Assessment of 83 pharmaceuticals in WWTP influent and effluent samples by UHPLC-MS/MS: Hourly variation

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Highlights

•Hourly variation of 83 pharmaceuticals in WWTP influent and effluent is presented.
•Pharmaceuticals belonging to different therapeutic classes were analysed.
•Some pharmaceuticals were detected in the influents in the μg/L range.
•The importance of the determination of metabolites and transformation products is highlighted.

Abstract

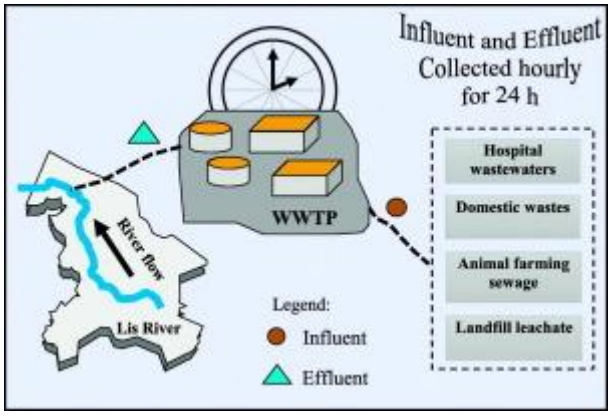
The removal efficiency of pharmaceuticals in wastewater treatment plants (WWTPs) is variable and some of these compounds pass these plants almost intact and others presenting a removal efficiency close to 100%. Their incomplete removal results in a continuous discharge of pharmaceuticals into the environment. To assess the profile of contamination of influents and effluents over a day, a set of 83 pharmaceuticals were evaluated hourly in a WWTP in Leiria, Portugal. The composite samples of the influent and effluent were also collected.

Concentrations varied from <MDL for ketoprofen, clarithromycin, ofloxacin, and diltiazem to 63.97 μg/L for caffeine in the WWTP influent composite sample and <MDL for clarithromycin, bupropion, and diltiazem to 2.01 μg/L for *O*-desmethylvenlafaxine for effluent composite sample. Concentrations in the range of μg/L were found for hydroxyibuprofen, salicylic acid, d,l-norephedrine, and caffeine in the WWTP influent, and diclofenac, carbamazepine, *O*-desmethylvenlafaxine in the WWTP effluents.

For the samples collected hourly, thirty-eight and twenty-nine pharmaceuticals were detected in at least one WWTP sample. In the WWTP influent the total concentration of detected pharmaceuticals was higher between 15 and 22 h and lower in the period from 23 to 10 h in the morning. In the WWTP effluent, a slight variation was noticed throughout the sampling hours.

Carbamazepine, fluoxetine, sertraline, atorvastatin, caffeine, simvastatin, and trazodone were the pharmaceuticals with risk quotient (RQ) >1 in WWTP influents, and carbamazepine, fluoxetine, sertraline the pharmaceuticals with an RQ > 1 in WWTP effluents.

Graphical abstract



Keywords

Wastewaters
Hourly sample collection
Mass spectrometry
Pharmaceuticals
Solid phase extraction
UHPLC-MS/MS

1. Introduction

Modern societies have benefited from the introduction of thousands of synthetic chemicals in the last century. However, the importance of their environmental fate has only been recognized in the last few decades, particularly in the case of micropollutants, such as pharmaceutical compounds (Rivera-Utrilla et al., 2013).

Little is known about the possible ecological risks of most of these pollutants. This lack of knowledge results in a substantial amount of ongoing efforts to develop data and approaches that may be useful in assessing the impact of pharmaceuticals on the environment (Ankley et al., 2007). The assessment of their presence in the aquatic environment, at very low levels (ng/L), has been possible due to the developments in analytical determination, such as the use of ultra-high performance liquid chromatography coupled to tandem mass spectrometry detection (UHPLC-MS/MS) (Paíga et al., 2015; Paíga et al., 2016; Petrovic et al., 2010). This method proved to be a robust and reliable instrument for monitoring pharmaceuticals in environmental samples (Paíga et al., 2016).

The massive use of pharmaceuticals for both human and veterinary purposes leads to the introduction of tons of these compounds in wastewaters, which is mainly attributed to the effluents of manufacturing processes, human and animal excretion, disposal of unused or expired pharmaceutical products, and unintentional shed through the manufacturing or distribution process (Diaz-Cruz et al., 2003).

After treatment in wastewater treatment plants (WWTPs), considerable amounts can be transferred to surface waters either due to insufficient removal efficiencies or, if high removals are attained, concentrations up to ng/L and μg/L can still be found, depending on the compounds' mass loadings (Paíga et al., 2015; Rivera-Utrilla et al., 2013). Although it is not legally required in Europe, the control of this type of substances in surface waters is crucial, because it may affect water quality and potentially impact drinking water supplies, ecosystems, and human health (EU_L78/40, 2015).

Outcomes of different studies showed that the concentrations of some pharmaceutical substances in wastewater and their treated effluents might fluctuate along the year (Fernández et al., 2014; Gago-Ferrero et al., 2017; Golovko et al., 2014; Vatovec et al., 2016). In Portugal, this fluctuation is coherent with the existent statistical data that refers a monthly sales variation of pharmacotherapeutic subgroups (INFARMED, 2018). The main reasons found for the seasonal variation of the presence of pharmaceuticals in wastewater were the changes in some substances/products consumption rate in response to each season characteristic diseases (respiratory infections, depression and allergies treatment drugs, etc.) (Gago-Ferrero et al., 2017; Golovko et al., 2014; Moreno-González et al., 2014; Sun et al., 2014; Vatovec et al., 2016), demographic characteristics (population age) associated or not with demographic mobility (areas strongly influenced by educational institutions, holyday period, tourism areas) (Moreno-González et al., 2014; Pereira et al., 2015; Vatovec et al., 2016) and weather variation (abundance/lack of precipitation, temperature changes, per capita domestic water consumption), all influencing the dilution rate (Diaz-Cruz et al., 2003; Fernández et al., 2014; Sun et al., 2014).

Some seasonal conditions, such as long periods of sunlight exposure of the effluent during the treatment were also referred as a cause to the reduction of some substances susceptible to photodegradation (Gago-Ferrero et al., 2017; Moreno-González et al., 2014). In addition to seasonal oscillation, weekly fluctuations in the concentrations of pharmaceutical substances in water courses were observed in several sampling points, associated with the same behavior in WWTP effluents, that occurred mainly between the weekend and the rest of the week (Moreno-González et al., 2014). Furthermore, daily variations were also noticed for some pharmaceutical products in wastewater, associated with daily drug administration patterns (Coutu et al., 2013; Plósz et al., 2010).

In a previous study, the occurrence of 33 pharmaceuticals and metabolites was evaluated along the Lis river (Leiria, Portugal) and in influents and effluents of two WWTPs located along the river (Paíga et al., 2016). In samples collected from August 2013 to June 2014, pharmaceuticals, such as ibuprofen, ketoprofen, carbamazepine and fluoxetine, and the metabolite salicylic acid showed 100% of detection frequency, at levels up to 1.3 μg/L for ibuprofen (Paíga et al., 2016).

The purpose of this study was to extend the number of pharmaceutical compounds analysed, using a new sampling campaign that took place in June 2017. Samples of one

WWTP (Leiria, Portugal) influent and effluent were collected hourly, for 24 h. Effluent samples were collected considering the WWTP hydraulic retention time. Flow proportional 24-h composite samples of the influent and the effluent were also collected. A set of 83 pharmaceuticals belonging to different therapeutic classes, including non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, antibiotics, anorectics, anxiolytics, beta-blockers, laxatives, antidiabetic drug, antipsychotic, calcium channel blocker, fibrate lipid lowering agent, stimulants, lipid regulator and cholesterol lowering statin drugs, proton pump inhibitor, and psychiatric drugs were assessed. The variation throughout the day of pharmaceutical concentrations, and the removal efficiency of the WWTP were characterized.

2. Materials and methods

2.1. Sampling site and sample collection

Leiria is a city and a municipality in the Centre Region of Portugal. Lis river is one of Leiria's most important resources. Almost 40 km long, the river drains in Vieira beach, after crossing the Lis fields, a wide farming area watered by its abundant flow (Vieira et al., 2012). Nowadays, after an extensive requalification project as part of the POLIS Programme, the riverbanks are the chosen place to exercise and play sports. Lis River also constitutes an important inland water resource for domestic, industrial and irrigation purposes (LeiriaMunicipality, 2018), thus it is imperative to prevent and control water pollution.

Hog farming located along the basin of the Lis river is known for being one of the sources of pollution in the river (Vieira et al., 2012). According to the news, Lis river basin has been subjected in the past 30 years to constant ecological disasters, mainly due to piggery untreated wastewater discharges (Vieira et al., 2012). Freshwater pollution problems are gaining attention regionally due to their social, economic, and health impacts. Moreover, the sources of contamination may be influenced by different geographical patterns of pharmaceuticals consumption (Vieira et al., 2012), and important fluctuations due to seasonal variations might also occur (Paíga et al., 2016).

The influents and effluents of a WWTP located along the Lis river are target of the present study. The wastewaters treated by the Coimbrão WWTP are domestic and hospital wastewaters, and landfill leachate. The WWTP also treats animal farming sewage (pigs manure), through the sludge treatment process, since the manure is discharged by trucks on the WWTP, going directly to anaerobic digesters, where that slurry joins the sludge removed from the liquid phase in the treatment process. The WWTP comprises primary, secondary (activated sludge), and tertiary (disinfection achieved by UV exposure) treatments. This WWTP is in operation since 2008 and has the capacity to treat about 37,997 m^3 of wastewater per day, corresponding to 248,685 inhabitants (Paíga et al., 2016). Of the total capacity volume, nearly 80% are domestic. The remaining volumes come mainly from industries and around 5% are swine effluent from farms located in the immediate vicinity of the WWTP. At the end of treatment, a portion of the treated effluent is reused for irrigation of the WWTP's green spaces and for washes and the other parcel is directly discharged to the Lis river (Website, 2015). The hydraulic retention time and the sludge retention time are 25 h and 18 d, respectively.

Influents and effluents were collected each hour, during a 24 h cycle, and a composite sample from 24 h was also analysed. Effluent samples were collected considering the WWTP hydraulic retention time. Polypropylene bottles (1 L) pre-rinsed with ultrapure water were used for the sample collection. Samples were kept at 4 °C until arrival to the laboratory. Then, the samples were vacuum filtered through 0.45 μm nylon membrane filters (Fioroni Filters, Ingré, France) and stored at −20 °C until extraction.

2.2. Reagents, solvents and materials

Methanol LC-MS and acetonitrile LC-MS grade were supplied by Scharlau (Barcelona, Spain), propanol LC-MS was obtained from Sigma-Aldrich (Steinheim, Germany), and formic acid (PA-ACS) and hydrochloric acid (HCl) 37% were supplied by Carlo Erba (Rodano, Italy). Ultrapure water (resistivity of 18.2 MΩ.cm) was produced using a Simplicity 185 system (Millipore, Molsheim, France).

Ethylenediaminetetraacetic acid disodium salt 2-hydrate (Na_2EDTA) (assay > 99.0%) was obtained from Panreac (Barcelona, Spain).

Pharmaceuticals, transformation products, metabolites, isotopically labelled internal standards (ILIS), CAS, molecular weight, formula, and supplier company are presented in Table SM1 (Supplementary material). All compounds were of high purity grade (≥98%).

Individual stock standard and ILIS solutions were prepared at a concentration of 1000 mg/L on a weight basis. Different solvents or mixture of solvents were used: acetonitrile, methanol, acetonitrile:methanol (1:1, v/v), acetonitrile:5% acetic acid in ultrapure water, methanol: ultrapure water (1:1, 2:1, v/v), and ultrapure water:10% acetic acid in ultrapure water (1:1, v/v) (Barry et al., 2004; Paíga et al., 2017a, Paíga et al., 2017b) (Table SM1, Supplementary material). All stock solutions were stored at −20 °C. Working standard solutions, containing all pharmaceuticals were prepared in acetonitrile:ultrapure water (30:70, v/v). A mixture with the seventeen ILIS was also prepared to be used for internal standard calibration.

Caffeine $^{13}C_3$ (1000 mg/L), carbamazepine-d10 (100 mg/L), *O*-desmethylvenlafaxine (100 mg/L), diazepam-d5 (1000 mg/L), norsertraline hydrochloride (100 mg/L), sibutramine hydrochloride (1000 mg/L), topiramate-d12 (100 mg/L), and venlafaxine-d6 (100 mg/L) were purchased as methanolic solutions.

All chromatographic solvents were filtered through a 0.22 μm nylon membrane filter (Fioroni Filters, Ingré, France) using a vacuum pump (Dinko D-95, Barcelona, Spain). The solvents were degassed for 15 min in an ultrasonic bath (Sonorex Digital 10P, Bandelin DK 255P, Germany). SPE cartridges Strata-X (200 mg, 3 mL) from Phenomenex, Inc. (California, USA) were used in the SPE extraction. Sample extracts were filtered through 0.22 μm PTFE syringe filters (Specanalitica, Carcavelos, Portugal) before the chromatographic analysis.

2.3. Sample extraction

In the previous work of the authors (Paíga et al., 2015, Paíga et al., 2016) SPE procedure was optimized for the extraction of 33 pharmaceuticals belonging to the

NSAIDs/analgesics, antibiotics, and psychiatric drugs. The optimized procedure was then extended for the extraction of 83 pharmaceuticals. In brief, SPE cartridges were conditioned and equilibrated with 5 mL of methanol, 5 mL of ultrapure water followed by 5 mL of ultrapure water at pH 2 using a vacuum system manifold (Chromabond, Düren, Germany). Chelating agent was added to the filtered samples. A suitable volume of a 0.1 M Na_2EDTA solution was added to the samples to achieve a final concentration of 0.1% (g solute/g solution). Volumes of 100 mL for the WWTP effluent and 50 mL for the WWTP influent samples were used, adjusted to pH 2 with concentrated HCl, and pre-concentrated on Strata-X cartridges. The cartridges were then rinsed with 5 mL (2 × 2.5 mL) of ultrapure water and dried under vacuum for 60 min to remove excess water. Then, a total of 10 mL of methanol (4 × 2.5 mL) were used in the elution step and the extracts were evaporated under a gentle stream of nitrogen. The residues were reconstituted with 500 μL of acetonitrile:ultrapure water (3:7, v/v) and 5 μL of a mixture of ILIS solutions was added. The final concentration of each ILIS in the standard solutions and in the WWTP effluents and influents samples is presented in Table SM2 (Supplementary Material).

2.4. UHPLC-MS/MS analysis

Chromatographic analysis was performed on a Shimadzu Nexera UHPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two solvent delivery pumps (LC-30 AD), a column oven (CTO-20 AC), an auto-sampler (SIL-30 AC), a degasser (DGU-20A 5R), and a system controller module (CBM-20A) coupled to a triple-quadrupole mass spectrometer (Ultra-Fast Mass Spectrometry series LCMS-8030, Shimadzu Corporation, Kyoto, Japan) operated in the electrospray ionization (ESI) mode. Lab Solutions software (Shimadzu Corporation, Kyoto, Japan) was used for control and data processing.

Kinetex™ C18 column (2.6 × 150 mm i.d.; 1.7 μm particle size) from Phenomenex, Inc. (California, USA) and Cortecs™ UPLC® C18+ column (100 × 2.1 mm i.d.; 1.6 μm particle size) from Waters (Milford, Massachusetts, USA) were the two columns used for the chromatographic separation.

From the preceding works of the authors, chromatographic separation, chromatographic columns, different mobile phases, mode of elution (isocratic or gradient), oven temperature, and flow rate were tested. The optimized programs were developed for NSAIDs/analgesics, antibiotics, psychiatric drugs (Paíga et al., 2017b) and anorectic, antiepileptic, anxiolytics, laxatives, and stimulants compounds (Paíga et al., 2017a).

32 new pharmaceuticals were added to the present study belonging to the therapeutic classes of antibiotics, antidiabetic drug, antipsychotic, calcium channel blocker, β-blockers, fibrate lipid lowering agent, lipid regulator and cholesterol lowering statin drugs, proton pump inhibitor, psychiatric drugs metabolites, and stimulant compounds. Thus, a total of 83 pharmaceuticals were analysed. Ampicillin, atorvastatin, atenolol, caffeine, chlortetracycline, chlorpromazine, citalopram *N*-oxide, didemethylcitalopram, demethylcitalopram, *O*-desmethylvenlafaxine, diltiazem, doxycycline, erythromycin, fenofibrate, lansoprazole, lomefloxacin, metformin, moxifloxacin, norfloxacin, oxytetracycline, propranolol, prulifloxacin, simvastatin, sulfathiazole, sulfamethizole, sulfaquinoxaline, and tetracycline were analysed in the positive ESI mode (Paíga et al., 2017b) using a Cortecs™ UPLC® C18+ column. Amoxicillin, citalopram propionic acid,

gemfibrozil, pravastatin, and potassium clavulanate, ionized in negative ESI, were introduced in the program developed for the analysis of pharmaceutical adulterants in plant food supplements (Paíga et al., 2017a) using a Kinetex™ C18 column.

The pharmaceuticals analysed in each program are described in Table SM3 (Supplementary material) and all chromatographic conditions and MS parameters are presented in Table SM4 (Supplementary material). Most of the pharmaceuticals have a good peak shape except for tetracycline group that show tailing. Ampicillin, amoxicillin, atenolol, chlorocycline, ephedrine, lomefloxacin, oxytetracycline, potassium clavulanate, pravastatin, propranolol, and tetracycline showed a lower sensitivity when compared with the remaining pharmaceuticals.

An overlay chromatogram of the studied pharmaceuticals in each program is presented in Fig. SM1 (Supplementary material). In program II, a large number of pharmaceuticals (50) is analysed making it difficult to view the peaks in the chromatogram. Therefore, in Fig. SM1 (Supplementary Material) three chromatograms for program II are presented in Fig. SM1 (Supplementary material) with a legend of b), c), and d). The antibiotic and psychiatric drugs, two families already studied by the authors in previous studies (Paíga et al., 2017b; Paíga et al., 2016), are shown in the chromatograms b) and c) and the new pharmaceuticals inserted in program II are presented in the chromatogram d) (Fig. SM1, Supplementary material).

The mass spectrometer was operated in multiple reaction monitoring mode (MRM) and two MRM transitions were monitored for each compound, being the most intense used as quantifier and the second one as qualifier. For the new pharmaceuticals, MRM settings were analyte-specific and were optimized by direct injection of individual standard solutions with a concentration of 100 mg/L. Optimized mass spectrometry parameters (precursor ions, quantifier and qualifier ions, and ion ratio), the optimum collision energies and cone voltages selected for each transition used for quantification and identification of each pharmaceutical are shown in Table SM5 (Supplementary material).

The auto-sampler was operated at 4 °C and the needle was rinsed before and after sample aspiration using acetonitrile:methanol:propanol (1:1:1, v/v/v). The injection volume was 5 µL and column oven was set at 30 °C. Argon was used as the collision induced dissociation gas (CID) at a pressure of 230 kPa.

2.5. Validation of the analytical method

A thorough and complete method validation of the studied compounds in WWTP influents and effluents was performed. The method was validated for linearity, method detection limits (MDLs), method quantification limits (MQLs), precision (intra- and inter-day), recovery, and matrix effect (ME).

The linearity of the method was established by setting calibration curves using linear regression analysis with twelve concentration levels in the range of 0.5 to 1000 µg/L (0.5, 1.0, 5.0, 10, 25, 50, 75, 100, 250, 500, 750, and 1000 µg/L). Solvent blanks containing acetonitrile were prepared to run after every ten samples for monitoring the instrumental background.

MDLs and MQLs were determined as the minimum amount detectable of analyte with a signal-to-noise ratio of 3 and 10, respectively.

Method precision was determined by repeated intra- and inter-day analysis and expressed as the relative standard deviation (RSD (%)). A standard mixture containing all the analytes at a final concentration of 50, 100, and 250 μg/L was used and six successive injections in one day and sextuplicate injections in three consecutive days were performed, respectively.

The influence of the ME was evaluated by the comparison of the matrix matched calibration curve and the calibration curve prepared in solvent, namely, acetonitrile:ultrapure water (30:70, v/v). For each compound, the ratio between its response in the wastewater effluents and influents and the response of the standard in solvent at the same concentration (250 μg/L) was taken as ME, and was calculated according to the Eq. (1) (Gros et al., 2012). A value of zero indicates that there is no ME, while for a positive value there is an ion enhancement signal and a negative % value indicates an ion suppression signal.(1)

Recovery was calculated by comparing the MRM peak area for samples spiked prior to SPE extraction (pre-spiked sample) with the MRM peak area for samples spiked after SPE extraction (post-spiked sample). Thus, for the WWTP influents and effluents a blank and a fortified experiment were carried out for the pre- and post-spiked sample. The pharmaceuticals extraction efficiencies were determined by analysis of three replicates with the following conditions for WWTP influent:

Level I (0.5 $\mu g_{pharmaceutical}/L_{sample}$*): 1 mL of 25 μg/L of fortified concentration using 50 mL of sample;*

Level II (1.0 $\mu g_{pharmaceutical}/L_{sample}$*): 1 mL of 50 μg/L of fortified concentration using 50 mL of sample;*

Level III (2.5 $\mu g_{pharmaceutical}/L_{sample}$*): 1 mL of 125 μg/L of fortified concentration using 50 mL of sample;*

and for WWTP effluents:

Level I (0.25 $\mu g_{pharmaceutical}/L_{sample}$*): 1 mL of 25 μg/L of fortified concentration using 100 mL of sample;*

Level II (0.5 $\mu g_{pharmaceutical}/L_{sample}$*): 1 mL of 50 μg/L of fortified concentration using 100 mL of sample;*

Level III (1.25 $\mu g_{pharmaceutical}/L_{sample}$*): 1 mL of 125 μg/L of fortified concentration using 100 mL of sample.*

2.6. Environmental risk characterization

The risk that the pharmaceuticals detected in the WWTP influents and effluents in the present study may represent to the aquatic environment was estimated through their risk quotient (RQ) at three representative trophic levels of the aquatic ecosystem (algae,

daphnia, and fish). The RQ depends not only on the concentration of each pharmaceutical but also on its ecotoxicity (Ginebreda et al., 2010). The RQs are defined as the ratio of potential exposure to the substance and the level at which no adverse effects are expected. According to EU guidelines (EMEA, 2006) the RQ was calculated for each substance according to the Eq. (2):(2)

where the MEC corresponds to the highest concentration of the pharmaceutical found in the analysed samples, while the PNEC was calculated dividing the lowest acute toxicity value (median effective or lethal concentration, EC50 or LC50) reported in the peer reviewed literature for the three selected trophic levels by the pertinent assessment factor (usually 1000) (EuropeanComission, 2003). ECOSAR predictive model (v1.11) (USEPA, 2012) was used for MEC/PNEC calculation.

A worst-case scenario approach was followed, and the maximum measured environmental concentration found in the WWTP influents and effluents in the Lis river was used. When the compound was detected in the samples but the concentration was below the method quantification limit (MQL) or the method detection limit (MDL), half of the MQL was considered.

The potential ecological risk of these chemicals was evaluated according to a frequently used risk ranking criterion (Rivera-Jaimes et al., 2018). If RQ is equal or above 1 there is a potential environmental risk, whereas for values lower than 1 it is not expected risk (Ginebreda et al., 2010). Moreover, Mendoza et al. (2015) mentioned that for RQ values between 0.1 and 1, a low or negligible risk can be expected, while for RQ values between 1 and 10 a medium risk can be expected. RQ values above 10 indicate a high ecological risk (Mendoza et al., 2015).

3. Results and discussion

3.1. Method performance

Following the European Union criteria of 2002 (2002/657/EC, 2002), the analytical methodology used was validated in terms of linearity, inter- and intra-day precision, recovery, sensitivity (MDL and MQL), and matrix effects. The obtained results have been summarized in Table 1 and discussed in the following paragraphs.

Table 1. Retention time (min), linearity, recoveries (%) at three levels of fortification for WWTP influents and WWTP effluents for all pharmaceuticals grouped in each chromatographic program (CP).

	Pharmaceuticals, degradation products, metabolites, and Isotopically-Labelled Internal Standards (ILIS)[a]	tr (%RSD) (min)	r^2	Influents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)	Effluents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)
				Level I	Level II	Level III			Level I	Level II	Level III		
CP	Salicylic acid	1.316 (2.19)	0.9997	89.5	85.9	89.7	9.90	33.1	89.3	74.1	95.9	1.13	3.76
	Acetylsalicylic acid	1.652 (2.34)	0.9997	73.9	88.1	85.2	0.10	0.20	88.0	73.1	73.2	0.02	0.06
	Acetaminophen	1.752 (1.76)	0.9990	37.2	39.8	52.6	10.5	35.0	5.2	43.6	44.6	0.10	0.34
	Carboxyibuprofen	2.678 (4.47)	0.9999	76.6	63.9	81.9	1.50	5.10	62.1	74.2	58.3	1.24	4.12
	Hydroxyibuprofen	2.781 (4.39)	0.9992	80.0	82.2	97.6	0.20	0.80	111	88.8	89.3	2.32	7.74
PI	Ketoprofen	3.933 (0.162)	0.9985	84.3	89.9	85.0	0.50	1.60	96.4	95.0	95.9	0.26	0.88
	Naproxen	3.979 (0.143)	0.9986	96.8	98.9	94.6	0.60	2.10	89.2	98.1	116	0.69	2.31
	Nimesulide	4.131 (0.0705)	0.9991	86.5	110	99.3	0.20	0.60	92.4	70.4	97.6	1.77	5.90
	Diclofenac	4.235 (0.0848)	0.9984	95.1	98.4	85.3	0.20	0.50	123	90.2	91.6	0.03	0.10
	Ibuprofen	4.300 (2.61)	0.9993	89.5	135	114	9.90	32.9	89.4	79.2	100	18.1	60.3
	Trimethoprim	2.274 (0.378)	0.9990	82.0	99.0	109	2.10	7.20	101	89.1	88.7	0.95	3.15
	Norfloxacin	2.289 (1.17)	0.9987	82.9	49.9	70.6	72.4	241	65.0	87.2	88.7	2.60	8.70
	Ofloxacin	2.292 (0.249)	0.9999	48.6	60.5	106	0.20	0.80	73.1	123	75.2	1.40	4.65
PII	Ciprofloxacin	2.305 (0.299)	0.9993	60.6	38.1	65.5	1.60	5.50	56.1	104	68.4	98.7	329
	Lomefloxacin	2.313 (0.319)	0.9990	91.4	37.2	122	6.30	21.0	92.1	71.3	84.9	3.15	10.5
	Enrofloxacin	2.333 (0.296)	0.9999	58.6	90.1	60.8	50.3	168	75.6	90.0	80.0	19.8	66.0
	Azithromycin	2.334(0.270)	0.9998	64.4	107	105	9.40	31.2	75.8	73.8	73.5	0.30	1.05
	Moxifloxacin	2.414 (0.255)	0.9985	98.8	79.5	70.3	167	557	48.5	109	72.0	9.50	31.7

CP	Pharmaceuticals, degradation products, metabolites, and Isotopically-Labelled Internal Standards (ILIS)[a]	tr (%RSD) (min)	r^2	Influents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)	Effluents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)
				Level I	Level II	Level III			Level I	Level II	Level III		
	Sulfadiazine	2.475 (0.440)	0.9993	52.2	77.0	93.6	0.30	0.90	58.6	77.4	84.2	0.10	0.35
	Sulfapyridine	2.509 (0.335)	0.9989	55.5	74.6	88.0	2.40	8.00	54.5	75.4	81.1	0.25	0.75
	Erythromycin	2.602 (0.299)	0.9990	80.4	52.8	99.2	10.0	33.3	76.6	61.5	81.1	0.80	2.75
	Sulfamethoxypyridazine	2.637 (0.279)	0.9983	78.1	75.9	86.9	7.00	23.5	73.8	80.8	77.7	0.20	0.65
	Sulfamethazine	2.637 (0.202)	0.9985	45.2	68.6	79.1	0.60	1.90	53.3	69.6	61.6	1.30	4.30
	Prulifloxacin	2.698 (0.718)	0.9998	58.6	80.8	68.6	1.40	4.80	85.1	86.7	71.8	0.90	2.95
	Clarithromycin	2.758 (0.165)	0.9985	73.2	86.7	81.0	0.10	0.30	92.4	65.9	98.0	0.05	0.10
	Sulfamethoxazole	2.840 (0.156)	0.9988	58.8	69.5	77.6	1.20	4.10	57.1	68.2	62.9	0.65	2.15
	Sulfadimethoxine	2.968 (0.173)	0.9994	48.0	64.8	86.7	7.30	24.2	53.1	68.2	84.0	1.30	4.35
	Venlafaxine	2.469 (0.197)	0.9986	91.6	99.8	107	0.10	0.40	102	92.8	96.7	0.15	0.50
	Trazodone	2.504 (0.148)	0.9989	82.0	78.7	90.2	0.30	1.00	100	96.0	90.9	0.10	0.30
	Citalopram	2.628 (0.183)	0.9987	92.7	85.9	99.3	6.30	21.0	107	98.9	102	3.15	10.5
	Paroxetine	2.694 (0.159)	0.9991	77.4	86.2	93.3	62.4	208.1	67.1	85.3	79.8	13.1	43.5
	Norfluoxetine	2.758 (0.291)	0.9985	78.6	80.5	61.4	8.20	27.30	122	106	113	5.90	19.6
	Norsertraline	2.782 (0.721)	0.9992	76.4	111	77.7	949	3162	66.7	105	103	21.6	71.8
	Fluoxetine	2.785 (0.143)	0.9989	85.7	89.3	74.4	1.30	4.50	91.2	107	83.1	0.25	0.80
	Sertraline	2.807 (0.158)	0.9994	57.3	74.9	80.2	0.40	1.30	68.7	76.8	80.4	0.60	2.05
	10,11-Epoxicarbamazepine	2.858 (0.143)	0.9988	65.0	89.8	79.1	1.10	3.60	74.8	87.3	86.8	0.05	0.10

CP	Pharmaceuticals, degradation products, metabolites, and Isotopically-Labelled Internal Standards (ILIS)[a]	tr (%RSD) (min)	r^2	Influents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)	Effluents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)
				Level I	Level II	Level III			Level I	Level II	Level III		
	Carbamazepine	3.076 (0.158)	0.9985	96.6	102	91.5	0.30	1.00	110	95.1	95.2	0.55	1.85
	Diazepam	3.490 (0.152)	0.9995	80.1	107	105	6.60	22.0	70.7	76.9	95.9	2.25	7.60
	Metformin	0.6309 (0.307)	0.9990	0.571	0.452	0.927	1.00	3.30	0.807	1.80	0.517	0.58	1.94
	Atenolol	0.8802 (0.112)	0.9993	32.5	20.2	18.1	0.20	0.80	19.3	9.68	19.4	0.03	0.10
	O-Demethylvenlafaxine	2.320 (0.198)	0.9990	95.2	99.0	111	37.2	124	81.7	89.8	101	0.10	0.32
	Oxytetracycline	2.330 (0.583)	0.9990	34.3	33.4	51.1	124	414	7.58	54.2	57.9	7.67	25.6
	Tetracycline	2.357 (0.147)	0.9998	72.6	33.3	73.3	15.6	52.0	34.0	51.6	34.9	15.5	51.8
	Doxcycline	2.357 (0.285)	0.9990	47.8	56.2	69.4	6.30	21.0	21.7	89.2	47.0	1.22	4.08
	Caffeine	2.447 (0.197)	0.9993	86.8	63.1	103	88.4	295	109	94.2	103	0.13	0.43
	Sulfathiazole	2.478 (0.181)	0.9989	63.5	84.0	85.8	3.20	10.8	11.1	1.08	2.31	2.44	8.12
	Chlorocycline	2.482 (0.352)	0.9990	13.8	18.8	52.7	0.40	1.40	23.1	34.9	28.7	1.74	5.80
	Propranolol	2.552 (0.140)	0.9988	78.5	111	90.4	15.9	52.9	160	84.7	85.8	4.87	16.2
	Didemethylcitalopram	2.594 (0.179)	0.9987	57.5	94.7	92.3	5.60	18.6	83.3	77.0	100	6.64	22.1
	Demethylcitalopram	2.618 (0.119)	0.9985	92.3	105	84.1	1.90	6.50	85.7	109	80.4	0.58	1.94
	Sulfamethizole	2.635 (0.151)	0.9985	39.2	72.6	85.9	4.50	15.1	6.97	6.72	4.33	0.98	3.26
	Diltiazem	2.652 (0.132)	0.9999	89.3	95.3	94.2	14.8	49.3	102	91.5	98.7	0.14	0.48
	Citalopram *N*-oxide	2.676 (0.132)	0.9999	79.9	121	101	14.3	47.6	91.5	105	106	1.70	5.65
	Ampicillin	2.790 (0.871)	0.9990	29.0	69.0	72.4	90.9	303	78.5	46.3	92.1	7.52	25.1

CP	Pharmaceuticals, degradation products, metabolites, and Isotopically-Labelled Internal Standards (ILIS)[a]	tr (%RSD) (min)	r^2	Influents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)	Effluents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)
				Level I	Level II	Level III			Level I	Level II	Level III		
PI II	Lanzoprazole	2.819 (0.0441)	0.9998	n.d.	n.d.	n.d.	183	610	n.d.	n.d.	n.d.	6.50	21.7
	Chlorpromazine	2.828 (0.173)	0.9990	51.1	64.8	62.7	20.2	67.4	0.59	0.698	0.81	0.10	0.33
	Sulfaquinoxaline	2.973 (0.0314)	0.9994	53.9	62.7	78.3	21.4	71.4	4.35	2.54	1.46	0.17	0.55
	Atorvastatin	3.494 (0.111)	0.9983	62.5	65.9	55.2	3.30	10.9	54.9	37.3	61.0	0.02	0.07
	Simvastatin	4.145 (0.0551)	0.9985	34.6	69.0	58.4	75.4	251	4.6	5.73	12.1	0.01	0.05
	Fenofibrate	4.318 (0.0195)	0.9990	63.1	57.1	41.5	1.20	3.90	12.3	70.0	56.3	0.63	2.10
	Potassium clavulanate	0.973 (1.85)	0.9996	n.d.	51.8	39.5	451	1505	87.5	36.2	22.9	87.1	290.2
	Amoxicillin	1.255 (0.432)	0.9987	n.d.	58.2	55.4	7.80	26.1	48.4	74.5	53.7	1.90	6.30
	Zonisamide	3.628 (0.308)	0.9994	n.d.	97.6	96.9	57.0	190	104	98.3	84.7	27.1	90.2
	Pravastatin	3.780 (0.123)	0.9994	n.d.	69.0	69.6	24.1	80.5	56.9	62.2	46.0	22.3	74.4
	Topiramate	4.142 (0.266)	0.99995	n.d.	117	88.1	9.70	32.50	105	85.1	91.8	0.70	2.25
	Phenolphthalein	4.446 (0.257)	0.9992	n.d.	123	80.3	15.5	51.70	99.1	77.3	81.3	0.05	0.10
	Citalopram propionic acid	4.744 (0.234)	0.9997	n.d.	120	89.4	0.50	1.70	92.5	85.1	81.5	1.20	4.00
	Gemfibrozil	5.847 (0.179)	0.9998	n.d.	90.3	83.9	0.10	0.30	99.1	92.6	96.1	2.80	9.35
PI V	Synephrine	1.284 (2.11)	0.9992	3.79	3.17	1.16	26.6	88.60	3.60	4.54	1.63	0.30	0.95
	Cathine	3.169 (0.598)	0.9996	10.3	10.6	9.06	11.4	38.00	6.81	7.09	11.6	5.30	17.6
	d,l-Norephedrine	3.171 (0.132)	0.9994	8.84	9.92	10.8	72.5	241.50	8.12	9.47	9.09	10.7	35.7
	Ephendrine	3.180 (0.676)	0.9997	19.1	22.6	19.8	2.40	7.90	17.8	24.3	21.1	0.50	1.75

CP	Pharmaceuticals, degradation products, metabolites, and Isotopically-Labelled Internal Standards (ILIS)[a]	tr (%RSD) (min)	r^2	Influents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)	Effluents samples Recoveries (n = 2)			MDL (ng/L)	MQL (ng/L)
				Level I	Level II	Level III			Level I	Level II	Level III		
	Phentermine	3.193 (0.900)	0.9992	43.7	42.9	46.2	16.0	53.4	46.4	46.5	41.8	5.95	19.9
	d,l-Methamphetamine	3.1931 (0.0900)	0.9987	43.2	38.5	47.1	26.1	87.1	41.5	41.5	44.0	11.5	38.4
	Anfepramone	3.205 (0.382)	0.9996	99.0	110	90.3	0.40	1.50	90.7	88.9	96.7	0.15	0.60
	Bupropion	3.253 (0.0895)	0.9987	89.2	108	87.1	2.00	6.70	104	111	96.9	0.10	0.30
	Mazindol	3.252 (0.0751)	0.9994	75.8	98.5	93.2	3.40	11.3	95.6	92.0	98.3	3.25	10.8
	Fenfluramine	3.265 (0.0715)	0.9992	96.6	118	89.2	9.40	31.4	99.5	82.5	93.0	0.10	0.35
	Clobenzorex	3.308 (0.0880)	0.9991	80.8	84.1	87.0	3.60	12.1	90.6	92.1	88.8	1.20	3.95
	Sibutramine	3.410 (0.105)	0.9996	71.3	80.8	79.1	2.90	9.70	74.2	76.4	88.8	0.20	0.70
	Lorazepam	3.493 (0.0677)	0.9997	85.1	83.8	84.8	0.20	0.70	86.6	87.7	87.4	0.05	0.15
	Alprazolam	3.523 (0.117)	0.9986	87.1	67.7	83.7	2.50	8.20	76.9	71.5	77.8	0.50	1.70
	Rimonabant	4.255 (0.0631)	0.9995	57.7	77.2	71.9	0.20	0.50	42.7	50.6	60.0	0.05	0.10
PI	**ILIS 1**-Salycilin acid-d_4	1.314 (2.11)											
PI	**ILIS 2**-Acetaminophen-d4	1.749 (0.153)											
PI	**ILIS 3**-Ibuprofen-d3	4.2998 (0.145)											
PII	**ILIS 4**-Topiramate-d12	4.118 (0.301)											
PIV	**ILIS 5**-Gemfibrazil-d6	5.833 (0.192)											
PIV	**ILIS 6**-d.l-Methamphetamine-d5	3.1925 (0.0494)											

CP	Pharmaceuticals, degradation products, metabolites, and Isotopically-Labelled Internal Standards (ILIS)[a]	tr (%RSD) r^2 (min)	Influents samples Recoveries (n = 2)					Effluents samples Recoveries (n = 2)				
			Level I	Level II	Level III	MDL (ng/L)	MQL (ng/L)	Level I	Level II	Level III	MDL (ng/L)	MQL (ng/L)
PI II	**ILIS 7-** Diazepam-d5[b]	3.476 (0.101)										
PI V	**ILIS 7-** Diazepam-d5[b]	3.705 (0.0633)										
PI I	**ILIS 8-** Ciprofloxacin-d8	2.304 (0.264)										
PI I	**ILIS 9-** Azithromycin-d3	2.333 (0.549)										
PI I	**ILIS 10-** Sulfamethoxazole-d4	2.837 (0.183)										
PI I	**ILIS 11-** Carbamazepine-d10	3.060 (0.174)										
PI I	**ILIS 12-** Fluoxetine-d5	2.782 (0.159)										
PI I	**ILIS 13-** Venlafaxine-d6	2.468 (0.198)										
PI I	**ILIS 14-** Atenolol-d7	0.8797 (0.331)										
PI I	**ILIS 15-** Propanolol-d7	2.551 (0.145)										
PI I	**ILIS 16-** Metformin-d6	0.6263 (0.386)										
PI I	**ILIS 17**-Caffeine $^{13}C_3$	2.446 (0.167)										

CP-Chromatographic program.

a

Pharmaceuticals organized in the table by their retention time in each chromatographic program.

b

Retention time of diazepam-d5 is different in accordance with the chromatographic method used.

Isotope-labelled standards were used as internal standards in order to improve the method precision, accuracy, and linearity (Maddela et al., 2017). However, ILIS are not always available or are very expensive. For the analysis and quantification of the 83 pharmaceuticals of the present study, seventeen ILIS were selected. A mixture with all ILIS was added to the standards for the construction of the internal standard calibration curves for all pharmaceuticals and added to each sample extract (WWTP effluents and influents), respectively.

As shown in Table 1 a slight difference in retention time between analytes and their corresponding ILIS was observed. All ILIS show a slightly earlier retention time when compared with the retention time of the analyte. Wang et al. (2007) demonstrated for the first time that a minimum difference in retention time between the analyte and the ILIS was caused by deuterium isotope effect (Wang et al., 2007). The phenomenon is explained due to the replacement of the carbon bound hydrogen with deuterium, which slightly alters the lipophilicity of the molecule, and hence the retention time of the deuterium labelled compound during reversed phase separations (isotope effect) (Wang et al., 2007).

Twelve calibration points were used for the construction of the internal standard calibration curves. Linear regression analysis over the concentration ranges shown in Table 1 presented good fits ($r^2 \geq 0.99$).

For the 83 pharmaceuticals, the validation of the chromatographic and SPE extraction methodologies developed (Paíga et al., 2017a, Paíga et al., 2017b) showed satisfactory performance in terms of repeatability (RSD below 10 and 15% for intra- and inter-day analyses), accuracy (62.7% and 63.9% of the 83 pharmaceuticals had recoveries above 75% in WWTP effluents and WWTP influents (Fig. 1)), and sensitivity (the lowest limits were $\leq$0.1 and $\leq$0.2 ng/L for MDL and MQL for both matrices). Considering all the pharmaceuticals, mean MDL and MQL values were calculated. Thus, 5.51 and 34.7 ng/L for MDL, and 18.4 and 116 ng/L for MQL in WWTP effluents and influents matrices, respectively, was obtained. Higher limits were observed for ampicillin, caffeine, enrofloxacin, lansoprazole, moxifloxacin, d,l-norephedrine, norfloxacin, norsertraline, oxytetracycline, paroxetine, potassium clavulanate, pravastatin, simvastatin, and zonisamide in WWTP influents. Potassium clavulanate was the pharmaceutical with the highest MDL and MQL for both matrices.

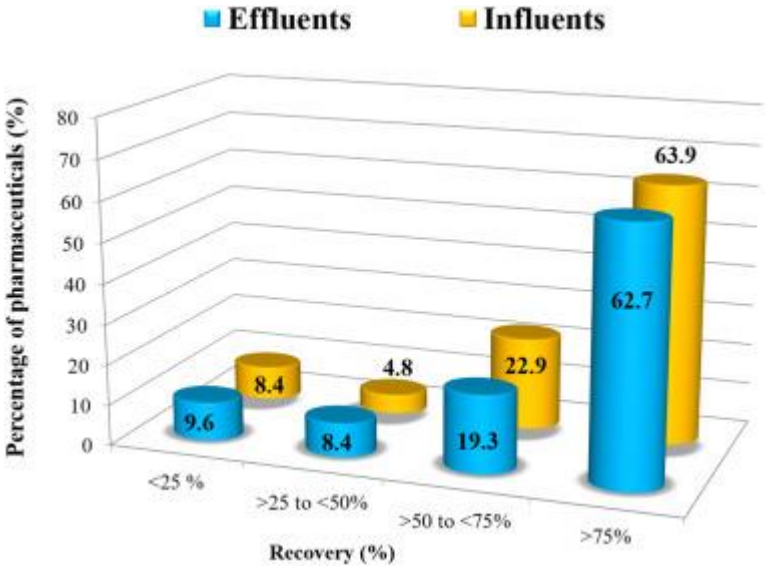


Fig. 1. Percentage of the pharmaceuticals obtained in each range of recovery in the WWTP effluent and influent samples. The present results refer to fortification at level III.

The results obtained at three different spiking levels for the two types of WWTP samples are presented in Table 1, and a good consistency of the recoveries was obtained in the three levels of fortification for most of the pharmaceuticals. For the majority of the studied pharmaceuticals, RSDs lower than 10% were found. The exception was observed for compounds such as cathine, d,l-norephedrine, ephedrine, synephrine, and d,l-methamphetamine, for which very low recoveries were also obtained, probably due to the higher hydrophilicity of these compounds.

Lansoprazole was not detected in all spiking levels and potassium clavulanate, amoxicillin, zonisamide, pravastatin, topiramate, phenolphthalein, citalopram propionic acid, and gemfibrozil were not detected at the lower level for WWTP influent. For lansoprazole the non-detection could be attributed to the fact that the extraction conditions used were not the most appropriate to retain this pharmaceutical onto the sorbent.

The good recoveries obtained using Strata-X and sample pH adjusted to 2 can be explained by the presence of acidic functional groups in the molecular structure of many pharmaceuticals. Therefore, lowering pH under their pKa values enhances the presence of neutral forms and their interaction with the reversed-phase sorbent.

Pharmaceuticals were gathered in four groups, namely (i) recoveries lower than 25%, (ii) recoveries between 25 and 50%, (iii) recoveries between 50 and 75%, and (iv) recoveries higher than 75% (Fig. 1). The average recovery was around 74.9% and 76.9%, for WWTP effluents and influents, respectively. As already mentioned, most of the studied pharmaceuticals presented recoveries above 75% and the smallest percentage of the pharmaceuticals was allocated to recoveries <25% and between 25 and 50%. Recoveries lower than 25% were achieved for potassium clavulanate in WWTP effluent, and atenolol, lansoprazole, metformin, ephedrine, cathine, synephrine, and d,l-norephedrine for both WWTP effluent and WWTP influent samples.

ME was evaluated for the two types of samples and results are presented in Figs. SM2 and SM3 (Supplementary material). The bar graphs were constructed by grouping the pharmaceuticals into their therapeutic classes. The group named "others" includes several therapeutic classes with few pharmaceuticals. Pharmaceuticals ordering in the bar graphs was performed from the highest to the lowest ME value. So, it should be noted that both the legends of these two figures and the numbering of each bar is different. For almost all pharmaceuticals ME were observed, in the studied matrices, expressed as an ion suppression. Pharmaceuticals belonging to the NSAIDs and to the stimulant, anorectics, anxiolytics, and laxatives groups, for both WWTP influent and effluent matrices, showed ion suppression except for caffeine in WWTP influent. Ion suppression was also observed for most of the pharmaceuticals included in the antibiotics, psychiatric drugs, and "others". A total of 11 out of 72 and 19 out of 64 pharmaceuticals had an ion enhancement signal for WWTP effluent and influent samples, respectively.

Acetaminophen, erythromycin, and lansoprazole, had remarkable ion suppression, having lansoprazole the biggest ME in WWTP effluents. On the other hand, for WWTP influents, more pharmaceuticals showed pronounced ion suppression (carboxyibuprofen, naproxen, nimesulide, ketoprofen, acetylsalicylic acid, salicylic acid, potassium clavulanate, bupropion, metformin, and d,l-methamphetamine), most of which are included in the NSAIDs/analgesics classes. In the case of ion enhancement, the highest ME was observed for atenolol, propranolol, and azithromycin in WWTP effluent, and for ciprofloxacin, atenolol, propranolol, and caffeine in the WWTP influents. It should be noted that atenolol and propranolol showed an ion enhancement signal in both wastewaters matrices.

Matrix effects in LC-MS/MS analysis are not easy to explain, from the chemical point of view, for a particular analyte. In a multi-residue method developed for a large number of compounds, with different physico-chemical properties, matrix effects will depend on the matrix composition (influent or effluent), the sample preparation method which will allow to eliminate several matrix components, while keeping others, the mobile phase and the ionization/detection conditions used.

Regarding NSAIDs, ion suppression both in influents and effluents was found for all the compounds (Figs. SM2 and SM3, Supplementary Material). Acetaminophen presented the lowest signal suppression in influents but the highest signal suppression in effluents when compared with the remaining NSAIDs. For six out of ten compounds, namely: carboxyibuprofen, naproxen, nimesulide, ketoprofen, acetylsalicylic acid, and salicylic acid, ion suppression increased in influent samples when compared with effluent samples. As also reported in the work of Gracia-Lor et al. (2012) the higher complexity of the influents leads to strong matrix effects (commonly ionization suppression), which can hamper the detection of some analytes at very low levels. Signal suppression for the majority of the NSAIDs was also observed in previous works of the authors (Paíga et al., 2016, Paíga et al., 2017b).

Antibiotics exhibited signal enhancement in a few cases and mainly signal suppression (Figs. SM2 and SM3, Supplementary Material). For influent samples, matrix effects were found to be related to the antibiotic family. For example, fluoroquinolones were the antibiotics for which signal enhancement was found. Regarding the antibiotics showing signal suppression, the lowest value was observed for trimethoprim, followed by sulfonamides, macrolides, tetracyclines, and, finally, the B-lactam antibiotics. The

highest signal suppression was found for potassium clavulanate. However, this pattern was not observed for the effluent samples, regardless of their less complex composition.

In the case of psychiatric drugs, and for influent samples, the parent compound and the corresponding metabolite or metabolites appear together in the sequence, meaning that they have similar matrix values (Fig. SM3, Supplementary Material). This was also found for all the benzodiazepine pharmaceuticals.

For the group "others", six and three in eleven compounds had signal enhancement in influent and effluent samples, respectively. As verified for antibiotics and psychiatric drugs, the pharmaceuticals were also grouped by their chemical family.

For caffeine an ion suppression signal was observed in the WWTP effluent and an ion enhancement signal for WWTP influent (Figs. SM2 and SM3, Supplementary material).

3.2. Occurrence of pharmaceuticals in WWTP influents and effluents

The effects of pharmaceuticals on aquatic ecosystems are the subject of increasing environmental concern (Richmond et al., 2016). The presence of pharmaceuticals in water may be associated with certain factors, including the pharmaceuticals' physicochemical properties that allow them to resist to biological, physical, and chemical processes (Brooks et al., 2005; Snyder, 2008), and are determinant for their behavior once introduced in the sewer system. The molecular weight, water solubility, partitioning values such as Log (Kow), which give an indication of the molecule's polarity, and pKa values will be decisive for their behavior together with the molecule stability.

Generally, compounds with higher water solubility values, and that are not (bio)degradable, may present lower removal percentages. Conversely, less polar compounds, even when they are resistant to (bio)degradation may be removed in the biological treatment due to sorption to suspend solids (Peng et al., 2012).

In the current study, pharmaceuticals were analysed in each WWTP sample, namely in grab samples (influent and effluent hourly collection, during one day) and in the composite WWTP (influent and effluent) samples. Regarding the composite samples, the concentration obtained for the detected pharmaceuticals is presented in Table SM6 (Supplementary material).

Results show that 25 and 20 pharmaceuticals were detected in the WWTP influent and effluent samples, respectively, most of them belonging to the NSAIDs/analgesic, antibiotics, and psychiatric drugs. In Table 2, the results of the present study are shown together with the results reported for the same pharmaceuticals in different countries in Europe (Afonso-Olivares et al., 2017; Aydin et al., 2017; Bahlmann et al., 2014; Baker and Kasprzyk-Hordern, 2013; Brunsch et al., 2018; Camacho-Muñoz et al., 2014; Evans et al., 2015; Gardner et al., 2013; Gracia-Lor et al., 2012; Kasprzyk-Hordern et al., 2009; Kasprzyk-Hordern et al., 2010; Kay et al., 2017; Kosma et al., 2014; Mendoza et al., 2015; Muz et al., 2012; Nakada et al., 2017; Petrie et al., 2017; Ternes, 1998; Urtiaga et al., 2013; Vasskog et al., 2006; Verlicchi et al., 2012; Wick et al., 2009), America (Conn et al., 2006; Fang et al., 2012; Gerrity et al., 2011; Lajeunesse et al., 2008; Lajeunesse et al., 2012; Nelson et al., 2011; Rivera-Jaimes et al., 2018; Spongberg and Witter, 2008; Writer et al., 2013; Yu et al., 2013; Zacarías et al., 2017), Asia (Archana et al., 2017;

Aydin et al., 2017; Behera et al., 2011; Chang et al., 2008; Hong et al., 2015; Muz et al., 2012; Nguyen et al., 2018; Shraim et al., 2017; Subedi et al., 2017; Suzuki et al., 2014; Zhang et al., 2018; Zhou et al., 2010), Africa (Madikizela and Chimuka, 2017), and Australia (Cardenas et al., 2016; Roberts et al., 2016; Watkinson et al., 2007; Watkinson et al., 2009).

Table 2. Measured concentrations (ng/L) for the target analytes in WWTP influent and effluent composite samples reported in this study and in the literature.

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
			Portugal	**683 (±4.1%)**	**n.d.**	Present study
			Spain	2300–14,900		(Mendoza et al., 2015)
			Spain	330–165,000		(Camacho-Muñoz et al., 2014)
		Europe	Italy	500–1200	12.0–58.0	(Verlicchi et al., 2012)
			Greece	n.d.–65,402.8	n.d.–1060.3	(Kosma et al., 2014)
			UK	68,107–482,687	<80.0–24,525	(Kasprzyk-Hordern et al., 2009)
			UK	171,875–512,813	692.0–2195	(Petrie et al., 2017)
NSAIDs/analgesic	Acetaminophen	America	México	2330–14,900	n.d.	(Rivera-Jaimes et al., 2018)
			Mexico	100–4300	100–1000	(Zacarías et al., 2017)
			Vietnam	11,000–30,000	n.d.–<LOQ	(Nguyen et al., 2018)
			Korea	843–7750		(Hong et al., 2015)
			China	739.9–8983.9	2.90–58.4	(Zhang et al., 2018)
		Asia	Saudi Arabia	3610–99,600	<LOD–90.5	(Shraim et al., 2017)
			India	<LOQ–30,000	<LOQ–11000	(Archana et al., 2017)
			India	2900–11,000	<LOQ–1200	(Subedi et al., 2017)
			Portugal	**449 (±6.6%)**	**1934 (±1.4%)**	Present study
	Diclofenac	Europe	Spain	600–2500		(Mendoza et al., 2015)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
			Spain	45.0–1605	n.d.–2240	(Afonso-Olivares et al., 2017)
			Spain	<LOD–1,67		(Camacho-Muñoz et al., 2014)
			UK	239.9–1881	239.4–521.2	(Petrie et al., 2017)
			Italy	360–480	220–330	(Verlicchi et al., 2012)
			Greece	n.d.–5164	n.d.–382.5	(Kosma et al., 2014)
			Germany		420–4880	(Brunsch et al., 2018)
			UK	57–1161	6.00–496	(Kasprzyk-Hordern et al., 2009)
			UK	175–1805	401–2830	(Kay et al., 2017)
		America	Mexico	560–2470	466–2180	(Rivera-Jaimes et al., 2018)
			USA		18.0–47.0	(Nelson et al., 2011)
		Asia	Korea	12.0–113		(Hong et al., 2015)
			China	128.6–1027.1	7.9–237.7	(Zhang et al., 2018)
		Australian	Australia	560	260	(Cardenas et al., 2016)
		Africa	South Africa	6400–16,00	1400–2,00	(Madikizela and Chimuka, 2017)
			Portugal	**421 (±6.2%)**	**217 (±0.13%)**	Present study
	Ibuprofen	Europe	Spain	400–2800		(Mendoza et al., 2015)
			Spain	1150–56,300	21.0–21,700	(Afonso-Olivares et al., 2017)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
			Spain	<LOD–220,000		(Camacho-Muñoz et al., 2014)
			Italy	930–1200	10.0–120	(Verlicchi et al., 2012)
			Greece	n.d–8890.1	n.d.–301.2	(Kosma et al., 2014)
			UK	968–6328	65.0–491	(Kasprzyk-Hordern et al., 2009)
			UK	4016–20,215	1746–3718	(Petrie et al., 2017)
			UK	76–14,231	863–4617	(Kay et al., 2017)
		America	Mexico	370–2835	n.d.	(Rivera-Jaimes et al., 2018)
		Asia	Vietnam	780–1700	n.d.–<LOQ	(Nguyen et al., 2018)
			India	<LOQ–2800	270–1940	(Subedi et al., 2017)
		Africa	South Africa	55,000–69,000	2100–4200	(Madikizela and Chimuka, 2017)
			Portugal	**<MDL**	**56.5 (±2.2%)**	Present study
			Spain	116–24,300	152–1170	(Afonso-Olivares et al., 2017)
	Ketoprofen	Europe	Spain	<LOD–1,65		(Camacho-Muñoz et al., 2014)
			Italy	130–190	56.0–110	(Verlicchi et al., 2012)
			UK	<4–346	<3.00–37.0	(Kasprzyk-Hordern et al., 2009)
			UK	n.d.	15.2–64.0	(Petrie et al., 2017)
		Asia	China	100.6–7881.0	37.7–1712.7	(Zhang et al., 2018)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
			Portugal	**28.6 (±7.3%)**	**n.d.**	Present study
		Europe	Spain	144–5140	50.0–872	(Afonso-Olivares et al., 2017)
			Spain	800–4200		(Mendoza et al., 2015)
			Spain	<LOD–33,400		(Camacho-Muñoz et al., 2014)
			Italy	780–910	100–210	(Verlicchi et al., 2012)
			Greece	n.d.–5899.9	n.d.–483.5	(Kosma et al., 2014)
	Naproxen		UK	400–3504	<2–703	(Kasprzyk-Hordern et al., 2009)
			UK	3800	8920	(Nakada et al., 2017)
			UK	6985–20,398	3291–6412	(Petrie et al., 2017)
		America	Mexico	825–4210	49–392	(Rivera-Jaimes et al., 2018)
			USA		11.0–41.0	(Nelson et al., 2011)
		Asia	Vietnam	60.0–170	n.d.	(Nguyen et al., 2018)
			Japan	30.0–430	10–90	(Suzuki et al., 2014)
		Australian	Australia	5280	n.d.	(Cardenas et al., 2016)
		Africa	South Africa	15,000–20,000	600–1100	(Madikizela and Chimuka, 2017)
			Portugal	**1099 (±7.9%)**	**107 (±4.9%)**	Present study
	Salicylic acid	Europe	Spain	<LOD–3295000		(Camacho-Muñoz et al., 2014)
			Italy	210–1100	110–130	(Verlicchi et al., 2012)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
Antibiotics	Azithromycin		Greece	<LOQ–89,133.5	n.d.–431.9	(Kosma et al., 2014)
			UK	1479–32,082	<1.00–497	(Kasprzyk-Hordern et al., 2009)
		America	Mexico	125–408	65–320	(Rivera-Jaimes et al., 2018)
		Asia	Vietnam	3700–19,000	n.d.–660	(Nguyen et al., 2018)
			Portugal	**402 (±7.2%)**	**283 (±4.0%)**	Present study
		Europe	Italy	10.0–330	70–180	(Verlicchi et al., 2012)
			UK	52.0–283.5	84.5–147.5	(Petrie et al., 2017)
		America	USA		124–385	(Nelson et al., 2011)
	Clarithromycin	Europe	**Portugal**	**<MDL**	**<MDL**	Present study
			Italy	110–780	260–310	(Verlicchi et al., 2012)
		Asia	Korea	9.00–85.0		(Hong et al., 2015)
			China	374.4–661.4	1.20–342.6	(Zhang et al., 2018)
	Ciprofloxacin		**Portugal**	**448 (±12%)**	**159 (±11%)**	Present study
		Europe	Spain	94.0–4.220	>MDL–89.0	(Afonso-Olivares et al., 2017)
			Spain	<LOQ–3260		(Camacho-Muñoz et al., 2014)
			Italy	1100–3700	290–1100	(Verlicchi et al., 2012)
		Asia	India	12,000–140,000	5000–58,000	(Archana et al., 2017)
		Australian	Australia	1100	1300	(Watkinson et al., 2009)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
	Ofloxacin	Europe	Australia	4600	720	(Watkinson et al., 2007)
			Australia	530	n.d.	(Cardenas et al., 2016)
			Portugal	**<MDL**	**147 (±12%)**	Present study
			Spain	43.0–2280	<MQL–>MQL	(Afonso-Olivares et al., 2017)
			Italy	450–2200	220–520	(Verlicchi et al., 2012)
	Sulfamethoxazole	Europe	**Portugal**	**600 (±5.4%)**	**n.d.**	Present study
			Spain	19.0–1150	n.d.–1520	(Afonso-Olivares et al., 2017)
			Spain	<LOQ–1030		(Camacho-Muñoz et al., 2014)
			Italy	280–740	170–240	(Verlicchi et al., 2012)
		America	Greece	n.d–2170.4	n.d.–72.9	(Kosma et al., 2014)
			UK	64.5–1154	23.0–188.8	(Petrie et al., 2017)
			Mexico	775–2010	440–1215	(Rivera-Jaimes et al., 2018)
			USA	600–1500	1150–1550	(Gerrity et al., 2011)
			USA		18–265	(Nelson et al., 2011)
		Asia	Japan	6.90–27.0	24.0–28.0	(Chang et al., 2008)
			Korea	n.d.–229		(Hong et al., 2015)
			China	214–982	25.0–366	(Zhang et al., 2018)
			India	<LOQ–690	n.d–420	(Subedi et al., 2017)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
		Australian	Australia	3000	230	(Watkinson et al., 2009)
			Australia	500	720	(Watkinson et al., 2007)
			Australia	3570	260	(Cardenas et al., 2016)
		Europe	**Portugal**	**n.d.**	**24.2 (±3.8%)**	Present study
			Spain	60.0–452	n.d.–31.0	(Afonso-Olivares et al., 2017)
			Spain	<LOQ–500		(Camacho-Muñoz et al., 2014)
			Italy	3.00–72.0	36.0–51.0	(Verlicchi et al., 2012)
			Greece	<LOQ–180.3	n.d.–111.2	(Kosma et al., 2014)
			UK	931.5–2124	554.0–1104	(Petrie et al., 2017)
	Trimethoprim	America	Mexico	125–790	135–395	(Rivera-Jaimes et al., 2018)
			USA	490–1100	50.0–200	(Gerrity et al., 2011)
			USA		<10.0–59.0	(Nelson et al., 2011)
		Asia	Japan	14.0–42.0	11.0–26.0	(Chang et al., 2008)
			Korea	3.00–38.0		(Hong et al., 2015)
			China	11.2–423.2	4.3–427.8	(Zhang et al., 2018)
			India	<LOQ–400	n.d.–25.0	(Subedi et al., 2017)
		Australian	Australia	4300	250	(Watkinson et al., 2009)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
Psychiatric drugs	Carbamazepine	Europe	Australia	930	320	(Watkinson et al., 2007)
			Australia	2350	260	(Cardenas et al., 2016)
			Portugal	**820 (±1.9%)**	**1059 (±6.2%)**	Present study
			Spain	281–3030	11.0–1770	(Afonso-Olivares et al., 2017)
			Spain	<LOQ–180		(Camacho-Muñoz et al., 2014)
			Italy	300–1170	280–440	(Verlicchi et al., 2012)
			Greece	<LOQ–354.7	n.d.–416.8	(Kosma et al., 2014)
			Germany	150	140	(Bahlmann et al., 2014)
			Germany	660 (median)	740 (median)	(Wick et al., 2009)
			Germany	n.d.	2100	(Ternes, 1998)
			Germany		170–2700	(Brunsch et al., 2018)
			UK	n.d–790	274–876	(Nakada et al., 2017)
			UK	168.6–367.0	134.7–175.8	(Petrie et al., 2017)
		America	Mexico	85–380	165–476	(Rivera-Jaimes et al., 2018)
			USA	34.0–350	nd–62.0	(Yu et al., 2013)
			USA	24.8–50.9	33.7–111.2	(Spongberg and Witter, 2008)
			USA	20–100	100–200	(Gerrity et al., 2011)
			USA		223–297	(Nelson et al., 2011)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
		Asia	Vietnam	30.0–190	<LOQ–0.05	(Nguyen et al., 2018)
			Korea	43.0–127	40.0–74.0	(Behera et al., 2011)
			Korea	14.0–58.0		(Hong et al., 2015)
			China	62.7–2499	43.4–672.5	(Zhang et al., 2018)
			India	240–750	290–770	(Subedi et al., 2017)
		Australian	Australia	1600	830	(Cardenas et al., 2016)
			Australia	589–685	685–702	(Roberts et al., 2016)
		Europe/Asia	Turkey	6.35–135.6	<LOD–245.13	(Aydin et al., 2017)
	Citalopram	Europe	**Portugal**	**149 (±1.6%)**	**148 (±0.68%)**	Present study
			UK	239.0–509.5	189.0–270.5	(Petrie et al., 2017)
	Demethylcitalopram	Europe	**Portugal**	**n.d.**	**364 (±7.7%)**	Present study
			UK	37.0–172.5	17.0–57.5	(Petrie et al., 2017)
	Fluoxetine	Europe	**Portugal**	**78.0 (±2.6%)**	**57.5 (±7.1%)**	Present study
			Spain	77.0–207	63.0–72.0	(Afonso-Olivares et al., 2017)
			Italy	55.0–190	10.0–63.0	(Verlicchi et al., 2012)
			UK	4.90–175.9	5.60–44.9	(Baker and Kasprzyk-Hordern, 2013)
			UK	36.0–436.5	33.0–66.5	(Petrie et al., 2017)
			Norway	0.400–2.40	n.d.–1.30	(Vasskog et al., 2006)
		America	USA		18.0–22.0	(Nelson et al., 2011)
			Canada	16.0–26.0	6.60–20.0	(Lajeunesse et al., 2012)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
		Australian	Australia	n.d.–51.1	n.d.–16.2	(Roberts et al., 2016)
		Europe/Asia	Turkey	<LOD–2.60	<LOD–2.70	(Aydin et al., 2017)
			Portugal	**275 (±1.5%)**	**484 (±6.5%)**	Present study
		Europe	Spain	40–520	60–300	(Gracia-Lor et al., 2012)
			UK	28.8–446.1	21.4–285.1	(Baker and Kasprzyk-Hordern, 2013)
	Venlafaxine		UK	119.2–642.9	170.5–251.4	(Petrie et al., 2017)
		America	USA	n.d.	<10.0–5500	(Writer et al., 2013)
			Canada	788–2987	600–2563	(Lajeunesse et al., 2012)
		Asia	India	n.d.–76.0	n.d–18.0	(Subedi et al., 2017)
		Australian	Australia	100–100	511–736	(Roberts et al., 2016)
			Australia	2000	1450	(Cardenas et al., 2016)
		Europe	**Portugal**	**865**	**2014 (±8.8%)**	Present study
	O-Desmethylvenlafaxine	America	Canada	345	330	(Lajeunesse et al., 2008)
			Portugal	**197 (±3.2%)**	**n.d.**	Present study
		Europe	Italy	<LOD–18.0	<LOD–10.0	(Verlicchi et al., 2012)
			UK	216.5–788.5	69.0–233.0	(Petrie et al., 2017)
Lipid regulator and cholesterol lowering statin drugs	Atorvastatin	America	USA	1560	240	(Ottmar et al., 2012)
		Asia	India	110–690	<LOQ–510	(Subedi et al., 2017)
		Australian	Australia	1000		(Cardenas et al., 2016)
	Gemfibrozil	Europe	**Portugal**	**57.0 (±6.4%)**	**13.2 (±7.5%)**	Present study

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
			Spain	126–45,200	8.00–20,100	(Afonso-Olivares et al., 2017)
			Spain	652–99,574	447–12,697	(Urtiaga et al., 2013)
			Spain	<LOQ–58,300		(Camacho-Muñoz et al., 2014)
			Greece	n.d.–733.2	n.d.–230.9	(Kosma et al., 2014)
		America	Mexico	20.0–225	20.0–380	(Rivera-Jaimes et al., 2018)
			USA		215–773	(Nelson et al., 2011)
			USA	3470–63,800	80.0–19,400	(Fang et al., 2012)
		Asia	China	4.70–220.3	0.300–6.90	(Zhang et al., 2018)
		Australian	Australia	1000		(Cardenas et al., 2016)
Calcium channel blocker	Diltiazem	Europe	**Portugal**	**<MDL**	**<MDL**	Present study
		Europe/Asia	Turkey	520–3300	n.d.–1120	(Muz et al., 2012)
			Portugal	**320 (±9.4%)**	**n.d.**	Present study
			Spain	54.0–695	n.d.–<MQL	(Afonso-Olivares et al., 2017)
			Spain	<LOQ–120		(Camacho-Muñoz et al., 2014)
β-Blockers	Propranolol	Europe	Italy	14.0–45.0	13.0–26.0	(Verlicchi et al., 2012)
			UK	60.0–638	93.0–388	(Gardner et al., 2013)
			UK	83.1–269.7	60.9–102.7	(Petrie et al., 2017)
			UK	n.d–29	1.00–1464	(Kay et al., 2017)
			Germany	40.0 (median)	40.0 (median)	(Wick et al., 2009)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
		America	USA		n.d.–25	(Nelson et al., 2011)
		Asia	China	3.40–60.9	1.90–17.2	(Zhang et al., 2018)
			Korea	4.00–19–0		(Hong et al., 2015)
			India	30–62	21.0–52.0	(Subedi et al., 2017)
		Australian	Australia	18.1–151	36.8–75.8	(Roberts et al., 2016)
			Australia	130	60.0	(Cardenas et al., 2016)
		Europe	**Portugal**	**1013 (±0.20%)**	**n.d.**	Present study
			UK	n.d.	n.d.	(Kasprzyk-Hordern et al., 2010)
	d,l-Norephedrine		UK	359.3	52.7	(Evans et al., 2015)
			UK	15.0–99.9	n.d.	(Baker and Kasprzyk-Hordern, 2013)
			UK	n.d.	n.d.	(Petrie et al., 2017)
			Portugal	**63,965 (±2.3%)**	**n.d.**	Present study
Stimulant, anorexics, anxiolytics, laxatives			Spain	14,000–145,000	17.0–3260	(Afonso-Olivares et al., 2017)
	Caffeine		Spain	360–72,400		(Camacho-Muñoz et al., 2014)
			Greece	n.d.–96,648.3	n.d.–1180.5	(Kosma et al., 2014)
			UK	1044.7150,413.6	148.4–34,198.3	(Baker and Kasprzyk-Hordern, 2013)
			UK	26,000–542,000	110–1370	(Nakada et al., 2017)
			UK	41,625–230,562	1125–18,688	(Petrie et al., 2017)
		America	USA	500 to 320,000		(Conn et al., 2006)

Therapeutic class	Pharmaceuticals	Continent	Country	WWTP influent (ng/L)	WWTP effluent (ng/L)	Reference
		Asia	China	50,000		(Zhou et al., 2010)
			China	3793.6–39,665.6	15.8–1790.9	(Zhang et al., 2018)
			Vietnam	12,140–25,000	<LOQ–1600	(Nguyen et al., 2018)
			Korea	887–5630		(Hong et al., 2015)
			India	132,000–373,000	86,000–232,000	(Archana et al., 2017)
			India	16,000–120,000	810–4400	(Subedi et al., 2017)

Pharmaceutical concentrations found in this study are discussed in the following subsections, and compared to the values reported in the literature in Table 2, where pharmaceuticals are presented by alphabetical order for the different therapeutic classes. For each compound, the results obtained in the different countries were grouped by continent.

3.2.1. NSAIDs/analgesics

NSAIDs/analgesics are a widely used therapeutic group not only by the Portuguese population but also worldwide. The Portuguese law defines NSAIDs/analgesics as prescription-only medicines or over-the counter pharmaceuticals, depending on the active ingredients and/or the dosages (Nunes et al., 2016). Literature refers that in countries where over-the-counter pharmaceuticals can be sold, NSAIDs/analgesics can be acquired outside of the pharmacies. Thus, the increase in NSAIDs/analgesics consumption and the decrease in professional counselling may pose a serious risk for a substantial increase in adverse effect occurrences in humans (Howard et al., 2007) and in the environment (He et al., 2017).

As can be seen in Table SM6 (Supplementary material) and Table 2, diclofenac, one of the pharmaceuticals in the EU watch list, was detected in both matrices. In WWTP influents, seven of the found pharmaceuticals belonging to this group presented a concentration that varied from <MDL (ketoprofen) to 2838 ng/L (hydroxyibuprofen). High concentration values were noted for three compounds, namely: acetaminophen, hydroxyibuprofen, and salicylic acid, the last two compounds being metabolites. For the WWTP effluents, 5 pharmaceuticals were detected with concentrations ranging from 56.5 (ketoprofen) to 1934 ng/L (diclofenac).

These results are in agreement with the literature (Table 2) where Mendoza et al. (2015) stated that acetaminophen, naproxen, diclofenac, and ibuprofen were the compounds that contributed most to the total concentrations of pharmaceuticals measured in their study (Mendoza et al., 2015). In the study performed in 2017 by Gros et al., acetaminophen, ibuprofen and diclofenac, were also found in concentration levels of μg/L in WWTP matrices (Gros et al., 2017). Afonso-Olivares and collaborators detected diclofenac in the range of n.d. to 3.91 μg/L, ketoprofen in the range of 0.116 to 24.3 μg/L, naproxen in the range of 0.077 to 5.14 μg/L, and ibuprofen in the range of 0.021 to 56.3 μg/L (Afonso-Olivares et al., 2017). The highest concentration reported by these authors was higher than the concentration found in the present study for ketoprofen, naproxen, and ibuprofen. Kasprzyk-Hordern et al. (2009) studied the fate of 55 emerging pollutants in two WWTPs in South Wales (UK) and reported an average acetaminophen concentration > 180 μg/L over a period of 5 months, demonstrating that the micropollutant concentrations were correlated with their usage/consumption patterns (Kasprzyk-Hordern et al., 2009).

The highest concentrations of salicylic acid were measured in industrial wastewater, reaching levels up to 3295 μg/L (Camacho-Muñoz et al., 2014).

3.2.2. Antibiotics

Overuse and misuse of antibiotics can promote the development of antibiotic-resistant bacteria. Antibiotics have attracted increasing concern due to their high human and veterinary use.

Sulfonamides, fluoroquinolones and macrolides were the three subclasses of antibiotics detected (Table SM6 (Supplementary material) and Table 2). Two of the three macrolides listed in the watch list were found either in effluent and influent samples. Concentrations in the range of <MDL (clarithromycin and ofloxacin) to 600 ng/L (sulfamethoxazole) in WWTP influents and in the range of <MDL (clarithromycin) to 283 ng/L (azithromycin) in WWTP effluents were obtained. Sulfamethoxazole and sulfapyridine (Table SM6, Supplementary material) were detected only in WWTP influent and trimethoprim was detected only in WWTP effluent. The highest concentration was observed for sulfamethoxazole (600 ng/L) in WWTP influents and azithromycin (283 ng/L) in WWTP effluents. As also reported by Rivera-Jaimes et al. (2018), sulfamethoxazole was found at higher levels compared to trimethoprim (Rivera-Jaimes et al., 2018).

In literature, concentrations in the range of: n.d. to 398 ng/L for trimethoprim (Afonso-Olivares et al., 2017; Kosma et al., 2014), >MQL to 2.28 μg/L for ofloxacin (Afonso-Olivares et al., 2017), >MDL to 4.22 μg/L for ciprofloxacin (Afonso-Olivares et al., 2017), and n.d. to 1.52 μg/L for sulfamethoxazole (Afonso-Olivares et al., 2017; Kosma et al., 2014) were observed (Table 2).

3.2.3. Psychiatric drugs

Psychiatric drugs are not completely metabolized by the human body and the unchanged parent compound, metabolites or conjugates are excreted (Heberer, 2002).

Concentrations in the μg/L range were obtained for carbamazepine and *O*-desmethylvenlafaxine in WWTP effluent (Table SM6 (Supplementary material) and Table 2). The lowest concentration was found for fluoxetine in WWTP influent and bupropion in WWTP effluent. Demethylcitalopram and bupropion (Table SM6, Supplementary Material) were detected only in WWTP effluents. Higher concentrations of carbamazepine, *O*-desmethylvenlafaxine, and venlafaxine were observed in WWTP effluent when compared with the WWTP influent.

In Portugal, according to the regulatory pharmaceuticals Agency, INFARMED, only venlafaxine is authorized as an active substance. To our knowledge, *O*-desmethylvenlafaxine is not an authorized pharmaceutical in Portugal, despite its use as an active substance is authorized in other countries. Therefore, in this study, it is assumed that the presence of *O*-desmethylvenlafaxine in the samples results from being the main venlafaxine metabolite.

Almost all the psychiatric drugs were detected at similar or higher concentrations in WWTP effluent than in WWTP influent (Table SM6, Supplementary material). This is in agreement with previously published data, where low or no removal of the psychiatric drugs carbamazepine, venlafaxine, and fluoxetine were described (Paíga et al., 2016). Similar results were observed in other studies (Gros et al., 2007; Kosma et al., 2014; Papageorgiou et al., 2016; Verlicchi et al., 2012) (Table 2).

Pharmaceuticals excreted as conjugates can be cleaved by enzymes during the wastewater treatment process, converting them again in the parent compound form (Bahlmann et al., 2014). The gradual release of psychiatric drugs adsorbed onto sludge during biological treatment, can also lead to an increase of these compounds in the WWTP effluents and, consequently, to a negative removal rate (Jelic et al., 2011).

Carbamazepine, diazepam, fluoxetine, lorazepam, and paroxetine are psychiatric drugs commonly detected in the environment (Aydin et al., 2017) (Table 2). Concentrations of carbamazepine in WWTP samples in different developed countries, mostly from Europe, averaged the μg/L levels (Verlicchi et al., 2012). In other continents, concentrations of carbamazepine of hundreds of ng/L were also found in recent years (Table 2). In the study conducted by Afonso-Olivares et al. (2017), concentrations in the range of n.d to 0.207 μg/L for fluoxetine and 0.011 to 3.03 μg/L for carbamazepine were obtained. Venlafaxine has been detected in concentrations in the μg/L range in the USA, Canada and Australia (Table 2).

3.2.4. Stimulants

Caffeine was included in the study because it is a central nervous system stimulant (Nehlig et al., 1992). Caffeine is often the compound reported with the highest frequency and concentration (Seiler et al., 1999; Spongberg et al., 2011) in similar studies and has previously been used as an indicator of anthropogenic contamination (Buerge et al., 2003; Daneshvar et al., 2012; Paíga and Delerue-Matos, 2017; Seiler et al., 1999). The abundant presence of caffeine is associated with the high consumption of coffee, tea, and soft drinks as well as the disposal of these items (Luo et al., 2014).

The high concentration (63.97 μg/L in WWTP influent) obtained in the present study (Table SM6, Supplementary material), is in accordance with other studies reported in the literature (Table 2). Caffeine was detected approximately at 50 μg/L in the raw sewage in three WWTPs in China (Zhou et al., 2010), between 0.012 and 145 μg/L in the study conducted by Afonso-Olivares et al. (2017), and in the range of <0.5 to 320 μg/L in a screening study in the U.S.A. (Conn et al., 2006). In Costa Rica, high concentrations were observed not only for caffeine, but also for pharmaceuticals (Spongberg et al., 2011). Caffeine had the maximum concentration of 1.1 mg/L, possibly due to coffee bean production facilities upstream. Pharmaceuticals were detected at alarmingly high levels with maximum concentrations of 74, 37, 17, 13, and 10 μg/L for doxycycline, ibuprofen, gemfibrozil, acetaminophen, and ketoprofen (Spongberg et al., 2011). Caffeine had the highest observed concentration of 373 μg/L in a study conducted in India (Archana et al., 2017).

3.2.5. Other therapeutic classes

The remaining pharmaceuticals detected (Table SM6, Supplementary material), belonging to the lipid regulator and cholesterol lowering statin drugs (atorvastatin and gemfibrozil), presented a concentration of 57.0 ng/L for gemfibrozil and 197 ng/L for atorvastatin in WWTP influents and 13.2 ng/L for gemfibrozil in WWTP effluents. Diltiazem (calcium channel blocker) was detected below the MDL for both WWTP matrices. Propranolol and d,l-norephedrine were both detected only in WWTP influent with a concentration of 320 ng/L and 1.013 μg/L (Table SM6, Supplementary material).

A literature review (Table 2) showed that propranolol was found in Spain in levels of <MQL to 695 ng/L (Afonso-Olivares et al., 2017; Urtiaga et al., 2013), and between 60 to 638 ng/L in WWTP influents and 93 to 388 ng/L in WWTP effluents in the UK (Gardner et al., 2013). Gemfibrozil was found at concentration values between 0.652 and 99.574 μg/L in WWTP influents and 0.447 and 12.697 μg/L in WWTP effluents in Spain (Urtiaga et al., 2013) and between 3.47 and 63.8 μg/L in WWTP influents and 0.08 and

19.4 μg/L in WWTP effluents in Texas (Fang et al., 2012). Atorvastatin was found in the USA with a concentration of 1.56 and 0.24 μg/L for WWTP influents and effluents (Ottmar et al., 2012). In 2012, diltiazem was detected in almost of all WWTP samples. Thus, a concentration between 0.52 and 3.30 μg/L in WWTP influents and in the range of n.d. to 1.12 μg/L in WWTP effluents was achieved in Turkey (Muz et al., 2012). In the UK, norephedrine was neither detected in WWTP influents (Kasprzyk-Hordern et al., 2010) nor in WWTP effluents (Baker and Kasprzyk-Hordern, 2013; Kasprzyk-Hordern et al., 2010). However, it was detected in other studies in UK at 359.3 ng/L in WWTP influents and 52.7 ng/L for WWTP effluents (Evans et al., 2015), and from 15.0 to 99.9 ng/L in WWTP influents (Baker and Kasprzyk-Hordern, 2013). To the best of our knowledge, this was the first time that norephedrine was found in WWTP samples in Portugal.

3.2.6. Metabolites and their parent pharmaceuticals

Studies have focused on the occurrence, fate, behavior, distribution, and toxicity of pharmaceuticals in wastewater influent and effluent (Gros et al., 2007), sludge (Radjenović et al., 2009), surface water (Rivera-Jaimes et al., 2018), and sediment and soil (Diaz-Cruz et al., 2003; Koba et al., 2018). Pharmaceuticals are released into the environment either as parent compound and as active/inactive metabolites (Christensen, 1998). Therefore, it is important to underline that not only the parent compound should be the target of the studies but also the transformation products and metabolites. Moreover, in 2017, Yin et al. mentioned that transformation products and metabolites are detected at higher concentrations than their parent compounds (Yin et al., 2017).

A total of eighteen compounds were analysed including seven pharmaceuticals, their transformation products, and their metabolites. The concentrations (ng/L) obtained are shown in Fig. SM4 (Supplementary material).

Concentrations ranged from n.d. to 2838 ng/L (hydroxyibuprofen) for WWTP influents and n.d. to 2014 ng/L (*O*-desmethylvenlafaxine) for WWTP effluents.

Carboxyibuprofen, acetylsalicylic acid, 10,11-epoxi carbamazepine, norfluoxetine, sertraline, norsertraline, citalopram propionic acid, citalopram *N*-oxide, citalopram didemethyl were not detected either in the WWTP influents and effluents. Only the parent compounds, carbamazepine and fluoxetine, were detected in both WWTP matrices. Carbamazepine was found with concentrations of 820 ng/L for WWTP influent, and 1059 ng/L for WWTP effluent and fluoxetine with 78 ng/L for WWTP influent, and 57 ng/L for WWTP effluent. Similar to the achievements in this study, Weston et al. (2003) reported fluoxetine levels up to 540 ng/L in two WWTP effluents and norfluoxetine was not detected (Weston et al., 2003).

Comparing the levels found for ibuprofen and its metabolites, a higher concentration was obtained for hydroxyibuprofen when compared with ibuprofen in both WWTP matrices. The highest concentration was observed for hydroxyibuprofen in WWTP influents (2838 ng/L) (Table SM6, Supplementary Material) and carboxyibuprofen was not detected.

95% of ibuprofen is excreted in the urine, of which 35% is excreted as hydroxyibuprofen (15% free, 20% conjugated), 51% as carboxyibuprofen (42% free, 9% conjugated), and

9% as ibuprofen (1% free, 8% conjugated) (Ternes et al., 2004). In the environment, hydroxyibuprofen was detected as the main component related to ibuprofen. It was also noted that ibuprofen metabolites can also be formed during the biodegradation of ibuprofen, but as hydroxyibuprofen is a more stable compound, showing lower removal percentages than carboxyibuprofen (Ferrando-Climent et al., 2012), it is probable that hydroxyibuprofen would be present at higher concentrations than ibuprofen (Ferrando-Climent et al., 2012), as was reported in the present study.

The non-detection of acetylsalicylic acid can be related to its hydrolysis. Acetylsalicylic acid, undergoes hydrolysis with the resultant transformation products being salicylic acid and acetic acid (Farrell, 2017). Therefore, salicylic acid in the environmental would be detected more frequently and with higher levels than acetylsalicylic acid. Concentrations of 1099 and 107 ng/L for WWTP influents and WWTP effluents were reached in the present study.

Venlafaxine undergoes extensive first-pass metabolism and <5% of the parent pharmaceutical is excreted in the urine. Most of it is metabolized in the liver to a major metabolite, *O*-desmethylvenlafaxine, and two minor, less active metabolites. In line with our results, Lajeunesse et al. (2008) showed that higher concentrations of *O*-desmethylvenlafaxine were determined in raw sewage and effluent in Montreal WWTP, with concentrations of 345 ng/L and 330 ng/L, respectively, approximately 1.5 times higher than that of the parent compound (Lajeunesse et al., 2008). The results found in the WWTP under study reveal concentrations between 275 and 484 ng/L for venlafaxine and between 865 and 2014 ng/L for *O*-desmethylvenlafaxine in WWTP influent and effluent samples, respectively.

Hence, some transformation products and/or metabolites are not completely removed in WWTPs, and together with their parent compounds will be simultaneously discharged through WWTP effluent and enter in surface water. Therefore, monitoring studies of pharmaceuticals in the environment should cover not only the detection of parent compounds, but also their transformation products and metabolites, considering the high concentrations found in several reported studies.

3.3. Hourly pharmaceutical determination in WWTP influent and effluent

WWTP influents and effluents were collected hourly for one day. A total of 24 samples for influent and 24 samples for effluent were collected, extracted, and analysed. Thirty-eight and twenty-nine pharmaceuticals were detected in at least one sample in WWTP influents and effluents (Fig. SM5, Supplementary material). Minimum, maximum, average, number of times that a pharmaceutical is detected and detection frequency for each detected pharmaceutical in WWTP effluent are shown in Table 3.

Table 3. Minimum, maximum, and average concentration, number of times that a pharmaceutical is detected and detection frequency for each detected pharmaceutical in WWTP influent and effluent samples.

	WWTP influents					Effluents				
Pharmaceuticals	**Minimum (ng/L)**	**Maximum (ng/L)**	**Average (ng/L)**	**Number of time detected in 24 samples**	**Detection frequency (%)**	**Minimum (ng/L)**	**Maximum (ng/L)**	**Average (ng/L)**	**Number of time detected in 24 samples**	**Detection frequency (%)**
Azithromycin	n.d.	453	283	11	45.8	207	316	257	24	100
Acetaminophen	n.d.	728	477	7	29.2	n.d.	n.d.	–		
Atorvastatin	n.d.	325	238	9	37.5	n.d.	n.d.	–		
Bupropion	n.d.	<MDL	a	4	16.7	n.d.	<MDL	a	16	66.7
Caffeine	6527	84,265	55,102	24	100	n.d.	n.d.	–		
Carbamazepine	462	1339	689	24	100.0	790	1427	1107	24	100
Ciprofloxacin	n.d.	684	579	5	20.8	n.d.	285	250	2	8.3
Citalopram	n.d.	200	167	13	54.2	n.d.	173	147	18	75.0
Citalopram propionic acid	n.d.	<MDL	a	2	8.3	n.d.	<MDL	a	1	4.2
Clarithromycin	n.d.	<MDL	a	13	54.2	n.d.	<MDL	a	18	75.0
Demethylcitalopram	n.d.	253	b	1	4.2	n.d.	385	315	18	75.0
***O*-Desmethylvenlafaxine**	459	1061	786	24	100	991	1876	1340	24	100
Diazepam	n.d.	56	b	1	4.2	n.d.	35	b	1	4.2
Diclofenac	41	2778	373	24	100	84	2922	1412	24	100
Diltiazem	n.d.	<MDL	a	13	54.2	n.d.	<MDL	a	22	91.7
Enrofloxacin	n.d.	359	b	1	4.2	n.d.	n.d.	–		
10,11-Epoxicarbamazepine	n.d.	<MDL	a	1	4.2	n.d.	<MDL	a	1	4.2
Fluoxetine	49	92	76	24	100	n.d.	79	67	23	95.8
Gemfibrozil	17	184	59	24	100	13	36	27	24	100
Hydroxyibuprofen	1812	22,909	7046	24	100	<MDL	577	269	24	100
Ibuprofen	127	7681	689	24	100	80	358	196	24	100
Ketoprofen	n.d.	<MDL	a	19	79.2	n.d.	n.d.	–		
Lomefloxacin	n.d.	n.d.	–			n.d.	<MDL	a	1	4.2
Lorazepam	n.d.	n.d.	–			n.d.	91	74	2	8.3

	WWTP influents					Effluents				
Pharmaceuticals	**Minimum (ng/L)**	**Maximum (ng/L)**	**Average (ng/L)**	**Number of time detected in 24 samples**	**Detection frequency (%)**	**Minimum (ng/L)**	**Maximum (ng/L)**	**Average (ng/L)**	**Number of time detected in 24 samples**	**Detection frequency (%)**
Moxifloxacin	n.d.	324	290	2	8.3	n.d.	n.d.	–		
Naproxen	n.d.	376	80	21	87.5	<MDL	122	66	24	100
d,l-Norephedrine	n.d.	2595	991	4	16.7	n.d.	n.d.	–		
Norsertraline	n.d.	n.d.	–			n.d.	228	b	1	4.2
Ofloxacin	n.d.	39	b	1	4.2	n.d.	233	174	9	37.5
Propranolol	n.d.	528	344	14	58.3	n.d.	n.d.	–		
Salicylic acid	n.d.	7014	1099	12	50.0	n.d.	172	115	16	66.7
Sertraline	n.d.	172	163	3	12.5	n.d.	100	96	5	20.8
Simvastatin	n.d.	485	b	1	4.2	n.d.	n.d.	–		
Sulfadiazine	n.d.	<MDL	a	1	4.2	n.d.	n.d.	–		
Sulfamethizole	83	83	.b	1	4.2	n.d.	n.d.	–		
Sulfamethoxazole	229	1117	489	13	54.2	n.d.	114	57	4	16.7
Sulfapyridine	n.d.	1442	576	14	58.3	n.d.	36	35	3	12.5
Sulfathiazole	220	220	b	1	4.2	n.d.	n.d.	–		
Trazodone	n.d.	504	294	19	79.2	155	234	191	24	100
Trimethoprim	n.d.	<MDL	a	1	4.2	n.d.	108	62	6	25.0
Venlafaxine	220	363	285	24	100	411	543	486	24	100

Pharmaceuticals organized in the table by alphabetic order.

a-Average was not performed due to the minimum value (n.d.) and maximum value (<MDL).

b-Pharmaceutical was detected in one sample of the 24 samples.

Diclofenac, ibuprofen, hydroxyibuprofen, carbamazepine, fluoxetine, venlafaxine, *O*-desmethylvenlafaxine, and gemfibrozil were the pharmaceuticals with detection frequency equal to 100% in both matrices. The pharmaceuticals only detected in WWTP influents were: caffeine (100%), naproxen (88%), propranolol (58%), azithromycin (46%), atorvastatin (38%), acetaminophen (29%), bupropion (17%), norephedrine (17%), moxifloxacin (8%), being enrofloxacin, ofloxacin, sulfadiazine, sulfathiazole, sulfamethizole, demethylcitalopram, diazepam, simvastatin the pharmaceuticals with the

lowest detection frequency (4%). Nonetheless, azithromycin (100%), demethylcitalopram (75%), bupropion (67%), ofloxacin (38%), lorazepam (8%), lomefloxacin (4%), norsertraline (4%), and diazepam (4%) were the pharmaceuticals only detected in WWTP effluents. The remaining pharmaceuticals were detected in both matrices. However, higher detection frequencies were achieved in WWTP influents when compared with WWTP effluents for: fluoxetine (100% vs. 96%), sulfapyridine (58% vs. 13%), sulfamethoxazole (54% vs. 17%), ciprofloxacin (21% vs. 8%), and citalopram propionic acid (8% vs.4%), while higher detection frequencies were found in WWTP effluents when compared with WWTP influents for: ketoprofen (100% vs. 79%), trazodone (100% vs. 79%), clarithromycin (75% vs. 54%), citalopram (75% vs. 54%), diltiazem (92% vs. 54%), salicylic acid (67% vs.5 0%), sertraline (21% vs. 13%), and trimethoprim (25% vs. 4%). For 10,11-epoxi carbamazepine, its presence was detected only in one sample of WWTP influent and effluent, and 4% of detection frequency was found.

Regarding the WWTP influents, the analysis of the individual pharmaceutical concentrations obtained for each sampling hour, and the total concentration considering all pharmaceuticals was performed with the objective to check one pattern of the pharmaceuticals consumption. For WWTP effluents, only the total concentration was analysed for each sampling hour, since the concentration achieved in the WWTP effluents depends not only on the pharmaceutical consumption but also on the efficiency of the WWTP treatment. In the WWTP effluent, the objective was to verify the total concentration of pharmaceuticals that is released to the environment. Thus, in Fig. 2, the obtained concentration (ng/L) versus collection hour of the WWTP influent for each pharmaceutical, is presented.

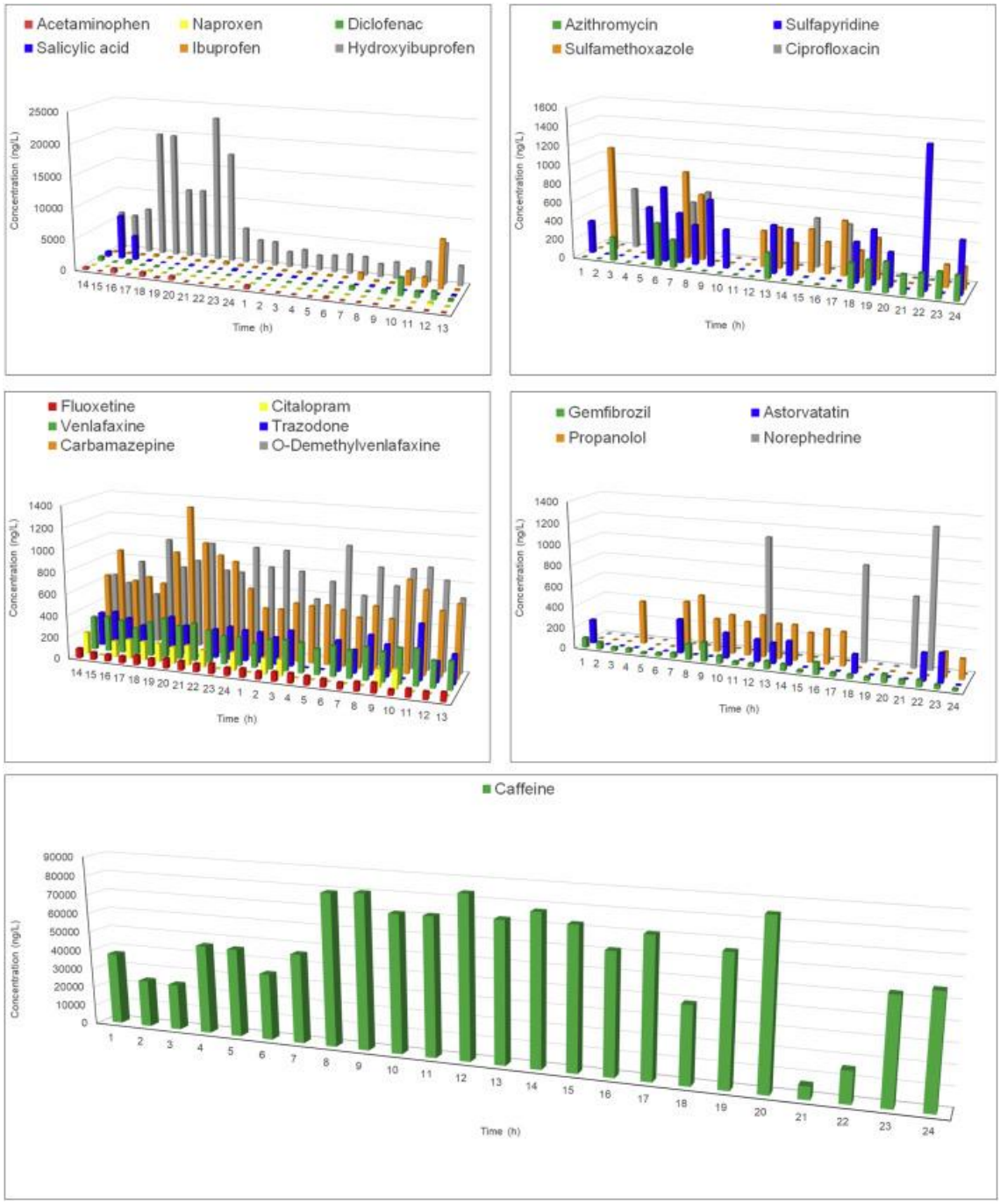


Fig. 2. Figure SM8-concentration (ng/L) versus hourly WWTP influent collection for the detected pharmaceuticals.

There were some pharmaceuticals only detected in one sample, or with concentrations below the MDL and thereby the bar graph was not plotted. These pharmaceuticals were: ketoprofen (concentration range from n.d to <MDL), enrofloxacin (one sample detected: 359 ng/L at 11 h), ofloxacin (one sample detected: 39 ng/L at 16 h), moxifloxacin (two samples detected: 324 ng/L (9 h) and 256 ng/L (12 h)), trimethoprim (concentration range from n.d to <MDL), sulfadiazine (concentration range from n.d to <MDL), sulfathiazole (one sample detected: 220 ng/L at 19 h), sulfamethizole (one sample detected: 83 ng/L at 19 h), clarithromycin (range from n.d to <MDL), 10,11-epoxi carbamazepine (concentration range from n.d to <MDL), sertraline (three samples detected: 172 ng/L

(23 h), 152 ng/L (04 h), and 164 ng/L (13 h)), citalopram propionic acid (concentration range from n.d to <MDL), demethylcitalopram (one sample detected: 253 ng/L at 22 h), diazepam (one sample detected: 56 ng/L at 24 h), bupropion (concentration range from n.d to <MDL), simvastatin (one sample detected: 485 ng/L at 21 h), and diltiazem (concentration range from n.d to <MDL).

For NSAIDS/analgesics, the highest concentration peak was achieved at: 16, 18, 20 and 01 h for acetaminophen, 10 h for diclofenac, 12 h for ibuprofen, 17, 18, 21, and 22 h for hydroxyibuprofen, 12 and 18 h for naproxen, and 15 and 16 h for salicylic acid. Diclofenac, ibuprofen, and hydroxyibuprofen were detected in all samples. For NSAIDs/analgesics, it could be seen that, diclofenac, ibuprofen and naproxen had the highest concentration in the end of the morning, salicylic acid in the middle of afternoon, hydroxyibuprofen between the end of the afternoon and the beginning of the night, and acetaminophen had several maxima but always in afternoon and at night, no values were found in the morning.

For the antibiotics group, ciprofloxacin, sulfapyridine, sulfamethoxazole, and azithromycin were the antibiotics present in the bar charts in the Fig. 2. It is estimated that 30–90% of an administered dose of most antibiotics, human and veterinary, may be excreted as active substances (Chen et al., 2006). Ciprofloxacin was detected only in 21% of the samples: (i) in the middle of the afternoon (16 h), (ii) at the beginning of the night (20 and 21 h), and (iii) during the night at 04 and 06 h, respectively. The concentrations obtained in these five points were very similar. For the other antibiotics, there is a different pattern for each antibiotic. More samples were detected between 01 and 08 h for sulfamethoxazole, between 08 and 13 h for azithromycin, and between 18 and 23 h for sulfapyridine. From 13 to 18 h, there were not many samples detected. However, the concentration of ciprofloxacin, sulfamethoxazole and azithromycin reached higher values in that period of time and the highest concentration found for sulfapyridine was observed at 11 h. The administration of an antibiotic depends largely on the clinical picture, the condition of the patient, and the availability (Enenkel and Stille, 1988). It is important to highlight that a pattern could not be noticed in the results due to the different dosage, time of administration, and type of antibiotic used for each disease.

According to literature, and the studies on the hourly variation of pharmaceuticals in WWTPs influents, several factors can contribute to these variations. Some of the most important are related to pharmaceuticals therapeutic class, posology and dosages. For instance, for antibiotics, Coutu et al. (2013) found a peak concentration in the morning and a second peak approximately 12 h later, which is in agreement with the typical patterns of consumption for some of these compounds. According to Zhang et al. (2018) the diurnal variations of antibiotics concentrations showed a very good consistency with the possible consumption timing and the citizens' movement between residence and working areas. The different hourly variation observed by Camacho-Muñoz et al. (2014) was found to be dependent on the pharmaceutical therapeutic group and the urban or industrial source. These authors report pharmaceutical concentrations during a 24 h period in accordance with their consumption and excretion patterns (Camacho-Muñoz et al., 2014). Regarding to other therapeutic families, the concentrations seem to be more consistent over time, according to some authors (Kay et al., 2017; Petrie et al., 2017), while for others the exact reason for the observed variability is unclear and further information on pharmacokinetics and consumer behavior would be necessary in order to give a definitive explanation (Gerrity et al., 2011; Hong et al., 2015; Nelson et al., 2011).

Weather conditions can also influence hourly concentrations, as after heavy rainfall events, a decrease in pharmaceuticals concentration was observed. Dilution effects due to the increase in wastewater flowrates related to working hours or in the beginning/end of the day may also explain a decrease in the concentrations in particular moments of the day (Brunsch et al., 2018; Hong et al., 2015). The physicochemical properties of the compounds along with the chemical conditions found between the households and the WWTPs can also contribute to distinct diurnal variations for different compounds.

From OECD (Organisation for Economic Co-operation and Development) Health Statistics 2017 (OECD, 2017), psychiatric drugs consumption increased twice in most countries between 2000 and 2015. Long-term use of psychiatric drugs, the constant release to the environment, and their persistency results in the detection of psychiatric drugs in all the analysed samples. For this group including carbamazepine and fluoxetine (Fig. 2) the obtained concentration was very similar, with exception of carbamazepine that had the highest peak at 20 h and at 11 h for trazodone. Carbamazepine, fluoxetine, venlafaxine, and *O*-desmethylvenlafaxine had 100% detection frequency and the highest concentration for the psychiatric drugs was noticed for the metabolite of venlafaxine.

Finally, propranolol, gemfibrozil, atorvastatin, caffeine, and norephedrine were also detected. For propranolol, most of the samples were grouped between 20 and 06 h of the morning. Caffeine and gemfibrozil had 100% of detection frequency. The highest concentrations of gemfibrozil were observed at 08 h and 09 h and for propranolol about 1 h earlier, which is in accordance with the findings of Camacho-Muñoz et al. (2014). The highest values obtained for caffeine were between 21 and 09 h. In Portugal, the consumption of coffee is a generalized practice throughout the day, what may explain these results. Fewer samples were detected in the case of atorvastatin and norephedrine. The highest value was observed at 20 h for atorvastatin and 01 and 11 h for norephedrine.

In the following paragraphs, a discussion of the results for WWTP influents and effluents samples are performed, not for each pharmaceutical as was done in the previous paragraphs for WWTP influents, but for all pharmaceuticals that are detected in that specific sampling hour. Thus, the total concentration found for each sampling hour and for each type of WWTP sample is shown in Fig. 3. Due to the high concentration of caffeine found in the WWTP influents, it was decided to exclude it to avoid concealing the total concentration.

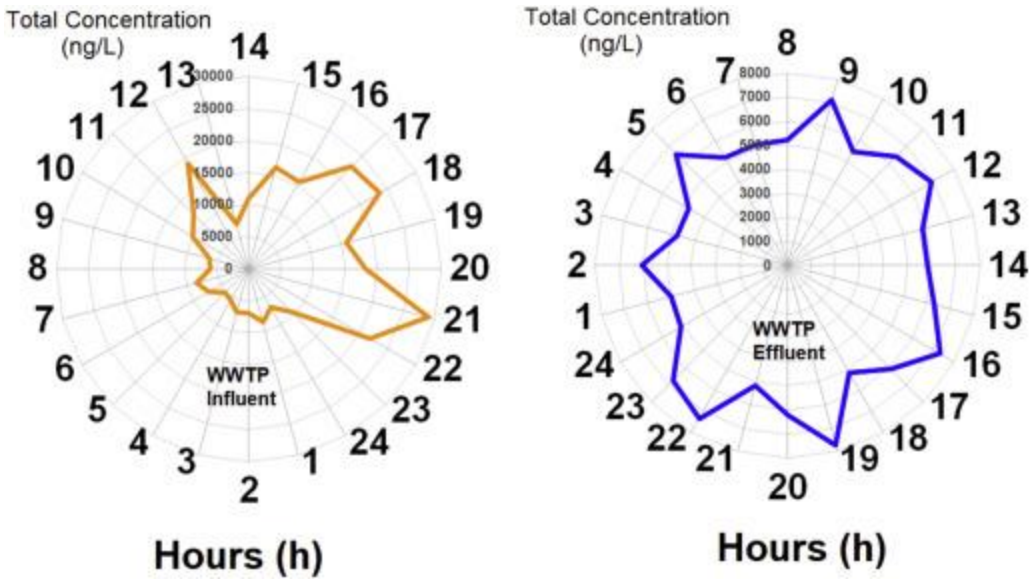


Fig. 3. Radar chart of the total concentration for all detected pharmaceuticals (ng/L) in each sampling hour.

Analysing each radar graph, it could be observed in the WWTP influent that the total concentration of detected pharmaceuticals is higher between 15 and 22 h and lowest in the period from 23 to 10 h in the morning. The total concentration starts to increase again until 12 h and then decreases at 13 h. In the WWTP effluent, the total concentration varies little throughout the sampling hours.

Fast-target analysis and hourly variation of 60 pharmaceuticals in wastewater using UPLC-High resolution mass spectrometry was the study of Hong et al. (2015). Of the target pharmaceuticals in wastewater of a sewage-treatment plant analysed on an hourly basis, only 17 compounds were detected, and others were lower than the method detection limits. Concentration profiles of acetaminophen, caffeine, acetylsalicylic acid, chlorphenylamine, diclofenac, and mefenamic acid showed a significant decrease at 8 h due to dilution of high wastewater flow. The authors stated that the reason for the hourly variation of the remaining pharmaceuticals is unclear, which is likely due to their varying source and intermittent consumption (Hong et al., 2015).

3.4. Environmental risk characterization

Due to the high number of pharmaceuticals, only the database of the ECOSAR (U.S. EPA Ecological Structure Activity Relationships) was consulted. The pharmaceuticals were input by their CAS number. If RQ > 1, harmful effects could be expected due to the presence of the pollutant in water. On the contrary, if RQ < 0.1, the environmental risk is low (Ccanccapa et al., 2016).

In order to ensure maximum protection, when the analytes were detected in the samples but the concentration was below either MDL or MQL, half of reported MQL was used as MEC to consider the worst-case scenario (Mendoza et al., 2015). Maximum measured concentration (ng/L), acute toxicity data (EC50/LC50) for all pharmaceuticals on fish, *Daphnia magna*, and algae, and the estimated RQs are presented in Table SM7 (Supplementary material).

RQs were calculated for 60 pharmaceuticals since no information was obtained for the remaining. Seven pharmaceuticals in WWTP influents and three pharmaceuticals in WWTP effluents present an RQs higher than 1. Carbamazepine, fluoxetine, and sertraline were the common pharmaceuticals with RQ > 1 in both WWTP matrices and atorvastatin, caffeine, simvastatin, and trazodone the pharmaceuticals with an RQ > 1 only in WWTP influents. Atorvastatin was the pharmaceutical with an RQ > 1 in the three trophic levels in the WWTP influent. The highest RQ value was observed for the stimulant caffeine in algae (WWTP influent).

For the pharmaceuticals that showed an RQ > 1, it is important to highlight that four out of seven pharmaceuticals in WWTP influents and three out of three pharmaceuticals in WWTP effluents belong to the group of psychiatric drugs. Two lipid regulator and cholesterol lowering statin drugs (atorvastatin and simvastatin) and the stimulant (caffeine) were the other pharmaceuticals with an RQ > 1 in WWTP influents.

Pharmaceuticals commonly prescribed to treat depression can affect aquatic insects, amphibians, and fishes (Richmond et al., 2016). Fluoxetine, sertraline, and their metabolites can bioaccumulate up to 1 μg/kg of fish in the brain, liver, and muscle tissue in several species (Brooks et al., 2003; Chu and Metcalfe, 2007). Conners et al. mentioned that fluoxetine and sertraline reduced the growth rates of tadpoles (Conners et al., 2009). Moreover, fluoxetine changed burrowing behavior of the freshwater bivalve at 22.3 μg/L (Hazelton et al., 2014) and induced spawning in zebra mussels at low concentrations (Fong, 1998). Citalopram induced foot detachment in freshwater gastropods at 405 pg/L and 4.05 μg/L (Fong and Hoy, 2012). The alga *Pseudokirchneriella subcapitata* showed to be more sensitive to citalopram and fluoxetine when compared to *Daphnia magna* (Christensen et al., 2007). Algae developed cell deformities when exposed to 13.6 and 27.2 μg/L of fluoxetine (Brooks et al., 2003). One of the most commonly used benzodiazepines, diazepam, has been shown to increase activity in zebrafish (Oggier et al., 2010) and pumpkinseed sunfish (Brandão et al., 2013) at μg/L concentrations, and exposure to mg/L of diazepam increased boldness in larval zebrafish (Richendrfer et al., 2012). Psychiatric drugs have been shown to reduce territorial aggression in coral reef fish (Perreault et al., 2003) and locomotion and aggression in Siamese fighting fish (Kohlert et al., 2012).

Many toxicological studies have been conducted in order to assess the effects of psychiatric drugs but mainly referring to acute toxicity, using pharmaceuticals concentrations that are several orders of magnitude higher than the ones that are found in natural environments. Therefore, there is the need for further research on long term effects (chronic toxicity), by subjecting the test organisms to pharmaceuticals levels found in the environment, and particularly, when several different active substances are present concomitantly at the μg/L level.

4. Conclusions

The overarching goal of this study was the evaluation of the presence of 83 pharmaceuticals belonging to different therapeutic classes, namely: non-steroidal anti-inflammatory drugs, analgesics, antibiotics, anorectics, anxiolytics, laxatives, antidiabetic drug, antipsychotic, calcium channel blocker, β-blockers, fibrate lipid lowering agent, stimulants, lipid regulator and cholesterol lowering statin drugs, proton pump inhibitor, and psychiatric drugs in WWTP influent and effluent samples of one WWTP in Leiria

(Portugal). Two WWTP influent and effluent samplings were performed, involving the sampling hourly in one day and its composites samples.

The average recovery of pharmaceuticals was around 74.9% and 76.9% for WWTP effluent and influent samples. Recoveries above 75% were achieved for the majority of the studied pharmaceuticals. Therefore, STRATA-X cartridge and sample adjusted to pH 2 shows a good choice for the extraction of the selected pharmaceuticals.

In the composite WWTP samples, most of pharmaceuticals detected belonging to the NSAIDs/analgesic, antibiotics, and psychiatric drugs. Higher concentrations were noticed for acetaminophen, hydroxyibuprofen, and salicylic acid in WWTP influent and diclofenac in WWTP effluents. Diclofenac, listed in the watch list, was detected in both matrices. Due to high human and veterinary use of antibiotics, concern and studies by the scientific community have been increasing. Two of the three macrolides listed in the watch list were found either in effluent and influent samples. Concentration obtained in antibiotics were between <MDL (clarithromycin and ofloxacin) to 600 ng/L (sulfamethoxazole) in WWTP influent and between <MDL (clarithromycin) to 283 ng/L (azithromycin) in WWTP effluent. Concentrations in the μg/L range were reached for carbamazepine and *O*-desmethylvenlafaxine in WWTP effluent. It is important to highlight that psychiatric drugs concentration in effluents were or higher or similar to the concentrations found in WWTP influent. The highest concentration obtained in the present study was found to caffeine. The obtained result is in line with other studies reported in literature. Atorvastatin and propranolol in WWTP influent and gemfibrozil and diltiazem in both WWTP matrices were found at concentration in the ng/L level. Finally, d,l-norephedrine was detected at μg/L in WWTP influent.

One of the important points of our study was not only monitor pharmaceuticals but also monitor its transformation products and its metabolites. Thus, a total of eighteen compounds among which, pharmaceuticals, transformation products, metabolites were analysed. Hydroxyibuprofen, salicylic acid, and *O*-desmethylvenlafaxine were found in both matrices. Concentration in μg/L were reached for hydroxyibuprofen and salicylic acid in WWTP influent and for *O*-desmethylvenlafaxine in WWTP effluents. Demethylcitalopram was found in WWTP effluent with 364 ng/L.

The main objective of the study proposed by the authors was to monitor the 83 compounds in samples collected hourly. Twenty-four samples were collected in the WWTP influents and effluents and after extracted and analysed in the UHPLC-MS/MS. 45.8 and 34.9% of the pharmaceuticals, more precisely 38 and 29 pharmaceuticals were detected in at least one WWTP sample in influents and effluents. The highest total concentration was reached between 15 and 22 h and lowest total concentration was found in the period from 23 to 10 h in the morning in the WWTP influents. In the other hand, no evidence highs and lows total concentrations are highlighted in WWTP effluents. The concentration over the analysed hours is very consistent and it could not be possible to define one profile for the total concentration.

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