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Investigating the presence, distribution and risk of pharmaceutically active compounds (PhACs) in wastewater treatment plants, river sediments and fish

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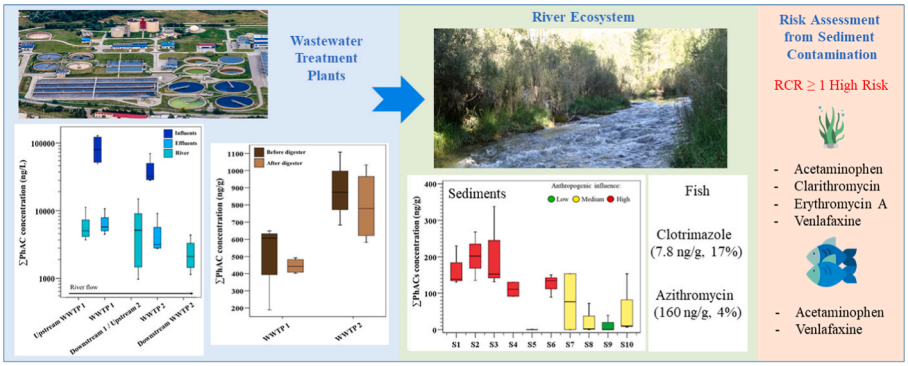
HIGHLIGHTS

- WWTP treatments can reach removal efficiencies above 75% for some PhACs.
- Treatments applied to the sludge were not effective in eliminating PhACs.
- WWTP effluents represent an increase in the PhACs pollution of the area studied.
- RCRs revealed risk in sediments, fish and sludge agricultural amendments.

GRAPHICAL ABSTRACT



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ABSTRACT

The increasing consumption of medicines and the lack of efficient technologies in wastewater treatment plants (WWTPs) can release pharmaceutically active compounds (PhACs) into any given river with the subsequent risk to the environment and human health. To assess the occurrence and transfer pathways of PhACs through the river ecosystem, 22 PhACs and one metabolite were analyzed in WWTPs, river sediments and fish collected alongside the Tagus River basin between 2020 and 2022. All the matrices presented at least two drugs being azithromycin the only one quantified in all of them. Analgesics, anti-inflammatories, antihypertensives, antidepressants and beta-blockers were the main PhACs in influents, with median concentrations up to 19 μg/L. In effluents, antihypertensives and antidepressants were the PhACs with the highest contribution. For acetaminophen, ibuprofen, ketoprofen, naproxen, atorvastatin, azithromycin, clarithromycin, sulfamethoxazole, trimethoprim, and valsartan WWTPs treatments reached removal efficiencies above 75%. Compounds with a high tendency to bind to organic matter were retained in sludge (clotrimazole, 96 ng/g before digester, 100%).

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However, results showed that applied treatments were not effective in removing PhACs from this matrix. Although the total mass balance revealed a high removal rate of some PhACs, many of them were still present in the effluent and their release into rivers became the main source of PhAC pollution of the aquatic ecosystem. The most hydrophobic ones (irbesartan, 24 ng/g, 61%), positively charged (*o*-desmethylvenlafaxine, 95 ng/g, 68%) and those with affinity to organic matter (clotrimazole, 21 ng/g, 61%) reached sediment samples. Only clotrimazole (7.8 ng/g) and azithromycin (160 ng/g) were found in fish samples. Risk assessment revealed a high risk for (i) acetaminophen, clarithromycin, erythromycin A, and venlafaxine in phototrophic organisms and (ii) acetaminophen and venlafaxine in fish.

1. Introduction

Pharmaceutically active compounds (PhACs) are substances used daily in the prevention and treatment of both human and veterinary diseases. These compounds can reach the environment through many pathways throughout their life cycle, but wastewater treatment plant (WWTP) effluents are undoubtedly one of the main sources of pollution to the aquatic environment (Jelíc et al., 2009). The constant release of contaminated effluents gives them a pseudo-persistent character (Mandaric et al., 2019; Zhou and Broodbank, 2014) which puts the entire river ecosystem at risk due to chronic exposure to these pollutants. These potential health and ecological risks have led the PhACs to be recognised as 'emerging' contaminants (Valdez-Carrillo et al., 2020). Studies on the environmental risks and toxic effects that PhACs can trigger in aquatic organisms are generally carried out on aqueous matrices without considering particulate matter and sediments (Sun et al., 2021) which should not be ignored (Maskaoui and Zhou, 2010). River sediments can act as a sink (Radović et al., 2015; Zhou and Broodbank, 2014) retaining part of the contaminant load carried by the aqueous phase and decreasing its bioavailability. However, changes in water composition, environmental conditions or biological activity can increase the mobility of these contaminants (Radović et al., 2015) and could promote their redispersion in water. Because knowledge about the fractionation of PhACs in sediments is still limited, it is necessary to develop studies that allow a better understanding of the absorption mechanisms and the risk that this contamination poses to organisms through this matrix.

Once introduced into the river ecosystem, PhACs remain bioactive molecules and can interact with non-target organisms. It has been shown, at least under laboratory conditions, that (i) antidepressants can cause growth problems related to endocrine disruption (Thompson and Vijayan, 2021), (ii) exposure to antifungals originated behaviour modification such as swimming rate reduction and decrease in the rate of feeding (Mefela and Nwani, 2021) or (iii) antibiotics, as well as favouring the emergence of antibiotic-resistant microorganisms, can cause liver damage in some fish (Akshaya et al., 2023). Even if the effects of pharmaceuticals can differ between species (Brodin et al., 2014), they can alter the inter-community behaviour and resilience of fishes (Polverino et al., 2021), and subsequently, the incorporation of PhACs into aquatic organisms may transfer potentially toxic concentrations via food chain to humans.

Not only the river ecosystem is threatened by PhACs pollution. Some pharmaceutical residues can be fractionated and transferred to the sludge generated in wastewater treatment carried out in WWTPs (Huber et al., 2016). If these drugs are not properly removed, they can reach other environmental compartments such as soil, terrestrial flora and fauna when treated sewage sludge is used as agricultural amendments. Currently, although drug concentrations in the order of hundreds or thousands of ng/g (Huber et al., 2016) have been found, there are no environmental quality standards or specific regulations regarding the maximum content of these substances in sewage sludge.

Considering the dangerous environmental effects that drug contamination can cause, it is not strange that since the first version, more than 40% of the compounds included in the Watch List of the Water Framework Directive (WFD) presented pharmacological activity. Nowadays, the Watch List (European Commission, 2022a) consists of 26 substances of which 11 are pharmacologically active compounds. Additionally, the proposal for amending the current WFD (European Commission, 2022b) proposes to include 9 PhACs in the List of Priority Substances and establishes their corresponding environmental quality standards (EQSs). In the present study, five out of nine PhACs included in the proposal were investigated contributing to improving knowledge of priority compounds for regulatory authorities. Together with those included in the WFD Watch List, other such antihypertensives, beta-blockers or lipid-modifying agents, have been of great concern because chronic cardiovascular diseases have become one of the main chronic health problems of recent decades at a global level (Spiteri and Brockdorff, 2019) and therefore their prevalence has increased markedly (OECD, 2021). In accordance with the Strategic Approach to Pharmaceuticals in the Environment of the European Union (European Commission, 2019), more monitoring data are still needed to understand the concentration levels and the risk posed by these substances. The Strategy also points to the importance of waste reduction and the need for investment to improve wastewater treatment.

To address this issue, the behaviour of 22 PhACs and one metabolite was investigated in WWTPs (influent, effluent and sewage sludge), by including removal efficiencies. In addition, investigations on occurrence and transfer pathways between the aquatic ecosystem compartments such as river sediments and fish were performed and related temporal variations and anthropogenic influence were assessed. To identify those substances that may pose the greatest risk to the aquatic ecosystem, Risk Characterization Ratios (RCRs) were evaluated for the target PhACs in the different environmental or river compartments.

2. Material and methods

2.1. Selection of target compounds

The 23 target pharmaceuticals were selected after a strengths, weaknesses, opportunities, and threats (SWOT) analysis conducted in the framework of the CEMEF project (CEMEF, 2023). The outcome of the SWOT analysis was a list of emerging pollutants that included antibiotics, antihypertensives, beta-blockers, psychiatric drugs such as antidepressants and antiepileptics, analgesics, anti-inflammatories, lipid regulators, fungicides, and anthelmintics. Subsequently, anhydroerythromycin, a non-active erythromycin metabolite, was added to the list. For additional information about target analytes, see Supplementary Material (SM) (Table S1).

2.2. Study area and sample collection

The Tagus River is the longest in the Iberian Peninsula (1092 km). Its watershed covers 80,100 km^2 of which 64% are in Spain and 36% in Portugal and supplies water resources to approximately 12% of the Spanish population. The basin is characterized by a Mediterranean climate, with cold winters and hot, dry summers, and a great diversity of flora and fauna. The river flows through natural and conservation reserves, areas with agricultural and livestock industries, and areas with a high population density such as Madrid (3.3 million inhabitants; INE, 2023a) or Toledo (86.070 inhabitants; INE, 2023b). Two WWTPs were

selected due to their representativeness (type and influent size, edification age and treatments implemented; see Table S2 for more details) in the basin. WWTP 1 is located 25 km upstream of WWTP 2 (Fig. S2) and there are no significant discharges between them (Ministry for the Ecological Transition and the Demographic Challenge, 2024). WWTP 1 receives 9000 m^3/day from an urban/industrial area and WWTP 2 collects 18,000 m^3/day of wastewater of exclusively urban character, reflecting the high anthropogenic load that the Tagus River Basin supports in the area. Both facilities have a primary treatment to remove suspended solids and oils followed by a secondary or biological process with activated sludge. Sewage sludge generated during primary and secondary treatment is taken to an anaerobic digester for nutrient removal (Fig. S1). Therefore, their selection was considered appropriate to evaluate the behaviour of PhACs in wastewater treatment plants and the contribution of their effluents to the river ecosystem. Influent and effluent samples were collected with polypropylene bottles and the sludge samples were assembled with amber glass bottles. A total of 16 wastewater (8 influent and 8 effluent) samples and 16 sludge (8 before and 8 after the anaerobic digesters) samples were collected quarterly during 2022 (Table S2). Samples for each quarter were taken on the same day. Sediment (n = 28) and fish (n = 24) samples were annually collected in amber glass bottles in ten sampling points located throughout the Tagus basin (Fig. S2) during autumn 2020, 2021 and 2022. Sediment samples were collected with grabs if the river was not fordable or scoops if it was fordable at a depth of 5–10 cm (Ministry for the Ecological Transition and the Demographic Challenge, 2022a). Fish samples were obtained by electrofishing following current regulations (Ministry of Agriculture, Food and Environment, 2015). Fish samples consisted of a pool of the whole organism, no organs, tissues or plasma were separated. Upon arrival at the laboratory, all samples were stored at −20 °C until analysis. Complete details about sampling locations are summarized in SM.

2.3. Sample preparation

Liquid samples (0.5 L) were filtered with glass fiber filters to remove particulate matter and avoid clogging of SPE cartridges. Then, samples were spiked with 50 ng of deuterated internal standards (acetaminophen-d3, atenolol-d7, gemfibrozil-d6, ibuprofen-d3, sulfamethoxazole-d4, and venlafaxine-d6) and loaded onto Oasis HLB SPE cartridges (6 mL, 500 mg; Waters, Milford, MA, USA) that were previously conditioned with 10 mL of methanol and 10 mL of Milli-Q water. After allowing the cartridges to dry for 1 h under vacuum, the PhACs were eluted with 10 mL of methanol and the extract was evaporated until 500 μL under a gentle nitrogen stream.

The sediment and sludge samples were extracted with a Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) procedure as described previously (Royano et al., 2024). Briefly, 1 g of spiked sample (50 ng of deuterated standards) was extracted with 2 mL of Milli-Q water and 4 mL of acidified acetonitrile (1% acetic acid) shaking for 30 min, following QuEChERS salts addition (0.8 g of magnesium sulfate and 0.2 g of sodium acetate). The mixture was vigorously stirred in a vortex to prevent coagulation of the magnesium sulfate, and then the blend was centrifuged (5 min, 5000 rpm) and the supernatant was evaporated to 1 mL with nitrogen, and filtered (0.45 μm PTFE vial filter) prior to instrumental analysis.

For the fish samples analysis, four different methodologies were tested, see SM for complete details (Table S4). Acidified acetonitrile extraction followed by SPE cleanup was the procedure for which the best recoveries and the highest number of compounds met validation requirements and therefore was the procedure selected. Freeze-dried sample was weighted (1 g), spiked with deuterated standards and extracted with 7 mL of acetate buffer (pH = 4)/acetonitrile (50:50, v/v) mixture followed by a SPE cleanup step using Oasis HLB SPE cartridges (6 mL, 200 mg; Waters, Milford, MA, USA) previously conditioned with 5 mL of methanol and 5 mL of Milli-Q water. Then, the supernatant was diluted (in 500 mL of Milli-Q water), loaded into the SPE cartridge, and washed with 6 mL of Milli-Q water. After drying for 30 min, the analytes were extracted with 6 mL of methanol and evaporated to 1 mL under a gentle stream of nitrogen. For fat content determination, 1 g of freeze-dried fish was extracted with 30 mL (3 cycles with 10 mL each time) of hexane/acetone (3:1). After vortexing for 1 min and centrifuging for 3 min at 4000 rpm, the supernatant was evaporated to dryness in TurboVap (TurboVap II, Zymark, OR, USA) to reach constant weight, and the fat percentage was determined by gravimetry. The average fat content was 7 ± 1% (4%–9% min-max, 20% RSD).

All the extracts (wastewater, sludge, sediments and biota) were spiked with 10 ng of clothianidin-d3 as injection standard before instrumental analysis.

2.4. Instrumental determination

UHPLC system (ExionLC; SCIEX, MA) equipped with a Luna omega 1.6 μm C18 100 Å column (100 × 2.1 mm i.d, Phenomenex) was coupled to a Triple Quad™ 3500 MS/MS System (SCIEX, MA) provided with a Turbo V™ ion source (SCIEX, MA). The mass spectrometer worked in multiple reaction monitoring (MRM) acquisition mode to acquire the two most intense transitions (SRM1 and SRM2; quantifier and qualifier) for each target analyte. Chromatographic conditions and MS parameters have been previously published (Royano et al., 2023).

2.5. QA/QC and statistical analysis

All analytical methods were validated in agreement with SANTE/2020/12830 (European Commission, 2020) and SANTE/11312/2021 (European Commission, 2021) performance criteria. Samples were fortified at different concentration levels (5 ng/L to 50 ng/L for wastewater samples, 1 ng/g to 50 ng/g for sediment and 5 ng/g to 50 ng/g for fish samples). According to SANTE, the lowest concentration level that fulfilled recovery (70–120%), precision (RSD ≤20%), and identification (MS/MS ion ratio within 30%) criteria, was established as the limit of quantification (LOQ). LOQs were set at 5 ng/L (25 ng/L for ibuprofen) for influent and effluent water samples (Table S6), from 1 ng/g to 10 ng/g (50 ng/g for ibuprofen) in sediments and sludge (Table S7) and from 5 ng/g to 50 ng/g in fish case (Table S8). Taking the signal-to-noise ratio of the qualifier transition (SRM2) from the LOQ, the limit of detection (LOD) was calculated as the concentration that gives a signal-to-noise ratio of 3. Linearity was assessed by calibration curves which were generated using linear regression analysis over the established concentration range. Procedural blanks (Milli-Q water and diatomaceous earth) were processed and analyzed as samples. Furthermore, solvent (methanol) injections were run between samples as instrumental blanks to check carryover contamination from the UHPLC-MS/MS system. All PhACs were below LODs in procedural and instrumental blanks and recoveries of surrogate standards ranged between 71 and 97%.

Statistical analyses were performed with SPSS 14.0 for Windows. For the descriptive statistical analysis, PhAC concentrations below LOQ but with total quantification frequencies (Qfs) above 30%, were replaced by LOQs divided by the square root of 2. Analyte concentrations were not normally distributed ($p < 0.05$, Shapiro-Wilk W and Kolmogorov-Smirnov tests). Therefore, to investigate bivariate relationships Spearman rank correlation coefficient was derived but in this case, only values above LOQs were included. Kruskal-Wallis H and Mann-Whitney U tests were run to evaluate differences between WWTP performance, geographic distribution and temporal variation.

2.6. Calculation of partition coefficients, mass flow rates and removal efficiencies

Sediment-water partition coefficients (K_D; L/Kg) were calculated as the ratio between the median concentration (ng/g) in the sediment and

in the river surface water (ng/L) for the same locations and dates as proposed by Castaño-Ortiz et al. (2024) (Eq. (1)).

$$K_D\left(\frac{L}{Kg}\right) = \frac{C_{sediment}\left(\frac{ng}{g}\right)}{C_{water}\left(\frac{ng}{L}\right)} \times 10^3 \quad (1)$$

WWTP mass flow rates were calculated from PhAC concentrations in the influent, effluent (ng/L), and sewage sludge (ng/g) and the inlet and outlet flows (Q, m^3/day for water and Kg/day for sludge; Table S2) of the evaluated WWTPs (Lindberg et al., 2010).

$$Mass\ flow\ rate = C_{PhAC}\left(\frac{ng}{L} or \frac{ng}{g}\right) \times Q\left(\frac{m^3}{day} or \frac{Kg}{day}\right) \times 10^{-6} \quad (2)$$

Removal efficiencies (REs) in the WWTPs were calculated considering the median concentration obtained for each analyte in the influents and the effluents (ng/L).

$$RE\ (\%) = \frac{C_{influent}\left(\frac{ng}{L}\right) - C_{effluent}\left(\frac{ng}{L}\right)}{C_{influent}\left(\frac{ng}{L}\right)} \times 100 \quad (3)$$

2.7. Risk assessment

In order to evaluate the environmental risk posed by the application of sewage sludge as an agricultural amendment, Risk Characterization Ratios (RCRs) were obtained as quotients between Predicted Environmental Concentrations (PEC) and Predicted No-Effect Concentration (PNEC) for the soil compartment and earthworms as organisms representing the bioaccumulation and introduction of contaminants into the terrestrial food chain with consequent potential secondary contamination (Navarro et al., 2018).

The PEC values and all the required parameters (equation (4) to equation (9)) were calculated following the Guidance on information requirements and Chemical Safety Assessment (ECHA, 2016a; 2016b). PEC estimation was conducted following a local scenario situation. PEC_{soil} was calculated following the equation:

$$PEC_{soil} = C_{soil}\ (0) \times \left[1 + \sum_{n=1}^{n-1} Facc^n\right] \quad (4)$$

where C_{soil} (0) was the initial concentration in the soil after the application of sludge (Eq. (5)) and Faac was the fraction of the initial concentration remaining in the top-soil layer after one year of application (Eq. (6)).

$$C_{soil}(0) = \frac{C_{sludge}\left(\frac{mg}{Kg}\right) \times APPL\left(\frac{Kg}{m^2}\right)}{DEPTH\ (m) \times RHO_{soil}\left(\frac{Kg}{m^3}\right)} \quad (5)$$

$$Faac = e^{-365k} \quad (6)$$

C_{sludge} was the PhAC concentrations measured in sludge samples, APPL corresponded to the dry sludge application rate, DEPTH was the depth of the soil-sludge mixture and RHO_{soil} was the bulk density of soil (see SM for details, Tables S9 and S10). The k parameter was the first order rate constant for removal from top soil and was calculated for each target compound according to equations (2)–(7) in SM.

$$k = k_{volatilisation} + k_{leaching} + kbio_{soil} \quad (7)$$

The prediction of the concentration contained in the earthworms was estimated following equation (8) (ECHA, 2016b). Both the amount of contaminant in the tissues and the corresponding amount of soil contained in the individual's gut were considered.

$$PEC_{earthworm} = \frac{BCF_{earthworms} \times C_{porewater} + PEC_{soil} \times F_{gut} \times CONV_{soil}}{1 + F_{gut} \times CONV_{soil}} \quad (8)$$

$BCF_{earthworm}$ was the bioconcentration factor for earthworms on wet weight basis, $C_{porewater}$ was the concentration in porewater, F_{gut} was the fraction of gut loading in worm and $CONV_{soil}$ was a conversion factor for soil concentration wet-dry weight soil (see SM for details).

As no ecotoxicological data for terrestrial organisms were found, $PNEC_{soil}$ values were provisionally calculated from $PNEC_{water}$ following the equilibrium partitioning theory (Eq. (9), ECHA, 2008). $PNEC_{water}$ values were previously published (Royano et al., 2023).

$$PNEC_{soil}\left(\frac{mg}{Kg}\right) = \frac{K_{soil-water}\left(\frac{m^3}{m^3}\right)}{RHO_{soil}\left(\frac{Kg}{m^3}\right)} \times PNEC_{water}\left(\frac{mg}{L}\right) \times 1000 \quad (9)$$

As information on ecotoxicological hazards of PhACs to bird or mammal organisms was not available, $PNEC_{earthworm}$ values could not be estimated.

The ecotoxicological risk that PhACs pose to aquatic organisms via sediments ingestion was evaluated for three trophic levels by calculating the RCRs between environmental concentration and $PNEC_{sediment}$ (Eq. (10)). To assess both the average situation and the worst scenario, the RCRs were calculated with the median (P50) and maximun environmental concentration (P95).

$$RCR_{sediment} = \frac{C_{sediment\,P50/P95}\left(\frac{mg}{Kg}\right)}{PNEC_{sediment}\left(\frac{mg}{Kg}\right)} \quad (10)$$

$PNEC_{sediment}$, as $PNEC_{soil}$, was calculated following the equilibrium partitioning theory (Eq. (11), ECHA, 2008).

$$PNEC_{sediment}\left(\frac{mg}{Kg}\right) = \frac{K_{susp-water}\left(\frac{m^3}{m^3}\right)}{RHO_{susp}\left(\frac{Kg}{m^3}\right)} \times PNEC_{water}\left(\frac{mg}{L}\right) \times 1000 \quad (11)$$

$K_{susp\text{-}water}$ is partition coefficient suspended matter-water and RHO_{susp} is the bulk density of wet suspended matter (see SM for details). $PNEC_{sediment}$ for PhACs with a log $K_{OW} > 5$ (atorvastatin, clotrimazole, and miconazole) was decreased by a factor of 10 in order to take uptake via ingestion of sediment into account (ECHA, 2008).

RCR values below 0.01 correspond to a negligible risk, RCR values between 0.01 and 1.00 represent a low to medium risk, and RCRs above 1.00 indicate a high risk to aquatic organisms. To characterize risk on the entire river ecosystem, the sum of the RCRs in both compartments (water and sediment) was calculated for each location as proposed by Kondor et al. (2022) for those sampling sites where water samples and sediments were taken at the same time.

3. Results and discussion

3.1. PhAC distribution in WWTPs

3.1.1. Wastewater line

Table S11 summarises influent and effluent concentrations found in WWTP 1 and 2. A total of 19 out of 23 target PhACs were quantified in at least one influent sample with individual concentrations ranging from 5.8 ng/L (thiabendazole) to 48 μg/L (acetaminophen) and median concentration up to 16 μg/L (acetaminophen) (Fig. 1). Effluents presented concentration levels from 5.1 ng/L (erythromycin A) to 4.5 μg/L (irbesartan). Despite the fact that the pollutant content of the influent water can be very heterogeneous, the samplings carried out provided similar PhAC concentrations to those described in other WWTPs worldwide (Čelić et al., 2019; Rivera-Jaimes et al., 2018; Semerjian

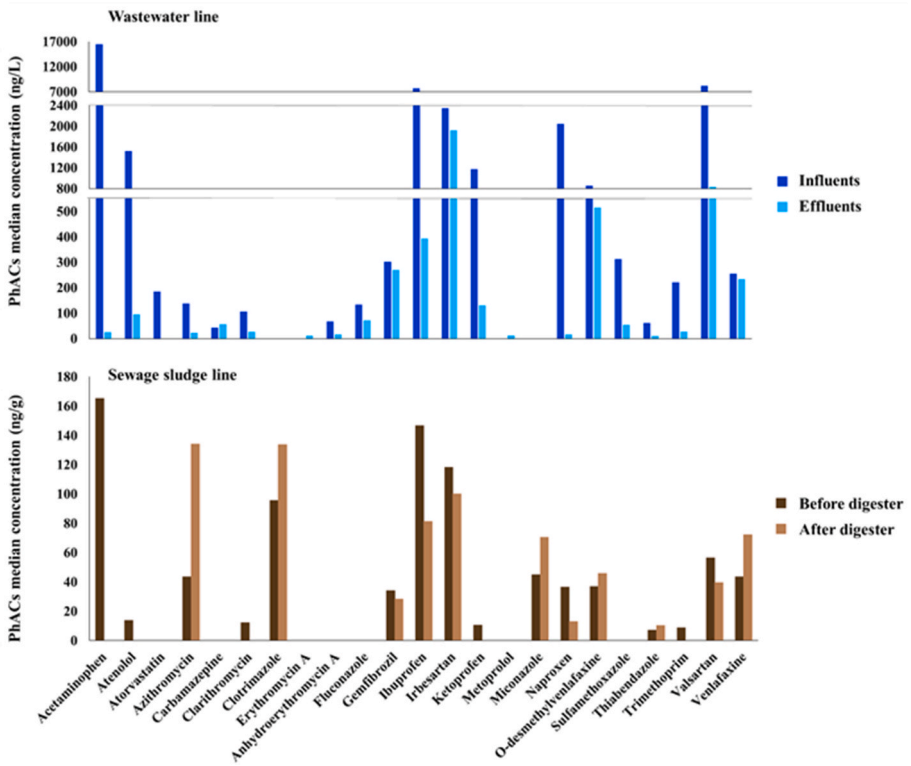

Fig. 1. PhACs median concentrations in influents, effluents (blue; ng/L), and sewage sludge before and after the anaerobic digesters (brown; ng/g). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2018; Yan et al., 2014). Interestingly, the metabolite anhydroerythromycin A, which is not usually included in monitoring activities, was quantified in all wastewater samples (influents and effluents). Erythromycin A is partially degraded in aqueous media (Wang et al., 2012) and therefore it was not strange to find its metabolite in these samples. On the other hand, clotrimazole and miconazole were below LOQ in all wastewater samples.

Analgesics and anti-inflammatories (acetaminophen, ibuprofen, ketoprofen and naproxen), antihypertensives (irbesartan and valsartan), antidepressants (*o*-desmethylvenlafaxine) and beta-blockers (atenolol) were the main PhACs in the influents with median concentrations ranging from 1.4 μg/L to 19 μg/L for WWTP 1 and from 546 ng/L to 16 μg/L for WWTP 2. Besides, influent water samples presented similar ($p > 0.05$, Mann Whitney H test) levels for acetaminophen, atenolol, atorvastatin, azithromycin, carbamazepine, clarithromycin, anhydroerythromycin, naproxen, *o*-desmethylvenlafaxine, sulfamethoxazole, thiabendazole, trimethoprim, valsartan, and venlafaxine in the two WWTPs investigated. On the opposite, levels of fluconazole, gemfibrozil, ibuprofen, irbesartan and ketoprofen reaching WWTP 1 were significantly higher ($p < 0.05$, Mann Whitney H test) than those quantified in WWTP 2. As mentioned before, WWTP 2 receives water from a higher area where more rainwater was collected by the urban sewage system. Therefore, to evaluate if this rainwater could trigger a dilution effect in WWTP 2 influents, concentrations were converted to mass flow rates (Table S12). After this conversion, WWTP differences only remain in the gemfibrozil (6.2 g/day and 0.8 g/day; WWTP1 and WWTP2) case.

Effluent samples from WWTPs did not show significant differences ($p > 0.05$, Mann Whitney H test) except for gemfibrozil (678 ng/L and 53 ng/L, WWTP 1 and 2). Again, mass flow rates calculation (Table S12) confirmed that both WWTPs discharge similar amounts of PhACs ($p > 0.05$, Mann Whitney H test) to the Tagus River. In this case, antihypertensives (irbesartan and valsartan) and antidepressants (*o*-desmethylvenlafaxine) were the main therapeutic groups quantified in all samples accounting for more than 74% of total PhACs ($\sum$PhACs) water release (Fig. S3). Similar results have been described in previous works (Čelić et al., 2019; Rivera-Jaimes et al., 2018; Semerjian et al., 2018; Yan et al., 2014; Salgado et al., 2012).

Up to 12 PhACs (acetaminophen, atenolol, atorvastatin, azithromycin, clarithromycin, anhydroerythromycin A, ibuprofen, ketoprofen, naproxen, sulfamethoxazole, trimethoprim and valsartan) presented removal efficiencies above 75% (Table S13) which highlights that even though WWTPs were designed to remove nutrients (nitrogen and phosphorous), pathogens, organic materials and suspended soils, they could also achieve a significant removal for some PhACs. It should be noted that calculations of mass removals did not consider the hydraulic residence time for the WWTPs. Nevertheless, similar behaviour has been previously described for acetaminophen and ibuprofen (Lopez et al., 2022; Čelić et al., 2019; Huber et al., 2016). Moreover, data generated in the present study revealed medium (≤55%) removal rates for antidepressants (*o*-desmethylvenlafaxine and venlafaxine) and the antiepileptic carbamazepine, while in the lower-end gemfibrozil was the PhAC with the lowest removal efficiencies (≤2%). Interestingly, although neither erythromycin A nor metoprolol were quantified in the influent samples, concentrations above their LOQs were found in the effluents. During metabolism some PhACs could form complexes with other molecules and be excreted as glucuronide or sulphated conjugates (De Jesus Gaffney et al., 2017), which will be deconjugated after treatment in WWTPs, allowing PhACs to be detected in effluents but not in influents (Gomes Moreira et al., 2022; Paíga et al., 2016).

Seasonal variations in the pharmaceutical residue load in WWTPs were investigated (Fig. 2). To avoid the dilution effect, the mass flow rate was depicted. As can be observed, the two WWTPs revealed a similar behaviour. In both cases, a decrease in the pollutant load was observed in the hottest and driest seasons (spring and summer) compared to the wettest and coldest seasons (winter and autumn). This effect was more pronounced in the case of influents. This result may be due to various factors such as i) the lower use of drugs used in seasonal diseases, ii) the decrease in the population in large cities during holiday

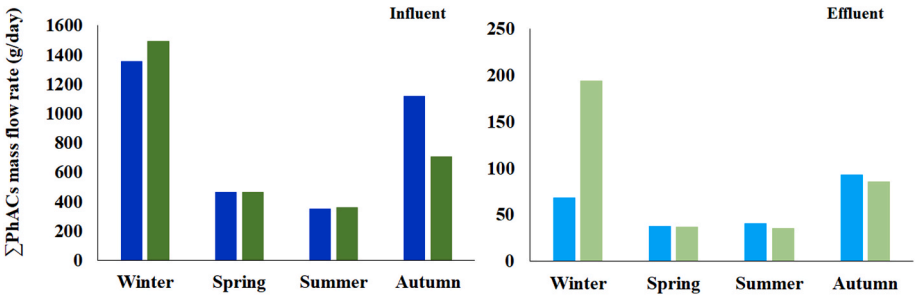

Fig. 2. Seasonal variation of the total load of pharmaceutical residues (g/day) in WWTP 1 (blue) and WWTP 2 (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

periods, or iii) a higher rate of environmental removal due to higher temperatures and longer daylight hours. This trend, although less pronounced, was also observed in effluents and may result from a higher rate of removal in WWTPs due to the effect of rising temperatures (Kosma et al., 2020).

To investigate the influence of WWTP discharges in the river surface water, our previously published data (Royano et al., 2023) obtained at downstream and upstream WWTPs was incorporated. Positive association ($r_s = 0.739$, $p < 0.01$; Table S14) were found between ∑PhACs in WWTP effluents and river water and PhAC concentrations downstream WWTPs were of the same order of magnitude than those quantified in the effluents of the WWTPs (Fig. 3). In addition, both matrices presented also a similar PhACs pattern. Irbesartan, valsartan and *o*-desmethylvenlafaxine were the most concentrated PhACs in WWTP effluents and surface water and the three compounds not detected in the formers (atorvastatin, clotrimazole and miconazole) were not observed in the river water either. However, it must be mentioned that ∑PhAC levels in surface water collected upstream WWTP 1 were comparable to those quantified after effluent discharge, which highlights that at this point, the Tagus River has already a high anthropogenic load and has received discharges from highly populated cities. Therefore, the effluents from the WWTPs studied represent an increase in the pollution of the area studied. Nevertheless, data should be studied with caution since important differences could be observed when PhAC profiles were calculated for each matrix. Interestingly, irbesartan presented a higher contribution to ∑PhACs (43% and 44%; WWTP1 and WWTP2; Fig. S3) in the effluent compared to the surface water collected downstream discharges (10% and 13%). This fact could suggest a dilution behavior however, pKa (4.1 and 4.3; Table S1) and $LogK_{OC}$ (6.11) values indicate that irbesartan will only exist partially in the cation form and it is expected to adsorb to suspended solids and sediments decreasing its contribution to ∑PhACs in surface waters.

3.1.2. *Sludge line*

A total of 16 PhACs were quantified above LOQs in sewage sludge samples (Table S15). Samples presented between 6 and 14 out of 23 of the target analytes. Clotrimazole was quantified in all sludge collected before the digester from WWTP 1 with the highest median concentration (127 ng/g) followed by acetaminophen (109 ng/g, 50%; median, Qf), irbesartan (90 ng/g, 100%), and miconazole (47 ng/g, 75%). In the case of WWTP 2 acetaminophen (213 ng/g, 50%), irbesartan (178 ng/g, 100%), and ibuprofen (174 ng/g, 50%) were the most concentrated contaminants and clotrimazole and miconazole were presented in more than 85% of the samples with median concentrations of 63 ng/g and 39 ng/g respectively.

As expected, PhACs with great tendency to sorption in the organic material ($logK_{OC} > 2.5$, Kearney et al., 1997) such as azithromycin, irbesartan, *o*-desmethylvenlafaxine, gemfibrozil, ibuprofen, naproxen, thiabendazole, and valsartan were partially retained in sludge samples. The fungicides clotrimazole and miconazole that possess two of the highest $logK_{OC}$ (6.43 and 5.74; Table S1) and a strong hydrophobic character ($logK_{OW} > 6$, Table S1) were frequently quantified in the sludge showing no tendency to mobilize towards the aqueous phase. Lindberg et al. (2010) studied the presence of antimycotics in water and

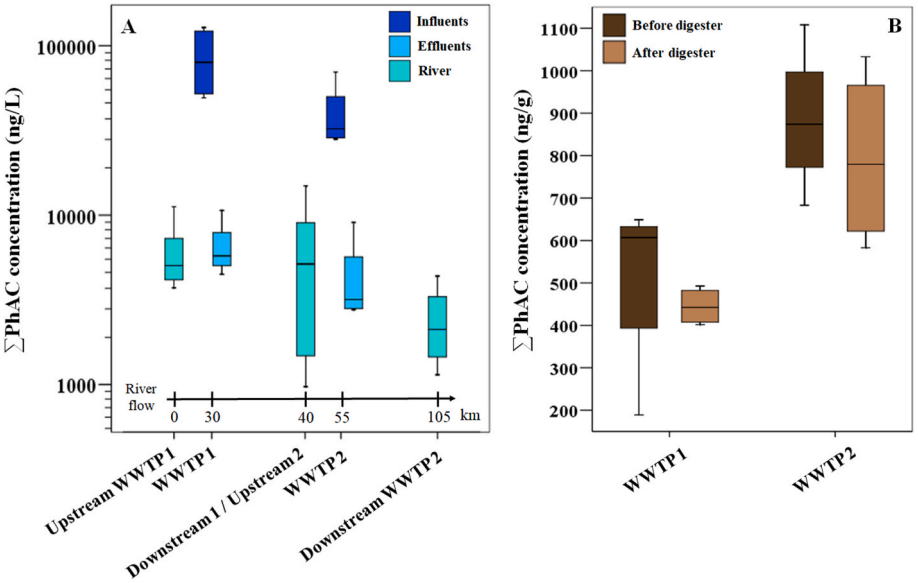

Fig. 3. Total PhAC concentration in (ng/L, logarithmic scale) (A) influents, effluents and Tagus River surface water upstream and downstream of the WWTPs, and (B) sludge before and after treatment in the anaerobic digesters (ng/g). Surface water data (river) were obtained for the same dates (Royano et al., 2023).

sewage sludge from Swedish WWTPs and, as in the present study, clotrimazole and miconazole were found in the sludge samples. On the contrary, fluconazole was not quantified in solid samples but was detected in the aqueous ones despite having a similar structure to miconazole. The alcohol group attached to its central carbon atom gives it greater polarity and therefore greater solubility in water (Table S1). Other PhACs with high water solubility such as acetaminophen (30 μg/mL) were detected (median: 165 ng/g, Fig. 1). However, collected sludge entering the digester presented a high water content (75–97%; Table S2) and therefore, more soluble compounds presented in the aqueous phase were quantified despite not being actually retained in the sludge. A clear positive correlation ($r_s = 0.723$, $p < 0.01$; Table S15) was obtained between PhACs level in the WWTP influents and the sludge collected before the digesters, evidencing that some PhACs are transferred from the water to the sludge line. After digestion the sludge water content decreased (70% drier) and correlation of ∑PhACs between WWTP effluent and sludge disappear (Table S14). According to Directive 91/271/EEC (European Commission, 1991), WWTPs exceeding 10,000 e.h. may discharge up to 35 mg/L suspended solids and therefore their effluents could incorporate PhACs bound to particulate, like clotrimazole and miconazole, reaching the river ecosystem.

Contrary to the case of the wastewater line, no statistically significant differences ($p > 0.05$, Mann Whitney H test) were found between median concentration for sludge collected before and after the digester in any of the WWTPs (Fig. 3) suggesting that the applied anaerobic treatment did not eliminate PhACs. Even so, considering the mass flow rates of effluent and sludge (Table S12), it appears that the treatments carried out in the plants were indeed successful in removing part of the PhACs present in the influent as the sum of the mass in effluent and sludge is lower than the mass in the influent.

Unlike the case of wastewater, no temporal variation was detected in the total load of pharmaceutical residues in the sewage sludge, either before or after the anaerobic digester (Fig. 4).

3.2. PhACs occurrence in Tagus Rives basin

3.2.1. Sediments

In sediment samples only 12 out of 23 target PhACs were quantified in at least one sample with individual concentrations ranging from 1.1 ng/g to 95 ng/g. Antidepressants presented the highest median concentration (47 ng/g for *o*-desmethylvenlafaxine and 24 ng/g for venlafaxine, Table S16) with Qfs above 68%, followed by fungicide clotrimazole (21 ng/g, 61%) and four antibiotics (clarithromycin, 15 ng/g, 61%; azithromycin, 14 ng/g, 61%; erythromycin, 14 ng/g, 11%; trimethoprim, 10 ng/g, 11%). It is not strange, that acetaminophen was only detected in one sediment sample, since this PhAC presented a much higher solubility in water than the rest of the target analytes (Table S1). Clotrimazole and miconazole were not detected in any surface water sample (Royano et al., 2023) but they were quantified in more than 57% of the sediments. In addition to their high tendency to bind to organic matter, river water pH keep both molecules deprotonated, decreasing their solubility in water and favouring the adsorption in sediments (Peschka et al., 2007).

In the case of antibiotics (azithromycin, clarithromycin, erythromycin A or trimethoprim) and antidepressants (*o*-desmethylvenlafaxine and venlafaxine) their pKa values indicate that in the aquatic environment they are found mainly in their cationic form, favouring the electrostatic interactions with the negatively charged sites present in the sediments. Similar results have been found for antibiotics in other regions with low quantification frequencies and concentrations ranging from <LOQs to tens of ng/g (Table S17). On the other hand, PhACs with low pKa values, such as gemfibrozil, ibuprofen, ketoprofen, naproxen or valsartan, are negatively charged at pH 5–9 and despite being relatively hydrophobic PhACs ($logK_{OW}>3$) were not found in the sediments. As happened in the sewage sludge, more polar compounds such as atenolol, carbamazepine, metoprolol or sulfamethoxazole were not detected or quantified at very low concentrations in sediments because of their higher water solubility and lower $logK_{OC}$ values. Those PhACs with a moderate or high hydrophobic character ($logK_{OW}>2.5$, Rogers, 1996), such as azithromycin, irbesartan, venlafaxine, clarithromycin or anhydroerythromycin, were quantified in more than 50% of the sediment samples.

Sediment-water partition coefficients (K_D) ranged between 1.3 L/kg (fluconazole) to 3400 L/kg (miconazole) (Table S18). Because clotrimazole and miconazole were not quantified in surface water, the K_D value could not be estimated according to Eq. (1). However, since their concentration in more than 50% of the sediments analyzed was above the LOQ, it was investigated and calculated using the Guidance on information requirements and Chemical Safety Assessment (ECHA, 2016a). In this way, these two fungicides and the macrolide antibiotics obtained the highest K_D values (miconazole: 3400 L/kg, erythromycin: 3300 L/kg; clotrimazole: 3050 L/kg, azithromycin: 517 L/kg; clarithromycin: 724 L/kg), coinciding with the values calculated in other studies (Li et al., 2018) and indicating their strong sorption affinity to the non-aqueous phase (Li et al., 2019). In contrast, fluconazole being one of the target compounds with the greatest solubility in water, presented the lowest value (1.3 L/kg). To investigate the potential relationship between the different aquatic matrices (sediments, fish and surface water), a correlation matrix was studied (Table S19; only data obtained at the same sampling points and dates were considered). Results revealed a positive relationship between the concentration in river water and sediments for antidepressants (*o*-desmethylvenlafaxine: $r_s = 0.661$, $p < 0.01$; venlafaxine: $r_s = 0.523$, $p < 0.05$) and antibiotics (azithromycin: 0.557, $p < 0.05$; clarithromycin: $r_s = 0.589$, $p < 0.05$), confirming the tendency of these compounds to transfer to the sediment from the aqueous phase slightly mitigating the contamination of the surface water.

The geographic distribution and temporal trends of sediment concentrations were explored and the results revealed that the most densely populated areas (red in Fig. 5) aroused higher pollution levels ($p < 0.05$ Kruskal-Wallis H test) than areas with less population, which identifies proximity to urban centres as the main contamination source. Moreover,

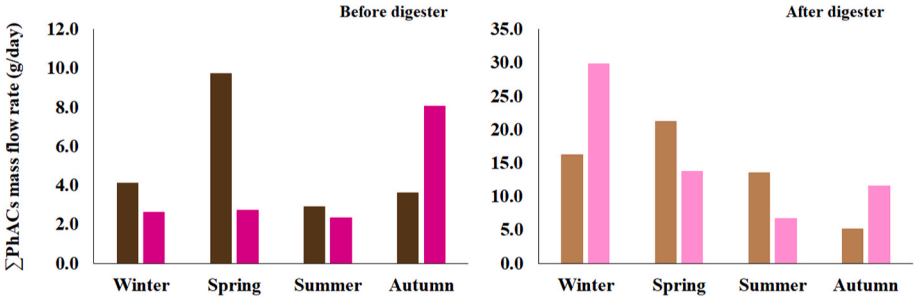

Fig. 4. Seasonal variation of the total load of pharmaceutical residues (g/day) in sewage sludge from WWTP 1 (brown) and WWTP 2 (pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

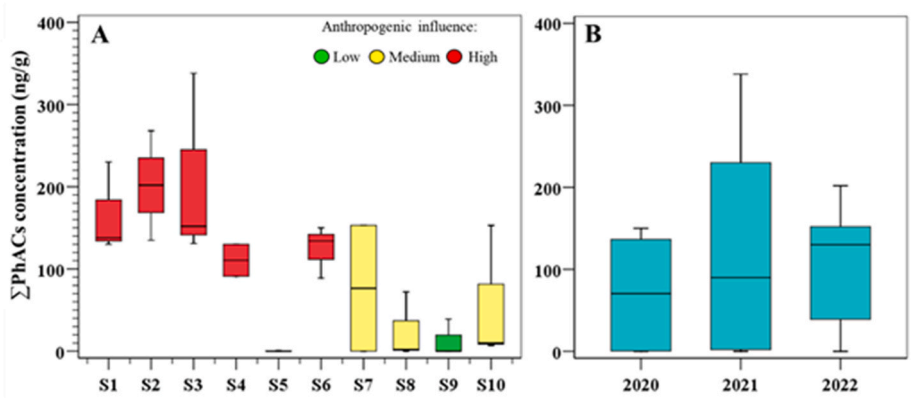

Fig. 5. Box and whisker plot of the total PhACs concentration (ng/g) in sediments at (A) each sampling point and at (B) different sampling years.

no temporal trends ($p > 0.05$, Kruskal Wallis H test) were found between sediments collected in 2020, 2021 and 2022 (Fig. 5), which seem to reflect the lack of variation in the river flow rate and changes in the population load of the area. However, this result should be corroborated in the future with a longer time series.

3.2.2. Fish

Despite their high quantification frequency in surface water, only 2 out of 23 target PhACs, were quantified in fish samples. Azithromycin was only quantified in one sample at 160 ng/g and clotrimazole was found in four samples at levels ranging between 5.8 and 9.5 ng/g (Table S16). Bioaccumulation factor of clotrimazole (BAF $3.9x10^5$ L/kg, Table S1) suggests that it has high potential to accumulate in aquatic organism (Gómez-Regalado et al., 2023; Government of CanadaMinister of Justice, 2000) and is in line with the criteria of the Regulation of the Registration, Evaluation, Authorization and Restriction of Chemical Substances (REACH) which indicates that substances with a bioconcentration factors (BCF) greater than 2000 are considered bioaccumulative (European Parliament, 2006) ($BCF_{clotrimazole}$ 6297 L/kg, U. S. EPA, 2012). Furthermore, fish samples that presented this compound were taken in locations with high anthropogenic influence (S2, S3, S4 and S6) and therefore with a higher level of contamination. Other studies (Carmona et al., 2017; Huerta et al., 2013) carried out on whole fish obtained similar results with low detection frequencies and concentrations from 5 ng/g (carbamazepine) to 32 ng/g (metoprolol) (Table S20). The fat-soluble nature of some PhACs may suggest that they tend to accumulate in fatty tissues and that is why most of the PhACs were detected in the brain, liver or kidney compared to the muscle or plasma (Table S20). However, in accordance with the requirements for monitoring contaminants in biota (fish) of the Ministry for the Ecological Transition and the Demographic Challenge (Ministry for the Ecological Transition and the Demographic Challenge, 2022b), the analysis in the laboratory must be carried out on the entire organism and on a pool of organisms, not separating the organs or mixing different species. Therefore, it is interesting to obtain the concentration in whole fish to facilitate comparison between studies and produce data useful to investigate the risk to secondary predators.

3.3. Environmental implications

The use of contaminated sewage sludge as agricultural amendments could introduce these pollutants into the soil where they can reach other environmental compartments such as crops, groundwater or terrestrial organisms such as earthworms (Montemurro et al., 2021) especially in soils with low organic matter content (García-Delgado et al., 2023). For RCRs estimation, the PEC values for soil and earthworm after one year sludge application were calculated (Tables S21 and S22). Although most of the target compounds can degrade and their half-lifes are approximately one year, some of them may remain in the environment for longer and could accumulate year after year throughout the amendment (Walters et al., 2010). In order not to disregard the long-term effects that these more persistent PhACs can cause on soil organisms, the PEC values after 10 and 25 years of annual amendment were evaluated. As expected, PhACs with the highest $logK_{OC}$ values such as clotrimazole, miconazole and irbesartan reached the highest PEC_{soil} levels due to their high tendency to remain in the soil, but none of these PhACs appeared to pose a risk to terrestrial organisms because their RCR_{soil} values were below 1 (Table S23). On the other hand, the RCRs could suggest a high risk for gemfibrozil, ibuprofen, and venlafaxine since the first year and for naproxen from year 10.

Regarding the PEC calculated in earthworms, gemfibrozil presented the highest PEC levels indicating possible risk to terrestrial organisms and their predators. Unfortunately, RCRs estimation for this compartment is hampered by the lack of ecotoxicológical information for worm-eating birds or mammals. However, given the possibility that PhACs reach terrestrial organisms, it is important to continue carrying out studies which evaluate the environmental risk that pharmaceutical compounds may pose to the terrestrial ecosystem.

In the freshwater ecosystem, the proposal for amending the current Water Framework Directive (European Commission, 2022b) establishes the EQSs as the annual average (AA) and maximum admissible concentration (MAC) for the substances proposed to enter the List of Priority Substances. It is the first time that pharmaceutical substances and their corresponding EQSs have been considered in this proposal, and interestingly 5 out of 9 PhACs included have been investigated in the present study. The reliable results obtained with the validated methodology presented in this work could therefore be useful in the advancement of this regulation. Although the WFD refers to surface water, the PhAC concentration in the effluent were lower than the established EQSs except for azithromycin (max: 110 ng/L) and ibuprofen (max: 458 ng/L). However, to evaluate the environmental impact of these pharmaceuticals on the aquatic ecosystem, $PNEC_{sediment}$ (Table S24) and RCRs (Table S25) for target PhACs were calculated at each location. The RCRs obtained in sediments indicated high risk for some of the compounds. In both the median and the worst scenario, phototrophic organisms turned out to be the organisms with the highest number of RCRs above 1 (acetaminophen, clarithromycin, erythromycin, and venlafaxine). Crustaceans did not obtain high risk for any compound in the median scenario and only anhydroerythromycin obtained high risk in one sampling location (S6) in the worst scenario. In the case of fish, two of the most water-soluble PhACs (acetaminophen and venlafaxine) presented high risk in both scenarios. Algae seem to be the organisms most threatened by sediment contamination since they grow in this compartment, while fish and crustaceans are more affected by the aqueous phase. Considering the total RCR as the sum of the RQs in water and RCRs sediments (Table S26), irbesartan was the only PhAC with

high risk for the three trophic levels studied. In the median scenario, 10 PhACs (acetaminophen, carbamazepine, clarithromycin, erythromycin, gemfibrozil, ibuprofen, irbesartan, ketoprofen, naproxen, and venlafaxine) posed a high risk to at least one organism in at least one sampling site. In the worst scenario, the number of compounds was greater, adding to those already mentioned azithromycin, anhydroerythromycin, fluconazole, and metoprolol. According to the field study conducted by Sun et al. (2021), the accumulation of PhACs depends on the ionic form of the compounds, and since most of the compounds studied are presented in ionic form, they are less bioavailable than if they were in their neutral form. Sun et al. (2021) concluded that lower organisms can incorporate these compounds from water, but metabolic decomposition throughout the food chain may contribute to the fact that fewer PhAC residues were found in higher organisms. This could explain why in this study few PhACs with low quantification frequencies and concentrations were found. Nonetheless, the bioaccumulation of drugs in fish should not be neglected, because although in small concentrations, Corcoran et al. (2014) demonstrated a certain persistence of some PhACs such as clotrimazole by observing that it was eliminated from the plasma after a period of purification but maintained appreciable levels in the liver.

Risk assessment was conducted according to the methodology proposed by ECHA facilitating the comparison of results. Nevertheless, it must be mentioned that the equilibrium partitioning method used for environmental risk assessment in sediments and soil compartments can lead to risk overestimation and these PhACs should be considered as substances requiring further testing on soil organisms and sediment-dwelling organisms.

4. Conclusions

This research is a very exhaustive study on the presence and transfer of PhACs in WWTPs and the freshwater ecosystem. In the WWTPs studied, analgesics, anti-inflammatories, antihypertensives, antidepressants and beta-blockers turned out to be the majority drugs in influents. Antihypertensives and antidepressants were also the main PhACs in the effluent with a contribution of up to 74% of the ∑PhACs. Acetaminophen, ibuprofen, ketoprofen, naproxen, atorvastatin, azithromycin, clarithromycin, sulfamethoxazole, trimethoprim, and valsartan obtained removal efficiencies above 75%, demonstrating that the WWTP treatments can degrade these compounds. PhACs with greater affinity to organic matter such as clotrimazole and miconazole were quantified in 85% of the sludge evidencing its transfer to this compartment, but results manifested that the treatments applied to the sludge were not effective in eliminating PhACs. The total mass balance of the effluent and sludge revealed that WWTPs are capable of degrading part of the PhACs but some are still present in the effluent and reach the river, becoming the main source of PhACs contamination of the freshwater ecosystem. Among all the drugs released into river water, those with greater hydrophobicity (e.g. irbesartan), positively charged (e.g. *o*-demethylvenlafaxine) or likely to bind to organic matter (e.g. clotrimazole and miconazole), were quantified in more than 57% of the sediments. However, only clotrimazole and azithromycin were found in fish. Risk assessment revealed that algae are the most threatened organisms by sediment contamination. The results obtained in this work could help to better understand the behaviour of these contaminants in other water bodies and ecosystems worldwide. Besides, information related to the transfer pathways and fate of pharmaceutical products in WWTPs could help to optimize and improve current technologies to achieve the complete elimination of these residues. The conclusions reached could have a global impact by contributing to the development and implementation of international policies related to the protection of water quality and both public and environmental health.

CRediT authorship contribution statement

Silvia Royano: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Irene Navarro:** Writing – review & editing, Validation, Methodology, Investigation. **Adrián de la Torre:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **María Ángeles Martínez:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143759.

Data availability

Data will be made available on request.

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