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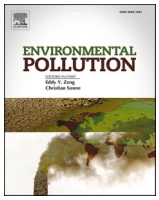
Environmental Pollution 311 (2022) 120016

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Prioritization based on risk assessment to study the bioconcentration and biotransformation of pharmaceuticals in glass eels (*Anguilla anguilla*) from the Adour estuary (Basque Country, France)☆,☆☆

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ARTICLE INFO

Keywords:
Glass eel
Contaminants of emerging concern
Bioconcentration
Biotransformation
Suspect screening
HPLC-HRMS

ABSTRACT

The presence of contaminants of emerging concern in the aquatic environment directly impacts water-living organisms and can alter their living functions. These compounds are often metabolized and excreted, but they can also be accumulated and spread through the food chain. The metabolized contaminants can also lead to the formation of new compounds with unknown toxicity and bioaccumulation potential. In this work, we have studied the occurrence, bioconcentration, and biotransformation of CECs in glass eels (*Anguilla anguilla*) using UHPLC-HRMS. To select the target CECs, we first carried out an environmental risk assessment of the WWTP effluent that releases directly into the Adour estuary (Bayonne, Basque Country, France). The risk quotients of every detected contaminant were calculated and three ecotoxicologically relevant contaminants were chosen to perform the exposure experiment: propranolol, diazepam, and irbesartan. An experiment of 14 days consisting of 7 days of exposure and 7 days of depuration was carried out to measure the bioconcentration of the chosen compounds. The quantitative results of the concentrations in glass eel showed that diazepam and irbesartan reached BCF ≈10 on day 7, but both compounds were eliminated after 7 days of depuration. On the other hand, propranolol's concentration remains constant all along with the experiment, and its presence can be detected even in the non-exposed control group, which might suggest environmental contamination. Two additional suspect screening strategies were used to identify metabolization products of the target compounds and other xenobiotics already present in wild glass eels. Only one metabolite was identified, nordiazepam, a well-known diazepam metabolite, probably due to the low metabolic rate of glass eels at this stage. The xenobiotic screening confirmed the presence of more xenobiotics in wild glass eels, prominent among them, the pharmaceuticals exemestane, primidone, iloprost, and norethandrolone.

1. Introduction

In the literature, we share the term emerging contaminants (ECs) or contaminants of emerging concern (CECs) for those compounds that are not included in any priority list of contaminants and whose effects on the environment are not yet known (Diamond and Burton Jr., 2021). This heterogeneous group includes chemicals with very different properties with two common characteristics: they are unregulated, and the scientific community cannot guarantee that they are not hazardous to the environment (Diamond and Burton Jr., 2021). Different sub-classifications can be defined according to the use (e.g., pharmaceuticals, personal-care products, industrial products …), common

☆ This paper has been recommended for acceptance by. Eddy Y. Zeng.☆☆ Contaminants of Emerging Concern in Glass Eel (Anguilla anguilla): Occurrence, Bioconcentration and Biotransformation.

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https://doi.org/10.1016/j.envpol.2022.120016
Received 29 June 2022; Received in revised form 8 August 2022; Accepted 17 August 2022
Available online 22 August 2022

physicochemical properties (e.g., persistent organic pollutants), or specific chemical families (e.g., per- and polyfluoroalkyl substances, polybrominated diphenyl ethers …). Due to the growing use of many chemicals and the wider occurrence of CECs shown in the literature, the effect of CECs on the environment and human health has become one of the most complex environmental problems of this decade (Landrigan et al., 2018). The aquatic environment is typically the destination for CECs and stands out as one of the most sensitive compartments. Some studies point out that wastewater treatment plants (WWTPs) can barely remove CECs efficiently from wastewater, making their effluents an unwanted source of aquatic contamination (Loos et al., 2013; Mijangos et al., 2018).

Undoubtedly, the presence of CECs in impacted ecosystems has a direct consequence on aquatic organisms and can alter their living functions (Duarte et al., 2020; McCallum et al., 2019; Merola et al., 2022; Vossen et al., 2020). These contaminants are often metabolized and excreted but they can also be accumulated and spread through the food chain. The occurrence of CECs has been reported in aquatic organisms that comprise from invertebrate species (Marigómez et al., 2013) to several fish species including apex predators (Álvarez-Muñoz et al., 2015; Chiesa et al., 2019; Chynel et al., 2021; Madenjian et al., 2020). Recognizing which of these compounds are truly a concern is a complex issue due to the lack of information about them.

The main assessments to approach this issue are the study of alarming chemical properties (e.g., bioconcentration or bioaccumulation) and the evaluation of potential effects. The environmental risk assessment of CECs in a sample is often addressed by measuring the risk quotients (RQ). These values are calculated for each compound as the ratio between the experimental concentration measured in the sample and the expected no-effect concentration. This strategy can be used to prioritize between CECs when measures need to be taken or further research is needed to confirm if those contaminants are indeed a concern (Lopez-Herguedas et al., 2021).

Bioconcentration refers to the intake from water, and retention of a given contaminant and is one of the core properties to perceive the environmental risk. Contaminants that are bioconcentrated in aquatic organisms can build up to higher trophic levels (i.e., biomagnification) and even reach humans in the worst scenario. This retention can be measured as the bioconcentration factor (BCF) which is defined as the concentration of the contaminant in an organism, divided by its equilibrium concentration in water. Many studies indicate that CECs can also be extensively biotransformed resulting in metabolites with equal or higher toxicity and bioaccumulation potential as the parent compound (Chen et al., 2021; Zind et al., 2021). Thus, the estimation of the bioconcentration focusing only on the parent contaminants could underestimate the true extent of the exposure.

In this work, we have studied the occurrence of CECs in a distinctive aquatic species, the European eel (*Anguilla anguilla*) by means of both target and non-targeted methods. The larvae of this species (*leptocephali*) cross from the Sargasso Sea to the European coast following ocean currents. At this stage, they accumulate the energy stores needed to metamorphose into glass eels and then migrate up estuaries to reach the river. Glass eel recruitment has drastically decreased since the early 1980s and the species is now below its safe biological limit. Among other confounding factors, several studies point out contamination as one of the causes behind this decrease (Palstra et al., 2006; Robinet and Feunteun, 2002). The accumulation of toxic substances including polychlorinated biphenyls (Freese et al., 2016; Maes et al., 2012), polycyclic aromatic hydrocarbons (Kammann et al., 2014), metals (Claveau et al., 2015; Figueiredo et al., 2018; Maes et al., 2012), etc., has been previously studied, especially in sub-adult (*yellow eel*) or adult (*silver eel*) stages, due to their diet (bottom-dwelling predators) and their high body fat contents. Glass eels are also likely exposed to chemicals in estuaries, which are considered a sink for various contaminants. In addition, it is now well accepted that all glass eels do not migrate up the estuary, some of them just settle in the estuary. These different patterns of migration could have a strong impact on the fate of the population because of the sex determinism in eels (Geffroy and Bardonnet, 2016; Tesch and Greenwood, 1977). It is, therefore, crucial to understand constraints on glass eels to either settle in or migrate up the marine/river continuum. A recent study suggested that glass eels exposed to methylmercury may present a lower propensity to migrate (Liu et al., 2019) and it becomes necessary to characterize the potentially hazardous compounds that glass eels are exposed to and understand their behaviour within their organism. In this study, therefore, we aim to gain further insight into the uptake of CECs by glass eels and elucidate their metabolization products. A risk assessment study was first conducted on the WWTP effluent that is released directly into the glass-eel habitat to address the most relevant CECs. The selected contaminants were used to perform a controlled exposure experiment with captured wild glass eels and evaluate their bioconcentration potential. Quantitative analyses of the targeted molecules and non-targeted analyses of their metabolites were carried out using a UHPLC-Q Exactive Orbitrap.

2. Procedure

2.1. Standards and reagents

Information regarding the analytical standards used in the targeted analysis is provided in the Supplementary Information (Table SI1). This list includes a wide range of CECs known to be frequently detected in WWTP's effluents and some of them are prone to be included in future monitoring programs. Working solutions containing all the target compounds and surrogates at 2 μg/g and 10 μg/g, respectively, were prepared in MeOH:H_2O (50:50, v/v; UHPLC-MS, Scharlab, Barcelona, Spain).

The solvents used in the SPE procedure were MeOH (HPLC, 99.9%, Sigma-Aldrich, St. Louis, MO, USA), ethyl acetate (HPLC, 99.9%, Sigma-Aldrich), ammonia (25%, Sigma-Aldrich) and formic acid (>98%, Panreac, Barcelona, Spain). For the UHPLC-q-Orbitrap analysis, formic acid, water, acetonitrile (UHPLC-MS grade, Fischer Scientific, Geel, Belgium), and ammonium acetate (≥99%, Scharlab) were used in the mobile phases.

2.2. Multi-target analysis of Bayonne's WWTP effluent

An automatic large volume solid-phase extraction system (LV-SPE, MAXX Mess-u. Probenahmetechnik GmbH, Rangendingen, Germany) was used to sample 17 L of Bayonne's (France) Pont de l'aveugle WWTP (111,667 population capacity, primary and secondary treatments) effluent in 12 h on the (November 30th, 2020). An in-house cartridge was prepared by filling a PFTE cartridge with 6 g Strata HR-X and 2 g of both Strata ZT-WAX and ZT-WCX, and then conditioned with 200 mL EtOAc:MeOH (50:50, v/v) followed by 200 mL Milli-Q. After the loading of the 17 L of the sample, the cartridge was dried under a nitrogen stream and eluted using 300 mL EtOAc:MeOH (50:50, v/v) with 2% ammonium hydroxide and 300 mL of EtOAc:MeOH (50:50, v/v) with 1.7% formic acid. The extracts were pooled and evaporated in a rotary evaporator (LABOROTA 4000, Heidolph Laborota 4000, Schwabach, Germany) to 15 mL obtaining a relative enrichment factor (REF) of 1133. An aliquot of 55 μL was evaporated to dryness, reconstituted in 250 μL MeOH:H_2O (50:50, v/v; REF 250), and subjected to a multi-target analysis including 284 CECs.

2.3. Environmental risk assessment

An Environmental Risk Assessment (ERA) was carried out following the RQ approach described by Lopez-Herguedas et al. (2021) Briefly, the measured environmental concentration (MEC) was divided by the minimum predicted no-effect concentration (PNEC) among three trophic levels to calculate the RQ of the ecosystem. RQ values > 1 indicate a high potential environmental risk, values between 0.1 and 1 indicate

moderate risks, and for RQs <0.1 the environmental risk was negligible. RQs were used to prioritize between CEC for further bioconcentration study. For each quantified compound, the available toxicity data (i.e. NOEC or EC50 values) was collected from ECOTOX (https://cfpub.epa.gov/ecotox/), NORMAN Network (https://www.norman-network.com/nds/) and Pesticide Properties databases (http://sitem.herts.ac.uk/aeru/ppdb/) or literature (Paíga et al., 2016; Papageorgiou et al., 2016) as annotated in Table SI. X. When no experimental toxicity data were available, ECOSAR™ v. 2.0 software was used to predict the NOEC.

2.4. Fish collection and exposure

Procedures used in this study have been validated by the ethics committee N°073 'Aquitaine Poissons-Oiseaux' (ref: APAFIS#28511-2020120213191896 v3). The experiment was carried out in strict accordance with the EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e., Directive, 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2013-118, February 1st, 2013).

Wild glass eels were captured near the mouth of the Adour estuary using a dip net at night during flood tide. Once in the laboratory, glass eels were kept in an aerated tank with water from the sampling site. For the next 48 h, the seawater was progressively diluted with fresh water. Fish were kept under 12 °C and a photoperiod of 12 L/12 D with a very low light intensity during the photophase (0.2–0.3 μW/cm^2).

After acclimatisation, glass eels were exposed to a continuous flow of a mix of diazepam, irbesartan, and propranolol at 3 ng mL^{-1}, 3 ng mL^{-1}, and 0.1 ng mL^{-1} nominal concentration respectively (i.e., concentrations found in WWTP effluent) as shown in Fig. SI. Two tanks with four eels each (n = 8) were used in the exposure experiment and 5 samplings times were fixed: One before the exposure (control group t_0), three after 30 h (t_{30h}), 5 days (t_{5d}), and 7 days (t_{7d}) of continuous exposure and the last one after 7 days of exposure followed by 7 days of depuration (t_{14d}). Sampled fish were killed using a lethal bath of anaesthesia (benzocaine, 0.05 mg L^{-1}), individually measured for the wet weight (±1.0 mg) and length (±0.5 mm), and then flash-frozen in liquid nitrogen and stored at −80 °C before analysis. Additionally, 100 mL of water from tanks was also sampled at each sampling time and stored at −20 °C for future determination of actual concentrations in water.

2.5. Sample treatment

The glass eels were homogenized and extracted in pools of 2 eels for each exposure condition (n = 4 × 2). The homogenization of the pools was carried out in 7 mL MeOH:H_2O (95:5, v/v) using FUSLE (focused ultrasound solid-liquid extraction) first, as described by Mijangos et al. (2019), and second, the Precellys 24 Tissue Homogenizer (3 × 60s-6400 rpm) under controlled cooled temperature (4 °C) (Cryolys, Bertin Technologies, Montigny-le-Bretonneux, France). The samples were then centrifuged for 15 min at 21,000 rpm (Centrifuge Allegra X-30 R, F2402H, Beckman Coulter, High Wycombe, UK) to get the supernatant which was evaporated to 1 mL under a gentle stream of nitrogen (Horizon Technology XcelVap, Lake Forest, CA, USA). The extraction of the samples was carried out following the method validated by González-Gaya et al. (2021) Briefly, samples were diluted to 6 mL in Milli Q water and loaded into homemade 0.5 g SPE cartridges (Strata HR-X/ZT-WCX/ZT-WAX, 3/1/1) previously conditioned with 6 mL MeOH and 6 mL Milli Q water. After loading the sample, the cartridge was rinsed with 5 mL of Milli-Q water and dried overnight under a vacuum. The cartridge was subsequently eluted first with 12 mL of EtOAc:MeOH (50:50, v/v) with 2% ammonium hydroxide and then 12 mL of EtOAc:MeOH (50:50, v/v) with 1.7% formic acid. Eluate was concentrated to dryness under nitrogen stream, and reconstituted in 250 μL MeOH:H_2O (50:50, v/v) spiked with 70 μg/L of azoxystrobin-d4 as internal standard. Water samples collected from each tank were also extracted following the same SPE procedure. Two additional pools of two glass eels, spiked with 50 μg/L of diazepam and irbesartan and 15 μg/L of propranolol, were also prepared to assess the suitability of the method and calculate the recoveries.

2.6. Instrumental analysis

WWTP effluent, exposure tank water, and glass eel extracts were injected using the same chromatographic and mass spectrometry conditions in a Thermo Scientific Dionex UltiMate 3000 UHPLC running an XB-C18 column (150 × 2.1 mm, 2.6 μm, Phenomenex Kinetex®, CA, USA) with a pre-filter (2.1 mm, 0.2 μm, Phenomenex Kinetex®, CA, USA), coupled to a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap mass spectrometer (UHPLC-q-Orbitrap) equipped with a heated ESI source (HESI, Thermo-Fisher Scientific, Waltham, MA, USA), operating in full scan−data dependent MS2 (Full MS-ddMS2) acquisition mode. Operating conditions described by Lopez-Herguedas et al. (2021) were followed for optimal chromatographic separation and data acquisition (SI.1).

2.7. Data handling

Four independent data managing strategies were implemented: i) multi-target screening of CECs for WWTP effluent, ii) target analysis of the selected contaminants in glass eels and exposure water, iii) suspect screening of potential metabolites of these selected contaminants in glass eels, and iv) suspect screening of CECs in glass eels.

For the quantitative analysis in target screening approaches, TraceFinder 5.1 software (Thermo-Fisher Scientific, Waltham, MA, USA) was used to process the experimental data. From the multi-target analysis, concentrations found in effluent water were used as MEC values for RQ calculation. BCFs of the exposure contaminants were calculated as the ratio between tissue and water concentrations.

The suspect screening of CECs was carried out in t_0 control samples using Compound Discoverer 3.3 (Thermo-Fisher Scientific, Waltham, MA, USA). Peak picking and peak alignment were conducted with mass tolerances of 5 ppm and maximum retention time deviations of 30s. Annotated features compared to the Norman list of CECs (https://www.norman-network.com/nds/) and MS2 fragmentation were contrasted with the mzCloud database (https://www.mzcloud.org/). Detailed workflows and feature filtering information are described in SI.2. When the standards of the candidates were available, experimental retention time was confirmed with an allowed error of ±0.1 min. If not, retention times predicted using the Retention Time Index (RTI) platform (http://rti.chem.uoa.gr/) were compared to the experimental data. Finally, candidates were classified according to Schymanski's (Schymanski et al., 2014) identification confidence level and only levels 1 and 2 were considered.

The suspect screening of potential metabolites in glass eel (iii) was addressed by two parallel strategies. The differences between both strategies lay in the way to predict the potential metabolites from the parent contaminant. Although both used in-silico predictions, the first strategy used BioTransformer 3.0 to predict the phase I and phase II metabolites and build the suspect list. The second strategy used the transformation prediction node in the Compound Discoverer workflow (Djoumbou-Feunang et al., 2019). Again, metabolites were searched in the mass lists with a tolerance of 5 ppm, peaks were manually checked and MS2 spectra were studied for confirmation in Compound Discoverer and MetFrag (Ruttkies et al., 2016) when PubChem ID was available for that candidate.

3. Results and discussion

3.1. Multi-target analysis of Bayonne's WWTP effluent

The method recoveries and quantification limits of all the target compounds are shown in Table SI1. Absolute recoveries of the method

were obtained from the validation of the method in a previous work by Gonzalez-Gaya et al. (González-Gaya et al., 2021), and the instrumental quantification limits were set as the lowest concentration level where the RSD <30% and the trueness between calculated and theoretical concentration >70% after the injection of three replicates of the calibration points.

The multi-target analysis carried out in the effluent of the WWTP detected the presence of 56 CECs. Their mean concentrations and standard deviations are included in Table 1. Pharmaceuticals represent 69% of the detected compounds, being antihypertensives the ones found at higher concentrations. A lower occurrence of fungicides, industrial chemicals, and herbicides was also detected (8%, 8%, and 6% respectively). These results are in line with our previous studies conducted on WWTPs from the Basque Country and Spain (Lopez-Hergueedas et al., 2021; Mijangos et al., 2018; Miossec et al., 2019). Broadly saying, the distribution of CECs follows similar patterns, since angiotensin-II receptor blockers (such as the sartans) and some antibiotics appear at the same levels and, on the contrary, metformin or gabapentin, follow very different ones. The high concentrations of the compounds found in the list agree with several studies that stress the poor removal efficiency of secondary treatments upon these compounds. Golovko et al. (2021), also reported the low removal efficiency of WWTP for most of the compounds in the top part of our list (e.g., sotalol, irbesartan, telmisartan, valsartan, tramadol, azithromycin, atenolol …).

Table 1
Results of the quantitative analysis and risk assessment of CECs in the WWTP effluent. RQs of the compounds detected in the WWTP effluent were calculated from the minimum PNEC value among three trophic levels (min. PNEC). *RQ value for Telmisartan considering fish PNEC was 0.4. ** RQ value for Diazepam considering fish PNEC was 0.52. Only diazepam and telmisartan had hazardous RQs for more than one trophic level. The RQs of those contaminants posing a high risk potential are coloured in red, whereas those with moderate risk potential are coloured in orange.

Compound	Family	Concentration (ng/L)			RQ	min. PNEC
Sotalol	β-blocker antihypertensive	3100	±	54	0.01	Invertebrate
Diazepam	Anxiolytic	3000	±	74	**0.72**	Invertebrate**
Irbesartan	Antihypertensive	2700	±	38	**1.5**	Fish
Telmisartan	Antihypertensive	2000	±	52	**1**	Green Algae*
Valsartan	Antihypertensive	1300	±	3.1	0.01	Fish
Tramadol	Analgesic	930	±	20	0.01	Fish
Azithromycin	Antibiotic	650	±	6.2	**34**	Green Algae
Atenolol	β-blocker antihypertensive	450	±	1.3	0.01	Invertebrate
Hydroxychloroquine	Malaria treatment	450	±	5.4	0.03	Invertebrate
Carbamazepine	Antidepressant	430	±	14	**0.17**	Invertebrate
Bisoprolol	β-blocker antihypertensive	410	±	3.8	0.01	Invertebrate
Cetirizine	Antihistaminic	380	±	6.1	0.03	Invertebrate
2-Hydroxybenzothiazole	Industrial chemical	270	±	2.4	0	Invertebrate
Losartan	Antihypertensive	260	±	5.6	0.09	Invertebrate
Gabapentin	Antiepileptic	250	±	0.5	0	Invertebrate
Metformin	Antidiabetic	200	±	0.5	0.05	Fish
Mycophenolic acid	Antibiotic	200	±	2.4	0	Fish
Ketoprofen	Anti-inflammatory	200	±	2.3	**0.1**	Green Algae
Metoprolol	β-blocker antihypertensive	170	±	2.1	0.02	Green Algae
Fluconazole	Antifungal	140	±	1.1	0.01	Fish
Amantadine	Antiviral	140	±	2.3	0.01	Invertebrate
Trimethoprim	Antibiotic	130	±	0.92	0.01	Fish
Lidocaine	Anaesthetic	130	±	0.77	0	Invertebrate
Caffeine	Stimulant	120	±	2.1	0.02	Fish
Propranolol	Anaesthetic	110	±	2.2	**0.18**	Fish
Sulphapyridine	Sulphonamide	110	±	3.7	0	Invertebrate
Bezafibrate	Lipid-regulator	100	±	1.4	0.01	Invertebrate
Omeprazole	Proton pump inhibitor	93	±	1.3	0.02	Invertebrate
Caprolactam	Industrial chemical	87	±	5.3	0	Invertebrate
Verapamil	Antiarrhythmic and antihypertensive	68	±	2.3	0.01	Invertebrate
Lorazepam	Anxiolytic	51	±	0.91	0	Fish
Cyclophosphamide	Anticarcinogenic	51	±	2.1	0	Fish
Diuron	Herbicide	46	±	0.4	**930**	Green Algae
Equilin	Hormone	45	±	3.2	0	Invertebrate
Amitriptyline	Antidepressant	42	±	0.74	0.04	Invertebrate
Imidacloprid	Insecticide	42	±	0.21	0	Fish
Terbutryn	Herbicide	35	±	0.33	**0.22**	Green Algae
EDDP	Fungicide	31	±	0.79	0.03	Invertebrate
Mirtazapine	Antidepressant	25	±	0.61	0	Fish
Propiconazole	Fungicide	21	±	0.21	0	Fish
4-Methylbenzophenone	Industrial chemical	21	±	0.23	0	Green Algae
Thiabendazole	Anthelminthic	18	±	0.3	0.02	Fish
Benzophenone-2	Ultraviolet absorber in cosmetics	13	±	1.1	0.07	Fish
Tebuconazole	Fungicide	13	±	0.23	0.01	Fish
Eprosartan	Antihypertensive	12	±	0.5	0.01	Invertebrate
Indomethacin	Anti-inflammatory	12	±	3.1	0	Invertebrate
4-Hydroxybenzophenone	Industrial chemical	11	±	0.2	0	Invertebrate
Propyphenazone	Anti-inflammatory	11	±	0.2	0.01	Fish
Triphenyl phosphate	Industrial chemical	9.2	±	0.62	0.02	Green Algae
Clomipramine	Antidepressant	7	±	0.44	0.02	Fish
Diphenhydramine	Antihistaminic	6	±	0.13	0	Invertebrate
Carbaryl	Insecticide	6	±	0.2	0.01	Green Algae
Glibenclamide	Antidiabetic	4.9	±	0.54	0	Green Algae
Desloratadine	Loratadine metabolite	4.3	±	0.33	0	Invertebrate
Ketoconazole	Antifungal	3.8	±	0.14	0	Invertebrate
Acetamiprid	Herbicide	3.5	±	0.29	0	Green Algae

This implies that the presence of these compounds is not an isolated case for this particular scenario, but probably many other migration sites for glass eels are also contaminated with these pharmaceuticals.

3.2. Environmental risk assessment

RQs calculated from the minimum PNEC values among the three taxonomic groups are summarized in Table 1. The RQs calculated from the most impacted taxonomic group allow us to get an idea of the whole ecosystem impact but does not address the individual comparison of the taxonomic groups. For that, the disaggregated RQs for each of the three taxonomic groups are shown in Table SI2. . Nine detected compounds showed environmental risk potential (high or moderate risk potential), being algae the most affected taxonomic group with three contaminants, telmisartan, azithromycin, and diuron, above the high potential risk limit (RQ > 1). Special attention must be given to the photosynthesis inhibitor diuron used precisely as an algicide, and herbicide in agriculture, which shows an unquestionable risk with a RQ almost 1000 times over the threshold. On the other hand, two contaminants threaten the invertebrate group with moderate RQs, diazepam, and carbamazepine. Finally, for the taxonomic group that specially concerned to this study, fish, the contaminants with the highest RQs were irbesartan, diazepam, telmisartan, and propranolol. Irbesartan (antihypertensive) was the only compound with a high-risk potential and the first candidate chosen to conduct the exposure experiment in glass eels. Telmisartan was the second contaminant with the highest RQ but, since contaminants with different mechanisms of action were sought, and telmisartan and irbesartan both belong to the same pharmaceutical family, diazepam (anxiolytic) and propranolol (anaesthetic) were the chosen instead. The three selected CECs have been previously reported in the literature as harmful to different fish species. For instance, endocrine disruption was reported by Overturf et al. (2016), in channel catfish after the exposure to diazepam, and the reproductive behaviour of fathead minnow was affected by this compound according to Lorenzi et al. (2016). Studies with zebrafish (Zuo et al., 2022) also report the impact of irbesartan on the hatching success and the heart rate. In the case of propranolol, this compound can affect to the energy metabolism of meagre (Duarte et al., 2020), and even the swimming pattern of some fish (Matus et al., 2018), two interesting findings that can be related to the ability of glass eels to migrate (Bureau Du Colombier et al., 2007).

3.3. Exposure experiments and accumulation in glass eels

Mortality or alteration of the well-being status of glass eels was not observed during the exposure experiments. Groups sampled at different times did not differ significantly in weight or length (ρ levels$\gg$0.05), as shown in the values collected in Table 2. The samples used for the optimization of the method showed recoveries of 91%, 99% and 71% for diazepam, irbesartan and propranolol respectively (Table SI3). The recoveries of the method were applied to calculate the concentration of real samples. Low RSD (<%5) were also obtained for each of the three compounds.

Diazepam, irbesartan, and propranolol concentrations in water during the uptake phase and in glass eels at different sampling times are presented in Fig. 1. Water concentrations at t_0 and t_{14d} were always below detection limits for the three compounds. However, propranolol was measured in glass eels at all exposure times, regardless the water levels were below the detection limits at t_0 and t_{14d}. On the contrary, diazepam and irbesartan levels in glass eels showed a constant accumulation up to ~60 ng g^{-1} and ~20 ng·g^{-1} respectively at t_{7d} followed by a decrease during the depuration time. This particular trend of propranolol was also described by Miller et al. (2017) In the same work, the authors also estimated that the half-life period for diazepam and metabolites was ~12 h, which would lead to the complete depuration of these contaminants as confirmed in our study. Therefore, we can conclude that the contamination of glass eels by these pollutants is not an irreversible problem, but their constant release would not allow their depuration.

Table 2
Biometric measurements (mean ± standard deviation) of glass eels sampled at the different exposure times. "n" refers to the sample size.

	Length (mm) $p = 0.36$	Weight (mg) $p = 0.92$	n
T0	240 ± 43	70 ± 4	8
T2d	240 ± 62	70 ± 5	10
T5d	200 ± 59	60 ± 5	10
T7d	240 ± 54	70 ± 5	10
T14d	200 ± 43	65 ± 4	10

The use of wild animals in bioaccumulation experiments has some implications that can hamper the results (i.e., unwanted contamination from environmental exposure) but, at the same time, they provide a real picture of the environmental issue that we must face. As mentioned, propranolol was found at low and constant concentrations over the experiment, even before exposure and after the depuration phase. At t_{2d} the concentration increased a 50%, an increment that was rapidly regulated by depuration or metabolization. Thus, propranolol was proven to be already bioconcentrated in advance from the wild and this hinders the calculation of its BCF within the experiment. This is not the first time that the presence of this pharmaceutical has been detected in fish, in fact, two studies reported high frequencies of detection in several wild fish species (Rojo et al., 2019; Xie et al., 2017).

Consequently, the evolution of BCFs overtime was only calculated for diazepam and irbesartan (Fig. 2). Both compounds showed similar trends that reached a maximum BCF of 10 at t_{7d}. In the case of irbesartan, we can say that the bioaccumulation has reached steady state but, for diazepam, this fact is not entirely clear. In a recent review, Duarte et al. (2022) studied the bioconcentration of numerous neuroactive pharmaceuticals in fish and reported a mean BCF of 10 for diazepam. On the contrary, McCallum and colleagues (McCallum et al., 2019) did not find any evidence of bioconcentration for irbesartan in sea trout. Thus, diazepam bioconcentration results agree with those found in the literature but, to the best of our knowledge, this is the first time that a bioconcentration factor for irbesartan has been disclosed in fish. There is no evidence to suggest that the BCFs obtained in this experiment is related to log K_{ow} values of the selected compounds (log $K_{ow,\ Irbe} = 4.23$, $K_{ow,\ Prop} = 3.48$ & $K_{ow,\ Diaz} = 2.91$), so the mechanism behind the accumulation of propranolol remains unknown.

3.4. Metabolite identification

The suspect screening of biotransformation products (TPs) revealed the presence of one diazepam metabolite in glass eel (Fig. 3). Structural assignments based on MS2 fragmentation data are available in SI.3. This metabolite corresponds to nordiazepam (*N*-methylation of the amine group) a well-known phase I metabolite that has been reported in many studies about diazepam pharmacokinetics (Greenblatt et al., 2021; Hooper et al., 1992; Zhou et al., 2020). These studies also describe two more active metabolites oxazepam and temazepam, but they were not detected in the analysis. Nordiazepam was identified within Schymanski's confidence level 2a (Schymanski et al., 2014) as no reference standard was available for confirmation. Thus, the quantification of this compound was not reached, but the transformation ratio was calculated based on the parent and the metabolite peak areas (Fig. 4). The metabolization ratio of diazepam/nordiazepam at t_{2d} indicates that for the first 2 days almost no biotransformation of diazepam occurs in glass eels, therefore, the impact of this pollutant in acute exposures would be mostly due to the effect of the parent compound. This is not the case in long exposures where this ratio can be seen to decrease and nordiazepam would start to become relevant. As said before nordiazepam is an active metabolite, and it has higher bioavailability (Vernau and LeCouteur, 2008) and a longer elimination half-life (Wang et al., 2022),

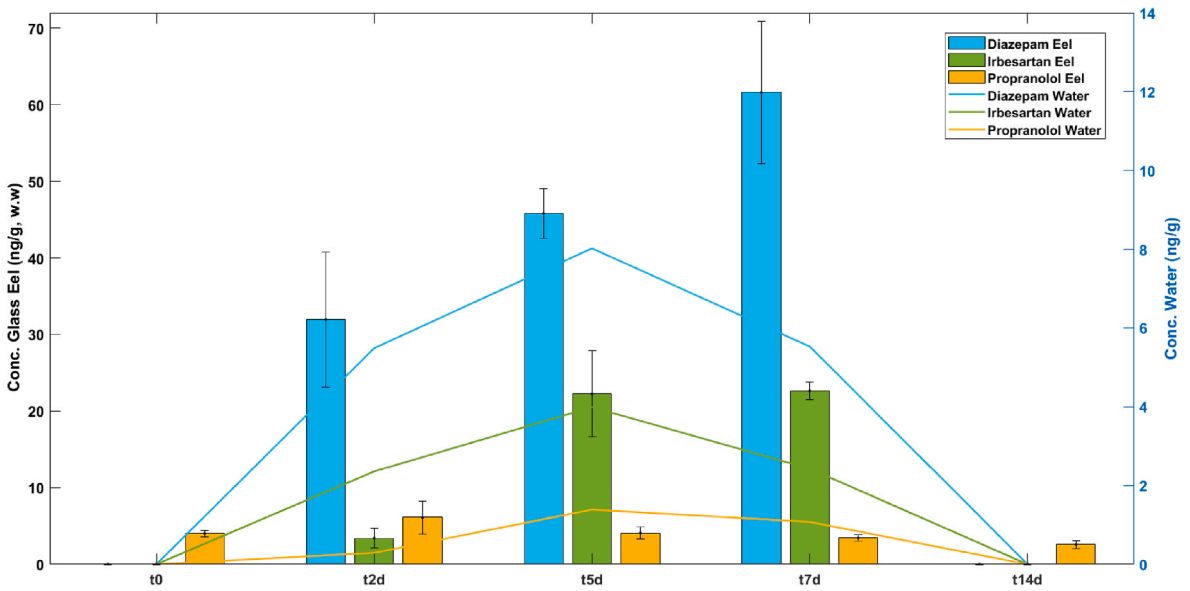

Fig. 1. Concentrations of the three target contaminants in water (right axis) and glass eel (left axis) at five sampling times.

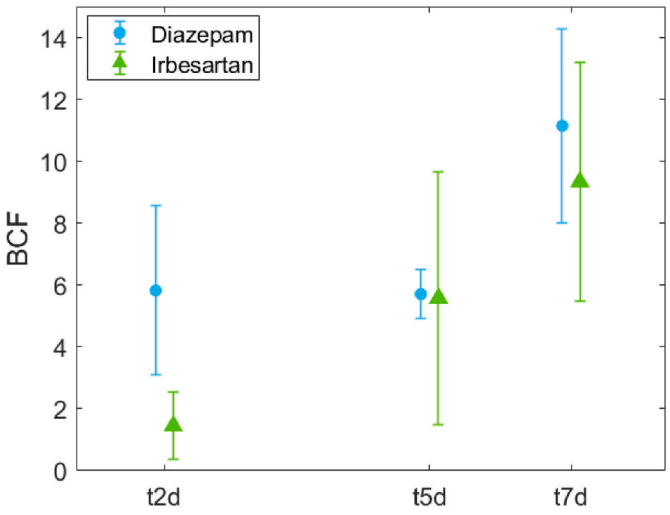

Fig. 2. BCF values of diazepam and irbesartan over the exposure time.

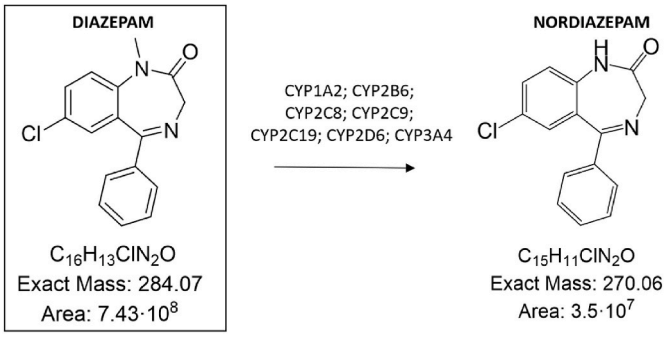

Fig. 3. Identified metabolites of the target compounds. Given areas indicate the maximum value found in the samples.

which highlights once again the importance of monitoring the transformation products of CECs.

Additionally, the suspect screening of TPs was also applied to the water samples of the tanks at different exposure times, but none of the mentioned metabolites was found above identification limits. However, we were able to identify one irbesartan TP which agrees with a minor surface water TP found by Boix et al. (2016) (SI.3), but its occurrence could not be related to biotransformation since its presence in glass eels was not detected.

3.5. Suspect screening of CECs in glass eel

Since we found propranolol in control samples, the analysis of other xenobiotic compounds that could be accumulated in wild glass eels gained interest. The suspect screening of CECs revealed a wide number of compounds, classified within *Schymanski*'s 1 and 2 classification levels (i.e., compounds confirmed with reference standards or structural library matching) (Table 3). Besides propranolol, the occurrence of two more pharmaceuticals was confirmed by reference

standards, primidone, and exemestane. It's worth mentioning that these two pharmaceuticals have been previously described as endocrine-disrupting chemicals (EDC) (Ismail et al., 2021; Jones-Lepp et al., 2015). In the case of exemestane, the exposure to this compound has been associated with female-to-male sex change in other fish species (Breton et al., 2019). Some studies point to a disparity between the distribution of females and males at different heights in estuaries (Harrison et al., 2014), an unexplained phenomenon for which we cannot rule out that exposure to endocrine disruptors has some kind of effect. To the best of our knowledge, most of the compounds identified in this suspect screening (e.g., Iloprost, Imazapic …) have never been reported in biological matrices. The chemical penetration enhancer 1-Dodecyl-2--pyrrolidinone was also identified in the samples (Godavarthy et al., 2009). Another benzodiazepine metabolite shows up within the identified compounds, 7-aminonimetazepam. Although they are structurally related, the metabolization of diazepam into this compound has not been described in the literature and it has only been related to nimetazepam and nitrazepam metabolization, which have no marketing authorization in France (Airagnes et al., 2019).

4. Conclusions

The exposure of glass eels to CECs was based on a previous prioritization of the most toxic and more abundant contaminants released from one of the main WWTPs of Bayonne. Based on the quantification of 56 CECs continuously discharged into the Adour estuary, we were able to calculate the risk quotients for three trophic levels. Our study

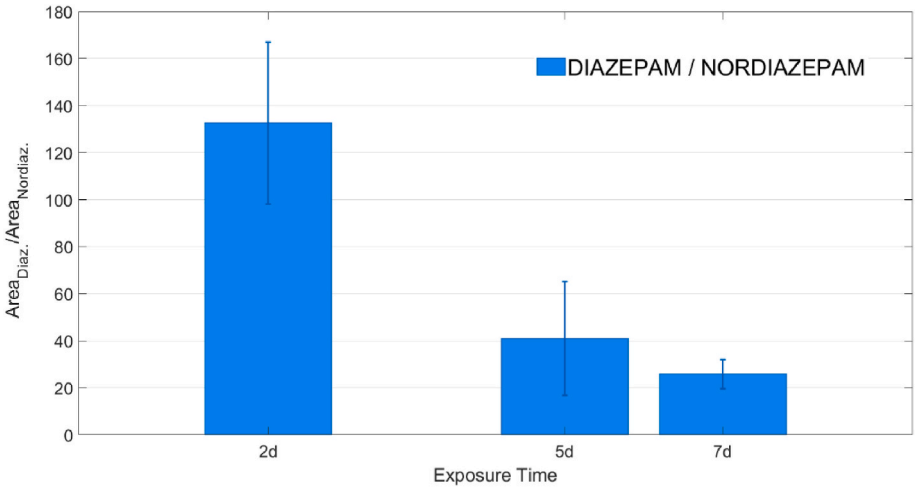

Fig. 4. Metabolization ratios of diazepam and nordiazepam at different exposure times.

Table 3
CECs identified in the suspect screening of t_0 control samples. Level 1 confirmation was only possible for the compounds included in our target list. (MW = Molecular Weight; rt = retention time).

Compound	Formula	MW	rt	Confidence level	Use
Exemestane	$C_{20}H_{24}O_2$	296.17763	5.463	**1**	Pharmaceutical/Antineoplastic
Primidone	$C_{12}H_{14}N_2O_2$	218.10553	3.567	**1**	Pharmaceutical/Anticonvulsant
Propranolol	$C_{16}H_{21}NO_2$	259.15723	5.299	**1**	Pharmaceutical/Beta blocking agent
1-Dodecyl-2-pyrrolidinone	$C_{16}H_{31}NO$	253.24056	10.309	**2a**	Personal care products/Cosmetics
2-(4-Morpholinyl) benzothiazole	$C_{11}H_{12}N_2OS$	220.06703	7.27	**2a**	Flame retardant
3-Hydroxybupivicaine	$C_{18}H_{28}N_2O_2$	304.21508	8.137	**2a**	Pharmaceutical/Anaesthetic
7-Aminonimetazepam	$C_{16}H_{15}N_3O$	265.12151	3.024	**2a**	Pharmaceutical/Benzodiazepine metabolite
Iloprost	$C_{22}H_{32}O_4$	360.23006	6.965	**2a**	Pharmaceutical/Antithrombic
Imazapic	$C_{14}H_{17}N_3O_3$	275.12699	2.074	**2a**	Herbicide
N, N′-Diphenylguanidine	$C_{13}H_{13}N_3$	211.11095	2.893	**2a**	Rubber component
Diisopropylethylamine	$C_8H_{19}N$	129.15175	2.206	**2a**	Plasticiser/Synthetic polymer
N-Ethylaniline	$C_8H_{11}N$	121.08915	5.663	**2a**	Industrial product
Norethandrolone	$C_{20}H_{30}O_2$	302.22458	13.188	**2a**	Anabolic steroid/Progestogen
Triethylene glycol monobutyl ether	$C_{10}H_{22}O_4$	206.15181	5.421	**2a**	Industrial product/Solvent

concludes that the Adour estuary is, at least, threatened by 4 CECs that pose a high environmental risk potential, including the algicide diuron which exceeds the limits by almost 1000 times being a serious concern for the health of the algae together with telmisartan and azithromycin. The antihypertensive irbesartan also exceeds this limit, in this case being a threat to fish. The environmental risk assessment results were then used to select the contaminants with the highest impact on fish to conduct an exposure experiment on our target species, the glass-eel. The exposure to diazepam and irbesartan showed that these compounds were bioconcentrated up to 10 times in glass eels. Since the depuration period was sufficient to remove these two compounds from the glass eel organism, we can state that this contamination problem is not irreversible, but to solve it, the continuous release of these contaminants must be stopped. Surprisingly, propranolol was found at t_0, which suggests that glass eels accumulate low levels of this drug that are not fully eliminated neither after the quarantine period prior to the experiment nor the depuration phase of 7 days, which suggests a much more worrying contamination problem than in the previous case. In addition, we searched for metabolization products that could occur in the glass eels by suspect screening, but only one diazepam metabolite was identified, nordiazepam, an active metabolite with longer half-life and higher bioavailability, which stresses the importance of the monitoring of biotransformation products when the effects of CECs are assessed in biota. Finally, a suspect screening of CECs was also carried out to identify further cases of environmental contamination in t_0 glass eels. Two more CECs were confirmed as Schymanski's confidence level 1, exemestane and primidone, both being endocrine disruptors that could affect the sex differentiation of glass eels. Eleven more CECs were also identified as level 2a, some of them pharmaceuticals that had never been reported in fish. In summary, this work exposes the contamination problem faced by glass eels during their migratory stage. The habitat of this species is highly threatened by many contaminants that pose high risk potential, some of them, such as diazepam and irbesartan, being able to accumulate several times in their organism. Once again, it has been demonstrated that at this stage the glass eels are specially affected by contamination since they are directly exposed to contaminants released to estuaries from human activity, and thus, the protection of this endangered species also relies on the evaluation of the contaminants that are now part of its habitat. With this work we have addressed the identification of the main threats to glass eels and the information will be soon used to assess the effects of these contaminants and to study the connection between the glass eel exposome, their migratory behaviour and the population decrease.

Author contributions

Iker Alvarez-Mora – Conceptualization – Methodology – Investigation – Formal Analysis - Writing, Original Draft – Writing, Review & Editing – Visualization. Valérie Bolliet – Conceptualization – Methodology – Investigation – Resources — Writing Review & Editing – Supervision – Project administration – Funding acquisition, Naroa Lopez-Herguedas – Methodology – Investigation – Formal Analysis - Writing, Review & Editing, Lyen Castro — Methodology – Investigation, Eneritz Anakabe — Data Curation - Writing Review & Editing, Mathilde

Monperrus – Resources – Conceptualization – Writing Review & Editing – Supervision - Project administration, Nestor Etxebarria – Conceptualization – Methodology – Investigation – Resources – Writing Original Draft – Writing Review & Editing – Supervision – Project administration – Funding acquisition.

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.

Data availability

Data will be made available on request.

Acknowledgements

Authors gratefully acknowledge financial support from the Agencia Estatal de Investigación (AEI) of Spain and the European Regional Development Fund through project CTM2017–84763-C3–1-R and the Basque Government through the financial support as a consolidated group of the Basque Research System (IT1213–19). Iker Alvarez is grateful to the University of the Basque Country and the Université de Pau et des Pays de L′ Adour for his cotutelle predoctoral scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120016.

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