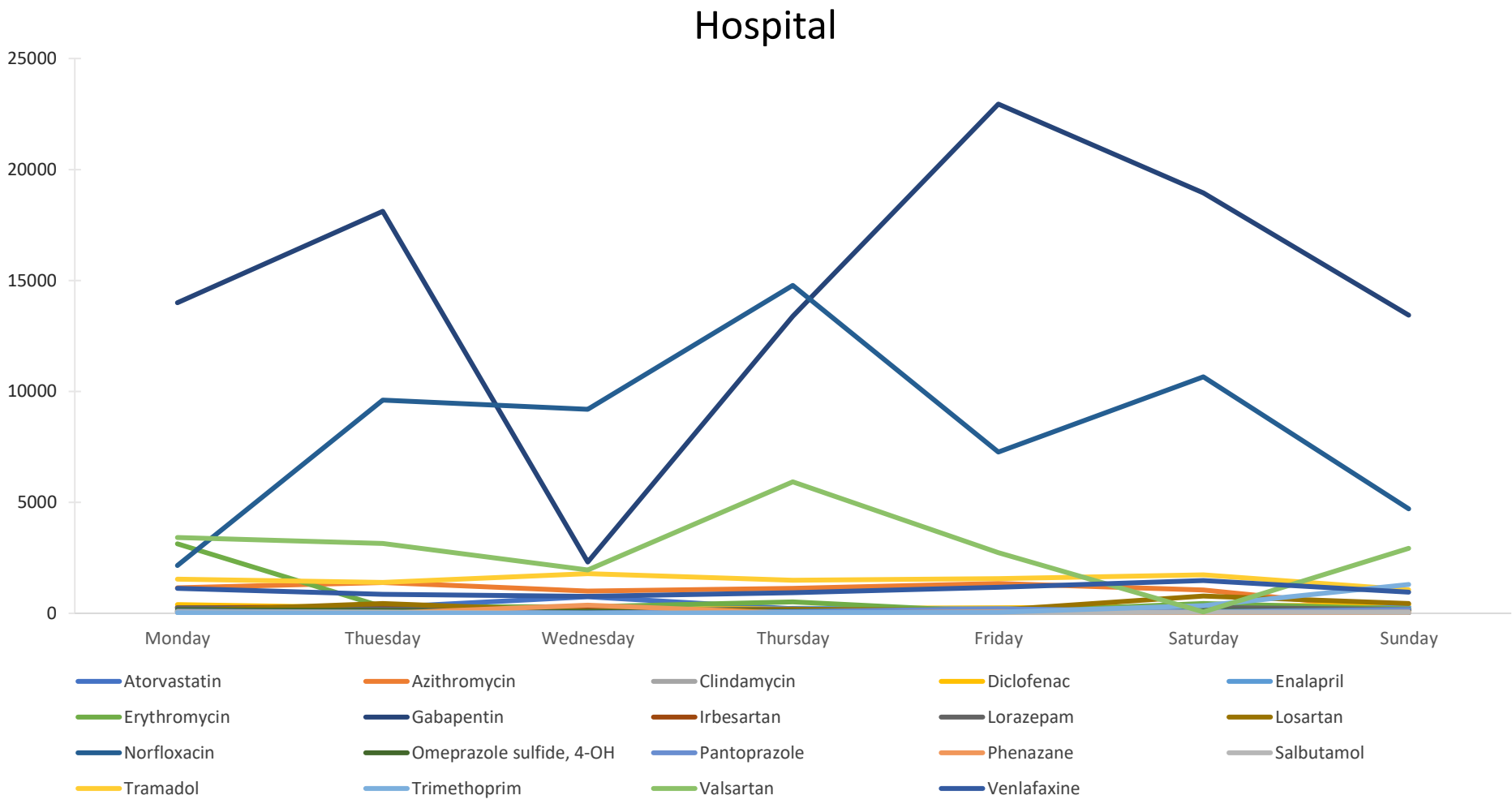

Figure 11. *Quantification trend graph of all drugs in the one-week Hospital samples except Acetaminophen and Ciprofloxacin.*