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

N. Lopez-Herguedas[1], B. González-Gaya[1,2], N. Castelblanco-Boyacá[1], A. Rico[3,4], N. Etxebarria[1,2], M. Olivares[1,2], A. Prieto[1,2], O. Zuloaga[1,2]

1. Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Leioa, Basque Country, Spain
2. Research Centre for Experimental Marine Biology and Biotechnology (PIE), University of the Basque Country (UPV/EHU), Plentzia, Basque Country, Spain
3. IMDEA Water Institute, Science and Technology Campus of the University of Alcalá, Alcalá de Henares, Madrid, Spain
4. Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Paterna, Valencia, Spain

KEYWORDS

Wastewater; suspect analysis; contaminants of emerging concern; pharmaceuticals; risk assessment

HIGHLIGTHS

- 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 40000 compounds
- RQs revealed that pharmaceuticals and pesticides pose high risk in the area
- WWTPs need to enhance their performance to decrease their discharges riskiness

ABSTRACT

The scientific and societal interest in contaminants of emerging concern (CECs) has increased during the last decades due to their continued emission and their 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 (more than 40000 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 pharmaceutical active ingredients, 21 % to pesticides and 3 % to industrial chemicals. Apart from the 82 compounds quantified during the target analysis, suspect screening increased the list to 297 annotated compounds. Significant differences in the CEC fingerprint were observed between the summer and autumn campaigns and between the WWTPs, being those serving the city of Alcalá de Henares the ones with the largest number of identified 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 of some substances in urban WWTPs. Furthermore, considering the complex mixtures emitted into the environment and the low dilution capacity of Mediterranean rivers such as those in the study area, 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 [1]. 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 [2]. 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.) [3–9].

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 [10–12]. Nevertheless, the list of anthropogenic compounds being detected in aquatic systems receiving urban, agricultural and industrial treated wastewaters is wider [13–15], 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 [4,8,16]. 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 [8,17–20]. 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 [1,8,21–23]. 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 [8,17,24,25]. Therefore, the occurrence of these micropollutants has to be controlled in surface waters [8,9,16,17,26–29] and in soil and sediments [30,31]. 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 [30,32–34] and plants [35]. However, the potential environmental hazard of CECs mixtures is still poorly understood [4,36]. 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 [37], which is particularly relevant in areas affected by water scarcity [38,39].

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 [40,41]. 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 [41,42].

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 [43].

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 Figure 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 Figure S1, while further information regarding the amount of sludge produced or detailed treatment steps can be obtained from Schell et al [44]. 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 dicharge 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 9,800 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^{o}C to the University of the Basque Country (UPV/EHU) in October of 2019 and kept t 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[19]. 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 ^{o}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 ^{o}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 x 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 70000 FWHM for a 200 *m/z* relation, and it was followed by three ddMS2 scans with a resolution of 17500 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 [19]. To avoid false positives, experimental tR window was limited to 60 second 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 Figure 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 (Thermo-Fisher 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 [45] 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 a more accurate value). 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 Gonzalez-Gaya and co-workers [19]. 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 [19], 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 taking into account 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 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 [46]. 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 [24,47] (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 [48]. The PNEC values were calculated considering the lowest chronic toxicity data (no observed effect concentration, NOEC) collected from the ecotoxicology knowledge-base (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 [24,49], 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 [24]. 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[50]. 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 [24,49] 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 LOI_{proc} and $LOQs_{proc}$ are comparable to those reported in previous European studies [23,24,49,51,52].

3.1.2. Recoveries and precision

As depicted in the box-whisker diagram in Figure 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 Figure 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-4,225 ng/L) and antiinflamatories (<LOQ-1,425 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 [53] and is often detected in wastewater[54]. Ranitidine (antihistaminic) 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[55], and like fluconazole, is one of the most common pharmaceuticals prescribed and used in common diseases, thus prone to be found in domestic wastewaters [56,57]. 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 (1,799-56,817 ng/L). Regarding pesticides, fungicides and herbicides showed similar occurrence regardless of the wastewater effluents analyzed, only standing out the concentrations of fenpropimorph (1,858 ng/L) and chlortoluron (7,445 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 [58] and grass weed[59], respectively, and due to the agricultural land use in the area [44] 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 [5,17,23,24,30,49,51,52]. A previous study performed in small rivers and streams within the area[60], 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[61] 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 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 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 Figure 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 [45]. 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 (Figure 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[62–65] and pose adverse effects to wild fauna, and even if some of them are regulated (such as metronidazole, banned in some countries)[65], 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 [66,67]. This reveals the need of the development of a more appropriate treatment for the urban wastewaters to eliminate these active and 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. Figure 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 (Figure 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. [44]), with a more agricultural influence, may be pointed as the reason for the separation of these latter in the PCA space.

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[68] 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 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. Figure 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 Figure 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.

3.5. Ecological risk assessment

RQs calculated based on the highest concentration detected for each compound among the five different WWTPs are summarized in Figure 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 [69,70]. The effects of pesticides, even non authorized ones (diuron, carbendazim), have been previously noticed in the area[68], and their occurrence in wastewater effluents and riverine waters[60]

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.

The aforementioned results are in line with recent literature for emerging contaminants in wastewaters [31,49], freshwaters [24,26,70] and marine waters [26,69], even if the higher RQs observed here are due to pesticides and not only posed by pharmaceuticals, as shown in former studies [4,13]. The above findings are a clear example of the need to optimize the elimination treatments of these emerging compounds in WWTPs, 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 (Figure 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 [38]. 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) [71]. The total effluent discharge of the five studied WWTPs (Figure 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 [38], 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.

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 fresh water organisms are yet unknown, potentially resulting in a biodiversity decline[72]. Thus, the enhancement of WWTPs processes to remove xenobiotics from the effluents in areas with low dilution capacity should be prioritized[4,70,73]. Additionally, it should be mentioned that the ERA performed here disregard possible synergic effects caused by complex CEC mixtures, which may increase the potential ecological risk posed to aquatic organisms.

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, antihistaminics 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 taking into account 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.

Acknowledgements

Authors acknowledge financial support from the Agencia Estatal de Investigación (AEI) of Spain and the European Regional Development Fund through project CTM2017-84763-C3-1-R project and the Basque Government through the financial support as consolidated group of the Basque Research System (IT1213-19). NLH is grateful to the Spanish Ministry of Economy, Industry and Competitivity for her predoctoral scholarship FPI 2018. BGG acknowledge an EHU/UPV postdoctoral fellowship. AR is supported by the Talented Researcher Support Programme - Plan GenT (CIDEGENT/2020/043) of the

Generalitat Valenciana. Finally, the authors acknowledge support from the AEI and the Ministry of Science, Innovation and Universities (MICIU) to support the Thematic Network of Excellence (NET4SEA) on emerging contaminants in marine settings (CTM2017-90890-REDT, MICIU/AEI/FEDER, EU).

REFERENCES

(1) Luo, Y.; Guo, W.; Hao, H.; Duc, L.; Ibney, F.; Zhang, J.; Liang, S.; Wang, X. C. A Review on the Occurrence of Micropollutants in the Aquatic Environment and Their Fate and Removal during Wastewater Treatment. *Science of the Total Environment* **2014**, *473–474*, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065.

(2) Dulio, V.; van Bavel, B.; Brorström-Lundén, E.; Harmsen, J.; Hollender, J.; Schlabach, M.; Slobodnik, J.; Thomas, K.; Koschorreck, J. Emerging Pollutants in the EU: 10 Years of NORMAN in Support of Environmental Policies and Regulations. *Environmental Sciences Europe* **2018**, *30* (1), 5. https://doi.org/10.1186/s12302-018-0135-3.

(3) Cruzeiro, C.; Rocha, E.; João, M. Determination of 54 Pesticides in Waters of the Iberian Douro River Estuary and Risk Assessment of Environmentally Relevant Mixtures Using Theoretical Approaches and Artemia Salina and Daphnia Magna Bioassays. *Ecotoxicology and Environmental Safety* **2017**, *145* (July), 126–134. https://doi.org/10.1016/j.ecoenv.2017.07.010.

(4) Fonseca, E.; Hernández, F.; Ibáñez, M.; Rico, A.; Pitarch, E. Occurrence and Ecological Risks of Pharmaceuticals in a Mediterranean River in Eastern Spain. *Environment International* **2020**, *144* (June), 106004. https://doi.org/10.1016/j.envint.2020.106004.

(5) Blasco, C.; Sancho, J. V; Picó, Y.; Hernández, F.; Masiá, A.; Ibá, M. Combined Use of Liquid Chromatography Triple Quadrupole Mass Spectrometry and Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry in Systematic Screening of Pesticides and Other Contaminants in Water Samples. *Analytica Chimica Acta* **2013**, *761*, 117–127. https://doi.org/10.1016/j.aca.2012.11.032.

(6) Hernández, F.; Calısto-ulloa, N.; Gómez-fuentes, C.; Gómez, M.; Ferrer, J. Occurrence of Antibiotics and Bacterial Resistance in Wastewater and Sea Water from the Antarctic. *Journal of Hazardous Materials* **2019**, *363* (July 2018), 447–456. https://doi.org/10.1016/j.jhazmat.2018.07.027.

(7) Maruya, K. A.; Dodder, N. G.; Sengupta, A.; Smith, D. J.; Lyons, J. M.; Heil, A. T.; Drewes, J. E. Multimedia Screening of Contaminants of Emerging Concern (CECs) in Coastal Urban Watersheds in Southern California (USA). *Environmental Toxicology and Chemistry* **2016**, *35* (8), 1986–1994. https://doi.org/10.1002/etc.3348.

(8) Lindholm-Lehto, P. C.; Ahkola, H. S. J.; Knuutinen, J. S.; Herve, S. H. Widespread Occurrence and Seasonal Variation of Pharmaceuticals in Surface Waters and Municipal Wastewater Treatment Plants in Central Finland. *Environmental Science and Pollution Research* **2016**, 7985–7997. https://doi.org/10.1007/s11356-015-5997-y.

(9) Alda, D.; Gil, A.; Gorga, M.; Valc, Y.; Navas, J. M.; Petrovic, M.; Barcel, D. Determining the Presence of Chemicals with Suspected Endocrine Activity in Drinking Water from the Madrid Region (Spain) and Assessment of Their Estrogenic , Androgenic and Thyroidal Activities. *Chemosphere* **2018**, *201*, 388–398. https://doi.org/10.1016/j.chemosphere.2018.02.099.

(10) European Parliament. *Commission Implementing Decision (EU) 2015/495 of 20 March 2015 Establishing a Watch List of Substances for Union-Wide Monitoring in the Field of Water Policy Pursuant to Directive 2008/105/EC of the European Parliament and of the*

649 *Council (Notified under Document C(2015) 1756) Text with EEA Relevance*; 2015; Vol.
650 078.
651 (11) European Parliament. Commission Implementing Decision (EU) 2018/ 840 - of 5 June
652 2018 - Establishing a Watch List of Substances for Union-Wide Monitoring in the
653 Field of Water Policy Pursuant to Directive 2008/ 105/ EC of the European
654 Parliament and of the Council and Repealing Commission Implementing Decision
655 (EU) 2015/ 495 - (Notified under Document C(2018) 3362). **2018**, 4.
656 (12) US EPA, O. Contaminants of Emerging Concern including Pharmaceuticals and Personal
657 Care Products https://www.epa.gov/wqc/contaminants-emerging-concern-including-
658 pharmaceuticals-and-personal-care-products (accessed 2020 -03 -19).
659 (13) Bijlsma, L.; Pitarch, E.; Fonseca, E.; Ibáñez, M.; Botero, A. M.; Claros, J.; Pastor, L.;
660 Hernández, F. Investigation of Pharmaceuticals in a Conventional Wastewater
661 Treatment Plant: Removal Efficiency, Seasonal Variation and Impact of a Nearby
662 Hospital. *Journal of Environmental Chemical Engineering* **2021**, 105548.
663 https://doi.org/10.1016/j.jece.2021.105548.
664 (14) Gosset, A.; Wiest, L.; Fildier, A.; Libert, C.; Giroud, B.; Hammada, M.; Hervé, M.; Sibeud,
665 E.; Vulliet, E.; Polomé, P.; Perrodin, Y. Ecotoxicological Risk Assessment of Contaminants
666 of Emerging Concern Identified by "Suspect Screening" from Urban Wastewater
667 Treatment Plant Effluents at a Territorial Scale. *Science of The Total Environment* **2021**,
668 *778*, 146275. https://doi.org/10.1016/j.scitotenv.2021.146275.
669 (15) Tröger, R.; Ren, H.; Yin, D.; Postigo, C.; Nguyen, P.-D.; Baduel, C.; Golovko, O.; Been, F.;
670 Joerss, H.; Boleda, M. R.; Polesello, S.; Roncoroni, M.; Taniyasu, S.; Menger, F.; Ahrens,
671 L.; Lai, F. Y.; Wiberg, K. What's in the Water? – Target and Suspect Screening of
672 Contaminants of Emerging Concern in Raw Water and Drinking Water from Europe and
673 Asia. *Water Research* **2021**, 117099. https://doi.org/10.1016/j.watres.2021.117099.
674 (16) Kapsi, M.; Tsoutsi, C.; Paschalidou, A.; Albanis, T. Environmental Monitoring and Risk
675 Assessment of Pesticide Residues in Surface Waters of the Louros River (N.W. Greece).
676 *Science of the Total Environment* **2019**, *650*, 2188–2198.
677 https://doi.org/10.1016/j.scitotenv.2018.09.185.
678 (17) Paíga, P.; Santos, L. H. M. L. M. Development of a Multi-Residue Method for the
679 Determination of Human and Veterinary Pharmaceuticals and Some of Their
680 Metabolites in Aqueous Environmental Matrices by SPE-UHPLC – MS / MS. *Journal of*
681 *Pharmaceutical and Biomedical Analysis* **2017**, *135*, 75–86.
682 https://doi.org/10.1016/j.jpba.2016.12.013.
683 (18) Letsinger, S.; Kay, P.; Rodríguez-mozaz, S.; Villagrassa, M.; Barceló, D.; Rotchell, J. M.
684 Spatial and Temporal Occurrence of Pharmaceuticals in UK Estuaries. *Science of the*
685 *Total Environment* **2019**, *678*, 74–84. https://doi.org/10.1016/j.scitotenv.2019.04.182.
686 (19) González-Gaya, B.; Lopez-herguedas, N.; Santamaria, A.; Mijangos, F.; Etxebarria, N.;
687 Olivares, M.; Prieto, A.; Zuloaga, O. Suspect Screening Workflow Comparison for the
688 Analysis of Organic Xenobiotics in Environmental Water Samples. *Chemosphere* **2021**,
689 *274*, 1–9. https://doi.org/10.1016/j.chemosphere.2021.129964.
690 (20) Bakker, J.; Bijlsma, L.; Boer, J. De; Botero-coy, A. M.; L, F. J. The Role of Analytical
691 Chemistry in Exposure Science : Focus on the Aquatic Environment. *Chemosphere* **2019**,
692 *222*, 564–583. https://doi.org/10.1016/j.chemosphere.2019.01.118.
693 (21) Botero-coy, A. M.; Martínez-pachón, D.; Boix, C.; Rincón, R. J.; Castillo, N.; Arias-marín,
694 L. P. An Investigation into the Occurrence and Removal of Pharmaceuticals in
695 Colombian Wastewater. *Science of the Total Environment* **2018**, *642*, 842–853.
696 https://doi.org/10.1016/j.scitotenv.2018.06.088.
697 (22) Mart, L.; Castro, A. J.; Mart, I.; Jos, J. Priority Organic Compounds in Wastewater e Ffl
698 Uents from the Mediterranean and Atlantic Basins Of. *Environmental Science: Processes*
699 *and Impacts* **2013**, 2194–2203. https://doi.org/10.1039/c3em00329a.

(23) Köck-schulmeyer, M.; Villagrasa, M.; López, M.; Alda, D.; Céspedes-sánchez, R.; Ventura, F.; Barceló, D. Occurrence and Behavior of Pesticides in Wastewater Treatment Plants and Their Environmental Impact. *Science of the Total Environment* **2013**, *460*, 466–476. https://doi.org/10.1016/j.scitotenv.2013.04.010.

(24) Paíga, P.; Santos, L. H. M. L. M.; Ramos, S.; Jorge, S.; Gabriel, J.; Delerue-matos, C. Presence of Pharmaceuticals in the Lis River (Portugal): Sources , Fate and Seasonal Variation. *Science of the Total Environment* **2016**, *573*, 164–177. https://doi.org/10.1016/j.scitotenv.2016.08.089.

(25) Hollender, J.; Rothardt, J.; Radny, D.; Loos, M.; Epting, J.; Huggenberger, P.; Borer, P.; Singer, H. Comprehensive Micropollutant Screening Using LC-HRMS / MS at Three Riverbank Fi Ltration Sites to Assess Natural Attenuation and Potential Implications for Human Health. *Water Research X* **2018**, *1*, 100007. https://doi.org/10.1016/j.wroa.2018.100007.

(26) Minguez, L.; Pedelucq, J.; Farcy, E.; Ballandonne, C.; Budzinski, H. Toxicities of 48 Pharmaceuticals and Their Freshwater and Marine Environmental Assessment in Northwestern France. *Environmental Science and Pollution Research* **2016**, 4992–5001. https://doi.org/10.1007/s11356-014-3662-5.

(27) Navarro-ortega, A.; Pic, Y.; Ccanccapa, A.; Masi, A.; Val, U. De. Pesticides in the Ebro River Basin : Occurrence and Risk Assessment. *Environmental Pollution* **2016**, *211*, 414–424. https://doi.org/10.1016/j.envpol.2015.12.059.

(28) Čelić, M.; Škrbić, B. D.; Insa, S.; Živančev, J.; Gros, M. Occurrence and Assessment of Environmental Risks of Endocrine Disrupting Compounds in Drinking, Surface and Wastewaters in Serbia. *Environmental Pollution* **2020**. https://doi.org/10.1016/j.envpol.2020.114344.

(29) Mijangos, L.; Ziarrusta, H.; Ros, O.; Kortazar, L.; Fernández, L. A.; Olivares, M.; Zuloaga, O.; Prieto, A.; Etxebarria, N. Occurrence of Emerging Pollutants in Estuaries of the Basque Country: Analysis of Sources and Distribution, and Assessment of the Environmental Risk. *Water Research* **2018**, *147*, 152–163. https://doi.org/10.1016/j.watres.2018.09.033.

(30) Carmona, E.; Andreu, V.; Picó, Y. Multi-Residue Determination of 47 Organic Compounds in Water , Soil , Sediment and Fish — Turia River as Case Study. *Journal of Pharmaceutical and Biomedical Analysis* **2017**, *146*, 117–125. https://doi.org/10.1016/j.jpba.2017.08.014.

(31) Picó, Y.; Alvarez-ruiz, R.; Alfarhan, A. H.; El-sheikh, M. A.; Alshahrani, H. O.; Barceló, D. Pharmaceuticals , Pesticides , Personal Care Products and Microplastics Contamination Assessment of Al-Hassa Irrigation Network (Saudi Arabia) and Its Shallow Lakes. *Science of the Total Environment* **2020**, *701*. https://doi.org/10.1016/j.scitotenv.2019.135021.

(32) Muir, D.; Simmons, D.; Wang, X.; Peart, T.; Villella, M.; Miller, J.; Sherry, J. Bioaccumulation of Pharmaceuticals and Personal Care Product Chemicals in Fish Exposed to Wastewater Effluent in an Urban Wetland. *Scientific Reports* **2017**, No. June, 1–11. https://doi.org/10.1038/s41598-017-15462-x.

(33) Lorenzo, M.; Campo, J.; Suárez-varela, M. M.; Picó, Y. Occurrence , Distribution and Behavior of Emerging Persistent Organic Pollutants (POPs) in a Mediterranean Wetland Protected Area. *Science of the Total Environment* **2019**, *646*, 1009–1020. https://doi.org/10.1016/j.scitotenv.2018.07.304.

(34) Ruhí, A.; Acuña, V.; Barceló, D.; Huerta, B.; Mor, J.; Rodríguez-mozaz, S.; Sabater, S. Bioaccumulation and Trophic Magnification of Pharmaceuticals and Endocrine Disruptors in a Mediterranean River Food Web. *Science of the Total Environment* **2016**, *540*, 250–259. https://doi.org/10.1016/j.scitotenv.2015.06.009.

(35) Picó, Y.; Alvarez-ruiz, R.; Alfarhan, A. H.; El-sheikh, M. A.; Alobaid, S. M.; Barceló, D. Uptake and Accumulation of Emerging Contaminants in Soil and Plant Treated with

Wastewater under Real-World Environmental Conditions in the Al Hayer Area (Saudi Arabia). *Science of the Total Environment* **2019**, *652*, 562–572. https://doi.org/10.1016/j.scitotenv.2018.10.224.

(36) Nilsen, E.; Smalling, K. L.; Ahrens, L.; Gros, M.; Miglioranza, K. S. B.; Picó, Y.; Schoenfuss, H. L. Critical Review: Grand Challenges in Assessing the Adverse Effects of Contaminants of Emerging Concern on Aquatic Food Webs. *Environmental Toxicology and Chemistry* **2019**, *38* (1), 46–60. https://doi.org/10.1002/etc.4290.

(37) Link, M.; von der Ohe, P. C.; Voß, K.; Schäfer, R. B. Comparison of Dilution Factors for German Wastewater Treatment Plant Effluents in Receiving Streams to the Fixed Dilution Factor from Chemical Risk Assessment. *Science of The Total Environment* **2017**, *598*, 805–813. https://doi.org/10.1016/j.scitotenv.2017.04.180.

(38) Abily, M.; Vicenç, A.; Gernjak, W.; Rodríguez-Roda, I.; Poch, M.; Corominas, L. Climate Change Impact on EU Rivers' Dilution Capacity and Ecological Status. *Water Research* **2021**, *199*, 117166. https://doi.org/10.1016/j.watres.2021.117166.

(39) Köck-Schulmeyer, M.; Ginebreda, A.; Postigo, C.; López-Serna, R.; Pérez, S.; Brix, R.; Llorca, M.; Alda, M. L. de; Petrović, M.; Munné, A.; Tirapu, L.; Barceló, D. Wastewater Reuse in Mediterranean Semi-Arid Areas: The Impact of Discharges of Tertiary Treated Sewage on the Load of Polar Micro Pollutants in the Llobregat River (NE Spain). *Chemosphere* **2011**, *82* (5), 670–678. https://doi.org/10.1016/j.chemosphere.2010.11.005.

(40) Altenburger, R.; Ait-Aissa, S.; Antczak, P.; Backhaus, T.; Barceló, D.; Seiler, T.-B.; Brion, F.; Busch, W.; Chipman, K.; de Alda, M. L.; de Aragão Umbuzeiro, G.; Escher, B. I.; Falciani, F.; Faust, M.; Focks, A.; Hilscherova, K.; Hollender, J.; Hollert, H.; Jäger, F.; Jahnke, A.; Kortenkamp, A.; Krauss, M.; Lemkine, G. F.; Munthe, J.; Neumann, S.; Schymanski, E. L.; Scrimshaw, M.; Segner, H.; Slobodnik, J.; Smedes, F.; Kughathas, S.; Teodorovic, I.; Tindall, A. J.; Tollefsen, K. E.; Walz, K.-H.; Williams, T. D.; Van den Brink, P. J.; van Gils, J.; Vrana, B.; Zhang, X.; Brack, W. Future Water Quality Monitoring — Adapting Tools to Deal with Mixtures of Pollutants in Water Resource Management. *Science of The Total Environment* **2015**, *512–513*, 540–551. https://doi.org/10.1016/j.scitotenv.2014.12.057.

(41) González-Gaya, B.; Lopez-Herguedas, N.; Bilbao, D.; Mijangos, L.; M. Iker, A.; Etxebarria, N.; Irazola, M.; Prieto, A.; Olivares, M.; Zuloaga, O. Suspect and Non-Target Screening: The Last Frontier in Environmental Analysis. *Analytical Methods* **2021**, *13* (16), 1876–1904. https://doi.org/10.1039/D1AY00111F.

(42) Gago-Ferrero, P.; Schymanski, E. L.; Hollender, J.; Thomaidis, N. S. Chapter 13 - Nontarget Analysis of Environmental Samples Based on Liquid Chromatography Coupled to High Resolution Mass Spectrometry (LC-HRMS). In *Comprehensive Analytical Chemistry*; Pérez, S., Eichhorn, P., Barceló, D., Eds.; Applications of Time-of-Flight and Orbitrap Mass Spectrometry in Environmental, Food, Doping, and Forensic Analysis; Elsevier, 2016; Vol. 71, pp 381–403. https://doi.org/10.1016/bs.coac.2016.01.012.

(43) Aalizadeh, R.; Alygizakis, N. A.; Schymanski, E. L.; Krauss, M.; Schulze, T.; Ibáñez, M.; McEachran, A. D.; Chao, A.; Williams, A. J.; Gago-Ferrero, P.; Covaci, A.; Moschet, C.; Young, T. M.; Hollender, J.; Slobodnik, J.; Thomaidis, N. S. Development and Application of Liquid Chromatographic Retention Time Indices in HRMS-Based Suspect and Nontarget Screening. *Anal. Chem.* **2021**. https://doi.org/10.1021/acs.analchem.1c02348.

(44) Schell, T.; Hurley, R.; Nizzetto, L.; Rico, A.; Vighi, M. Spatio-Temporal Distribution of Microplastics in a Mediterranean River Catchment: The Importance of Wastewater as an Environmental Pathway. *Journal of Hazardous Materials* **2021**, *420*, 126481. https://doi.org/10.1016/j.jhazmat.2021.126481.

(45) Schymanski, E. L.; Jeon, J.; Gulde, R.; Fenner, K.; Ru, M.; Singer, H. P.; Hollender, J. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Con Fi Dence. **2014**, 2097–2098.
(46) European Parliament. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (Text with EEA relevance)Text with EEA relevance https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A02006R1907-20190702 (accessed 2020 -03 -19).
(47) Alygizakis, N. A.; Gago-Ferrero, P.; Borova, V. L.; Pavlidou, A.; Hatzianestis, I.; Thomaidis, N. S. Occurrence and Spatial Distribution of 158 Pharmaceuticals, Drugs of Abuse and Related Metabolites in Offshore Seawater. *Sci. Total Environ.* **2016**, *541*, 1097–1105. https://doi.org/10.1016/j.scitotenv.2015.09.145.
(48) European Commission. Joint Research Centre. Institute for Health and Consumer Protection. *Assessment of Mixtures: Review of Regulatory Requirements and Guidance.*; Publications Office: LU, 2017.
(49) Papageorgiou, M.; Kosma, C.; Lambropoulou, D. Seasonal Occurrence , Removal , Mass Loading and Environmental Risk Assessment of 55 Pharmaceuticals and Personal Care Products in a Municipal Wastewater Treatment Plant in Central Greece. *Science of the Total Environment* **2016**, *543*, 547–569. https://doi.org/10.1016/j.scitotenv.2015.11.047.
(50) European Commission Joint Research Centre, I. (IT). Technical Guidance Document on Risk Assessment in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for Existing Substances, and Directive 98/8/EC of the European Parliament and of the Council Concerning the Placing of Biocidal Products on the Market. EUR 2003.
(51) Barco-bonilla, N.; Romero-González, R.; Plaza-bolaños, P.; Martínez, J. L.; Garrido, A. Systematic Study of the Contamination of Wastewater Treatment Plant Ef Fl Uents by Organic Priority Compounds in Almeria Province (SE Spain). *Science of the Total Environment* **2013**, *447*, 381–389. https://doi.org/10.1016/j.scitotenv.2013.01.027.
(52) Fernández-López, C.; Guillén-Navarro, J. M.; Javier, J.; Parsons, J. R. Comparison of the Removal Efficiencies of Selected Pharmaceuticals in Wastewater Treatment Plants in the Region of Murcia, Spain. *Ecological Engineering* **2016**, *95*, 811–816. https://doi.org/10.1016/j.ecoleng.2016.06.093.
(53) Grant, S. M.; Clissold, S. P. Fluconazole. *Drugs* **1990**, *39* (6), 877–916. https://doi.org/10.2165/00003495-199039060-00006.
(54) Kahle, M.; Buerge, I. J.; Hauser, A.; Müller, M. D.; Poiger, T. Azole Fungicides: Occurrence and Fate in Wastewater and Surface Waters. *Environ. Sci. Technol.* **2008**, *42* (19), 7193–7200. https://doi.org/10.1021/es8009309.
(55) Grant, S. M.; Langtry, H. D.; Brogden, R. N. Ranitidine. *Drugs* **1989**, *37* (6), 801–870. https://doi.org/10.2165/00003495-198937060-00003.
(56) Gómez, M. J.; Petrović, M.; Fernández-Alba, A. R.; Barceló, D. Determination of Pharmaceuticals of Various Therapeutic Classes by Solid-Phase Extraction and Liquid Chromatography–Tandem Mass Spectrometry Analysis in Hospital Effluent Wastewaters. *Journal of Chromatography A* **2006**, *1114* (2), 224–233. https://doi.org/10.1016/j.chroma.2006.02.038.
(57) Gros, M.; Petrović, M.; Barceló, D. Development of a Multi-Residue Analytical Methodology Based on Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) for Screening and Trace Level Determination of Pharmaceuticals in Surface and

Wastewaters. *Talanta* **2006**, *70* (4), 678–690. https://doi.org/10.1016/j.talanta.2006.05.024.
(58) FAO. *Pesticide Residues in Food 1995 - Report 1995: Joint FAO/WHO Meeting on Pesticide Residues*; Estudio FAO: Producción y Protección Vegetal; FAO and WHO: Rome, Italy, 1996.
(59) Moro, C. V.; Bricheux, G.; Portelli, C.; Bohatier, J. Comparative Effects of the Herbicides Chlortoluron and Mesotrione on Freshwater Microalgae. *Environmental Toxicology and Chemistry* **2012**, *31* (4), 778–786. https://doi.org/10.1002/etc.1749.
(60) Rico, A.; Arenas-Sánchez, A.; Alonso-Alonso, C.; López-Heras, I.; Nozal, L.; Rivas-Tabares, D.; Vighi, M. Identification of Contaminants of Concern in the Upper Tagus River Basin (Central Spain). Part 1: Screening, Quantitative Analysis and Comparison of Sampling Methods. *Science of The Total Environment* **2019**, *666*, 1058–1070. https://doi.org/10.1016/j.scitotenv.2019.02.250.
(61) MAGRAMA. Spanish Ministry of Food, Agriculture and Fisheries. Plant protection products registration office https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/menu.asp (accessed 2021 -09 -27).
(62) Jayaraj, R.; Megha, P.; Sreedev, P. Organochlorine Pesticides, Their Toxic Effects on Living Organisms and Their Fate in the Environment. *Interdiscip Toxicol* **2016**, *9* (3–4), 90–100. https://doi.org/10.1515/intox-2016-0012.
(63) Arias, M.; Chevallier, O. P.; Graham, S. F.; Gasull-Gimenez, A.; Fodey, T.; Cooper, K. M.; Crooks, S. R. H.; Danaher, M.; Elliott, C. T. Metabolomics Reveals Novel Biomarkers of Illegal 5-Nitromimidazole Treatment in Pigs. Further Evidence of Drug Toxicity Uncovered. *Food Chemistry* **2016**, *199*, 876–884. https://doi.org/10.1016/j.foodchem.2015.12.075.
(64) Wu, M.; Liu, S.; Hu, L.; Qu, H.; Pan, C.; Lei, P.; Shen, Y.; Yang, M. Global Transcriptomic Analysis of Zebrafish in Response to Embryonic Exposure to Three Antidepressants, Amitriptyline, Fluoxetine and Mianserin. *Aquatic Toxicology* **2017**, *192*, 274–283. https://doi.org/10.1016/j.aquatox.2017.09.027.
(65) Tiboni, G. M.; Marotta, F.; Castigliego, A. P. Teratogenic Effects in Mouse Fetuses Subjected to the Concurrent in Utero Exposure to Miconazole and Metronidazole. *Reproductive Toxicology* **2008**, *26* (3), 254–261. https://doi.org/10.1016/j.reprotox.2008.09.005.
(66) Hug, C.; Ulrich, N.; Schulze, T.; Brack, W.; Krauss, M. Identification of Novel Micropollutants in Wastewater by a Combination of Suspect and Nontarget Screening. *Environmental Pollution* **2014**, *184*, 25–32. https://doi.org/10.1016/j.envpol.2013.07.048.
(67) Singer, H. P.; Mcardell, C. S.; Fenner, K. Rapid Screening for Exposure to " Non-Target " Pharmaceuticals from Wastewater Effluents by Combining HRMS-Based Suspect Screening and Exposure Modeling. *Environmental Science & Technology* **2016**. https://doi.org/10.1021/acs.est.5b03332.
(68) Arenas-Sánchez, A.; Rico, A.; Rivas-Tabares, D.; Blanco, A.; Garcia-Doncel, P.; Romero-Salas, A.; Nozal, L.; Vighi, M. Identification of Contaminants of Concern in the Upper Tagus River Basin (Central Spain). Part 2: Spatio-Temporal Analysis and Ecological Risk Assessment. *Science of The Total Environment* **2019**, *667*, 222–233. https://doi.org/10.1016/j.scitotenv.2019.02.286.
(69) Čelić, M.; Jaén-Gil, A.; Briceño-Guevara, S.; Rodriguez-Mozaz, S.; Gros, M.; Petrović, M. Extended Suspect Screening to Identify Contaminants of Emerging Concern in Riverine and Coastal Ecosystems and Assessment of Environmental Risks. *Journal of Hazardous Materials* **2020**, 124102. https://doi.org/10.1016/j.jhazmat.2020.124102.
(70) Kuzmanović, M.; Ginebreda, A.; Petrović, M.; Barceló, D. Risk Assessment Based Prioritization of 200 Organic Micropollutants in 4 Iberian Rivers. *Science of The Total Environment* **2015**, *503–504*, 289–299. https://doi.org/10.1016/j.scitotenv.2014.06.056.

(71) Ministry for Ecological Transitiona and Demographical challenge. Estaciones de aforo en ríos https://sig.mapama.gob.es/WebServices/clientews/redes-seguimiento/default.aspx?nombre=ROAN_ESTACION_AFORO_RIOS&claves=COD_HIDRO%7CCOD_SITUACION_ESTACION&valores=3062%7C4&origen=1008# (accessed 2021 -07 -20).

(72) Marshall, M. M.; McCluney, K. E. Mixtures of Co-Occurring Chemicals in Freshwater Systems across the Continental US. *Environmental Pollution* **2021**, *268*, 115793. https://doi.org/10.1016/j.envpol.2020.115793.

(73) Arnold, K. E.; Brown, A. R.; Ankley, G. T.; Sumpter, J. P. Medicating the Environment: Assessing Risks of Pharmaceuticals to Wildlife and Ecosystems. *Phil. Trans. R. Soc. B* **2014**, *369* (1656), 20130569. https://doi.org/10.1098/rstb.2013.0569.

Figure Captions

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

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

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

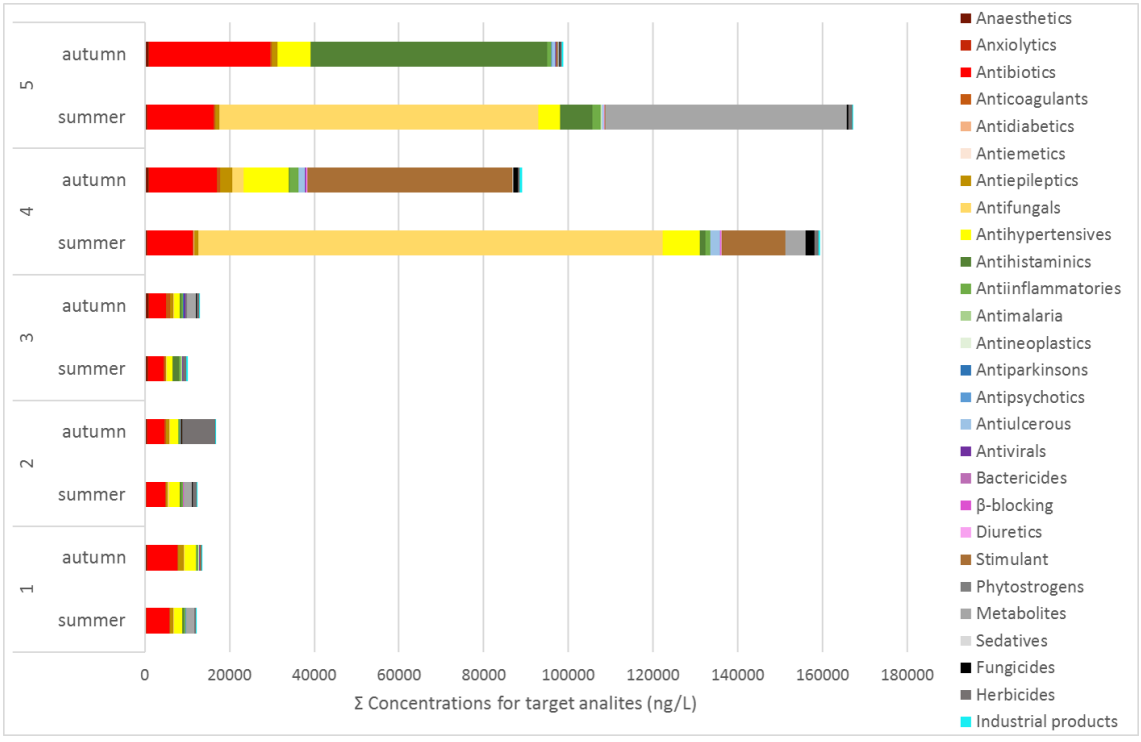
Figure 3. PCA biplot showing the suspect analysis results in the (a) positive and (b) negative mode. PC1 and PC2 show the differences between WWTPs.

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

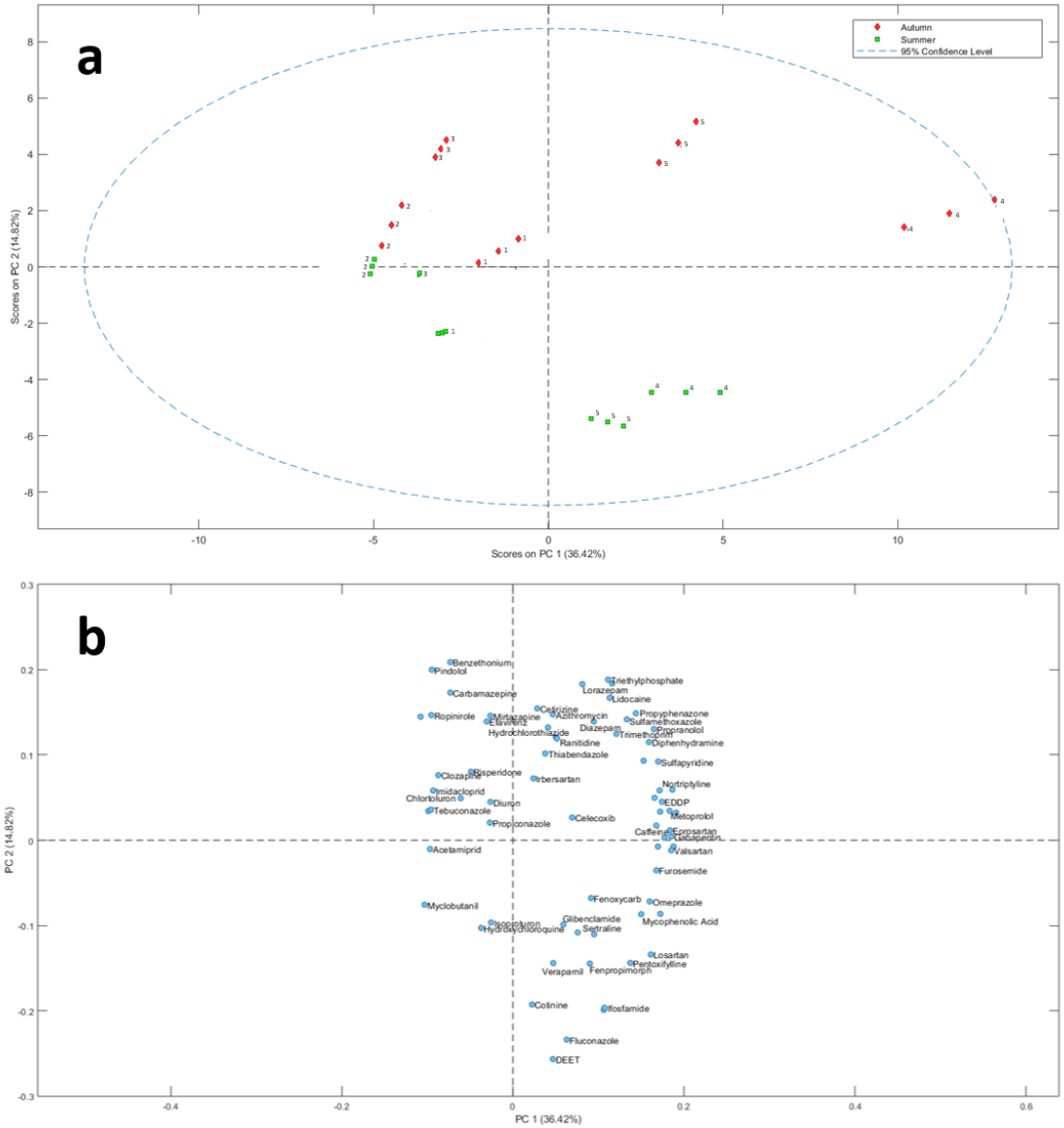
Figure 5. Calculated RQs for each detected compound in the target analysis considering the maximum measured concentration. The compounds are sorted alphabetically from acetaminophen to hydrochlorothiazide (a), and followed by hydroxychloroquine to verapamil (b).

Table 2. RIs of each individual WWTP per season.

Fig. 1



940 Fig. 2



941

942

943

944

945

946

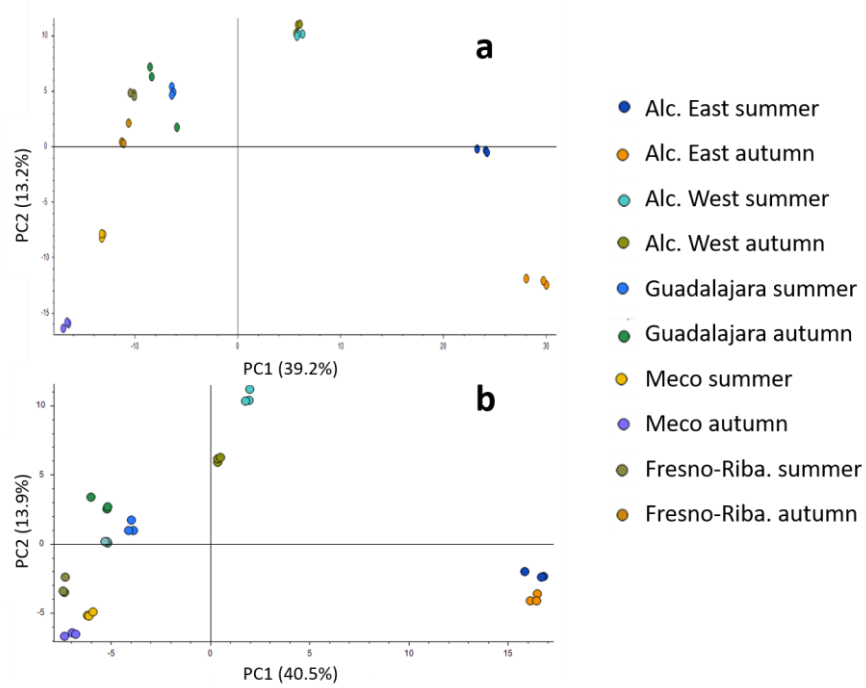
947

948

949

950

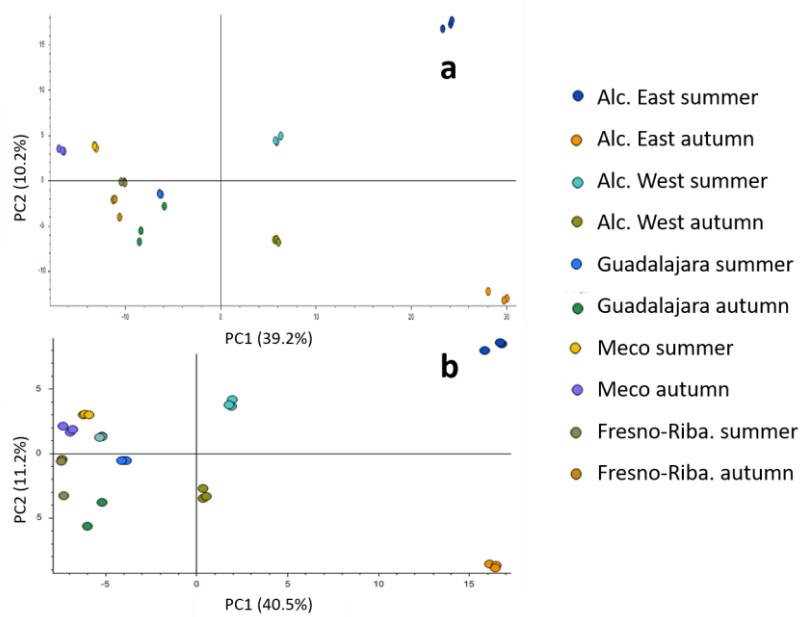
951 Fig. 3



952

953

954 Fig. 4



955

956

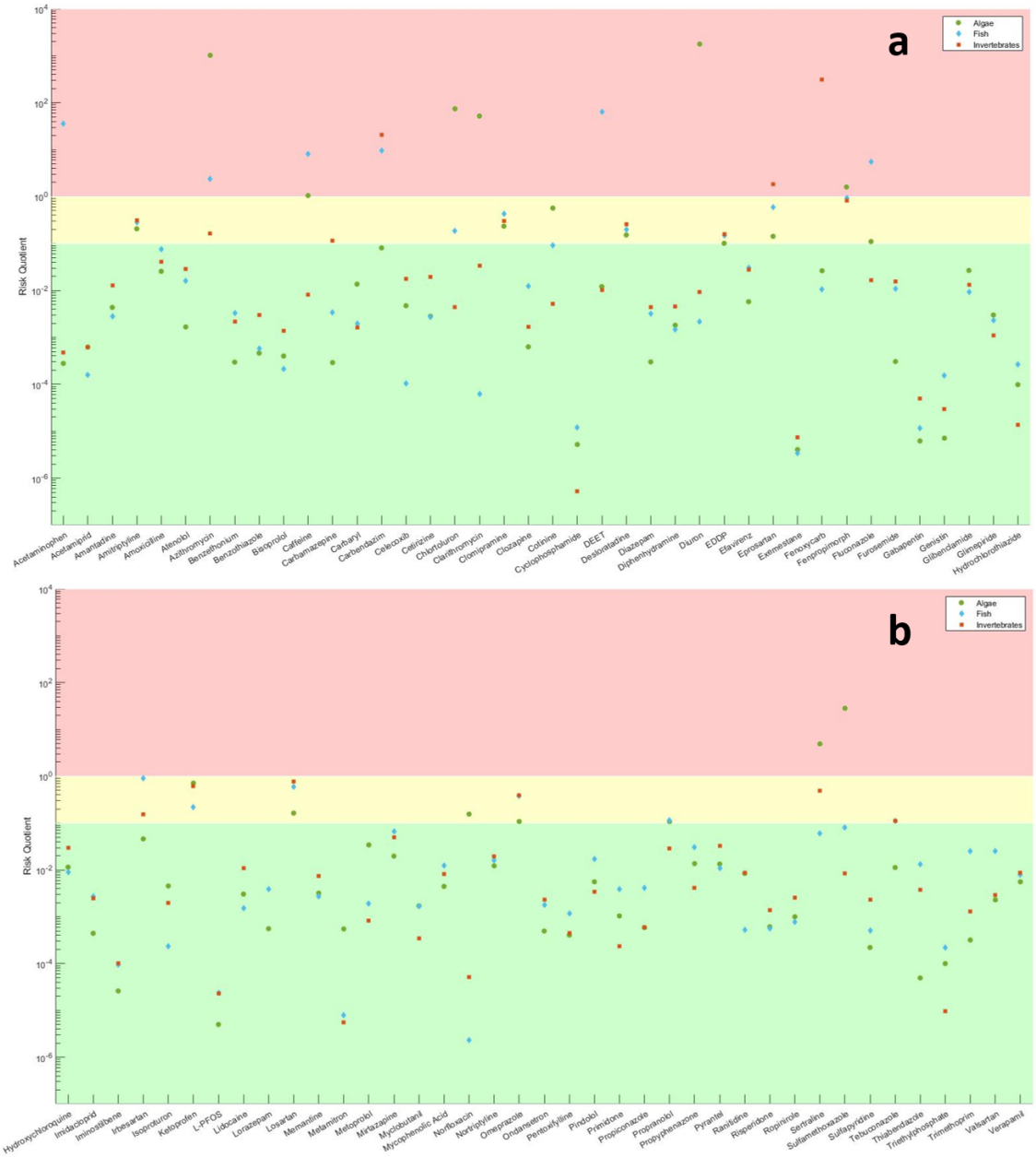
957

958

959

960

Fig. 5


a
Algae
Fish
Invertebrates
Risk Quotient
Acetaminophen
Acetamiprid
Amantadine
Amitriptyline
Amoxicillin
Atenolol
Azithromycin
Benzethonium
Benzothiazole
Bisoprolol
Caffeine
Carbamazepine
Carbaryl
Carbendazim
Celecoxib
Cetirizine
Chlortoluron
Clarithromycin
Clomipramine
Clozapine
Cotinine
Cyclophosphamide
DEET
Desloratadine
Diazepam
Diphenhydramine
Diuron
EDDP
Efavirenz
Eprosartan
Exemestane
Fenoxycarb
Fenpropimorph
Fluconazole
Furosemide
Gabapentin
Genistin
Glibendamide
Glimepiride
Hydrochlorothiazide
b
Algae
Fish
Invertebrates
Risk Quotient
Hydroxychloroquine
Imidacloprid
Iminostilbene
Irbesartan
Isoproturon
Ketoprofen
L-PFOS
Lidocaine
Lorazepam
Losartan
Memantine
Metamitron
Metoprolol
Mirtazapine
Mycobutanil
Mycophenolic Acid
Norfloxacin
Nortriptyline
Omeprazole
Ondansetron
Pentoxifylline
Pindolol
Primidone
Propiconazole
Propranolol
Propyphenazone
Pyrantel
Ranitidine
Risperidone
Ropinirole
Sertraline
Sulfamethoxazole
Sulfapyridine
Tebuconazole
Thiabendazole
Triethylphosphate
Trimethoprim
Valsartan
Verapamil