Mikropoluitzaileak araztegietan pandemiaren ondoren

COVID-19aren itxialdian, asko handitu zen botika terapeutikoen erabilera, birusaren aurkako tratamendu berezirik ez zegoelako. Botika horiek, askotan, uretara joaten dira, eta hondakin-uren tratamendu-zentroetan azaltzen dira, uraren analisi kimikoan. Substantzia horiek berriak dira araztegietan, eta metodo berriak bilatu beharko dira urak garbitzeko. Arazte-teknika berriak eskuragarri daudenean, haiek inplementatzeko baliabideak jarri beharko dira.

Pandemian zehar, Europan araztegietako urak analizatu izan ziren, bai birusaren hedapen geografikoari jarraitzeko, bai eta ustezko mikropoluitzaile berriak bilatzeko ere. Euskadin ere egin zuten; ikertzaile-talde batek bi araztegitako urak analizatu zituen, Crispijanakoa Araban eta Galindokoa Bizkaian, 2020ko apiriletik uztailera bitartean.

Zehaztasun handiko analisien bitartez, substantzia askoren aztarnak detektatu zituzten, hondakin-urak tratatzeko instalazioak ez baitziren eraginkorrak substantzia horiek desagerrarazteko. Kasu batzuetan, substantzien kontzentrazioa ohi baino handiagoa zen; medikamentu antibiralena eta antimikrobianoena, adibidez. Gainera, analisi horien emaitzek adierazten dute antsiolitikoen erabilerak gora egin zuela, bai eta legez kontrako zenbait drogarenak ere. Egoera berriak substantzia berriak ekarri ditu araztegietara, eta arriskua dago substantzia horiek ez detektatzeko. Beraz, analisi-teknikak berak ere aztertu behar izan dira, ahalik eta mikropoluitzaile gehien atzeman ahal izateko.

Pandemiaren eragin kimikoa

Crispijanako eta Galindoko araztegietan, ur-laginak hartu, iragazi eta molekulak oso zehaztasun handiz identifikatzen dituen teknika batez aztertu ziren: substantziak likido-kromatografo batez banatu eta gero, banaka identifikatu ziren masa-espektroskopiaren bitartez.

Bi araztegietan antzeko substantziak topatu dituzte. Gehienbat, produktu farmazeutikoak dira, baina bestelako substantzia asko ere badaude: estimulatzaileak, pestizidak, hormonak, produktu industrialak, suaren kontrako produktuak eta abar. Ohiko medikamentu batzuk ohi baino kontzentrazio altuagoetan azaldu dira, azetaminofenoa (parazetamola), metformina (diabetesaren kontrako botikarik ohikoena) eta kafeina, adibidez. Beste batzuk lehen aldiz azaldu dira Euskadiko araztegi batean. Arreta berezia jaso du COVID-19a tratatzeko ahaleginen ondorioak. Ustezko tratamendu berriei lotutako substantziak agertu dira lehen aldiz araztegietan, hidroxiklorokina eta lopinavir esate baterako. Gainera, antipsikotikoen kopuruak ere handitu dira, eta gauza bera gertatu da legez kanpoko droga batzuekin ere, anfetaminarekin eta ketaminarekin adibidez.

«Araztegietan botiken aztarnak areagotu dira, eta arriskua dago substantzia horiek ez detektatzeko»

Aztertu behar da substantzia horiek ingurumenean kalte egiten ote duten; alegia, haien biotoxikotasuna neurtzeko irizpideak ezarri behar dira. Oro har, molekula bakoitzak berezko inpaktua du.

Araztegietan, helburua da substantzia horiek uretatik kentzea. Hain zuzen ere, arazte-teknikak garatu, eta etorkizunean teknika horiek inplementatzeko baliabideak jarri beharko dira.

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Comprehensive micropollutant characterization of wastewater during Covid-19 crisis in 2020: suspect screening and environmental risk prioritization strategy

N. Lopez-Herguedas[1,2], M. Irazola[1,2], I. Alvarez-Mora[1,2], G. Orive[3,4], U. Lertxundi[4,5], M. Olivares[1,2], O. Zuloaga[1,2], A. Prieto[1,2]

[1] Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country (UPV/EHU); [2] Research Centre for Experimental Marine Biology and Biotechnology (PIE), University of the Basque Country (UPV/EHU); [3] NanoBioCel Group, Laboratory of Pharmaceutics, Faculty of Pharmacy, University of the Basque Country UPV/EHU; [4] Bioaraba, NanoBioCel Research Group; [5] Bioaraba Health Research Institute; Osakidetza Basque Health Service, Araba Mental Health Network, Araba Psychiatric Hospital, Pharmacy Service

ABSTRACT: Micropollutants monitoring in wastewater can serve as a picture of what is consuming society and how it can impact the aquatic environment. In this work, a suspect screening approach was used to detect the known and unknown contaminants in wastewater samples collected from two wastewater treatment plants (WWTPs) located in the Basque Country (Crispijana in Alava, and Galindo in Vizcaya) during two weekly sampling campaigns, which included the months from April to July 2020, part of the confinement period caused by COVID-19. To that aim, high-resolution mass spectrometry was used to collect full-scan data-dependent tandem mass spectra from the water samples using a suspect database containing >40000 chemical substances. The presence of more than 80 contaminants was confirmed (level 1) and quantified in both WWTP samples, while at least 47 compounds were tentatively identified (2a). Among the contaminants of concern, an increase in the occurrence of some compounds used for COVID-19 disease treatment, such as lopinavir and hydroxychloroquine, was observed during the lockdown. A prioritization strategy for environmental risk assessment was carried out considering only the compounds quantified in the effluents of Crispijana and Galindo WWTPs. The compounds were scored based on the removal efficiency, estimated persistency, bioconcentration factor, mobility, toxicity potential and frequency of detection in the samples. With this approach, 33 compounds (e.g. amantadine, clozapine or lopinavir) were found to be considered key contaminants in the analyzed samples based on their concentration, occurrence and potential toxicity. Additionally, antimicrobial (RQ-AR) and antiviral (EDRP) risk of certain compounds was evaluated, where ciprofloxacin and fluconazole represented medium risk for antibiotic resistance (1>RQ-AR>0.1) in the aquatic ecosystems. Regarding mixture toxicity, the computed sum of toxic unit values of the different effluents (>1) suggest that interactions between the compounds need to be considered for future environmental risk assessments.

* **Corresponding author / Harremanetan jartzeko:** N. Lopez-Herguedas, Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country UPV/EHU, Leioa, Basque Country, Spain. e-mail: naroa.lopez@ehu.eus. **Contributed by** Jon Zarate **editoreak egindako ekarpena**.

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1. Introduction

The year 2020 was marked by the onset of the global pandemic triggered by the SARS-CoV-2 virus, causing millions of deaths all over the world (WHO, 2021). This situation led most of the countries to introduce several measures (e.g. cancellation of public events, closure of schools and various businesses, curfews and lockdowns) in order to avoid the spread of the virus. This standstill of the countries severely affected the health, the economy and the social life of most of citizens all over the world.

During the pandemic period wastewater was used in many research studies to monitor the spread of the virus considering its excretion from infected people (de Araújo *et al.*, 2022; Godini *et al.*, 2021; Kuroda *et al.*, 2021) but also to determine whether people lifestyle changed. In fact, the lack of specific therapeutic treatments to combat COVID-19 led to an unprecedented consumption of different therapeutic drugs (Cappelli *et al.*, 2022; Kuroda *et al.*, 2021), which could end-up in environmental waters (Bandala *et al.*, 2021; Cappelli *et al.*, 2022; Domingo-Echaburu *et al.*, 2022). Particularly, during the confinement time, high amounts of antiviral and/or antimicrobial pharmaceuticals were prescribed for COVID-19 treatment and their inefficient elimination in wastewater treatment plants (WWTPs) led to detect such compounds in wastewater effluents and environmental waters (Nannou *et al.*, 2020). Moreover, the potential presence of antivirals and antimicrobials in environmental waters may increase the development of antiviral (Kuroda *et al.*, 2021; Nannou *et al.*, 2020) and antimicrobial resistance (Knight *et al.*, 2021; Usman *et al.*, 2020). In this regard, it is known that the environment constitutes one of the main sources of gene resistance to pathogens (Bengtsson-Palme and Larsson, 2016), but such resistance is not considered in the current regulatory systems (Boxall *et al.*, 2012). Even though efforts have been done to determine the minimum inhibitory concentration (MIC) of certain compounds with antimicrobial activity (Bengtsson-Palme and Larsson, 2016; Booth *et al.*, 2020), adverse effects even below the MIC values have been reported in the literature (Andersson and Hughes, 2012; Gullberg *et al.*, 2014), pointing out the lack of comprehensive knowledge about the effects of the unknown chemicals' cocktail can pose on the environment and human health (Fonseca *et al.*, 2020; Markert *et al.*, 2020; Nilsen *et al.*, 2019).

The potential of wastewater monitoring to get epidemiological information on human consumption and exposure to chemical residues has been widely demonstrated in many research works, where wastewater-based epidemiology (WBE) approach was used (Alygizakis *et al.*, 2021; Been *et al.*, 2021; Galani *et al.*, 2021; Nason *et al.*, 2022; Perkons *et al.*, 2022; Reinstadler *et al.*, 2021; Wang *et al.*, 2020). By monitoring wastewater samples during the pandemic period, for example, variations in benzoylecgonine use in European countries (Been *et al.*, 2021), increase of methamphetamine consumption (Reinstadler *et al.*, 2021), increase of benzodiazepines (psychoactive pharmaceuticals with anxiolytic activity) use (Alygizakis *et al.*, 2021) and no-alteration of certain pharmaceuticals consumption (Wang *et al.*, 2020) was determined using WBE approach.

As far as Spain is concerned, the monitoring of emerging contaminants (ECs) in wastewaters of WWTPs is widely done using mainly multi-targeted analytical methods (Afonso-Olivares *et al.*, 2017; Díaz-Garduño *et al.*, 2017; Martín *et al.*, 2012; Solaun *et al.*, 2021) and also applying WBE approach (Bijlsma *et al.*, 2021; Estévez-Danta *et al.*, 2022; Montes *et al.*, 2020). Although the unquestionable adequacy of target screening for the monitoring of a fixed set of micropollutants, the unknowns that may occur in the aquatic environment depends on many factors (e.g., land use, proximity to industry, type of sewer system, WWTP processes, population demographics, etc.) and contaminants end up being overlooked. Those limitations move scientists towards the use of more flexible and easily adaptable suspect screening studies that allow (i) addressing a larger amount of micropollutants and/or (ii) performing risk assessment (Cappelli *et al.*, 2022; Gago-Ferrero *et al.*, 2016; González-Gaya *et al.*, 2021; Li *et al.*, 2018; Perkons *et al.*, 2022). The use of those

analytical strategies to analyze wastewater samples can serve to determine as many as possible unknown chemicals which could provide hint information about what the population is consuming in a specific period of time.

Within this context, the main aim of this work was to evaluate the presence of micropollutants via suspect screening, and the subsequent confirmation through a validated target analysis in the influents and effluents of two WWTPs located in the Basque Country (Crispijana, Alava, and Galindo, Vizcaya) during two weekly sampling campaigns (from April to July 2020), in part of the period of confinement caused by COVID-19. The identification of the main potential toxicity drivers based on a prioritization strategy including different categories was assessed. Moreover, antimicrobial and antiviral compounds risks were also evaluated.

2. Experimental section

2.1. *Reagents and materials*

All chemicals and laboratory materials used in this work are provided in section S1 and the Supporting Information (SI) of Lopez-Herguedas *et al.* (Lopez-Herguedas *et al.*, 2022).

2.2. *Sampling*

Sampling was carried out 1 or 2 times per week, from April to July 2020 (Figure 1), collecting 24-hour composite aqueous samples (influent and effluents) from two WWTPs located in Vizcaya and Alava, Galindo and Crispijana, respectively (see details in section S2 in SI). Samples began to be collected after the peak incidence of Covid-19 cases in the Basque Country (Spain).

At the Galindo WWTP, composite samples were collected from the influent (IWW), primary treatment (EWW1), secondary treatment (EWW2) and tertiary treatment (EWW3), while at the Crispijana WWTP, the influent (IWW) and effluent after secondary treatment (EWW) were collected. All samples were stored and frozen at −20 °C until their analysis, which was carried out 2 months after their collection.

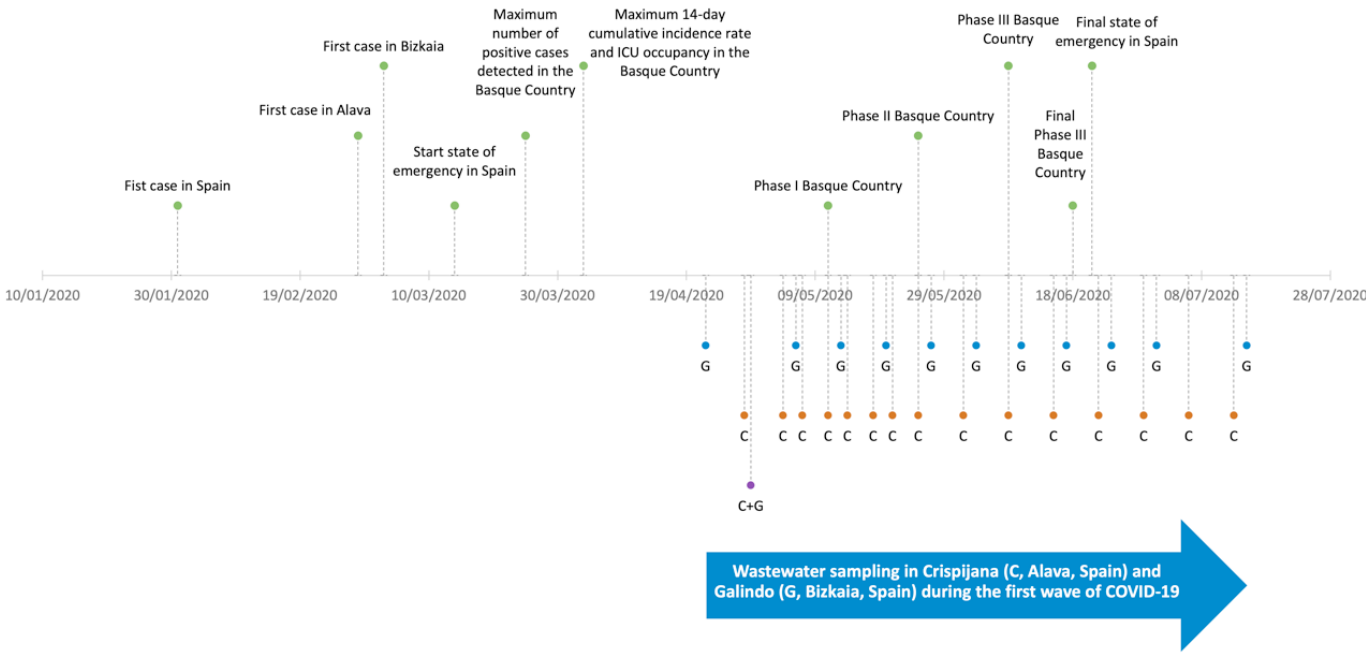

Figure 1. Timeline of Covid-19 situation in its first wave and sampling dates of composite water samples in both WWTPs (G: Galindo, C: Crispijana)

2.3. *Sample treatment*

The water samples were thawed and once at room temperature, all samples were filtered through cellulose filters (0.7 µm, 90 mm, Whatman; Maidstone, UK). Three replicates of 250 mL (effluent) or 100 mL (influent) were processed according to a previously validated method in our research group (González-Gaya *et al.*, 2021) (see details in section S2 in SI). Briefly, the samples were extracted with 500 mg solid-phase extraction (SPE) cartridges consisting of cation exchange (100 mg, ZT-WCX), anion exchange (100 mg, ZT-WAX) and reverse phase (300 mg, HRX) sorbents for effluent samples, and with 250 mg SPE cartridges containing half of the above-described amounts for influent samples. The cartridges were conditioned using 5 mL of MeOH:EtOAc and 5 mL of Milli-Q water. Subsequently, each sample aliquot was loaded and were left to dry under vacuum before analytes elution using 12 mL of a MeOH:EtOAc mixture (1:1) containing 2% ammonia and 12 mL of a MeOH:EtOAc mixture with 1.7% formic acid. Both extracts were combined, evaporated to dryness using a Turbovap (Zymark, Hopkinton, USA) under a gentle nitrogen stream and reconstituted in 250 µL of MeOH:Milli-Q water (1:1, v:v).

2.4. *Analysis by UHPLC-q-Orbitrap*

Extracts were analyzed on a Thermo Scientific Dionex Ulti-Mate 3000 UHPLC coupled to a Thermo Scientific Q Exactive Focus quadrupole-Orbitrap mass spectrometer (UHPLC-q-Orbitrap) equipped with a heated electrospray ionisation source (HESI, Thermo-Fisher Scientific, CA, USA) based on the previously developed methods (González-Gaya *et al.*, 2021; Lopez-Herguedas *et al.*, 2022) detailed in section S3 of the SI.

2.5. *Quality assurance of the analytical method*

The analytical protocol used in this work was thoroughly optimized in a previous work of our research group and is described elsewhere (González-Gaya *et al.*, 2021) (see section S4 in SI). Anyhow the QA/QC criteria of the analyses conducted in this work were assured for 231 compounds in terms of identification limits and apparent recoveries (see Table S1).

2.6. *Suspect analysis*

Suspect analysis data treatment was carried out using the Compound Discoverer 3.2 (Thermo-Fisher Scientific) and the workflow previously reported by González-Gaya *et al.* (González-Gaya *et al.*, 2021) (see detailed information in SI). Only Lorentzian peaks were considered and they were manually checked. The SusDat NORMAN database (40,059 compounds, www.norman-network.net, DOI:10.5281/zenodo.2664077) was used as a suspect list with a fixed error lower than ± 5 ppm in the exact mass. The molecular formulas suggested by the software were only accounted for if MS1 was satisfactorily matched (SFit> 30% and isotopic profile > 70%). Minimum peak areas considered were set at 10e6 units for both negative and positive ionization modes. Additionally, only peaks 10 times larger in the samples than in the blanks and with a relative standard deviation (% RSD) lower than 30% within injection replicates (n=3) were further studied. MS2 spectra were compared with mzCloud database (https://www.mzcloud.org/), and a match of over 70% was set for the identification of the feature. When the standards of the candidates were available, experimental retention time was confirmed with an allowed error of ± 0.1 min. If not available, retention times were estimated from the Retention Time Index (RTI) platform (http://rti.chem.uoa.gr/) and candidates were rejected or accepted depending on whether there was a statistical difference or not with the estimated value within the uncertainty of the model built. Finally, identification criteria according to Schymanski and coworkers (Schymanski *et al.*, 2014) was noted to provide the candidates with a tentative code from 1 to 3 levels of identification. Although this scale is numbered from one to five, in this work we annotated compounds up to level 3 being level 1 the one with the highest confidence level (features with their structure identified and confirmed by reference standard acquisition) and three the least one (features identified as potential

candidates with known structure but more than one candidate is provided since they are potential isomers).

2.7. *Quantification and multivariate data analysis*

Quantitative data analysis of the suspects annotated as level 1 (target analysis) was performed using Tracefinder 4.2 software (Thermo-Fisher Scientific). Target compounds and their instrumental characteristics including molecular formula, ionization mode, retention time (Rt) and experimental MS/MS fragments were added to the software library according to studies previously performed by the research group (Lopez-Herguedas *et al.*, 2022). To avoid false positives, the experimental retention time window was limited to 60 seconds around the retention time of the pure standard, a mass error equal to or less than 5 ppm, isotopic profile matching at more than 70% and mass accuracy for fragments equal to or less than 5 ppm were considered. Peak integration and calibration curves were checked manually.

Once obtained the data, principal component analysis (PCA) was carried out with PLS toolbox (8.7.1 version, Eigenvector Research, Wenatchee, USA) in the Matlab programming environment (R2019b, Mathworks Inc., Natick, USA). Mean-centering and variance scaling was carried out prior to multivariate statistical analysis. Leave-one-patient-out cross-validation was used to validate and optimize the PCA model.

2.8. *Prioritization strategy for environmental risk assessment*

Risk assessment was accomplished through a prioritization strategy of suspects annotated as level 1 following the approach described by Gros *et al.* with slight modifications (Gros *et al.*, 2017). Six category classes were set to prioritize the most environmentally relevant compounds identified in each WWTP effluent including: (a) removal efficiency (RE, %), (b) estimated persistency (half-life time in days, DT50), (c) bioconcentration factor (BCF), (d) mobility, (e) toxicity potential and (f) frequency of detection in the samples (Table 1). Each micropollutant was scored with a value between 1-5 in each category (a-e) summed up to obtain a total score, being the compounds showing the lowest value the ones posing the highest environmental risk. Compounds that were never detected above the LOQ were excluded in order to avoid overestimation of risks by including compounds that were likely to be absent. Similarly, compounds present at levels < LOQs in the influent samples were not considered since the calculated RE would be biased leading to an overestimation of the risk.

Table 1
Criteria and scoring system for prioritization of identified micropollutants

Criteria	Score				
	1	2	3	4	5
Removal efficiency (RE)	<40%		40-60%		>60%
Biodegradation (predicted half-life time in days)	>180	>60	>37.5	>15	<15
Bioaccumulation (BCFpred)	>10,000	>1000	>100	>10	<10
Mobility (log K_{ow})	<2.5		2.5-4.0		>4.0
Risk Quotient (RQ)	>1	>0.1	>0.01	>0.001	<0.001
Frequency of detection (%) in effluent	100%	>75%	>50%	>25%	<25%

RE (%) of individual ECs was estimated considering their concentrations in wastewater before and after wastewater treatment (Golovko *et al.*, 2021; Li *et al.*, 2018) (see **Equation 1**). Independent two samples t-test was performed at a 95% confidence level to evaluate significant differences among the concentrations quantified in influent and effluent samples for each contaminant to avoid comparison between influent and effluent pairs that do not really show significant differences and their comparison may lead to misleading results. Considering the high variability of the observed values between days, the scoring system for the RE relied on 3 values that were established as follows: (i) effectively removed compounds with RE values higher than 60%, (ii) moderately removed compounds with RE values between 40% and 60%, and (iii) not eliminated compounds with RE values lower than 40% and/or compounds for which influent and effluent mean concentrations are indistinguishable (e.g. DEP shows a RE of 65% in Galindo WWTP but the t-test reveals that values in the IWW and EWW3 are not significantly different).

$$RE(\%) = \left(\frac{\left([Influent] - [Effluent] \right)}{[Influent]} \right) \times 100 \qquad (1)$$

The biodegradation potential (due to biological activity, chemical reactivity or physical degradation) of the compounds is a good indicator of their persistence in the environment. The bioaccumulation potential refers to the ability that some chemical compounds have to accumulate in a living organism and can be predicted by the lipophilicity of the chemical. The values for both categories were defined based on Gros *et al.* (Gros *et al.*, 2017), which were established according to the European legislation for chemicals of concern, REACH (EC 1907/2006). In the present work, half-life times (DT50) and BCFs were retrieved from the CompTox Chemical Dashboard (https://comptox.epa.gov/dashboard/) relying on the OPERA models (Finckh *et al.*, 2022; Mansouri *et al.*, 2018).

The capability of a compound to diffuse the source to other environmental compartments is given by its mobility. Considering that log K_{ow} serves as a measure of the relationship between lipophilicity (fat solubility) and hydrophilicity (water solubility) of a substance, it was used to score the mobility pattern of compounds using the following criteria: (i) compounds with log K_{ow} < 2.5 were considered to be highly mobile, (ii) compound with log K_{ow} values between 2.5-4.0 were considered to show medium mobility, and compounds with log K_{ow} > 4.0 were considered to be low mobile (Dimitrov *et al.*, 2019; Jones-Lepp and Stevens, 2007; Roveri and Lopes Guimarães, 2023).

The toxicity potential was expressed in terms of risk quotients (RQ), calculated for each compound according to the European Union technical Guidance Document (European Parliament, 2006) as the ratio of the measured environmental concentration (MEC) in WWTP effluents and predicted no-effect concentration (PNEC). 95th percentiles of the measured concentrations for each compound were used as MEC values. The PNEC values were calculated as described by Lopez-Herguedas *et al.* (Lopez-Herguedas *et al.*, 2022) (see details in section S5 in SI).

Considering the sudden increase in the discharge of antimicrobials, including antibiotics and antivirals, to the environment the potential risk of the mentioned compounds was also determined. The Antibiotic Resistance (AR) was assessed based on the RQ metric (RQ-AR) as described by Bengtsson-Palme and Larsson (Bengtsson-Palme and Larsson, 2016). The PNECs for the selection of AR (PNEC-AR) were derived considering the MICs of the antibiotic compounds, which are the lowest concentrations of antibiotic for inhibiting bacterial growth, and the application of an appropriate assessment factor to the MIC (Bengtsson-Palme and Larsson, 2016; Cappelli *et al.*, 2022). On the other hand, the antiviral resistance was determined by the calculation of the Environmentally acquired antiviral Drug Resistance Potential (EDRP) as described by Kuroda and coworkers (Kuroda *et al.*, 2021) (Equation 2):

$$EDRP = Min\left(\frac{\mathrm{MEC\ 95^{th}\ perc}}{\mathrm{vEC_{50}\ or\ vIC_{50}}}, \frac{\mathrm{vEC_{50}\ or\ vIC_{50}}}{\mathrm{MEC\ 95^{th}\ perc}} \right) \qquad (2)$$

Where, vIC_{50} and vEC_{50} refer to the antiviral drug concentration which determines the 50% of the vi-

ral growth inhibition expressed as the half maximal inhibitory (IC_{50}) and effective (EC_{50}) concentrations, respectively. Those values were compiled from (Kuroda *et al.*, 2021). EDRP values vary between 0 and 1, being a value equal to 1 the maximum risk potential.

Given that the environmental samples are constituted by myriads of contaminants, mixture toxicity was also evaluated using the sum of toxic units (STU) approach based on CA (representing the worst-case scenario) in order to avoid an overestimation of the real risk as suggested by Backhaus and Faust, 2012 (Backhaus and Faust, 2012) (Equation 3):

$$RQ_{STU} = max\left(STU_{algae}, STU_{daphnids}, STU_{fish}\right) \times AF = max\left(\sum_{i=1}^{n}\frac{MEC}{EC50_{i,algae}}, \sum_{i=1}^{n}\frac{MEC}{EC50_{i,daphnids}}, \sum_{i=1}^{n}\frac{MEC}{EC50_{i,fish}}\right) \times AF \quad (3)$$

In this study, more conservative NOEC values corresponding to selected BQE instead of EC_{50} values were considered as reference concentrations for the calculation of STU to assess the impact on the aquatic ecosystem likewise for the calculation of individual RQ values. When experimental chronic NOEC values were not available, EC_{50} experimental values prevail over predicted NOEC values. In each case, an appropriate AF was applied (see section S5 in SI).

A dilution factor (DF) was applied to effluent concentrations to perform a more representative risk assessment caused by chemical exposure (Keller *et al.*, 2014). In both WWTPs, a minimum DF value was applied to simulate "the worst-case scenario"; thus, 10- and 50-fold effluent dilutions were considered for Crispijana and Galindo WWTP, respectively.

3. Results and discussion

The observations obtained in this work were based on a three-step workflow. First, the samples were analyzed using a suspect screening approach in order to detect the largest amount of contaminants present. Then, those candidates annotated as level 1 (i.e., standards available in the lab) were quantified. To end, those chemicals detected in secondary and tertiary effluent samples were ranked according to their potential hazards based on a prioritization strategy that included six relevant categories (see section 2.8).

3.1. Occurrence of ECs in analyzed samples

3.1.1. Suspect screening

The compounds identified and annotated at levels 1-3 by means of the workflow previously described (see section 2.6) are included in Table 2, where complete information about the annotation as well as the occurrence is compiled. In the case of Crispijana WWTP, among the identified candidates, the presence of 79 compounds was confirmed by chemical standards (level 1) (see section 3.2.1. and Table 2), while additionally, 47 candidates were tentatively identified as probable structures (level 2a) (29 candidates in IWW and 18 in EWW), and 4 tentative candidates (level 3) (only in IWW). Among the vast number of candidates identified some compounds stood out as the most frequently identified in Crispijana WWTP: (i) the pharmaceuticals lidocaine (anaesthetic), carbamazepine (anticonvulsant) and tramadol (analgesic) identified at level 1, and febuxostat (uric acid lowering agent) and rosuvastatin (antilipidemic) identified at level 2a; (ii) some transformation products identified at level 2a such as O-desmethylnaproxen, carbamazepine 10,11-epoxide and 11-ketotestosterone; and (iii) illicit drugs identified at level 2a such as ketamine and cocaine. Overall, more compounds with higher chromatographic areas were identified in influent wastewater, pointing out that the treatments implemented at the WWTPs partially removed chemicals present in wastewater.

Regarding the wastewaters from Galindo WWTP (see section 3.2.2. and Table 2), a total of 88 compounds were annotated as level 1, 53 candidates were annotated as level 2a (29 of them in the set of IWW and EWW1, 9 in the EWW2 and the remaining 15 in the EWW3), and 12 candidates (9 in the set of IWW and EWW1, 1 in the EWW2 and 2 in the EWW3) were tentatively identified (level 3). Compared to Crispijana WWTP, an increase in the number of identified compounds and chromatographic

areas was observed in the Galindo WWTP, a fact that may be related to the location (i.e. more populated area) and the influent volume (i.e., Galindo WWTP treats almost twice the flow that Crispijana WWTP treats). This is the case, for example, of methylparaben, nonylphenol, pyrantel or finasteride; compounds that were not identified in any sample from the Crispijana WWTP, but most of which were found in all influent samples belonging to Galindo. On the other hand, the tendency to find higher signals in IWW samples compared to the treated ones (EWW1, EWW2 and EWW3) remained constant, suggesting again a certain removal efficiency of the treatments implemented in the WWTPs.

3.1.2. Quantification of compounds annotated as level 1

The suspects annotated as level 1 were quantified using the chemical standards and following the QA/QC criteria described in section 2.5. The concentrations in ng/L found in all the studied samples (n = 32 and n = 47, in Crispijana and Galindo WWTPs, respectively) are detailed in Table 3 (see Tables S2 and S3 in SI for more detailed information). Multivariate data analysis was performed by means of PCA aiming to detect differences among the WWTPs studied as well as the different effluent treatments (see section S6 and Figure S1 in SI).

Among all the wastewater samples belonging to Crispijana WWTP, 80 compounds were quantified at ng/L level, whereas, 88 were the total compounds quantified in Galindo WWTP.

Overall, pharmaceutical products (PPs), stimulants, pesticides, phthalates, hormones, industrial agents, perfluorinated compounds and flame retardants were quantified at ng/L levels in both untreated and treated samples (i.e. IWW and EWW regarding Crispijana WWTP, IWW, EWW1, EWW2 and EWW3 regarding Galindo WWTP), being in both WWTPs the group of PPs the most abundant (around 59% and 65% of the detected compounds, respectively) (see Tables S2 and S3 in SI). Moreover, as it is summarized in Table 3, most of the compounds detected in Crispijana WWTP were also detected in Galindo WWTP. Following the trend observed in suspect screening, the highest concentration levels were found in IWW samples suggesting the removal efficiency of the treatments for some of the detected compounds. Concretely, the pharmaceuticals acetaminophen, (also known as paracetamol, an anti-inflammatory used to treat headaches), metformin (a drug to treat diabetes) and mycophenolic acid (an antibiotic usually used as an immunosuppressant drug, in organ transplants or for the treatment of certain autoimmune diseases), as well as the plasticizer caprolactam or the stimulant caffeine were determined at high ng/L levels in IWW samples of both WWTPs (see Table 3). Although caprolactam, for example, can be degraded up to 40% in 28 days by the action of certain microorganisms (López Rocha *et al.*, 2020), the adequate elimination of ECs in WWTPs is a crucial issue especially if they are present at such high concentration levels. On the other hand, it has to be mentioned that metformin (recently included in the WL-3) (Gomez Cortes *et al.*, 2020) is by far the most popular diabetes medication worldwide, which has been demonstrated to be hardly metabolized in the human body (Krentz and Bailey, 2005). As a result, it is excreted unaltered and dispersed in wastewater, as has been observed in several studies where the concentration of metformin was non-negligible (Alvarez-Mora *et al.*, 2022; Čelić *et al.*, 2021; Finckh *et al.*, 2022; Golovko *et al.*, 2021). According to the German Umweltbundesamt (UBA) database, such high levels of mycophenolic acid have never been reported, being up to now a concentration of 650 ng/L in surface waters (Franquet-Griell *et al.*, 2017) the highest detected value (https://www.umweltbundesamt.de/en/database-pharmaceuticals-in-the-environment-0, accessed October 2022). The detected large amount of caffeine in untreated samples could be attributed to its high consumption in beverages, as an excipient in a wide variety of drugs and cosmetics. Caffeine concentrations up to 20000 ng/L were reported in the literature (Ebrahimzadeh *et al.*, 2021), but it is eliminated during biological treatment reported (Qi *et al.*, 2015) as it was observed also in this work (> 90% of elimination rate).

Table 2
Target analysis of features identified as level 1 in Crispijana and Galindo WWTPs

Compounds	Abbre-viations	LOQproc (ng/L)	IWW Crispijana WWTP					EWW Crispijana WWTP					IWW Galindo WWTP				
			Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)
2-Hydroxybenzothiazole	OBT	15.4	14	245	845	414	365	15	88	271	146	137	11	1270	2950	1772	1591
4-tert-octylphenol		138.5	5	235	905	470	360	4	159	2238	812	425	0	<LOD	<LOD		
Acetaminophen		2.9	16	7548	24,098	17,275	17,827	3	163	484	293	233	11	31,269	58,474	44,466	47,978
Amantadine		3.3	14	15	31	23	24	15	20	49	35	37	11	45	91	66	62
Amitriptyline		5.4	13	702	2232,000	1081	1019	9	38	1902	482	219	8	22	47	34	33
Atenolol		6	16	173	435	316	311	15	118	236	193	203	11	556	964	770	805
Azithromycin		17.2	0	<LOQproc	<LOQproc			11	25	73	46	43	0	<LOQproc	<LOQproc		
Bendiocarb		6.5	15	9	52	27	24	0	<LOQproc	<LOQproc			9	9	51	22	19
Bentazone		6.2	12	7	9	8	8	13	7	21	14	14	4	20	38	26	23
Benzophenone		0	6	26	53	41	40	0	<LOQproc	<LOQproc			10	39	252	166	165
Bezafibrate		2.9	13	8	16	13	12	16	4	24	14	14	11	181	293	237	252
Bicalutamide		5.4	12	6	15	9	8	16	15	72	45	51	10	6	25	19	20
Bis(2-ethylhexyl) phthalate	DEHP	138.5	6	315	2340	920	733	8	438	1405	1004	984	6	2225	42,815	14,194	7029
Bisoprolol		3.3	7	18	39	25	24	16	21	92	61	59	11	230	327	272	273
Bisphenol A	BPA	15.1	15	362	2709	1719	1727	15	44	400	149	115	10	1098	2702	1717	1696
Bupropion		4.7	0	<LOD	<LOD			0	<LOD	<LOD			0	<LOQproc	<LOQproc		
Caffeine		338.3	16	9587	28,480	20,860	20,858	0	<LOQproc	<LOQproc			11	30,315	82,035	59,811	62,439
Caprolactam		31.9	15	702	2232	1147	1021	8	32	329	152	116	11	18,054	72,388	34,602	29,917
Carbamazepine		6.6	16	20	33	25	24	16	31	176	113	118	11	54	86	68	66
Carbendazim		7.6	16	20	83	52	52	15	15	53	32	29	11	28	104	60	61
Celecoxib		4.2	12	5	11	7	6	15	7	15	10	10	6	10	20	16	18
Cetirizine		4.5	13	5	173	87	90	16	55	252	146	149	4	165	214	196	202
Ciprofloxacin		19.8	13	52	203	118	109	14	29	185	63	56	11	144	327	228	200
Clarithromycin		5.5	0	<LOQproc	<LOQproc			6	40	334	122	83	1	14	14	14	14
Clopidogrel		6.8	3	8	8	8	8	9	8	13	10	10	11	10	19	15	16
Clozapine		3.2	0	<LOQproc	<LOQproc			16	16	99	53	56	2	12	13	13	13
Cotinine		6.2	16	434	1529	971	1023	15	48	264	182	201	11	1626	3288	2381	2381
Dibutyl phthalate	DBP	28.3	13	595	1411	981	971	10	58	286	139	143	11	1262	3263	2093	2041
Diethyl phthalate	DEP	130.6	10	233	1373	674	724	8	326	10,897	4759	2544	11	1819	42,444	6973	3252
Diethyl Toluamide	DEET	6.5	16	24	264	113	75	11	11	86	38	28	11	60	279	165	153
Dioctyl phthalate	DOP	45	6	323	2398	942	751	8	449	1439	1029	1008	6	1186	37,220	12,233	6232
Diuron		5.8	16	34	105	65	68	16	42	206	140	154	11	60	287	109	96
Efavirenz		6.6	6	10	23	15	14	16	18	74	48	51	10	22	63	41	38
Eprosartan		7.4	8	253	834	547	497	14	8	252	73	59	11	1755	3111	2473	2589
Estriol		55.6	9	68	112	90	87	1	72	72	72	72	4	56	145	106	111
Ethyl-S,S-diphenyldithiophosphate	EDDP	3.7	0	<LOD	<LOD			0	<LOD	<LOD			1	8	8	8	8
Finasteride		3.2	0	<LOD	<LOD			0	<LOD	<LOD			10	8	32	18	13
Fluconazole		2.9	15	52	172	108	106	7	36	421	211	167	11	293	1321	658	579
Furosemide		6.5	15	240	623	409	410	14	51	407	232	258	10	186	559	351	331
Gabaspentin		15.3	13	906	3788	2164	2201	15	110	657	453	494	11	2646	5013	4028	3943
Genistein		338.3	13	495	1316	829	777	0	<LOQproc	<LOQproc			11	854	6278	3009	3236
Genistin		6.4	16	64	342	160	149	0	<LOQproc	<LOQproc			9	174	293	222	195

Compounds	EWW1 Galindo WWTP					EWW2 Galindo WWTP					EWW3 Galindo WWTP				
	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)
2-Hydroxybenzothiazole	12	1270	3055	2152	2153	12	135	349	210	211	6	21	142	79	74
4-tert-octylphenol	0	<LOD	<LOD			0	<LOD	<LOD			0	<LOD	<LOD		
Acetaminophen	12	34,750	85,774	63,685	63,832	10	127	380	243	238	9	83	195	140	133
Amantadine	12	71	292	186	200	12	39	68	59	63	12	6	38	15	11
Amitriptyline	7	21	195	81	65	12	34	65	49	51	4	8	19	13	12
Atenolol	12	630	1766	1277	1288.5	12	196	369	303	320	12	126	341	206	195
Azithromycin	0	<LOQproc	<LOQproc			12	390	965	693	719	5	45	547	356	409
Bendiocarb	6	32	99	72	78.5	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Bentazone	7	10	89	29	19	4	7	14	10	10	1	11	11	11	11
Benzophenone	12	179	1029	439	365	12	33	155	94	96	4	55	103	73	67
Bezafibrate	12	154	361	267	263	12	44	104	76	73	8	6	62	32	27
Bicalutamide	8	19	63	41	39	12	25	53	42	43	12	35	58	47	48
Bis(2-ethylhexyl) phthalate	7	75	4528	1854	644	4	49	12,759	3269	133	2	185	1113	649	649
Bisoprolol	12	235	933	627	680.5	12	225	589	415	400	12	67	527	228	202
Bisphenol A	10	1084	2612	2097	2280	12	134	409	283	298	12	151	341	239	252
Bupropion	4	8	14	11	11	12	6	14	11	11	11	6	18	9	8
Caffeine	12	37,859	136,871	95,781	105,386.5	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Caprolactam	12	28,020	158,832	89,859	93,068	12	77	2399	619	474	12	189	955	525	488
Carbamazepine	12	86	290	193	214	12	103	204	149	147	5	33	86	56	54
Carbendazim	12	41	222	139	159.5	12	40	82	62	65	4	12	46	22	15
Celecoxib	7	6	15	11	12	12	8	13	10	10	3	6	7	7	7
Cetirizine	4	116	192	157	160	12	120	226	167	158	3	34	57	43	38
Ciprofloxacin	12	20	241	94	71	12	53	116	79	78	3	51	58	55	57
Clarithromycin	2	18	26	22	22	12	16	42	27	28	3	10	17	14	15
Clopidogrel	12	21	63	40	35.5	12	10	19	14	15	0	<LOQproc	<LOQproc		
Clozapine	11	5	116	64	52	12	85	200	132	131	3	10	18	14	14
Cotinine	12	1268	4218	2864	2626	12	164	251	215	225	12	115	218	166	160
Dibutyl phthalate	12	1318	5420	2763	2378	12	108	724	366	371	12	50	403	186	144
Diethyl phthalate	12	1737	37,408	16,053	16,889.5	11	174	7378	2262	903	11	545	11,397	2501	1181
Diethyl Toluamide	12	112	609	322	257.5	12	32	135	77	75	12	29	128	64	53
Dioctyl phthalate	3	849	2064	1626	1965	1	6528	6528	6528	6528	1	148	148	148	148
Diuron	12	62	310	143	139	12	57	97	78	80	12	11	63	35	33
Efavirenz	10	29	57	44	44.5	12	35	57	47	48	9	22	46	34	34
Eprosartan	12	3231	10,449	7012	7235	12	148	470	307	293	4	8	123	64	63
Estriol	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Ethyl-S,S-diphenyldithiophosphate	0	<LOQproc	<LOQproc			12	9	18	14	14	10	6	15	9	8
Finasteride	9	13	46	25	23	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Fluconazole	12	262	1839	934	873.5	12	218	413	337	355	12	199	701	496	557
Furosemide	1	416	416	416	416	11	127	397	266	266	2	19	24	22	22
Gabaspentin	12	5472	16,4408	11,126	11,636.5	12	442	967	670	663	11	76	672	165	132
Genistein	12	1157	14,506	7036	5866.5	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Genistin	12	117	1216	526	506.5	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		

Compounds	Abbre-viations	LOQproc (ng/L)														
			Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)
Hydrocortisone		4.4	0	<LOD	<LOD			0	<LOD	<LOD			5	268	631	425	421
Hydroxychloroquine		9.4	0	<LOQproc	<LOQproc			3	32	71	53	57	2	116	132	124	124
Imidacloprid		9.6	3	13	22	17	16	12	12	29	20	20	2	27	47	37	37
Indomethacin		14	9	9	21	14	12	12	17	61	37	35	0	<LOQproc	<LOQproc		
Irbesartan		5.3	6	15	97	64	69	16	80	252	175	183	9	342	582	413	399
Ketoprofen		6	15	115	249	201	205	16	9	103	52	48	11	295	632	525	565
Lidocaine		6	16	15	52	34	35	16	30	166	98	91	11	25	141	94	106
Lopinavir		6.8	4	9	20	14	13	16	7	33	15	12	7	22	121	49	36
Lorazepam		3.2	16	82	203	142	148	7	82	743	354	235	11	264	372	319	324
Losartan		2.9	14	196	550	351	347	7	25	145	87	84	11	449	718	611	639
Mebendazole		3	13	10	32	18	16	15	13	42	29	30	11	35	85	58	59
Mecoprop		5.4	16	56	192	89	82	16	21	489	158	142	0	<LOD	<LOD		
Medroxyprogesterone		3.4	14	41	195	91	82	0	<LOQproc	<LOQproc			6	277	561	388	329
Memantine		9.1	0	<LOD	<LOD			0	<LOD	<LOD			0	<LOQproc	<LOQproc		
Metformin		6.1	14	3930	8780	5953	6193	5	143	287	227	230	11	12,583	21,454	18,525	19,183
Methylparaben		65.7	0	<LOD	<LOD			0	<LOD	<LOD			11	2686	5553	3788	3752
Metoprolol		4.1	1	11	11	11	11	7	11	22	15	14	11	59	392	165	129
Monobutyl phthalate	MBP	16.1	8	268	579	438	472	10	13	592	305	273	1	165	165	165	165
Mycophenolic acid		3.1	15	1211	2701	1762	1670	3	49	55	52	51	11	1819	3457	2993	3103
Naproxen		31.1	0	<LOD	<LOD			0	<LOD	<LOD			11	3739	8712	7461	7969
Nonylphenol		189.5	0	<LOD	<LOD			0	<LOD	<LOD			6	189.5	287	242	252
Norfloxacin		29.6	13	99	350	178	164	13	30	328	139	94	3	2597	10,008	5678	4430
Ofloxacin		1.8	0	<LOD	<LOD			0	<LOD	<LOD			11	80	124	96	92
Omeprazole		3.2	7	10	92	49	27	16	35	123	77	85	7	29	88	56	59
Pentoxifylline		6.3	16	25	65	36	33	8	9	41	18	15	11	111	229	166	161
Perfluorobutanesulfonic acid	PFBS	3	3	7	10	9	9	15	9	602	59	21	10	7	120	23	12
Perfluorooctanoic acid	PFOA	4.5	8	63	109	82	82	14	70	262	167	178	0	<LOD	<LOD		
Pravastatin		6.8	11	146	354	250	251	0	<LOQproc	<LOQproc			0	<LOD	<LOD		
Primidone		5.2	0	<LOQproc	<LOQproc			7	124	422	247	221	0	<LOQproc	<LOQproc		
Propamocarb		17.9	0	<LOD	<LOD			0	<LOD	<LOD			11	93	554	250	221
Propiconazole		5.8	15	7	24	13	11	6	19	89	44	34	4	7	25	13	9
Propyphenazone		2.6	14	5	8	7	7	15	10	26	19	20	11	21	57	36	33
Pyrantel		4	0	<LOD	<LOD			0	<LOD	<LOD			4	22	76	46	44
Ritonavir		32.5	1	38	38	38	38	0	<LOQproc	<LOQproc			7	40	105	72	69
Ropinirole		5.9	1	6	6	6	6	6	9	29	17	14	0	<LOQproc	<LOQproc		
Sertraline		4.4	1	4	4	4	4	14	7	17	10	10	0	<LOQproc	<LOQproc		
Sotalol		5.9	16	9	20	15	15	15	14	24	20	22	11	235	858	465	437
Sulfadiazine		6.1	0	<LOD	<LOD			0	<LOD	<LOD			5	8	32	20	14
Sulfamethoxazole		4.6	0	<LOD	<LOD			0	<LOD	<LOD			11	92	5301	855	183
Sulfapyridine		6.8	4	18	22	21	22	4	18	26	22	22	8	18	44	29	30
Telmisartan		6.1	13	120	376	243	232	16	6	903	643	701	1	1216	1216	1216	1216
Terbutryn		2.9	15	7	40	21	19	8	11	65	31	23	8	63	115	83	84
Testosterone		2.9	15	36	91	49	48	0	<LOQproc	<LOQproc			11	131	468	239	196
Thiabendazole		4.6	0	<LOQproc	<LOQproc			14	6	17	12	12	11	9	34	17	17
Tramadol		16.7	16	142	326	242	233	16	317	1294	819	851	11	726	1805	1404	1436
Triethylphosphate		2.9	4	4	19	10	8	3	7	153	58	14	10	3	233	62	37
Trimethoprim		2.9	14	11	31	23	25	14	16	60	39	41	11	53	1604	314	93
Triphenylphosphate		6.3	16	16	38	28	28	16	8	14	11	11	3	10	18	13	12
Valsartan		16	16	112	1422	1000	1021	15	53	368	163	182	11	5114	9538	6976	6663

Compounds	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)	Times detected	Min Conc. (ng/L)	Max Conc. (ng/L)	Mean (ng/L)	Median (ng/L)
Hydrochlorothiazide	1	231	231	231	231	6	226	231	281	275	7	18	146	79	69
Hydrocortisone	2	186	286	236	236	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Hydroxychloroquine	11	122	372	213	169	12	65	140	118	126	1	82	82	82	82
Imidacloprid	5	22	42	35	38	11	16	32	26	27	11	15	51	29	27
Indomethacin	0	<LOQproc	<LOQproc			11	11	22	14	13	0	<LOQproc	<LOQproc		
Irbesartan	9	226	554	368	329	12	201	381	335	352	11	6	291	109	63
Ketoprofen	12	279	780	579	602.5	12	68	155	115	106	12	8	64	37	31
Lidocaine	12	63	406	263	270	12	54	145	102	103	5	7	60	29	30
Lopinavir	7	12	68	31	25	12	10	68	23	18	12	9	57	19	14
Lorazepam	12	271	978	619	640.5	12	382	892	605	591	8	47	731	260	221
Losartan	12	590	2064	1365	1412	12	198	439	326	320	5	14	353	118	40
Mebendazole	12	68	128	96	99	12	19	29	24	24	4	7	17	13	13
Mecoprop	0	<LOD	<LOD			0	<LOD	<LOD			0	<LOD	<LOD		
Medroxyprogesterone	3	181	309	258	285	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Memantine	10	32	101	72	72	12	56	128	91	91	4	41	83	58	54
Metformin	12	19,439	83,111	51,417	54,757	12	1216	3921	2173	2079	11	13	7338	1185	269
Methylparaben	12	2899	11,642	7375	7203.5	1	443	443	443	443	2	132	151	142	142
Metoprolol	12	74	889	295	256.5	12	39	129	59	56	9	5	51	23	21
Monobutyl phthalate	5	5	650	272	199	12	5	590	150	110	12	31	270	72	49
Mycophenolic acid	12	2591	10,739	7157	7975.5	10	47	170	91	81	4	4	5	4	4
Naproxen	12	4164	9640	7370	7558.5	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Nonylphenol	9	189.5	440	316	340	10	203	240	219	216	12	200	305	255	256
Norfloxacin	3	1805	15,929	7152	3723	9	44	2373	446	107	12	30	4986	624	86
Ofloxacin	3	64	82	73	74	10	33	74	56	58	0	<LOQproc	<LOQproc		
Omeprazole	12	35	123	72	72	12	29	106	58	51	1	9	9	9	9
Pentoxifylline	12	151	693	465	521	12	46	139	98	111	12	20	198	93	95
Perfluorobutanesulfonic acid	11	3	335	49	21	11	6	49	12	8	12	7	37	11	8
Perfluorooctanoic acid	0	<LOD	<LOD			0	<LOD	<LOD			0	<LOD	<LOD		
Pravastatin	0	<LOD	<LOD			0	<LOD	<LOD			0	<LOD	<LOD		
Primidone	0	<LOQproc	<LOQproc			12	77	319	188	179	12	38	384	232	273
Propamocarb	12	58	534	234	202	2	38	51	45	45	0	<LOQproc	<LOQproc		
Propiconazole	6	11	45	20	17.5	9	7	16	11	12	12	8	28	15	14
Propyphenazone	12	41	115	83	90	12	19	41	32	33	0	<LOQproc	<LOQproc		
Pyrantel	12	35	166	96	102	12	42	86	65	64	6	14	46	27	25
Ritonavir	8	33	102	57	50.5	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Ropinirole	0	<LOQproc	<LOQproc			10	7	21	11	9	7	7	14	10	8
Sertraline	0	<LOQproc	<LOQproc			12	10	25	18	18	0	<LOQproc	<LOQproc		
Sotalol	12	185	681	438	482	12	79	113	99	100	2	56	68	62	62
Sulfadiazine	3	10	36	19	12	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Sulfamethoxazole	12	115	7306	1525	311.5	12	55	1354	308	83	1	62	62	62	62
Sulfapyridine	4	36	51	42	40	1	18	18	18	18	0	<LOQproc	<LOQproc		
Telmisartan	0	<LOQproc	<LOQproc			12	1111	1658	1469	1493	11	26	1088	476	269
Terbutryn	6	90	326	210	235	12	66	126	94	95	3	8	26	15	11
Testosterone	10	111	375	214	205	9	16	692	180	128	0	<LOQproc	<LOQproc		
Thiabendazole	11	14	59	36	37	12	14	42	28	28	8	6	30	17	16
Tramadol	12	1350	6444	4011	4035.5	12	1402	2537	2087	2127	9	33	1020	370	129
Triethylphosphate	12	16	269	99	72	12	54	141	93	94	12	34	110	77	80
Trimethoprim	12	29	1334	281	83	12	37	598	166	68	1	16	16	16	16
Triphenylphosphate	2	7	13	10	10	0	<LOQproc	<LOQproc			0	<LOQproc	<LOQproc		
Valsartan	12	3425	9402	6092	5822	12	102	501	268	243	12	80	746	290	200

Table 3
Qualitative comparison between compounds detected during COVID-19 lockdown and pre-pandemic in the secondary effluent of Galindo WWTP

Class of compound	Compounds detected during COVID-19	Use	Identification level	Detected pre-COVID-19
Drugs used in COVID-19 treatment	Acetaminophen	Pharmaceutical/Analgesic	1	Yes
	Azithromycin	Pharmaceutical/Antibiotic	1	Yes
	Hydroxychloroquine	Pharmaceutical/Antimalarial	1	No
	Lopinavir	Pharmaceutical/Antiretroviral	1	No
	Darunavir	Pharmaceutical/Antiretroviral	2a	Yes
Other related pharmaceuticals	Amantadine	Pharmaceutical/Antiviral	1	Yes
	Amitriptyline	Pharmaceutical/Antidepressant	1	Yes
	Atenolol	Pharmaceutical/Antihypertensive	1	Yes
	Bisoprolol	Pharmaceutical/Antihypertensive	1	Yes
	Candesartan	Pharmaceutical/Antihypertensive	2a	No
	Carbamazepine	Pharmaceutical/Anticonvulsant	1	Yes
	Celiprolol	Pharmaceutical/Antihypertensive	2a	No
	Ciprofloxacin	Pharmaceutical/Antibiotic	1	No
	Citalopram	Pharmaceutical/Antidepressant	3	Yes
	Clarithromycin	Pharmaceutical/Antibiotic	1	No
	Clozapine	Pharmaceutical/Antipsychotic	1	No
	Doxylamine	Pharmaceutical/Anti-inflammatory	2a	Yes
	Efavirenz	Pharmaceutical/Antiretroviral	1	Yes
	Enalaprilat	Pharmaceutical/Antihypertensive	2a	Yes
	Eprosartan	Pharmaceutical/Antihypertensive	1	No
	Fluconazole	Pharmaceutical/Antifungal	1	Yes
	Indomethacin	Pharmaceutical/Anti-inflammatory	1	No
	Irbesartan	Pharmaceutical/Antihypertensive	1	Yes
	Ketoprofen	Pharmaceutical/Anti-inflammatory	1	No
	Lacosamide	Pharmaceutical/Anticonvulsant	2a	Yes
	Lorazepam	Pharmaceutical/Anxiolytic	1	Yes
	Lormetazepam	Pharmaceutical/Anxiolytic	2a	Yes
	Losartan	Pharmaceutical/Antihypertensive	1	Yes
	Metoprolol	Pharmaceutical/Antihypertensive	1	Yes
	Mexedrone	Pharmaceutical/Antidepressant	2a	No
	Minoxidil	Pharmaceutical/Antihypertensive	2a	No
	Mycophenolic acid	Pharmaceutical/Antibiotic	1	Yes

Class of compound	Compounds detected during COVID-19	Use	Identification level	Detected pre-COVID-19
Other related pharmaceuticals *(Cont.)*	Nalbuphine	Pharmaceutical/Analgesic	2a	No
	Norfloxacin	Pharmaceutical/Antibiotic	1	No
	Oxazepam	Pharmaceutical/Anxiolytic	3	Yes
	Ofloxacin	Pharmaceutical/Antibiotic	1	No
	Primidone	Pharmaceutical/Anticonvulsant	1	No
	Propyphenazone	Pharmaceutical/Anti-inflammatory	1	Yes
	Sertraline	Pharmaceutical/Antidepressant	1	Yes
	Sotalol	Pharmaceutical/Antihypertensive	1	Yes
	Sulfamethoxazole	Pharmaceutical/Antibiotic	1	Yes
	Sulpiride	Pharmaceutical/Antidepressant	2a	No
	Telmisartan	Pharmaceutical/Antihypertensive	1	Yes
	Temazepam	Pharmaceutical/Anxiolytic	2a	Yes
	Tiapride	Pharmaceutical/Antipsychotic	2a	No
	Tramadol	Pharmaceutical/Analgesic	1	Yes
	Trazodone	Pharmaceutical/Antidepressant	2a	Yes
	Trimethoprim	Pharmaceutical/Antibiotic	1	Yes
	Valsartan	Pharmaceutical/Antihypertensive	1	Yes
	Venlafaxine	Pharmaceutical/Antidepressant	2a	Yes
Other related compounds	Amphetamine	Illicit drug	3	Yes
	Cocaine	Illicit drug	2a	No
	Cotinine	Nicotine metabolite	1	No
	Ketamine	Illicit drug	2a	Yes
	Metamphetamine	Illicit drug	3	Yes

After the secondary treatments a removal rate higher than 50% was determined for 22 and 30 compounds (in Crispijana and Galindo WWTP, respectively), and the efficiency of the tertiary treatment from Galindo WWTP was evidenced. By the use of the tertiary treatment, a large number of compounds (n = 32) were significantly removed (see Table S4 in SI). A non-significant elimination rate was observed through the secondary treatment for the rest of identified compounds (i.e., 45 compounds), so that they can be cathegorized as "pseudo-persistent" contaminants that are continuously released into the aquatic ecosystem (see Table S4 in SI).

3.2. *Influence of the COVID-19*

The lack of knowledge of the virus and the need to rapidly find some effective treatments to combat the virus led to the massive use of several pharmaceutical compounds (or combinations) with antiviral and/or antimicrobial activity (Costanzo *et al.*, 2020). In this work, suspect analysis enabled the identification (at level 1 and 2a) of some of those drugs that were massively used for COVID-19 treatment early in the pandemic thereby increasing their occurrence in wastewaters (see Table 4) (Alygizakis *et al.*, 2021; Cappelli *et al.*, 2022;

Galani *et al.*, 2021). Based on some previous occurrence data get in sampling campaigns before COVID-19 time in secondary effluent of Galindo WWTP (González-Gaya *et al.*, 2021), the analgesic acetaminophen, the antibiotic azithromycin, the antivirals darunavir and lopinavir, and the antimalarial hydroxychloroquine are some of those drugs with significant occurrence during the pandemic time.

As can be observed in Table 4, there is no prior evidence of the occurrence of the compounds hydroxychloroquine and lopinavir above detection limits, being the first time that the presence of hydroxychloroquine was registered in Basque environmental waters (Domingo-Echaburu *et al.*, 2022). Hydroxychloroquine, typically used for malaria, lupus and rheumatoid arthritis treatment (Drug Bank Online, 2020), was considered as a possible efficient drug to treat COVID-19 disease (either alone or in combination with azithromycin) at the beginning of the pandemic (Gautret *et al.*, 2020). The use of lopinavir (an antiviral often prescribed with ritonavir to treat HIV (Osborne *et al.*, 2020) as an effective virus-fighting agent was also revealed by its high occurrence in wastewaters during the pandemic period. In fact, according to the UBA, the concentration found for lopinavir in the analyzed samples was the highest registered at the European level (https://www.umweltbundesamt.de/en/database-pharmaceuticals-in-the-environment-0, accessed October 2022). Acetaminophen, typically used in WBE to predict disease outbreaks because it is a short-term application analgesic that can be consumed without prescription (Halwatura *et al.*, 2022), was also used to control some of the COVID-19 symptoms, and hence, its occurrence was detected during the pandemic time but also before that period (see Table 4) (González-Gaya *et al.*, 2021). A similar trend was also observed for the previously highlighted azithromycin and darunavir compounds, which were detected during and before pandemic time (González-Gaya *et al.*, 2021).

Regarding the antibiotics detected in samples collected in this study, although their occurrence is positively correlated with the COVID-19 metrics and it is known that they were massively administered during lockdown (Cappelli *et al.*, 2022; Galani *et al.*, 2021; Gonzalez-Zorn, 2021), the presence of broad-spectrum class antibiotics in wastewaters could be a consequence of seasonal diseases. Heterogeneous trend in pharmaceuticals for other therapeutic purposes (e.g. antihypertensives, anti-inflammatories, anticonvulsants) consumption during the pandemic has been reported. On the other hand, post-traumatic stress, depression, insomnia, fear and/or frustration, among others suffered by citizens during the lockdown (Brooks *et al.*, 2020) (Singh *et al.*, 2020) could led to the consumption of illicit drugs. Qualitative comparison of compounds' occurrence before (González-Gaya *et al.*, 2021) and during the pandemic time (this study) revealed negligible differences in the presence of most of the compounds detected in this study at the Galindo WWTP, with only 20 (e.g. hydroxychloroquine, lopinavir, clarithromycin, clozapine, sulpiride and tiapride, among others) compounds more detected in samples collected during the lockdown (see Table 4); particularly, new pharmaceuticals have emerged in Galindo WWTP effluent (e.g., candesartan, clozapine, eprosartan or primidone, among others). In line with other studies (Alygizakis *et al.*, 2021; Nason *et al.*, 2022; Wang *et al.*, 2020), a higher number of antipsychotic drugs (including antidepressants) have been observed compared to the non-COVID-19 period, which, as aforementioned, would give more insight into the mental health of the Basque citizens provoked by the different measures applied. Furthermore, certain illicit drugs considered as biomarkers in WBE studies (Alygizakis *et al.*, 2021; Been *et al.*, 2021; Reinstadler *et al.*, 2021) such as amphetamine or ketamine were also detected (see Table 4).

Unfortunately, the lack of previous studies hindered the comparison of the values detected at the Crispijana WWTP. However, an increase in hospital drug consumption of certain selected drugs during the first wave pandemic was previously discussed (Domingo-Echaburu *et al.*, 2022).

3.3. *Prioritization strategy for environmental risk assessment*

A prioritization strategy for environmental risk assessment was carried out using the compounds quantified in the effluents of Crispijana and Galindo WWTPs. The compounds were scored based on the (a) removal efficiency (RE, %), (b) estimated persistency (half-life time in days, DT50), (c) bioconcentration factor (BCF), (d) toxicity potential and (e) frequency of detection in the samples (see section 2.8). Those compounds with the lowest total score value were set as the potential drivers of toxicity.

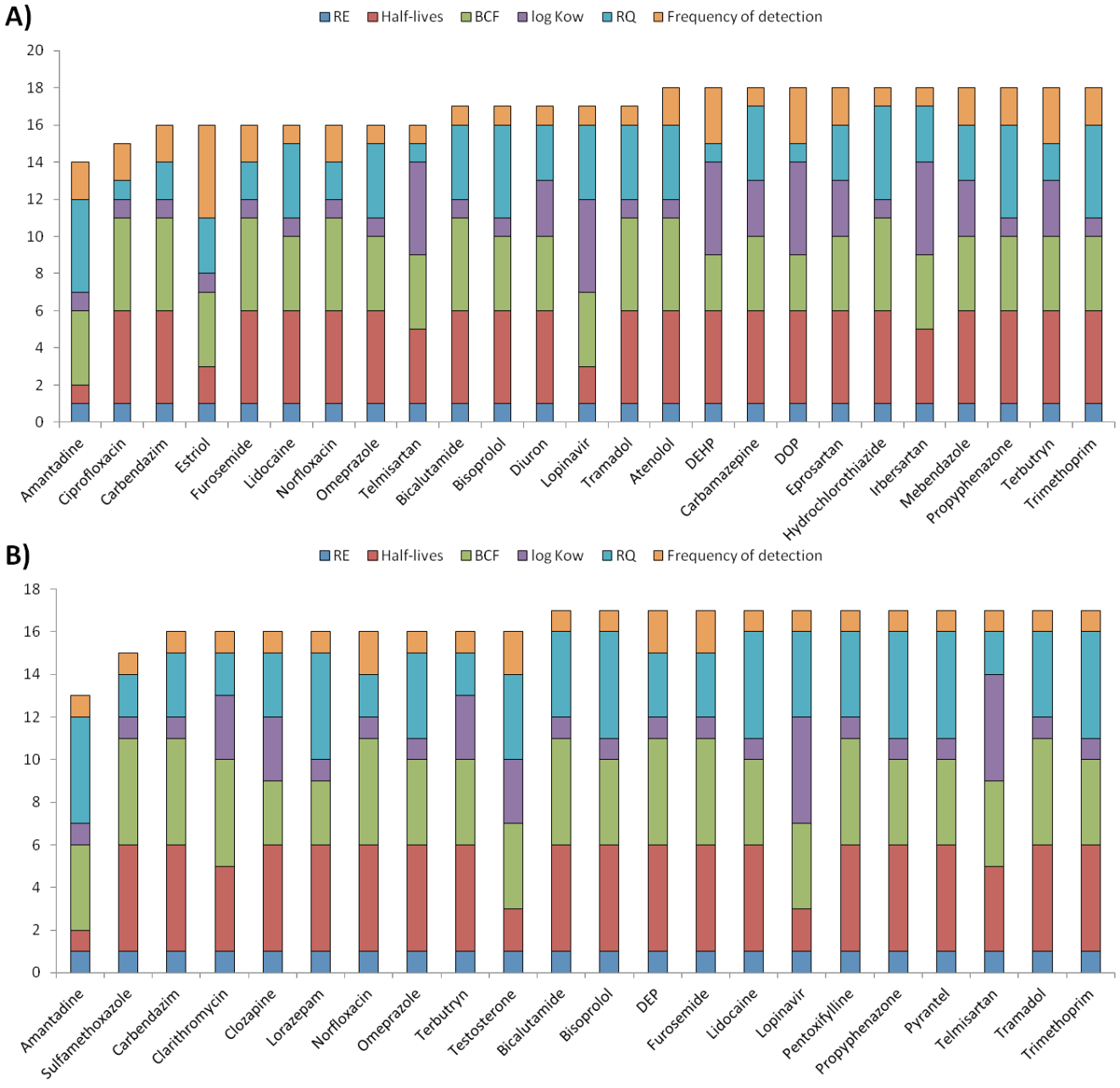

Figure 2. Total scores of the top risk drivers found in the secondary effluent of Crispijana (A) and Galindo WWTPs (B)

Among the compounds quantified in both WWTPs, the list of the most concerning compounds is constituted by 25 and 22 micropollutants in Crispijana and Galindo, respectively. Pharmaceutical compounds dominated both priority lists (> 70% of the total in both WWTPs), while, lower total scores were obtained in wastewaters from Galindo WWTP for the prioritized contaminants (total score ≤ 17 vs 18) (see Figure 2, Table S6 in SI). Several compounds identified as priority compounds in this work have already been considered hazardous elsewhere such as the ones included in WFD priority list (DEHP, diuron and terbutryn) (European Commission, 2013) and the ones included in the current Watch List to be considered for future prioritization (clarithromycin and sulfamethoxazole) (European Commission, 2015; Gomez Cortes *et al.*, 2020). Moreover, some of the compounds considered in here as priority compounds were also pointed out as key chemicals in environmental toxicity studies. In the work of Gros and coworkers, for example, lidocaine (included in both priority rankings) was pointed out as one of the top-risk drivers of Swedish wastewaters, followed by diuron (included in the priority list of Crispijana WWTP) to a lower extent (higher total scores) (Gros *et al.*, 2017). Carbamazepine, irbesartan, sulfamethoxazole and ciprofloxacin were identified as relevant chemicals for marine organisms in the area of Ebro Delta (Spain) in the work of Čelić and coworkers, where a similar prioritization strategy to the one used in the present work was done (Čelić *et al.*, 2019). After the assessment of 52 European WWTPs, Finckh *et al.* pointed out carbendazim, terbutryn and diuron as toxicity-driver compounds (Finckh *et al.*, 2022). Moreover, other recent studies based on the calculation of RQs in WWTP effluents (Figuière *et al.*, 2022; Lopez-Herguedas *et al.*, 2022; Solaun *et al.*, 2021), freshwater (Figuière *et al.*, 2022) and riverine and coastal ecosystems (Čelić *et al.*, 2021) highlighted the need to prioritize some of the concerning compounds pointed out in the present work.

Secondary treatments implemented in both analyzed WWTPs seemed to be not efficient enough to remove completely all the prioritized contaminants (score of 1). The poor elimination rate of the detected organic micropollutants through conventional secondary treatments implemented in WWTPs is widely reported in the literature (Golovko *et al.*, 2021; Jelic *et al.*, 2011; Köck-Schulmeyer *et al.*, 2013; Kovalova *et al.*, 2012; Le Corre *et al.*, 2012). The associated matrix effect that can result in signal suppression is usually the argument used to explain these "negative" removals. However, typical retransformation of conjugated compounds into the original compound through biological processes, improper sample collection (lack of correlation between influent and effluent samples due to a bad timely collection) or the release of the compounds from fecal particles due to microbial breakdown can also be considered to report negative compound removals (Fernández-López *et al.*, 2016; Köck-Schulmeyer *et al.*, 2013).

Amantadine (score 1) and lopinavir (score 2) stood out as the most persistent compounds in both WWTPs, showing DT50 values exceeding 60 days, with the addition of estriol (Crispijana WWTP, score 2) and testosterone (Galindo WWTP, score 2). The persistency of the remaining compounds was lower (< 37.5 days), suggesting that most of the top compounds were easily degradable (see Figure 2, Table S6 in SI). DEHP and DOP in Crispijana WWTP and clozapine and lorazepam in Galindo WWTP were the compounds showing the highest predicted BCF values, however, none of the detected compounds could be considered as highly bioaccumulative (BCF < 100). Additionally, it is important to note that statements made considering biodegradation and bioaccumulation of the compounds are fully based on predicted values due to the lack of experimental values and contradictions may exist, as was observed when comparing half-life times and REs. Thus, there could be an overestimation of the real risk. In consequence, these categories should not share the same weight as categories based on experimental data in future prioritization strategies.

In terms of mobility, prioritized compounds showed, overall, low log K_{ow} values, suggesting a high mobility potential, with the exception of DOP, irbesartan, lopinavir and telmisartan (see **F**igure 2, Table S6 in SI).

Individual RQs were calculated to assess the maximum concentration at which the ecological status of the ecosystem is preserved. To that aim, predicted values based on in-silico tools (i.e. ECOSAR) for baseline toxicity were considered, since there is a lack of experimental toxicity data available for the assessed compounds (see Table S5). In this case, experimental toxicity values were found for around 50 and 60% of the prioritized compounds for PNEC calculation in Crispijana and Galindo WWTPs, respectively. Estimated individual toxicities highlighted that although most of the detected compounds do not pose a relevant environmental risk, some compounds should be closely tracked, especially ciprofloxacin, telmisartan, DEHP and DOP (RQ > 1), and sulfamethoxazole, clarithromycin, norfloxacin and terbutryn (RQ > 0.1), in a lesser extent. Furthermore, the over/underestimation of the environmental risk led by the use of predicted ecotoxicological data rather than experimental (i.e. NOEC and/or EC_{50}) for the calculation of RQs emphasizes the need for more empirical evidence to provide more reliable results.

Both priority rankings include compounds that have not been identified in previous studies as concerning and which may be related in some way to COVID-19 disease. Lopinavir, as aforementioned, has been used in combination with ritonavir to combat the virus, suggesting that its massive use during this particular period is responsible for increasing the potential environmental risk it may pose. On the other hand, the potential risk of the psychoactive compounds clozapine and lorazepam could be correlated with their raised prescription rates to overcome mental illnesses caused by the lockdown.

Comparing both secondary effluents with the tertiary effluent of Galindo WWTP, slightly higher total scores of the top-ranked contaminants were obtained in the latter (see section S7 in SI).

Table 4
Potential antimicrobial and antiviral activity of the drugs of interest in both analyzed WWTPs

Compounds	PNEC-AR (µg/L) (Bengtsson-Palme and Larsson, 2016	vIC50/vEC50 (µg/L) (Kuroda *et al.*, 2021)	Crispijana WWTP		Galindo WWTP	
			RQ-AR	EDRP	RQ-AR	EDRP
Ciprofloxacin	0.064		0.1742	—	0.0347	—
Clarithromycin	0.25		0.0676	—	0.00314	—
Fluconazole	0.25		0.1411	—	0.032644	—
Hydroxychloroquine		242	—	0.000025	—	1.14339E-05
Lopinavir		1088	—	2.96415E-06	—	9.26471E-07
Norfloxacin	0.5		0.05105	—	0.065858	—
Ofloxacin	0.5		—	—	0.002938	—
Ritonavir		6222	—	—	—	1.04468E-07
Sulfamethoxazole	16,000		—	—	0.001536438	—
Trimethoprim	0.5		0.0105	—	0.023326	—

Considering the high loads of pharmaceuticals with antimicrobial and antiviral activity released into the environment due to the COVID-19 disease, the concern of the development of resistance in the aquatic environment has increased (Knight *et al.*, 2021; Kuroda *et al.*, 2021). The antimicrobial and antiviral potential activity of the drugs of interest was determined with the calculation of RQ-AR and EDRP (see section 2.8). The risk indices determined (see Table 5) suggest that none of the detected compounds might pose a relevant activity, since RQ-AR and EDRP values did not exceed the threshold of

> 1. However, in the case of antimicrobial activity, ciprofloxacin and fluconazole reached concentrations of medium antimicrobial resistance risk (1 > RQ-AR > 0.1). Our findings, considering the antimicrobial activity, were contrary to those observed by Cappelli and coworkers, as in that case both azithromycin and ciprofloxacin exceeded the RQ-AR = 1 threshold, posing a high potential for developing antimicrobial resistance (Cappelli *et al.*, 2022). Nevertheless, it should be highlighted that any DF (see section 2.8) was applied in that study, representing the worst-case scenario. On the other hand, the negligible risk of EDRP determined in this study is in line with other studies (Cappelli *et al.*, 2022; Kuroda *et al.*, 2021). However, regardless of the determined low RQ-AR and EDRP values, a reduction of antiviral and antimicrobial drug residues is suggested in order to avoid the disruption of natural biological systems as well as the development of resistance in aquatic systems (Kuroda *et al.*, 2021; Usman *et al.*, 2020).

Once the priority list of contaminants was defined, mixture toxicity was assessed via the calculation of STU (see section 2.8). All effluent samples exceeded the threshold of 1 (Figure 3) obtaining the highest mixture risk (STU = 11.1) for the secondary effluent of Crispijana WWTP being DOP the main contributor of the mixture toxicity (72% of the total) followed by DEHP and telmisartan (STU values of 1.28 and 1.11, respectively). In the case of the secondary effluent of Galindo WWTP, the risk was almost halved to an STU value of 6.8, predominated by DEHP which contributed to around 90% of the total mixture risk, while more than the remaining mixture toxicity was attributed to norfloxacin. Similarly to the individual risk assessment, the lowest STU value was estimated for the tertiary effluent of Galindo WWTP (STU = 1.6). In this latter case, any of the compounds exceeded the threshold of 1 being DEHP and norfloxacin the most influential compounds in the mixture risk both with moderate risks (0.63 and 0.79, respectively).

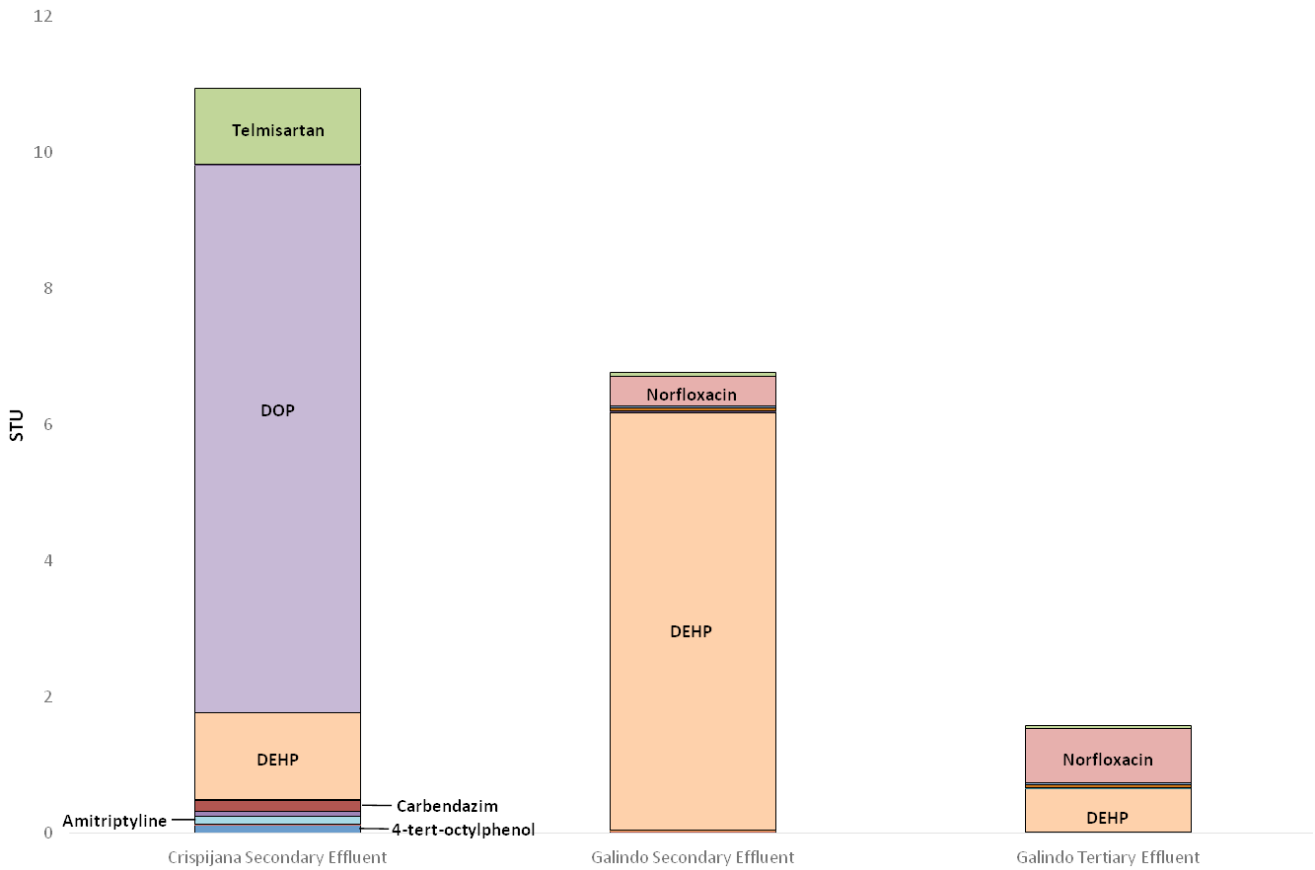

Figure 3. STU values for analyzed effluent samples including the main contributors

Chronic ecotoxicological data was considered rather than acute data when possible for the mixture toxicity assessment (see section 2.8). As indicated by Markert *et al.* the choice of acute or chronic toxicity data will have a clear impact on the calculated risks of the mixture, and they recommend that the risk assessment of the mixture should be based not only on the commonly applied acute toxicity data but also on the chronic toxicity data (Markert *et al.*, 2020). In fact, with many of the contaminants, it is known that it is the long-term risks that will really affect the environment. However, the use of fixed ratios for the extrapolation from acute to chronic toxicity is problematic, because some chemicals show different modes of action (MoA) under short- and long-term conditions (Ahlers *et al.*, 2006). In addition, the biological mechanisms of action differ from species to species.

4. Conclusions

A previously validated suspect screening workflow was used for the identification of emerging contaminants present in two different WWTPs located in the Basque Country (Crispijana and Galindo) during COVID-19 confinement. Pharmaceutical compounds used for COVID-19 disease treatment were detected in both WWTP samples including the antivirals ritonavir/lopinavir (level 1) and darunavir (level 2a), the antimalarial hydroxychloroquine (level 1) and the antibiotic azithromycin (level 1). Moreover, other pharmaceuticals used for therapeutic purposes were also detected (e.g. amitriptyline, clozapine, lorazepam, primidone and valsartan, among others), suggesting a positive correlation with the mental illnesses caused by the lockdown. Despite the differences between the number and concentrations of the compounds found in both WWTPs due to their different locations, the population of influence and the treatments implemented, they both coincide in not being able to eliminate most of the drugs found in their influents with any of the treatments implemented.

A prioritization strategy for the ECs detected in WWTP effluent samples was carried out in order to point out the major contributors to environmental risk. Although several compounds were considered of concern, both prioritization lists consisted mostly of pharmaceutical compounds (e.g. amantadine, telmisartan, lopinavir, clarithromycin, clozapine) highlighting the need for monitoring and thereby concluding whether they should be considered for future regulation. On the other hand, the lack of measured data (e.g. degradation, bioaccumulation and toxicity) for many frequently detected compounds leaves no alternative but to make use of reference QSARs or other in-silico tools for data prediction, which leads to high uncertainty in the affirmations made. Although the values determined to assess antimicrobial and antiviral resistance activity for the compounds of interest were low (RQ-AR and EDRP values < 1), the results of the antimicrobial risk index showed medium environmental concern for the detected levels of ciprofloxacin and fluconazole, demonstrating the need to include these endpoints in current regulatory systems.

Thus, the development of new technologies in the wastewater treatments is required to improve the removal efficiency of those compounds so the potential environmental risk they may pose in receiving water ecosystems decreases. On the other hand, more efforts need to be made to fill the gaps by prioritizing chemicals for effect testing and evaluating the mixture effects (i.e. synergic or antagonistic effects) of the contaminants.

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