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Biomonitoring emerging hazards of pharmaceuticals in river water using gut microbiome and behavioural *Daphnia magna* responses

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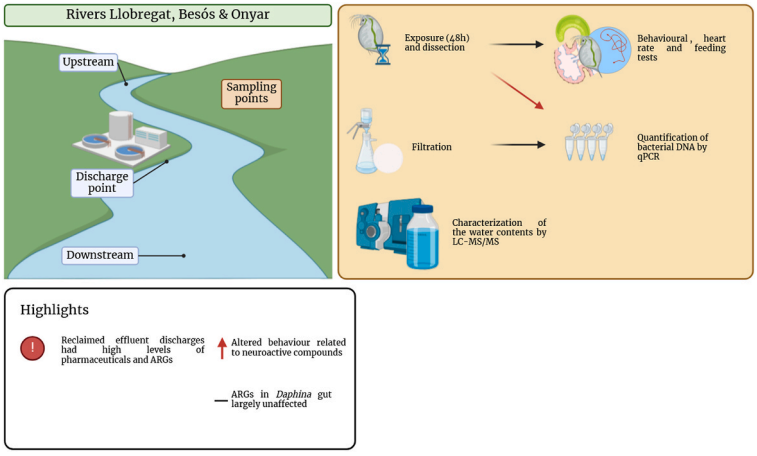
HIGHLIGHTS

- *Daphnia magna* testing was applied to identify emerging hazards in river water.
- Antibiotic resistant gene prevalence in water was related with bactericides in water.
- Behavioural defects in D. magna were related to neuroactive contaminants.
- *Daphnia* behaviour was sensitive enough to be altered by neurochemical mixtures.

GRAPHICAL ABSTRACT



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ABSTRACT

A cost-effective *Daphnia magna* testing framework was applied to identify emerging hazards such as neurological and cardiovascular defects as well as antibiotic resistant genes (ARGs), related to pharmaceuticals present in waste water treated (WWTP) effluent discharged into rivers. *D. magna* juveniles were exposed during 48 h to water samples from three rivers in the vicinity of Barcelona (NE Spain), Besós, Llobregat and Onyar, upstream and downstream of WWTP discharging points. The analyses included measuring levels of 80 pharmaceutical residues in water samples by HPLC-MS, determination of the loads of different clinically relevant antibiotic resistant genes (ARGs) in both water samples and exposed animals, and assessment of toxic effects in feeding, heartbeat responses, and behavioural indicators. ARG prevalence in water, but not in gut microbiomes, was associated with the presence of bactericides in water. These results suggest that their levels were high enough to put a selective pressure over river microbial populations, but that *Daphnia* guts were not easily populated by environmental bacteria. Toxic effects were found in 20–43% of water samples, depending on the river, and

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related to water quality parameters and to pollutant levels. For example, heartbeats were correlated with salinity, whereas feeding impairment did so with high loads of suspended solids. In contrast, behavioural alterations were associated to the concentration of neuroactive chemicals. Accordingly, we hypothesize that measured neuroactive chemicals have caused the observed effects. If this also applies to local invertebrate populations, the environmental consequences may be severe and unpredictable.

1. Introduction

Wildlife is exposed to low-level concentrations of pharmaceutical mixtures, which are potentially dangerous to ecosystems and ultimately to human health (König et al., 2017). Among them, neuroactive chemicals are of special concern due to their ability to interact with the central nervous system (CNS) and hence disrupt not only cognitive responses but also key organismic physiological processes such as metabolism, survival, growth and reproduction (Leuthold et al., 2019). On the other hand, the excessive use of antibiotic and disinfectants have also promoted emerging human health hazards such as the raise of antibiotic resistance bacteria and/or genes (ARGs) (Singh et al., 2019). Treated wastewater treatment plants (WWTP) discharges are an important pollution source for both neuroactive pharmaceuticals and ARGs in surface waters (David et al., 2018; García et al., 2020; Styrishave et al., 2011). This situation is aggravated in Mediterranean and other semi-arid regions where WWTP effluents are an important part of the river flow (Petrovic et al., 2011). Furthermore, in these semi-arid regions reclaimed water is increasingly used as a recycling water source for drinking water, irrigation, industry and ecological purposes (Munné et al., 2023). Thus, there is an urgent need to monitor contaminant and ARGs hazards present in reclaimed water using reliable cost-effective methods (Múrria et al., 2024; Stankiewicz et al., 2024).

In real field conditions, chemical cocktails include the parental compounds but also their degradation products and metabolites (Nerin et al., 2013). Furthermore, water physico-chemical conditions like pH, hardness and conductivity may change the speciation of chemicals (Neuwoehner and Escher, 2011; Pinheiro et al., 2021). Current one-chemical-based hazard approaches suffer from a lack of exposure realism since they evaluate one chemical at a time, often in laboratory exposures, and hence do not consider chemical speciation, transformation, metabolism and interaction (Drakvik et al., 2020). In addition, current hazard assessment methods are based on standardized toxicity tests that lack sensitive responses to assess emerging hazards, such as neurological defects or the presence of ARGs.

The use of sentinel species for environmental monitoring using toxicological procedures upgraded with neurological sensitive responses may improve our ability to identify neuroactive hazardous chemicals. The freshwater crustacean *Daphnia magna* is a world-wide used sensitive sentinel species for environmental monitoring, because of its key role in the aquatic food web and sensitivity to toxic pollution (Abdullahi et al., 2022). Recently, the development of behavioural and cardiovascular tests in *D. magna* has allowed to better evaluate cognitive and cardiovascular defects of a large set of neuroactive chemicals under laboratory conditions (Bedrossiantz et al., 2020, 2021, 2023; Bellot et al., 2021). *Daphnia magna* has also the ability to feed on algae and bacteria, and thus it has been also used in antibiotic resistance related studies mostly in lab conditions (Carrillo et al., 2023; Olanrewaju et al., 2019; Tang et al., 2010). So far, the development and application of field and lab assays in *D. magna* has allowed to monitor lethal and sub-lethal (i.e. feeding inhibition) mediated effects of pesticides, metals and other industrial contaminants in the field (Barata et al., 2007; Damasio et al., 2008,2011a; Puértolas et al., 2010). Field assays include short term transplant in situ assays where animals are exposed to river water in special designed cages (Barata et al., 2007; Damasio et al., 2008; Puértolas et al., 2010), whereas in lab assays animals are exposed to field-collected water (Abdullahi et al., 2022; Múrria et al., 2024; Rivetti et al., 2015). In both assays, post-exposure effects are assessed by combining individual, biochemical and/or molecular responses.

The aim of this study is to evaluate neurological, cardiovascular and feeding mediated effects of pharmaceuticals, as well as the presence of ARGs, in WWTP discharges in three Mediterranean rivers along the Catalonian region (NE Spain) using lab exposure procedures. The study is focused on a tertiary treated reclaimed water effluent discharge used to re-establish the ecological water flow of the Llobregat river (Munné et al., 2023) and in two conventional secondary WWTP effluents that discharge in the Besós and Onyar rivers. The Llobregat and Besós rivers are affected by high industrialized areas surrounded by heavily populated regions including the metropolitan area of Barcelona (Munné et al., 2023), whereas the Onyar river is situated in a less populated area but suffers from agricultural and industrial activities around the city of Girona (North Catalonia) (Córdoba-Ariza et al., 2024).

It is well known that many pharmaceutical residues, bactericides, and ARGs (or/and ARG-carrying bacteria) are not completely removed by WWTP and hence they are released into the rivers (Domínguez-García et al., 2024a; Khasawneh and Palaniandy, 2021; Wang et al., 2020). To assess the importance of this combined environmental impact, we combined chemical analysis, ARG quantification techniques, and a large battery of behavioural assays in *D. magna* individuals. More precisely, our goal is to determine whether or not current pharmaceutical and bactericidal levels in rivers are high enough to elicit physiological responses, either in invertebrate populations or in their gut microbiomes, using *Daphnia* as suitable and convenient model organism.

2. Material and methods

2.1. Sampling

Samples were obtained from three distinct rivers located in Catalonia (Spain) through grab sampling techniques: The Besòs (B), Llobregat (LL) and Onyar (O) rivers. Most sites from Besós and Llobregat rivers were sampled twice between January and March 2023. During each sampling campaign, water was collected in three locations: upstream, at the effluent discharge point and downstream. Sampling locations in the Besòs River were chosen near the Montornès del Vallès WWTP, which processes 28,000 m^3 of domestic and industrial wastewater daily from up to 200,000 inhabitants. In the Llobregat River, the sampling sites were selected to examine the impact of using reclaimed water to recharge the aquifer and to maintain the river flow. This reclaimed water originates from the El Prat de Llobregat WWTP, situated 20 km away from the recharging point, and serving a population of up to 2 million people in the metropolitan area of Barcelona (Munné et al., 2023) Onyar River sampling points were situated close to a small WWTP from the Girona suburbs that treats agricultural and domestic effluents of 600 m3/day from about 4400 inhabitants. Fig. 1 shows a satellite view of the sampling sites, and Table S1 (Supplementary Material, SM) provides a description of the 14 samples.

Sampling was performed in amber glass bottles of 2 L. Samples (6 L) were refrigerated until toxicological evaluations and processing for physico-chemical and ARG analyses, which were carried out the very same day. In each site, samples were collected at the discharge point and immediately upstream and downstream of it, three samples per campaign in total (Fig. 1).

2.2. Water physico-chemical and ARGs analysis

For each water sample, pH, conductivity, oxygen levels, suspended solids, ARGs and up to 80 chemical residues were determined. Briefly, pH, conductivity, and dissolved oxygen concentration were measured in situ using a WTW Multi 340i handheld meter (Wissenschaftlich-Technische Werkstätten [GmbH], Weilheim, Germany). Total suspended solids were measured in the laboratory according to standard methods (Eaton et al., 1995).

For ARG analyses, duplicated water samples of 0.5 L were sequentially filtered through 3 μm and 0.2 μm membrane filters (Isopore, polycarbonate and Omnipore, PTFE respectively, Merck-Millipore), to retain both particle-attached and free-living bacteria. Filters were preserved at −20 °C until being processed for DNA extraction.

For chemical residue determinations, the analytical methodology followed previous studies (Domínguez-García et al., 2024a, b; Gómez-Canela et al., 2021). It is focused on preserving and extracting pharmaceuticals from river waters for the subsequent analysis by LC-MS/MS. Prior to analysis river water was filtered using 0.45 μm nylon filters (Phenomenex, Torrance, CA, USA) to remove solid particles. The LC-MS/MS analysis employed a CORTECS T3 column with a mobile phase gradient, in the positive electrospray ionization (ESI+) mode. Details on optimization parameters, fragmentation, accuracy and recovery have been published elsewhere (Domínguez-García et al., 2024a).

2.3. Experimental animals and culture conditions

Parthenogenetic cultures of the *D. magna* clone F were used for this study. This clone has been maintained for over 20 years in our lab (Barata and Baird, 1998). Animals were cultured under a 16 h light: 8 h dark photoperiod cycle, and at 20 ± 1 °C. Several bulk cultures of 10 adult *Daphnia* females were maintained in 2 L of laboratory-prepared water, i.e. ASTM hard synthetic water (APHA, 1995) complemented with a 0.25 μm filtered food additive mixed into the water, which is a seaweed extract (Marinure, UK) using a food ratio of 5×10^5 cells/mL of *Chlorella vulgaris* that was cultured in semi-axenic conditions (Barata and Baird, 1998). Culture media were changed every other day. Groups of 50 third brood neonates collected within the first 12 h of being released by their mothers from the adult bulk cultures were reared in 1.5 L of media as previously described, during 4 days (hereafter referred as 4-day old juveniles).

2.4. Exposures

Daphnia magna 4-day old juveniles were exposed in duplicate to 0.5 L of the tested water samples in groups of 60 individuals during 48 h in an orbital incubator set up at 1 rpm to prevent the sedimentation of suspended solids. Daphnids maintained in ASTM hard water were also incubated as laboratory control. Twenty four, 10 and 18 individuals from each treatment (including controls) were used immediately for behavioural, cardiovascular and feeding tests, respectively; the remaining ones were depurated for an additional 8 h in filtered clean ASTM hard water and preserved in RNAlater until further processing for ARG determination.

2.5. Feeding, behavioural and heartbeat tests

Post-exposure feeding responses were measured following previous procedures with minor modifications (Rivetti et al., 2015). After 48 h of exposure, 18 individuals for each treatment, plus the control group, were distributed in 6 beakers (3 individuals per beaker) containing 40 mL ASTM water with *C. vulgaris* (5×10^5 cells/mL). Two beakers without daphnids were used as blanks. Beakers were kept in darkness during 4 h and feeding rates were measured as the reduction in algal cells relative to the blanks, measured as optical density at 665 nm. Proportional feeding responses were reported as % of feeding rates relative to controls.

Three tests were performed to study the effects in *D. magna* basal swimming activity in darkness, visual motor, habituation and phototactic responses to light stimuli, using a DanioVision Observation Chamber (DVOC-0040) complemented with am EthoVision XT video tracking software (Noldus, Netherlands) and following previous procedures (Bedrossiantz et al., 2020; Bellot et al., 2021, 2022; Savva et al.,

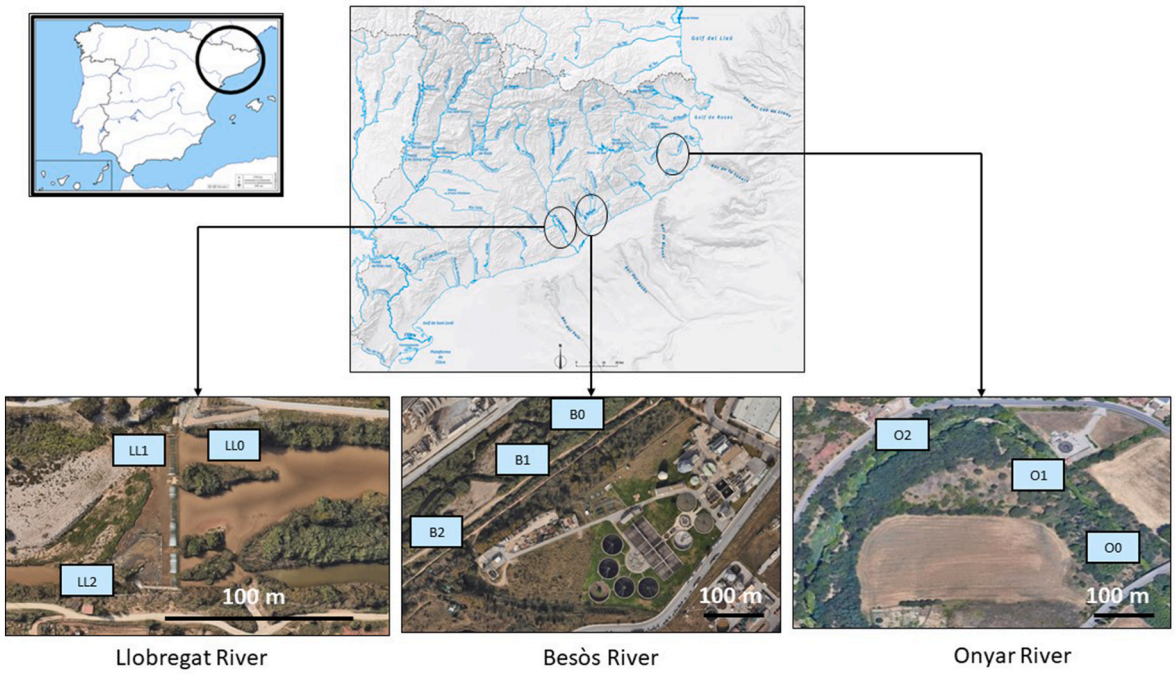


Fig. 1. Study sites and satellite view of the Besós, Llobregat and Onyar (labeled "B", "LL", and "O", respectively) sampling spots. "0", "1"and "2" indicate sampling points upstream, close to, and downstream the corresponding discharge points.

2023). Those tests are based on the exposure to light that, in the studied *D. magna* clone, promotes different anti-predatory escape responses, like increasing locomotion/swimming activity and negative phototactic behaviour upon light stimuli (Bedrossiantz et al., 2020; Bellot et al., 2022).

Before the test, individuals were kept in darkness for acclimatization during 20 min and trials were performed in 24 well plates at 20 °C. The first test was based on monitoring basal locomotion activity during 5 min of darkness and visual motor responses upon the exposure to light during 5 min (Bellot et al., 2021). In the second test, individuals were exposed to 30 consecutive light flashes (1 s-light followed by 4 s of dark) to assess non-associative learning responses such as the maximal response to first stimuli and habituation to repetitive light stimuli (Bedrossiantz et al., 2020). Lastly, the third test was performed also in a 24 multi-well plate covered in its base in a special acrylic grid opaque to visible light such that half of the well was protected from the light and half exposed, thus allowing to evaluate the percentage of time individuals preferred to stay in the light or the dark side of the well (phototaxis) (Bellot et al., 2022). Videos were recorded during all the tests, and responses were measured as distance of movement (for the first and second test) or percentage of time spent in the dark zone (for the third test). Each treatment included 24 individual replicates, which were randomly distributed across the different well plates assayed within a day.

Daphnia individuals were directly positioned in lateral view in methylcellulose and the cardiac activity of each daphnia was video recorded for 30 s with a GigE camera (UI–5240CP-NIR-GL, Imaging Development Systems, Germany) mounted onto a stereomicroscope (Motic SMZ-171, Wetzlar, Germany) (Faria et al., 2022). Videos of each individual *Daphnia* were analyzed using DanioScope™ software (Noldus, Wageningen, The Netherlands). About ten individual replicates per treatment were used.

2.6. DNA extraction

To study the ARGs associated to the *D. magna* microbiome, ten entire *D. magna* guts (foregut, midgut with the hepatic diverticulum, and hindgut) previously preserved in in RNAlater at 4 °C during 24h, were carefully dissected and stored in DNA lysis buffer. DNA extraction followed the phenol-chloroform method described before (Cerro-Gálvez et al., 2020) adding few modifications, such as including a previous step of three freeze and thaw cycles and performing homogenization step using a TissueLyser® (Qiagen, Germantown, MA, USA). The inclusion of sequential freeze and thawing steps improves the DNA extraction efficiencies.

DNA from membrane filters obtained from water samples was extracted using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), and eluted in 50 μl of kit elution buffer (10 mM Tris-HCl pH 8.5). DNA extracts obtained from 3 μm to 0.2 μm membrane filters of a same water sample were pooled for further analyses.

The quality and quantity of total DNA was determined using NanoDrop Spectrophotometer 8000 (Thermo Fisher Scientific, Inc). Extracted DNA samples were stored at −20 °C.

2.7. Quantification of bacterial DNA genes by qPCR

Absolute quantification was performed for the 16S rRNA gene, *intI*1 (class 1 integron-integrase) and for the five most abundant ARGs found in wastewater treated effluents of Catalonia (SE, Spain) (Leiva et al., 2021; Sanz et al., 2021, 2022). ARGs included *sul*1 (dihydropteroate synthetase, conferring resistance to sulphonamide), *qnr*S1 (a Pentapeptide Repeat Protein family member that inhibits the effect of quinolones), *tetM* (a ribosomal protection protein, which confers tetracycline resistance by binding to the ribosome and preventing drug interaction with its binding site), bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$ (two β-lactamases conferring resistance to beta-lactamic antibiotics such as cephalosporins, monobactams, and carbapenems).

Primer sequences are listed in Sanz et al. (2021). Ten ng/μl of total DNA preparations was used for ARG quantification by real time qPCR in a LightCycler 480 II (A F. Hoffmann–La Roche AG, Inc), in 20 μL reaction volumes on 96-well plates. Optimal primer concentrations were 300 nM for all genes. All samples were run as technical duplicates along with the quantification curve to reduce variability between assays. Plasmids used for the quantification curves were pNORM1 conjugative plasmid (Gat et al., 2017) for *intI1, Sul1, qnrS1, tetM,* bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$ and individual pUC19 plasmids for *tetM* (Laht et al., 2014; Szczepanowski et al., 2009; Tamminen et al., 2011). Quantification limits (LOQ) were established as the minimum amount of plasmid that could be detected without interference from the negative control. LOQ values were 100 copies/μL for *Intl1, Suil1, qnrS1,* $bla_{CTX\text{-}M\text{-}32}$; 1000 copies/μL for *tetM* and bla_{TEM}. The quality criteria within the standard curve was a $R^2 > 0.99$, and a slope between −3.1 and −3.4. The accepted efficiency of the reactions ranged from 97% to 100%. Melting curves were obtained to confirm amplification specificity. Dynamo ColorFlash SYBR Green (Thermo Scientific, Inc.) was used for *tetM* quantification; all the other genes were quantified with LightCycler 480 SYBR Green I Master (A F. Hoffmann–La Roche AG, Inc). Amplification protocol was adapted following manufacturers guidelines and an annealing temperature of 60 °C was used.

Copy numbers per gene were calculated by extrapolation from the standard curves, and expressed in relation to the number of individuals (*Daphnia* samples) or per mL (filtered water samples). ARG prevalence was calculated relative to the total amount of 16S rRNA gene.

2.8. Statistical analyses

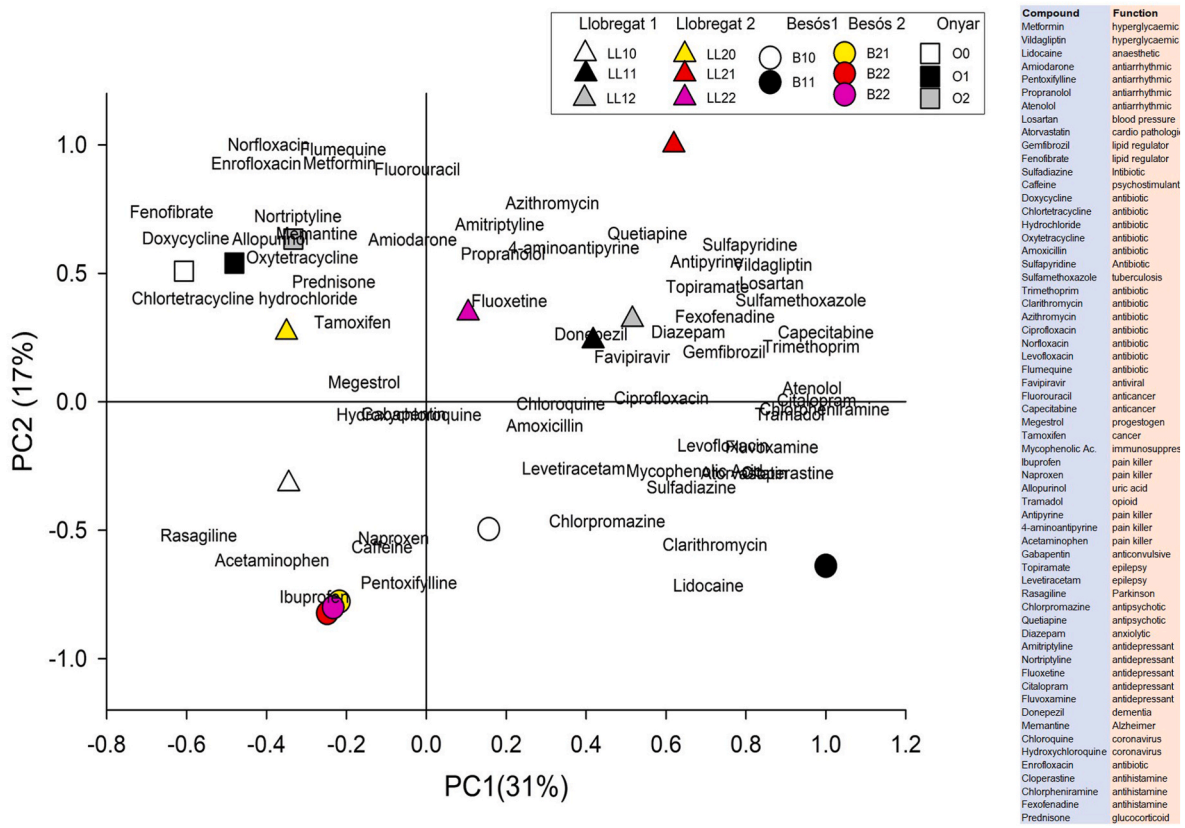
This study aimed to asses sublethal effects of contaminant residues present in the studied water samples in pre-exposed *D. magna* individuals, as well as to determine whether or not *D. magna* individuals were able to accumulate/retain ARGs from water in their gut microbiomes. One-way ANOVA was used to compare value distribution for the different parameters (behaviour, heart beats and ARG prevalence) among sites, sub-sites, and control-exposed animals. Post-hoc Dunnet's tests were performed to compare exposure treatments to solvent controls. Prior to analyses data was tested to meet ANOVA assumptions of normality and variance homoscedasticity. If required, percentage responses were arc-sin transformed and the rest of variables log transformed. Significance levels were set at $p < 0.05$. Tests were performed with the IBM- SPSS Statistics software version 27.

Associations between physico-chemical variables, including contaminant residue levels, and the measured biological responses were studied by Pearson correlation and Principal Component analyses.

3. Results

3.1. Physicochemical water variables

Except for one water sample from the Besós River (B21), all water samples were saturated with oxygen (Tables S2 and SM). Conductivity was relatively high across the studied water samples, varying from almost 2500 μS/cm in the Llobregat reclaimed effluent to near 1100 μS/cm in the Onyar WWTP effluent discharge. pH varied little across samples (7.5–8.2; Tables S2 and SM). Suspended solids were moderate in Llobregat upstream samples (19–26 mg/L), high in one sample from Besós WWTP effluent discharge (51 mg/L) and relatively low in the rest of samples (1.7–9 mg/L). Fifty out of the 80 pharmaceutical residues studied were found in 70% of the water samples (Table S3). Maximal residue levels were found for several antibiotics, NSAIDs, psychostimulants/psychiatric drugs, anticancer, blood pressure, lipid regulator and glucocorticoid drugs (>1 μg/L) (Tables S3 and SM). To summarize the distribution of measured chemical residue levels across water samples, a Principal Component Analysis (PCA) was performed on standardized chemical residue levels across water samples. Fig. 2 shows a bi-



Compound	Function
Metformin	hyperglycaemic
Vildagliptin	hyperglycaemic
Lidocaine	anaesthetic
Amiodarone	antiarrhythmic
Pentoxifylline	antiarrhythmic
Propranolol	antiarrhythmic
Atenolol	antiarrhythmic
Losartan	blood pressure
Atorvastatin	cardio pathologi
Gemfibrozil	lipid regulator
Fenofibrate	lipid regulator
Sulfadiazine	Intibiotic
Caffeine	psychostimulant
Doxycycline	antibiotic
Chlortetracycline	antibiotic
Hydrochloride	antibiotic
Oxytetracycline	antibiotic
Amoxicillin	antibiotic
Sulfapyridine	Antibiotic
Sulfamethoxazole	tuberculosis
Trimethoprim	antibiotic
Clarithromycin	antibiotic
Azithromycin	antibiotic
Ciprofloxacin	antibiotic
Norfloxacin	antibiotic
Levofloxacin	antibiotic
Flumequine	antibiotic
Favipiravir	antiviral
Fluorouracil	anticancer
Capecitabine	anticancer
Megestrol	progestogen
Tamoxifen	cancer
Mycophenolic Ac.	immunosuppres
Ibuprofen	pain killer
Naproxen	pain killer
Allopurinol	uric acid
Tramadol	opioid
Antipyrine	pain killer
4-aminoantipyrine	pain killer
Acetaminophen	pain killer
Gabapentin	anticonvulsive
Topiramate	epilepsy
Levetiracetam	epilepsy
Rasagiline	Parkinson
Chlorpromazine	antipsychotic
Quetiapine	antipsychotic
Diazepam	anxiolytic
Amitriptyline	antidepressant
Nortriptyline	antidepressant
Fluoxetine	antidepressant
Citalopram	antidepressant
Fluvoxamine	antidepressant
Donepezil	dementia
Memantine	Alzheimer
Chloroquine	coronavirus
Hydroxychloroquine	coronavirus
Enrofloxacin	antibiotic
Cloperastine	antihistamine
Chlorpheniramine	antihistamine
Fexofenadine	antihistamine
Prednisone	glucocorticoid

Fig. 2. Bi-plot of the first two Principal Components of the measured drug residues across the studied water samples. Score symbols are explained in the legend being L, B and O Llobregat, Besós and Onyar, respectively; the first number following L or B indicates first or second sampling campaign and the second one identify upstream (0), effluent (1) or downstream (2). The therapeutic role (function) of the detected drugs is also included.

plot of the first two PC components, explaining almost 50% of the variance. PCA scores defined loose, but mutually separated clusters of water samples from Llobregat, Besós and Onyar, which were associated to distinct profiles of pharmaceutical residues (Fig. 2). Llobregat water sample scores were associated with antiarrhythmic and brood pressure drug loadings, whereas Onyar samples with lipid regulators and immunosuppressants (Fig. 2). Besós water sample scores from the first sampling date were related with antibiotics, immunosuppressants, antihistaminic and psychiatric drug residue loadings, whereas samples from the second sampling associated with pain killers (Fig. 2).

3.2. Biological effects

Four out of the five studied ARGs plus the integron *int1* were found in the studied waters of Llobregat and Besós (Fig. 3A). Seven out of 10 comparisons depicted in Fig. 3A show an increase in ARG loads at the discharge point, demonstrating the impact of WWTP effluent in the ARG content of both rivers (dark grey bars in Fig. 3A), whereas the remaining three comparisons did not show significant differences among the subsites (Fig. 3A). Downstream samples (light grey bars in the figure) showed a heterogeneous behaviour, from values even higher than those of the corresponding discharge points to values identical to upstream levels (Fig. 3A).

Daphnia magna individuals cultured in lab water and those exposed to the studied river waters showed quantificable levels of *sul*1 and bla-TEM. Additionally individuals exposed to Besós river waters had also qnrS1 (Fig. 3B). Absolute values together with the abundance of 16S rDNA are also depicted in Fig. S1 (SM) and their stats in Table S6 (SM).

The seven measured behavioural, feeding and heartbeat responses were significantly affected by at least one water sample (Table S5, Fig. 4). Heartbeat was the most affected response (in 57.1 % of the 14 water samples) and feeding the least affected (7.1% of cases). Water samples from Besós affected *D. magna* behavioural, feeding and/or cardiac responses in 40 % of occasions, followed by Onyar (33.3%) and Llobregat (23.8 %). Significant effects of the studied waters were inhibitory for basal activity, habituation and feeding responses, whereas for the remaining response in most cases were stimulatory.

3.3. Associations between environmental factors and biological responses

The prevalence of bla$_{TEM}$ in the studied water samples was positively correlated with the residue levels of five antibiotics, several of them exclusively used in veterinary (enrofloxacin, ciprofloxacin, flumequine) (Fig. 5). The prevalence of the ARG *qnr*S1 and of the integron *int1* were negatively related with the quinolone antibiotic ciprofloxacin and amoxicillin, respectively. Other than antibiotics, 13 pharmaceutical residues belonging to different therapeutical classes showed positive or negative correlations with the prevalence of bla$_{TEM}$, *qnrS1, int1, Sul*1 and *tetM*.

Twenty-seven contaminant residues and three physicochemical water variables were significantly correlated with the studied *D. magna* behavioural, heartbeat and feeding responses (Fig. 5). Heartbeat rates were positively correlated with water conductivity and oxygen levels, whereas feeding rates were inversely related with suspended solids. Basal activity was also positively related with pH. To account for potential effects of water conductivity we studied how heartbeats responded to increasing NaCl levels added to ASTM water. Fig. S2 shows that heartbeats increased with water conductivity in a quadratic manner and that only the water sample from the WWTP effluent discharge of Besós from the second sampling period was situated outside the 95 % confidence limits. Phototaxis followed by heartbeats, basal activity, max, habituation and feeding responses were significantly related with

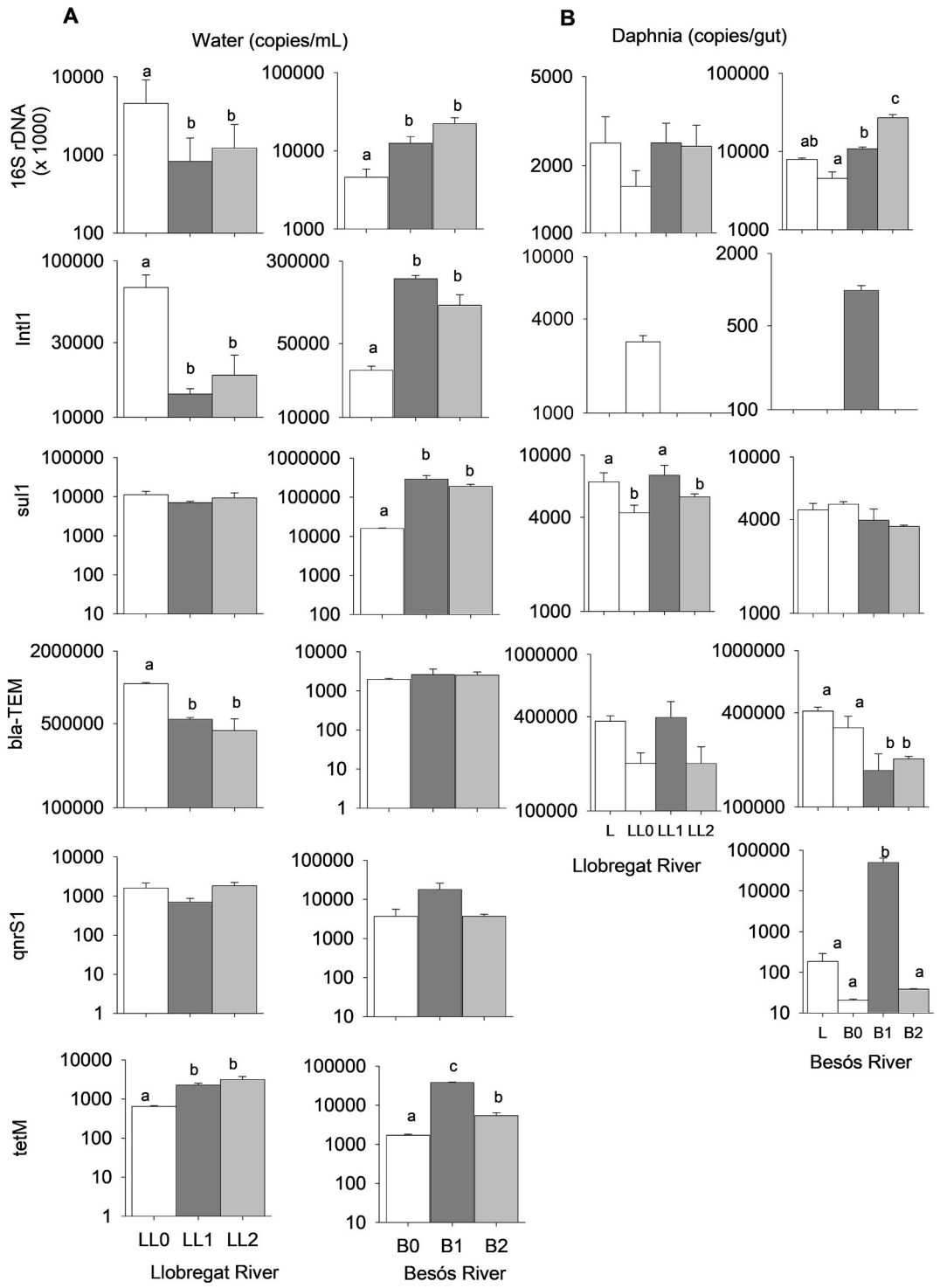


Fig. 3. Prevalence of antibiotic resistant genes (ARGs) and of integron *int1* (Mean, SE, N = 2–6) across the studied water samples of Llobregat and Besós (A) and in the guts of exposed *Daphnia magna* juveniles (B). Different letters indicated significant differences among samples following ANOVA and Tukey's post-hoc tests. L, LL and B are laboratory, Llobregat and Besós waters, respectively and numbers 0, 1 and 2 means upstream, effluent discharge and downstream, respectively.

11, 7, 4, 4, 1 and 1 drug residues, respectively. Interestingly phototaxis, heartbeats and basal activity were related significantly with 6 neuroactive chemicals such as β-blockers, anticholinergic, antidepressants, anxiolytics, mono amino oxidase (MAO) inhibitors and anticonvulsants. Five antibiotic residues were associated with basal, max activities and phototaxis; the antiviral flavipiravir and the progestogen megestrol were positively related with heartbeats and two anticancer drugs (DNA synthesis inhibitors) correlated negatively with phototaxis and heartbeats. Additionally, two NSAIDs were positively related with phototaxis and the uric acid regulator allopurinol was positively related with max and basal activities.

4. Discussion

This study aimed to show that the aquatic ecotoxicological model species *D. magna* could be used to identify hazardous pharmaceutical

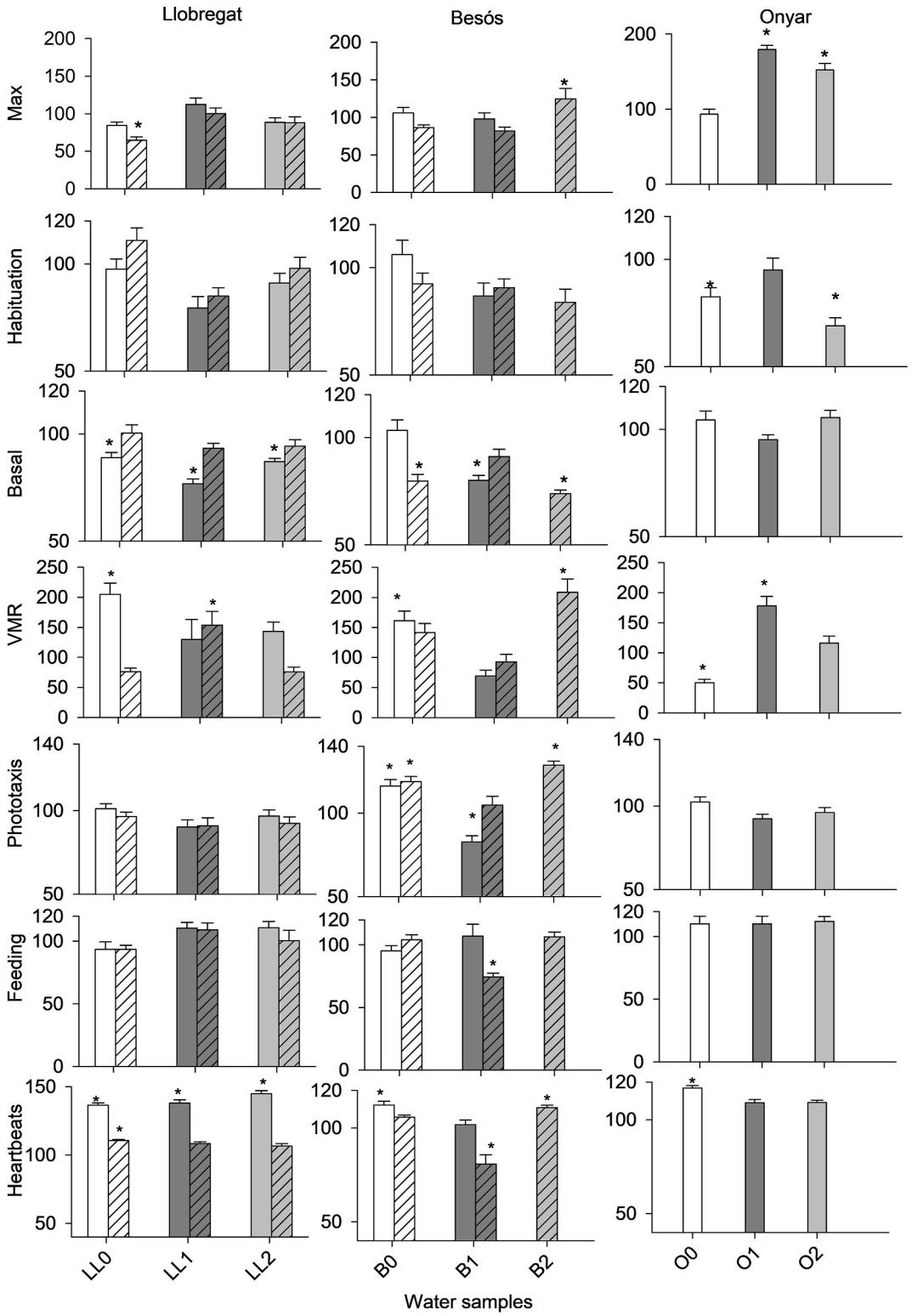


Fig. 4. Behavioural, feeding and heartbeat responses of *D. magna* juveniles relative to lab control treatments (Mean ± SE, N = 10–14) following exposures to the water samples. Grated bars indicate a second sampling, * indicates significant ($P < 0.05$) differences of samples from laboratory controls following ANOVA and Dunnett's post-hoc tests. Responses include maximal distance moved per second (Max) and its habituation upon 30 consecutive light stimuli; the mean activity in darkness (Basal) and during light (Visual Motor Responses, VMR); negative phototaxis, % of algae consumed (feeding) and heartbeats Water sampling codes are described in Fig. 1.

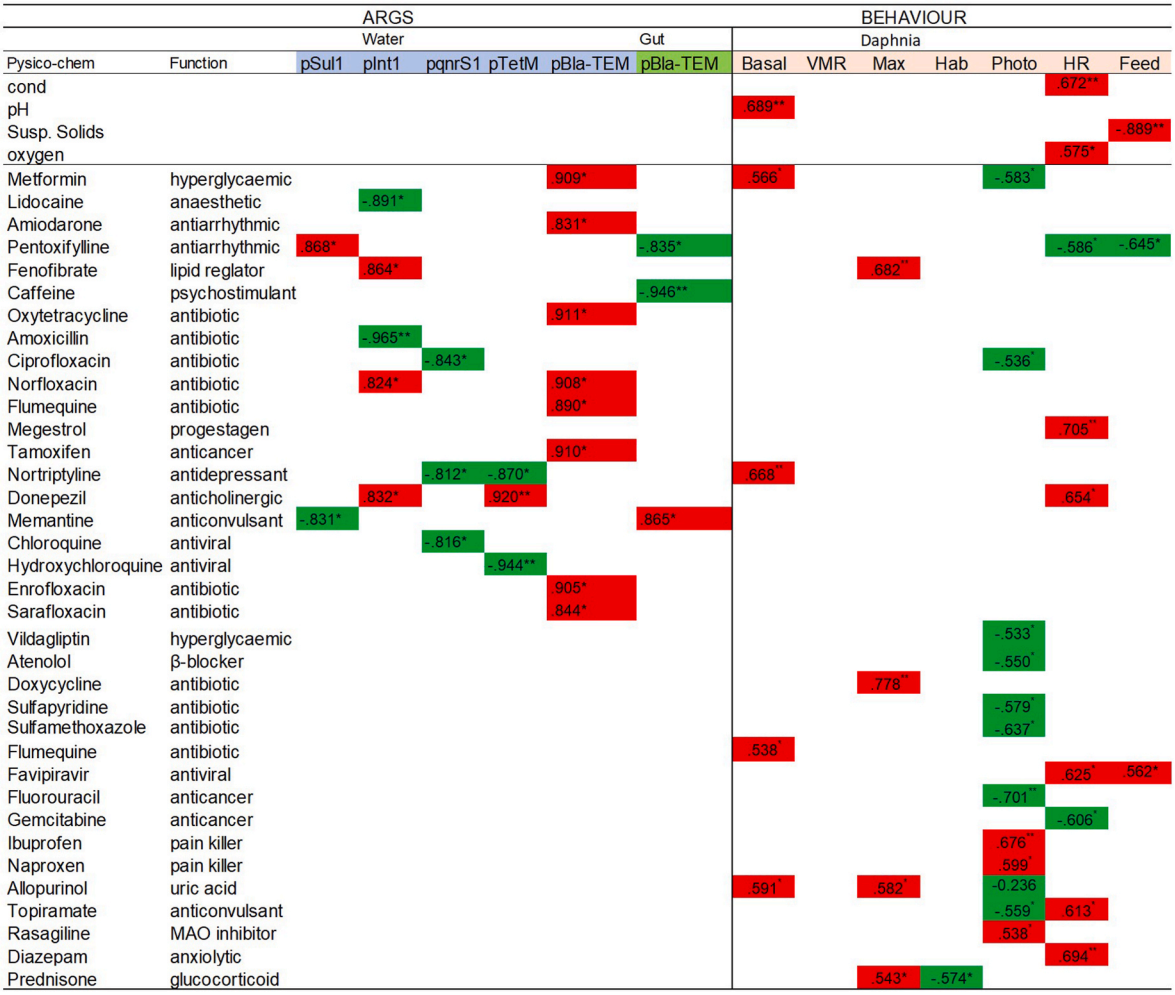


Fig. 5. Heatmap of correlations (Pearson) between biological variables (ARGs in water and in guts, behavioural, feeding and heartbeats responses in *Daphnia magna*) and physico-chemical water parameters. Only significant correlations are depicted. Positive and negative values are depicted in red and green, respectively. Drug functions are also depicted.

present in real water samples. One of the objectives of this work was to test whether the *D. magna* gut microbiome ARGs content could also reflect that of the exposed water samples. Results indicated that the prevalence of the studied ARGs was higher in river water receiving WWTP discharges in 5 out of 8 comparisons, whereas in *D. magna* microbiome the same pattern was only observed in 1 out of 5 comparisons. This means that the ARG content of the *D. magna* gut microbiome is a poor representative of the studied river waters. This is consistent with previous findings that indicated the gut microbiomes in general and, in particular, for *D. magna* are quite stable and depend to a greater extent on the host rather than on their surrounding environment (Akbar et al., 2022; Tang et al., 2010). Interestingly, the gut microbiome of animals maintained in lab conditions contained *Sul1*, Bla_{TEM} and also residual levels of qnr*S*1. This may be related to the presence of certain ARGs and mobile gene elements that promote the transfer ARGs across bacteria in the semi-axenic culture lab media of *D. magna* (Carrillo et al., 2023). The observed greater levels of most studied ARGs at WWTP discharge points compared to those obtained from upstream sampling points support the argument that WWTP effluents are the principal sources of ARGs (Nnadozie and Odume, 2019; Taylor et al., 2011).

The second objective of this study was to identify neurological hazardous pharmaceutical present in real water samples using behavioural *D. magna* responses. Water samples from Besós affected *D. magna* behavioural, feeding and/or cardiac responses in 40 % of cases, followed by Onyar (33.3%) and Llobregat (23.8 %). These results indicated that WWTP effluents from Besós were the most hazardous ones, whereas the Onyar effluents showed lower toxic activity, which may reflect the relatively smaller urban population and industrial users served by the Onyar WWTP. The relatively good results from the Llobregat WWTP, which serves a large urban and industrial area, may be related the application of a quaternary treatment to the effluent before its release into the river.

The heartbeat was the physiological parameter affected by more samples (eight), followed in decreasing order by visual motor responses (six), phototaxis and maximal locomotion activity (four), habituation (two) and feeding (one). Heartbeats are affected by salinity (Figs. S2 and SM), which varied considerably across the studied water samples. The lower part of Llobregat and Besós basin are affected by salinization due to the active exploitation of salt mines upstream (Damásio et al., 2007, 2011; Prat et al., 2002, 2013; Prat and Munné, 2000), which means that heartbeat's results should be interpreted with caution due to the confounding effect of salinity. Heartbeats also showed a negative relationship with oxygen levels, which can be related with the reduced *D. magna* heartbeats observed in animals exposed to a single sample from the Besós effluent (B21), that had low oxygen levels. Feeding was negatively related to high loads of suspended solids, which were quite high in some locations. There is ample information that contaminants bound to suspended solids may affect *D. magna* feeding (Barata et al., 2008).

Inhibition of feeding rates is closely linked with fitness as resource acquisition determines survival, growth and reproduction in *D. magna*

and in most animals (Barata et al., 2008). Furthermore, previous field studies have established a link between *D. magna* post exposure feeding inhibition and the ecological quality stage of macroinvertebrate communities along the Llobregat and Besos rivers (Damasio et al., 2008). This link has been associated to the fact that many macroinvertebrate species are grazers or filter feeders and hence share a *D. magna* similar foraging behaviour (Damasio et al., 2008). This means that the observed low occurrence of feeding inhibition responses across the tested water samples may indicate low ecological detrimental effects of treated effluent discharges. Heartbeats in *Daphnia* facilitate the correct distribution of oxygen along tissues (Pirow et al., 2001), therefore a decrease in metabolic rate is predicted in those animals exposed sub-chronically based on the decrease in heart rate and thus oxygen delivery (Dzialowski et al., 2006). Alternatively, *D. magna* exposed to hypoxia conditions, which often occurs when it migrates to deeper waters during daylight to avoid fish predation, increases heartbeats to maintain respiration rates (Pirow et al., 2001). In both cases observed increasing and decreasing heartbeats across the studied water samples are likely to be metabolically costly and hence detrimental to fitness. *Daphnia magna* scape responses to light stimuli such as its maximal locomotion activity and its visual responses to longer exposures will determine its chances to escape to the attack of visual predators such as fish (Bedrossiantz et al., 2020; Bellot et al., 2021). Having a negative phototaxis is also advantageous to visual predators since will diminish light exposure and hence the chances to be detected by them (Bedrossiantz et al., 2021). Finally, individuals more prone to habituation could also experience mortality costs by wrongly habituating to a dangerous predator (Rodríguez-Prieto et al., 2010). This means that the observed changes in behavioral traits may be detrimental for *D. magna* in the field.

Fifty out of the 80 pharmaceutical residues studied were found in 70% of the water samples. Maximal residue levels were found for several antibiotics, pain killers, psychostimulants/psychiatric drugs, anticancer, blood pressure, lipid regulator and glucocorticoid drugs. River water samples affected by WWTP or reclaimed effluent discharges in Besós and Onyar/Llobregat rivers, respectively, contained between 9-10 and 2–4 times greater residual contaminant levels than upstream and downstream locations. From the 36 contaminant residues significantly related with the studied responses, 17 were bactericides. ARGs prevalence in water was associated with 9 of those bactericides (oxytetracycline, amoxicillin, ciprofloxacin, norfloxacin, flumequine, tamoxifen, chloroquine, hydroxychloroquine, enrofloxacin). There is reported information that tetracyclines, sulphonamides and quinolones, such as those found in the present study, are related with the prevalence of the ARGs *qnrS1, ul1*, bla_{TEM}, TetM (Xu et al., 2015; Zheng et al., 2022). The class 1 mobile element *int1* was also abundant in WWTP effluent discharges and it is related with ARG horizontal transfer (Chen et al., 2015).

Seven out of 10 compounds correlated with behavioural responses were neuroactive chemicals (topiramate, rasagiline, diazepam, donepezil, atenolol, nortriptyline, memantine). Memantine and diazepam, are known to modulate the studied behavioural responses in *D. magna* (Bedrossiantz et al., 2020). The antidepressant fluoxetine that like nortriptyline increases serotonin brain levels, also affects the studied *D. magna* behaviour traits (Bedrossiantz et al., 2020). There is also reported information that β-blockers such as atenolol modulate cardiac and other behavioural responses in *Daphnia* (Jeong et al., 2018). The remaining neuromodulators (topiramate, rasagiline donepezil) disrupt behavioural responses in zebrafish that are equivalent to those measured in this study for *Daphnia* (Berghmans et al., 2007; Best et al., 2008, 2008b; Vaz et al., 2020). Thus, there is reported experimental evidence indicating that the seven prioritized neuroactive chemicals may impair cognitive and cardiac responses in *D. magna*. There are, however, other chemical contaminants including their transformation products not analyzed in the present study that may have also contributed to the observed effects. Future investigations using drug exposures need to be performed to confirm those associations.

5. Conclusion

In summary, the reported results showed that the *D. magna* behaviour was sensitive enough to be altered by field collected river water samples, whereas the ARG gut microbiome composition was only marginally affected by them. The correlation study evidenced associations between mixtures of neuroactive compounds in river water with behaviour responses and between antibiotics and ARGs in water. The above-mentioned correlations, however, need to be further tested in the lab to correctly establish a causal-effect relationship. Furthermore, the studied waters are likely to contain mixtures of hundreds of different contaminants and/or their transformation products, which may have also contributed to the observed effects. The use of non-target chemical analyses and probably of sample fractionation procedures combined with toxicity bioassays such as those proposed in this study may help to elucidate more robust associations in the future (Altenburger et al., 2019). Examples of the above mentioned approaches, termed effect-direct analyses, that also used D. magna bioassays, include the identification of toxic compounds such as nonylphenol in stormwater (Thomas et al., 2001), pesticides in water (Fang et al., 2017) or cyanotoxins in suspended solids (Rivetti et al., 2015).

This study was focused on only one crustacean species, which may not represent the species biodiversity of the riparian communities composed of many invertebrate and vertebrate species where insect larvae, molluscs, annelids and fish are an important part of them. This means that the inclusion of additional species more representative of insects, molluscs and fish, for example, will improve the study conclusions. Nevertheless many of the molecular drug targets and the neurological signalling pathways that control the studied behavioural and cardiovascular responses in *D. magna* are phylogenetically conserved across arthropods, fish and even humans (Bedrossiantz et al., 2020, 2023; Bellot et al., 2024; Gunnarsson et al., 2008; Rivetti et al., 2023; Spanier et al., 2022). Indeed comparative -omics data from cellular and developmental studies across genomic model species suggest a high degree of conservation in regulatory pathways in the fly *Drosophila*, the worm *Caenorhabditis elegans* and humans (Hodges et al., 2018). This means that the identified neurological hazardous compounds may also threat local macrobenthonic invertebrates many of them insect larvae, as well as fish species.

CRediT authorship contribution statement

Hugo Moro: Writing – original draft, Methodology, Investigation. **Raquel Vaya:** Methodology, Investigation. **Marta Casado:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Benjamín Piña:** Writing – review & editing, Formal analysis, Data curation. **Pol Domínguez-García:** Methodology, Investigation. **Cristian Gómez-Canela:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Carlos Barata:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143612.

Data availability

Data will be made available on request.

References

Abdullahi, M., Li, X., Abdallah, M.A.-E., Stubbings, W., Yan, N., Barnard, M., Guo, L.-H., Colbourne, J.K., Orsini, L., 2022. Daphnia as a sentinel species for environmental health protection: a perspective on biomonitoring and bioremediation of chemical pollution. Environ. Sci. Technol. 56, 14237–14248. https://doi.org/10.1021/acs.est.2c01799.

Akbar, S., Gu, L., Sun, Y., Zhang, L., Lyu, K., Huang, Y., Yang, Z., 2022. Understanding host-microbiome-environment interactions: insights from Daphnia as a model organism. Sci. Total Environ. 808. https://doi.org/10.1016/j.scitotenv.2021.152093.

Altenburger, R., Brack, W., Burgess, R.M., Busch, W., Escher, B.I., Focks, A., Mark Hewitt, L., Jacobsen, B.N., de Alda, M.L., Ait-Aissa, S., Zonja, B., Krauss, M., 2019. Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. Environ. Sci. Eur. 31. https://doi.org/10.1186/s12302-019-0193-1.

Barata, C., Baird, D.J., 1998. Phenotypic plasticity and constancy of life-history traits in laboratory clones of Daphnia magna straus: effects of neonatal length. Funct. Ecol. 12, 442–452. https://doi.org/10.1046/j.1365-2435.1998.00201.x.

Barata, C., Damasio, J., López, M.A., Kuster, M., De Alda, M.L., Barceló, D., Riva, M.C., Raldúa, D., 2007. Combined use of biomarkers and in situ bioassays in Daphnia magna to monitor environmental hazards of pesticides in the field. Environ. Toxicol. Chem. 26 (1). https://doi.org/10.1897/06-209R.

Barata, C., Alañon, P., Gutierrez-Alonso, S., Riva, M.C., Fernández, C., Tarazona, J.V., 2008. A Daphnia magna feeding bioassay as a cost effective and ecological relevant sublethal toxicity test for Environmental Risk Assessment of toxic effluents. Sci. Total Environ. 405, 78–86. https://doi.org/10.1016/j.scitotenv.2008.06.028.

Bedrossiantz, J., Martínez-Jerónimo, F., Bellot, M., Raldua, D., Gómez-Canela, C., Barata, C., 2020. A high-throughput assay for screening environmental pollutants and drugs impairing predator avoidance in Daphnia magna. Sci. Total Environ. 740. https://doi.org/10.1016/j.scitotenv.2020.140045.

Bedrossiantz, J., Fuertes, I., Raldua, D., Barata, C., 2021. Pharmacological modulation of fish-induced depth selection in D. magna: the role of cholinergic and GABAergic signalling. Sci. Rep. 11. https://doi.org/10.1038/s41598-021-98886-w.

Bedrossiantz, J., Faria, M., Prats, E., Barata, C., Cachot, J., Raldúa, D., 2023. Heart rate and behavioral responses in three phylogenetically distant aquatic model organisms exposed to environmental concentrations of carbaryl and fenitrothion. Sci. Total Environ. 865. https://doi.org/10.1016/j.scitotenv.2022.161268.

Bellot, M., Faria, M., Gómez-Canela, C., Raldúa, D., Barata, C., 2021. Pharmacological modulation of behaviour, serotonin and dopamine levels in daphnia magna exposed to the monoamine oxidase inhibitor deprenyl. Toxics 9. https://doi.org/10.3390/toxics9080187.

Bellot, M., Gómez-Canela, C., Barata, C., 2022. Phototactic behaviour and neurotransmitter profiles in two Daphnia magna clones: vertical and horizontal responses to fish kairomones and psychotropic drugs. Sci. Total Environ. 830. https://doi.org/10.1016/j.scitotenv.2022.154684.

Bellot, M., Soria, F., López-Arnau, R., Gómez-Canela, C., Barata, C., 2024. Daphnia magna an emerging environmental model of neuro and cardiotoxicity of illicit drugs. Environ. Pollut. 344 (1), 123355. https://doi.org/10.1016/j.envpol.2024.123355.

Berghmans, S., Hunt, J., Roach, A., Goldsmith, P., 2007. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res. 75, 18–28. https://doi.org/10.1016/j.eplepsyres.2007.03.015.

Best, J.D., Berghmans, S., Hunt, J.J.F.G., Clarke, S.C., Fleming, A., Goldsmith, P., Roach, A.G., 2008. Non-associative learning in larval zebrafish. Neuropsychopharmacology 33, 1206–1215. https://doi.org/10.1038/sj.npp.1301489.

Carrillo, M.P., Sevilla, M., Casado, M., Piña, B., Pastor López, E., Matamoros, V., Vila-Costa, M., Barata, C., 2023. Impact of the antibiotic doxycycline on the D. magna reproduction, associated microbiome and antibiotic resistance genes in treated wastewater conditions. Environmental Pollution 334. https://doi.org/10.1016/j.envpol.2023.122188.

Cerro-Gálvez, E., Roscales, J.L., Jiménez, B., Sala, M.M., Dachs, J., Vila-Costa, M., 2020. Microbial responses to perfluoroalkyl substances and perfluorooctanesulfonate (PFOS) desulfurization in the Antarctic marine environment. Water Res 171, 115434. https://doi.org/10.1016/j.watres.2019.115434.

Chen, B., Liang, X., Nie, X., Huang, X., Zou, S., Li, X., 2015. The role of class I integrons in the dissemination of sulfonamide resistance genes in the Pearl River and Pearl River Estuary, South China. J. Hazard Mater. 282, 61–67. https://doi.org/10.1016/j.jhazmat.2014.06.010.

Córdoba-Ariza, G., Batalla, R.J., Sabater, S., Mas-Pla, J., 2024. Disclosing the effects of climate, land use, and water demand as drivers of hydrological trends in a Mediterranean river basin. Limnética 43, 333–352. https://doi.org/10.23818/limn.43.22.

Damásio, J.B., Barata, C., Munné, A., Ginebreda, A., Guasch, H., Sabater, S., Caixach, J., Porte, C., 2007. Comparing the response of biochemical indicators (biomarkers) and biological indices to diagnose the ecological impact of an oil spillage in a Mediterranean river (NE Catalunya, Spain). Chemosphere 66, 1206–1216. https://doi.org/10.1016/j.chemosphere.2006.07.055.

Damasio, J., Tauler, R., Teixido, E., Rieradevall, M., Prat, N., Riva, M.C.C., Soares, A.M.V.M., Barata, C., 2008. Combined use of Daphnia magna in situ bioassays, biomarkers and biological indices to diagnose and identify environmental pressures on invertebrate communities in two Mediterranean urbanized and industrialized rivers (NE Spain). Aquatic toxicology 87, 310–320. https://doi.org/10.1016/j.aquatox.2008.02.016.

Damásio, J., Fernández-Sanjuan, M., Sánchez-Avila, J., Lacorte, S., Prat, N., Rieradevall, M., Soares, A.M.V.M., Barata, C., 2011. Multi-biochemical responses of benthic macroinvertebrate species as a complementary tool to diagnose the cause of community impairment in polluted rivers. Water Res. 45, 3599–3613. https://doi.org/10.1016/j.watres.2011.04.006.

David, A., Lange, A., Tyler, C.R., Hill, E.M., 2018. Concentrating mixtures of neuroactive pharmaceuticals and altered neurotransmitter levels in the brain of fish exposed to a wastewater effluent. Sci. Total Environ. 621, 782–790. https://doi.org/10.1016/j.scitotenv.2017.11.265.

Domínguez-García, P., Aljabasini, O., Barata, C., Gómez-Canela, C., 2024a. Environmental Risk Assessment of pharmaceuticals in wastewaters and reclaimed water from Catalan main river basins. Sci. Total Environ. 949 (1), 175020. https://doi.org/10.1016/j.scitotenv.2024.175020.

Domínguez-García, P., Fernández-Ruano, L., Báguena, J., Cuadros, J., Gómez-Canela, C., 2024b. Assessing the pharmaceutical residues as hotspots of the main rivers of Catalonia, Spain. Environ. Sci. Pollut. Control Ser. 31, 44080–44095. https://doi.org/10.1007/s11356-024-33967-7.

Drakvik, E., Altenburger, R., Aoki, Y., Backhaus, T., Bahadori, T., Barouki, R., Brack, W., Cronin, M.T.D., Demeneix, B., Hougaard Bennekou, S., Öberg, M., Bergman, 2020. Statement on advancing the assessment of chemical mixtures and their risks for human health and the environment. Environ. Int. 134. https://doi.org/10.1016/j.envint.2019.105267.

Dzialowski, E.M., Turner, P.K., Brooks, B.W., 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists in Daphnia magna. Arch. Environ. Contam. Toxicol. 50, 503–510. https://doi.org/10.1007/s00244-005-0121-9.

Eaton, A.D., Clesceri, L.S., Greenberg, A.E., 1995. Standard Methods for the Examination of Water and Wastewater, 19th Edition. American Public Health Association, Washington DC.

Fang, B., Guo, J., Li, F., Giesy, J.P., Wang, L., Shi, W., 2017. Bioassay directed identification of toxicants in sludge and related reused materials from industrial wastewater treatment plants in the Yangtze River Delta. Chemosphere 168, 191–198. https://doi.org/10.1016/j.chemosphere.2016.10.046.

Faria, M., Bellot, M., Bedrossiantz, J., Ramírez, J.R.R., Prats, E., Garcia-Reyero, N., Gomez-Canela, C., Mestres, J., Rovira, X., Barata, C., Llebaria, A., Raldua, D., 2022. Environmental levels of carbaryl impair zebrafish larvae behaviour: the potential role of ADRA2B and HTR2B. J. Hazard Mater. 431. https://doi.org/10.1016/j.jhazmat.2022.128563.

García, J., García-Galán, M.J., Day, J.W., Boopathy, R., White, J.R., Wallace, S., Hunter, R.G., 2020. A review of emerging organic contaminants (EOCs), antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in the environment: increasing removal with wetlands and reducing environmental impacts. Bioresour. Technol. 307. https://doi.org/10.1016/j.biortech.2020.123228.

Gat, D., Mazar, Y., Cytryn, E., Rudich, Y., 2017. Origin-Dependent variations in the atmospheric microbiome community in eastern mediterranean dust storms. Environ. Sci. Technol. 51, 6709–6718. https://doi.org/10.1021/acs.est.7b00362.

Gómez-Canela, C., Edo, S.S., Rodríguez, N., Gotor, G., Lacorte, S.S., Gomez-Canela, C., Edo, S.S., Rodriguez, N., Gotor, G., Lacorte, S.S., 2021. Comprehensive characterization of 76 pharmaceuticals and metabolites in wastewater by LC-MS/MS. Chemosensors 9, 1–19. https://doi.org/10.3390/chemosensors9100273.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42, 5807–5813. https://doi.org/10.1021/es8005173.

Hodges, G., Gutsell, S., Taylor, N., Brockmeier, E., Butler, E., Rendal, C., Colbourne, J., 2018. Invertebrate model species in AOP development. A Systems Biology Approach to Advancing Adverse Outcome Pathways for Risk Assessment. https://doi.org/10.1007/978-3-319-66084-4_5.

Jeong, T.Y., Yoon, D., Kim, S., Kim, H.Y., Kim, S.D., 2018. Mode of action characterization for adverse effect of propranolol in Daphnia magna based on behavior and physiology monitoring and metabolite profiling. Environmental Pollution 233, 99–108. https://doi.org/10.1016/j.envpol.2017.10.043.

Khasawneh, O.F.S., Palaniandy, P., 2021. Occurrence and removal of pharmaceuticals in wastewater treatment plants. Process Saf. Environ. Protect. 150, 532–556. https://doi.org/10.1016/j.psep.2021.04.045.

König, M., Escher, B.I., Neale, P.A., Krauss, M., Hilscherová, K., Novák, J., Teodorović, I., Schulze, T., Seidensticker, S., Kamal Hashmi, M.A., Ahlheim, J., Brack, W., 2017. Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environmental Pollution 220, 1220–1230. https://doi.org/10.1016/j.envpol.2016.11.011.

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., Kisand, V., 2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. PLoS One 9. https://doi.org/10.1371/journal.pone.0103705.

Leiva, A.M., Piña, B., Vidal, G., 2021. Antibiotic resistance dissemination in wastewater treatment plants: a challenge for the reuse of treated wastewater in agriculture. Rev. Environ. Sci. Biotechnol. 20, 1043–1072. https://doi.org/10.1007/s11157-021-09588-8.

Leuthold, D., Klüver, N., Altenburger, R., Busch, W., 2019. Can environmentally relevant neuroactive chemicals specifically Be detected with the locomotor response test in zebrafish embryos? Environ. Sci. Technol. 53, 482–493. https://doi.org/10.1021/acs.est.8b04327.

Munné, A., Solà, C., Ejarque, E., Sanchís, J., Serra, P., Corbella, I., Aceves, M., Galofré, B., Boleda, M.R., Paraira, M., Paraira, M., Molist, J., 2023. Indirect potable water reuse to face drought events in Barcelona city. Setting a monitoring procedure to protect aquatic ecosystems and to ensure a safe drinking water supply. Sci. Total Environ. 866. https://doi.org/10.1016/j.scitotenv.2022.161339.

Múrria, C., Maceda-Veiga, A., Barata, C., Gomà, J., Faria, M., Antich, A., Arnedo, M.A., Bonada, N., Prat, N., 2024. From biomarkers to community composition: negative effects of UV/chlorine-treated reclaimed urban wastewater on freshwater biota. Sci. Total Environ. 912. https://doi.org/10.1016/j.scitotenv.2023.169561.

Nerin, C., Alfaro, P., Aznar, M., Domeño, C., 2013. The challenge of identifying non-intentionally added substances from food packaging materials: a review. Anal. Chim. Acta 775, 14–24. https://doi.org/10.1016/j.aca.2013.02.028.

Neuwoehner, J., Escher, B.I., 2011. The pH-dependent toxicity of basic pharmaceuticals in the green algae Scenedesmus vacuolatus can be explained with a toxicokinetic ion-trapping model. Aquat. Toxicol. 101, 266–275. https://doi.org/10.1016/j.aquatox.2010.10.008.

Nnadozie, C.F., Odume, O.N., 2019. Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. Environmental Pollution 254. https://doi.org/10.1016/j.envpol.2019.113067.

Olanrewaju, T.O., McCarron, M., Dooley, J.S.G., Arnscheidt, J., 2019. Transfer of antibiotic resistance genes between Enterococcus faecalis strains in filter feeding zooplankton Daphnia magna and Daphnia pulex. Sci. Total Environ. 659, 1168–1175. https://doi.org/10.1016/j.scitotenv.2018.12.314.

Petrovic, M., Ginebreda, A., Acuña, V., Batalla, R.J., Elosegi, A., Guasch, H., de Alda, M. L., Marcé, R., Muñoz, I., Navarro-Ortega, A., Navarro, E., Vericat, D., Sabater, S., Barceló, D., 2011. Combined scenarios of chemical and ecological quality under water scarcity in Mediterranean rivers. TrAC, Trends Anal. Chem. 30, 1269–1278. https://doi.org/10.1016/j.trac.2011.04.012.

Pinheiro, J.P.S., Windsor, F.M., Wilson, R.W., Tyler, C.R., 2021. Global variation in freshwater physico-chemistry and its influence on chemical toxicity in aquatic wildlife. Biol. Rev. 96, 1528–1546. https://doi.org/10.1111/brv.12711.

Pirow, R., Bäumer, C., Paul, R.J., 2001. Benefits of haemoglobin in the cladoceran crustacean Daphnia magna. J. Exp. Biol. 204, 3425–3441.

Prat, N., Munné, A., 2000. Water use and quality and stream flow in a Mediterranean stream. Water Res. 15, 3876–3881.

Prat, N., Munné, A., Solà, C., Casanovas, R., Vilas, M., Bonada, N., Jubany, J., Miralles, M., Plans, M., Rieradevall, M., 2002. Ecological quality of Llobregat, Besòs, foix and tordera rivers. Survei 10. Deputació de Barcelona. Area de Medi Ambient (Estudis de Qualitat Ecològica dels Rius). Barcelona.

Prat, N., Rieradevall, M., Barata, C., Munné, A., 2013. The combined use of metrics of biological quality and biomarkers to detect the effects of reclaimed water on macroinvertebrate assemblages in the lower part of a polluted Mediterranean river (Llobregat River, NE Spain). Ecol Indic 24. https://doi.org/10.1016/j.ecolind.2012.06.010.

Puértolas, L., Damásio, J., Barata, C., Soares, A.M.V.M., Prat, N., 2010. Evaluation of side-effects of glyphosate mediated control of giant reed (Arundo donax) on the structure and function of a nearby Mediterranean river ecosystem. Environ. Res. 110, 556–564. https://doi.org/10.1016/j.envres.2010.05.004.

Rivetti, C., Gómez-Canela, C., Lacorte, S., Díez, S., Lázaro, W.L., Barata, C., 2015. Identification of compounds bound to suspended solids causing sub-lethal toxic effects in Daphnia magna. A field study on re-suspended particles during river floods in Ebro River. Aquat. Toxicol. 161. https://doi.org/10.1016/j.aquatox.2015.01.021.

Rivetti, C., Houghton, J., Basili, D., Hodges, G., Campos, B., 2023. Genes-to-Pathways species conservation analysis: enabling the exploration of conservation of biological pathways and processes across species. Environ. Toxicol. Chem. https://doi.org/10.1002/etc.5600.

Rodríguez-Prieto, I., Martín, J., Fernández-Juricic, E., 2010. Habituation to low-risk predators improves body condition in lizards. Behav. Ecol. Sociobiol. 64, 1937–1945. https://doi.org/10.1007/s00265-010-1004-2.

Sanz, C., Casado, M., Navarro-Martin, L., Tadić, Đ., Parera, J., Tugues, J., Bayona, J.M., Piña, B., 2021. Antibiotic and antibiotic-resistant gene loads in swine slurries and their digestates: implications for their use as fertilizers in agriculture. Environ. Res. 194. https://doi.org/10.1016/j.envres.2020.110513.

Sanz, C., Casadoi, M., Tadic, Pastor-López, E.J., Navarro-Martin, L., Parera, J., Tugues, J., Ortiz, C.A., Bayona, J.M., Piña, B., 2022. Impact of organic soil amendments in antibiotic levels, antibiotic resistance gene loads, and microbiome composition in corn fields and crops. Environ. Res. 214. https://doi.org/10.1016/j.envres.2022.113760.

Savva, K., Farré, M., Barata, C., 2023. Sublethal effects of bio-plastic microparticles and their components on the behaviour of Daphnia magna. Environ. Res. 236. https://doi.org/10.1016/j.envres.2023.116775.

Singh, R., Singh, A.P., Kumar, S., Giri, B.S., Kim, K.-H., 2019. Antibiotic resistance in major rivers in the world: a systematic review on occurrence, emergence, and management strategies. J. Clean. Prod. 234, 1484–1505. https://doi.org/10.1016/j.jclepro.2019.06.243.

Spanier, K., De Meester, L., Aerts, S., Orsini, L., 2022. Of Men and Water Fleas: Gene Regulatory Network Discovery in Human Cancer and in an Ecological Context.

Stankiewicz, K., Boroń, P., Prajsnar, J., Żelazny, M., Heliasz, M., Hunter, W., Lenart-Boroń, A., 2024. Second life of water and wastewater in the context of circular economy – do the membrane bioreactor technology and storage reservoirs make the recycled water safe for further use? Sci. Total Environ. 921. https://doi.org/10.1016/j.scitotenv.2024.170995.

Styrishave, B., Halling-Sørensen, B., Ingerslev, F., 2011. Environmental risk assessment of three selective serotonin reuptake inhibitors in the aquatic environment: a case study including a cocktail scenario. Environ. Toxicol. Chem. 30, 254–261. https://doi.org/10.1002/etc.372.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.-H., Gützkow, T., Eichler, W., Pühler, A., Schlüter, A., 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology (N. Y.) 155, 2306–2319. https://doi.org/10.1099/mic.0.028233-0.

Tamminen, M., Karkman, A., Lohmus, A., Muziasari, W.I., Takasu, H., Wada, S., Suzuki, S., Virta, M., 2011. Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. Environ. Sci. Technol. 45, 386–391. https://doi.org/10.1021/es102725n.

Tang, K.W., Turk, V., Grossart, H.-P., 2010. Linkage between crustacean zooplankton and aquatic bacteria. Aquat. Microb. Ecol. 61, 261–277. https://doi.org/10.3354/ame01424.

Taylor, N.G.H., Verner-Jeffreys, D.W., Baker-Austin, C., 2011. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol. Evol. 26, 278–284. https://doi.org/10.1016/j.tree.2011.03.004.

Thomas, K.V., Hurst, M.R., Matthiessen, P., Sheahan, D., Williams, R.J., 2001. Toxicity characterisation of organic contaminants in stormwaters from an agricultural headwater stream in South East England. Water Res. 35, 2411–2416. https://doi.org/10.1016/S0043-1354(00)00535-2.

Vaz, R.L., Sousa, S., Chapela, D., van der Linde, H.C., Willemsen, R., Correia, A.D., Outeiro, T.F., Afonso, N.D., 2020. Identification of antiparkinsonian drugs in the 6-hydroxydopamine zebrafish model. Pharmacol. Biochem. Behav. 189. https://doi.org/10.1016/j.pbb.2019.172828.

Wang, J., Chu, L., Wojnárovits, L., Takács, E., 2020. Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: an overview. Sci. Total Environ. 744. https://doi.org/10.1016/j.scitotenv.2020.140997.

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., Zhang, Y., Li, X., Meng, W., 2015. Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. Chemosphere 119, 1379–1385. https://doi.org/10.1016/j.chemosphere.2014.02.040.

Zheng, S., Wang, Y., Chen, C., Zhou, X., Liu, Y., Yang, J., Geng, Q., Chen, G., Ding, Y., Yang, F., 2022. Current progress in natural degradation and enhanced removal techniques of antibiotics in the environment: a review. Int J Environ Res Public Health 19. https://doi.org/10.3390/ijerph191710919.