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Research Paper

Removal efficiency for emerging contaminants in a WWTP from Madrid (Spain) after secondary and tertiary treatment and environmental impact on the Manzanares River

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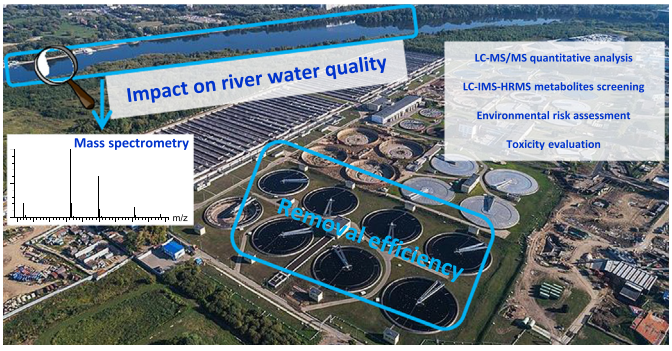
HIGHLIGHTS

- 40 pharmaceuticals and 7 pesticides monitored in wastewater and river water.
- Tertiary treatment notably improved the removal efficiency of pharmaceuticals.
- 7 pharmaceuticals detected in the river showed moderate or high environmental risk.
- 4 antibiotics and 7 pesticides investigated were included in the EU Watch List.
- The results demonstrated the environmental impact of the treatment plant.

GRAPHICAL ABSTRACT



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ABSTRACT

The effluents from wastewater treatment plants (WWTPs) can be an important contamination source for receiving waters. In this work, a comprehensive study on the impact of a WWTP from Madrid on the aquatic environment has been performed, including a wide number of pharmaceuticals and pesticides, among them those included in the European Watch List. 24-h composite samples of influent (IWW) and effluent wastewater after secondary (EWW2) and after secondary + tertiary treatment (EWW3) were monitored along two campaigns. Average weekly concentrations in IWW and EWW2 and EWW3 allowed estimating the removal efficiency of the WWTP for pharmaceutical active substances (PhACs). In addition, the impact of EWW3 on the water quality of the Manzanares River was assessed, in terms of PhAC and pesticide concentrations, through analysis of the river water collected upstream and downstream of the discharge point. After a preliminary risk assessment, a detailed evaluation of the impact on the aquatic environment, including a toxicological study and screening of pharmaceutical metabolites, was made for the seven most relevant PhACs: sulfamethoxazole, azithromycin and clarithromycin (antibiotics), metoprolol (antihypertensive), diclofenac (anti-inflammatory/analgesic), irbesartan (antihypertensive), and the antidepressant venlafaxine. Among selected PhACs, irbesartan,

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clarithromycin and venlafaxine presented moderate or high risk in the river water downstream of the discharge. Albeit no acute toxicity was detected, more detailed studies should be carried out for these substances, including additional toxicological studies, to set up potential sublethal and chronic effects on aquatic organisms.

1. Introduction

The extensive contamination of the aquatic environment with anthropogenic micropollutants, among them those known as "emerging contaminants" (ECs), is a matter of concern in the last years. ECs comprise many different compounds, emphasizing pharmaceutical active substances (PhACs) and personal care products. Among PhACs, antibiotics are of particular concern (Voigt et al., 2020). The presence of these compounds in waters can pose threats to aquatic organisms, such as cyanobacteria, algae, crustaceans, fish, etc., and promote antibiotic resistance, which could be associated to inappropriate use and disposal of these medicines (Felis et al., 2020; Kumar et al., 2019; Kümmerer, 2009).

The main source of PhAC residues in the aquatic environment is human excretion, and consequently the widespread presence of pharmaceuticals in environmental samples is most likely to occur from wastewater treatment plants (WWTPs), which commonly do not completely remove these compounds (Burns et al., 2018). Besides the wastewater treatment applied in a WWTP, physicochemical characteristics of each compounds also affect their elimination in WWTPs, and their ability to interact with solid particles also plays an important role. Thus, compounds with low sorption coefficients tend to remain in the aqueous phase, favouring their mobility through the WWTPs and into the receiving water (Behera et al., 2011; Rosal et al., 2010). Most WWTPs are not equipped for dealing with complex pharmaceuticals, pesticides or personal care products, as they were built and upgraded with the aim of removing biodegradable carbon, nitrogen and phosphorus compounds and pathogens. So, many ECs are able to pass non-degraded and unhindered through conventional treatments, turning the WWTPs in significant sources of pharmaceuticals to the environment, including parent compounds, metabolites, as well as transformation products (TPs) (Fonseca et al., 2020; Lindholm-Lehto et al., 2016; Pereira et al., 2020a). The use of advanced analytical methodologies has notably contributed to the increase of knowledge on the occurrence of these compounds in the aquatic environment (Hernández et al., 2015, 2019a).

Pharmacokinetic data provide understanding the environmental occurrence and behavior of PhACs in the water compartment (Almeida et al., 2014). After consumption, pharmaceuticals are metabolized and primarily excreted in urine and faeces as a mixture of the parent compound and its metabolites. The proportion of compounds excreted as the original components and as conjugates (glucuronide and sulphate) is of great relevance (Pereira et al., 2020a). The excretion rateand the consumption data contribute to either a greater or lesser environmental impact and is related to the reported occurrence of the parent compound and its metabolites in the aquatic compartment. Although only data on concentrations of parent compounds are commonly reported in the literature, information on the presence of metabolites in the aquatic environment is of relevance to provide for a realistic overview of the current situation (Ibáñez et al., 2021).

Although no legal limits have been established in water, several compounds have been included in the different European Union (EU) Watch Lists (WLs) to obtain more EU-wide monitoring data, with the final goal to better regulate priority pollutants in the aquatic environment. The last WL (European Comission 2020/1161, 2020) comprises 8 PhACs, and for the first time, a metabolite, *O*-desmethyl-venlafaxine, revealing the increasing concern on the presence of PhAC-related compounds. The identification of PhACs residues in the water environment and their prioritization are important goals to be accomplished for future regulatory updates in order to minimize the impact of pharmaceuticals into the aquatic environment. This requires robust monitoring campaigns, including seasonal or annual sampling, covering a wide range of compounds, with reasonable spatial resolution (Burns et al., 2018).

In the light of the scientific literature, it can be assumed that there is a risk to the aquatic ecosystems derived from the presence of many ECs in the aquatic environment. Consequently, the implementation of additional purification stages in conventional WWTPs is an urgent need (Nidheesh et al., 2021; Voigt et al., 2020). Removal rates in WWTPs are highly variable between treatment types (Kasprzyk-Hordern et al., 2009; Luo et al., 2014), seasons (Golovko et al., 2014), and even within treatment plants themselves (Verlicchi et al., 2014). Despite the increasing number of publications dealing with the occurrence of ECs in wastewater, removal rates have only been estimated for a small fraction of the total number of pharmaceuticals in use (Botero-Coy et al., 2018; Boxall et al., 2014; Burns et al., 2018; Gracia-Lor et al., 2012).

The environmental impact of PhACs has been recognized worldwide. Obviously, their use cannot be avoided, and therefore an appropriate risk assessment derived from their presence and impact on the environment is a key issue. The potential ecotoxicological effects of PhACs, even at sublethal concentrations, on the aquatic environment is a matter of concern. However, the ecotoxicological risks associated to the ubiquitous occurrence of pharmaceuticals in aquatic ecosystems are far from being fully known (Pereira et al., 2020b). Measured environmental concentrations (MECs) combined with predicted no effect concentration (PNECs) as proposed by European Commission (European Comission, 2003), are commonly used to screen compounds with potential environmental risks in surface water (Desbiolles et al., 2018; Houtman et al., 2014; Mendoza et al., 2014; Palma et al., 2020; Thomaidi et al., 2017; Thomatou et al., 2013).

Chronic exposure to trace levels of PhACs can affect the aquatic environment (Fent et al., 2006) and also to human health (de Jesus Gaffney et al., 2015), with possible consequences such as antibiotic resistance (Hernández et al., 2019b; Posada-Perlaza et al., 2019; Qiao et al., 2018; Sabri et al., 2020) or endocrine disruption (Fent et al., 2006). European Guidelines recommend the calculation of the PNEC using chronic toxicity endpoints as crucial for the assessment of environmental risks of pharmaceuticals to non-target aquatic organisms at a large scale. However, the acute toxicity data still play an important role in risk assessment processes because these data are common requirements under many regulatory frameworks to provide classification and labelling warning, or the possible consequence of exposure to a chemical. In addition, many of the PhACs tend to have short half-lives in water, so in prolonged exposures, the concentration of these substances in the exposure water tends to decrease, and this would not be a very realistic scenario of what we would find in the water of the Manzanares River.

The objective of this work was to carry out a comprehensive study of the impact and performance of an important WWTP in Madrid focused on emerging contaminants. To this aim, the different aspects included in this multidisciplinary research were considered. The specific objectives were: 1) To evaluate the removal efficiency of the WWTP "Viveros de la Villa" from Madrid for the removal of a wide group of PhACs, after application of a conventional secondary treatment and an advanced tertiary process. To this aim weekly monitoring of PhACs concentrations in influent wastewater (IWW) and effluent wastewater after secondary (EWW2) and tertiary (EWW3) treatment were performed. 2) To estimate the discharges of the EWW3 for a wide group of emerging contaminants, including PhACs and pesticides, with special attention to those compounds included in the European WL. 3) To evaluate the impact of the EWW3 on the water quality of the Manzanares River, through the analysis of surface water (SW) collected upstream and downstream of the discharge point. 4) To Perform a risk assessment and acute toxicity study on the most relevant compounds, as well as a suspect screening of their metabolites and TPs in the river water. To reach these objectives, two powerful analytical techniques were applied, liquid chromatography coupled to tandem mass spectrometry

(LC-MS/MS) for quantitative analysis and LC coupled to quadrupole-time of flight mass spectrometry (LC-QTOF MS) for screening of metabolites, including quality control samples to ensure the reliability of data reported.

2. Experimental

2.1. Description of the WWTP and the Manzanares River

The WWTP "Viveros de la Villa" (coordinates 40°27′1"N 3°44′38"W) is located in the North of Madrid (Spain) and treats, partially or totally, wastewater from the districts of Fuencarral-el Pardo, Chamartín, Tetuán and Moncloa, as well as from other municipalities such as Majadahonda, las Rozas and Pozuelo de Alarcón. The plant also has a complementary water regeneration facility to supply the North-West network, which has a treatment capacity of 31,200 m^3/day. The facility has a treatment capacity of 151,200 m^3/day of wastewater and benefits a population of approximately 700,000 inhabitants.

The water line has a biological activated sludge with phosphorus removal, secondary settling with recirculation of sludge, tertiary treatment based on a microfiltration system using textile mesh, an advanced oxidation treatment with ozone generators and an ultraviolet (UV) disinfection system (see **Fig. S1** in the Supplementary Material (**SM**)). In the sludge line, thickening, anaerobic digestion, dehydration and cogeneration are applied.

The effluent wastewater after tertiary treatment is partly discharged into the Manzanares River (see Fig. 1) and partly used to irrigate parks and green areas of Madrid City, as well for as other minor uses such as filling ornamental hydraulic installations, cleaning the sewage network or cleaning wastebaskets. The quality parameters established for the effluent wastewater, according to the design of the tertiary treatment, are shown in **Table S1** in the **SM**.

The Manzanares River is born in the centre of the Iberian Peninsula, in the Sierra de Guadarrama (2258 m), a natural place recognized as a biosphere reserve by UNESCO. The river runs 30 km from the city of Madrid (3.3 million inhabitants) where it has been channelled through the built-up areas of the town. Since the first human settlements, due to its passage through the city, it has been used for irrigation and as a receiver of wastewater from the city. Currently, six WWTPs discharge their effluents into the Manzanares River.

2.2. Collection of samples

In the present study, only the dissolved phase of wastewater was analyzed, while the particulate material was not included in the analyses. As the objective was to evaluate the impact of the WWTP on aquatic environment, the effluent wastewater was of primary importance. In addition, most of the compounds studied were of medium-high polarity, and therefore scarcely sorbed onto the sewage sludge. Three types of wastewater samples (24-h composite) from the WWTP "Viveros de la Villa" were collected: IWW, EWW after secondary treatment (EWW2) and EWW after secondary plus tertiary treatment (EWW3). Firstly, two campaigns were carried out in March–April 2019 and June 2019, in which IWW, EWW2 and EWW3 samples were collected over seven consecutive days. In two subsequent campaigns (October 2019 and January 2020), only one EWW3 sample was taken in each campaign to confirm and support data obtained in the previous campaigns. **Table S2** gives detailed information about wastewater samples collection.

Manzanares River was monitored along the four campaigns (see Fig. 1), collecting three SW samples before (AA) and three after (AB) the discharge point of the WWTP into the river over three consecutive days. Full details are given in **Table S3**.

All samples were collected in high-density polyethylene bottles, stored immediately at < −20 °C, and shipped to the laboratory when the monitoring campaign was completed. Once received in the laboratory, analyses were carried out within a maximum period of 48 h after receipt of the sample. If this was not possible, the samples were stored at < −20 °C until analysis. The time the samples were exposed to the light was minimized as much as possible to reduce the possibilities of photo-degradation.

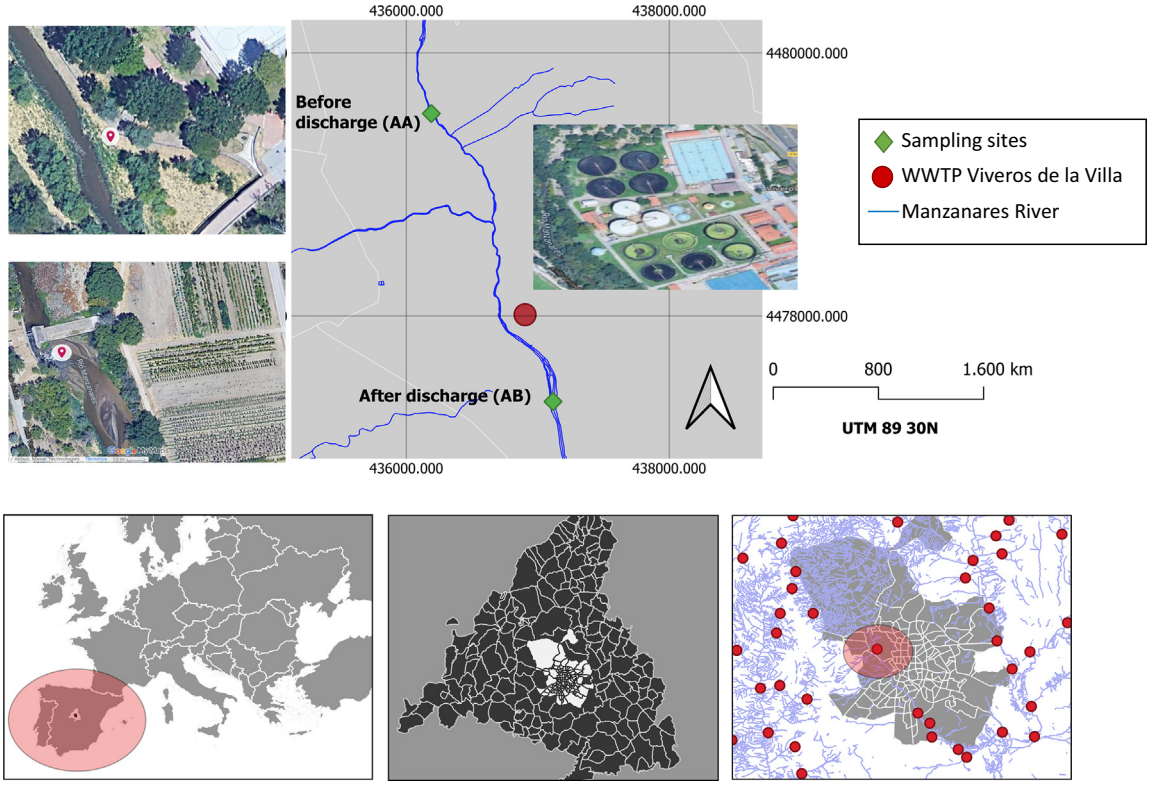

Fig. 1. Map of the sampling location.

2.3. Study design

To achieve the objectives proposed in this research, a comprehensive study was designed in two well differentiated parts. The first one consisted on two monitoring campaigns described as described in section 2.2 (March–April and June 2019) in order to obtain the concentrations of selected PhACs in influent and effluent wastewater (IWW, EWW2 and EWW3). In parallel, SW samples from the Manzanares River were also analyzed. The goal was to estimate the removal efficiency of the WWTP after secondary and tertiary treatments for the wide group of PhACs selected, and to evaluate the impact of treated wastewater (EWW3) into the river, performing a preliminary risk assessment as well. The final expected output was the selection of the most relevant PhACs as a function of their removal in the WWTP and their impact on the river water quality, in order to perform a subsequent and more detailed study on risk assessment and toxicity. Pesticides included in the current WL were also monitored in treated wastewater (EWW2, EWW3) and in surface water (upstream and downstream of the discharge) to evaluate the impact of the wastewater discharges on the water quality of the river.

In the second part of the study, two additional monitoring campaigns (October 2019 and January 2020) were carried out for the PhACs selected in the first part of the study. The concentrations of these compounds in EWW3 and in SW (upstream and downstream) allowed complementary data on environmental impact, as these campaigns were performed in different seasons to those of the first part of the study. A wide suspect screening of metabolites was also performed in the river water for a more complete insight of the impact of PhACs. A screening-level risk assessment for the selected PhACs, and a further analysis of toxicity in aquatic organisms were carried out as well. Finally, the toxicity of the water collected at the sites with the highest risk quotient was evaluated in order to obtain a comprehensive overview of the topic.

2.4. Analysis

2.4.1. Quantitative analysis by LC-MS/MS (pharmaceuticals)

In total, 40 PhACs were quantitatively investigated (see **Table S4**). Four out of the five antibiotics included in the EU WL in force at the moment of performing this work (European Comission 2018/840, 2018) were included in the target list (azithromycin, clarithromycin, erythromycin and ciprofloxacin). The WL was updated while preparing this article, with 8 PhACs (4 antibiotics, 3 azole fungicides and 1 antidepressant) and 1 metabolite (European Comission 2020/1161, 2020) included in the new list. 5 out of the 9 PhACs from the 2020 WL were included in our study (ciprofloxacin, sulfamethoxazole, trimethoprim, venlafaxine, *O*-desmethyl-venlafaxine).

The determination of PhACs was carried out by direct injection of samples without any pre-concentration step (Bijlsma et al., 2021a; Botero-Coy et al., 2018; Fonseca et al., 2020). The only sample treatment was a dilution with Milli-Q water in order to reduce matrix complexity (IWW and EWW were five-fold and two-fold diluted, respectively). Eighteen isotope-labelled internal standard (ILIS) were used for matrix effects correction. Finally, 50 μL of diluted samples were injected into the LC-MS/MS system with a triple quadrupole (Xevo TQ-S™, Waters Corp.). **Tables S4** and **S5** show MS/MS conditions for PhACs and their ILIS, respectively.

PhACs quantification was performed using internal standard (when analyte-ILIS was available) or external standard method (ILIS was not available) with calibration curves prepared in solvent. The lowest calibration level (LCL) was taken as the limit of quantification (LOQ) (see **Table S6**). A compound was considered as "detected" when its concentration was below the LCL and at least one q/Q ratio was accomplished ensuring its reliable identification. For the constructions of graphs, the cut-off value used for detected compounds was half the LCL. The method had been previously validated in different water samples by analysis of a notable number of quality control (QC) samples. For further details, see **SM** and (Bijlsma et al., 2021a; Botero-Coy et al., 2018; Fonseca et al., 2020).

2.4.2. Quantitative analysis by LC-MS/MS (pesticides)

The seven pesticides (methiocarb, metaflumizone, imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid) included in the EU WL in force at the time of performing this work (European Comission 2018/840, 2018) were also investigated in EWW and SW (**Table S7**). The quantitative determination was performed by LC-MS/MS with triple quadrupole (TQD, Waters Corp.) previous solid phase extraction (SPE) with Oasis HLB Prime cartridges. This allowed to reach the low detection limits established in the WL. Six ILIS were used. **Tables S7** and **S8** show MS/MS conditions for pesticides and ILIS, respectively. Quantification was performed using internal standard method, except for metaflumizone for which the ILIS was not available.

The analytical methodology applied for pesticides was validated in SW and EWW samples fortified, in triplicate, at 10 ng/L (**Tables S9** and **S10**). The LOQs were lower than the detection limits established in the WL (2 ng/L for methiocarb, 65 ng/L for metaflumizone and 8.3 ng/L for the rest of pesticides). For further details, see **SM**.

2.4.3. Screening of metabolites by LC-QTOF MS

In order to improve the sensitivity of the screening methodology, a SPE extraction with Oasis HLB cartridges was applied (400-fold preconcentration). Then, 5 μL of sample extract were injected into a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) coupled to a VION high-resolution mass spectrometer equipped with ion mobility separation (IMS-QTOF), with electrospray ionization (ESI) operating in positive ionization mode. For further details, see (Celma et al., 2020) and **SM**.

A database was built, containing the metabolites and degradation products reported in the literature for the seven selected drugs: the antibiotics sulfamethoxazole and clarithromycin, the antihypertensives irbesartan and metoprolol, the non-steroidal anti-inflammatory drug diclofenac and the antidepressant venlafaxine. This database contained a total of 148 compounds (**Table S11**).

A compound was considered as fully identified (Level 1 of confidence identification (Celma et al., 2020)) when the protonated molecule and at least one fragment ion, both with a mass error of less than 5 ppm, were observed in the sample. Additionally, the experimental chromatographic retention time (RT) should differ less than 0.1 min from the RT of the reference standard, and the collision cross section (CCS) error (value obtained by ion mobility) should be less than 2%. So, this was only possible when the analytical reference standard was available. However, for most of the metabolites and TPs reported in the literature there are no commercially available reference standards. In these cases, to consider a compound as tentatively identified (Level 2 of confidence (Celma et al., 2020)), the observed fragment ions were justified on the basis of the compound's structure shared with the original molecule, or on data reported in the literature (e.g. Boix et al., 2016). In all cases, the mass error of the protonated molecule and the fragment ions used for the tentative identification should be less than 5 ppm. When less information was available to support the identification, the compounds could only be identified at higher levels of confidence (up to Level 4) (more details in section 3.5).

2.4.4. Estimation of removal efficiency

The removal efficiency (RE) in WWTPs can be estimated either by comparing the concentration of the contaminant in inlet and outlet waters or by using the total daily loads when flow rates (m^3/24 h) of the corresponding streams are available. In this work, RE was estimated for each analyte from the average weekly concentrations (i.e. average calculated from the daily concentrations of the 7 samples analyzed in one week) (Eq. 1)

$$\mathrm{RE}\ (\%) = \frac{c_I - c_E}{c_I} \times 100 \quad (1)$$

where c_I and c_E are the average concentration (ng/L) in IWW and EWW samples, respectively.

In the case of loads, the RE was estimated with the average weekly loads (see Eq. 2, where q_I and q_E are the average daily loads of pharmaceutical (g/24 h) in IWW sand EWW sample, respectively).

$$\mathrm{RE}\ (\%) = \frac{q_I - q_E}{q_I} \times 100 \quad (2)$$

When a compound was detected but could not be quantified (since its concentration was below the LOQ), a concentration equivalent to half the quantification value (or the daily load corresponding to this concentration value) was considered in order to calculate the removal efficiency.

2.5. Risk assessment

The potential risk for the aquatic organisms/environment was assessed based on the Hazard Quotient (HQ), calculated as HQ = MEC/PNEC (MEC is the measured environmental concentration and PNEC the predicted no effect concentration). Values of HQ < 0.1 lead to a risk classified as negligible. For 0.1 < HQ < 1 the risk is considered as low, suggesting that the compound is less likely to cause hazardous effects in aquatic environment, but small-scale adverse effect should be considered. Values 1 < HQ < 10 suggest a moderate risk, and adverse effects on aquatic species are possible. Finally, for HQ > 10 high hazard is predicted.

PNEC is the concentration of a substance, below which adverse effects will most likely not occur during short or long term exposure (European Comission, 2003). Accordingly, PNEC was calculated for each substance using toxicity endpoints on representative organisms of three trophic levels. In the present study, experimental data on algae or aquatic plants (phototrophic level), crustaceans (invertebrates) and fish (vertebrates) were used. Firstly, values for chronic non adverse effect concentrations (NOAECs) and for the effective concentration with reproduction or growth effects for 10% (EC10) or 50% (EC50) of organisms, were selected from literature. When data were not available, the lethal concentrations for 50% of organisms (LC50) were used. In the absence of such experimental toxicity data, acute toxicity values were obtained through the application of QSAR (Quantitative Structure-Activity Relationship) or ECOSAR (Ecological Structure Activity Relationships) tools. According to the EU guidelines (European Comission, 2003), to overcome the uncertainty related to the raw toxicity data and to derive the PNEC, an assessment factor (AF) was used: i) 10 was applied when chronic values are available for all three trophic levels; ii) 50 was given when chronic data are available for two of the three trophic levels; iii) 100 was applied when at least one chronic NOAEC value is available for any of the trophic level organisms; and iv) value of 1000 was established when at least one acute toxicity value (LC50) is available for each of the species evaluated **(Table S12)**.

2.6. Toxicity

Toxicity for the Manzanares River water was evaluated according to the OECD Test Guideline for testing of chemicals (TG) n203: Fish, Acute Toxicity Testing. Seven juveniles (weight 3.41 ± 1.8 g and length 7.2 ± 0.7 cm) of goldfish (*Carassius auratus*) were randomly distributed (loading 0.5 g wet weigh fish/L) in glass aquarium (20*L*) filled with water from the Manzanares River, and dechlorinated and filtered tap water as control, in triplicate. The tests were performed semi-statically for 96 h, without feeding. Following the guideline, part of the water was renewed (25–30%) daily, and water test conditions of temperature (21.0 ± 1.3 °C), pH (7.9 ± 0.4) and dissolved oxygen (80.3 ± 1.9%) were verified, before and after partial water renewal. Mortalities and visible fish abnormalities related to appearance and behavior were daily recorded during 96 h of exposure. Before the test, fish was acclimated for 11 days in the same conditions in big rectangular tanks (50 L) and was daily fed following the recommendations of food provider.

Experimental procedure with fish was conducted according to the Spanish regulation (RD 53/2013, 2013) and the European animal directive (Directive 2010/63/EU, 2010) for the protection of animals used in experiments and other scientific purposes. Procedures used were approved by the Ethics and Animal Welfare Committees of "Instituto de Acuicultura de Torre de la Sal" (IATS), The Spanish National Research Council (CSIC) and "Generalitat Valenciana" (reference: 2020/VSC/PEA/0150). Experiments were carried out in the registered installation facility of IATS (code ES120330001055).

3. Results and discussion

3.1. Analytical quality control

In this work, special attention was paid to the quality control of analysis in order to ensure the reliability of the results. Thus, QC samples at two fortification levels were included in all analysis sequences. For more details, see **SM**.

As an example, **Tables S13–16** show the recoveries obtained for QCs corresponding to the determination of PhACs in IWW, EWW2, EWW3 and SW samples, respectively. For the less complex-matrix samples (EWW2, EWW3 and SW), most of analytes presented satisfactory recoveries (60–140%, for individual recoveries in QC analysis) (Sante, 2019), with the exceptions of alprazolam and lorazepam (with some recoveries lower than 60%) and gabapentin, metronidazole and sulfadiazone (some recoveries higher than 140%). Despite their greater complexity and matrix effect, the QCs recoveries for IWW was satisfactory for 28 out of the 40 PhACs investigated. When the recoveries for the QCs were outside the 60–140% range, the concentrations obtained were corrected considering the mean recovery obtained for the QCs injected in the sample analysis sequence. For a few analytes, especially in IWW and EWW2 samples, recoveries could not be calculated because of their high concentration in the "blank" real-world samples used for QC preparation. **Tables S12–16** also show relative standard deviation (RSD) values calculated for the four QC recoveries. In general, most of RSDs were ≤ 20% and only a few values were higher than 30%, being EWW2 and EWW3 the samples with more number of cases (around 12%).

Tables S17–18 show the QCs recoveries for pesticides in EWW3 and SW samples, respectively. It can be seen that all were satisfactory except for metaflumizone. The low recovery for this compound could not be corrected because its analog ILIS was not available. In any case, this compound was not detected in any of the samples, and so no concentration data were reported for this pesticide. **Tables S17–18** also show RSD values for the four QC recoveries which all of them below 20%.

3.2. Removal efficiency of pharmaceuticals after secondary and tertiary treatment

Concentrations obtained for the 42 wastewater samples (14 IWW, 14 EWW2 and 14 EWW3) collected along the two first sampling campaigns are shown in **Tables S19-S24**. Additionally, the daily loads of each compound (g/24 h) were calculated from daily pharmaceutical concentration data (ng/L) and flow rate (m^3/24 h) (**Tables S25-S30**). In both campaigns, the behavior and trends observed in terms of elimination of PhACs in the WWTP were similar using either concentration or daily load data. Therefore, the estimation of RE using daily loads was selected for the discussion of the obtained results.

Fig. 2 illustrates the mean RE of PhACs after the secondary and the tertiary treatments applied in the WWTP.

Regarding the secondary treatment, less than half of the PhACs detected (14 out of 33) were almost completely removed, with RE higher than 70% (six of them above 95%). For other PhACs, the elimination was less efficient, but greater than 40% (diclofenac, levamisole, primidone, sulfadiazine and trimethoprim). Another ten compounds were partially removed (RE < 40%) (azithromycin, clarithromycin, clindamycin, erythromycin, irbesartan, metoprolol, metronidazole, pantoprazole, tramadol and venlafaxine). The three remaining PhACs (carbamazepine, lorazepam and phenazone) did not seem to be eliminated with RE values near 0%. Our results are in line with those reported in the current literature, where highly

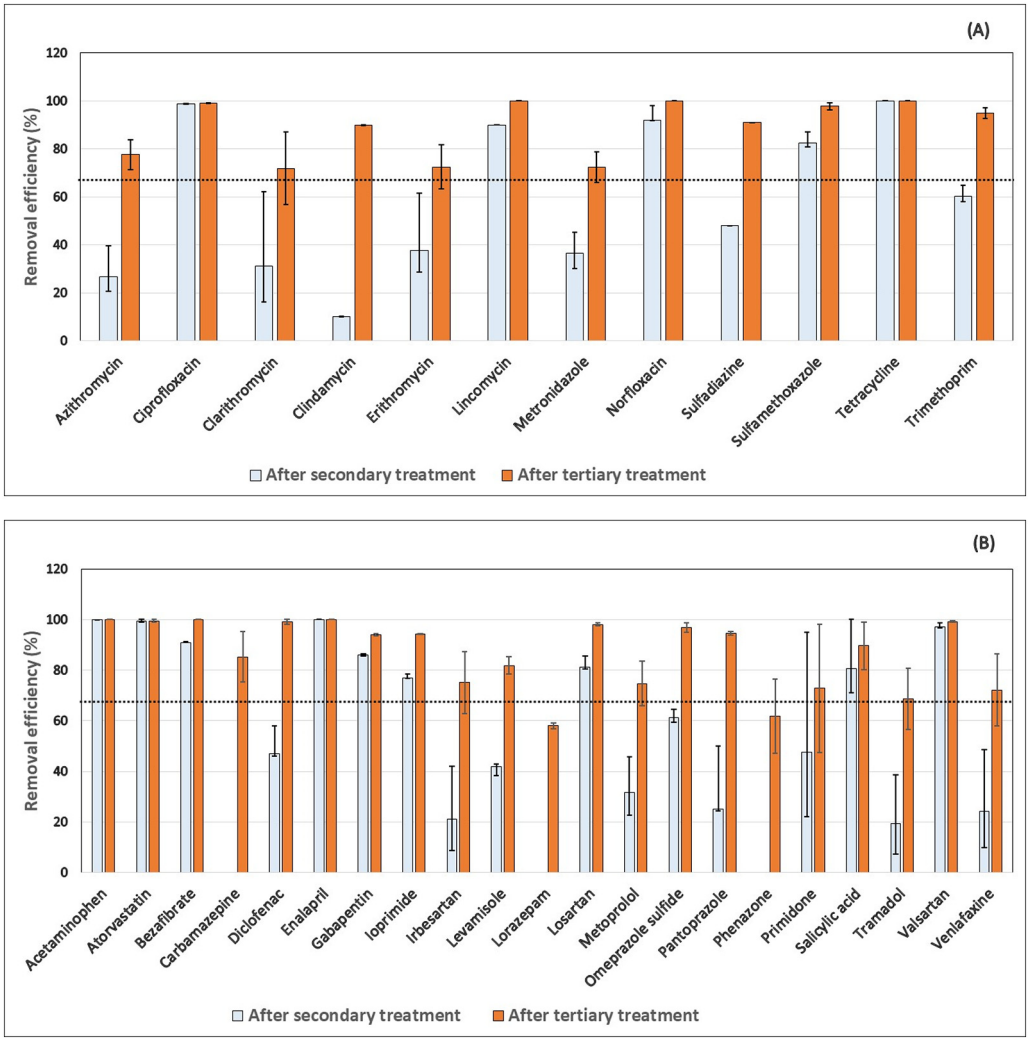

Fig. 2. Mean removal efficiency (%) calculated from total daily loads for antibiotics (A) and the rest of pharmaceuticals (B) in the WWTP after the secondary and the tertiary treatment. Data obtained from the results of the 1st and the 2nd monitoring campaigns. The absence of a bar indicates RE near or below 0%.

varying values of RE have been estimated for PhACs, ranging between negative and high rates (Pereira et al., 2020a). Anti-inflammatories family has been one of the most investigated group, and despite the high variability, average removal rates are between 77% and 95%, with the exception of diclofenac (around 34%) (Luo et al., 2014; Qian et al., 2015). The antiepileptic carbamazepine has been often found to be resistant to wastewater treatments, with RE usually below 18% (Grujić et al., 2009; Leclercq et al., 2009; Qian et al., 2015). Other groups, such as antibiotics, also showed highly variable behavior from low to high removal rates (Behera et al., 2011; Bijlsma et al., 2021a; Karthikeyan and Meyer, 2006; Seifrtová et al., 2010; Zuccato et al., 2010), with average removal rates for the macrolides (azithromycin, clarithromycin and erythromycin) near 30%.

The results obtained in the present study showed that pharmaceutical elimination was more efficient after applying an additional tertiary treatment consisting on a microfiltration system using textile mesh, an advanced oxidation treatment with ozone generators and UV disinfection. As expected, RE after tertiary treatment (see Fig. 2) was better as nearly all PhACs presented efficiencies higher than 70% (15 of them above 95%). Only three compounds (lorazepam, phenazone and tramadol) were not completely eliminated, but the RE was greater than 50%. The literature reports that UV treatment has allowed to partially remove some pharmaceuticals, although it does not completely eliminate them (Homem and Santos, 2011; Pereira et al., 2020a). On the other hand, ozonation alone promotes the partial oxidation of pharmaceuticals, so the use of the combination UV/H_2O_2 appears a common alternative for the treatment of ECs. Sun et al. (2019) and Santos et al. (de Santos et al., 2015) found this combination an important role in removing some antibiotics, such as norfloxacin.

WWTPs generally employ a primary, a secondary and an optional tertiary treatment process, the last one being always associated with a high treatment cost. It seems quite evident that the elimination of many PhACs after conventional treatments in WWTPs is not complete, and it is not exclusively related neither to the physicochemical properties nor to the type of treatment processed (Pereira et al., 2020a). While primary and secondary treatments do not commonly remove efficiently PhACs, tertiary treatments such as advanced oxidation processes, ultraviolet radiation (UV) or ozonation (Gao et al., 2012; Luo et al., 2014) are more efficient for that purpose.

3.3. Impact of treated wastewater on the quality of the river water. Pesticides and PhACs (1st and 2nd campaigns)

The impact of treated wastewater from the WWTP on the water quality of the Manzanares River was investigated for the 40 PhACs and the 7 pesticides included in the 1st and the 2nd monitoring campaigns. To this aim, the concentrations in EWW3 samples and in SW samples from the river were compared. The results for the 14 EWW3 samples are summarized in **Tables S23–24** (PhACs) and **Tables S31–32** (pesticides), respectively. The results for the 12 SW samples analyzed are summarized in **Tables S33–34** (PhACs) and **Tables S35–36** (pesticides).

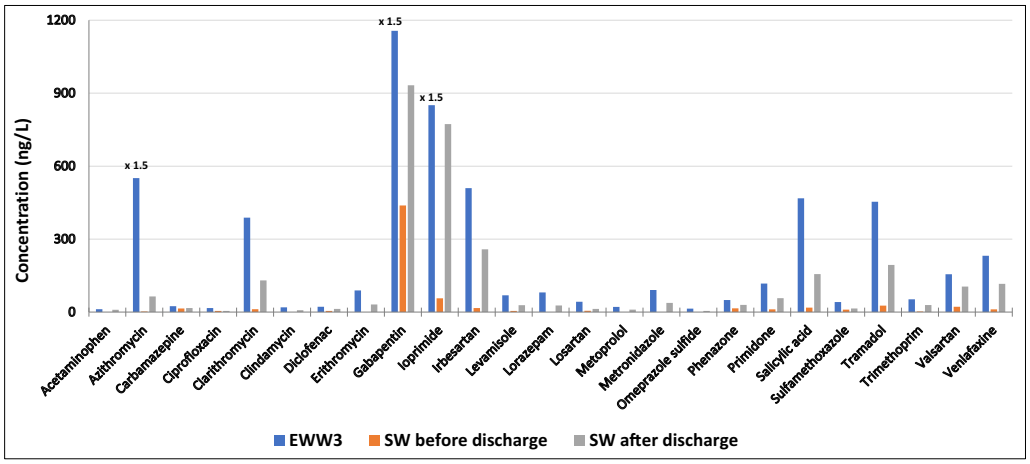

Fig. 3. Mean concentrations of pharmaceuticals detected in EWW3, SW before discharge and SW after discharge samples. The annotation (x1.5) on the bars indicates that concentration level is 1.5 times higher than the level presented in the graphic.

Fig. 3 shows the average concentrations of the pharmaceuticals found in the river samples collected after the discharge from the WWTP. These compounds were also found in EWW3 samples although at much higher concentration levels. Only two analytes exceeded the average value of 500 ng/L in SW after the discharge: the antiepileptic gabapentin (933 ng/L) and the X-ray contrast agent iopromide (773 ng/L). For another six compounds the concentrations were higher than 100 ng/L (clarithromycin, irbesartan, salicylic acid, tramadol, valsartan and venlafaxine) and for the rest were lower than 65 ng/L. Among the 25 PhACs found after the discharge point, 18 of them were also found in the samples taken before the discharge but at much lower concentrations (below 60 ng/L). Only gabapentin was present above 100 ng/L (439 ng/L). This finding is in agreement with other studies in Mediterranean rivers (Fonseca et al., 2020), where gabapentin stood out for its high concentration levels (up to 1.9 μg/L), especially in the samples collected downstream WWTP discharges.

Four of the antibiotics (azithromycin, ciprofloxacin, clarithromycin and erythromycin) identified in the Manzanares River were included in the European WL in force at that moment (European Comission 2018/840, 2018). Three of them surpassed the detection limit indicated in the WL (19 ng/L) in more than 80% of the river samples collected after the discharge in the two campaigns: azithromycin (11–130 ng/L), clarithromycin (69–201 ng/L) and erythromycin (13–49 ng/L). Special attention should be paid to the fact that WWTPs could constitute hotspots for antibiotic emissions, contributing to the enrichment of resistance genes in surface water ecosystems.

Regarding pesticides, Fig. 4 shows the average concentrations in the Manzanares River. Two pesticides (imidacloprid and acetamiprid) were found in EWW3 and also in SW after discharge, but only imidacloprid was also present in the samples before the discharge point. However, its concentration was 3 ng/L, which was notable lower than that found after discharge (21 ng/L). These two neonicotinoid insecticides were found at concentrations above the detection limit established in the WL (8.3 ng/L) (European Comission 2018/840, 2018) in around 70% of samples collected in the river after the discharge: acetamiprid (5–19 ng/L) and imidacloprid (7–37 ng/L).

Imidacloprid has been recently prohibited for outdoor agricultural applications in the European Union (European Comission 2018/783, 2018). Its presence in the river water might be explained by its high persistence in sediments and soils (Bonmatin et al., 2015). The two neonicotinoid insecticides found in Manzanares River have been also reported in other monitoring river catchments in Spain, where agriculture plays an important role (Bijlsma et al., 2021b; Ccanccapa et al., 2016; Fonseca et al., 2019; Kuster et al., 2008; Masiá et al., 2015; Rubirola et al., 2017).

The results obtained illustrate the impact of the wastewater effluents to the river in terms of PhACs and pesticides occurrence. In the near

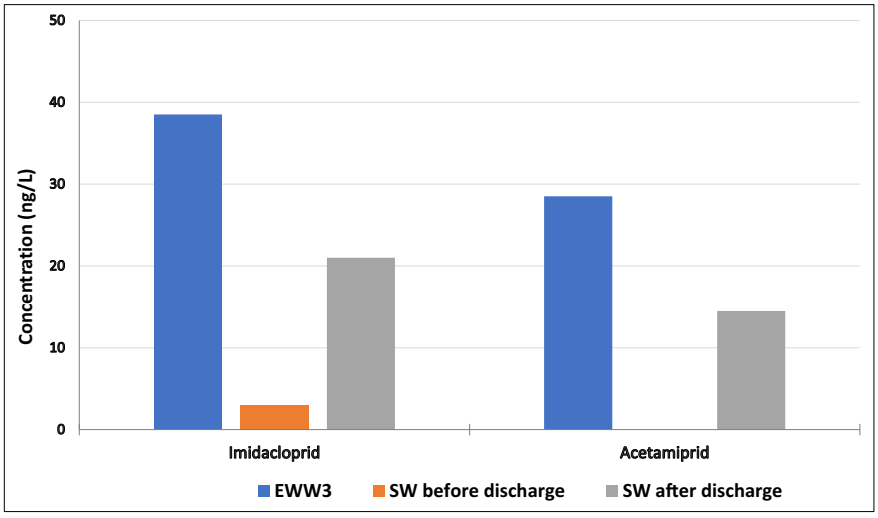

Fig. 4. Mean concentrations of pesticides found in EWW3 and in SW before and after the discharge point of the treated wastewater.

Table 1
Hazard Quotient (HQ) estimated for compounds evaluated in SW samples during 1st campaign (March–April 2019).

	28/03/2019	29/03/2019	01/04/2019	28/03/2019	29/03/2019	01/04/2019
	AA_28_03	**AA_29_03**	**AA_01_04**	**AB_28_03**	**AB_29_03**	**AB_01_04**
Imidacloprid	0.07	0.04	0.04	0.10	0.23	0.19
Acetamiprid	n.d.	n.d.	n.d.	0.04	0.07	0.04
Azithromycin	d.	d.	n.d.	1.46	2.50	2.17
Carbamazepine	0.01	0.01	0.01	0.00	0.00	0.00
Clarithromycin	0.69	0.69	0.77	2.65	4.50	3.38
Clindamycin	n.d.	d.	n.d.	n.d.	d.	n.d.
Diclofenac	n.d.	n.d.	n.d.	n.d.	0.18	n.d.
Erythromycin	n.d.	n.d.	n.d.	0.01	0.03	0.02
Gabapentin	0.00	0.00	0.00	0.00	0.00	0.00
Iopromide	0.00	0.00	0.00	0.00	0.00	0.00
Irbesartan	0.19	0.18	0.20	0.72	2.09	1.16
Levamisole	n.d.	n.d.	n.d.	0.02	0.03	0.02
Lorazepam	n.d.	n.d.	n.d.	0.00	0.00	n.d.
Losartan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Metoprolol	n.d.	n.d.	n.d.	0.25	0.46	0.33
Metronidazole	n.d.	n.d.	n.d.	0.00	0.00	0.00
Omeprazole	n.d.	n.d.	n.d.	n.d.	d.	n.d.
Phenazone	0.01	0.01	0.01	0.01	0.03	0.01
Primidone	0.00	0.00	0.00	0.00	0.00	0.00
Salicylic acid	n.d.	n.d.	n.d.	0.01	n.d.	n.d.
Sulfamethoxazole	0.05	0.05	0.05	0.03	0.08	0.04
Tramadol	0.01	0.01	0.01	0.02	0.04	0.03
Trimethoprim	d.	d.	d.	d.	0.00	d.
Valsartan	n.d.	n.d.	0.00	0.01	0.02	0.01
Venlafaxine	1.27	1.09	1.27	4.36	10.55	6.64

d.: detected (not quantified); n.d.: not detected; gray = negligible risk; green = low risk; yellow = moderate risk; red = high risk. AA: before discharge; AB: after discharge.

future, the requirements of water quality will surely be modified and become stricter, especially in relation to discharges from WWTPs, since the quality of wastewater effluents, one of the main sources of contamination to receiving surface water, is of great relevance (Delgado et al., 2012).

3.4. Selection of relevant compounds after a preliminary risk assessment

Tables 1 and 2 show the hazard quotients estimated in three sampling points before (AA) and after (AB) the discharge of the effluent wastewater during the 1st and 2nd campaigns. Among the 27 compounds detected, imidacloprid, acetamiprid, azithromycin, clarithromycin, diclofenac, irbesartan, metoprolol, sulfamethoxazole and venlafaxine showed risk. Based on risk assessment in both spatial and temporal SW sampling points, the seven most relevant pharmacological compounds were sulfamethoxazole (antibiotic), metoprolol (antihypertensive) and diclofenac (analgesic) with low hazard; azithromycin, clarithromycin (antibiotics) and irbesartan (antihypertensive) which denoted moderate hazard; and the psychotropic drug venlafaxine with the highest level of risk in all the sampling points registered.

3.5. Monitoring of selected PhACs in EWW3 and in SW samples. Screening of metabolites (3rd and 4th campaigns)

The seven selected PhACs were monitored in EWW3 and SW from the river during the 3rd (October 2019) and 4th (January 2020) campaigns, collecting one EWW sample and six SW samples in each campaign. In this way, a whole picture for these compounds along one-year period could be obtained. The concentrations of pharmaceuticals found in these samples are shown in **Tables S37–38**.

All pharmaceuticals were detected in EWW3 and SW collected after the discharge point (at lower concentrations in SW, as expected). However, in the SW collected before the discharge, the antibiotics azithromycin and clarithromycin were not detected and the anti-inflammatory diclofenac was only found in the samples collected in winter (4th campaign). In agreement with data found in the first and second campaigns, the PhACs concentrations were much lower in the river samples collected upstream than in the samples collected downstream of the discharge, evidencing the impact of the WWTP.

In addition, a screening of metabolites of the seven selected PhACs was performed for a comprehensive overview on the presence and impact of PhACs and because of the generalized lack information on occurrence of metabolites and TPs of PhACs. These compounds, which can potentially be as hazardous or even more than the parent compound, can be present in different aquatic bodies at a higher concentration than original molecules (Boix et al., 2016; Ibáñez et al., 2021; Kosjek et al., 2009; Pereira et al., 2020a).

The different treatments applied in WWTPs can affect not only the PhAC removal efficiency but also the metabolites and TPs generated. This supports the need for the evaluation of metabolites and TPs, and to direct the evaluation of such treatments towards the complete mineralization of ECs (Kosjek et al., 2009; Pereira et al., 2020a). These facts, together with the increasing number of reported metabolites and TPs in the water cycle, allow for the conclusion that monitoring campaigns should not be only limited to parent compounds but also to their main metabolites/TPs.

Table 3 shows the results obtained in the screening of two EWW3 samples and two SW samples after the discharge point (campaigns October 2019 and January 2020). It can be seen that numerous metabolites and TPs were found in all samples analyzed, illustrating the wide presence of these compounds in the aquatic environment.

Table 2
Hazard Quotient (HQ) estimated for compounds evaluated in SW samples during 2nd campaign (June 2019).

	19/06/2019	20/06/2019	21/06/2019	19/06/2019	20/06/2019	21/06/2019
	AA_19_06	**AA_20_06**	**AA_21_06**	**AB_19_06**	**AB_20_06**	**AB_21_06**
Imidacloprid	n.d.	0.09	n.d.	0.33	0.42	0.54
Acetamiprid	n.d.	n.d.	n.d.	0.14	0.16	0.17
Azithromycin	n.d.	n.d.	n.d.	0.42	0.21	0.71
Carbamazepine	0.01	0.01	0.01	0.01	0.01	0.01
Clarithromycin	d.	d.	d.	6.31	5.58	7.73
Clindamycin	n.d.	n.d.	n.d.	0.00	0.00	0.00
Diclofenac	d.	d.	d.	0.22	0.30	0.50
Erythromycin	n.d.	n.d.	n.d.	0.04	0.04	0.05
Gabapentin	0.00	0.00	0.00	0.00	0.00	0.00
Iopromide	0.00	0.00	0.00	0.00	0.00	0.00
Irbesartan	0.07	0.09	0.08	2.25	2.52	3.37
Levamisole	d.	d.	d.	0.03	0.03	0.04
Lorazepam	n.d.	n.d.	n.d.	0.00	0.00	0.01
Losartan	0.00	d.	0.00	0.01	0.01	0.01
Metoprolol	n.d.	n.d.	n.d.	0.42	0.54	0.67
Metronidazole	n.d.	n.d.	n.d.	0.00	0.00	0.00
Omeprazole	n.d.	n.d.	n.d.	0.02	0.02	0.04
Phenazone	0.01	0.01	0.01	0.02	0.03	0.04
Primidone	0.00	0.00	0.00	0.00	0.00	0.00
Salicylic acid	0.00	n.d.	0.00	0.00	0.00	n.d.
Sulfamethoxazole	0.12	0.12	0.14	0.17	0.19	0.26
Tramadol	n.d.	0.01	0.01	0.06	0.06	0.08
Trimethoprim	n.d.	n.d.	n.d.	0.00	0.00	0.00
Valsartan	n.d.	n.d.	n.d.	0.00	0.00	0.01
Venlafaxine	0.91	1.00	0.91	12.00	13.09	17.00

d.: detected (not quantified); n.d.: not detected; gray = negligible risk; green = low risk; yellow = moderate risk; red = high risk. AA: before discharge; AB: after discharge

In particular, 5 TPs of azithromycin, 3 of clarithromycin, 6 of venlafaxine, 5 of irbesartan, 6 of metoprolol, 1 of diclofenac and 1 of sulfamethoxazole were detected. Four of them (*O*-desmethyl-venlafaxine, *N*-desmethyl-venlafaxine, *N*-desmethyl-clarithromycin and *N*-acetylsulfamethoxazole), as well as the 7 parent compounds, were confirmed with their corresponding available reference standard (Level 1). Illustrative is the fact that *O*-desmethyl-venlafaxine has been recently included in the 2020 WL (European Comission 2020/1161, 2020). The remaining TPs found in the screening were classified as Level 2b, 3 or 4 (Celma et al., 2020). Level 2b (probable structure) indicates that an exact structure could be proposed based on experimental evidence. Between brackets, the number of fragment ions justified based on the compound structure is indicated. Regarding Level 3 (tentative candidate), different chemical structures are compatible with the empirical RT, CCS and MS data. Finally, Level 4 (unequivocal molecular formula) indicates that only MS, RT and CCS information, without fragmentation information, is collected.

TPs can often find at higher levels than the original compounds. Although not very usual, transformation process may lead to the formation of intermediates that can be more toxic than the parent compound (Kostopoulou and Nikolaou, 2008). Even in the case of antibiotics, some TPs can also preserve the activity for which the original compounds were designed, retaining chemical groups that confer said antimicrobial activity (Majewsky et al., 2014). Data found in the present work are in agreement with previous findings. Regarding diclofenac, its hydroxylated metabolite (4-hydroxy-diclofenac) has been the most frequently analyzed and it has been detected in river water (García-Galán et al., 2016; López-Serna et al., 2012) and in EWW (García-Galán et al., 2016). As regards antibiotics, sulfamethoxazole metabolites can be highlighted. Specifically, *N*-acetylsulfamethoxazole has been detected in EWW in Castellon province (Spain) at concentrations close to 1 μg/L (Gracia-Lor et al., 2014). Clarithromycin metabolite (*N*-desmethylclarithromycin) has been also found in surface water but at lower concentration level (Gracia-Lor et al., 2014). Within the group of β-blockers, metaprolol metabolites have been detected in EWW, with maximum concentration of metoprolol acid of 2 μg/L (García-Galán et al., 2016). Regarding psychiatric medication, numerous metabolites have been also detected for compounds such as venlafaxine. *N*-desmethylvenlafaxine has been found in EWW and SW samples in Valencia region (Spain) (Boix et al., 2016). In the case of antihypertensives, irbesartan stands out, of which up to 5 metabolites have been detected in effluents and surface water from different points of the Valencian region (Spain) (Boix et al., 2016).

Data obtained in this work can be used in the near future for updating the list of target compounds included in monitoring pharmaceuticals in the aquatic environment.

3.6. Risk assessment

Tables S40 and S41 summarize the results obtained in the monitoring and hazard characterization of the seven selected PhACs for the SW samples collected before and after the discharge point, during the 3rd and 4th campaigns. All evaluated substances presented risk in the water samples collected downstream, highlighting venlafaxine with high risk in both sampling points (up and downstream). According to our results, venlafaxine has been previously categorized as high risk in several studies (Birch et al., 2015; Fernández-Rubio et al., 2019; Gros et al., 2012). Diclofenac and clarithromycin have been also reported to present high and moderate risk, respectively (Palma et al., 2020). The antihypertensive irbesartan has also been identified as high risk (Mijangos et al., 2018). It is notable that the absence of experimental toxicological data for irbesartan make risk assessment difficult, resulting in more probabilistic

Table 3
Results obtained in the screening of metabolites by LC- IMS-HRMS in EWW3 and SW from the Manzanares river (after discharge).

Compound	Elemental composition	RT (min)	3rd campaign		4th campaign	
			EWW3 31_10	SW AB 31_10	EWW3 22_01	SW AB 22_01
Azithromycin	C38H72N2O12	5.5	√	√	√	√
Azithromycin TP1	C37H70N2O12	5.5	Level 2(2)			
Azithromycin TP3	C30H57NO10	7.9	Level 2(2)	Level 2(2)	Level 2(3)	Level 2(2)
Azithromycin TP4	C30H58N2O19	2.6	Level 2(1)	Level 3	Level 3	Level 3
Azithromycin TP5	C22H43NO7	4.3	Level 2(4)	Level 2(6)	Level 2(6)	Level 2(2)
Azithromycin TP6	C8H17NO4	7.9			Level 3	
Clarithromycin	C38H69NO13	9.3	√	√	√	√
14-OH-Clarithromycin/ Clarithromycin-N-oxide	C38H69NO14	7.5	Level 3	Level 3	Level 3	Level 3
14-OH-Clarithromycin/ Clarithromycin-N-oxide	C38H69NO14	9.8	Level 2(1)	Level 2(1)	Level 2(1)	Level 2(1)
N-Desmethyl Clarithromycin	C37H67NO13	8.2			√ (*m/z*, RT, CCS, no frag)	√ (*m/z*, RT, CCS, no frag)
Venlafaxine	C17H27NO2	5.7	√	√	√	√
O-Desmethyl venlafaxine	C16H25NO2	3.7	√	√	√	√
VB2/Venlafaxine_N oxide/ Venlafaxine_TP3	C17H27NO3	8.0	Level 3	Level 3	Level 3	Level 3
VB3a/VB3b/Venlafaxine_TP4	C17H27NO3	2.2	Level 3	Level 3		
VB3a/VB3b/Venlafaxine_TP4	C17H27NO3	2.8	Level 2(1)	Level 3	Level 3	Level 3
Venlafaxine_TP2 = N-desmethylvenlafaxine	C16H25NO2	5.8	√ (m/z, RT, CCS, no frag)	√ (m/z, RT, CCS, no frag)	√ (m/z, RT, CCS, no frag)	√ (m/z, RT, CCS, no frag)
Metoprolol TP10/Didesmethyl-venlafaxine	C15H23NO2	4.4	Level 2(1)	Level 3	Level 3	Level 3
Irbesartan	C25H28N6O	10.1	√	√	√	√
Irbesartan M1	C25H28N6O3	6.8	Level 2(1)	Level 2(1)	Level 2(2)	Level 2(1)
Irbesartan M2/M3	C25H26N6O3	7.4	Level 2(4)	Level 2(4)	Level 2(5)	Level 2(5)
Irbesartan M6	C25H26N6O2	8.7	Level 2(4)	Level 2(4)	Level 2(4)	Level 2(4)
Irbesartan M6	C25H26N6O2	9.2	Level 2(5)	Level 2(5)	Level 2(5)	Level 2(5)
SR49498/ISW1b/ISW1a	C25H30N6O2	9.4	Level 2(3)	Level 2(3)		
Metoprolol	C15H25NO3	4.2	√	√	√	√
Metoprolol acid	C14H21NO4	2.5		Level 2(2)	Level 2(4)	Level 2(3)
Metoprolol TP7	C15H23NO4	2.5				Level 2(1)
Metoprolol TP8	C13H19NO3	2.1	Level 3	Level 3	Level 2(3)	Level 2(2)
Metoprolol TP8	C13H19NO3	8.0	Level 3	Level 3		207
Metoprolol TP10/ Didesmethyl-venlafaxine	C15H23NO2	4.4	Level 2(1)	Level 3	Level 3	Level 3
Metoprolol TP16	C14H21NO3	8.3	Level 2(1)	Level 2(3)	Level 2(1)	Level 2(3)
Diclofenac	C14H11Cl2NO2	11.5				√
3-OH/4-OH/5-OH/TP2/TP23/TP3/ TP4-diclofenac	C14H11Cl2NO3	10.0			Level 2(1)	Level 2(1)
Sulfamethoxazole	C10H11N3O3S	3.8			√	√
N-acetylsulfamethoxazole	C12H13N3O4S	5.1	√ (m/z, RT, CCS, no frag)	√ (m/z, RT, CCS, no frag)	√ (m/z, RT, CCS, no frag)	√ (m/z, Rt, CCS, no frag)

The level of identification is indicated for all the detected compounds. For metabolites/degradation products without reference standard, the number of fragment ions shared with the unaltered compound is also included in brackets.
RT: Retention time; CCS: Collision Cross Section.
Level 1: Confirmed with reference standard.
Level 2(X): Probable structure. Between brackets the number of fragment ions justified based on the structure.
Level 3: Tentative candidate (different chemical structures are compatible with the empirical RT, CCS and MS data).
Level 4: Unequivocal molecular formula (only MS, RT and CCS information, without fragmentation information, is collected).

approximations when PNEC values are estimated using tools such as QSAR (Busch et al., 2016).

Table 4 summarizes the HQ estimated in the four monitoring campaigns for the substances (venlafaxine, irbesartan, clarithromycin) that presented moderate or high risk in SW collected after the WWTP discharge point, highlighting once again that venlafaxine was the compound of highest concern. It must be taken into account that the variability in the river flow rate over time, as well as the type of sediment in the sampling point, are important parameters that influence the amount of substance in the dissolved phase of the water. In our study, metoprolol and diclofenac showed an increase in risk in October (autumn) and January (winter). The continued presence of irbesartan in SW emphasizes the need for toxicological studies on this substance in order to make a more accurate risk characterization. Some studies (Palma et al., 2020; WHO, 2017) reported that concentrations generally increased during dry periods when the sediments are composed mainly of sand, while in areas where the sediments are made up of fine particles, the highest concentrations were detected after periods of heavy rain, due to the re-suspension of substances from the sediments. It can be concluded that the time factor and periods of drought have a direct influence on the degree of exposure to these types of substances. These trends are of concern and need to be further studied, especially in regions with increasing trends in PhACs use. It should not be forgotten that drought phenomena are closely linked to the regions most affected by the adverse effects of climate change.

3.7. Toxicity study

No fish mortality was detected after 96 h of exposure to water from the Manzanares River. No incidents or visible abnormalities were observed during exposure, which allowed to conclude that the water assayed did not have fish acute effects. However, other sub-lethal effects could be expected due to the presence of venlafaxine, azithromycin, clarithromycin, irbesartan, diclofenac, metoprolol and sulfamethoxazole, as these compounds present some risk at the point downstream of the treatment plant, especially venlafaxine, with high risk in the samples from the Manzanares river. As highlighted above, the continued presence of

Table 4
Hazard Quotient (HQ) obtained in the four monitoring campaigns for the PhACs that represented moderate or high risk downstream.

After discharge		Venlafaxine	Irbesartan	Clarithromycin
March-April 2019	AB_28_03	4.36	0.72	2.65
	AB_29_03	10.55	2.09	4.50
	AB_01_04	6.64	1.16	3.38
June 2019	AB_19_06	12.00	2.25	6.31
	AB_20_06	13.09	2.52	5.58
	AB_21_06	17.00	3.37	7.73
October 2019	AB_28_10	11.36	2.48	5.12
	AB_29_10	10.36	2.14	5.35
	AB_30_10	12.55	2.10	5.81
January 2020	AB_22_01	13.82	3.42	9.92
	AB_23_01	13.00	3.58	8.88
	AB_24_01	11.36	3.15	9.38

green = low risk; yellow = moderate risk; red = high risk. AA: before discharge; AB: after discharge.

irbesartan in the surface water emphasizes the need for further toxicological studies. The worldwide occurrence of PhAC residues in aquatic environments requires monitoring their effects on fish and other aquatic organisms. The acute toxicity data still play an important role in risk assessment of all types of chemicals. Nonetheless, there are limited studies related to the assessment of toxicity in fish on PhACs. The lethal acute ecotoxicity effect of PhACs in aquatic organisms generally occurs at concentrations greater than 1 mg/L (Cunningham et al., 2006).

However, in ecosystems with continuous exposure and high rates of introduction of effluents with PhACs, it is necessary to characterize their chronic and sublethal effects on aquatic life (Conners et al., 2009; Zeilinger et al., 2009).

Among the biological effects of PhACs, behavioral (Painter et al., 2009), histological (Schultz et al., 2013), hematological (Li et al., 2011) and biochemical changes occur more quickly and sensitively, and might be a valuable early warning of pollution. These sublethal effects could allow an integrated measurement of bioavailable contaminants causing biochemical responses (Martínez-Morcillo et al., 2020). More detailed studies should be carried out for these substances, including additional toxicological studies, to set up potential sublethal and chronic effects after of the discharge points of the WWTPs on aquatic organisms.

4. Conclusions

A comprehensive investigation on the impact of a WWTP on the Manzanares River (Madrid, Spain) has been performed for 40 PhACs, as well as for 7 pesticides included in the EU Watch List (2018). Data showed that WWTP removal efficiency notably improved after a tertiary advanced treatment consisting on a microfiltration system using textile mesh, an advanced oxidation treatment with ozone generators and ultraviolet (UV) disinfection. The impact of wastewater effluents into Manzanares River was evidenced by the increase of pharmaceutical and pesticide concentrations, and in the number of compounds detected in samples collected downstream of WWTP discharge, where only two pharmaceuticals (gabapentin and iopromide) exceeded 500 ng/L and another six compounds (clarithromycin, irbesartan, salicylic acid, tramadol, valsartan and venlafaxine) exceeded 100 ng/L. As regards pesticides, only imidacloprid and acetamiprid were found in the samples downstream of the discharge.

After a risk assessment of the river water, seven pharmaceuticals were identified (azithromycin, clarithromycin, venlafaxine, irbesartan, metoprolol, diclofenac and sulfamethoxazole), emphasizing irbesartan, clarithromycin and venlafaxine, which presented moderate or high risk in the river water downstream of the discharge. An additional HRMS-based screening was applied to the river water samples, and allowed to identify a notable number of metabolite/TPS of the above indicated pharmaceuticals, illustrating the need to include not only the parent compounds but also they derivatives in environmental monitoring studies. No mortality was observed in the acute toxicity study performed with goldfish using water from the Manzanares River, and no incidents or visible abnormalities were observed during exposure (96 h), concluding that the water assayed did not have fish acute effects. However, other sub-lethal effects might occur because several PhACs presented some type of risk at the point downstream of the treatment plant. Additional studies on the occurrence, risk assessment and toxicity are necessary in the near future for the most relevant PhACs and metabolites to have a complete picture of the impact of WWTPs on the aquatic environment. Since wastewater effluents are among the main sources of contamination to receiving waters, their quality requirements in relation to the presence and concentrations of emerging contaminants should be strictly considered.

Declaration of competing interest

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

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Appendix A. Supplementary data

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