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Active biomonitoring of river pollution using an *ex-situ* exposure system with two model species

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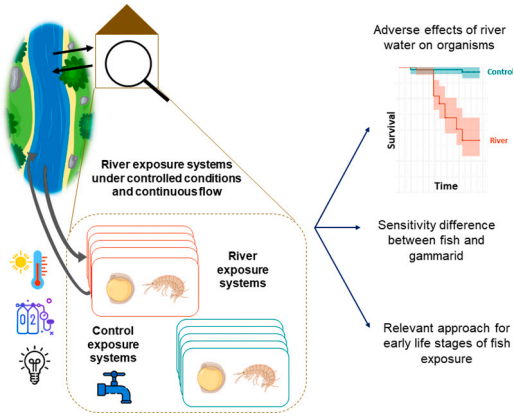
HIGHLIGHTS

- The approach used has proven effective in exposing fish embryos to river water.
- Fish early life stages were significantly impacted by the river's water quality.
- River contamination had different effects on Japanese medaka and gammarids.

GRAPHICAL ABSTRACT



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ABSTRACT

In the context of increasing pollution pressure on aquatic ecosystems, it is essential to improve our knowledge of habitat quality and its suitability for organisms. It is particularly relevant to better integrate early life stages of fish into pollution biomonitoring programs, as they are reliable indicators of ecosystem integrity and because of their high sensitivity to pollutants. To avoid the influence of environmental parameters on their development, a lab-on-field approach, called the *ex-situ* exposure method, was developed. Aquatic organisms were exposed to a continuous flux of water under semi-controlled temperature, oxygen, and photoperiod conditions to avoid the influence of these confounding factors when interpreting the results. To investigate the potential role of water contamination, this active biomonitoring method was applied to the Garonne River (Southwest France), where migratory fish populations have declined. Two model species from different taxa were used: embryos of the

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Life traits
Biomarkers

Japanese medaka (*Oryzias latipes*) and adults of the crustacean *Gammarus fossarum*. The results showed a significant impact of water quality on embryo mortality and early hatching in two separate experiments on Japanese medaka. In addition, an induction of feeding rate was observed in exposed gammarids, but no impact on their embryo survival, suggesting differences in sensitivity between the two species selected. Chemical and biological analyses did not identify trace metals, pesticides, or microorganisms as potential sources of toxicity in medaka embryos or *G. fossarum*. These results raise concerns about the quality of the water in the Garonne River and its toxicity to aquatic organisms.

1. Introduction

Numerous pollutants including pharmaceutical products, pesticides, trace metals, and industrial products are released into aquatic ecosystems (Häder et al., 2020; Reid et al., 2019). Their variety, persistence, bioavailability, and toxicity constitute an increasing threat to biodiversity that needs to be addressed (Sigmund et al., 2023). The European Union Water Framework Directive (WFD) aims to achieve good chemical status for EU water bodies (European Commission, 2000). Currently, chemical assessments are carried out on 45 priority substances that remain limited. The emissions of novel compounds into the environment have surpassed our current monitoring and assessment capacities, thereby exceeding the planetary threshold for new chemicals (Persson et al., 2022). Consequently, there is a significant gap in our understanding of the presence and impacts of these substances on ecosystems. To address this issue, new approaches to ecosystem contamination monitoring have been developed over the last few decades, including biomonitoring approaches (Brack et al., 2019; Wepener, 2013). Passive biomonitoring (PBM) involves the use of indigenous organisms as water quality indicators. However, studying the responses of organisms to water quality using molecular biomarkers is challenging and using life traits as proxies for toxicity is difficult. Additionally, the capture of wild organisms is limited by their presence in the environment, and their past exposure remains unknown, limiting their use (Catteau et al., 2021; Smolders et al., 2003). More recently, active biomonitoring (ABM) approaches have been developed, which are defined as the use of living organisms translocated from a reference site to a contaminated site. Studying their health status and biomarker responses allows for the assessment of water quality while integrating a wide variety of pollutants present in ecosystems (Gagnaire et al., 2015; Marchand et al., 2024; Wepener, 2013). Moreover, the use of calibrated farmed animals with known life histories facilitates their use in risk assessment. Standardised ABM methods already exist for several species, especially invertebrates, such as gammarids and mollusc bivalves (Besse et al., 2013; OSPAR Commission, 2013).

Fish are suitable species for biomonitoring and risk assessment (Roset et al., 2007). Fish species are present worldwide and crucial for maintaining ecosystem equilibrium. They are regularly used in ABM to assess water quality and responses to several contamination sources, such as wastewater treatment plants (WWTPs) (Catteau et al., 2021; Cavallin et al., 2021), mines (Barbee et al., 2008; Bougas et al., 2016), industrial effluents (Al-Arabi et al., 2005; Santos et al., 2006) as well as agriculture (Vieira et al., 2017; Whitehead et al., 2005) and urban runoff waters (Camargo and Martinez, 2007). 'Early life stages' or eleutheroembryos refer to embryos and larvae for which exogenous feeding has not yet begun (Balon, 1975). These stages of development are considered critical for population recruitment and health and have gained increasing interest in chemical and environmental risk assessments (Jonsson and Jonsson, 2014; Kamler, 1992). Moreover, the early life stages of fish are increasingly being used in laboratory studies, and there are currently several guidelines for toxicity assays (OECD, 1998, 2018). Their sensitivity to pollutants makes them relevant for environmental monitoring, but other factors, such as temperature, significantly impact their survival or their development, posing a challenge to their use in ABM (Bancel et al., 2024; McKim, 1977).

To overcome this limitation, the purpose of this study was to (i) develop and validate an exposure method that allows the exposure of early life stages of fish and (ii) determine the potential impact of pollution in a river where fish populations have significantly declined over the last two decades. For the first objective, we set up a lab-on-a-field experimentation device that allows the direct exposure of fish embryos to water from various aquatic systems and monitors their responses. The *ex-situ* exposure method involves exposing organisms to continuous water flow under semi-controlled temperature, oxygen, and photoperiod conditions. Two model species for which toxicity test guidelines were available were used. The Japanese medaka, *Oryzias latipes*, is a freshwater fish species native to Southeast Asia that is widely used in laboratory toxicity tests (Shima and Mitani, 2004). The survival and development of medaka exposed to water from the Garonne River were monitored daily from embryos one day post-fertilisation to the end of the sac-fry stage. Experiments on the freshwater amphipod *Gammarus fossarum* were also conducted using the *ex-situ* exposure approach. This species is common in rivers throughout Europe and is frequently used in biomonitoring studies, leading to the normalisation of these tests in France (AFNOR XP T90-722-2, 2020; AFNOR XP T90-722-3, 2020). For the second objective, this exposure method was used in the Garonne River, Southwest France, where several diadromous fish populations have declined over the last few decades, raising concerns regarding contamination and water quality (Castelnaud, 2011; Delage et al., 2014; Rougier et al., 2012). Historically, trace metals, such as cadmium, zinc, lead, and copper have been detected in the basin at high concentrations owing to mining and industrial activities; however, this contamination has significantly reduced since the 1980s (Audry et al., 2003; Coynel et al., 2009; Grousset et al., 1999; Pougnet et al., 2022). However, trace metals and PAHs are still present at moderate or high concentrations in the sediments of the Garonne and Lot Rivers (Barjhoux et al., 2017). Several types of pesticides, including herbicides such as metolachlor, insecticides, fungicides, and their associated metabolites, are regularly detected in the basin, depending on the land use in the area (Bernard et al., 2019, 2023).

The aim of this study was to evaluate the suitability of the water quality in the spawning grounds of several species for the early development of fish and hence for fish population recruitment and survival. Experiments were conducted in the spring of 2023, which is the reproductive period for numerous freshwater fish species.

2. Material and methods

2.1. Description of the study site and experimental setup

The Garonne Basin (southwest France) covers approximately 56,000 km^2 and represents 10 % of France's mainland surface area. It comprises two main urban areas (Bordeaux and Toulouse), and most of the land use is dedicated to agriculture, mainly for cereal crops, fruit trees, and vineyards (Bernard et al., 2019). The Garonne River flows 580 km from its source, located in the Pyrenees, to the Gironde Estuary on the Atlantic coast. A portion of the Garonne's water is diverted from Malause (France) to supply Golfech's hydroelectric and nuclear power plants. This diversion is approximately 15 km long and the diverted water reconnects with the Garonne at Lamagistère (France). We conducted our experiments in Golfech, immediately upstream of the hydrodam and approximately 100 km downstream of the city of Toulouse (Fig. 1).

A "field lab" was installed at the hydroelectric power plant of Golfech (44°06′35.6″N 0°51′15.6″E), 40 m above the dam. Water was pumped directly and continuously from the canal (at a flow rate of 250–300 L/h) into a filtration and decantation tank outside the field lab (Fig. 2). This device consisted of a 300-L tank in which water passed through blocks of filtration foam (Ø 2–4-mm pores) to prevent large particles from entering and blocking the water circulation in the system. The filtration blocks were rinsed with tap water every two days. Then, water was supplied inside the "field lab" into a first distribution tank (80 L), from which it was distributed to the fish embryos and gammarids exposure aquariums. The exposure system and water parameter monitoring for Japanese medaka embryos and gammarids are detailed below. Water exiting the exposure systems was collected in a final tank (exit tank) equipped with a block of filtration foam (Ø 1–2-mm pores) to prevent the escape of organisms before returning to the river. The photoperiod was maintained at a light/dark cycle of 14:10 h.

2.2. Water analysis: pesticides, trace metals and microbiology

2.2.1. Pesticides analysis

Two distinct deployments of a Polar Organic Chemical Integrative Sampler (POCIS) of two weeks each were performed in April during the same period as the two Japanese medaka exposures and *G. fossarum* feeding assays. The first deployment of POCIS was from 4/5/2023 to 4/20/2023, and the second deployment was from 4/20/2023 to 5/4/2023. Two sets of POCIS were deployed simultaneously in the "exit tank" to account for contaminants that passed through exposure systems. Two types of passive sampler were used in each deployment. POCIS-HLB (containing an oasis hydrophilic–lipophilic phase) was used to monitor 44 neutral pesticides. POCIS-MAX (containing an Oasis Mixed-mode, strong anion-exchange) was used to monitor 19 anionic pesticides. A total of 63 chemical compounds were investigated. The methods and analyses were performed as described by Bernard et al. (2019). For each POCIS deployment, chemical analysis provided the average concentration in water of each contaminant ($\mu g.L^{-1}$). Controls consisting of POCIS (blank) exposed to air at the same time as the POCIS exposure were used.

2.2.2. Trace metals analysis

Regarding metals concentrations in the Garonne water, 15 trace metals have been monitored (V, Cr, Co, Ni, Cu, Zn, As, Sr, Mo, Ag, Cd, Sn, Sb, Ba and Pb). Every day, 12 mL of water was sampled from each condition directly in fish embryo exposure incubators, filtered (Ø <0.2 µm; Sartorius-Minisart®), and acidified with nitric acid (2 %; HNO_3 Ultrapure J.T. Baker®). Analyses were performed for all samples collected under the Garonne condition and at T0, T6, and T14 (beginning, middle, and end of the experiment, respectively) for the control condition in each batch. Analyses were conducted using TQ-ICP-MS (Thermo Scientific ICAP TQ-ICP-MS). External calibration was performed in a matrix similar to the samples (*i.e.* HNO_3; 2 %). A certified reference material (SLRS-6; Yeghicheyan et al., 2019) was used to determine the accuracy of the trace metal analyses. The obtained concentrations showed good recovery.

2.2.3. Microbiological analysis

Water samples (50 mL) were collected in triplicate for each condition on the first and last days of each experiment. Additional samples were collected on the days when high embryo mortality was observed. All samples were then transferred to the Microbiology R&D platform (Aquitaine Microbiology, Bordeaux University) for microbiota analysis

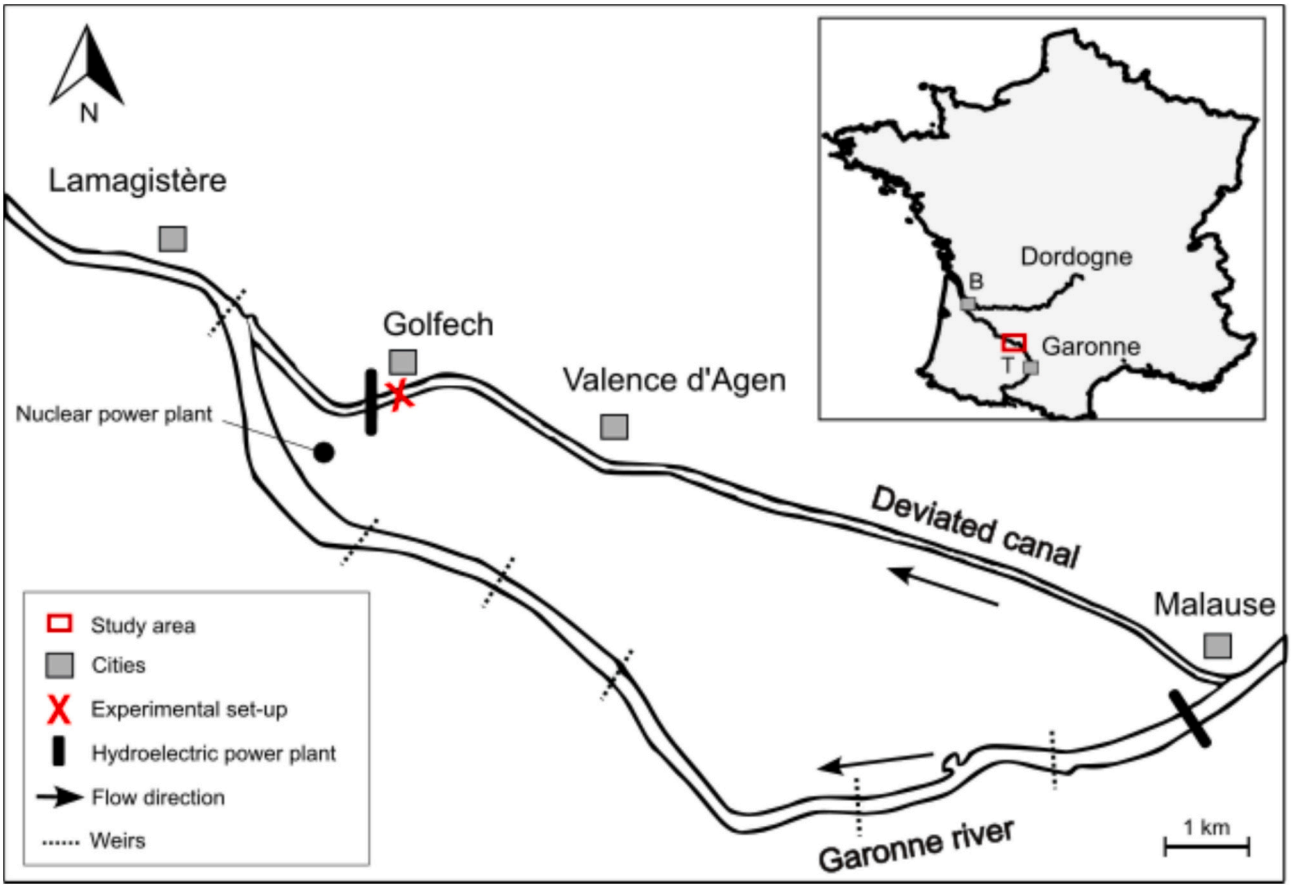

Fig. 1. Map of the study area (adapted from MIGADO and Paumier et al., 2019). There are two dams on each section: Malause and Toulouse). On the map of France, city legends are B for Bordeaux and T for Toulouse.

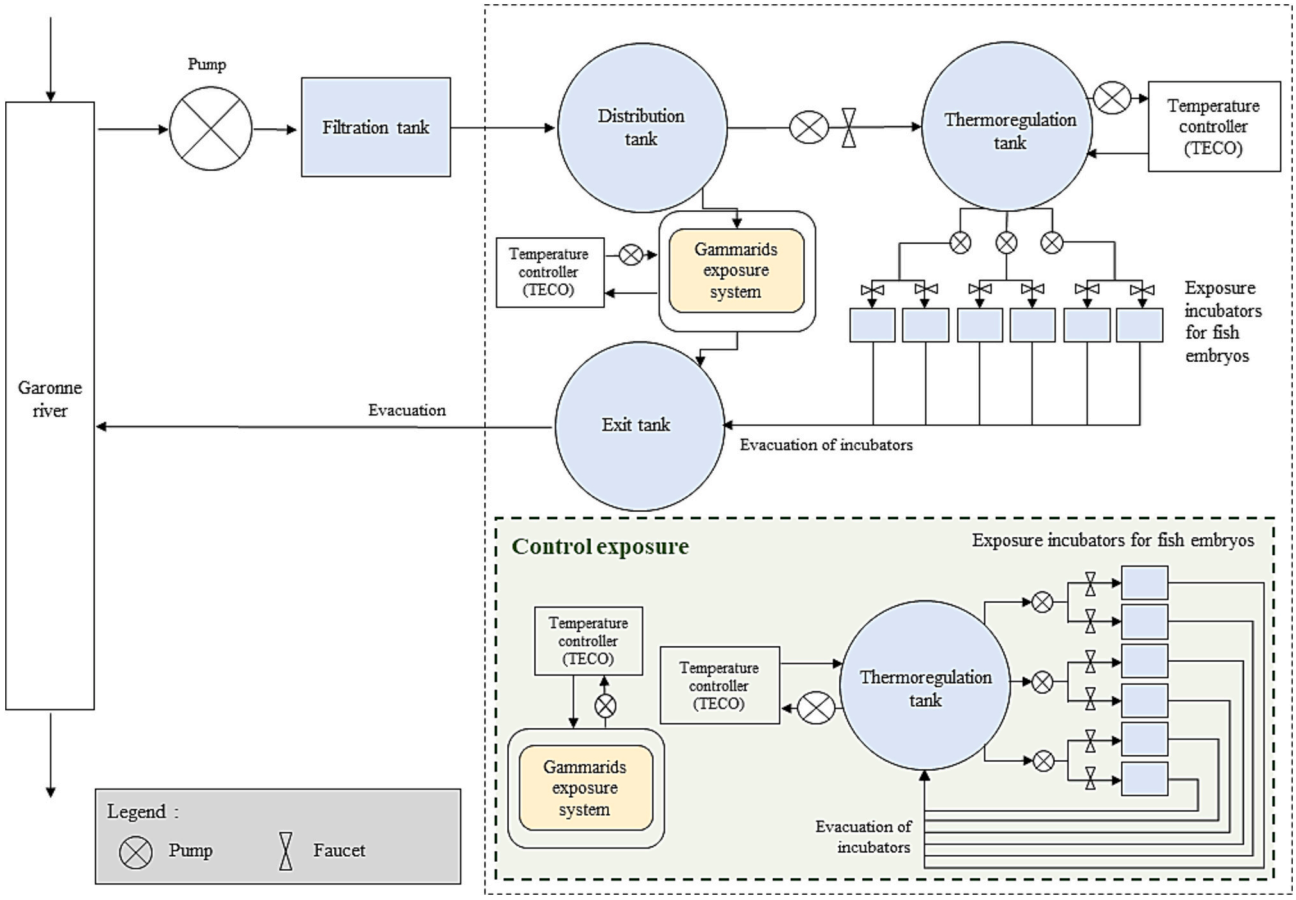

Fig. 2. Diagram of the *ex-situ* experimental device for the exposure of early life stages of Japanese medaka and adults of gammarid to the Garonne water and the control circuit. About 20 m separate the field lab (represented by the grey dotted rectangle) from the river. Within the field lab, the green dotted rectangle represents the control exposure circuit and gammarids' exposure system is represented in yellow.

by mean of culturomics based on previously published protocols (Lagier et al., 2015, 2018). Every sample was divided into several aliquots upon reception in the microbiological laboratory. The aliquots were then subjected to serial dilutions and all dilutions were then cultured in various agar media and growth conditions recommended for the recovery of aerobic, microaerophilic and anaerobic microorganisms presents in the sample. The incubation time was up to one week for anaerobic microorganisms. Reading of the agar plates was carried out daily and all isolated colonies were identified by mass spectrometry (MALDI-TOF MS, Microflex, Bruker, France). The results were then analysed by two veterinarians specialized in fish pathologies.

2.3. Test with gammarids

Adults of *Gammarus fossarum* were collected in ponds through 2- and 2.5-mm sieves using nets in Saint-Maurice-de-Remens (France). The organisms were then acclimatised for 14 d in the laboratory in drilling water at 12 ± 1 °C and fed *ad libitum* with alder leaves (*Alnus glutinosa*). Gammarid toxicity was evaluated using reproductive parameters (reprotoxicity assays) and feeding assays. The tests were conducted as described previously (Geffard et al., 2010; Leprêtre et al., 2023) and according to the French standard protocols AFNOR XP T90-722-2 and AFNOR XP T90-722-3. Reprotoxicity test consists of exposing females in A stage carrying embryos that have just been fertilised. Exposure is stopped just before the embryos are released from the marsupium and the viable embryos are then counted. Feeding rate test consists of exposing a known number of gammarids with alder leafs of known surface. Exposure lasts 2 weeks and the number of surviving gammarids and the surface area consumed were assessed, making it possible to calculate a feeding rate in terms of surface area of leaf consumed per individual per day. Two distinct reprotoxicity assays were conducted: A (4/6/2023–4/26/2023) and B (4/26/2023–5/17/2023). Two feeding assays were conducted in April: A (4/6/2023–4/19/2023) and B (4/19/2023–5/3/2023).

In the "field lab", water from the distribution tank was pumped directly into buckets (placed in a crase) containing exposure systems (Fig. 2). Water was then collected by overflow in the crase where it was thermoregulated at 12.5 ± 1 °C using temperature controllers (TECO TK 1000). The temperature was recorded hourly using a TinyTag Aquatic 2 data logger. Organisms were placed in exposure systems (punctured polypropylene cylinders) and exposure began when the systems were placed in buckets. For the feeding assays, five replicates (systems) with 15 males of the same size were placed on 20 alder leaf disks of a known diameter for a two-week exposure period (14 d). For the reprotoxicity assays, four replicates with seven females in the A-B moulting stage were subjected to a three-week exposure (21 d) (Geffard et al., 2010). For the reprotoxicity assessment, females were fed *ad libitum* with alder leaves. The biological responses (male survival, feeding rate, and number of viable embryos per female) were assessed at the end of exposure. Controls were conducted in the laboratory using drilled water at the same temperature, with the same number of individuals and replicates as in the Garonne exposure condition.

2.4. Test with medaka embryos

Two distinct batches of Japanese medaka (*O. latipes*) embryos of the Carbio strain provided by INRAE LPGP (Rennes, France) were used for two independent experiments (Experiment 1: 4/6/2023 to 4/19/2023,

and Experiment 2: 4/20/2023 to 5/3/2023).

2.4.1. Exposure design

To expose medaka embryos to the Garonne River water, water from the distribution tank was pumped to a second 80-L tank for thermoregulation and oxygenation (Fig. 2). The temperature was set within the optimal range for medaka embryo–larval development, *i.e.* 24 ± 2 °C (Iwamatsu, 2004; OECD, 1998). For the same reason, oxygen saturation was maintained above 90 % using an air pump and oxygen diffusion systems. Water was then pumped into six exposure incubators (1 L), as previously described by Delage et al. (2014). The incubators were made of modified polyethene terephthalate glycol (PETg) with an added stainless grid, where the embryos were laid to ensure bottom-up circulation of water. The exposure incubators are also equipped with a grid with holes of <1 mm to prevent leaks of organisms in the environment. The control exposure was conducted in parallel in a field laboratory using a closed exposure circuit. As in the Garonne exposure circuit, an 80-L tank was filled with dechlorinated tap water for thermoregulation and oxygen saturation. The targeted temperature was adjusted to 24 ± 2 °C also using thermocontrollers (TECO TK). Water was pumped into six modified incubators for embryo exposure. The flow rate in the incubators for control and Garonne conditions was between 12 and 18 L. h^{-1}.

For both conditions, five incubators were used as replicates for embryo exposure, and one to monitor water parameters (Tinytag Aquatic 2 data loggers for temperature and multiparameter probe WTW Multi-Line® Multi 3630 IDS for pH, oxygen saturation rate, and conductivity). The temperature was recorded once per hour, and the pH, conductivity, and oxygen saturation rate were recorded once per day in the morning. Nitrogen forms (NH_4^+, NO_3^-, and NO_2^-) and phosphates (PO_4^{3-}) were also monitored using kits (JBL Pro Aquatest Kits®). NO_2^- was monitored daily, and NH_4^+, NO_3^-, and PO_4^{3-} were monitored every two days. Turbidity was monitored daily by sampling the water and measuring it with a portable turbidimeter (Hach 2100).

2.4.2. Embryotoxicity test

Exposure began 24 h post-fertilisation (hpf). Between 57 and 65 embryos in gastrula were randomly placed on a stainless-steel grid in each incubator throughout development until hatching. During the experiment, survival and hatching were monitored daily and dead individuals were removed. Embryonic survival was calculated by dividing the number of viable embryos at the end of the experiment by the total number of viable embryos at the beginning of the experiment. Hatching success was calculated by dividing the total number of hatched embryos by the total number of viable embryos at the beginning of the experiment. Half-hatched larvae were counted if at least one part of the larval body was outside the chorion. The percentage of half-hatched larvae was calculated by dividing the number of half-hatched larvae by the number of viable embryos at the beginning of the experiment.

Exposure was stopped two days after the hatching peak (in order to stay on eleutheroembryonic stages, according to Balon, 1975) under at least one of the two conditions, meaning that more than half of the organisms had hatched in more than half of the replicates. As far as possible, ten hatched larvae per replicate were used for biometric measurements, corresponding to 50 larvae for control at the end of the two experiments and 48 and 23 larvae for experiments 1 and 2 (respectively) in the Garonne condition. Images were captured with a Dino Lite® digital microscope (magnification of 32.5×) and measurements (total length, head length, yolk sac) were obtained using the ©ImageJ software. Larvae presenting developmental abnormalities (including oedema and skeletal, cardiovascular, and yolk sac abnormalities) were also counted in each replicate. The percentage of malformed individuals was calculated by dividing the number of larvae with at least one malformation by the number of larvae sampled at the end of the experiment.

2.4.3. Quantitative analysis of transcription levels

At the end of the first experiment, five larvae per replicate were sampled under each condition. They were immediately frozen in dry ice for lipid peroxidation analyses (thiobarbituric acid-reactive substances (TBARS)) and stored at −80 °C until analysis. For transcriptomic analyses, larvae were placed in a fridge with 200 μL of RNA Later for 24 h and then stored at −20 °C until analysis. Only the larvae from the first experiment were used for TBARS and transcriptomic analyses, as their survival at the end of the exposure was higher than that in the second experiment.

Total RNA extraction from medaka larvae was carried out on single larvae, with five larvae per replicate (five replicates for control and four for Garonne) using the SV Total RNA Isolation system (Promega) following the supplier's instructions. First-strand cDNA was synthesised from 300 ng of total RNA using the GoScript Reverse Transcription System (Promega). The cDNA was then diluted 15-fold. Finally, PCR reactions were performed using a LC480 (Roche), with the following conditions: 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Each 15-μL reaction contained 7.5 μL of GoTaq qPCR Master mix (Promega), 5 μL of template, and the specific primer pairs at a final concentration of 200 nM each. In order to investigate the potential effects of the Garonne water on fish, the transcription level of 20 genes involved in fish development, immunity, energy metabolism and toxicity management was analysed (Table 1). Reaction specificity was determined from the dissociation curve of the PCR product. Relative quantification of gene transcription was performed by the concurrent amplification of *actb1*, *eef1a* and *mob4* endogenous controls (Hu et al., 2016; McCurley and Callard, 2008). Total RNA was quantified, and the same quantity was used for reverse transcription. During subsequent qPCR amplifications, the output cycles of endogenous controls were examined. No significant difference was observed in the output cycles of *actb1* and *eef1a* among fish, in contrast to mob4. Relative quantification of the expression of each gene was normalised based on the mean Ct value of the first two genes. The amplification efficiencies for all primer sets were calculated and found to be sufficient for direct comparison.

2.4.4. Lipid peroxidation (TBARS)

Pools of five larvae were homogenised using a G50 Tissue Grinder (Coyote Bioscience Company) at 3000 rpm for 20 s in a 4 °C chilled phosphate buffer (0.1 M; pH 7.4). The supernatant S9 fraction was obtained by centrifuging samples for 20 min at 9000*g* (4 °C). Lipid peroxidation was estimated by measuring TBARS according to the method by Buege and Aust (1978). The protocol described by Weeks Santos et al. (2019) was adapted for the microplate reader. Briefly, Solutions of 20 % trichloroacetic acid and 2 % butylated hydroxytoluene were added to the S9 fraction and centrifuged for 10 min at 9000*g*. Then, hydrochloric acid (0.6 N) and TRISbase (25 mM)–Thiobarbituric acid (100 mM) were added to the supernatant and heated at 80 °C for 15 min. Absorbance was measured at 530 nm using a spectrophotometer microplate reader (Fluostar Optima, BMG Labtech). Results are expressed in nmoles of TBARS per millilitre of solution (nmoles.mL^{-1}). As the quantity of lipids depends on larval size, the results were normalised for each replicate by the mean total length of the larvae in each replicate.

2.5. Statistical analysis

Statistical analyses were performed using the R software (http://cran.r-projet.org/). Statistical significance level was set at 5 %. There were no differences between the replicates and all comparisons were made between the control and Garonne conditions for each experiment.

For Japanese medaka embryonic mortality and hatching kinetics, log-rank test (on Kaplan–Meier analysis) was performed to compare the control and Garonne conditions. Hatching events and embryonic mortality (for embryonic survival and hatching kinetics, respectively) were considered in the analysis. The number of half-hatched larvae, lipid peroxidation, and relative gene expression were compared between the

Table 1
: Names, biological functions and accession numbers of the genes analysed by quantitative RT-PCR. Specific primer pairs: F, forward; R, reverse.

Gene	Name	Primer sequences	Function	Accession number
ahr	Aryl hydrocarbon receptor	F_CCTGGAAATCCGCACAAAG R_CGGACGTGGTTTTCGGC	Defense, xenobiotic receptor	NM_001104678.1
gstm3	Glutathione S-transferase Mu 3	F_CTGGCGCCAAGTATGAGGAA R_CTTCAGTTTCCCCGCAAAGC	Antioxidant defense	XM_004070400.3
mt	Metallothionein	F_AGGGAAGACCTGCGACACTA R_TGGACAAATGGCACGTTAAA	Metal detoxification	NM_001104785.1
rad51	DNA repair protein RAD51 homolog 1	F_AGTGTGGAATCAGCTCCAGC R_CCGACCTTCTCTGGTGGAAC	DNA repair	XM_004083901.3
tp53	Cellular tumor antigen p53	F_ACTGTAAATGAGCCCACCGG R_CGTCACAGACTTTGCAGTGC	Apoptosis	XM_020711402.1
apex1	DNA-(apurinic or apyrimidinic site) endonuclease	F_AGGAGCATGACAAAGAGGGC R_CCTGGTGGGCTACATTGAGG	DNA damage, oxidative stress	XM_004082982.3
line1	Long interspersed nuclear element-1	F_GAGGGAAATGAAATGGCTGA R_AACCAGTGTGTCCCATCCTC	Genome stability	Helmprobst et al., 2021
ache	Acetylcholinesterase	F_CCTCCTCTTCCTCATCCCCA R_GCATTAGGGTAGGAGTCGGC	Nervous system, neurotoxicity marker	XM_020711765.1
C3–1	Complement component C3–1	F_CGCATGGAGCCACTAGAGAG R_CTCTCCTGTGGGTTGCTCTG	Immunity	NM_001105082.2
il1b	Interleukin 1B	F_AAAACGTCTCCTCTGGCGAG R_GACCGTCATCCCACTGTTGT	Immunity	XM_020707411.1
chgh	Choriogenin H	F_CTCAGCCCCCTCAGAATCCT R_TCCACAGGGGACTCTCACAT	Endocrine system, estrogenic activity	NM_001104807.2
dmy	DM-domain gene on the Y-chromosome	F_TCCTATTATGGAAACCTGCACAACTAC R_GAAGGAGTGCATGCGGTACTG	Endocrine system, sex determination	XM_020700511.1
cox1	Cytochrome *c* oxidase subunit1	F_TTCCCCCAACACTTCTTCTTAGGC R_TGTGGCTGTTAGTTCGACTGA	Mitochondria, energy metabolism	NP_739817.1
cs	Citrate synthase	F_GTTGTTGCCCAAAGCTCCAG R_TGTGATCGCAGCACTGAACT	Mitochondria, energy metabolism	XM_004070888.3
dnmt3ba	DNA (cytosine-5-)-methyltransferase	F_GGCGATCAGACTGCATCGTA R_CACCAGACCGGAGAGCTTTT	Embryogenesis, cell differentiation	XM_011477502.2
tet2	Methylcytosine dioxygenase TET2	F_TCACCCATCCCCATGCAATC R_GGGCTGGTATCGAGGATTGG	Embryogenesis, cell differentiation	XM_004065914.3
nkx2.5	NK2 homeobox 5	F_CTGGAGCAGAACCAAGACGT R_CAGAGGTAAAGCTGAGCGCA	Embryogenesis, vasculogenesis	NM_001104912.1
vegfab2	Vascular endothelial growth factor A	F_CAAGAGTGCCCACATACCGA R_CAGCATCTCGTCATTGCAGC	Embryogenesis, vasculogenesis	XM_020714411.1
mlc2	Myosin regulatory light chain	F_ACAGAGACGGCATCATCAGC R_GGGGTCCAGGACTTTGAAGG	Embryogenesis, cardiogenesis	XM_004071182.3
sox4	SRY-box transcription factor 4a	F_CAAGAGACCCATGAACGCCT R_CTGGGCCGGTACTTGTAGTC	Embryogenesis, neurogenesis	NM_001164881.1
eef1a	Elongation factor 1-alpha, somatic form	F_GACAAAAGCCACTGCAGCTG R_CTGGCGTTGGTGAGTTTGAG	Endogenous control	NM_001104662.1
actb1	Actin, cytoplasmic 1	F_GCGTCATCTTTTCTCTGTTAGCC R_GGGCCAGAAAGACAGCTATGT	Endogenous control	XM_004065978.3
mob4	MOB family member 4	F_TAGGAGGAATCGGCCTGGAA R_GTGACACAGGAACGTCTCGT	Endogenous control	XM_020702103.1

two conditions using a non-parametric test (Mann–Whitney). For biometric measurements (total length, head length, yolk sac surface, and head/total length ratio), there was no homogeneity of variance between conditions for all variables (Levene test), so non-parametric tests (Mann-Whitney) were performed to compare conditions.

For *G. fossarum* biological responses, non-parametric (Mann–Whitney) tests were performed to compare the exposure conditions.

3. Results

3.1. Exposure conditions

3.1.1. Water parameters

The physicochemical parameters of the Japanese medaka embryos and *G. fossarum* exposure are detailed in Table 2. At the end of the experiments with Japanese medaka, the mean temperature was within the optimal range for embryo–larval development under both conditions. The conductivity (between 255 and 312 μS cm^{-1} for all conditions and experiments), pH (7.9–8.4), oxygen saturation rate (above 90.0 % for all conditions in both experiments), and turbidity (less than ten FNU (Formazine Nephelometric Unit)) were also within the ranges allowing for the optimal development of medaka embryos. Additionally, the nitrogen forms and phosphate values did not reach levels that could be toxic to embryos. For the gammarid experiments, the exposure temperature was within the target range of 12.5 ± 1 °C for all experiments (Table 2).

3.1.2. Water quality analysis

Among the 63 pesticides investigated, 14 were present in quantifiable concentrations in Garonne water, as revealed by the POCIS results (maximum concentrations are presented in Table 3. Fifteen trace metals were analysed, all of which were quantified (Table 3). An exhaustive list of the monitored compounds, quantification limits, and detailed results for both pesticides and trace metals are provided in Supplementary Materials 1, 2 and 3. Table 3 also includes the results obtained from the Adour-Garonne Agency as part of the WFD framework (https://adour-garonne.eaufrance.fr/data/) for the 2022–2023 period at the closest station (Lamagistere, station number 05117000). Additionally, the available predictive-no-effect concentrations (PNECs) for freshwater species, no-effect concentrations (NOEC, here only regarding mortality for early life stages of fish), and the percentage of the maximum concentration retrieved during our study compared with the PNEC are also presented.

Microbiological analyses by culture were performed at three time points in both experiments in the Garonne River treatment (Supplementary Material 4). Our analyses highlighted a large variety of bacteria

Table 2
Physico-chemical parameters measured during experiments for both species (experiments 1 and 2 for Japanese medaka exposures, and experiments A and B for *G. fossarum* exposures). Results for temperature, pH, conductivity, oxygen rate are expressed by mean ± standard deviation and maximal values for nitrogen forms, $PO4^{3-}$ and turbidity.

	Experiment condition		Experiment 1		Experiment 2	
			Control	Garonne	Control	Garonne
Japanese medaka embryo exposure	Temperature (°C)	Mean ± SD	23.4 ± 0.7	23.7 ± 0.8	24.8 ± 0.2	24.8 ± 0.3
	pH	Mean ± SD	8.2 ± 0.1	8.3 ± 0.1	8.1 ± 0.1	7.9 ± 0.1
	Conductivity ($\mu S.cm^{-1}$)	Mean ± SD	278.4 ± 6.4	300.4 ± 7.7	301.4 ± 5.4	279.5 ± 12.9
	Oxygen saturation rate (%)	Mean ± SD	96.3 ± 1.2	104.0 ± 2.0	98.6 ± 0.8	98.9 ± 2.2
		Min	94.4	100.8	96.9	95.3
		Max	98.2	106.4	99.5	103.4
	$NH4^{+}$ ($mg.L^{-1}$)	Max value	<0.05	<0.05	<0.05	<0.05
	$NO3^{-}$ ($mg.L^{-1}$)	Max value	1.0	1.0	1.0	1.0
	$NO2^{-}$ ($mg.L^{-1}$)	Max value	0.0	0.1	0.1	0.1
	$PO4^{3-}$ ($mg.L^{-1}$)	Max value	<0.02	<0.02	<0.02	<0.02
	Turbidity (FNU)	Min value	–	1.0	0.2	1.7
		Max value	–	3.9	0.4	8.5

	Experiment condition		Experiment A		Experiment B	
			Control	Garonne	Control	Garonne
G. fossarum exposure	Reprotoxicity	Temperature (°C, mean ± SD)	12.0 ± 0.1	12.3 ± 0.04	12.4 ± 0.2	12.2 ± 0.2
	Feeding assay	Temperature (°C, mean ± SD)	12.0 ± 0.1	12.4 ± 0.1	12.3 ± 0.3	12.3 ± 0.1

Table 3
Maximum concentrations of pesticides and trace metals in the Garonne River during the experiments compared to mean concentrations in water at the closest station monitored for the Water Framework Directive (Lamagistère, station number 05117000, https://adour-garonne.eaufrance.fr/data/ficheStation?stq=05117000&panel=eco). When not indicated, data are expressed in $\mu g.L^{-1}$. Data were also compared to the Predicted No Effect Concentration (PNEC) and No Effect Concentrations (NOEC) for mortality of fish embryos available. References for PNEC pesticides are the French Agency for Food, Environmental and Occupational Health and Safety (https://www.anses.fr/fr) and the European Chemical Agency for trace metals (https://echa.europa.eu/fr/home). References for NOECs are available in Supplementary Material 7.

	Compound	Maximum concentration in the Garonne River	Mean concentration (AEAG)	NOEC	PNEC for freshwater	Max concentration / PNEC (%)
Pesticides ($\mu g.L^{-1}$)	Alachlore	0.003	< LQ	1100	0.25	1.2
	Dimethanamide	0.002	< LQ	4100	1.78	0.12
	IPPU	0.002	–	–	–	–
	Metolachlore	0.006	–	5000	6.7	0.1
	2,4-D	0.04	< LQ	1240	30	0.13
	Dicamba	0.006	< LQ	14,000	45	0.01
	MCPA	0.02	< LQ	50,000	50	0.03
	Mecoprop	0.01	< LQ	LOEC (deformities) = 25,000	44	0.03
	Mesotrione	0.03	< LQ	7.5	0.77	4.22
	Metalochlor OA	0.1	–	–	–	–
	Metolachlor ESA	0.01	0.14 ± 0.1	–	–	–
	Metsulfuron	0.006	< LQ	LC50 > 1200 $mg.L^{-1}$	0.11	5.44
Trace metals ($\mu g.L^{-1}$)	Vanadium (V)	0.6	–	480	17.8	–
	Chrome (Cr)	0.2	< LQ	12,000	3.4	5.9
	Cobalt (Co)	0.1	–	60	1.06	9.4
	Nickel (Ni)	0.5	0.6 ± 0.2	250	–	–
	Copper (Cu)	0.8	1.5 ± 0.8	90	4	20.0
	Zinc (Zn)	1.5	5.1 ± 5.2	–	6.5	23.1
	Arsenic (As)	1.2	1.6 ± 0.7	7380	5.6	–
	Strontium (Sr)	122.0	–	–	2900	4.2
	Molybdenum (Mo)	0.3	–	–	11,900	0.0
	Silver (Ag)	0.00	–	500	0.86	0.0
	Cadmium (Cd)	0.01	0.02	20	0.19	5.3
	Tin (Sn)	0.01	–	–	–	–
	Antimony (Sb)	0.2	–	–	–	–
	Baryum (Ba)	28.0	–	–	–	–
	Lead (Pb)	0.1	0.2 ± 0.1	48	2.4	4.2

(29 species) in the Garonne River's water. In the first experiment, bacterial diversity increased over time (5 species at T0, 10 at T6, and 13 at T13). In contrast, in the second experiment, the number of bacterial species remained constant over time (6 species at T0, 5 at T6, and 6 at T13). In addition, ten species were detected at one or two sampling times, and only three species were found more frequently: *Bacillus cereus* (5/6), *Staphylococcus epidermidis* (4/6), and *Fictibacillus halophilus* (4/6). It is noteworthy that the majority of microorganisms recovered have been frequently reported in aquatic environments. To the best of our knowledge, none of the detected bacteria were pathogenic to fish. However, the level of antimicrobial resistance has not yet been determined.

3.2. Toxicity for G. fossarum

Fig. 3 shows the results of the feeding assay and reprotoxicity tests of *G. fossarum,* conducted for each experiment. For both experiments, there were no significant differences in the mean survival of adult male gammarids (Fig. 3A), with values of 85.3 ± 8.7 % and 86.7 ± 4.7 % for experiment A, and 73.3 ± 9.4 % and 68 ± 13.7 % for experiment B (mean ± SD for control and Garonne conditions, respectively). The mean feeding rates were significantly different between the control and Garonne conditions in both experiments (Fig. 3B). Gammarids exposed to the Garonne River water showed higher feeding rates, with an induction of 41.3 % in Experiment A and 29.2 % in Experiment B compared with the control. Regarding embryotoxicity, the mean number of viable embryos per female was not significantly different between the conditions in either experiment (Fig. 3C).

3.3. Toxicity for Japanese medaka embryos

3.3.1. General survival and hatch rates

Fig. 4 shows the overall results for all replicates of survival and hatching for the two embryo toxicity tests conducted on Japanese medaka. Under the control conditions, the mean survival of embryos and larvae was consistent across all replicates in both experiments. Total survival exceeded 80 % for all control replicates, including the number of larvae and viable embryos to hatch. The hatching rate was satisfactory in all control replicates in both experiments, with mean hatching rates of 82.7 % in Experiment 1 and 77.3 % in Experiment 2. Larval mortality was low, at 0 % and 5.4 % for Experiment 1 and 0.3 % and 4.1 % for Experiment 2 under control and Garonne conditions, respectively. Most mortalities occurred during the embryonic period in the Garonne condition, with mortality rates of 55.4 % and 85.3 % for Experiments 1 and 2, respectively, resulting in lower hatching rates of 33.4 % and 11.9 % for Experiments 1 and 2, respectively.

3.3.2. Embryonic survival

Fig. 5 shows that embryos incubated under the control condition had a significantly higher probability of survival than those incubated under the Garonne condition ($p < 0.001$ for both experiments; log-rank analysis). The survival rates under the two conditions were similar during the first five days of the experiments. However, a significant increase in embryonic mortality was observed in both experiments, starting at approximately 150 h post-fertilisation.

3.3.3. Dynamic of hatchings

Fig. 6 shows a significantly lower percentage of hatched larvae under the Garonne condition than under the control condition ($p = 0.0033$ and $p < 0.001$ for Experiments 1 and 2, respectively). Additionally, hatching began earlier under the Garonne condition than under the control in each experiment (144 hpf in Garonne for Experiments 1 and 2; 240 and 216 hpf under the control for Experiments 1 and 2, respectively). The mean percentage of half-hatched larvae was higher under the Garonne condition in both experiments (2.3 % and 11.2 % under Experiments 1 and 2, respectively) than under the control condition (0 % in both experiments), but this difference was only significant in experiment 2 (p-value = 0.07 and 0.007 for Experiments 1 and 2, respectively) (Table 4).

3.3.4. Biometrics

At the end of the exposure period, 50 larvae were considered in each condition for the biometric study in Experiment 1 (10 larvae per replicate), and 48 and 23 (control and Garonne, respectively) in Experiment 2 (Table 4). Only three malformed larvae (skeletal deformities) were observed at the end of Experiment 1 under the Garonne condition, representing 6.25 % of the total number of larvae sampled at the end of the experiment. No developmental malformations were observed under the control condition in Experiment 1 or under either condition in Experiment 2. The total body and head lengths of the larvae, as well as the head/total length ratio, were significantly different between the control and Garonne conditions, but only in the second experiment (Table 4). The results indicated that larvae exposed to Garonne water had a shorter body length and longer head compared with control larvae in Experiment 2. Significant differences in yolk sac surfaces were observed between the two conditions for both experiments, with smaller yolk sacs for larvae exposed to the Garonne conditions. However, the differences between the two conditions are not particularly significant.

3.3.5. Gene expression

The 20 targeted genes were chosen to investigate several functions or physiological parameters: detoxification and antioxidant defense (*ahr, gstm3*, and *met*), neurotoxicity (*ache*), DNA repair (*apex1* and *rad51*), DNA damage (*line1*), apoptosis (*tp53*), immunity (*C3-1* and *il1b*), endocrine system (*chgh* and *dmy*), mitochondrial metabolism (*cox* and *cs*), embryogenesis (*dnmt3ba*, *sox4*, and *tet2*), and specifically cardiogenesis (*mlc2*) and vascularisation (*nkx2.5* and *vegfab2*). The results (ratios compared with the control and significance levels) are shown in Supplementary Materials 5 and 6. Of the 20 genes monitored, 9 showed significant differences between the control and Garonne conditions:

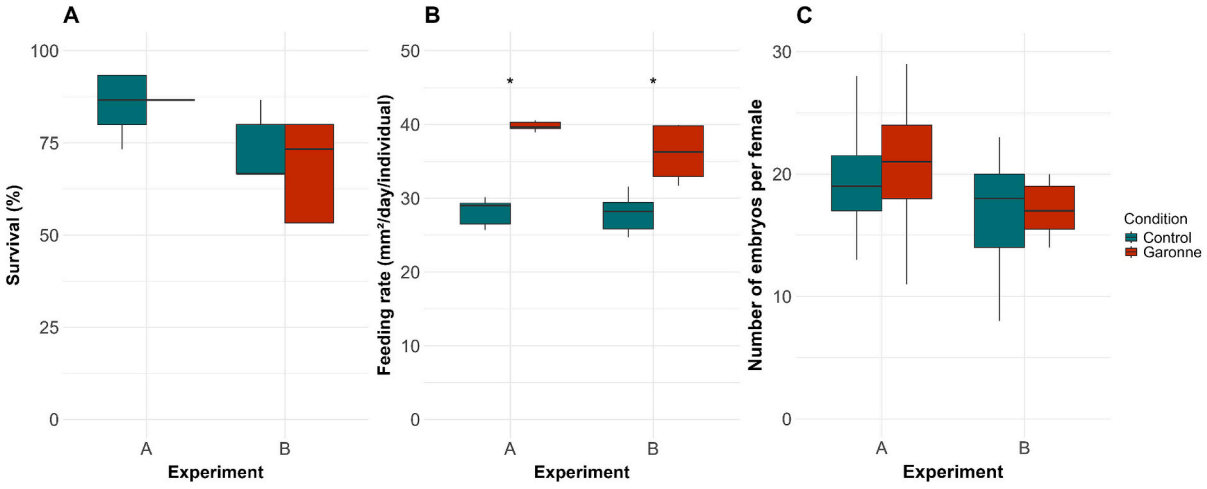

Fig. 3. Results of toxicity tests with *G. fossarum* for each condition and for each experiment (A or B). Experiments were conducted in the *ex-situ* exposure system on the Garonne river in spring 2023. A: Mean percentage of survival for each studied condition. $N = 5$. B: Mean feeding rate (mm^2/individual/day) for each studied condition. N = 5C: Mean number of embryos per female for each studied condition. $N = 4$. For each experiment, asterisks represent significant differences between the control and the Garonne conditions (Mann-Whitney test, $p < 0.05$).

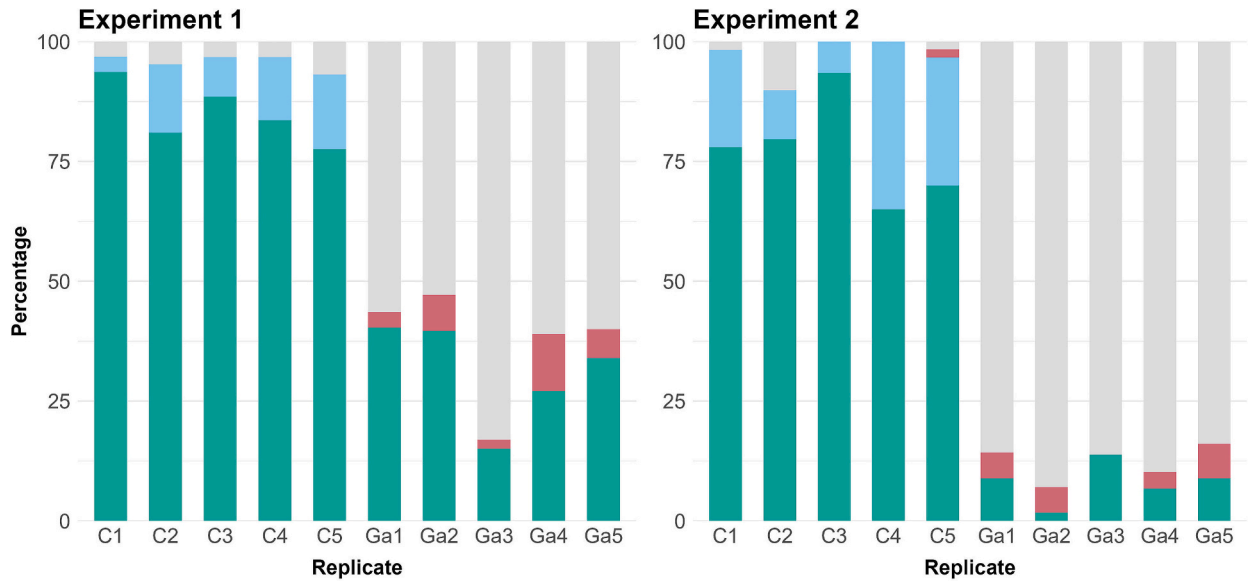

Fig. 4. Final survival and mortality of Japanese medaka embryos and larvae for each replicate and each studied condition. Two independent experiments were conducted in the *ex-situ* exposure system on the Garonne river in spring 2023 (Experiment 1 and 2). Detail of replicates: Control condition: C1, C2, C3, C4, C5; Garonne condition: Ga1, Ga2, Ga3, Ga4, Ga5. Legend: grey corresponds to the cumulated embryonic mortality, pink to cumulated larval mortality, blue: remaining viable embryos, green: larval survival.

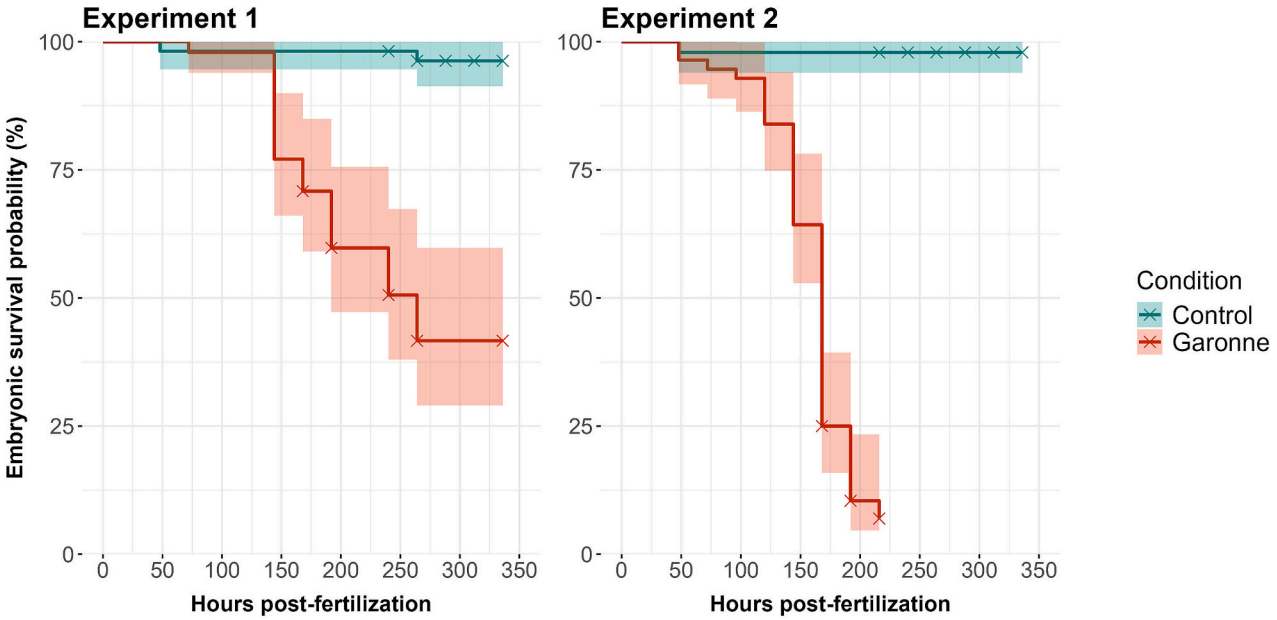

Fig. 5. Embryonic survival probability of Japanese medaka embryos for each studied condition. Two independent experiments were conducted in the *ex-situ* exposure system on the Garonne river in spring 2023 (Experiment 1 and 2). Values are mean ± confidence interval. *P*-value <0.001 for both experiments (log-rank analysis between both conditions). Crosses show events of hatch that occurred during the experiment.

ache, cox, dnmt3ba, il1b, mlc2, nkx2.5, rad51, sox4 and *vegfab4*. Among the six genes involved in embryonic development, five showed different transcript levels under these two conditions (*dnmt3ba, mlc2, nkx2.5, sox4*, and *vegfab2*). Genes involved in the neurotoxicity response (*ache*), mitochondrial metabolism (*cox*), immunity (*il1b*), cardiogenesis (*mlc2*), and vascularisation (*vegfab2*) were more highly expressed in the control group than in the Garonne group. On the other hand, *dnmt3ba* (embryogenesis), *nkx2.5* (vascularisation) and *rad51* (DNA repair) were more highly expressed in larvae exposed to Garonne than in the controls. For the *gstm3* gene (detoxification), there was no significant difference; however, the results showed higher variability in responses under the Garonne condition than under the control (Supplementary Material 6). However, the differences in expression remained quite low (fold-changes ranged from 0.62 to 1.87). Sex differentiation in Japanese medaka is strictly genetic, with an XX/XY system similar to that in mammals (Chakraborty et al., 2016). The *dmy* gene, which we monitored in our study, is present only on the Y chromosome, and its expression begins just before hatching in the embryo (Kobayashi et al., 2004). As the transcription analyses were conducted on single larvae (rather than pools), it was possible to determine the number of genetic males and females (XY for males and XX for females) according to the expression of the gene (*dmy* expression was not detectable in females). In experiment 1, there were 8 males in the control group of the 25 larvae analysed and 13 of the 20 larvae analysed in the Garonne group. This

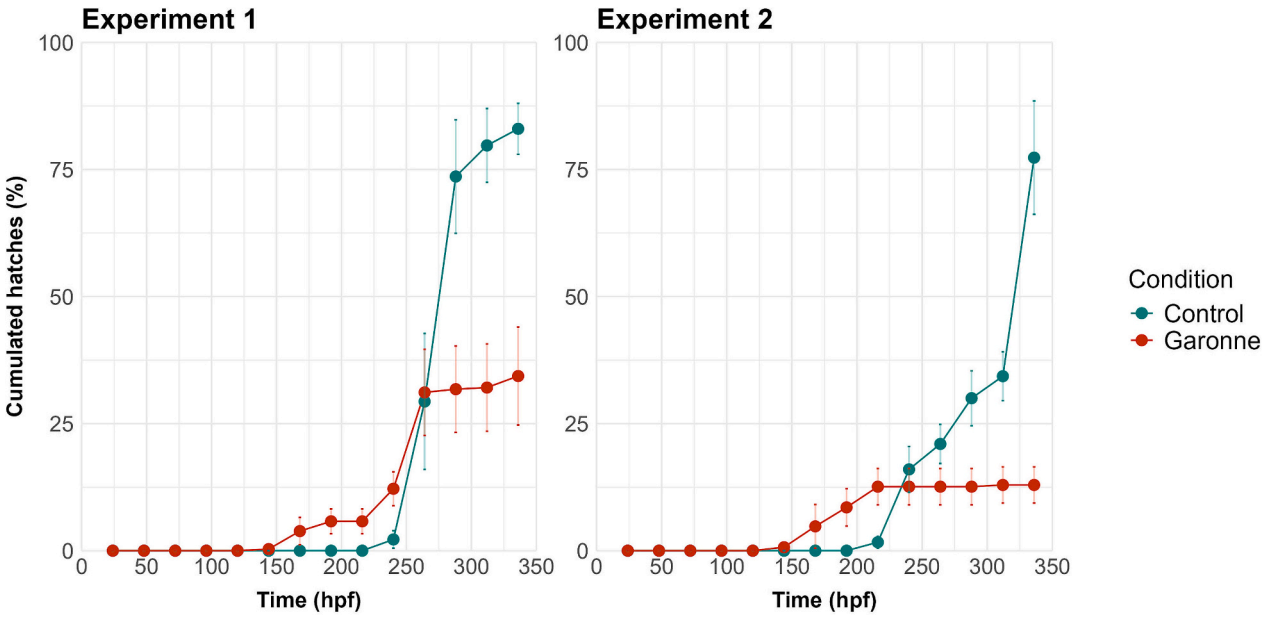

Fig. 6. Cumulated hatchings of medaka embryos for each studied condition. Two independent experiments were conducted in the *ex-situ* exposure system on the Garonne river in spring 2023 (Experiment 1 and 2). Values are mean ± SD. P-value <0.0033 for experiment 1 and <0.001 for experiment 2 (log-rank analysis for each experiment between both conditions).

Table 4
Sub-lethal endpoints in larvae of Japanese medaka after exposure to water from the Garonne compared with the control. Asterisks and bold indicate significant differences between the two conditions for each experiment: p-value <0.05 (Mann-Whitney for percentage of half-hatched and malformed larvae, head and total length, head/total length ratio and yolk-sac surface).

	Experiment 1		Experiment 2	
	Control	Garonne	Control	Garonne
Number of individuals for biometric analyses	50	48	50	23
Half-hatched (%, Mean ± sd)	0 ± 0	2.3 ± 1.5	**0 ± 0**	**11.2 ± 3.8***
Malformed larvae (%)	0	6.25	0	0
Total length (mm, Median (IQR))	5.02 (0.26)	4.98 (0.33)	**5.18 (0.50)**	**4.87 (0.15)***
Head length (mm, Median (IQR))	1.22 (0.18)	1.22 (0.18)	**1.22 (0.18)**	**1.31 (0.23)***
Ratio head/total length (Median (IQR))	0.25 (0.04)	0.25 (0.04)	**0.24 (0.03)**	**0.27 (0.06)***
Yolk-sac surface (mm^2, Median (IQR))	**0.33 (0.06)**	**0.29 (0.13)** *	**0.39 (0.11)**	**0.27 (0.06)***

represented 32 % of genetic males under the control *versus* 65 % under the Garonne condition, showing a significant difference in the sex ratio between the two conditions (two proportions z test, $P = 0.02$).

3.3.6. Lipid peroxidation (TBARS)

Larvae exposed to Garonne River water did not show any significant differences in thiobarbituric acid reactive substance (TBARS) concentrations compared with the control (data not shown). The mean concentrations, normalised by the mean size of larvae (± SD), were 0.7 ± 0.03 in both conditions.

4. Discussion

4.1. Applicability of the exposure method

During exposure of Japanese medaka embryos, temperatures were maintained within the targeted ranges of approximately 23–25 °C. Temperature is a critical factor determining the duration and quality of embryonic development (Kamler, 2002; Schiemer et al., 2002). The mean daily exposure temperature was within the optimal range for the development of medaka embryos and larvae (OECD, 1998). In each experiment, temperatures were comparable between the two conditions, enabling reliable comparisons. Other water parameters, including the oxygen saturation rate, pH, conductivity, and concentrations of nitrogen forms, which could influence embryo responses, were similar between control and Garonne conditions. As water temperature was controlled, it is important to closely monitor parameters that could be influenced by temperature changes in both conditions (such as pH or nitrogen forms) to ensure it does not reach toxic values for embryos. Additionally, it would have been interesting to monitor physico-chemical parameters directly in the river to ensure that these values are not too importantly modified by temperature changes.

In both experiments, embryonic survival and total hatching rates in control were close to 80 % which is largely satisfactory in our experimental design taking into consideration that experiments were not carried out as in ideal laboratory conditions (OECD, 1998). This could also explain differences of hatch between the two experiments. With regard to gammarids, there were no differences in survival between the experimental conditions. Water temperature is known to influence the feeding behaviour of *G. fossarum* (Coulaud et al., 2011). In both experimental feeding assays, the temperature was within optimal ranges for the species and was similar between conditions (range value: 12.5 ± 1 °C), allowing comparisons between the control and Garonne exposures.

4.2. Toxicity for organisms

Exposure to Garonne water induced very high embryonic mortality in medaka embryos across all experimental replicates in both experiments. Fish embryonic stages, from fertilisation to the yolk sac larval stage, are extremely sensitive to environmental factors and contaminants (Murl Rolland, 2000), which can lead to toxicity in developing embryos (McKim, 1977; Von Westernhagen, 1988). Because the monitored water parameters were similar in both conditions for embryonic development during both experiments, this high mortality rate suggests that other factors are linked to water quality. Given that these results

were obtained from two independent experiments, they may indicate chronic toxicity of Garonne River water, at least during the spring season. The dynamics of embryonic mortality showed a high survival rate for embryos exposed to Garonne water during the first five days after the start of exposure. However, a significant increase in embryonic mortality was observed in both series, starting between 125 and 150 h post-fertilisation. This suggests a specific window of sensitivity within the embryonic development of the Japanese medaka. While the egg envelope can act as a barrier to exogenous compounds, the chorion's toughness decreases starting at approximately 6 d post-fertilisation (dpf) at 20–25 °C in Japanese medaka embryos, which reduces its ability to protect the embryo from exogenous compounds (Suga, 1963). This period corresponds to the beginning of the mortality peak observed in our experiments. According to the species development table, this period corresponds to the organogenesis stage, more specifically to stages 34–36, which involve the heart and blood vessel development (Iwamatsu, 2004). The periods during which organs develop and begin to function are considered critical periods of sensitivity in developing embryos exposed to chemical contaminants (Von Westernhagen, 1988). In a previous study conducted in a nearby area (a few kilometres upstream), exposure to a tributary of the Garonne River significantly increased DNA damage in adult crucian carp (*Carassius carassius*). Moreover, the genotoxicity of the water samples was higher in the spring than in the winter, which is consistent with the chemical analysis of pesticides in the water (Polard et al., 2011). Our results also showed that embryos exposed to Garonne water hatched earlier when they survived than controls, despite similar exposure temperatures under both conditions. Embryos passively undergo variations in environmental factors and in the case of stress, premature hatching can occur (Cowan et al., 2024; Schiemer et al., 2002). This has been shown in some species under conditions of reduced oxygen saturation and non-optimal temperatures (Kamler, 2002). Several studies have also reported that exposure to metals or organic compounds can lead to premature hatching, such as copper in brook trout (*Salvelinus fontinalis*), PAHs (Polycyclic Aromatic Hydrocarbon) in Atlantic cod (*Gadus morhua*), and mixtures of PAHs and PCBs (Polychlorinated biphenyl) in brown trout (*Salmo trutta* f. *fario*) (Hansen et al., 2019; Luckenbach et al., 2003; McKim and Benoit, 1971). The higher number of half-hatched embryos observed under the Garonne condition may also be a consequence of premature hatching. At the end of embryonic development, embryos produce chorionase, which helps soften the chorion and facilitate its rupture. Incomplete hatching can occur if the embryos hatch too early and do not produce sufficient chorionase (Jezierska et al., 2009). Further analyses could be conducted, such as cardiac frequency and oxygen consumption of organisms, and may permit to more precisely study a potential modification of embryonic metabolism and acceleration of hatching induced by the river water contamination (*e.g.* Bedrossiantz et al., 2024; Kozal et al., 2023).

Gene expression is a highly sensitive and dynamic parameter (van der Oost et al., 2003). However, in our study, only moderate (*i.e.* fold-changes ranged from 0.62 to 1.87), effects were observed on the target gene transcription level between larvae exposed to the river water compared with the controls. Transcription analyses were conducted on the larvae at the end of the experiment and not during the observed mortality peak. Thus, monitoring gene expression just before the mortality peak would have provided a more integrative and sensitive response, allowing the investigation of which genes involved in specific developmental pathways were differentially expressed. Moreover, the difference in hatching timing between the two conditions may explain the significant differences in the expression of genes involved in embryogenesis. The higher expression of *rad51* under the Garonne condition compared with the control could indicate the exposure of larvae to genotoxic compounds, leading to the activation of the transcription of genes involved in DNA repair (*rad51*). The different sex ratios in our experiment between conditions (32 % genetic males in the control *versus* 65 % in the Garonne group) could indicate selection on organisms favouring the survival of males over females in the Garonne group. This effect could be triggered by the exposure to endocrine disruptors. It is commonly known that organisms respond differently to stress according to their sex (Moisan, 2021). Differences in responses between males and females have already been observed following exposure to endocrine-active chemicals, such as PCBs (Rypel et al., 2007), DDT (dichlorodiphenyltrichloroethane) metabolites, chlordecone (Carlson et al., 2000), and pulp mill effluents (Afonso et al., 2003).

The measurement of the feeding rate revealed that gammarids exposed to water from the Garonne consumed more food than those in the control group. Similar to the increase in yolk sac consumption observed in larvae, an increase in the feeding rate can indicate a stress response linked to exposure to the Garonne's water. To enhance their immune and/or detoxification defences, organisms must increase their food intake. These results may also be associated with an overall increase in metabolism, potentially triggered by specific chemical compounds (Sokolova, 2013). Interestingly, the exposure of *G. fossarum* females to water from the Garonne River did not lead to embryotoxicity. In *G. fossarum*, embryonic development and the female moult stage are synchronised (Geffard et al., 2010). Freshly fertilised gammarid embryos were simultaneously exposed to the marsupium of females in stage A and continued to develop until the females reached stage D2. There was no difference in the number of viable embryos between the two conditions, indicating a difference in sensitivity between the fish and gammarids. Fish and invertebrates have been shown to bioaccumulate pollutants differently, raising questions about the pathways of exposure and entry of xenobiotics into organisms and, consequently, the toxicity of pollutants present in aquatic habitats (Grabicova et al., 2022). In addition, the hormonal systems of crustaceans and fishes differ. Fish have a hormonal system similar to that of other vertebrates (*e.g.* oestrogens and androgens), whereas crustaceans share similar hormone systems with ecdysozoans (*e.g.* ecdysteroids and terpenoids) (LeBlanc, 2007; Milla et al., 2011; Rodríguez et al., 2007). If endocrine-disrupting substances are present in the Garonne River, it is possible that only one of the two taxa is affected, resulting in high embryotoxicity in fish, but not in *G. fossarum*, an assumption supported by the biased sex ratio observed in fish exposed to water from the Garonne River.

4.3. Water quality of the Garonne River

The control water for the fish experiments was dechlorinated tap water intended for human consumption and regularly monitored by the Regional Health Agency of Occitanie (https://www.occitanie.ars.sante.fr/). Water quality monitoring consists of the chemical analyses of several persistent pollutants, including trace metals and pesticides. During our experimental period, tap water in this area was considered suitable for human consumption, indicating that the pollutant concentrations were significantly lower than the limits set by European Directive 2020/2184 on the quality of water intended for human consumption (European Commission, 2020).

The results of the water analysis showed the presence of several trace metals and pesticides in the Garonne River. These results are consistent with the Adour-Garonne Water Agency (AEAG) data obtained at the closest monitoring station (Table 3). However, only seven compounds were quantified in 2023 at this station in the Garonne River: metolachlor EAS, Nickel, Copper, Zinc, Arsenic, Cadmium, and Lead. The quantification of a compound in our analysis may not always imply a higher concentration because of variations in the analytical techniques and quantification limits between our study and AEAG monitoring. However, none of these compounds were present at toxic concentrations. For pesticides, passive samplers integrate the potential concentration fluctuations in water, providing an average concentration over the exposure time and smoothing potential contamination peaks (Bernard et al., 2019). Microbiological analyses indicated that there were no pathogens in the river and control samples across all sampling dates and in both experimental batches. The concentrations of the chemicals for which we obtained the toxicity data (NOECs) for fish embryos in the Garonne

River were also lower than the NOEC values estimated for fish embryo mortality (references for NOECSs are available in Supplementary Material 7). The PNEC is an indicator frequently used in environmental risk assessments to set a threshold concentration of a chemical in an environment below which no discernible deleterious effects on living organisms are expected. This concentration is frequently determined using toxicity models and experiments, in which a safety factor is applied (Backhaus and Faust, 2012). None of the substances (trace metals and pesticides) quantified for which PNEC data were available reached the PNEC threshold (Table 3).

Pesticides and trace metals were monitored because of the dominant anthropogenic activities (agricultural and industrial) upstream of the study site. Their presence poses a low, but non-negligible, toxic risk to the aquatic ecosystems in the basin (Faggiano et al., 2010). However, despite monitoring an exhaustive list of pesticides and trace metals (Supplementary Material 1), chemical analysis of the water did not identify any single chemical compound that could solely account for the toxicity observed in medaka embryos and *G. fossarum*. Given the estimated number of compounds currently on the market, monitoring only 78 pesticides and trace metals (out of at least 100,000 estimated in the European market) is insufficient to fully characterise the overall contamination of the Garonne River (European Environment Agency, 2019). Over time, the concentrations of some pollutants, such as perfluoroalkyl substances (PFASs) (Macorps et al., 2022; Munoz et al., 2015) and rare earth elements, such as Gadolinium (Lerat-Hardy et al., 2019), are increasing in the Garonne Basin. A wide variety of PFASs have been detected in the Garonne River, which flows through two major urban areas: Toulouse, approximately 100 km upstream of our study site, and the Gironde Estuary (Munoz et al., 2019). High human population density also leads to the release of hormones and drugs from human use into aquatic environments (Hughes et al., 2013). Some of these drugs, such as antibiotics, anxiolytics, and nonsteroidal anti-inflammatory drugs, are regularly detected downstream of Toulouse and upstream of Golfech (Destrieux et al., 2017). As fish and mammals share similar endocrine and immune systems, they may be adversely affected by these pollutants (Brodin et al., 2014; Corcoran et al., 2010). Moreover, analysis of contaminants bioaccumulated in organisms can be used to identify the contaminants to which they have been exposed (van der Oost et al., 2003). It is also very likely that the toxicity of the Garonne River water is due to a combination of multiple compounds. Therefore, toxic unit approaches can be useful if sufficient toxicity data are available. A recent study demonstrated a potential toxic risk (using the msPAF approach) in the Garonne River for fish species, particularly during their early life stages. Trace metals, personal care products, and industrial pollutants are the main contributors to the potential toxicity (Bellier et al., 2024). However, these approaches do not account for the cocktail effect, which has been demonstrated to have a significant impact on the health of living organisms (Backhaus and Faust, 2012).

5. Conclusion

In summary, this study demonstrated the usefulness of the *ex-situ* exposure approach, particularly in terms of controlling water parameters and utilising aquatic organisms that are highly sensitive during their early life stages, even non-native model species. The experiments revealed a significant impact of the Garonne River's water quality on the early development of a model fish species, but not on the crustacean *G. fossarum*, highlighting different sensitivities across taxa. Further research and monitoring are required to determine the presence and extent of pollutants in rivers. Moreover, the findings emphasise the usefulness of the *ex-situ* exposure method, allowing the exposure of critical developmental stages and a more comprehensive investigation of the effects of water quality on organisms. This approach constitutes a significant advancement in environmental monitoring, enabling the exposure of suitable aquatic organisms and providing deeper insights into the effects of anthropogenic activities on global biodiversity decline.

CRediT authorship contribution statement

Sarah Bancel: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Olivier Geffard:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Cécile Bossy:** Writing – review & editing, Investigation. **Christelle Clérandeau:** Writing – review & editing, Investigation, Formal analysis. **Alexandra Coynel:** Writing – review & editing, Investigation. **Flore Daramy:** Writing – review & editing, Investigation. **Nicolas Delorme:** Writing – review & editing, Investigation. **Laura Garnero:** Writing – review & editing, Investigation. **Nicolas Mazzella:** Writing – review & editing, Investigation, Formal analysis. **Debora Millan-Navarro:** Writing – review & editing, Investigation. **Fatima Mzali:** Writing – review & editing, Investigation. **Fabien Pierron:** Writing – review & editing, Investigation, Formal analysis. **Rémi Recoura-Massaquant:** Writing – review & editing, Investigation. **Eric Rochard:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Jérôme Cachot:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Ethical statement

We declare that all experiments were conducted according to the regulations for embryos and yolk sac larvae, which are exempt from the EU's 2010/63/EU animal testing regulations.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Sarah Bancel reports financial support was provided by Regional Water Agency Adour-Garonne. If there are other authors, they 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.scitotenv.2024.178159.

Data availability

Data will be made available on request.

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