Occurrence and ecological risks of pharmaceuticals in a Mediterranean river in Eastern Spain

Eddie Fonseca[1,2], Félix Hernández[1], María Ibáñez[1], Andreu Rico[3], Elena Pitarch[1,*], Lubertus Bijlsma[1,*]

[1]Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water (IUPA), University Jaume I, Avda. Sos Baynat s/n, E-12071, Castellón, Spain

[2]Centro de Investigación en Contaminación Ambiental (CICA), Universidad de Costa Rica, P.O. 2060, San José, Costa Rica

[3]IMDEA Water Institute, Science and Technology Campus of the University of Alcalá, Avenida Punto Com 2, 28805, Alcalá de Henares, Madrid, Spain

**Corresponding authors: epitarch@uji.es (Elena Pitarch); bijlsma@uji.es (Lubertus Bijlsma).*

ABSTRACT

Pharmaceuticals are biologically active molecules that may exert toxic effects to a wide range of aquatic organisms. They are considered contaminants of emerging concern due to their common presence in wastewaters and in the receiving surface waters, and the lack of specific regulations to monitor their environmental occurrence and risks. In this work, the environmental exposure and risks of pharmaceuticals have been studied in the Mijares River, Eastern Mediterranean coast (Spain). A total of 57 surface water samples from 19 sampling points were collected in three monitoring campaigns between June 2018 and February 2019. A list of 40 compounds was investigated using a quantitative target UHPLC-MS/MS method. In order to complement the data obtained, a wide-scope screening of pharmaceuticals and metabolites was also performed by UHPLC-HRMS. The ecological risks posed by the pharmaceutical mixtures were evaluated using species sensitivity distributions built with chronic toxicity data for aquatic organisms. In this study, up to 69 pharmaceuticals and 9 metabolites were identified, out of which 35 compounds were assessed using the quantitative method. The highest concentrations in water corresponded to acetaminophen, gabapentin, venlafaxine, valsartan, ciprofloxacin and diclofenac. The compounds that were found to exert the highest toxic pressure on the aquatic ecosystems were principally analgesic/anti-inflammatory drugs and antibiotics. These were: phenazone > azithromycin > diclofenac, and to a lower extent norfloxacin > ciprofloxacin > clarithromycin. The monitored pharmaceutical mixtures are expected to exert severe ecological risks in areas downstream of WWTP discharges, with the percentage of aquatic species affected ranging between 65% and 82% in 3 out of the 19 evaluated sites. In addition, five antibiotics were found to exceed antibiotic resistance thresholds, thus potentially contributing to resistance gene enrichment in environmental bacteria. This work illustrates the wide use and impact of pharmaceuticals in the area under study, and the vulnerability of surface waters if only conventional wastewater treatments are applied. Several compounds included in this study should be incorporated in future water monitoring programs to help in the development of future regulations, due to their potential risk to the aquatic environment.

Keywords

57 Pharmaceuticals and metabolites

58 Surface water

59 Screening

60 Liquid chromatography mass spectrometry

61 Environmental impact

62 Ecological risk assessment

1. INTRODUCTION

The prevention of water bodies deterioration is an urgent issue nowadays. Among other matters, it is necessary to accurately monitor the presence of a wide variety of organic contaminants in order to preserve the ecological status of aquatic ecosystems. In this context, pharmaceuticals are of current concern due to their widespread use and frequent detection in the water cycle. Pharmaceuticals can reach water bodies from different sources, such as a human consumption (Botero-Coy et al., 2018; García-Galán et al., 2016), landfill leachates (Lu et al., 2016; Masoner et al., 2014), use of wastewater treatment plants (WWTPs) sludge as fertilizers (Behera et al., 2011), effluents from hospitals (Della-Flora et al., 2019; Verlicchi et al., 2012) or improper disposal of unused or expired medicines (Bashaar et al., 2017; Tong et al., 2011). Due to the poor removal efficiency of most conventional WWTPs (Al-Odaini et al., 2010; Behera et al., 2011), it is not surprising that pharmaceuticals are found in treated effluents and reach receiving surface waters (Botero-Coy et al., 2018; Collado et al., 2014; Gao et al., 2012; Gracia-Lor et al., 2012; Hernández et al., 2019a; Ibáñez et al., 2017; Paíga et al., 2017; Picó et al., 2020; Rico et al. 2016) and even drinking water sources (Boleda et al., 2014; Bruce et al., 2010; Praveena et al., 2019).

Pharmaceuticals are biologically active molecules designed to target a varied range of human receptors and that display different toxicological modes of action, depending on the biological endpoint that is evaluated. A recent review on the environmental exposure and toxicity data for 22 pharmaceuticals shows that hormones, antiepileptics, anti-inflammatories and antibiotics are generally the TCs posing the highest ecotoxicological risks (Pereira et al., 2020). However, consumption patterns and removal efficiencies vary across different river basins, which result in diverse complex mixtures, that need to be evaluated case-by-case (Altenburger et al., 2015).

A significant amount of research has been carried out on the occurrence of pharmaceuticals in surface waters, but only data from parent compounds are normally reported (Boix et al., 2015; Ferrer et al., 2010; Grabic et al., 2012; Gracia-Lor et al., 2011; Huntscha et al., 2012; Ibáñez et al., 2009). However, there are more and more data available evidencing that the unaltered compounds are just the “top of the iceberg”, because they usually represent a small part of the total amount of the compounds

excreted in urine (Hernández et al., 2019a). In the last few years, several papers have reported the occurrence of many metabolites in surface and wastewaters (Boix et al., 2016; Della-Flora et al., 2019; Gracia-Lor et al., 2014; Ibáñez et al., 2017; Langford and Thomas, 2011; Rúa-Gómez and Püttmann, 2012). Apart from analytical drawbacks, such as the lack of reference standards and the absence of priority compounds lists, the evaluation of the toxicity of metabolites and transformation products (TPs) involves considerable effort (Lindholm-Lehto et al., 2016). However, it is of importance as they can be as persistent and/or toxic as the parent compound and can have negative effects on different aquatic organisms (Rivera-Jaimes et al., 2018). For this reason, they should be gradually included in analytical methods and in aquatic risk assessments (Hernández et al., 2019a; Santana-Viera et al., 2016).

Until recently, environmental regulations barely included maximum allowable concentration levels for pharmaceuticals in surface waters. The European Commission (European Commission, 2018) establishes a Watch List of substances that must be followed up as part of public policies. The objective of that list is to collect data from the Member States about the concentration levels of the included pharmaceuticals in the water bodies and to decide, in a later stage, whether they can be considered as priority substances in the regular monitoring of water quality. Five antibiotics (i.e. the fluoroquinolone ciprofloxacin, the penicillin amoxicillin and the macrolides azithromycin, clarithromycin, erythromycin) have already been included in the current Watch List. Recent studies indicate that the aquatic risk of pharmaceuticals, such as carbamazepine and ciprofloxacin, has increased from 10 to 20 times in the last 20 years due to the demographic concentration in urban areas and the low dilution capacity of surface waters in (semi-)arid areas (Oldenkamp et al., 2019). The presence of antibiotics in the environment is of special concern, as it can lead to the development of bacterial resistance genes, a fact that has already been observed even in pristine areas such as the Antarctic (Hernández et al., 2019b) and which may represent a serious problem in fighting some diseases (Mokh et al., 2017). Recent investigations show that urban WWTPs constitute hotspots for antibiotic emissions, contributing to the enrichment of resistance genes in surface water ecosystems (Buelow et al., 2020). In this regard, threshold concentrations for antibiotic resistance have been proposed for a wide range of

antibiotics to aid the assessment of their respective resistance development risks (Bengtsson-Palme and Larsson, 2016; Rico et al., 2017), and to prioritize compounds and management practices that should be implemented at a watershed scale.

One of the main reasons for the increase of data on the presence of pharmaceuticals in water is the relevant role of modern environmental analytical chemistry (Hernández et al., 2019a). Most data reported nowadays are based on target quantitative methods commonly using ultra high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), which offers excellent sensitivity, selectivity and robustness (Beccaria and Cabooter, 2020; Campos-Mañas et al., 2017; García-Galán et al., 2016; van Nuijs et al., 2010). However, the application of target methodologies may provide incomplete results as other compounds present in the sample could remain ignored in the analysis. Then, a screening based on high resolution MS (HRMS) becomes necessary in order to identify as many contaminants as possible, even when reference standards are not available at the laboratory (Aceña et al., 2015; Boix et al., 2016; Hernández et al., 2015a, 2015b).

The aim of this study was to assess the occurrence and ecological risks of a wide variety of pharmaceuticals and metabolites in the Mijares River, located in Eastern Mediterranean Spain. A total of 57 surface water samples were collected in three different campaigns over one year. Samples were quantitatively analyzed by UHPLC-MS/MS for the determination of 40 target pharmaceuticals. Additionally, a screening by UHPLC-HRMS was performed in order to complement the quantitative results obtained. The results of the quantitative analysis were used to perform a probabilistic risk assessment for aquatic organisms, which helped to highlight individual compounds and pharmaceutical mixtures that are posing an ecotoxicological risk. Moreover, the monitored antibiotics were evaluated in regards to their resistance development risks. Overall, this study contributes to the identification of pharmaceutical compounds that need to be further monitored and that are candidates to be included in future updates of the Water Framework Directive and regional monitoring plans.

2. EXPERIMENTAL

2.1 Chemicals and materials

See **Supplementary Material (S.M.)**

2.2 Description of the sampling sites and sample collection

The Mijares River originates in Aragón (at 1.600 m in Sierra de Gúdar, in the municipality El Castellar, province of Teruel) and ends in the Mediterranean Sea, Castellón, Eastern Spain (see **Figure 1**). It is 156 km long with a 5.466 km^2 wide basin, which represents 13% of the total demarcation of the Jucar Hydrographic Confederation. The river is an important source of irrigation water in the lower basin, which is an important agricultural area with predominance of citrus crops (Garófano-Gómez et al., 2013).

Water samples were taken at 19 different points (see **Figure 1**), covering almost all the Mijares River, from its source until its estuary: points 1-6 are sited in the upper section of the river, 7-14 in the middle section, 15-18 in the lower section, and point 19 in the river mouth. All sampling sites were selected based on different characteristics and/or accessibility (**Table S1**). In the municipality of Sarrión (Teruel), three sampling points were considered due to their proximity to a fertilizer factory (point 2) or to a fish farm (points 3 and 4). The potential contribution of small towns in terms of emerging contaminants might be attributed to four WWTPs discharging their effluents into the river. For this reason, several sampling sites were selected downstream of the WWTPs: points 9 and 10, near Montanejos (WWTP flow 627 m^3/day; population served 1.513 p.e); 11 near Toga (WWTP flow 21 m^3/day; population served 66 p.e); 17 near Vila-real (WWTP flow 3.666 m^3/day; population served 16.449 p.e); and 18 near Almassora (WWTP flow 7.386 m^3/day; population served 34.337 p.e) (**Table S2**) (EPSAR, 2020). Also, two sampling sites (13 and 14), located downstream of a solid waste treatment plant (SWTP) near Onda (Castellón), were included in this study. Waters from three reservoirs located in the Mijares River were also sampled: 5 (Toranes reservoir, Teruel), 7 and 8 (Arenós reservoir, Castellón) and 12 (Sitjar reservoir, Castellón).

Three sampling campaigns were conducted in order to monitor pharmaceuticals concentrations along different periods: June 2018 (1st campaign, summer), September 2018 (2nd campaign, autumn) and February 2019 (3rd campaign, winter). In every

campaign, 19 surface water samples, one from each sampling point, were collected in polyethylene bottles, transported to the laboratory on the same day (within max. 6h) in refrigerated isothermal containers, and stored at -20 °C until analysis.

2.3 Sample treatment

2.3.1. Quantitative analysis

The procedure applied for quantitative UHPLC-MS/MS analysis was based on methodology previously developed by our research group (Boix et al., 2015; Botero-Coy et al., 2018) using direct injection of the sample, without any pre-concentration. Briefly, 2 mL of water was centrifuged at 12.000 rpm for 10 min. Subsequently, 50 µL of isotopically labelled internal standard (ILIS) mix solution of 1 µg/L was added to 950 µL of the centrifuged water sample (final ILIS concentration in the sample injected, 50 ng/L) . Finally, 50 µL was injected into the UHPLC-MS/MS system.

2.3.2. Screening analysis

The UHPLC-HRMS screening required a previous generic sample extraction and pre-concentration. This was performed by solid-phase extraction (SPE), following the procedure described by Pitarch et al. (2016). **Figure S1** shows a flowchart of the extraction procedure. Briefly, 100 mL of water was passed through an Oasis® HLB (150 mg) cartridge. After elution, the extract was reconstituted with 100 µL of methanol:water (10:90, v/v) and 20 µL were injected into the UHPLC-QTOF MS.

2.4 Instrumentation

Quantitative analyses was performed using a Waters ACQUITY UPLC® (Waters Corp.) equipped with a binary pump system was interfaced to a Xevo TQ-S™ triple quadrupole (QqQ) mass spectrometer (Waters Corp.). For qualitative screening a Waters ACQUITY UPLC® (Waters Corp.) interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters) was used. For more details related to the instrumentation used see **S.M**.

2.5 Quantitative LC-MS/MS analysis and quality assurance

In total, 40 pharmaceuticals (**Table 1**) from different therapeutical classes were selected for target quantitative analysis by LC-MS/MS (QqQ). The experimental conditions are

shown in **Table S3**. At least, seven-point calibration curves (0.005-20 μg/L) were injected at the beginning and the end of each sequence. As the samples were analysed by direct injection, without any pre-concentration step, the lowest calibration level (LCL) was taken as the limit of quantification in samples (**Table S3**). A compound was considered as "detected" when its concentration was below LCL and at least one q/Q ratio was accomplished allowing in this way its reliable identification. For the constructions of graphs, risk assessment evaluation, and for discussion of results obtained, the cut-off value used for detected positives was half of their LCL.

Quality control (QC) samples, consisting on three surface waters each fortified at three concentration levels (0.01, 0.1 and 1 μg/L), were analysed together with the samples (see **Table S4**). QCs recoveries between 60 and 140% were considered satisfactory (SANTE, 2019). For many compounds, the corresponding ILIS was used for matrix effects correction, ensuring an accurate quantification (**Table S3**). The ratio between the qualitative and quantitative transitions (q/Q ratio) as well retention time deviation (± 0.1 min) were used for the reliable identification of positive findings (SANTE, 2019).

2.6. UHPLC-HRMS screening

A great number of organic micro-pollutants were investigated by screening based on UHPLC-QTOF MS. Accurate-mass data generated at low and high collision energy were processed by ChromaLynx™ Application Manager (within MassLynx) in combination with a home-made database, containing a large number of pharmaceuticals and their main metabolites. In total, the presence of more than 900 compounds was investigated (see **Table S5** in **S.M.**). This software applies a "post-target" processing method by monitoring exact masses of the suspect analytes and obtains the corresponding narrow-window Extracted Ion Chromatogram (nw-EICs).

The database included, at least, the name and elemental composition of the parent compounds (occasionally adducts). Information on retention time (Rt), main fragment ions and adducts was also added when reference standards were available, which greatly helped to facilitate and support the identification process.

When a chromatographic peak was observed at the corresponding exact mass but the reference standard was not available, the characteristic isotope pattern (if chlorine or

bromine atoms were present) as well as fragment ions were evaluated and their compatibility with the chemical structure of the suspect compound was assessed. Tentative identification was reinforced by agreement with MS/MS product ions reported in literature or available databases (preferably in exact mass). For more information see (Hernández et al., 2015a, 2015b).

2.7. Ecological risk assessment

The probability that exposure concentrations result in unacceptable effects for aquatic organisms was calculated based on the Species Sensitivity Distributions (SSD) approach (Posthuma et al. 2002). The Potentially Affected Fraction (PAF) was calculated for individual compounds, and the multi-substance Potentially Affection Fraction (msPAF), for contaminant mixtures, following the methods described by de Zwart and Posthuma (2005). Risks were calculated using the SSDs provided by Posthuma et al. (2019) for chronic exposure. In their study, the SSD parameters μ (median of the log-transformed toxicity values) and σ (standard deviation of log-transformed toxicity values or slope) were calculated using a log-normal distribution on the basis of chronic toxicity data (primarily No Observed Effect Concentrations, NOECs) for bacteria, algae, invertebrates and fish. Since for some compounds chronic toxicity data is very limited, acute-to-chronic extrapolation techniques and read-across (i.e., Quantitative-Structure Activity Relationships, QSARs) was often applied for their derivation. The robustness of the SSD parameters was evaluated on the basis of the methods described by Posthuma et al. (2019), which consider four quality aspects: (1) the availability of a sufficient number of data to calculate the SSD μ and σ, (2) the biodiversity coverage, (3) the origin of the toxicity data (i.e., experimental, extrapolated or read-across), and (4) the type of extrapolation (in case the data was extrapolated). The SSD parameters of the compounds that were detected at least once in this study are provided in **Table S6** together with their quality scores, while a detailed description of the quality scores is provided in **Table S7.** When there was no chronic toxicity data for a specific compound, the μ was derived by subtracting 1 to the μ of the SSD built with acute toxicity data (i.e., assuming an acute-to-chronic extrapolation factor of 10 for the species assemblage), and using a σ of 0.7. A σ of 0.7 was also applied to the chronic SSDs that had a σ that was considered too large or

too low according to the criteria established by Posthuma et al. (2019). The σ value of 0.7 is the average SSD slope for the 12386 chemicals evaluated by Posthuma et al. (2019).

The monitored pharmaceuticals were classified into eleven Therapeutic Classes (TCs). Then, the toxic pressure of the compounds within each of the TCs and their mixtures was calculated for each sample. First, the Hazard Unit (HU) was calculated for each compound in each sampling site by dividing the logarithm of the measured concentration by the SSD μ. These HUs are used to adjust for differences in the potency of the evaluated compounds. Next, the concentration addition model was used to calculate the msPAF corresponding to each TC ($msPAF_{TC}$) in each sample using the Microsoft Excel © function (Eq. 1).

$$\mathrm{msPAF_{TC}} = NORM.DIST\ (HU_{TC}, 0, \sigma_{TC}, 1) \qquad \text{Eq. 1}$$

Where HU_{TC} is the sum of the HUs for each compound in the TC, and σ_{TC} is the average σ for all compounds in the TC.

After obtaining the $msPAF_{TC}$ for each TC, the total toxicity of the sample ($msPAF_{Total}$) was calculated using the response addition model (Eq. 2).

$$\mathrm{msPAF_{Total}} = 1 - \prod_{i=1}^{n}\left(1 - \mathrm{msPAF_{TC,i}}\right) \qquad \text{Eq. 2}$$

Finally, the $msPAF_{Total}$ for each sample was represented with the relative contribution of each TC to the total toxic pressure. In our study, the PAF and the $msPAF_{Total}$ represent the fraction of species of the aquatic ecosystem that will be affected (i.e., the NOEC is exceeded) by the chronic exposure to an individual compound or the pharmaceutical mixture, respectively. In this study, PAF and $msPAF_{Total}$ values between 5% and 25% were considered to result in moderate ecological risks, while values above 25% were considered to induce severe risks (see section 3.4 for rationale).

2.8 Antibiotic resistance risks

The risks of promoting antibiotic resistance in environmental bacteria were calculated using the resistance Predicted No Effect Concentrations (PNECs) proposed by Bengtsson-Palme and Larsson (2016) for all the evaluated antibiotics except furaltadone, oxolinic acid and sulfadiazine, for which resistance PNECs are not available. Risk Quotients (RQs) were calculated by diving the measured antibiotic concentrations by the resistance

PNECs, so that a RQ quotient larger than one indicates a potential risk of antibiotic resistance development.

3. RESULTS AND DISCUSSION

3.1 Quantitative analysis by UHPLC-MS/MS (QqQ)

3.1.1 Quality control samples

Especial emphasis was made on QCs evaluation in order to support the reliability of quantitative data reported. **Table S4** shows the average results obtained for 9 QCs analysed (one QC per spiking level and per sampling campaign, this is, 3 replicates per each spiking level). It should be noted that QCs at lowest fortification level were only performed in the first campaign (n=3). Recoveries were generally between 60 and 140% (SANTE, 2019), and mostly in the 80-120% range. The use of analyte-ILIS and the absence of complex sample treatment process surely facilitated obtaining satisfactory quality controls, with a few exceptions. The most relevant were for the antibiotics ciprofloxacin and norfloxacin, whose recovery values were slightly above 200% and poorly reproducible. The lack of sensitivity of our instrumentation in negative mode prevented the determination of the drugs measured under this mode (bezafibrate, gemfibrozil, ketoprofen and naproxen) at the low fortification levels tested and only QC recoveries at 1 μg/L could be calculated for these compounds. The antibiotics clarithromycin and roxithromycin showed unsatisfactory recoveries in some cases, especially at the highest level of fortification, probably because their analyte-ILIS was not available and therefore matrix effects could not be corrected. Regarding data reported in this paper for water samples, the unsatisfactory QCs recoveries only affected to ciprofloxacin and norfloxacin, and therefore those values must be taken as semi-quantitative. The reason might be the low ILIS concentration used (50 ng/L). In fact, in subsequent works performed in our group we increased the amount of ILIS added to the samples obtaining a significant improvement in the results.

3.1.2. Analysis of surface water samples

A total of 57 river water samples (19 per campaign) were analysed by LC-MS/MS (QqQ) for 40 target pharmaceuticals. The compounds were selected based on their frequent occurrence in effluent wastewater and surface water samples analysed in previous

studies (Botero-Coy et al., 2018; Hernández et al., 2015a, 2015b). The concentrations found in the samples analysed are included in **Tables S8**, **S9** and **S10,** corresponding to the first (June 2018, summer), second (September 2018, autumn) and third (February 2019, winter) campaigns. **Table 1** shows the frequency of detection (% positive samples) of the pharmaceuticals investigated. As indicated in section 2.5, the cut-off value used for the compounds detected was half of their LCL.

Thirty-five out of the 40 compounds evaluated in this study were measured at least once in the samples. The analgesic acetaminophen was the most frequently detected (65% of samples above the cut-off value 2. 5 ng/L). The antiepileptic gabapentin (42% above 2.5 ng/L), the antidepressant venlafaxine (40% above 2.5 ng/L), the antihypertensive valsartan (39% above 2.5 ng/L), the antibiotic ciprofloxacin (33% above 25 ng/L) and the anti-inflammatory drug diclofenac (33% above 2.5 ng/L) were also frequently found. A notable amount of pharmaceuticals (66% of the compounds detected) exceeded, in at least one of the samples, the concentration level of 0.1 μg/L (value set by European Union countries). The compounds with the highest percentage of exceedances were primidone, gabapentin, valsartan and diclofenac. Seven drugs (tramadol, azithromycin, ciprofloxacin, gabapentin, irbesartan, valsartan and phenazone) slightly surpassed 1 μg/L, particularly in the sites 17 and 18, but never exceeded 2 μg/L. Some of the pharmaceuticals detected in the Mijares River are currently included in the Watch List of substances for European-wide monitoring in the field of water policy (European Commission, 2018), such as the antibiotics ciprofloxacin, clarithromycin, erythromycin and azithromycin. As an example, **Figure S2** shows the positive findings of losartan (antihypertensive), diclofenac (NSAID) and erythromycin (antibiotic) in three surface water samples investigated.

The spatial distribution along the Mijares River, expressed as the sum of the average concentration of the 3 campaigns of each individual pharmaceutical, is shown in **Figure 2**. As expected, the upper section was the less contaminated (< 100 ng/L for total pharmaceuticals), even in the points near the fertilizer factory (site 2) and the fish farm (sites 3 and 4), which presented a similar pattern to the rest of upper sites demonstrating no relevant contribution of pharmaceutical residues into the Mijares River.

As regards to the middle section, most of the sampling points showed mean concentrations of pharmaceuticals lower than 100 ng/L (7: upstream Arenoso reservoir;

11: Toga; 12: Sitjar reservoir; 12-13: Onda SWTP). It is worth noticing the sample collected downstream Montanejos WWTP (point 10), with a total concentration of pharmaceuticals above 5000 ng/L and high number of positives (up to 27 pharmaceuticals in the 1st campaign). On the contrary, the sampling site 11 (downstream WWTP Toga) did not appear to be very contaminated, which may be explained by the small size of this village with only 100 inhabitants. Moreover, in the sample collected downstream of the SWTP located in Onda (points 13 and 14) very few pharmaceuticals were found (< 100 ng/L), indicating that no relevant pollution in terms of pharmaceuticals comes from this plant. This is in agreement with data reported on groundwater from that area, where pesticides were found as the most relevant contaminants due to the intensive agriculture in the surrounding area, focused on citrus crops (Pitarch et al., 2016).

As expected, the lower section of the river was the most contaminated, especially in the area nearest to the estuary. The most polluted sites (total concentration >5000 ng/L) were located downstream of the two WWTPs, near Vila-real (point 17) and Almassora (point 18). Surface water collected in these two sampling sites presented the highest number of positives (between 20 and 30, depending on the campaign). The last sampling site, near the river mouth into the Mediterranean Sea (19, Gola Almassora), also presented a notable pharmaceuticals pollution, but with mean total concentrations below 5000 ng/L.

3.2. Seasonal variation

The total concentration for the different pharmaceutical families in each sampling campaign is shown in **Figure 3**. Antihypertensive, anti-inflammatory agents and antibiotics presented the highest concentrations. No clear trends were observed as a function of the sampling season, although a slight increase in concentrations of antihypertensives, antidepressants, antibiotics and analgesics seemed to occur in winter (3rd sampling). This fact is not surprising in the case of antibiotics due to the increase of their consumption to treat respiratory infections in colder periods (Letsinger et al. 2019).

Due to the higher pollution observed in sampling sites 10, 17 and 18, specific data from these samples were evaluated to highlight possible seasonal trends. The antibiotics azithromycin, clarithromycin and trimethoprim were present at higher concentrations in winter at the three sampling sites. Other compounds were also found at higher

concentrations in winter, at least in 2 out of the 3 sampling sites: the antibiotics clindamycin, erythromycin, sulfamethoxazole and metronidazole; the antihypertensives irbesartan, losartan and valsartan; the benzodiazepine alprazolam; the antiepileptic primidone; and the analgesic tramadol. The fact that pharmaceuticals presented higher concentrations in winter is in agreement with other river monitoring campaigns (Conley et al., 2008; Daneshvar et al., 2010; Lindholm-Lehto et al., 2016). Moreover, during cold periods, there is less degradation of the compounds in the WWTPs due to the low temperatures and irradiation, which result in higher analyte concentration levels in the effluent wastewater and, therefore, in the receiving surface water (Azzouz and Ballesteros, 2013; Golovko et al., 2014; Lindholm-Lehto et al., 2016).

3.3 Screening of pharmaceuticals and metabolites

A qualitative screening using UHPLC-QTOF MS was applied to samples collected in the second campaign to complement quantitative data and obtain information about other compounds that could be present in the samples. **Table S11** shows the detection frequency of pharmaceuticals. In total, 41 pharmaceuticals were detected, and up to 35 were confirmed with reference standards. Six more compounds were tentatively identified on the basis of the interpretation of accurate-mass data acquired, but could not be confirmed because the reference standard was not available at our laboratory.

Compounds with the highest detection frequency were acetaminophen and venlafaxine, identified in 4 out of the 19 samples. Six pharmaceuticals (azithromycin, carbamazepine, diclofenac, irbesartan, lidocaine and sulfamethoxazole) were found in 3 samples (16%). As expected, the upper section (points 1-6) presented the lowest number of findings, illustrating the little anthropogenic influence on this area. Regarding sites located downstream of the SWTP in Onda (13 and 14), no analytes were found indicating that no relevant pharmaceutical pollution comes from this plant, which is in agreement with quantitative results obtained in the three campaigns. As expected, the highest number of findings corresponded to water samples collected WWTP downstream, especially near Vila-real (point 17) and Almassora (point 18).

Figure 4 shows a summary of the results obtained in the screening, grouped by pharmaceutical families. Antihypertensives and non-steroidal anti-inflammatory drugs (NSAIDs) were most frequently detected, each representing 20% of the findings, followed

by antibiotics (12%). The remaining families were below 10%. Other compounds, mainly identified in points 17 and 18, were amisulpride (antipsychotic), cetirizine (antihistamine), dimetridazole (antiparasitic), iopromide (X-ray contrast agent), rimantadine (antiviral agent), each one with 2.2%, and lidocaine (anesthetic, 4.4%). Most of the compounds identified by HRMS screening have been often reported in surface water by the scientific literature (Gómez et al., 2010; Hernández et al., 2015b; Ibáñez et al., 2009; López et al., 2014; Masiá et al., 2013).

From the 41 pharmaceuticals identified in the screening, 16 were already included in the target quantitative method applied in this work (marked with √ in **Table 1**). It must be taken into account that the quantitative UHPLC-MS/MS method offer much better sensitivity than the screening methodology, as it was optimized for a limited number of compounds and the TQS instrument has higher sensitivity than our QTOF instrument. It is therefore noteworthy that the detection frequency depends, not only on the concentration of the compound, but on the sensitivity of the method towards that particular compound. Hence, a lower detection frequency should not necessarily be associated to lower presence. The results from this screening will be useful to update the analytical methodology, by adding the compounds identified in the screening to the list of target analytes for quantitative UHPLC-MS/MS analysis.

The excellent potential of UHPLC-HRMS also allowed to investigate pharmaceutical metabolites with the aim to generate useful data for future monitoring, including relevant metabolites detected in surface water. The screening of metabolites was focused on the most contaminated samples (i.e. those collected in sampling sites 10, 17, 18 and 19) to facilitate their detection and identification. **Table 2** shows the nine metabolites (tentatively) identified in surface water. 6 out of 9 metabolites could be confirmed with reference standards.

4-acetylaminoantipyrine (4-AAA) and 4-formylaminoantipyrine (4-FAA), metabolites of the antipyretic drug dipyrone (metamizole), were identified in the 4 samples analysed. Furthermore, 4-OH omeprazole sulphide, carbamazepine-10,11-epoxide and clopidogrel carboxylic acid were also found in 2 out of the 4 samples, while 4-aminoantipyrine (4-AA) (another metabolite of dipyrone) was only identified in 1 of the surface water samples.

These metabolites have also been found in surface water in previous studies performed by our group (Boix et al., 2016, 2014; Gracia-Lor et al., 2014).

Three metabolites could only be tentatively identified as the reference standards were not available at our laboratory. The potential of QTOF MS for investigation of metabolites is illustrated in **Figure S3,** which shows the tentative identification of nordiazepam (N-desmethyldiazepam) in a sample that also contained the parent compound diazepam (for more details, see **S.M.**)

3.4. Ecological risk assessment

The results of the ecological risk assessment performed with SSDs built with chronic NOECs show that the majority of the sampling sites are exposed to a low mixture toxic pressure ($msPAF_{Total}$ below 5%; **Figure 5**). However the site 19 was considered to be moderately impacted, with msPAFs ranging between 5% and 25%; and sites 10, 17 and 18 were severely impacted, with calculated $msPAF_{Total}$ above 25%. Particularly, in sites 17 and 18 (in all sampling campaigns), and in 10 (in summer), the percentage of affected aquatic species ranged between 65% and 82%, indicating a very high ecotoxicological risk (**Figure 5**). In all cases, toxicity was dominated by the analgesic/anti-inflammatory TC ($msPAF_{TC}$ 15-81%). Within this TC, toxicity was clearly dominated by phenazone, although diclofenac also had an important contribution (**Tables S12-14**). The second TC with the highest calculated toxicity were the bactericides (antibiotics), with a $msPAF_{TC}$ ranging between 5% and 12% in sampling sites 10 (all sampling campaigns), 17 (summer and winter) and 18 (autumn and winter). Within this TC, toxicity was dominated by azithromycin in autumn and winter (in sites 10, 17 and 18). In summer, the toxicity of this TC was dominated by norfloxacin, although other antibiotics such as ciprofloxacin and clarithromycin also contributed to the toxicity of the mixture. Regarding each of the monitored compounds in isolation, the highest ecological risks were established for phenazone > azithromycin > diclofenac, with individual PAFs above 10% in at least one sampling site; and to a lower extent norfloxacin, ciprofloxacin and clarithromycin, with individual PAFs above 1% in at least one sampling site (**Tables S12-S14).**

The method based on SSDs, and the calculated msPAFs, is a more ecological relevant approach when compared to other methods (e.g. Toxic Unit) to assess the risk of chemical mixtures to aquatic ecosystems. This is basically because it integrates toxicity data for as

485 many taxa as possible and accounts for their sensitivity differences on the basis of a
486 statistical distribution. The capacity of the SSD approach to represent ecosystem effects
487 has been evaluated on the basis of field monitoring studies and micro- and mesocosm
488 experiments performed mainly with pesticides (e.g. Schäfer et al. 2013; Rico et al. 2018).
489 Due to the absence of validation studies performed with pharmaceuticals, it is somewhat
490 difficult to characterize the level of impact caused by each of the established risk
491 categories. We expect that in the sites classified with severe risks (PAF or $msPAF_{Total}$ above
492 25%), the NOEC exceedances contributes to a loss of species that results in significant
493 indirect ecological effects and in effects on important ecological functions. However,
494 further investigations should be performed to quantify these effects and to validate the
495 SSD method with pharmaceutical compounds.

496 One of the major drawbacks of the SSD approach for its implementation in
497 pharmaceutical risk assessment is the limited amount of experimental chronic toxicity
498 data available. In this way, chronic SSDs often need to be based on extrapolated or read-
499 across toxicity data. For example, the μ of the chronic SSD for phenazone were based on
500 the extrapolation of the μ for the acute one (2.5 μg/L), which was in turn constructed
501 with a limited number of QSAR-based toxicity data (Posthuma et al. 2019; **Table S7**).
502 Toxicity studies performed with other non-steroidal anti-inflammatory drugs, such as
503 diclofenac, have shown cellular toxicity, genotoxicity, immunodepression, growth
504 inhibition and estrogenic effects on fish at environmentally relevant concentrations
505 (Hoeger et al., 2005; Hong et al., 2007; Xu et al., 2019). Therefore, experiments aimed at
506 assessing the chronic toxicity of phenazone on fish are highly recommended. Regarding
507 the other high priority compounds, the SSDs for azithromycin, ciprofloxacin and
508 clarithromycin were based on a relatively large number of toxicity data, but relied on
509 acute-to-chronic toxicity data extrapolations, while the SSD for norfloxacin was based on
510 available chronic toxicity data (**Table S7**). Previous studies show that these compounds
511 are highly toxic to aquatic microorganisms, including cyanobacteria and some diatoms
512 (Guo et al., 2015). Therefore, their ecotoxicological risks may be associated to the
513 alteration of the structure of microbial communities and primary producers, most likely
514 those associated to hard substrates, downstream of areas with significant WWTP
515 influence (i.e., Montanejos, site 10, and in the mouth of the river, sites 17 and 18).

Furthermore, several studies show that ecosystem functions mediated by these microorganisms (e.g. nitrification, denitrification, anaerobic ammonium oxidation) can be affected by prolonged exposure to concentrations similar to those that have been found in this study (Roose-Amsaleg and Laverman, 2016).

Although a large number of pharmaceuticals have been monitored in this study, the results of the aquatic risk assessment show that only a very limited number of compounds has a potential contribution to the total toxicity of the sample. This is in line with other studies evaluating the potential ecotoxicological of pharmaceutical mixtures, which demonstrate that usually a reduced number of compounds (≤5) significantly contribute to the total toxicity of the sample (Schäfer et al. 2013; Arenas-Sánchez et al., 2019). In our study, two TCs were the main responsible for the toxicity observed in the most polluted sites (i.e., analgesic/anti-inflammatory drugs and antibiotics). In principle, effects other than additive or antagonistic between these pharmaceutical groups are not expected on the impacted ecosystem, as they affect species in well separated trophic levels (i.e., cyanobacteria and fish). In addition, toxicity studies assessing the effects of non-steroidal anti-inflammatory drug mixtures on fish and other aquatic organisms (Cleuvers, 2004; Sehonova et al., 2017), or antibiotic mixtures on algae (González-Pleiter et al., 2013) generally demonstrate additivity, confirming that the concentration addition model used in this study for chemicals within the same TC is not expected to underestimate, neither overestimate, the calculated risks.

3.5 Antibiotic resistance risks

RQs exceeding the value of 1 were calculated in 3 out of the 19 evaluated sampling sites of the Mijares River (sites 10, 17 and 18). Resistance PNECs were exceeded by five antibiotics (see **Figure 6**), being ciprofloxacin the compound with the highest RQ (17.3), followed by azithromycin (6.5), norfloxacin (1.9), trimethoprim (1.5) and clarithromycin (1.3). In some samples, exceedance of resistance thresholds occurred for more than one antibiotic (e.g. ciprofloxacin and norfloxacin; azithromycin and clarithromycin). Overall the antibiotics with the highest resistance development risk belong to the fluoroquinolone and the macrolide classes, which are classified as antibiotics of critical importance for human health (WHO, 2019). This study shows that WWTPs discharges into the Mijares River are contributing to environmental concentrations that may contribute

to the enrichment of resistance genes in aquatic bacterial communities. However, the link between these indicators and the risks to the human population are not that straightforward. The assessment of the human transmission risks depends on the exposure levels (via bathing, irrigation, drinking), and require a complementary *in-situ* evaluation of fecal contamination, resistant bacteria, genes and mobile genetic elements (Huijbers et al., 2019), which is out of the scope of this study. At this stage, however, this study evidences that antibiotics in the EU Watch List (and others co-occurring with them) should be evaluated, not only regarding their potential ecotoxicological side-effects, but also regarding their contribution to antibiotic resistance development in the environment.

4. CONCLUSIONS

A comprehensive investigation has been made on the occurrence and risks of pharmaceuticals in the Mijares River (Eastern Spain). Up to 35 pharmaceuticals were quantified in the water samples analyzed. The impact of wastewater effluents was evidenced by a notable increase of pharmaceutical concentrations as well as in the number of compounds detected in the samples collected downstream of WWTP discharges. The effect of the WWTP was observed even for small populations located along the river. The compounds most frequently found were acetaminophen, gabapentin, venlafaxine, valsartan, ciprofloxacin and diclofenac.

The complementary use of target quantitative methodology and qualitative wide-scope screening, allowed to have a more complete overview on the pharmaceuticals present in water. Accurate-mass data acquired by UHPLC-HRMS also allowed to investigate the presence of metabolites, leading to the identification of nine compounds, of which 4-acetylaminoantipyrine (4-AAA), 4-formylaminoantipyrine (4-FAA), 4-OH omeprazole sulphide, carbamazepine-10,11-epoxide and clopidogrel carboxylic acid were the most detected. Further studies on the occurrence and risks of these metabolites are recommended.

A probabilistic risk assessment for aquatic organisms has been performed, indicating moderate-to-severe ecological risks in four sampling points downstream of WWTP discharges. The toxicity of the pharmaceutical mixture was dominated by analgesic/anti-inflammatory drugs and antibiotics, and the compounds with the highest contribution to the toxicity were phenazone > azithromycin > diclofenac > norfloxacin, ciprofloxacin > clarithromycin. Out of these six compounds, only three are currently included in the EU Watch List. Out of the 13 antibiotic compounds evaluated in this study, 5 were found to exceed threshold concentrations for antibiotic resistance, particularly in the sampling sites downstream of WWTP discharges. Therefore, this study supports the advancement of water sanitation methods to minimize ecological and antibiotic resistance risks in the Mijares River.

Acknowledgements

Eddie Fonseca acknowledges University of Costa Rica for his pre-doctoral grant. Authors would like to thank Joan Vicens Vallés for his support on preparing the maps and to Carlos Sales for his collaboration in water sampling. The authors acknowledge University Jaume I (project UJI-B2018-55) and Ministry of Science, Innovation, and University, Spain (Ref RTI2018-097417-B-I00) for the financial support of our research on emerging contaminants. Andreu Rico is supported by a postdoctoral grant provided by the Spanish Ministry of Science, Innovation, and University (IJCI-2017-33465). The authors also acknowledge the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of LC-MS/MS instrumentation.

References

Aceña, J., Stampachiacchiere, S., Pérez, S., Barceló, D., 2015. Advances in liquid chromatography - high-resolution mass spectrometry for quantitative and qualitative environmental analysis. Anal. Bioanal. Chem. 407, 6289–6299. https://doi.org/10.1007/s00216-015-8852-6

606 Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., Surif, S., 2010. Multi-residue analytical method
607 for human pharmaceuticals and synthetic hormones in river water and sewage
608 effluents by solid-phase extraction and liquid chromatography-tandem mass
609 spectrometry. J. Chromatogr. A 1217, 6791–6806.
610 https://doi.org/10.1016/j.chroma.2010.08.033

611 Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barceló, D., Seiler, T.B., Brion, F.,
612 Busch, W., Chipman, K., de Alda, M.L., de Aragão Umbuzeiro, G., Escher, B.I.,
613 Falciani, F., Faust, M., Focks, A., Hilscherova, K., Hollender, J., Hollert, H., Jäger, F.,
614 Jahnke, A., Kortenkamp, A., Krauss, M., Lemkine, G.F., Munthe, J., Neumann, S.,
615 Schymanski, E.L., Scrimshaw, M., Segner, H., Slobodnik, J., Smedes, F., Kughathas,
616 S., Teodorovic, I., Tindall, A.J., Tollefsen, K.E., Walz, K.H., Williams, T.D., Van den
617 Brink, P.J., van Gils, J., Vrana, B., Zhang, X., Brack, W., 2015. Future water quality
618 monitoring - Adapting tools to deal with mixtures of pollutants in water resource
619 management. Sci. Total Environ. 512–513, 540–551.
620 https://doi.org/10.1016/j.scitotenv.2014.12.057

621 Arenas-Sánchez, A., Rico, A., Rivas-Tabares, D., Blanco, A., Garcia-Doncel, P., Romero-
622 Salas, A., Nozal, L., Vighi, M., 2019. Identification of contaminants of concern in the
623 upper Tagus river basin (central Spain). Part 2: Spatio-temporal analysis and
624 ecological risk assessment. Sci. Total Environ. 667, 222–233.
625 https://doi.org/10.1016/j.scitotenv.2019.02.286

626 Azzouz, A., Ballesteros, E., 2013. Influence of seasonal climate differences on the
627 pharmaceutical, hormone and personal care product removal efficiency of a
628 drinking water treatment plant. Chemosphere 93, 2046–2054.
629 https://doi.org/10.1016/j.chemosphere.2013.07.037

630 Bashaar, M., Thawani, V., Hassali, M.A., Saleem, F., 2017. Disposal practices of unused
631 and expired pharmaceuticals among general public in Kabul. BMC Public Health 17,
632 1–8. https://doi.org/10.1186/s12889-016-3975-z

633 Beccaria, M., Cabooter, D., 2020. Current developments in LC-MS for pharmaceutical
634 analysis. Analyst 145, 1129–1157. https://doi.org/10.1039/c9an02145k

635 Behera, S.K., Kim, H.W., Oh, J.E., Park, H.S., 2011. Occurrence and removal of antibiotics,

636 hormones and several other pharmaceuticals in wastewater treatment plants of
637 the largest industrial city of Korea. Sci. Total Environ. 409, 4351–4360.
638 https://doi.org/10.1016/j.scitotenv.2011.07.015

639 Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to
640 select for resistant bacteria: Proposed limits for environmental regulation. Environ.
641 Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015

642 Boix, C., Ibáñez, M., Sancho, J. V., Parsons, J.R., Voogt, P. de, Hernández, F., 2016.
643 Biotransformation of pharmaceuticals in surface water and during waste water
644 treatment: Identification and occurrence of transformation products. J. Hazard.
645 Mater. 302, 175–187. https://doi.org/10.1016/j.jhazmat.2015.09.053

646 Boix, C., Ibáñez, M., Sancho, J. V., Rambla, J., Aranda, J.L., Ballester, S., Hernández, F.,
647 2015. Fast determination of 40 drugs in water using large volume direct injection
648 liquid chromatography-tandem mass spectrometry. Talanta 131, 719–727.
649 https://doi.org/10.1016/j.talanta.2014.08.005

650 Boix, C., Ibáñez, M., Zamora, T., Sancho, J. V., Niessen, W.M.A., Hernández, F., 2014.
651 Identification of new omeprazole metabolites in wastewaters and surface waters.
652 Sci. Total Environ. 468–469, 706–714.
653 https://doi.org/10.1016/j.scitotenv.2013.08.095

654 Boleda, M.R., Alechaga, É., Moyano, E., Galceran, M.T., Ventura, F., 2014. Survey of the
655 occurrence of pharmaceuticals in Spanish finished drinking waters. Environ. Sci.
656 Pollut. Res. 21, 10917–10939. https://doi.org/10.1007/s11356-014-2885-9

657 Botero-Coy, A.M., Martínez-Pachón, D., Boix, C., Rincón, R.J., Castillo, N., Arias-Marín,
658 L.P., Manrique-Losada, L., Torres-Palma, R., Moncayo-Lasso, A., Hernández, F.,
659 2018. 'An investigation into the occurrence and removal of pharmaceuticals in
660 Colombian wastewater.' Sci. Total Environ. 642, 842–853.
661 https://doi.org/10.1016/j.scitotenv.2018.06.088

662 Bruce, G.M., Pleus, R.C., Snyder, S.A., 2010. Toxicological Relevance of Pharmaceuticals
663 in Drinking Water. Environ. Sci. Technol. 44, 5619–5626.
664 https://doi.org/10.1021/es1004895

665 Buelow, E., Rico, A., Gaschet, M., Lourenço, J., Kennedy, S.P., Wiest, L., Ploy, M.-C.,
666 Dagot, C., 2020. Hospital discharges in urban sanitation systems: Long-term
667 monitoring of wastewater resistome and microbiota in relationship to their eco-
668 exposome. Water Res. X 7, 100045. https://doi.org/10.1016/j.wroa.2020.100045

669 Campos-Mañas, M.C., Plaza-Bolaños, P., Sánchez-Pérez, J.A., Malato, S., Agüera, A.,
670 2017. Fast determination of pesticides and other contaminants of emerging
671 concern in treated wastewater using direct injection coupled to highly sensitive
672 ultra-high performance liquid chromatography-tandem mass spectrometry. J.
673 Chromatogr. A 1507, 84–94. https://doi.org/10.1016/j.chroma.2017.05.053

674 Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac,
675 ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. 59, 309–315.
676 https://doi.org/10.1016/S0147-6513(03)00141-6

677 Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J.,
678 Rodriguez-Roda, I., Buttiglieri, G., 2014. Pharmaceuticals occurrence in a WWTP
679 with significant industrial contribution and its input into the river system. Environ.
680 Pollut. 185, 202–212. https://doi.org/10.1016/j.envpol.2013.10.040

681 Conley, J.M., Symes, S.J., Schorr, M.S., Richards, S.M., 2008. Spatial and temporal
682 analysis of pharmaceutical concentrations in the upper Tennessee River basin.
683 Chemosphere 73, 1178–1187. https://doi.org/10.1016/j.chemosphere.2008.07.062

684 Daneshvar, A., Svanfelt, J., Kronberg, L., Prévost, M., Weyhenmeyer, G.A., 2010.
685 Seasonal variations in the occurrence and fate of basic and neutral
686 pharmaceuticals in a Swedish river-lake system. Chemosphere 80, 301–309.
687 https://doi.org/10.1016/j.chemosphere.2010.03.060

688 de Zwart, D., Posthuma, L., 2005. Complex mixture toxicity for single and multiple
689 species: Proposed methodologies. Environ. Toxicol. Chem. 24, 2665–2676.
690 https://doi.org/10.1897/04-639R.1

691 Della-Flora, A., Wielens Becker, R., Frederigi Benassi, S., Theodoro Toci, A., Cordeiro,
692 G.A., Ibáñez, M., Portolés, T., Hernández, F., Boroski, M., Sirtori, C., 2019.
693 Comprehensive investigation of pesticides in Brazilian surface water by high
694 resolution mass spectrometry screening and gas chromatography–mass

spectrometry quantitative analysis. Sci. Total Environ. 669, 248–257. https://doi.org/10.1016/j.scitotenv.2019.02.354

EPSAR, 2020. Entitat de Sanejament d'aigües. Generalitat Valenciana [WWW Document]. URL http://www.epsar.gva.es/instalaciones/buscador-edar.aspx (accessed 1.10.20).

European Commission, 2018. Commission Implementing Decision (EU) 2018/840 of 5 June 2018 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 Council and repealing Commission Implementing (EU) 2015/495. Off. J. Eur. Union 141, 9–12.

Ferrer, I., Zweigenbaum, J.A., Thurman, E.M., 2010. Analysis of 70 Environmental Protection Agency priority pharmaceuticals in water by EPA Method 1694. J. Chromatogr. A 1217, 5674–5686. https://doi.org/10.1016/j.chroma.2010.07.002

Gao, P., Ding, Y., Li, H., Xagoraraki, I., 2012. Occurrence of pharmaceuticals in a municipal wastewater treatment plant: Mass balance and removal processes. Chemosphere 88, 17–24. https://doi.org/10.1016/j.chemosphere.2012.02.017

García-Galán, M.J., Petrovic, M., Rodríguez-Mozaz, S., Barceló, D., 2016. Multiresidue trace analysis of pharmaceuticals, their human metabolites and transformation products by fully automated on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry. Talanta 158, 330–341. https://doi.org/10.1016/j.talanta.2016.05.061

Garófano-Gómez, V., Martínez-Capel, F., Bertoldi, W., Gurnell, A., Estornell, J., Segura-Beltrán, F., 2013. Six decades of changes in the riparian corridor of a mediterranean river: A synthetic analysis based on historical data sources. Ecohydrology 6, 536–553. https://doi.org/10.1002/eco.1330

Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in wastewater treatment plant. Environ. Sci. Pollut. Res. 21, 7578–7585. https://doi.org/10.1007/s11356-014-2654-9

725 Gómez, M.J., Gómez-Ramos, M.M., Malato, O., Mezcua, M., Férnandez-Alba, A.R., 2010.
726 Rapid automated screening, identification and quantification of organic micro-
727 contaminants and their main transformation products in wastewater and river
728 waters using liquid chromatography-quadrupole-time-of-flight mass spectrometry
729 with an accurate-mass . J. Chromatogr. A 1217, 7038–7054.
730 https://doi.org/10.1016/j.chroma.2010.08.070

731 González-Pleiter, M., Gonzalo, S., Rodea-Palomares, I., Leganés, F., Rosal, R., Boltes, K.,
732 Marco, E., Fernández-Piñas, F., 2013. Toxicity of five antibiotics and their mixtures
733 towards photosynthetic aquatic organisms: Implications for environmental risk
734 assessment. Water Res. 47, 2050–2064.
735 https://doi.org/10.1016/j.watres.2013.01.020

736 Grabic, R., Fick, J., Lindberg, R.H., Fedorova, G., Tysklind, M., 2012. Multi-residue
737 method for trace level determination of pharmaceuticals in environmental samples
738 using liquid chromatography coupled to triple quadrupole mass spectrometry.
739 Talanta 100, 183–195. https://doi.org/10.1016/j.talanta.2012.08.032

740 Gracia-Lor, E., Ibáñez, M., Zamora, T., Sancho, J. V., Hernández, F., 2014. Investigation of
741 pharmaceutical metabolites in environmental waters by LC-MS/MS. Environ. Sci.
742 Pollut. Res. 21, 5496–5510. https://doi.org/10.1007/s11356-013-2464-5

743 Gracia-Lor, E., Sancho, J. V., Hernández, F., 2011. Multi-class determination of around 50
744 pharmaceuticals, including 26 antibiotics, in environmental and wastewater
745 samples by ultra-high performance liquid chromatography-tandem mass
746 spectrometry. J. Chromatogr. A 1218, 2264–2275.
747 https://doi.org/10.1016/j.chroma.2011.02.026

748 Gracia-Lor, E., Sancho, J. V., Serrano, R., Hernández, F., 2012. Occurrence and removal
749 of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean
750 area of Valencia. Chemosphere 87, 453–462.
751 https://doi.org/10.1016/j.chemosphere.2011.12.025

752 Guo, J., Boxall, A., Selby, K., 2015. Do Pharmaceuticals Pose a Threat to Primary
753 Producers? Crit. Rev. Environ. Sci. Technol. 45, 2565–2610.
754 https://doi.org/10.1080/10643389.2015.1061873

Hernández, F., Bakker, J., Bijlsma, L., de Boer, J., Botero-Coy, A.M., Bruinen de Bruin, Y., Fischer, S., Hollender, J., Kasprzyk-Hordern, B., Lamoree, M., López, F.J., Laak, T.L. te., van Leerdam, J.A., Sancho, J. V., Schymanski, E.L., de Voogt, P., Hogendoorn, E.A., 2019a. The role of analytical chemistry in exposure science: Focus on the aquatic environment. Chemosphere 222, 564–583. https://doi.org/10.1016/j.chemosphere.2019.01.118

Hernández, F., Calısto-Ulloa, N., Gómez-Fuentes, C., Gómez, M., Ferrer, J., González-Rocha, G., Bello-Toledo, H., Botero-Coy, A.M., Boıx, C., Ibáñez, M., Montory, M., 2019b. Occurrence of antibiotics and bacterial resistance in wastewater and sea water from the Antarctic. J. Hazard. Mater. 363, 447–456. https://doi.org/10.1016/j.jhazmat.2018.07.027

Hernández, F., Ibáñez, M., Botero-Coy, A.M., Bade, R., Bustos-López, M.C., Rincón, J., Moncayo, A., Bijlsma, L., 2015a. LC-QTOF MS screening of more than 1,000 licit and illicit drugs and their metabolites in wastewater and surface waters from the area of Bogotá, Colombia. Anal Bioanal Chem 407, 6405–6416. https://doi.org/10.1007/s00216-015-8796-x

Hernández, F., Ibáñez, M., Portolés, T., Cervera, M.I., Sancho, J. V., López, F.J., 2015b. Advancing towards universal screening for organic pollutants in waters. J. Hazard. Mater. 282, 86–95. https://doi.org/10.1016/j.jhazmat.2014.08.006

Hoeger, B., Köllner, B., Dietrich, D.R., Hitzfeld, B., 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f. fario). Aquat. Toxicol. 75, 53–64. https://doi.org/10.1016/j.aquatox.2005.07.006

Hong, H.N., Kim, H.N., Park, K.S., Lee, S.K., Gu, M.B., 2007. Analysis of the effects diclofenac has on Japanese medaka (Oryzias latipes) using real-time PCR. Chemosphere 67, 2115–2121. https://doi.org/10.1016/j.chemosphere.2006.12.090

Huijbers, P.M.C., Flach, C.F., Larsson, D.G.J., 2019. A conceptual framework for the environmental surveillance of antibiotics and antibiotic resistance. Environ. Int. 130, 104880. https://doi.org/10.1016/j.envint.2019.05.074

Huntscha, S., Singer, H.P., McArdell, C.S., Frank, C.E., Hollender, J., 2012. Multiresidue

analysis of 88 polar organic micropollutants in ground, surface and wastewater using online mixed-bed multilayer solid-phase extraction coupled to high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1268, 74–83. https://doi.org/10.1016/j.chroma.2012.10.032

Ibáñez, M., Borova, V., Boix, C., Aalizadeh, R., Bade, R., Thomaidis, N.S., Hernández, F., 2017. UHPLC-QTOF MS screening of pharmaceuticals and their metabolites in treated wastewater samples from Athens. J. Hazard. Mater. 323, 26–35. https://doi.org/10.1016/j.jhazmat.2016.03.078

Ibáñez, M., Guerrero, C., Sancho, J. V., Hernández, F., 2009. Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. J. Chromatogr. A 1216, 2529–2539. https://doi.org/10.1016/j.chroma.2009.01.073

Langford, K., Thomas, K. V., 2011. Input of selected human pharmaceutical metabolites into the Norwegian aquatic environment. J. Environ. Monit. 13, 416–421. https://doi.org/10.1039/c0em00342e

Letsinger, S., Kay, P., Rodríguez-Mozaz, S., Villagrassa, M., Barceló, D., Rotchell, J.M., 2019. Spatial and temporal occurrence of pharmaceuticals in UK estuaries. Sci. Total Environ. 678, 74–84. https://doi.org/10.1016/j.scitotenv.2019.04.182

Lindholm-Lehto, P.C., Ahkola, H.S.J., Knuutinen, J.S., Herve, S.H., 2016. Widespread occurrence and seasonal variation of pharmaceuticals in surface waters and municipal wastewater treatment plants in central Finland. Environ. Sci. Pollut. Res. 23, 7985–7997. https://doi.org/10.1007/s11356-015-5997-y

López, S.H., Ulaszewska, M.M., Hernando, M.D., Martínez-Bueno, M.J., Gómez, M.J., Fernández-Alba, A.R., 2014. Post-acquisition data processing for the screening of transformation products of different organic contaminants. Two-year monitoring of river water using LC-ESI-QTOF-MS and GCxGC-EI-TOF-MS. Environ. Sci. Pollut. Res. 21, 12583–12604. https://doi.org/10.1007/s11356-014-3187-y

Lu, M.C., Chen, Y.Y., Chiou, M.R., Chen, M.Y., Fan, H.J., 2016. Occurrence and treatment efficiency of pharmaceuticals in landfill leachates. Waste Manag. 55, 257–264. https://doi.org/10.1016/j.wasman.2016.03.029

Masiá, A., Ibáñez, M., Blasco, C., Sancho, J. V., Picó, Y., Hernández, F., 2013. 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. Anal. Chim. Acta 761, 117–127. https://doi.org/10.1016/j.aca.2012.11.032

Masoner, J.R., Kolpin, D.W., Furlong, E.T., Cozzarelli, I.M., Gray, J.L., Schwab, E.A., 2014. Contaminants of emerging concern in fresh leachate from landfills in the conterminous United States. Environ. Sci. Process. Impacts 16, 2335–2354. https://doi.org/10.1039/c4em00124a

Mokh, S., El Khatib, M., Koubar, M., Daher, Z., Al Iskandarani, M., 2017. Innovative SPE-LC-MS/MS technique for the assessment of 63 pharmaceuticals and the detection of antibiotic-resistant-bacteria: A case study natural water sources in Lebanon. Sci. Total Environ. 609, 830–841. https://doi.org/10.1016/j.scitotenv.2017.07.230

Oldenkamp, R., Beusen, A.H.W., Huijbregts, M.A.J., 2019. Aquatic risks from human pharmaceuticals - modelling temporal trends of carbamazepine and ciprofloxacin at the global scale. Environ. Res. Lett. 14, 34003. https://doi.org/10.1088/1748-9326/ab0071

Paíga, P., Santos, L.H.M.L.M., Delerue-Matos, C., 2017. Development of a multi-residue method for the determination of human and veterinary pharmaceuticals and some of their metabolites in aqueous environmental matrices by SPE-UHPLC–MS/MS. J. Pharm. Biomed. Anal. 135, 75–86. https://doi.org/10.1016/j.jpba.2016.12.013

Pereira, A., Silva, L., Laranjeiro, C., Lino, C., Pena, A., 2020. Selected Pharmaceuticals in Different Aquatic Compartments: Part I—Source, Fate and Occurrence. Molecules 25. https://doi.org/10.3390/molecules25051026

Picó, Y., Alvarez-Ruiz, R., Alfarhan, A.H., El-Sheikh, M.A., Alshahrani, H.O., Barceló, D., 2020. Pharmaceuticals, pesticides, personal care products and microplastics contamination assessment of Al-Hassa irrigation network (Saudi Arabia) and its shallow lakes. Sci. Total Environ. 701. https://doi.org/10.1016/j.scitotenv.2019.135021

Pitarch, E., Cervera, M.I., Portolés, T., Ibáñez, M., Barreda, M., Renau-Pruñonosa, A.,

Morell, I., López, F., Albarrán, F., Hernández, F., 2016. Comprehensive monitoring of organic micro-pollutants in surface and groundwater in the surrounding of a solid-waste treatment plant of Castellón, Spain. Sci. Total Environ. 548–549, 211–220. https://doi.org/10.1016/j.scitotenv.2015.12.166

Posthuma, L., Traas, T.P., Suter GW, editors. 2002. Species sensitivity distributions in risk assessment. Boca Raton (FL): CRC Press, 564 p.

Posthuma, L., van Gils, J., Zijp, M.C., van de Meent, D., de Zwartd, D., 2019. Species Sensitivity Distributions for Use in Environmental Protection, Assessment, and Management of Aquatic Ecosystems for 12 386 Chemicals. Environ. Toxicol. Chem. 38, 703–711. https://doi.org/10.1002/etc.4373

Praveena, S.M., Mohd Rashid, M.Z., Mohd Nasir, F.A., Sze Yee, W., Aris, A.Z., 2019. Occurrence and potential human health risk of pharmaceutical residues in drinking water from Putrajaya (Malaysia). Ecotoxicol. Environ. Saf. 180, 549–556. https://doi.org/10.1016/j.ecoenv.2019.05.051

Rico, A., Arenas-Sánchez, A., Pasqualini, J., García-Astillero, A., Cherta, L., Nozal, L. and Vighi, M., 2018. Effects of imidacloprid and a neonicotinoid mixture on aquatic invertebrate communities under Mediterranean conditions. Aquatic toxicology, 204, pp.130-143.

Rico, A., Jacobs, R., Van den Brink, P.J., Tello, A., 2017. A probabilistic approach to assess antibiotic resistance development risks in environmental compartments and its application to an intensive aquaculture production scenario. Environ. Pollut. 231, 918–928. https://doi.org/10.1016/j.envpol.2017.08.079

Rico, A., Van den Brink, P.J., Leitner, P., Graf, W., Focks, A., 2016. Relative influence of chemical and non-chemical stressors on invertebrate communities: a case study in the Danube River. Sci. Total Environ. 571, 1370–1382. https://doi.org/10.1016/j.scitotenv.2016.07.087

Rivera-Jaimes, J.A., Postigo, C., Melgoza-Alemán, R.M., Aceña, J., Barceló, D., López de Alda, M., 2018. Study of pharmaceuticals in surface and wastewater from Cuernavaca, Morelos, Mexico: Occurrence and environmental risk assessment. Sci. Total Environ. 613–614, 1263–1274.

https://doi.org/10.1016/j.scitotenv.2017.09.134

Roose-Amsaleg, C., Laverman, A.M., 2016. Do antibiotics have environmental side-effects? Impact of synthetic antibiotics on biogeochemical processes. Environ. Sci. Pollut. Res. 23, 4000–4012. https://doi.org/10.1007/s11356-015-4943-3

Rúa-Gómez, P.C., Püttmann, W., 2012. Impact of wastewater treatment plant discharge of lidocaine, tramadol, venlafaxine and their metabolites on the quality of surface waters and groundwater. J. Environ. Monit. 14, 1391–1399. https://doi.org/10.1039/c2em10950f

Santana-Viera, S., Montesdeoca-Esponda, S., Sosa-Ferrera, Z., Santana-Rodríguez, J.J., 2016. Cytostatic drugs in environmental samples: An update on the extraction and determination procedures. TrAC - Trends Anal. Chem. 80, 373–386. https://doi.org/10.1016/j.trac.2015.08.016

SANTE, 2019. Guidance document on analytical quality control and method validation procedures for pesticide residues analysis in food and feed. Directorate-General for Health and Food Safety.

Schäfer, R.B., Gerner, N., Kefford, B.J., Rasmussen, J.J., Beketov, M.A., de Zwart, D., Liess, M. and von der Ohe, P.C., 2013. How to characterize chemical exposure to predict ecologic effects on aquatic communities?. Environmental science & technology, 47(14), pp.7996-8004.

Sehonova, P., Plhalova, L., Blahova, J., Doubkova, V., Prokes, M., Tichy, F., Fiorino, E., Faggio, C., Svobodova, Z., 2017. Toxicity of naproxen sodium and its mixture with tramadol hydrochloride on fish early life stages. Chemosphere 188, 414–423. https://doi.org/10.1016/j.chemosphere.2017.08.151

Tong, A.Y.C., Peake, B.M., Braund, R., 2011. Disposal practices for unused medications around the world. Environ. Int. 37, 292–298. https://doi.org/10.1016/j.envint.2010.10.002

van Nuijs, A.L.N., Tarcomnicu, I., Simons, W., Bervoets, L., Blust, R., Jorens, P.G., Neels, H., Covaci, A., 2010. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13

top-prescribed pharmaceuticals in influent wastewater. Anal. Bioanal. Chem. 398, 2211–2222. https://doi.org/10.1007/s00216-010-4101-1

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci. Total Environ. 430, 109–118. https://doi.org/10.1016/j.scitotenv.2012.04.055

WHO, 2019. World Health Organization. Critically important antimicrobials for human medicine [WWW Document]. URL https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf (accessed 4.20.20).

Xu, C., Niu, L., Guo, H., Sun, X., Chen, L., Tu, W., Dai, Q., Ye, J., Liu, W., Liu, J., 2019. Long-term exposure to the non-steroidal anti-inflammatory drug (NSAID)naproxen causes thyroid disruption in zebrafish at environmentally relevant concentrations. Sci. Total Environ. 676, 387–395. https://doi.org/10.1016/j.scitotenv.2019.04.323

Table 1. Target pharmaceuticals and results obtained by UHPLC-MS/MS (QqQ) quantitative analysis of water samples collected in the three campaigns. Percentages were calculated from a total number of 57 samples. Lowest calibration level (LCL), used as limit of quantification. The value of LCL/2 was taken as the cut-off reference for detection frequency.

Family	Compound	Positive samples (%)	Positive samples > 0.1 μg/L (%)	Maximum level found (μg/L)	LCL (ng/L)
Analgesics	Acetaminophen √	65	2	0.20	5
	Tramadol √	17	14	1.9	5
Anthelmintic agents	Levamisol	16	2	0.11	5
Antibiotics	Clindamycin	16	4	0.13	5
	Sulfadiazine	5	0	0.020	5
	Sulfamethoxazole √	19	9	0.20	5

	Tetracycline	9	0	0.011	5
	Trimetroprim	12	7	0.72	5
	Azithromycin* √	16	10	1.6	50
	Ciprofloxacin*[a]	33	5	1.1	50
	Clarithromycin* √	14	12	0.33	5
	Erythromycin*	17	2	0.12	5
	Furaltadone	0	0	-	5
	Lincomycin	9	0	0.011	5
	Metronidazole	10	2	0.11	5
	Nalidixic acid	2	2	d	5
	Norfloxacin[a]	25	5	0.94	50
	Oxolinic acid	19	0	d	5
	Roxithromycin	0	0	-	5
Antidepressants	Venlafaxine √	40	14	0.80	5
Antiepileptics	Gabapentin √	42	16	1.9	5
	Carbamazepine √	19	0	0.026	5
	Primidone	26	17	1.0	5
Antihipertensives	Enalapril	0	0	-	5
	Irbesartan √	23	12	1.7	5
	Losartan √	19	12	0.68	5
	Valsartan √	39	16	1.6	5
Antiulcer drugs	Omeprazole sulfide-4-hydroxy √	19	7	0.15	5
	Pantoprazole	14	0	0.013	5
Benzodiazepines	Alprazolam	19	0	0.020	5
	Lorazepam √	16	0	0.094	10
Beta-blocker agents	Metoprolol	14	0	0.057	5
	Salbutamol	17	0	0.023	5
Hypolipidemic agents	Atorvastatin	12	2	0.21	5
	Bezafibrate[b]	9	0	d	1000
	Gemfibrozil[b]	0	0	-	1000
Nonsteroidal anti-inflammatory	Diclofenac √	33	16	0.94	5
	Ketoprofen[b] √	0	0	-	1000
	Naproxen[b] √	14	0	d	1000
	Phenazone	21	14	2.0	10

*Compounds included in the Watch List of the Commission Decision 2018/840

√ Compounds also detected in the UHPLC-QTOF MS screening[a] Results in positive samples should be taken as guidance values since accurate quantification could not be made

[b] Compounds with LCL higher than 0.1 μg/L, so positive samples > 0.1 μg/L is not applicable

d, detected: concentration below LCL and at least one q/Q ratio was accomplished

Table 2. Metabolites and/or transformation products of pharmaceuticals identified in surface water samples by UHPLC-QTOF MS.

Compounds	**Samples**			
	10b	**17b**	**18b**	**19b**
4-AA (4-Aminoantipyrine)	✓	–	–	–
4-AAA (4-Acetylaminoantipyrine)	✓	✓	✓	✓

4-FAA (4-Formylaminoantipyrine)	✓	✓	✓	✓
Carbamazepine-10,11-epoxide	−	✓	✓	−
Clopidogrel carboxylic acid	−	✓	✓	−
O-Desmethyl venlafaxine	−	t	t	−
4-OH Omeprazole sulphide	−	✓	✓	−
Losartan carboxylic acid	−	t	t	−
Nordiazepam (N-desmethyldiazepam)	−	t	t	−

✓: confirmed with reference standard, ((de)protonated molecule and at least one fragment ion were observed at the expected retention time).
t: tentative identification ((de)protonated molecule was observed and at least one ion fragment was justified).

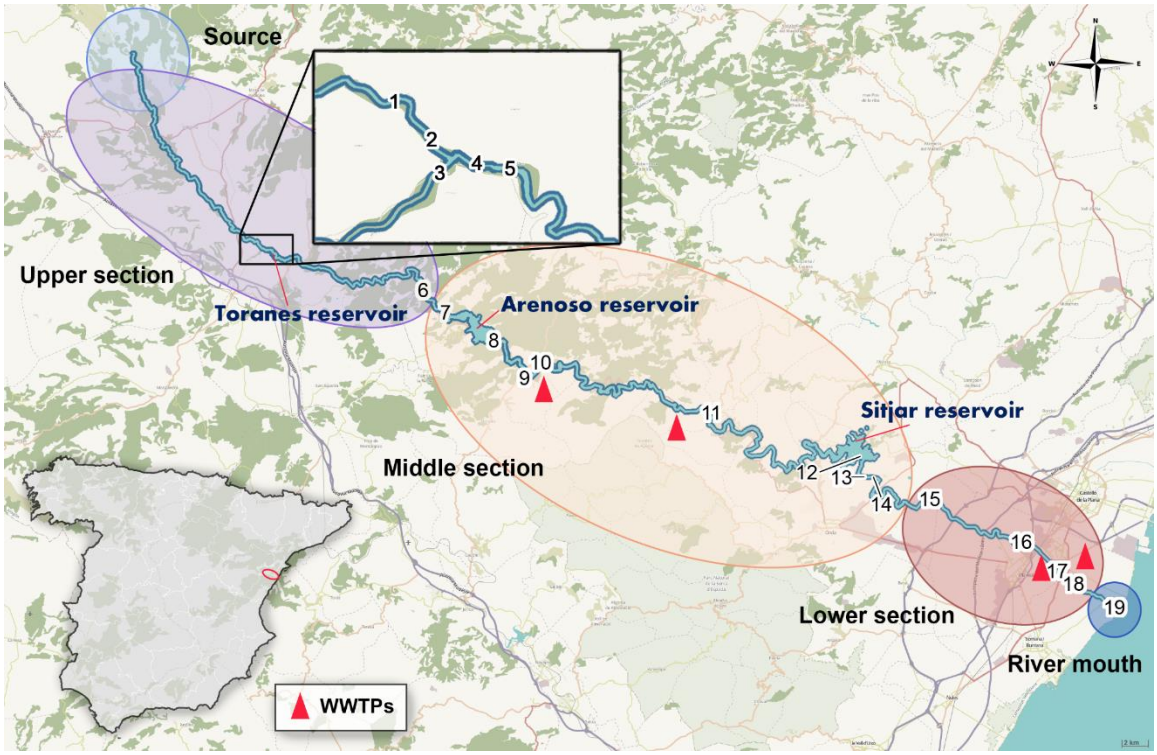


Figure 1. Location of the sampling sites and WWTPs along the Mijares River

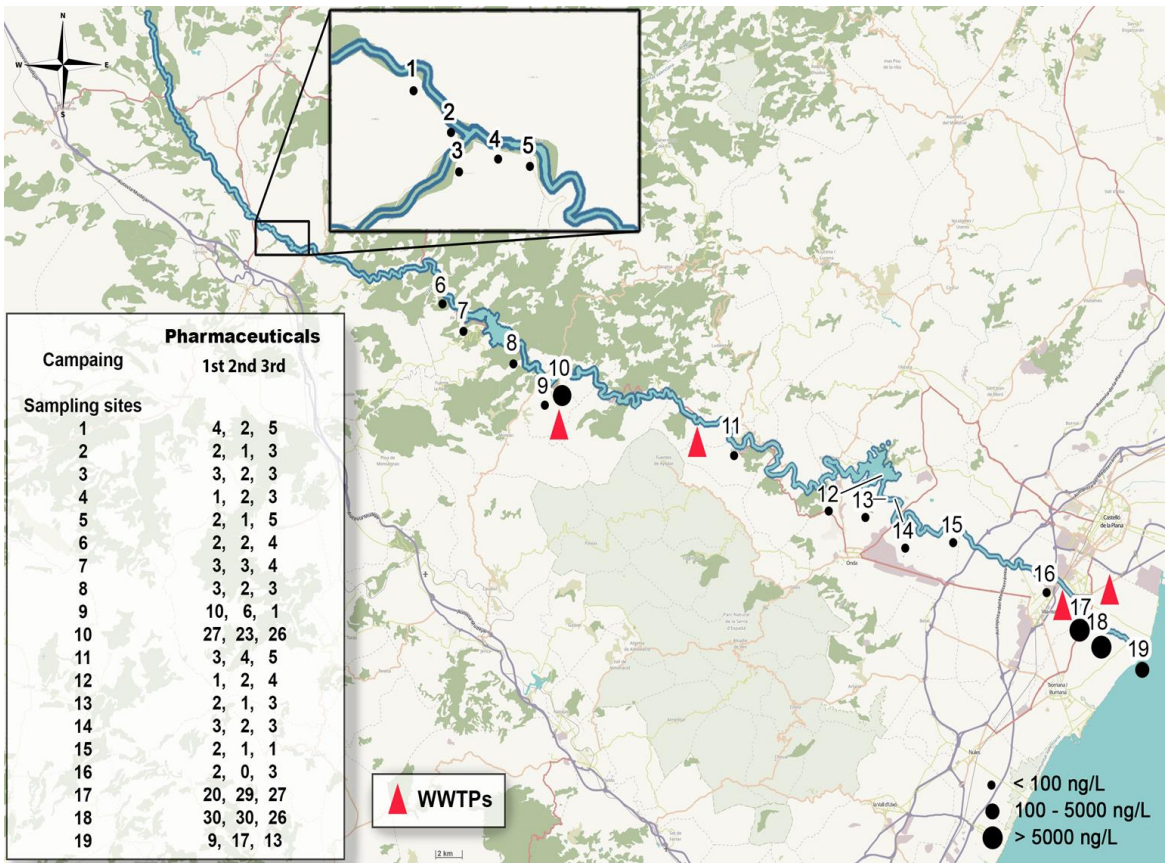
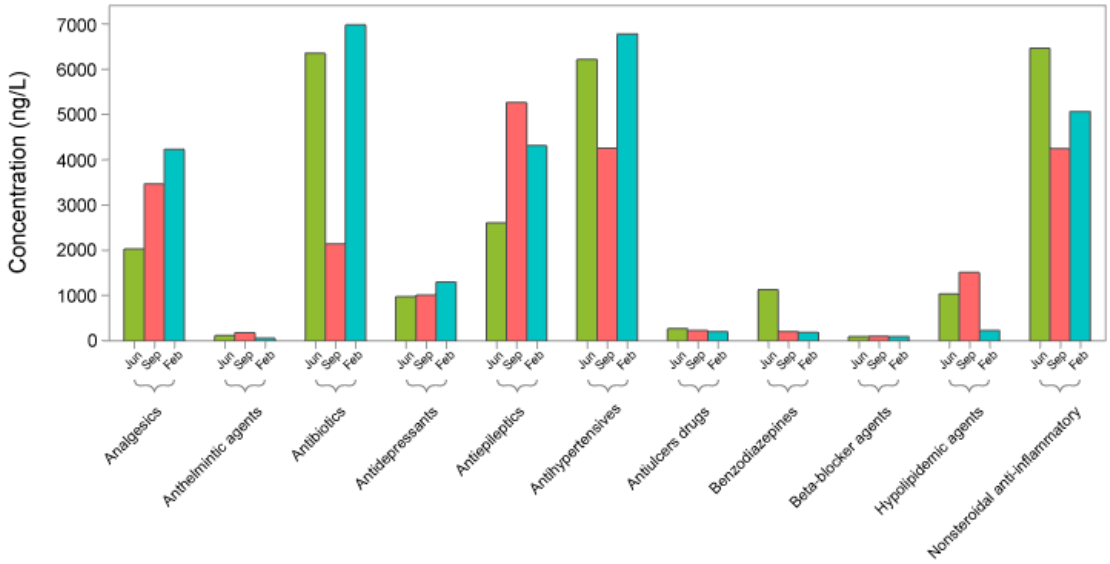


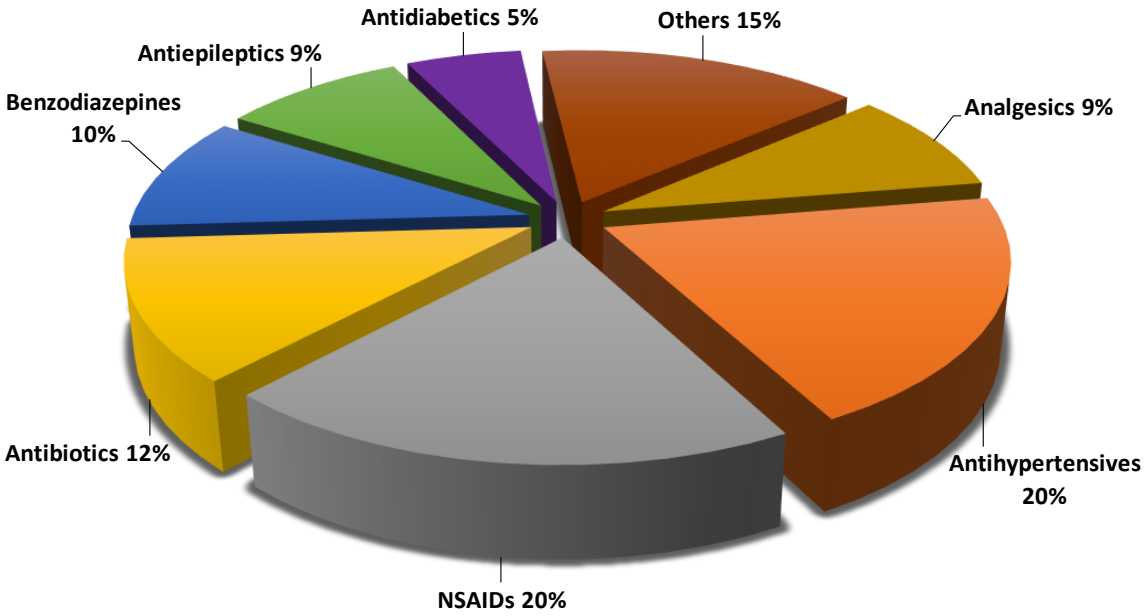
Figure 2. Spatial distribution as total average concentration of pharmaceuticals in Mijares River. In the left side, the number of pharmaceuticals found in each sampling site per campaign is shown (1st: June 2018; 2nd: September 2018; 3rd: February 2019)



959

960 **Figure 3**. Total pharmaceutical concentrations (µg/L) (grouped by families) in the Mijares River in every
961 sampling campaign (1st campaign: June 2018; 2nd campaign: September 2018; 3rd campaign: February
962 2019). NSAIDs: Nonsteroidal anti-inflammatory drugs

963



964

965 **Figure 4.** Percentages of the different families of pharmaceuticals identified
966 in the Mijares River by UHPLC-QTOF MS screening. NSAIDs: Nonsteroidal
967 anti-inflammatory drugs. The "Others" category includes the following
968 types of pharmaceuticals: anesthetics, antihistamines, antiparasitics,
969 antipsychotics, antiviral and X-ray contrast agents

970

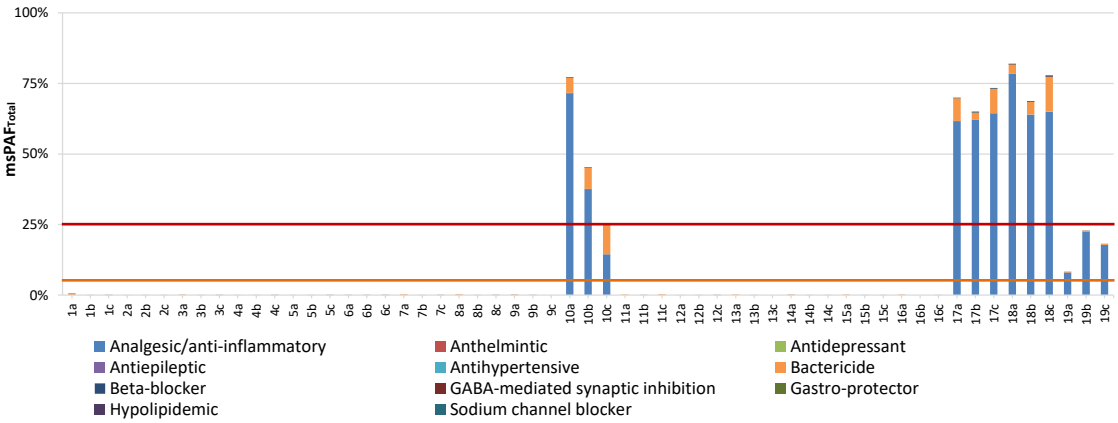


Figure 5. Calculated total chronic toxicity (msPAFTotal) for each sample and relative contribution of each specific therapeutic class to the total toxic pressure. The orange line indicates an msPAFTotal of 5%, and the red line an msPAFTotal of 25%. a, b, c refer to the samples taken in the first, second and third sampling campaigns, respectively (1st campaign: June 2018; 2nd campaign: September 2018; 3rd campaign: February 2019)

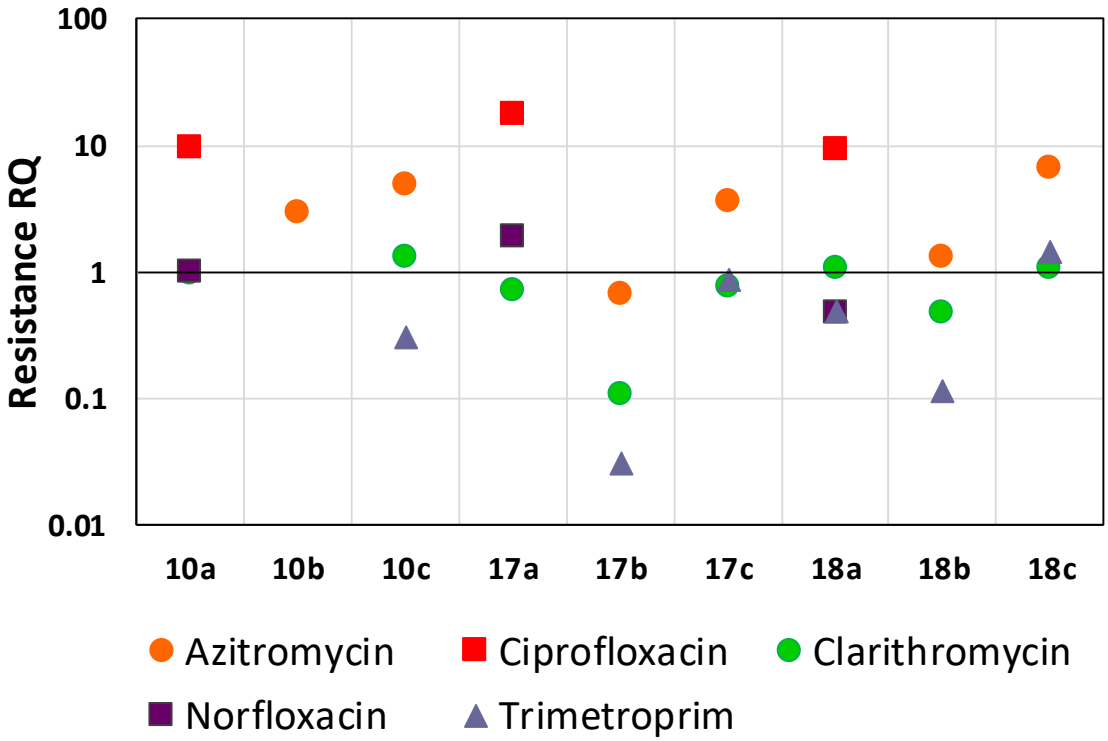


Figure 6. Calculated RQs for the antibiotics that are expected to result in antibiotic resistance risks (RQ>1) in at least one of the samples. Only the sites with RQs higher than

one are represented. a, b, c refer to the samples taken in the first, second and third sampling campaigns, respectively (1st campaign: June 2018; 2nd campaign: September 2018; 3rd campaign: February 2019