



Pharmaceutical footprint in the river ecosystem: Suspect screening approach with high resolution mass spectrometry

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ABSTRACT

Pharmaceutically active compounds (PhACs) are recognized as important emerging pollutants in aquatic environments. Despite growing concern, an increasing number of new pharmaceuticals are being authorized each year, reaching the environment and compromising the quality and health of ecosystems. However, comprehensive multi-matrix investigations integrating water, sediments, biota, and wastewater systems remain scarce. In this study, a comprehensive HRMS-based suspect screening workflow was implemented using ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS). Through a multi-compartment approach involving water ($n = 89$), sediments ($n = 28$), fish ($n = 24$), wastewater ($n = 16$), and sewage sludge ($n = 16$) matrices collected during 2020–2022, this study identified 153 PhACs (136 active ingredients and 17 metabolites or transformation products). Cardiovascular, psychotropic, pain management, and other nervous system drugs were the dominant therapeutic families, accounting for 63% of all identifications. Surface water contained the highest number of compounds (122), followed by effluents (85). Although influents presented fewer compounds, they showed significantly higher total input ($p < 0.05$). Solid matrices such as sediments and sludge accumulated highly hydrophobic substances (e.g., telmisartan, citalopram), whereas aqueous matrices contained more polar compounds (e.g., tramadol) and metabolites. Fish exhibited the lowest number of PhACs (11). Regarding spatial distribution, sites more strongly influenced by human activities displayed higher ($p < 0.05$) normalized areas and number of compounds. Overall, this study provides one of the most comprehensive multi matrix suspect screening assessments of PhACs to date. The holistic evaluation of the riverine ecosystem highlights the value of HRMS based workflows for identifying and prioritizing emerging contaminants, supporting the development of regulatory strategies to control pharmaceutical pollution and enabling early detection of newly marketed drugs.

1. Introduction

Pharmaceutically active compounds (PhACs) represent one of the most relevant groups of emerging contaminants in aquatic environments. They have played a fundamental role in extending human life expectancy and improving quality of life through the prevention, control, and treatment of numerous diseases. However, in contrast to other chemical substances such as pesticides, PhACs are not subject to comprehensive environmental risk assessment requirements prior to market approval (European Parliament, 2025). As a result, their release into natural ecosystems can generate unintended consequences for non-target organisms and may alter ecological processes at different

trophic levels (Kergoat et al., 2021; Oaks et al., 2004), ultimately threatening environmental sustainability. Despite these concerns, there is still limited understanding of the real extent, distribution, and environmental behavior of PhACs across interconnected aquatic compartments.

Pharmaceutical residues can enter the environment at multiple stages of their life cycle, from production to disposal. Nevertheless, the dominant route of release is associated with human and veterinary consumption. Most of the administered dose, whether in its original or metabolized form, is excreted through the kidneys or liver (Talevi and Bellera, 2024; Yin and Wang, 2016). These residues reach wastewater treatment plants (WWTPs), which operating mechanism was not

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originally designed to effectively remove such compounds. Consequently, their effluents contain a broad spectrum of PhACs that are discharged into receiving rivers. The magnitude of this problem is amplified by the global rise in pharmaceutical consumption, which has been increasing steadily over recent decades and is projected to continue expanding at a rate of 2.3% per year worldwide until 2028 (IQVIA, 2024). Once in aquatic ecosystems, PhACs may exert a variety of adverse effects. Antibiotics, for example, can contribute to the spread of antibiotic-resistant bacteria, posing a serious threat to both ecosystem and human health (European Commission, 2017). Antidepressants and psychoactive drugs may alter fish or crayfish behavior, causing increased aggression, greater exposure to risky situations, and decreased access to food (Hubená et al., 2021; Klaminder et al., 2019; Reisinger et al., 2021; Wilson et al., 2023). Hormones are well known for their potential to induce endocrine disruption, promoting the feminization of certain species, altering their development, and leading to population declines in aquatic organisms (Bizarro et al., 2014; Jobling et al., 2006; Teal et al., 2023). These findings reinforce the urgency of continuously monitoring and assessing the presence of pharmaceutical products in riverine ecosystem. In response to these environmental risks, the Water Framework Directive (WFD) has incorporated various groups of drugs into its successive Watch Lists, including antibiotics, anti-inflammatories, antidepressants, beta-blockers, hormones, anti-fungals, and antidiabetics. The list currently in force, approved in 2025, includes the largest number of drugs to date, with a total of 11 PhACs (European Commission, 2025). In addition, nine pharmaceutical substances have been proposed for inclusion in the List of Priority Substances and for the establishment of Environmental Quality Standards (European Commission, 2022), highlighting the need of investigating and prioritizing these compounds in aquatic ecosystems. Yet, regulatory monitoring frameworks still lack comprehensive analytical tools capable of capturing the full diversity of pharmaceuticals and their transformation products present in the environment.

To meet regulatory requirements and monitor these hazardous compounds, the analysis of PhACs in the environment has traditionally relied on targeted HPLC-MS/MS methods (An et al., 2022; Castaño-Ortiz et al., 2024b; Ebele et al., 2020; Köck-Schulmeyer et al., 2021; Kondor et al., 2022; Mastrángelo et al., 2022; Ramírez-Morales et al., 2021), which quantify specific compounds based on a preselected list and availability of reference standards. While accurate, these approaches are inherently limited, particularly as new pharmaceuticals continuously enter the market, creating a temporal gap in monitoring capacity and regulatory responses. To update regulations more effectively, analytical strategies capable of detecting a broader range of pharmaceutical compounds are needed.

In recent years, scientific efforts have therefore focused on developing non-target and suspect screening strategies. These methodologies enable the identification of a wide variety of compounds without requiring commercial standards, thereby greatly expanding the scope of environmental monitoring. In this regard, advances in high-resolution mass spectrometry (HRMS) have facilitated the characterization of a broad spectrum of chemicals with different levels of confidence (Schymanski et al., 2014) such as pesticides (Fernández-Fernández et al., 2025; Selwe et al., 2024; Serasinghe et al., 2022), per- and poly-fluoroalkyl substances (PFAS) (Ng et al., 2022), phthalates (Selwe et al., 2024) or tire-related compounds (Escobar-Arnanz et al., 2026). These approaches typically generate complex datasets that require substantial processing time and expert interpretation. In this sense, prioritization and data reduction procedures, often based on comparison with library spectra (Schymanski et al., 2014), are valuable tools for optimizing data processing and improving the screening of environmental contaminants (Zweigle et al., 2025). Besides, HRMS based methods offer notable advantages, including the ability to conduct retrospective analyses (Assres et al., 2019), perform qualitative comparisons across different matrices (Chen et al., 2025), and to prioritize newly identified compounds that may pose potential risks to ecosystem health (Tarábek et al., 2024).

Nevertheless, comprehensive multi-matrix studies applying harmonized HRMS workflows remain scarce, limiting our ability to compare contamination patterns across compartments and fully understand the environmental fate of pharmaceuticals.

To overcome methodological limitations, and to reduce the time required to identify both known and new pharmaceuticals in the aquatic ecosystem, this study established a comprehensive workflow through a multi-compartment approach based on suspect screening using ultra-performance liquid chromatography coupled with quadrupole time-of-flight high-resolution mass spectrometry (UPLC-QTOF HRMS). The methodology incorporates systematic data processing strategies, applicable to abiotic matrices (surface water, sediments, wastewater, and sludge) and biotic samples (fish) facilitating the identification of a wide range of pharmaceuticals in the studied aquatic ecosystem. By combining advanced analytical techniques with a comprehensive sampling design, this study strengthens our understanding of the environmental fate, transfer and pharmaceutical burden in river ecosystems. This enables accelerated environmental assessment and contributes to the design of more effective environmental policies.

2. Material and methods

2.1. Study area and sample collection

This study was conducted in the Tagus River basin, the third largest basin on the Iberian Peninsula. The basin is characterized by a strongly continental Mediterranean climate, with hot, dry summers and cold winters. Rainfall is seasonally distributed, with spring and fall being the wettest periods. To compensate for drier periods, the basin has a high flow-regulation capacity, enabling it to store up to 20% of its overall volume (Cátedra del Tajo, 2024). The basin originates in the central region of the peninsula, where the Tagus River, the main water source, and its tributaries cross areas with intensive livestock activity and densely populated urban centers such as the Madrid metropolitan area (3.3 million inhabitants, INE, 2025a) and Toledo (0.71 million inhabitants, INE, 2025b). This combination produces a significant anthropogenic impact that can lead to the release of numerous PhACs from both human and veterinary origin.

To assess all potential emission sources, surface water, sediment, fish, wastewater and sewage sludge samples were collected at multiple sites across the basin (Fig. S1). A total of 89 surface water samples were collected in polypropylene bottles from 13 sampling points distributed along the basin's main rivers. These sites were classified according to their anthropogenic influence (high, medium, or low) based on population density, number and capacity of WWTPs, land use, and presence of livestock industries (Table S1). Surface water samples were taken annually during 2020, 2021, and 2022. In addition, during 2022, quarterly sampling was carried out in winter, spring, summer, and autumn, with the aim of evaluating temporal trends and seasonality. Sediment ($n = 28$) and fish ($n = 24$) samples were collected annually at 10 of the 13 sites using dredging and electrofishing, respectively.

Wastewater and sewage sludge samples were obtained from two WWTPs located upstream (WWTP 1) and downstream (WWTP 2) of the city of Toledo. WWTP 1 collects both industrial and urban wastewater, while WWTP 2 receives mainly urban effluents. Treatment processes used in the WWTPs are detailed in Text S1. These facilities are representative of the anthropogenic load affecting the studied basin, and their locations (Fig. S1) allow to correlate the contribution of their effluents with the results observed at the surrounding sampling points (S7 - S10), as no major discharges occur between them (MITECO, 2025). A total of eight influent samples were collected after the desanding step, while eight effluent samples were taken at the WWTP outlet, representing the discharge point to the receiving environment. Simultaneously, sludge samples were collected in amber glass bottles before ($n = 8$) and after ($n = 8$) the anaerobic digestion step. Once in the laboratory, all samples were stored at -20°C until analysis.

2.2. Sample preparation

All samples were processed following validated methods described in previous studies (Royano et al., 2023; Royano et al., 2024a; Royano et al., 2024b). Water samples from surface water and WWTPs were filtered with glass fiber filters under negative pressure, spiked with 50 ng of deuterated internal standards (acetaminophen-d3, atenolol-d7, gemfibrozil-d6, ibuprofen-d3, sulfamethoxazole-d4, and venlafaxine-d6; Text S2) and extracted by solid-phase extraction (SPE). Briefly, Oasis HLB cartridges (6 mL, 500 mg; Waters, Milford, MA, USA) were preconditioned with 10 mL of methanol and 10 mL of Milli-Q water. Then, 1 L of surface water or 0.5 L of wastewater was loaded onto the cartridge and let dry for 1 h. Afterwards, analytes were eluted with 10 mL of methanol and concentrated to 300 μ L under a gentle nitrogen stream.

Sediment and sewage sludge were dried at room temperature, spiked with the deuterated internal standards, and extracted using a QuEChERS method. Concisely, 1 g of sample was treated with 2 mL of Milli-Q water and 4 mL of acetonitrile (1% acetic acid) and shaken for 30 min. Then, QuEChERS salts (0.8 g of magnesium sulfate and 0.2 g of sodium acetate) were added and the mixture was immediately vortexed and centrifuged (5 min, 5000 rpm). The supernatant was concentrated to 1 mL and filtered through 0.45 μ m polytetrafluoroethylene vial filter.

Freeze-dried pooled fish samples (1 g) were spiked with deuterated internal standards and extracted with 7 mL of acetate buffer (pH 4)/acetonitrile (50:50, v/v). Then, the mixture was shaken for 30 min and centrifuged (5 min, 5000 rpm). The supernatant was diluted to 500 mL in Milli-Q water before a SPE cleanup step, using Oasis HLB cartridges (6 mL, 200 mg; Waters) preconditioned with 5 mL of methanol and 5 mL of Milli-Q water. After loading the samples, the cartridges were washed with 6 mL of Milli-Q water and dried for 30 min. Finally, analytes were eluted with 6 mL of methanol and the extract was concentrated to 1 mL.

2.3. Instrumental analysis

Samples were retrospectively analyzed by ultra-high performance liquid chromatography (UPLC) coupled to HRMS. Chromatographic separation was carried out using a ExionLCTM AC system (SCIEX, MA, USA) equipped with a C18 SecurityGuard cartridge (2.1 $\times$ 4.6 mm) and a Luna[®] Omega C18 column (1.6 μ m, 100 $\text{\AA}$, 100 $\times$ 2.1 mm i.d.; Phenomenex, CA, USA). The composition of the mobile phase and the chromatographic conditions are detailed in Table S2.

The chromatographic system was coupled to a quadrupole time-of-flight mass spectrometer (X500R QTOF MS/MS system; SCIEX), provided with a Turbo VTM ion source with electrospray ionization (ESI) interface. Mass spectra were acquired in positive ionization mode (ESI⁺) using data-independent acquisition (DIA) mode. TOF-MS spectra were obtained in the range of 100–1100 Da, while TOF-MS/MS scans were carried out between 40 and 1100 Da distributed across 23 windows. Collision energy (CE) was set at 35 V with a spread of ± 15 V. All operating conditions for the mass spectrometer are listed in Table S3.

2.4. Data processing

Both the acquisition and processing of chromatograms and mass spectra were performed using SCIEX OS software (version 3.1.6.44). A processing method for the screening of suspect contaminants based on MS/MS library spectral match was developed. The libraries used were All-in-one HR-MS/MS version 2.0 and NIST 17 MS/MS Spectral Library, resulting in a suspect list of 2056 compounds, including active substances and metabolites. Each suspect was defined by its name, monoisotopic mass, and molecular formula.

Experimental fragment ion spectra (MS/MS) were automatically compared to library spectra, and features which obtained a match of more than 70% were identified with confidence level of 2a, following Schymanski et al. (2014) classification. However, to ensure high

confidence, compounds were considered identified when the most intense chromatographic peak showed a mass error ≤ 5 ppm, isotopic pattern difference $< 20\%$, and $\geq 50\%$ formula prediction agreement (Abafe et al., 2023; Gago-Ferrero et al., 2015; Rico et al., 2019; Wang et al., 2020). Further details about algorithm used in data processing are provided in Text S3.

Finally, a combined score was calculated weighting each identification criterion according to its relevance (Table S4). Peaks with a combined score $\geq 75\%$ were considered identified with a confidence level of 2a.

To optimize compound identification, filtering and prioritization strategies were applied, considering only features with signals at least 10 times higher than blanks (Krauss et al., 2019), peak height $> 10,000$ cps, and peak width at half height ≤ 0.20 Da, thus discarding minor and poorly resolved peaks. Additionally, the identification of a suspect feature was supported when the relative standard deviation (RSD) of the retention time obtained in all samples was below 20%.

To qualitatively estimate compound abundance, normalized peak areas were used since standard solutions were unavailable (Escobar-Arnanz et al., 2026; Rehnstam et al., 2024). Since raw peak areas can vary due to instrumental fluctuations, each suspect feature obtained after blank subtraction was normalized to the mean area of the deuterated internal standards within the same sample (Peter et al., 2022). This approach allows comparison of relative abundances across samples under consistent analytical conditions. Only internal standards present in more than 50% of samples per matrix were used for normalization.

2.5. QA/QC and statistical analysis

To evaluate the analytical space covered in this study, a scatter plot of mass-to-charge ratio (m/z) versus retention time (RT, min) was generated. As shown in Fig. 1, the developed suspect screening methodology enabled the detection of a broad range of compounds, from the most polar and low-molecular-weight species to lipophilic and high-molecular-mass compounds, confirming the suitability of the analytical conditions (m/z range, chromatographic gradient and total analysis time).

In addition, to ensure the quality of the results, concentrations reported in a previous quantitative study (Royano et al., 2023) for 23 compounds were compared with the normalized areas obtained in this work. The strong similarity between the pollutant profiles (Fig. S2) confirms the suitability of the identification criteria and the use of normalized areas for non-targeted pollution assessment.

To assess possible contamination during the analytical process, field blanks were processed in the same way as the samples. In addition, instrumental blanks (methanol or acetonitrile injections), were included before and after each sample in order to evaluate the memory effect.

Statistical analysis was performed using SPSS 31.0 for Windows and Microsoft Excel 2016. Given that most variables did not follow a normal distribution according to the Shapiro–Wilk and Kolmogorov–Smirnov statistical tests ($p < 0.05$), and to maintain methodological consistency in the analyses, non-parametric tests of Kruskal–Wallis and Mann–Whitney were used to analyze the differences in the amount of compounds present in each matrix, the influence of population load, seasonal differences, temporality, and the performance of different WWTPs.

3. Results and discussion

3.1. Identification and environmental distribution of PhACs

After applying the developed instrumental methodology to surface water, sediments, fish, wastewater, and sewage sludge samples, datasets containing up to 1500 features were obtained. In total, 153 compounds were identified at confidence level 2a from the initial list of 2056 suspect substances, including 136 active ingredients and 17 metabolites or TPs.

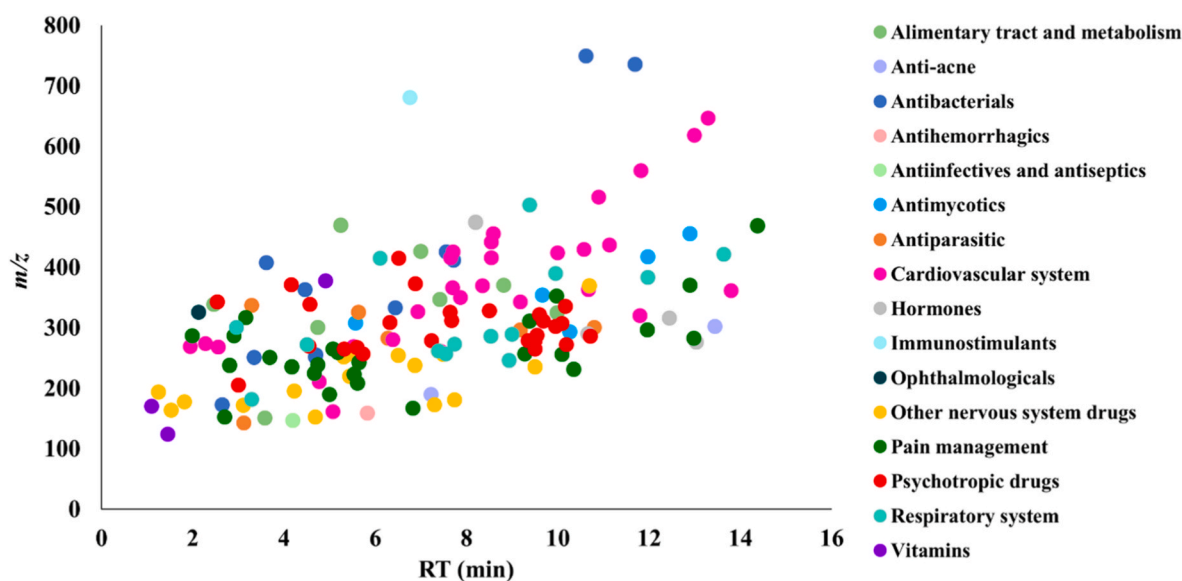


Fig. 1. Scatter plot of the m/z value versus the retention time of the identified compounds. Each dot represents an individual PhAC. Colors indicate different PhAC families. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The identified compounds were grouped into 16 therapeutic categories or families (Table S5).

The therapeutic family with the highest number of identified compounds was cardiovascular drugs ($n = 28$), followed by psychotropic ($n = 26$), pain management ($n = 25$), and other nervous system drugs ($n = 18$), which included antiepileptics, psychoanaleptics (anti-dementia drugs), antiparkinsonian agents, psychostimulants, and others. Similar results were obtained in other studies (Fabregat-Safont et al., 2021; Castaño-Ortiz et al., 2024a). Together, the number of identified compounds of these four families accounted for 63% of the total (Fig. 2). Fig. S3 depicts the number of compounds within all subfamilies in each pharmaceutical class, highlighting antidepressants as the most numerous subfamily (14 PhACs), followed by antibacterials (11) and opioid analgesics (10).

As shown in Fig. 3A, the matrix with the highest number of compounds was surface water, with a total of 122 substances, 49 of which were detected in more than 50% of the samples. The number of compounds per sample in this matrix ranged from 1 to 86 (Fig. 3B). This

variability could be explained by the fact that sites with higher population density exhibited a significantly greater number of identified PhACs ($p < 0.01$, Kruskal–Wallis test) compared to less populated areas, consistent with the findings reported by Tarábek et al. (2024).

Secondly, a total of 85 compounds were identified in the WWTP effluents, with up to 64 compounds detected in a single sample. Interestingly, the WWTP influents contained fewer compounds, with 68 PhACs identified in total and a maximum of 64 detected in a single sample. This phenomenon could be attributed to the deconjugation of metabolites bound to glucuronic acid or sulfates during the treatment process, which may lead to a higher detection of the parent compounds in the effluent compared to the influent. The higher identification frequency in effluents of compounds such as lamotrigine (Gurke et al., 2015), phenazone (Yu et al., 2025), levorphanol (Prommer, 2007), doxylamine (Skov et al., 2023), and temazepam (Styszko et al., 2021) supported this hypothesis.

The physicochemical properties of these substances play a crucial role in their distribution across environmental matrices. Compounds

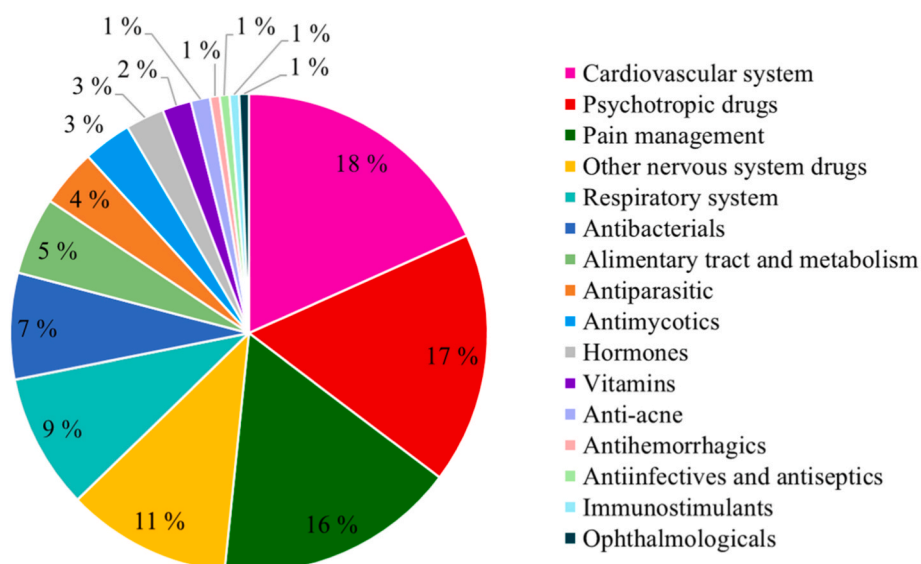


Fig. 2. Contribution by families to the total number of PhACs identified. The families are listed in descending order according to their abundance (percentage).

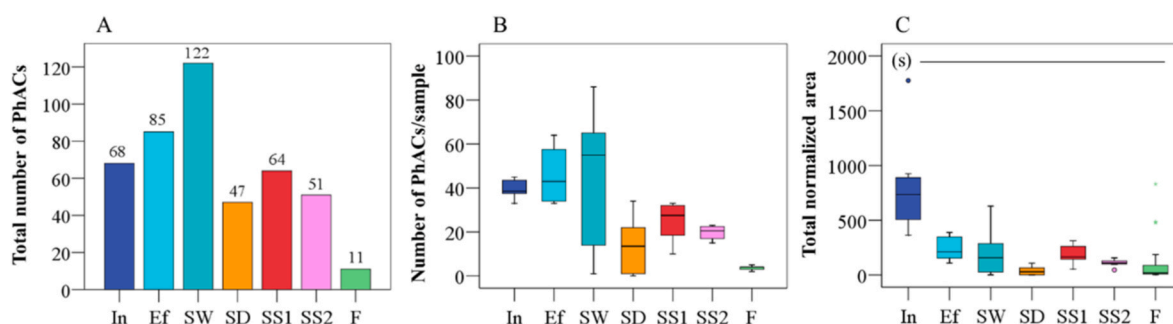


Fig. 3. (A) Total number of PhACs identified in each matrix. (B) Number of PhACs identified per sample. (C) Total normalized area per sample. (s) indicates statistically significant differences. In: influents, Ef: effluents, SW: surface water, SD: sediments, SS1: sewage sludge (before digester), SS2: sewage sludge (after digester), F: fish.

with a higher affinity for organic matter ($\log K_{oc} > 2.5$, Rogers, 1996; e.g., amitriptyline, citalopram, fluoxetine, telmisartan, tramadol; Table S5) were preferentially identified in river sediments and sewage sludge. A total of 47 compounds were detected in sediments, with detection frequencies (Df %) ranging from 4% (e.g., fexofenadine) to 70% (e.g., flecainide, citalopram). Up to 34 compounds were detected in the most contaminated samples (i.e. citalopram, flecainide, telmisartan). Sewage sludge contained 64 compounds before the digestion

process, reaching Df % of 100% for amitriptyline, adenosine, and telmisartan. Although the sludge collected after the anaerobic digester contained fewer compounds (51), no statistically significant differences were observed ($p > 0.05$, Mann–Whitney test) in the number of compounds per sample, indicating that overall contamination levels remained comparable. In some cases, the retention of pharmaceuticals in these solid matrices was so strong that certain compounds were not detected in the aqueous phase (e.g., amiodarone, diphenhydramine,

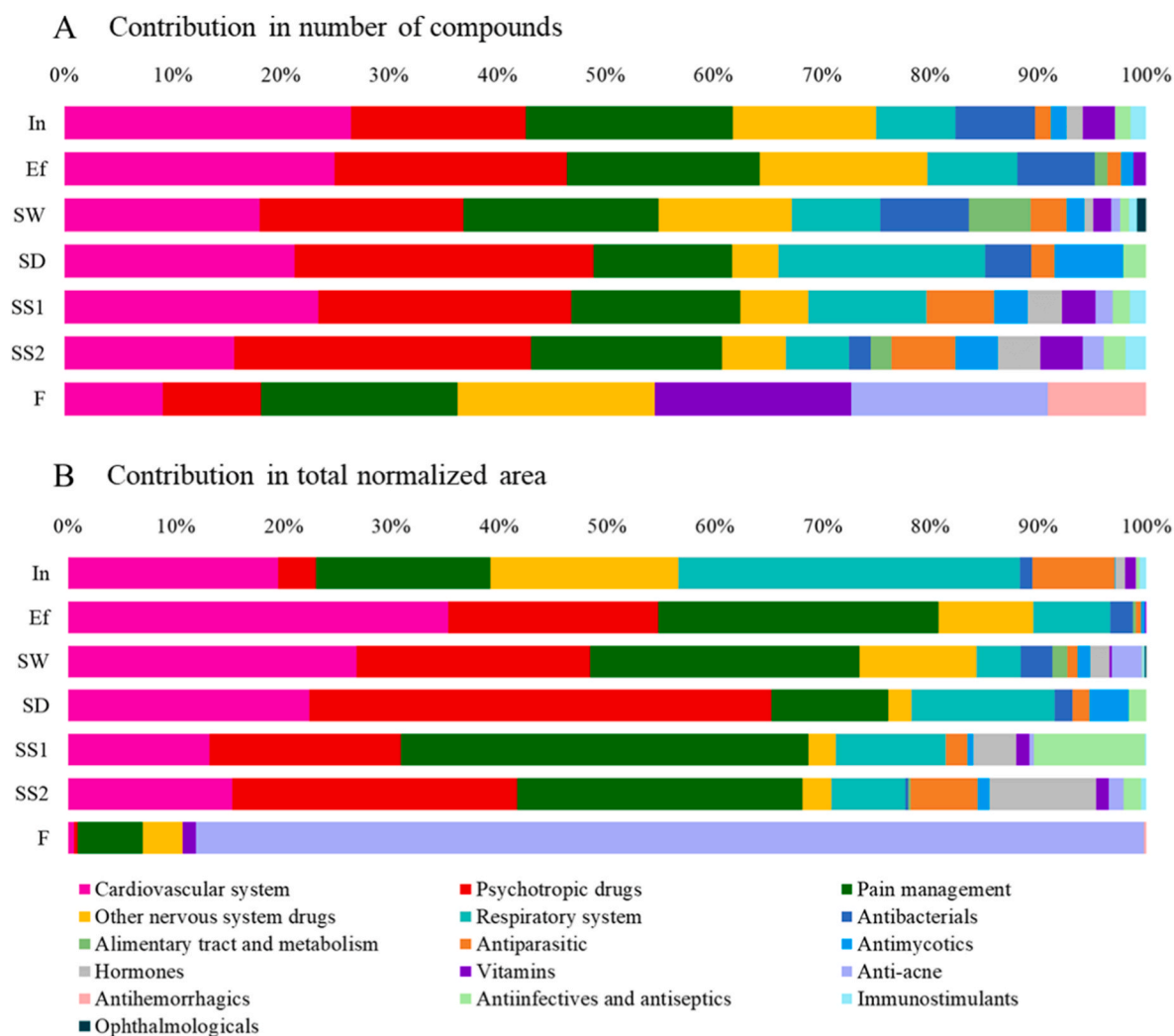


Fig. 4. Contribution of PhAC families to each matrix in terms of (A) number of compounds and (B) normalized area. In: influents, Ef: effluents, SW: surface water, SD: sediments, SS1: sewage sludge (before digester), SS2: sewage sludge (after digester), F: fish.

fenofibrate, fenticonazole, hydroxychloroquine, nortriptyline). This phenomenon may lead to an underestimation of PhAC occurrence in riverine ecosystems when only surface water is considered (Ferreira da Silva et al., 2011).

Lastly, fish represented the matrix with the lowest number of identified PhACs, with a total of 11 compounds and a maximum of five detected per sample. Only azelaic acid, L-tryptophan, and riboflavin were found in more than 50% of the samples.

3.2. Abundance of PhACs in the river ecosystem

The number of PhACs per sample was considered a valid indicator of pollution levels in the study area (Tarábek et al., 2024). However, to estimate the relative abundance of each drug in each matrix, peak areas were normalized, allowing for the identification of the predominant PhACs. Based on both detection frequency and normalized area, drugs for cardiovascular conditions, psychotropic treatments, pain management, other nervous system disorders, and respiratory illness led in almost all matrices (Fig. 4). Interestingly, antibiotics contribution in total normalized area suggested a lower predominance within the studied fluvial ecosystem. Nevertheless, the occurrence of antibiotics remains concerning due to their potential contribution to the spread of antimicrobial resistance (European Commission, 2017). Notably, antibiotics such as clindamycin and ofloxacin are included in the WFD Watch List (European Commission, 2025), while clarithromycin and erythromycin have been proposed for inclusion in the Priority Substances List (European Commission, 2022).

The chemical profile in fish differed from that of the other matrices due to the low number of contaminants observed. Anti-acne pharmaceuticals were predominant in fish, both in number of compounds and in normalized area, with azelaic acid and 13-cis-retinoic acid standing out among them. Azelaic acid is a dicarboxylic acid naturally present in cereals such as barley, wheat, and rye (Kumar et al., 2020), while 13-cis-retinoic acid is an isomer of retinoic acid, an active metabolite of vitamin A (Takahashi et al., 2011). Although both are components of various pharmaceutical formulations for acne and cosmetic products, their occurrence in the environment is likely to be influenced by biological sources as fish themselves can produce these compounds endogenously (Ekman et al., 2015; Gesto et al., 2012; Krupa et al., 2025). This consideration extends to other detected compounds, such as adenosine, which was identified in six out of eight matrices investigated. Its widespread presence is consistent with its role as an endogenous purine nucleoside found in all body cells (AEMPS, 2023), and therefore its ubiquity in the ecosystem is reasonable. Similarly, cotinine was particularly prevalent in influents (88%) and surface waters (85%). However, cotinine is a metabolite of nicotine, which, although occasionally used as an active pharmaceutical ingredient, is primarily associated to tobacco consumption. Given that approximately one-third of the Spanish population regularly uses tobacco (Ministry of Health, 2023), nicotine presence in water is likely to be substantially influenced by non-pharmaceutical sources. The high variability in area ratios between nicotine and cotinine (RSD >20%, Table S6) further supports the hypothesis of multiple origins. Likewise, the detection of caffeine, a compound widely present in food and beverages, may reflect its everyday consumption and its use as an excipient or adjuvant in certain medicines, making it difficult to attribute its presence exclusively to pharmaceutical inputs. Such variable sources may explain its consistent detection across all matrices analyzed.

Although a greater number of compounds were detected in surface water, the total normalized area of PhACs was higher ($p < 0.01$, Kurskal-Whallis test) in the influents (Fig. 3C). This fact could be expected, since influents receive the entire PhAC load from anthropogenic activities and have not yet been purified. Based on the predominant compounds identified in each matrix (those with the highest median normalized area and detection frequency), some of the main PhACs in the influents were caffeine (91.8, 100%), theophylline (38.0, 75%), and telmisartan

(17.9, 100%) (Fig. S4, Table S7). Although caffeine exhibited one of the highest normalized areas and detection frequencies, its elevated levels are likely influenced by non-pharmacological sources. The high presence of analgesics and non-steroidal anti-inflammatory drugs is consistent with their widespread use, as many of these medications are available over-the-counter (Fornasari et al., 2025; Mulka-Gierek et al., 2022). Similarly, given that hypertension is the second most prevalent chronic health condition in Spain (Ministry of Health, 2025), it is unsurprising that agents acting on the renin-angiotensin system such as telmisartan, commonly prescribed for this condition (AEMPS, 2024), were among the main compounds detected in the influents. Although codeine (136, median normalized area), noscapine (86.9), and quinine (63.8) exhibited high median normalized areas, their detection frequencies were lower (<50%). In the effluents, flecainide (9.0, 100%), and telmisartan (9.9, 100%) were two of the most predominant compounds (Fig. S5, Table S7) in line with results found in other studies (Celic et al., 2021). Some substances such as telmisartan and flecainide did not showed significant differences between influents and effluents ($p < 0.05$, Mann-Whitney test, Fig. S6), indicating limited removal efficiency for these substances. Another compound with a significant normalized area in the effluent was the opioid analgesic tramadol (10.6, 75%). This compound can be metabolized and excreted as its metabolites, including the demethylated isomers, which were also identified (6.4, 100%).

The compound profile observed in surface water was very similar to that found in the WWTP effluents. Tramadol (11.7, 84%) and its demethylated isomers (8.6, 72%), flecainide (7.7, 75%), telmisartan (6.5, 74%), tapentadol (5.3, 79%), and sulpiride (4.1, 88%) stood out among the PhACs with the highest normalized areas (Fig. S7, Table S7). This strong similarity confirms that WWTP effluents are the primary source of pharmaceutical emissions into the river environment. Furthermore, the pronounced hydrophobic character and the high affinity for organic matter of some compounds, support the idea that PhACs such as telmisartan ($\log K_{oc} = 5.61$; $\log K_{ow} = 8.42$) exhibited a greater tendency to accumulate in sediments (Fig. S8, Table S7) and sludge (Figs. S9 and S10, Table S7). Similarly, the antidepressant citalopram ($\log K_{oc} = 3.23$; $\log K_{ow} = 3.74$) also revealed a high prevalence in sediments (4.9, 71%), sludge before digestion (10.0, 75%), and sludge after digestion (9.7, 50%). Other compounds such as tramadol, sulpiride, hydro-morphine, oxycodone, 8-hydroxyquinoline, and codeine, with lower affinity for organic matter ($\log K_{oc} \leq 2.32$) and lower hydrophobicity ($\log K_{ow} \leq 2.68$), were also identified in these solid matrices, with variable Df % ranging from 13% to 100%. This behavior can be explained by their pKa values (9.1–13.1; Table S5), which result in their protonation under environmental pH conditions (pH 5–9). The resulting cationic forms can generate a cation exchange being adsorbed at negatively charged sites in sediment and sludge, promoting their retention in these solid matrices (Al-Khazrajy and Boxall, 2016). These adsorption processes play a key role in the removal of these compounds and pave the way for future research aimed at improving removal efficiency in wastewater treatment plants such as membrane reactor technology (Nguyen et al., 2025) and developing new bioremediation strategies (Nguyen et al., 2024). However, attention must be paid to the fate of these solids used for the elimination of pharmaceuticals from aqueous matrices. For instance, unlike wastewater, where the applied treatments appeared to be more effective for certain compounds such as psychostimulants (e.g., caffeine), antiepileptics (e.g., levetiracetam), agents acting on the renin-angiotensin system (e.g., eprosartan, losartan, perindopril), and lipid-modifying agents (e.g., fenofibric acid) (Fig. S6), the treatments applied to sludge did not reduce the concentration of any compound as none showed statistically significant differences ($p > 0.05$) between the areas measured before and after the digester (Fig. S11). The subsequent use of these sludges as agricultural amendments may promote the transfer of drugs to terrestrial ecosystems. Therefore, it is important to consider both the presence in the effluent and in the sludge when evaluating of removal efficiency. In some cases, such as clozapine,

a high removal efficiency from wastewater could be inferred, as the compound was not detected in the effluents. However, the data suggest that this apparent removal may not reflect the true efficiency of the treatment process but rather the possible transfer of the contaminant to the sludge (5.4, 13% before digestion; 7.9, 13% after digestion).

In fish, only azelaic acid (9.3, 62%), L-tryptophan (1.13, 58%), and riboflavin (2.95, 79%) were identified in more than 50% of the samples (Fig. S12, Table S7). Additionally, 13-cis-retinoic acid, was the most prominent compound in this matrix in terms of normalized area (464, 21%, Table S7). Although these were the most abundant PhACs in fish, they may have a biological origin. In contrast, other notable compounds such as buprenorphine (17.3, 17%) and gabapentin (10.5, 42%) appear to have a purely pharmacological origin, since they were primarily detected at sampling points with high (S2–S5, S7) or medium (S12) anthropogenic influence. Special attention should be given to these two compounds, which were identified in fish despite their moderate or low bioconcentration potential ($BCF_{\text{buprenorphine}} = 72$, U.S. EPA, 2012; $BCF_{\text{gabapentin}} = 3$, NIH, 2025).

To provide a straightforward illustration of the relationship among the different matrices, Table S8 presents the Spearman correlation matrix calculated using the median normalized areas obtained at each sampling point. The analysis focused on correlations among surface water, sediment, and fish. A positive correlation was observed for most therapeutic families, as well as for the number of compounds per sample and the total area in surface water and sediment. These results are

consistent with the physicochemical properties and partitioning mechanisms previously described. In contrast, correlations with biotic samples did not reach statistical significance.

Transformation products (TPs) of parent pharmaceuticals, either from direct metabolism or environmental transformations were also included in the original suspect list. Up to 17 TPs or groups of compounds were identified as shown in Fig. 5. The higher number of detected compounds and normalized areas indicates a greater tendency of these compounds to mobilize and remain in aqueous matrices (Park et al., 2018). Exceptions include desmethyldiazepam, desmethyldiazepam, and norverapamil, which, due to their physicochemical properties ($\log K_{oc} \geq 3.14$; $\log K_{ow} \geq 3.53$), preferentially accumulate in sediments and sewage sludge (Table S9). Different behaviour was observed for parent compounds and TPs due to their specific physical-chemical properties. For example, fenofibric acid, 10,11-dihydro-10-hydroxycarbamazepine, and carbamazepine epoxide display lower affinity for organic matter and higher water solubility compared to their parent compounds (Tables S5 and S9), which have been identified in sediments and sludge.

Interestingly, some TPs showed higher contribution than their parent compounds, such as carboxylic acids derived from zolpidem and the metabolite cotinine. Moreover, they can be detected despite the absence of the parent compound (e.g. quetiapine metabolite 2O, -2H and hydroxybupropion, Fig. 5 and Table S9). These findings highlight the importance of including both parent and TPs in environmental screening

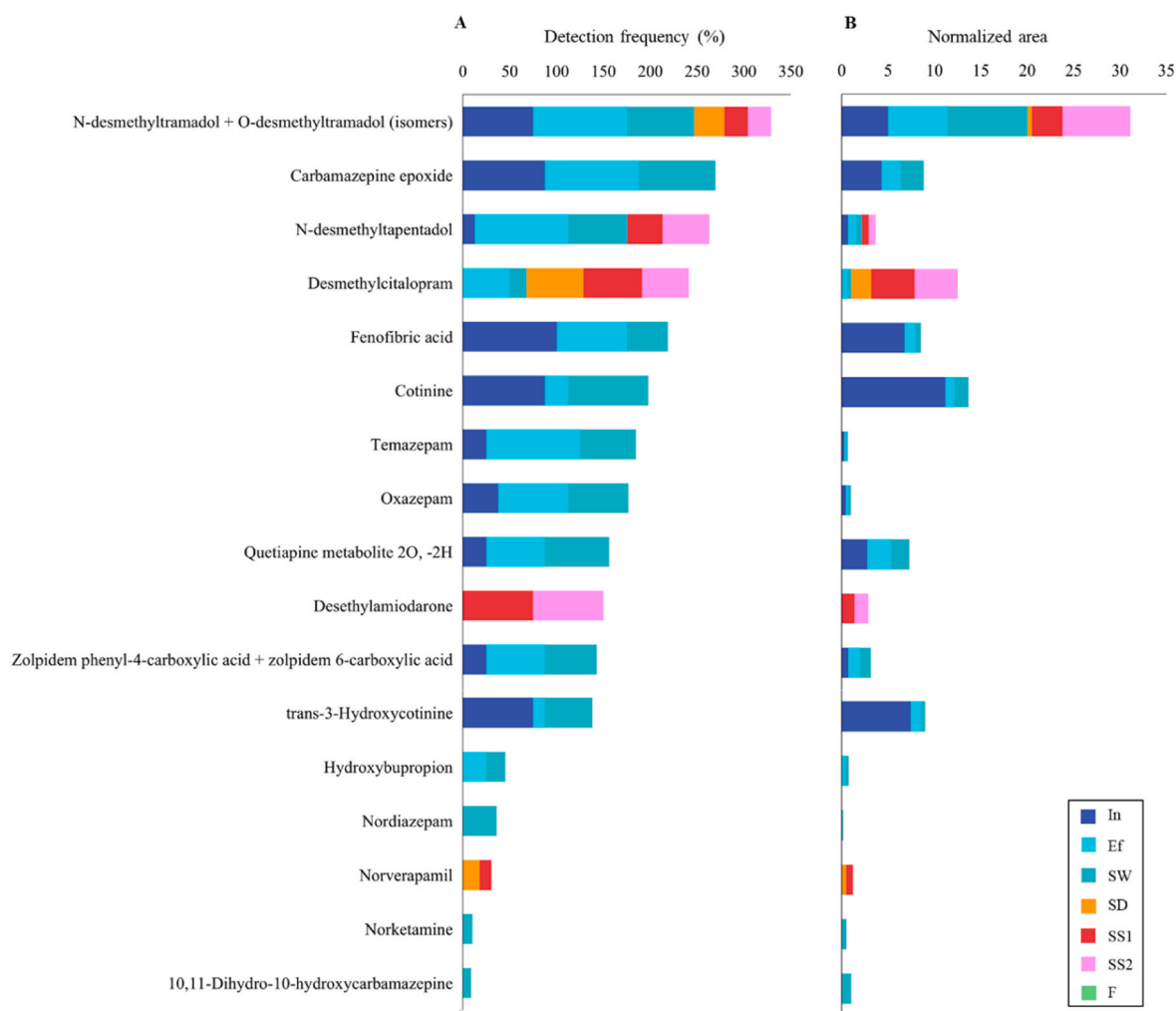


Fig. 5. Transformation products (TPs) identified in the different matrices according to their (A) detection frequency and (B) normalized area. In: influents, Ef: effluents, SW: surface water, SD: sediments, SS1: sewage sludge (before digester), SS2: sewage sludge (after digester), F: fish.

to avoid underestimating their environmental impact.

3.3. Spatio-temporal distribution

To evaluate anthropogenic influence on pharmaceutical pollution, those samples collected from same locations along the basin (surface water, sediment and fish) were compared. Results indicated that both the total normalized area and the number of PhACs per sample were significantly lower ($p < 0.05$) in areas classified as having low anthropogenic influence compared to areas with medium or high impact, in both surface water (Fig. S13A) and sediments (Fig. S13B). However, compounds such as procaine showed no differences ($p > 0.05$). Their detection in less urbanized areas may reflect their use in livestock industry (AEMPS, 2025). The results observed in fish were more variable, and no significant differences were observed ($p > 0.05$, Fig. S13C), likely due to the predominance of compounds of non-pharmacological origin. Regarding temporal patterns, no significant trends ($p > 0.05$) were observed for the number of compounds or total normalized area in any matrix (Fig. S14), nor were any seasonal differences detected in surface water samples collected at different times of the year (Fig. S15). Although a greater impact of pharmaceutical pollution was expected during the driest months, the strong flow regulation in the basin mitigates the effect of low rainfall, preventing excessive concentration of pollutants in the river.

The results obtained with this screening approach also allowed the comparison of the treatment efficiency of the two WWTPs studied. Both treatment plants exhibited similar removal performance, as no statistically significant differences ($p > 0.05$) were observed in the total effluent area between WWTP 1 and WWTP 2. Nevertheless, WWTP 2 appeared to release a greater number of compounds, which is expected given that it receives wastewater from a larger area with a higher population. Consequently, the use of medicines is more heterogeneous resulting in a greater variety of drugs. Correspondingly, higher normalized areas ($p < 0.05$) were observed for a few compounds such as losartan and telmisartan at WWTP 2.

4. Conclusions

The methodology developed in this study provided a comprehensive assessment of PhACs in a river ecosystem through a suspect screening approach based on UPLC-QTOF HRMS. A total of 153 PhACs were identified across multiple matrices, revealing the ubiquity and complexity of pharmaceutical contamination in fluvial systems. The dominant therapeutic groups included cardiovascular, psychotropic, pain management, and nervous system drugs. Surface waters and WWTP effluents showed the highest number of PhACs, confirming wastewater discharge as the primary source of contamination. Hydrophobic compounds such as telmisartan and citalopram accumulated preferentially in sediments and sewage sludge, whereas polar and more mobile compounds, including tramadol, predominated in aqueous matrices. Statistical analyses indicated significantly higher contamination in areas with intense anthropogenic activity, while no temporal or seasonal trends were observed, likely due to the strong hydrological regulation in the basin. Comparable removal performance between WWTPs highlights the limited efficiency of conventional treatments in eliminating persistent pharmaceuticals. The detection of several metabolites, sometimes at higher levels than their parent compounds, underlines the importance of including TP in environmental assessments. These findings demonstrate the capability of HRMS-based suspect screening to detect a broad spectrum of PhACs without the need for reference standards, highlighting its value for contaminant prioritization, early warning of newly introduced substances, and supporting regulatory decision-making. This approach also provides a robust basis for future ecotoxicological studies and design mitigation and remediation measures such as optimizing removal treatment processes or improving sludge management practices. Additionally, this approach supports retrospective analyses

that encompass extended timeframes. Integrating such methodologies into monitoring frameworks will enhance understanding of pharmaceutical behavior, facilitate the identification of high-risk substances, and contribute to the development of more effective environmental protection policies.

CRedit authorship contribution statement

Silvia Royano: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Juan Escobar-Arnanz:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Irene Navarro:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization. **Adrián de la Torre:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, 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.envpol.2026.127795>.

Data availability

Data will be made available on request.

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