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Characterization of the contamination fingerprint of wastewater treatment plant effluents in the Henares River Basin (central Spain) based on target and suspect screening analysis

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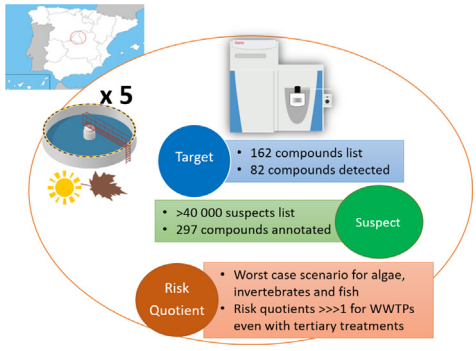
HIGHLIGHTS

- Five wastewater treatment plants were sampled in summer and autumn in central Spain.
- Target analysis revealed 82 out of 162 emerging pollutants.
- Suspect screening annotated 297 chemicals from a suspect list over 40,000 compounds.
- RQs revealed that pharmaceuticals and pesticides pose high risk in the area.
- WWTPs need to enhance their performance to decrease their discharges riskiness.

GRAPHICAL ABSTRACT



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ABSTRACT

The interest in contaminants of emerging concern (CECs) has increased lately due to their continued emission and potential ecotoxicological hazards. Wastewater treatment plants (WWTPs) are generally not capable of eliminating them and are considered the main pathway for CECs to the aquatic environment. The number of CECs in WWTPs effluents is often so large that complementary approaches to the conventional target analysis need to be implemented. Within this context, multitarget quantitative analysis (162 compounds) and a suspect screening (>40,000 suspects) approaches were applied to characterize the CEC fingerprint in effluents of five WWTPs in the Henares River basin (central Spain) during two sampling campaigns (summer and autumn). The results indicated that 76% of the compounds quantified corresponded to pharmaceuticals, 21% to pesticides and 3% to industrial chemicals. Apart from the 82 compounds quantified, suspect screening increased the list to 297 annotated compounds. Significant differences in the CEC fingerprint were observed between summer and autumn campaigns and between the WWTPs, being those serving the city of Alcalá de Henares the ones with the largest number of compounds and concentrations. Finally, a risk prioritization approach was applied based on risk quotients (RQs) for algae, invertebrates, and fish. Azithromycin, diuron, chlortoluron, clarithromycin, sertraline and sulfamethoxazole were identified as having the largest risks to algae. As for invertebrates, the compounds having the largest RQs were carbendazim, fenoxycarb and eprosartan, and for fish acetaminophen, DEET, carbendazim, caffeine, fluconazole, and azithromycin. The two WWTPs showing higher calculated Risk Indexes had tertiary treatments, which points towards the need of increasing the removal efficiency in urban

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WWTPs. Furthermore, considering the complex mixtures emitted into the environment and the low dilution capacity of Mediterranean rivers, we recommend the development of detailed monitoring plans and stricter regulations to control the chemical burden created to freshwater ecosystems.

1. Introduction

The group of contaminants of emerging concern (CECs) constitute an heterogeneous group of substances, including pharmaceuticals and personal care products (PPCPs), pesticides, steroid hormones and industrial chemicals, among others (Schymanski et al., 2014). The growth of the global population and enhancement of industrial, agricultural, health and sanitary systems over the last century has led to an increase in their production and emission to the environment (González-Gaya et al., 2021a). Despite most CECs are found at trace levels in aquatic and terrestrial ecosystems, some are susceptible to cause ecotoxicological effects and potential hazards for human health (e.g. endocrine disruption, antibiotic resistance, mutagenicity, etc.) (Cruzeiro et al., 2017; US EPA, n.d.; Letsinger et al., 2019; Köck-schulmeyer et al., 2013; Muir et al., 2017; Gago-Ferrero et al., 2016; Papageorgiou et al., 2016).

Different public bodies such as the European Environment Agency (EEA) and the US Environmental Protection Agency (EPA), or international regulations like the European Water Framework Directive (WFD) have included some of these compounds in their monitoring programs. Among the candidates to enter the EU WFD and EPA monitoring list, some antibiotics (e.g., azithromycin, clarithromycin, erythromycin, amoxicillin and ciprofloxacin), natural and synthetic hormones (e.g., estrone (E1), 17 beta-estradiol (E2), 17-alpha-ethinylestradiol (EE2), norethindrone), non-steroidal anti-inflammatories (e.g., diclofenac), several pesticides (e.g., acrolein) and pesticide by-products (e.g., 3-hydroxycarbofuran), perfluoroalkyl substances (e.g., perfluorooctanoic acid and perfluorooctane sulfonic acid) and plasticisers (e.g., nonylphenols) can be found (Rico et al., 2019; MAGRAMA et al., n.d.; Marshall & McCluney, 2021). Nevertheless, the list of anthropogenic compounds being detected in aquatic systems receiving urban, agricultural and industrial treated wastewaters is wider (Parliament, 2018; Čelić et al., 2020a; Ministry for Ecological Transitional and Demographical Challenge, n.d.), and no regulation or agreed monitoring programs are stablished for them.

Although there are many routes of entrance of CECs into the aquatic environment, including landfill leachates or agricultural runoff, wastewater treatment plants (WWTPs) have been described as one of the main pathways for CECs into aquatic ecosystems (Köck-schulmeyer et al., 2013; Link et al., 2017; Gago-Ferrero et al., 2016). Conventional processes implemented in WWTPs are mainly designed to remove the organic load of urban wastewaters, and are not effective to achieve the complete elimination of CECs (Alda et al., 2018; Minguez et al., 2016; González-Gaya et al., 2021b; Gago-Ferrero et al., 2016; Gros et al., 2006). Therefore, the role of WWTPs in the elimination of CECs and the implementation of more efficient monitoring and management procedures have become a challenge. The polar nature of many of these compounds facilitates their spread in the aquatic environment, reaching different environmental compartments and making their presence ubiquitous (Bijlsma et al., 2021; Köck-Schulmeyer et al., 2011; Gago-Ferrero et al., 2016; Schymanski et al., 2014; European Commission, 2017). Several factors such as the flow rate of the receiving water bodies, the sorption capacity to sediments, the microbial degradation processes, and photodegradation and other abiotic transformation reactions can affect the concentration of CECs in the aquatic environment (Lorenzo et al., 2019; Gago-Ferrero et al., 2016; Gros et al., 2006; FAO, 1996). Therefore, the occurrence of these micropollutants has to be controlled in surface waters (Cruzeiro et al., 2017; Tröger et al., 2021; Link et al., 2017; Gago-Ferrero et al., 2016; European Commission Joint Research Centre, 2003; Barco-bonilla et al., 2013; Grant et al., 1989; Gros et al., 2006) and in soil and sediments (Gosset et al., 2021; Wu et al., 2017). In addition, the chronic exposure of CECs in aquatic ecosystems can foster their bioaccumulation in aquatic organisms, such is so that CECs have been detected in wild fauna (Gosset et al., 2021; Schell et al., 2021; Kahle et al., 2008; Hug et al., 2014) and plants (Arias et al., 2016). However, the potential environmental hazard of CECs mixtures is still poorly understood (Köck-schulmeyer et al., 2013; Gómez et al., 2006). Moreover, the risk posed by the discharge of several WWTP effluents into rivers next to urbanized/industrialized areas with low dilution capacity is an issue of major concern (Aalizadeh et al., 2021), which is particularly relevant in areas affected by water scarcity (Dulio et al., 2018; Abily et al., 2021).

Besides, traditional analytical techniques cannot cope with the myriads of substances present in WWTPs effluents, and thus a new paradigm independent of biased or directed analysis is needed (Fonseca et al., 2020; Navarro-ortega et al., 2016). Recent studies based on non-target and suspect screening have revealed the enormous potential for discovery of CEC's in such complex matrixes, and point them as a promising tool for monitoring and regulatory purposes (Paíga et al., 2016; Navarro-ortega et al., 2016).

In this context, the main objective of this study was to evaluate the presence and exposure concentrations of a wide variety of CECs in effluents of 5 different WWTPs located in the Henares River basin (central Spain) during two sampling campaigns (summer and autumn) using both target and suspect screening approaches. Moreover, we aimed to identify the substances expected to pose an ecotoxicological hazard and that should be further monitored and controlled in WWTPs. An integrative assessment of the general risk of these mixtures was performed and the lack of information about their potential side effects in freshwater ecosystems with low dilution capacity is discussed. This study highlights the need of coupling novel analytical approaches, such as non-targeted analysis, with risk assessment information on vulnerable aquatic ecosystems exposed to WWTPs effluent discharges and water scarcity.

2. Materials and methods

2.1. Reagents and materials

The list of 162 target compounds included in the present study, comprising PPCPs, pesticides, and industrial products, and is provided in the Supplementary Information (SI, Table S1). The list includes substances of a wide variety of applications and chemical characteristics, known to be frequently detected in WWTP's effluents and some of them prone to be included in future monitoring programs due to their semi persistence or under study effects in biota (see Section 2.8). The table includes the information about the supplier, molecular formula, purity, solvent used for stock preparation and surrogate applied for analyte recovery correction. Working solutions containing all the target compounds and surrogates at 3 μg/g and 10 μg/g, respectively, were prepared in methanol (MeOH, UHPLC-MS, Scharlab, Barcelona, Spain). For the chromatographic confirmation in the suspect analysis through the Retention Time Index platform (http://rti.chem.uoa.gr/, see Section 2.5) a mix with the calibration compounds was also used (Luo et al., 2014).

The preconcentration and extraction of the samples was performed with home-made triphasic solid phase extraction (SPE) cartridges using

the following sorbents: reverse phase (Chromabond® HRX, 85 μm, 55–65 Å, Macherey-Nagel, Düren, Germany), anionic exchange (Sepra ZT-WAX, 30 μm, 85 Å, Phenomenex, California, USA) and cationic exchange (Sepra ZT-WCX, 30 μm, 85 Å, Phenomenex, California, USA). Frits and polypropylene cartridges (12 mL) were purchased to Supelco (Bellefonte, PA, USA). Solvents used at the SPE were MeOH (HPLC, 99.9%, Sigma-Aldrich, St. Louis, MO, USA), ethyl acetate (HPLC, 99.9%, Sigma-Aldrich), ammonia (25%, Sigma-Aldrich) and formic acid (HCOOH, >98%, Panreac, Barcelona, España).

During the chromatographic separation step, formic acid, water and acetonitrile (UHPLC-MS grade) and ammonium acetate (NH_4OAc, ≥99 %) provided by Fischer Scientific (Geel, Belgium) and Scharlab, respectively, were used in the mobile phase.

2.2. Sampling

Water samples were collected from the effluent discharge point of the five WWTPs noted in Fig. S1 (SI) in central Spain in two different sampling campaigns: July and November of 2017. One liter water samples were collected and stored in amber glass bottles, which were subsequently transported to the laboratory and stored at −20 °C. The wastewater treatment capacity and type of treatment used by each of the WWTPs included in this study is provided in Fig. S1, while further information regarding the amount of sludge produced or detailed treatment steps can be obtained from Schell et al. (Singer et al., 2016). WWTPs 1, 4 and 5 discharge their effluents directly into the Henares River and treat wastewaters from cities with a noteworthy industry and high population density. WWTPs 2 and 3 correspond to smaller installations for lower equivalent habitants, and discharge their effluents into the Torote and Monjas streams, respectively, both tributaries of the Henares River. In turn, the Henares River is one of the biggest tributaries of the Jarama River, which flows into the Tagus River between the Madrid and Castilla La Mancha autonomies in central Spain. The area of Alcalá de Henares is well-known as being one of the most industrialized areas in Spain, also called “Corredor del Henares”, composed by 33 municipalities between Madrid and Guadalajara with a population over 600,000 inhabitants, where approximately 9800 companies are located. These companies embrace different fields including technological industry, heavy (e.g. iron and steel) and light (e.g. food) industries and chemical industries (e.g. laboratories, cosmetic and perfume manufacturing) are located, among others.

An extra sample was gathered in April 2018 in the Galindo WWTP (Biscay, Basque country, North Spain) and used for the validation of the analytical method applied here.

2.3. Sample treatment

Samples were transported at −20 °C to the University of the Basque Country (UPV/EHU) in October of 2019 and kept at that temperature until processing. The stability of the monitored compounds was ensured with freezing and maintained storage until processing, but the degradation of other less stable compounds cannot be neglected, being thus the detection done here in the lower edge of the original pollution status. Once thawed, water was filtered (cellulose filters 0.7 μm, 90 mm, Whatman) and spiked with a deuterated standard mix (Table S1, SI) at 250 ng/L and processed according to a method previously validated in our research group (Minguez et al., 2016). Briefly, three replicates of 500 mL were extracted using in-house made SPE cartridges containing 100 mg of cationic exchange (ZT-WCX), 100 mg of anionic exchange (ZT-WAX) and 300 mg reverse phase (HRX) sorbents from bottom to top. Conditioning was done with 10 mL of MeOH: ethyl acetate (1:1, *v*/v) and 10 mL Milli-Q water, and after sample loading, the cartridges were eluted with 12 mL of MeOH: ethyl acetate (1:1, v/v) containing 2% ammonia and 12 mL of MeOH: ethyl acetate (1:1, *v*/v) 1.7% formic acid. Both extracts were combined, evaporated on a Turbovap (Zymark, Hopkinton, USA) at 40 °C under a gentle N_2 flow and reconstituted on 250 μL MeOH: Milli-Q water (1:1, v/v). Final extracts were filtered with syringe filters (PP, 0.22 μm, 13 mm, Jasco Analítica, Madrid, Spain) onto amber chromatography vials and were kept at −20 °C until their analysis, always in less than one week time.

The sample used for method validation purposes (see Section 2.6) was processed likewise, but spiked with the full list of standards (162) detailed in Table S1 (SI) prior to sample treatment (200 ng/L in original sample). Moreover, three procedure blanks using Milli-Q water and three replicates of Milli-Q water spiked with the full list of standards were processed together with the full set of samples.

2.4. Chemical analysis

The analysis was carried out with a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo Scientific Q Exactive Focus quadrupole-Orbitrap mass spectrometer (UHPLC-q-Orbitrap) equipped with a heated ESI source (HESI, Thermo-Fisher Scientific, CA, USA).

Extracts were injected on an ACE UltraCore XB-C18 (2.1 mm × 150 mm, 1.7 μm) chromatographic column with a pre-filter (2.1 mm ID, 0.2 μm) from Phenomenex. Concerning the mobile phase, Milli-Q water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (HCOOH), were used for the positive ionization mode. For the negative ionization mode, 5 mM of ammonium acetate were added to both solvents. The LC gradient started at 87% A and it stayed constant for 30 s. Then, it had a linear increase to 50% A at 10 min followed by another increase at 13 min to 5% A with a hold of 0.5 min. Finally, it returned to the initial conditions at 19 min and it ended a hold of 2 min. Flow rate was set to 0.3 mL/min, column temperature was 50 °C and 5 μL were injected three times maintaining the automatic sampler at 5 °C.

The q-Orbitrap was operated in full scan – data dependent MS2 (Full MS-ddMS2) discovery acquisition mode for both positive and negative ionizations. The intensity threshold and dynamic exclusion for the data dependent were respectively 8.0×10^3 and 8s. The scan range was *m/z* 70–1050, the Full MS had a resolution of 70,000 FWHM for a 200 *m/z* relation, and it was followed by three ddMS2 scans with a resolution of 17,500 FWHM with an isolation window of 3 *m/z*.

The stepped normalized collision energy (NCE) in the higher-energy collision dissociation (HCD) cell was set at 10–30–70 eV and10–45–90 eV for the positive and negative mode respectively, the MS2 was a sum of the fragmentations obtained with the different energies. Positive and negative HESI source parameters were set to 3.5 kV spray voltage, 300 °C capillary temperature, 40 arbitrary units (au) sheath gas (nitrogen), 15 au auxiliary gas, 280 °C auxiliary gas heater and S-lens RF level 55.0. Pierce LTQ ESI Calibration Solutions (Thermo-Fisher Scientific) were used for external calibration of the instrument every three days. The software used was Xcalibur 4.0 (Thermo-Fisher-Scientific).

2.5. Data treatment

The TraceFinder 5.1 (Thermo-Fisher Scientific) software was used for target analysis. Target compounds and their instrumental characteristics including molecular formula, ionization mode, retention time (tR) and experimental MS/MS fragments were added to the software library according to studies previously performed by the research group (Minguez et al., 2016). To avoid false positives, experimental tR window was limited to 60 s around the pure standard tR, mass error for parent and fragments was set as lower to 5 ppm and the isotopic profile match over 70%. Calibration curves and peak integration were manually checked and peaks with a base width smaller than 0.1 min were rejected.

For the suspect analysis, the Compound Discoverer 3.1 (Thermo-Fisher Scientific) software was applied. Filters and workflow applied is summed up in Fig. S2, SI. Only Lorentzian peaks were manually accounted. The NORMAN database (40059 compounds, www.norman-network.net) was used as suspect list with a fixed error lower than ±5 ppm in the exact mass. The molecular formula suggested by the software were only accounted if MS1 was satisfactorily matched

(SFit > 30% and isotopic profile > 80%). Minimum peak areas considered were set at $1e^6$ and $25e^6$ units for negative and positive ionization modes, respectively. Additionally, only peaks 30 times larger than the blanks and with a relative standard deviation (% RSD) lower than 25% within injection replicates were further studied. MS2 spectra was compared with mzCloud database (https://www.mzcloud.org/), and a match over 70% was set for the positive identification of the feature. In the case that the MS2 was not available in mzCloud database, *in-silico* fragmentation was performed with the massFrontiers tool (ThermoFisher Scientific) implemented in Compound Discoverer 3.1, and a positive identification was considered when at least the 70% of the largest fragments were explained. When standards of the candidates were available, experimental retention time was confirmed with an allowed error of ±0.1 min. If not available, retention times were estimated from the Retention Time Index (RTI) platform (http://rti.chem.uoa.gr/) and candidates were rejected or accepted depending on whether there was a statistical difference or not with the estimated value within the uncertainty of the model built. Finally, identification criteria according to Schymanski and coworkers (Arenas-Sánchez et al., 2019) was noted providing the candidates with a tentative code from 1 to 3 levels of identification. This scale is numbered from one to five being one the highest confidence level (features with their structure identified and confirmed by reference standard acquisition), and five the least one (only the exact mass of the compound can be provided). Two was assigned when a probable structure was found, and three, when a tentative candidate was identified.

2.6. Analytical method quality parameters

Calibration curves prepared in MeOH:Milli-Q water (1:1, *v*/v) were built within the instrumental limit of quantification (LOQ_{inst}) and 500 ng/g range (given in mass concentration units as the standards were prepared weighting all the solutions for obtaining more accurate values). Calibration points in the 0.1–50 ng/g range were injected in triplicate to calculate the LOQ_{inst}. The LOQ_{inst} were set as the lowest concentration level that, after triplicate injection, rendered RSD < 30% and trueness > 70% between the theoretical concentrations and the concentrations estimated from the external calibration curve, and can be found in a previous work by González-Gaya et al. (Minguez et al., 2016). LOQ_{proc} values were stablished as the theoretical concentration measurable and quantifiable in the original water sample taking into account the LOQ_{inst}, the absolute recoveries and the preconcentration factor, and are included in Table S3 (SI). As previously defined elsewhere (Minguez et al., 2016), the instrumental limits of identification (LOI_{inst}) were estimated as the lowest concentration for which the experimental and theoretical MS2 spectra match was equal or greater than 70% and the retention time difference was lower than ±0.1 min. Similarly, to LOQ_{proc}, procedural LOIs (LOQ_{proc}) were estimated considering the LOI_{inst}, the absolute recoveries and the preconcentration factor (see Table S3).

Blank and spiked Milli-Q water samples, as well as spiked effluent water samples from Galindo (200 ng/L in original sample) were processed together with the studied samples to calculate the apparent recoveries of the analytical method. Apparent recoveries, used to evaluate the trueness of the concentrations reported for each analyte (including matrix effect and ion suppression/enhancement evaluation), were calculated after the correction of the analyte concentration with the corresponding isotopically labelled surrogate. The surrogate used for each target analyte is defined in Table 1S, SI. In the case of negatively ionized compounds, the recoveries are absolute recoveries since no standard for correction was available.

2.7. Statistical analyses

Principal Component Analysis (PCA) was used to identify the underlying factors (e.g. water load, sampling period), which would allow to distinguish the chemical fingerprinting of the different WWTP effluent samples studied here. The PCA was run in the PLS Toolbox 8.9.1 (2020, Eigenvector Research, Inc., Manson, WA USA) implemented in MatLAB R2019b software (Mathworks, Natick, NA), and the PCA models were built with auto scaled data (mean centered divided by standard deviation) and were validated using full cross validation. LOQ_{proc} values were used for those compounds that were found at concentrations lower than the LOQ. The compounds that were not detected in any of the analyzed samples were not considered in the PCA.

Likewise, the list of suspects annotated in this work were analyzed through PCA. In this case, the areas provided by the software per each feature were studied using the tools available for multivariate data analysis in Compound Discoverer 3.1. software. The data was auto-scaled and centered before performing the PCA.

2.8. Ecological risk assessment

An Ecological Risk Assessment (ERA) was carried out following a risk quotient (RQ) approach according to the European Union technical Guidance Document (Jayaraj et al., 2016). In this study, RQs for chronic effects were calculated for each compound as the ratio of the measured environmental concentration (MEC) and the predicted no-effect concentration (PNEC).

Maximum concentrations for each compound measured among all the analyzed effluent samples were used as MEC values, which represent the "worst-case scenario" for this area of the Henares basin, assuming limited or no dilution capacity (Blasco et al., 2013; FAO, 1996) (Table S2). Moreover, an individual ERA for the chemical mixtures contained in each WWTP effluent was calculated based on the Risk Index (RI) approach, calculated as the sum of the RQs for the individual substances and assuming concentration addition (Bakker et al., 2019). The PNEC values were calculated considering the lowest chronic toxicity data (no observed effect concentration, NOEC) collected from the ecotoxicology knowledgebase (ECOTOX database, https://cfpub.epa.gov/ecotox/) for several target species representing different trophic levels (algae/bacteria, invertebrates and fish), divided by an assessment factor (AF). Values of any compound not available in this site were obtained from the literature (FAO, 1996; Moro et al., 2012), the Pesticides Properties (http://sitem.herts.ac.uk/aeru/ppdb/) and NORMAN Network data bases (https://www.norman-network.com/nds/) or calculated *in-silico* using the QSAR models included in the ECOSAR™ v. 2.0 software (ECOlogical Structure Activity Relationship), in which the lowest toxicity prediction for each taxon was chosen (FAO, 1996). The AFs reflect the degree of uncertainty in the extrapolation from laboratory toxicity test data for a limited number of species to species-rich ecosystems. The AF applied for long-term tests was reduced when number of species tested increased (Paíga & Santos, 2017). An AF of 100 was set if only one long-term NOEC value was available, and an AF of 50 and 10 was used if two or three NOECs were available, respectively. Acute toxicity values (EC_{50} lowest value) were used for the calculation of the PNECs (FAO, 1996; Moro et al., 2012) when no chronic NOEC values were found, by applying an AF of 1000. When the calculated RQ was ≥1, a high potential environmental risk was indicated. RQ values between 0.1 and 1 were considered to result in moderate risks, and when RQs were <0.1, the environmental risk was considered to be negligible.

3. Results and discussion

3.1. Analytical method quality parameters

3.1.1. Linearity, LOI and LOQs

Linearity of the calibration curves was confirmed with linear regression determination coefficient values (r^2) ≥ 0.96 in both, positive and negative ionization modes, except for the pharmaceutical terbinafine, with a r^2 higher than 0.95.

Of the 162 xenobiotic compounds included in this study (Table S3, SI), 144 showed LOI_{proc} values lower than 25 ng/L concentration in the sample. LOI_{proc} values of the remaining compounds (18) were between 30 and 151 ng/L. The vast majority compounds included in this study showed LOQ_{proc} values below 30 ng/L, except for the pharmaceuticals amiodarone and amoxicillin, which exhibited LOQ_{proc} values of 84 and 134 ng/L, respectively. These $LOIs_{proc}$ and $LOQs_{proc}$ are comparable to those reported in previous European studies (Parliament, 2015; Mart et al., 2013; Köck-Schulmeyer et al., 2011; FAO, 1996; Moro et al., 2012).

3.1.2. Recoveries and precision

As depicted in the box-whisker diagram in Fig. S3 and Table S3 (SI), adequate apparent recoveries were obtained in case of Galindo WWTP effluent with respect to the lower absolute recoveries obtained without any correction, proving that the use of selected isotopically labelled surrogates corrects the matrix effect in both the extraction and detection steps. The apparent recovery of 74% of the studied compounds ranged between 60 and 140%. The rest of the compounds (remaining 26% of the total compounds) showed worse apparent recovery values due to the lack of a corresponding isotope labelled standard to be used as surrogate. Moreover, the presence of some studied compounds in the sample at similar or higher concentrations as spiked ones hampered the calculation of their apparent recoveries.

It must be highlighted that the use of isotopically labelled surrogates improved the calculated precision as well as the RSD of the studied compounds, obtaining, in general terms, values lower than 30%, except for the antibiotic ofloxacin (RSD = 34%).

3.2. Target analysis of CECs in WWTPs

Mean concentrations and the corresponding RSD values of the xenobiotic compounds found in the different WWTPs are summarized in Fig. 1. A total of 82 xenobiotic compounds were detected in different sampling points, from which 62 of them were pharmaceuticals (76%), 17 pesticides (21%) and 3 industrial products (3%) (Table 1). Among the most widely detected pharmaceuticals, antifungals (<LOQ–109,480 ng/L), antibiotics (<LOQ–19,459 ng/L), antihistaminic (<LOQ–55,638 ng/L), antihypertensives (<LOQ–4225 ng/L) and antiinflamatories (<LOQ–1425 ng/L) were included. It is noteworthy the fluconazole (antifungal) concentrations in the effluents of both WWTPs 4 and 5, with values around 100 μg/L. Fluconazole is used against oropharyngeal/esophageal candidiasis, and thus frequently prescribed for female treatments and regular immunodeficiency (Mijangos et al., 2018) and is often detected in wastewater (Nilsen et al., 2019). Ranitidine (antihistamine) was found as well at high concentrations (up to 56,000 ng/L) in those two WWTPs, especially in autumn. It is used to reduce stomach acidity in ulcer and gastric reflux by regulating histamine (Carmona et al., 2017), and like fluconazole, is one of the most common pharmaceuticals prescribed and used in common diseases, thus prone to be found in domestic wastewaters (Hollender et al., 2018; Picó et al., 2020). In addition, there were high levels of caffeine in all WWTPs (30–48,508 ng/L), and particularly in 4, as well as cotinine, a nicotine metabolite, detected during summer in WWTPs 4 and 5 (1799–56,817 ng/L). Regarding pesticides, fungicides and herbicides showed similar occurrence regardless of the wastewater effluents analyzed, only standing out the concentrations of fenpropimorph (1858 ng/L) and chlortoluron (7445 ng/L) in WWTP 4 during summer and in WWTP 2 in winter, respectively. Both substances are of wide use in cereals crops for the control of fungi (Botero-coy et al., 2018) and grass weed (Grant & Clissold, 1990), respectively, and due to the agricultural land use in the area (Singer et al., 2016) transport of those to the WWTPs by atmospheric deposition, rainwater and run off cannot be excluded. In the case of industrial products, PFOS was only detected in the WWTP 5 at 7 ng/L, while the compounds benzothiazole and triethyl phosphate were found in all the samples in a concentration range between 50 and 450 ng/L in both sampling campaigns. Levels of compounds detected in this study are in agreement with others reported for the analysis of CECs in wastewater effluents (Parliament, 2015; US EPA, n.d.; Gosset et al., 2021; Mart et al., 2013; Köck-Schulmeyer et al., 2011; Gros et al., 2006; FAO, 1996; Moro et al., 2012). A previous study performed in small rivers and streams within the area (Tiboni et al., 2008), reports likewise the presence of many pharmaceuticals (i.e. acetaminophen, carbamazepine, valsartan) and remarks the occurrence of several pesticides, including the same detected in this study (i.e. imidacloprid, chlortoluron, propiconazole, tebuconazole) and even non authorized ones for agricultural use (European Parliament, n.d.) such as diuron and carbendazim.

As a general trend, a major presence of emerging contaminants was detected in the WWTPs 4 and 5, regardless of the sampling

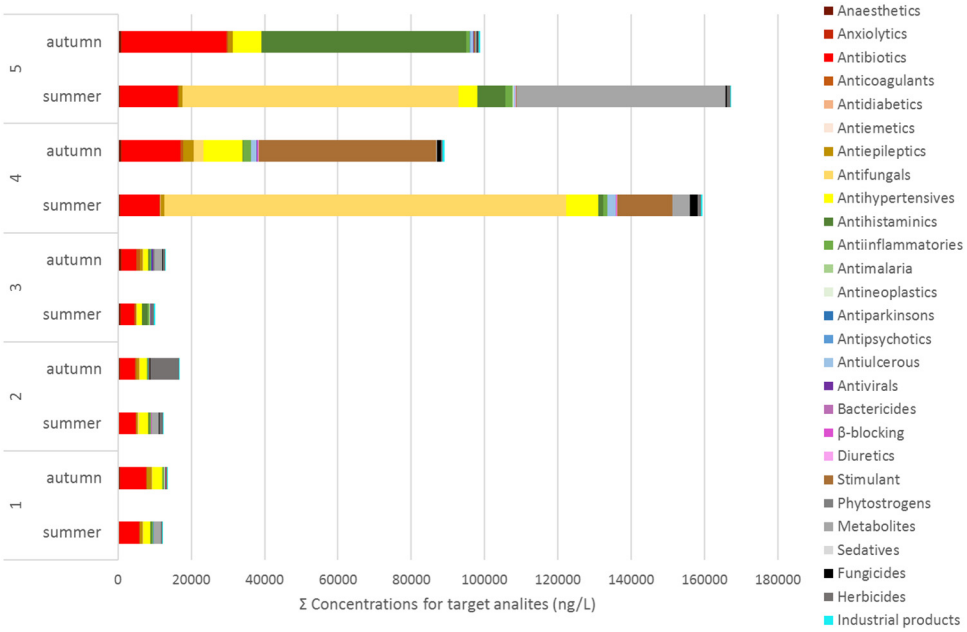

Fig. 1. Sum of concentrations (ng/L) of all the quantified target compounds by application. Compounds <LOQ were not accounted in the sum.

period (i.e., summer and autumn). On the contrary, the effluents of WWTP 3 collected in summer and of WWTP 2 collected in autumn were the ones with a lower number of contaminants detected and at the lowest concentrations. This was expected as WWTPs 4 and 5 are the largest in size, are located in the metropolitan area of Madrid, and cope with the treatment of greater wastewater volumes and higher demographic concentration. Likewise, the concentrations of pesticides detected among the different WWTPs depicts that 5, 3 and 2 were the WWTPs with the largest occurrence of pesticides in summer. In addition, the general prevalence of pesticides in summer must be pointed out, in lieu of the case of WWTP 1, showing just the opposite.

3.3. Suspect screening of the compounds present in the WWTPs

Suspect screening was performed to further elucidate the presence of CECs in the WWTP effluent samples. Apart from the 82 compounds quantified using target analysis, a vast number of candidates were identified by means of the workflow described in Fig. S2. They are included in Tables S4 and S5 in the SI for compounds annotated at levels 2–3, in the positive and negative ionization modes, respectively. Among them, 176 tentatively identified as probable structures (level 2a or 2b) and 39 as tentative candidates (level 3), according to Schymanski and co-workers classification (Arenas-Sánchez et al., 2019). Tables S4 and S5 in SI include the detailed information of each annotated as well as their occurrence in the analyzed samples.

Similar to the target analysis, WWTPs 4 and 5 provided not only a higher number of compounds (Fig. S4, SI) but also the greatest areas for the detected compounds in both seasons, confirming consequently, the relation with the size, population and industrialization of the located area of both mentioned WWTPs (Alcalá de Henares). Among the annotated compounds, xenobiotics such as dimetridazole and metronidazole, used as antifungals or antiparasitics, and the pesticide carbetamide were registered. Also, PPCPs like the cosmetic ingredient panthenol, the plasticizer/surfactant PEG monolaurate, the antidepressant mianserin, the β-blocker oxprenolol, and few sedatives such as nordiazepam and clomethiazole were annotated as well as other non-regulated substances like pentedrone, an illegal drug. Most of them have been reported to be toxic (Maruya et al., 2016; Picó et al., 2019; Kuzmanović et al., 2015; Arnold et al., 2014) and pose adverse effects to wild fauna, and even if some of them are regulated (such as metronidazole, banned in some countries)(Kuzmanović et al., 2015), they are not included in regular monitoring programs.

A wide range of compounds that differ in physicochemical properties were detected in this study in addition to other studies performed in effluents from other WWTPs (Ruhí et al., 2016; Čelić et al., 2020b). This reveals the need of the development of a more appropriate treatment for the urban wastewaters to eliminate these active and

Table 1
Individual concentrations of all the quantified target compounds by application in the five WWTPs in summer (June, J) and autumn (November, N).

		1 (J)		1 (N)		2 (J)		2 (N)		3 (J)		3 (N)		4 (J)		4. (N)		5 (J)		5 (N)	
	Compound	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)	C (ng/L)	RSD (%)
Pharmaceuticals and active compounds																					
	Anesthetics																				
1	Lidocaíne	320	3	340	20	300	5	360	19	590	2	700	3	470	8	720	4	460	2	760	1
2	Propranolol	20	2	30	14	30	3	30	1	30	3	50	3	50	11	70	8	30	3	50	5
	Anxiolytics																				
3	Lorazepam	150	1	230	13	190	4	240	12	180	3	240	2	180	9	260	5	220	4	240	3
	Antibiotics																				
4	Amoxicilline	4540	1	5280	10	3790	9	3570	18	3450	3	3520	11	8120	10	13100	12	4090	4	4950	4
5	Azithromycin	460	6	1310	23	190	9	170	1	30	14	70	14	< LOQ	29	< LOQ	< LOQ	10330	11	19460	12
6	Clarithormycin	40	3	110	5	80	5	220	1	80	2	110	4	100	9	390	10	100	6	630	12
7	Micophenolic acid	10	9	<5	30	<5	3	ND	ND	20	27	40	1	2000	10	1480	10	190	5	190	8
8	Norfloxacin	40	1	ND	ND	< LOQ	< LOQ	ND	ND	ND	ND	110	7	< LOQ	< LOQ	ND	ND	610	4	1900	30
9	Sulfamethoxazole	70	2	140	3	180	4	80	6	90	8	40	5	200	8	550	9	90	5	850	1
10	Sulfapyridine	60	2	120	3	< LOQ	< LOQ	30	14	< LOQ	< LOQ	70	3	90	12	230	4	40	7	110	2
11	Trimethoprim	30	3	40	14	30	2	30	16	ND	ND	ND	ND	70	10	180	8	70	6	400	6
	Anticoagulants																				
12	Pentoxifylline	80	3	<5	9	<5	6	10	28	10	8	<5	9	60	9	100	5	100	1	40	2
13	Amitriptyline	30	5	50	26	50	4	50	28	50	3	100	2	120	12	370	2	110	9	70	10
14	Bupropion	< LOQ	< LOQ	< LOQ	< LOQ	ND	ND	ND	ND	ND	ND	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
15	Carbamazepine	110	1	140	9	130	3	130	6	210	1	290	3	90	10	120	9	90	4	110	4
16	Clomipramine	10	5	10	23	< LOQ	< LOQ	< LOQ	< LOQ	ND	ND	20	7	< LOQ	< LOQ	< LOQ	< LOQ	40	6	160	12
17	Iminostilbene	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< LOQ	< LOQ	<5	1	< LOQ	< LOQ	< LOQ	< LOQ
18	Mirtazapine	10	9	30	8	10	4	10	29	<5	13	660	5	10	17	70	6	10	7	20	14
19	Nortriptyline	<5	8	10	8	<5	4	10	14	<5	4	10	8	10	17	30	17	10	21	10	15
20	Sertraline	30	3	40	19	30	7	30	28	60	2	30	2	40	14	50	9	50	8	40	4
	Antidiabetics																				
21	Glibenclamide	ND	ND	ND	ND	30	12	10	>30	10	27	10	7	20	12	20	16	60	13	40	17
22	Glimepiride	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<5	3	ND	ND
	Antiemetics																				
23	Ondansetron	<5	5	<5	11	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	10	15
	Antiepileptics																				
24	Gabapentin	190	3	220	29	260	10	240	1	220	5	90	2	490	4	1210	13	480	3	500	6

25	Primidone	620	4	1050	5	150	5	530	9	90	32	580	2	590	9	1740	7	390	2	740	10
	Antifungics																				
26	Fluconazole	140	3	280	7	60	2	90	7	130	2	150	2	109480	9	2710	6	75330	6	ND	ND
27	Ketoconazol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< LOQ	< LOQ	ND	ND	ND	ND
28	Pirantel	20	5	20	6	300	7	110	5	60	6	20	8	< LOQ	< LOQ	10	9	10	4	20	5
	Antihypertensive																				
29	Atenolol	160	2	200	12	140	6	110	2	200	2	190	2	460	11	1040	2	400	2	320	5
30	Eprosartan	100	1	50	3	140	4	20	10	30	4	20	1	1500	7	2020	1	760	6	1800	5
31	Hydrochlorthiazide	130	5	250	3	350	2	290	11	150	3	170	5	200	2	250	5	260	2	510	3
32	Irbesartan	1130	3	1610	4	1200	4	1210	11	550	1	740	4	720	7	1290	3	910	3	1100	5
33	Losartan	200	2	210	8	80	1	30	9	90	5	60	1	2240	8	1520	7	1260	9	880	6
34	Metoprolol	10	2	20	8	50	5	10	7	50	1	20	4	160	12	250	5	90	4	200	3
35	Pindolol	30	3	60	2	100	7	60	21	60	15	80	2	30	23	40	15	< LOQ	< LOQ	60	23
36	Valsartan	70	7	130	7	360	2	110	13	70	5	40	7	3540	1	4230	4	1390	2	2980	2
37	Verapamil	10	5	20	23	<5	5	10	1	10	9	10	4	10	23	10	16	20	2	<5	13
	Antihistamínico																				
38	Cetirizine	110	2	140	8	120	5	140	14	130	2	270	2	130	8	170	4	180	4	180	10
39	Difenhidramine	10	4	20	17	10	6	10	3	20	2	20	7	10	20	50	2	20	4	20	8
40	Ranitidine	300	1	ND	ND	200	8	ND	ND	1390	28	180	19	1210	18	ND	ND	7430	3	55640	34
	Antiinflammatory																				
41	Acetaminophen	200	3	290	15	290	2	330	11	200	13	390	6	370	28	500	8	710	6	250	11
42	Celecoxib	50	7	90	2	< LOQ	< LOQ	ND	ND	100	2	< LOQ	< LOQ	70	3	80	13	< LOQ	< LOQ	70	1
43	Ketoprofen	110	4	200	7	190	5	70	10	50	6	ND	ND	640	8	1430	5	1280	6	640	5
44	Propylphenazone	<5	6	10	15	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	<5	8	20	7	<5	5	30	1
	Antimalaria																				
45	Hidroxychlorquine	60	13	ND	ND	ND	ND	ND	ND	490	>30	ND	ND	30	2	ND	ND	230	15	ND	ND
	Antineoplastic																				
46	Cyclophpsphamide	10	8	10	12	<5	3	<5	1	<5	8	10	4	10	1	10	3	20	2	10	15
47	Exemestane	< LOQ	< LOQ	ND	ND	< LOQ	< LOQ	<5	24	< LOQ	< LOQ	ND	ND	< LOQ	< LOQ	<5	14	ND	ND	<5	10
	Antiparkinson																				
48	Memantine	10	8	10	5	50	4	30	2	10	2	50	10	10	7	10	10	10	3	10	2
49	Ropinirole	<5	9	<5	11	10	21	10	14	20	2	30	1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	<5	13
	Antipsychotics																				
50	Clozapine	40	7	40	28	10	4	20	1	20	4	30	3	ND	ND	<5	6	10	15	20	14

non-regulated compounds as they can be found nearly in all aquatic ecosystems with unknown adverse effects in most of the cases. In addition to pharmaceutical compounds (the ones detected with more frequency), pesticides, including herbicides and fungicides, PCPs and industrial chemicals were also detected in WWTP effluents.

3.4. Temporal and spatial analysis

Possible correlations between sampling location, season or WWTP treatment were assessed by means of a PCA of the data obtained from wastewater effluent samples.

3.4.1. Target analysis

In the case of target analysis, concentrations of the detected compounds among the five WWTPs were taken into account. Fig. 2 depicts the scores (2a) and loadings plot (2b) of the two main principal components (PCs), explaining almost 50% of the total explained variance. Based on the scores plot, the location of the WWTP is separated based on PC1 (explaining the 36% of the total variance), being the WWTPs 4 and 5 the most different ones with respect of the others. As mentioned in the previous sections, they receive the wastewaters of an area with higher population density and industry, and consequently, are the WWTPs with the largest load of CECs. It must be highlighted that the area of Alcalá de Henares exceeds in population density with 194,000 inhabitants the other sampling points, and thus, those WWTPs are the ones with higher water capacity, 31,000 and 75,000 m^3/day, respectively for WWTP 4 and 5 (Fig. S2). As it can be observed in the loadings plot, most of the compounds are correlated with the samples collected in WWTPs 4 and 5, prevailing pharmaceutical compounds including different antihypertensives (e.g., metoprolol, eprosartan, atenolol and valsartan), antibiotics (sulfapyridine, mycophenolic acid, trimethoprim), antifungals (fluconazole), anticonvulsants (gabapentin) and antiinflamatories (ketoprofen), among others. In addition, stimulant compound caffeine or industrial catalyzer triethyl phosphate also contribute as hidden important variables to the separation observed among the studied samples. Conversely, compounds directly related with samples from WWTPs 1, 2 and 3, are the ones in the negative part of the loadings plot, standing out pesticides such as myclobutanil, acetamiprid, tebuconazole and imidacloprid, and to a lower extent, some pharmaceuticals (e.g., clozapine, memantine, ropinirole or pindolol). The different land use and origin of the wastewaters (a map and brief description of the area can be found in Schell et al. (Singer et al., 2016)), with a more agricultural influence, may be pointed as the reason for the separation of these latter in the PCA.

On the other hand, PC2 (explaining the 15% of the total variance) is mainly related to the seasonal variability among the gathered wastewater effluent samples. The river flow is significantly lower in late summer as compared to spring or autumn, so lower dilution capacity and higher potential ecological risks during this season, as shown in a former study (Hernández et al., July 2018) was expected. Samples corresponding to the summer sampling campaign are grouped at the bottom of the scores plot, while the ones collected in autumn are projected in the positive

51	Risperidone	< LOQ	< LOQ	<5	15	ND	ND	20	2	< LOQ	< LOQ	< LOQ	< LOQ	ND	ND	ND	ND	<5	1	<5	158
	Antiulcerous																				
52	Omeprazol	<5	24	80	7	ND	ND	ND	ND	ND	ND	ND	ND	2210	8	1480	5	420	12	860	12
	Antiviral																				
53	Amantadine	50	2	80	14	10	5	20	1	50	4	60	8	100	8	210	6	50	3	100	1
54	Efavirenz	10	18	ND	ND	10	30	10	17	10	5	400	6	30	21	20	11	20	7	40	12
	Bactericide																				
55	Benzethonium	<5	3	<5	10	<5	14	<5	22	<5	6	<5	10	<5	3	<5	21	ND	ND	<5	26
	β-blocking																				
56	Bisoprolol	30	5	30	9	40	4	40	9	< LOQ	< LOQ	< LOQ	< LOQ	60	8	110	7	20	7	70	6
	Diuretic																				
57	Furosemide	50	1	100	2	30	10	10	5	< LOQ	< LOQ	10	8	160	2	210	4	30	2	50	3
	Stimulant																				
58	Caffeine	30	3	50	8	60	4	170	1	ND	ND	ND	ND	15200	8	48510	8	240	4	240	3
	Phytostrogen																				
59	Genistin	< LOQ	< LOQ	ND	ND	< LOQ	< LOQ	< LOQ	< LOQ	ND	ND	310	9	ND	ND	ND	ND	ND	ND	10	6
	Metabolite																				
60	Cotinine	1740	19	ND	ND	1800	25	ND	ND	ND	ND	2160	16	4700	15	ND	ND	56820	1	ND	ND
61	Desloratadine	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	10	2	< LOQ	< LOQ	ND	ND	ND	ND	ND	ND	120	2	520	5
	Sedative																				
62	Diazepam	10	4	10	5	< LOQ	< LOQ	< LOQ	< LOQ	10	8	20	3	< LOQ	< LOQ	20	5	10	7	10	14
	Pesticides																				
	Fungicides																				
63	Carbendazim	30	7	20	27	140	6	80	3	60	12	40	5	180	6	620	16	240	2	120	2
64	EDDP	10	4	20	14	20	5	20	24	20	5	40	3	60	14	190	6	40	5	40	13
65	Fenpropimorph	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1860	17	420	8	140	26	ND	ND
66	Myclobutanil	30	11	ND	ND	30	2	<5	21	30	5	<5	26	<5	12	ND	ND	10	16	ND	ND
67	Propiconazole	10	6	20	11	10	7	<5	14	10	23	10	6	<5	21	10	12	10	4	10	7
68	Tebuconazole	<5	18	<5	20	110	3	100	14	<5	2	ND	ND	ND	ND	ND	ND	<5	23	< LOQ	< LOQ
69	Tiabendazole	10	5	10	5	10	4	10	26	20	3	10	2	10	5	10	6	10	2	10	4
	Herbicides																				
70	Acetamiprid	10	7	20	13	30	4	60	9	30	1	10	4	10	14	10	15	20	5	10	1
71	Chlortoluron	< LOQ	< LOQ	10	4	160	4	7450	4	ND	ND	< LOQ	< LOQ	ND	ND	ND	ND	ND	ND	10	4
72	Diflufenican	ND	ND	< LOQ	< LOQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
73	Diuron	30	5	30	12	10	7	20	9	90	1	90	1	30	12	40	4	40	8	20	2
74	Isoproturon	< LOQ	< LOQ	< LOQ	< LOQ	20	4	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	10	13	10	9	10	4	10	3
75	Metamitron	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	10	15	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
76	Carbaril	< LOQ	< LOQ	< LOQ	< LOQ	ND	ND	ND	ND	10	4	10	7	ND	ND	< LOQ	< LOQ	ND	ND	ND	ND
77	Diethyl Toluamide	240	2	50	1	250	3	20	3	340	1	140	3	680	8	180	3	780	7	140	6
78	Fenoxycarb	ND	ND	ND	ND	ND	ND	10	11	10	1	10	5	10	9	10	1	10	16	10	2
79	Imidacloprid	70	5	60	1	440	3	140	4	280	1	230	3	130	15	100	7	90	3	130	4
	Industrial products																				
80	Benzothiazole	60	7	80	13	40	22	50	30	90	5	60	23	190	24	450	17	130	14	200	10
81	L-PFOS	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	10	19	ND	ND
82	Triethyl phosphate	100	5	150	13	80	2	130	1	180	3	230	3	160	11	230	8	130	7	240	2

axis of PC2. Based on the loadings plot, samples collected in autumn are characterized by higher loads of compounds, including mainly pharmaceuticals and pesticides. However, concerning samples from the WWTPs 2 and 3, the separation among samples collected in summer and autumn based on PC1–PC2 scores plot is not that evident. This can be explained by the size of the treatment plant itself - being those the smallest ones - or a consequence of other factors such as consumption patterns, climatology or detected analytes, among others.

3.4.2. Suspect screening

In the case of the results obtained in the suspect screening, areas of the identified compounds in the wastewater effluents were considered. Fig. 3 shows the PC1 vs. PC2 score plot for the compounds detected in the positive and negative modes, respectively. The first two PCs explained the 52% and 54% of the total variance for the results obtained in positive and negative modes, respectively. Similarly, to the observations found for the multivariate data analysis using target results, PC1 of the scores plot is related to the distribution of the samples according to the location of the treatment plants, showing the difference between the wastewater effluent samples from WWTPs 4 and 5, and the rest of the samples. In addition, seasonality is observed based on the PC2 of the scores plot. In the case of the suspect screening, the seasonal variation is more evident when plotting PC3 versus PC1, as can be observed in Fig. 4 for the results obtained in positive and negative ionization modes, respectively (49% and 52% of the total explained variance). WWTP 4 shows the largest differences between seasons, followed by 5, while number 3 exhibits a lower variability.

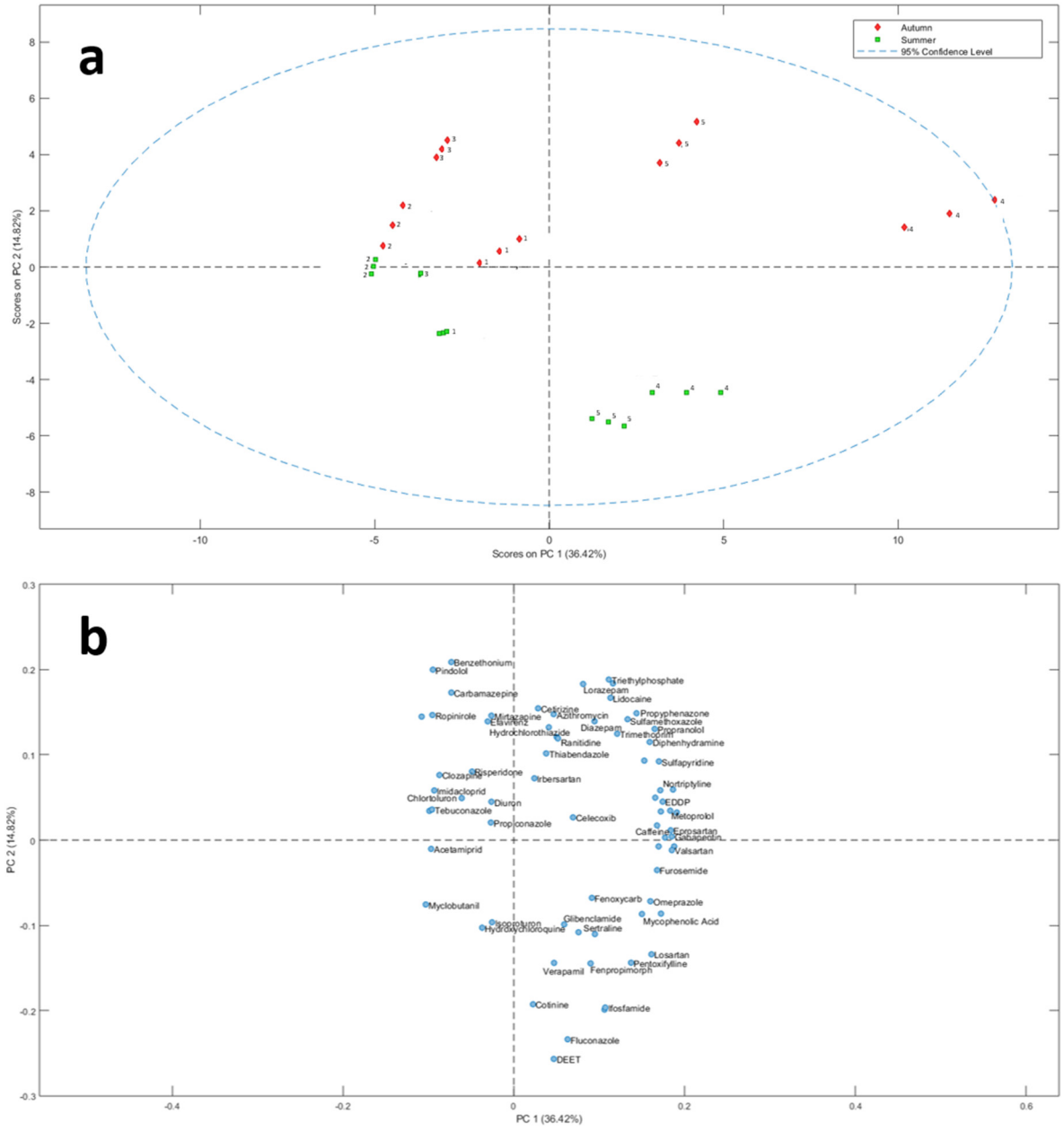

Fig. 2. PCA biplot for target compounds based on sample scores (a) and compound loadings (b).

3.5. Ecological risk assessment

RQs calculated based on the highest concentration detected for each compound among the five different WWTPs are summarized in Fig. 5. Several xenobiotic compounds exhibited RQs > 1 for the three representative taxonomic groups, indicating a potential ecological risk. According to the results obtained, algae seemed to be the organism groups with the highest potential risk, being different compounds the principal contributors (RQ > 1), namely the antibiotic azithromycin and the pesticide diuron, which exhibited the highest RQs, followed by chlortoluron and clarithromycin. Moreover, the antibiotic sulfamethoxazole, the pesticide fenpropimorph and the antidepressant sertraline, among others, also indicate a moderate risk for algae. In general, the calculated RQs for invertebrates were lower as compared to the other taxonomic groups. However, RQs higher than one were calculated for the pesticides carbendazim and fenoxycarb, and the antihypertensive eprosartan. RQs obtained for fish present a great environmental concern attributable, mainly, to the analgesic acetaminophen and the pesticide DEET, and to a lower extent, to the pesticide carbendazim, the stimulant caffeine, the antifungal fluconazole and the antibiotic azithromycin. It is noteworthy that the effect of pesticides and herbicides (unexpectedly found in the effluents, as they might come from agriculture, or from urban parks and gardens), pose a high risk to non-target fauna once released into freshwater ecosystems, even after wastewater treatments, as suggested by other authors (Kapsi et al., 2019; Altenburger et al., 2015). The effects of pesticides, even non authorized ones (diuron, carbendazim), have been previously noticed in the area (Hernández et al., July 2018), and their occurrence in wastewater effluents and riverine waters (Tiboni et al., 2008) demonstrates the need of the evaluation of their use and more restrictive controls. Moreover, the risk posed by pharmaceuticals of different groups such as antibiotics, antidepressants or antihypertensives, should be further examined in order to achieve more effective removal methods in urban WWTPs.

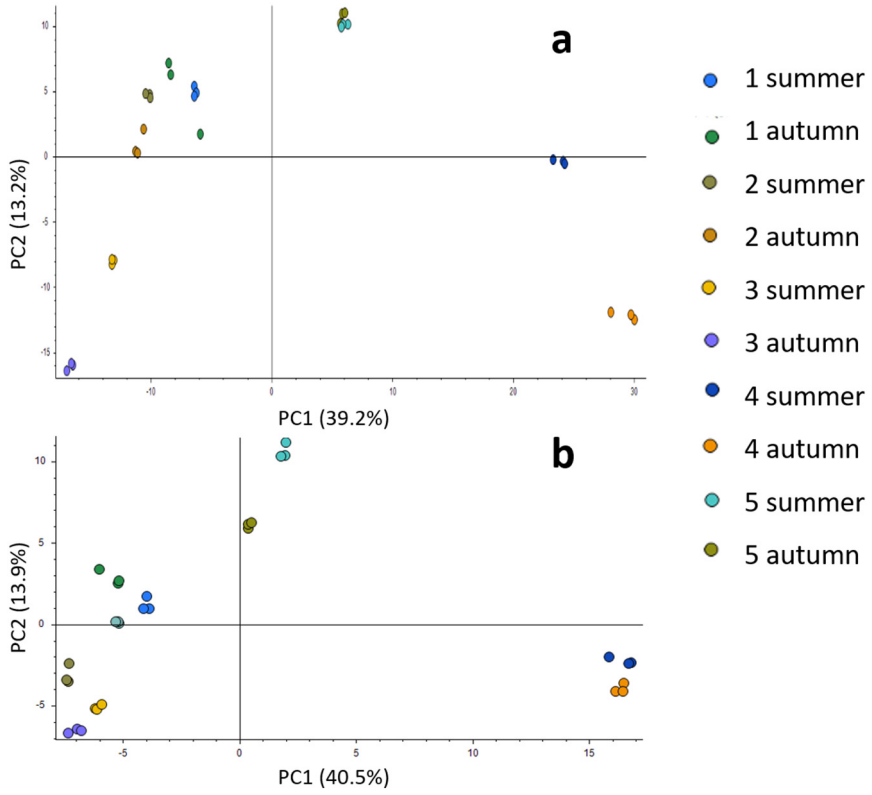

Fig. 3. PCA biplot showing the suspect analysis results in the (a) positive and (b) negative modes. PC1 and PC2 show the differences between WWTPs.

The aforementioned results are in line with recent literature for emerging contaminants in wastewaters (Moro et al., 2012; Wu et al., 2017), freshwaters (Altenburger et al., 2015; Barco-bonilla et al., 2013; FAO, 1996) and marine waters (Kapsi et al., 2019; Barco-bonilla et al., 2013), even if the higher RQs observed here are due to pesticides and not only posed by pharmaceuticals, as shown in former studies

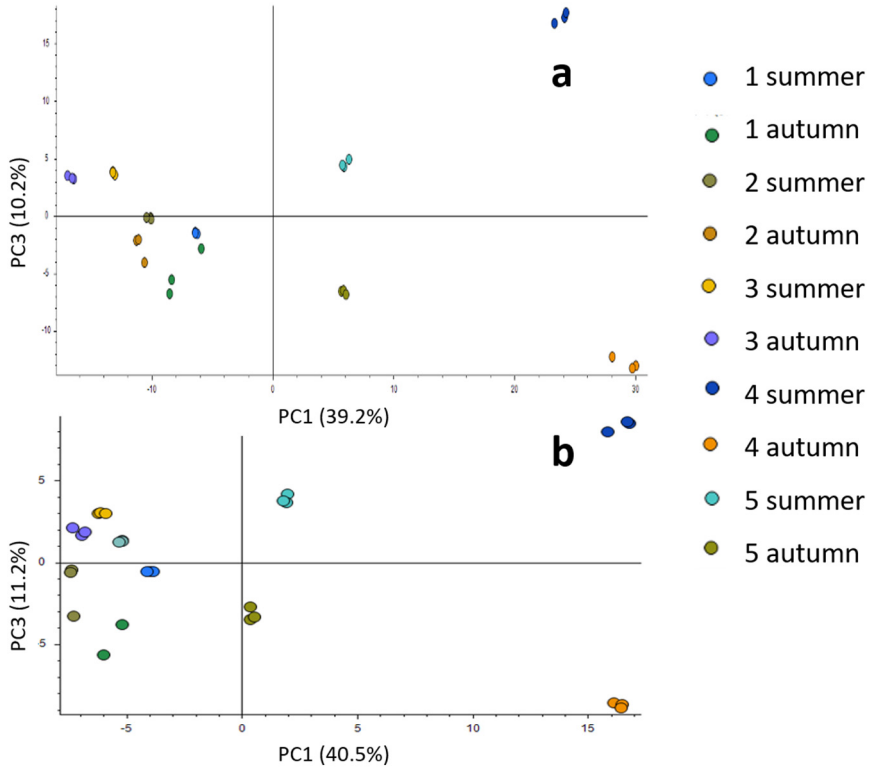

Fig. 4. PCA biplot showing the suspect analysis results in the (a) positive and (b) negative modes. PC1 and PC3 show the temporal differences.

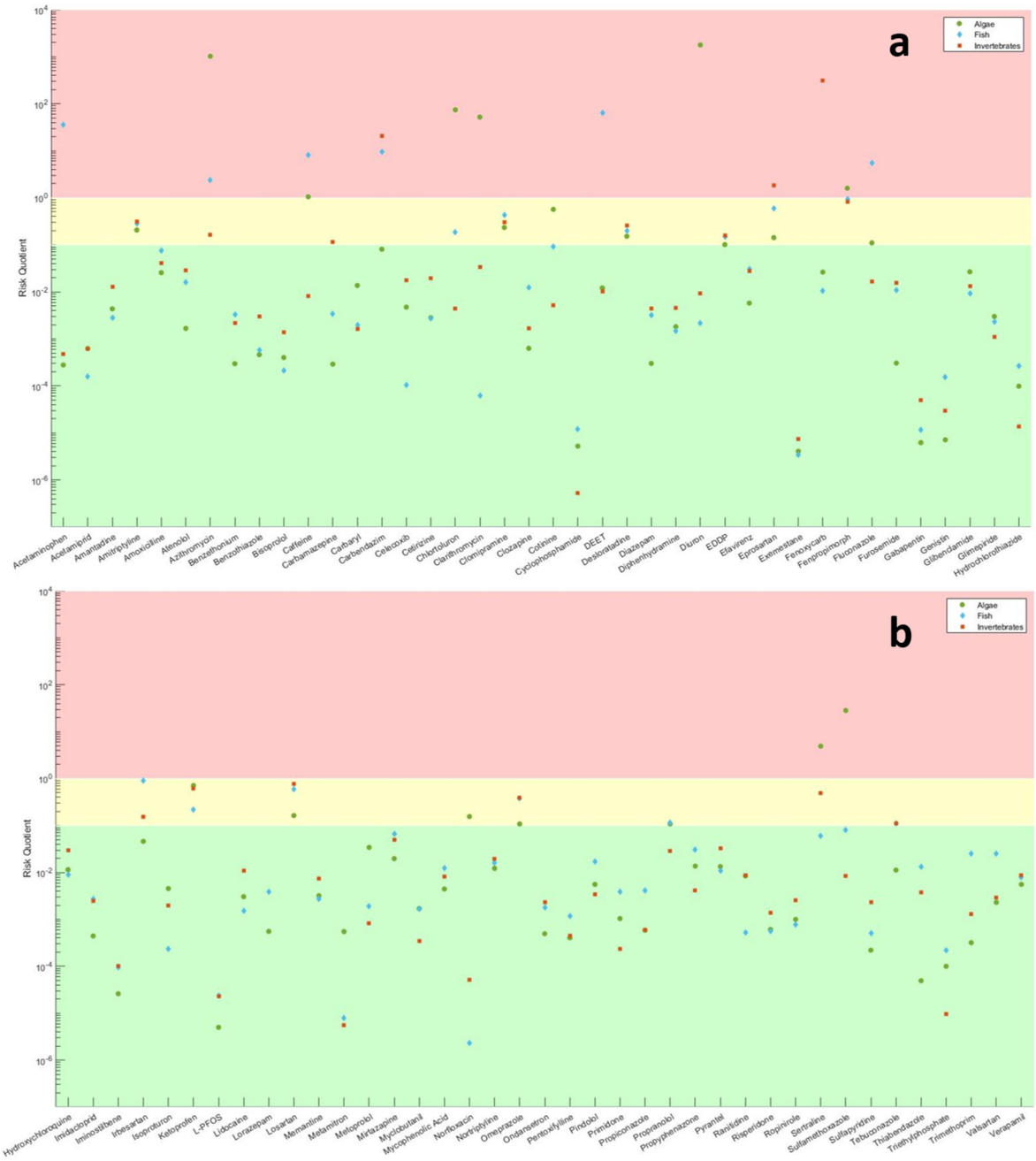

Fig. 5. Calculated RQs for each detected compound in the target analysis considering the maximum measured concentration. The compounds are sorted alphabetically from acetaminophen to hydrochlorothiazide (a), and followed by hydroxychloroquine to verapamil (b).

(Parliament, 2018; Köck-schulmeyer et al., 2013). The above findings are a clear example of the need to optimize the elimination treatments of these emerging compounds in WWTPs and to develop continued chemical and biological monitoring.

The combined RIs of each individual WWTP per season can be seen in Table 2. The mixture of compounds is expected to result in high risks for algae in WWTPs 3 and 5, mostly attributed to the generally high concentration of CECs of different classes in WWTP 3, and mainly due to the high concentration of the herbicide diuron found in both campaigns in WWTP 3. Even if these two WWTPs are the only ones with tertiary treatments, including sand filtration and phosphorous elimination (Fig. S2, SI), the concentration levels of CECs emitted into surface waters are expected to pose some environmental risks. WWTPs 1 and 2 exhibit the lowest RIs, being, nevertheless, all higher than one and thus posing a relevant risk for algae, invertebrates and fish in the receiving waters. It should be highlighted that in this study no dilution factors from the rivers have been applied (Dulio et al., 2018). Just to notice, the average annual flow in the first water gauging station after the effluents, located right after the Torote's river confluence with the Henares, is 10.5 m^3/s (1.2–55.6 m^3/s annual range between 1912 and 2017, the whole dataset available) (Fernández-López et al., 2016). The total effluent discharge of the five studied WWTPs (Fig. S2, SI) accounts for approximately the 20% of the mean annual discharge, meaning that the average dilution factor to consider would be about 5. However, the high seasonality of the smaller Torote and Monjas' streams, which may be exacerbated under the global climate change (Dulio et al., 2018), makes the approximation of this worst-case scenario very close to the actual situation posed by the combined WWTPs, remaining most of the values over 1 in the most optimistic calculations.

Table 2
RIs of each individual WWTP per season.

		Risk Index (RI)		
	WWTP	algae	invertebrates	fish
Summer	1	618	2	32
	2	243	5	39
	3	1717	204	39
	4	688	289	90
	5	1402	323	111
Autumn	1	715	2	20
	2	474	250	20
	3	1800	238	33
	4	764	268	62
	5	1556	231	31

Colors range from low RIs in green to highest RIs in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The combined effects of the detected pollutants should be further studied, paying special attention to potential synergisms among them. Moreover, the long-term effects of these contaminant mixtures on freshwater organisms are yet unknown, potentially resulting in a biodiversity decline (Alygizakis et al., 2016). Thus, the enhancement of WWTPs processes to remove xenobiotics from the effluents in areas with low dilution capacity should be prioritized (Lindholm-Lehto et al., 2016; Köck-schulmeyer et al., 2013; Altenburger et al., 2015).

4. Conclusions

Target analysis and suspect screening of contaminants of emerging concern was carried out in effluents of five WWTPs in the upper Tagus River basin at two different sampling campaigns in summer and autumn. Antibiotics, antifungals, antihypertensives, antihistamines and anti-inflammatories were among the pharmaceuticals quantified at the highest concentration, while pesticides and other industrial compounds, including benzothiazole, triethyl phosphate or PFOS were detected at trace levels. Suspect screening resulted in an efficient complementary tool to increase the number of compounds detected from the 82 analytes followed in the target analysis to up to 176 and 39 xenobiotics annotated at levels 2a–2b (probable structure found) and 3 (tentative candidates), respectively. According to the obtained results non-regulated pharmaceuticals such as mianserin, nordiazepam, clomethiazole or oxprenolol, personal care product compounds like panthenol or PEG monolaurate and pesticides such as dimetridazole, or metronidazole, to mention a few of the toxic compounds found with the non-targeted analysis, should be included in future quantitative analyses. The results of both the target and suspect screening allowed to find clear differences between effluent wastewater samples from largest WWTPs named 4 and 5, and the other three assessed stations. Moreover, temporal differences were observed, and further research should be performed to confirm those in future sampling campaigns, since this only corresponded to a one-year period. The environmental risk assessment carried out clearly showed the need to implement new technologies in WWTPs for a further elimination of contaminants of emerging concern. The most relevant compounds in terms of their ecotoxicological risk assessment were identified. The highest risk values (≫1) were obtained for azithromycin, diuron, chlortoluron, fenoxycarb, acetaminophen and DEET, affecting algae, invertebrates, and fish according to the calculated RQs. Interestingly, many pesticides drive the general risk even in WWTP effluents. The combination of the risk posed by the five WWTPs in the study area, even considering an averaged dilution factor, is of high concern for the Henares River basin. Thus, these results support the need of a wider regulation of compounds and the enhancement of the WWTPs performance and the monitoring conditions (non-directed approaches, mixtures assessment, accumulative effects in basins with low dilution capacity or highly vulnerable to global climate change) to protect the aquatic environment from xenobiotics.

CRediT authorship contribution statement

NLH Investigation, Formal analysis, Writing - original draft, Visualization.

BGG Investigation, Formal analysis, Writing - original draft, Visualization, Writing - review & editing.

NCB Investigation, Formal analysis, Writing - original draft.

AR Sample acquisition, Conceptualization, Writing review.

NE, Supervision, Resources, Funding acquisition, Writing review.

MO, Supervision, Methodology, Conceptualization, Formal analysis, Writing review.

AP Supervision, Methodology, Conceptualization, Formal analysis, Writing review.

OZ Supervision, Methodology, Conceptualization, Formal analysis, Writing review.

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.scitotenv.2021.151262.

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