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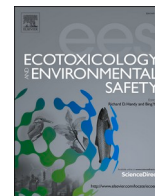
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## A psychiatric drug found in waste-water plant effluents alters the migratory behavior of critically endangered *Anguilla anguilla* juveniles

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### ABSTRACT

Migratory fishes cross or settle in several environments potentially polluted. Psychiatric drugs, which represent one growing pollution and are found in discharges from waste-water treatment plants, may alter individual behaviors. Here, we assessed behavioral alterations in the upstream migratory behavior of *Anguilla anguilla* caused by diazepam, an anxiolytic. We monitored the swimming activity, swimming behavior, and boldness to assess whether diazepam impacts them or not. Our 7-day behavioral follow-up allowed us to test the kinetics of the potential effects of diazepam. We found diazepam reduced swimming activity and altered individual swimming behavior, with fewer individuals swimming against the current, so swimming upstream. Those effects varied over time and were stronger at the end of our monitoring, suggesting chemical pollutants encountered in estuaries may act as a chemical burden for individuals, despite metabolisation. We also found diazepam favored bolder behavior in glass eels. Our results provide new knowledge on chemical pollution and psychiatric drugs inducing behavioral alterations. Those alterations may have ecological and evolutionary consequences for glass eels, by diminishing predator avoidance and impacting spatial colonization, and thus, local density.

### 1. Introduction

Chemical pollution is a growing concern for aquatic ecosystems due to discharges from various sources, including wastewater treatment plants (WWTPs). Effects of chemical pollution on individuals are usually assessed by comparing the survival of contaminated individuals to control ones (Jacquin et al., 2020; Saaristo et al., 2018), but pollutants can also alter behavior. Animals' primary response to chemical pollution is often behavioral, such as avoiding contaminated areas (Saaristo et al., 2018). For example, metal pollution has been found to alter the migration routes of salmon (Saunders and Sprague, 1967). Studying the effects of chemical pollution on behavior can help identify the immediate causes of behavioral changes and aid in the conservation of species (Candolin and Wong, 2019; Cooke et al., 2022; Jacquin et al., 2020; Saaristo et al., 2017).

Migratory fish face a variety of environmental stressors as they navigate through different environments, including chemical pollution. One growing concern is the presence of pharmaceutical drugs in the water, which are often not effectively removed by wastewater treatment plants. Benzodiazepines, oxazepam, and diazepam are among the anxiolytic drugs that have been found in the outlet of WWTP (Argaluz et al., 2021) and in multiple rivers worldwide (Fick et al., 2017; Mendoza et al., 2014; Wilkinson et al., 2022), especially because they are not efficiently removed during wastewater treatment processes (Samal et al., 2022). Fick et al. (2017) found that at least one benzodiazepine was detected in 86% of their 138 samples from 30 rivers in Europe. These drugs have been the focus of several studies investigating their effects on fish behavior leading to find they may cause behavioral alterations in fish (Argaluz et al., 2021; Gebauer et al., 2011; Hellström et al., 2016; Huerta et al., 2016; Sundin et al., 2019). Understanding the

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impacts of these pharmaceutical contaminants on migratory fish behavior is crucial for their conservation and management.

The implications of altered behavioral traits caused by anxiolytic drugs on fishes are multifaceted and may affect not only individual survival and fitness but also ecological processes. Behavioral alterations caused by anxiolytic drugs may involve evolutionary changes through direct survival or fitness differences between contaminated and healthy individuals (Jacquin et al., 2020; Klaminder et al., 2019; Saaristo et al., 2017, 2018). Previous studies found such pollutants reduced avoidance behavior in fishes (Klaminder et al., 2019; Saaristo et al., 2017) and raised the probability of predation in a migratory context for salmon smolts (Klaminder et al., 2019). Increased boldness or impacted activity (Brodin et al., 2017, 2014; Cooke et al., 2022; Gebauer et al., 2011; Jacquin et al., 2020; Sundin et al., 2019) are among the several effects on fish behaviors already highlighted with contaminated individuals migrating faster than control individuals in an in-field experiment on salmon smolts (Hellström et al., 2016). The observed changes in fish behavior may also have implications for the functioning of ecosystems, as the behaviors of fish can influence nutrient cycling and predator-prey dynamics (Conrad et al., 2011; Cooke et al., 2022; Dochtermann and Dingemans, 2013; Réale et al., 2007; Saaristo et al., 2018; Sih et al., 2004, 2012). Therefore, understanding the impact of anxiolytic drugs on fish behavior and their ecological consequences is crucial for conservation efforts and the management of aquatic ecosystems.

Glass eels of *Anguilla anguilla* are a suitable biological model to investigate behavioral alterations caused by anxiolytic drugs since this juvenile stage corresponds to individuals arriving at the continental shelf and migrating upstream into river systems (Cresci, 2020; Harrison et al., 2014). Individuals may either settle in estuaries or migrate further upstream (Daverat et al., 2006, 2005; Tsukamoto et al., 1998). Numerous studies have focused on the mechanisms associated with and triggering the upstream migration within estuaries (Bolliet et al., 2017; Bolliet and Labonne, 2008; Bolliet et al., 2007; Cresci, 2020; Harrison et al., 2014; Liu et al., 2019). For instance, it is now well admitted that glass eels use the tidal stream to migrate faster and easier into rivers and are more active at night (Bardonnat et al., 2003; Beaulaton and Castelnaud, 2005; Cresci, 2020; Gascuel, 1986; McCleave and Kleckner, 1982). On the other hand, upstream migration in rivers above the salt-water limit, where the influence of tides is absent, is often overlooked, as the majority of recruitment monitoring takes place at sites near the river mouths (Cresci, 2020; Harrison et al., 2014; ICES, 2020). Therefore, investigating the effects of anxiolytic drugs on glass eels during their in-river migration could provide valuable information on how these contaminants affect their survival and ecological roles. From here, we focus on this in-river migration and related behaviors only.

In a tank that mimics a continuous river flow, we assessed alterations of migratory behavior in glass eels induced by a diazepam contamination at an environmentally relevant concentration. Diazepam is an anxiolytic drug that has been the subject of several previous fish-related studies, providing numerous outcomes for comparison and discussion (de Abreu et al., 2014; Chen, 2021; Gebauer et al., 2011; Giacomini et al., 2016; Lorenzi et al., 2016). First, behavioral traits such as boldness and exploration are usually interrelated in behavioral syndromes (Conrad et al., 2011; Dochtermann and Dingemans, 2013; Réale et al., 2007; Sih et al., 2004, 2012). In fishes, boldness and exploration traits may be positively associated within the boldness-exploratory syndrome (Cote et al., 2010; Sutrisno et al., 2011). Boldness-exploratory syndrome is typically evaluated by measuring the time it takes for individuals to emerge from their shelter when placed in a new, unfamiliar environment (Brown et al., 2005; Conrad et al., 2011; Fraser et al., 2001). While we targeted alterations of migratory behavior, so in an exploratory context, we assessed the drug's effects on boldness by measuring the latency for individuals to exit the gravel bed of the tank, which served as their shelter. We hypothesized that contaminated individuals would exit their shelter and begin swimming more quickly than control (uncontaminated) individuals, as diazepam is known to promote boldness in fish (de

Abreu et al., 2014; Cote et al., 2010; Gebauer et al., 2011; Hellström et al., 2016; Sundin et al., 2019; Sutrisno et al., 2011), even at environmental concentrations (Brodin et al., 2017). Second, to investigate the alterations of migratory behavior, we evaluated two behavioral traits that compose it: swimming activity and swimming direction (i.e., against or with the current). While individuals should preferentially swim against the current to “migrate upstream”, diazepam contamination could either diminish or increase their activity level, which we measured by the number of swimming sequences observed (Brodin et al., 2014, 2017; Hellström et al., 2016). For example, individuals may exhibit an initial increase in swimming activity (boldness trait) but then decrease or stop their activity, leading to an overall decrease in its activity level due to the contamination. Therefore, it is necessary to consider both boldness and activity level when evaluating the effects of contamination on behavior. Third, effects on activity levels may vary over time and could potentially be mitigated or reversed after depuration (Sundin et al., 2019; Alvarez-Mora et al., 2022), which refers to the removal of contaminants from an organism's system. Our third analysis was thus to test whether diazepam effects on behavior vary across our 7-day behavioral monitoring. Due to depuration, we hypothesized a reduction of the potential diazepam effects.

## 2. Methods

### 2.1. Ethics, capture, and handling of fish

The procedures applied to the individuals were validated by the ethics committee N°073 “Aquitaine Poissons-Oiseaux” (ref: APAFIS #32100-2021062317263102) and were carried out in accordance with EU legal frameworks and French legislation governing the ethical treatment of animals (i.e., Directive 2010/63/EU and Decret no. 2013-118, February 1st, 2013).

The sampling site was the river mouth of the Courant d'Huchet, located at Mollets beach on the west coast of France (43.857822, -1.390253, map available in Valérie Bolliet et al., 2017). Sampling occurred at night on January 18th, 2022, during the rising tide. We used two fine mesh sieves placed against the waves and raised them after each wave to capture individuals. We maintained the individuals at 12°C (the sea water temperature at catch) in a tank (20-liter) with aerated water from the sampling site. During the following week, we replaced one-sixth of the water daily with filtered freshwater from the nearby Nivelle river. The freshwater was filtered using a sand and carbon filter and aerated for 24 h before use. After one week, we transferred the individuals to a larger tank (40-liter) containing filtered freshwater at 12°C, with a photoperiod of 10 h and very low light intensity during the photophase ( $1.00e^{-4} \mu W/cm^2$ ) since glass eels are lucifugal.

After 24 h of acclimation, we randomly selected 80 individuals in batches of 10 and anesthetized them by immersion in a solution of benzocaine at 0.01 mg/L. We measured the wet weight (using a Sartorius CP 153,  $\pm 1.0$  mg) and length ( $\pm 1.0$  mm) of each anesthetized individual and tagged them with a unique combination of two or three hypodermic spots of three different colors of Visible Implant Elastomer (VIE Tag) on their back (Bolliet et al., 2007). After being tagged and handled, each individual was released into a 10-liter bucket (with 40 individuals per bucket) to recover overnight before being subjected to chronic chemical contamination.

### 2.2. Chemical chronic contamination

We performed the chronic contamination under the conditions of light intensity, photo-period, and temperature described in the previous paragraph. Then, two tanks with 40 individuals (one for the control and one for contamination, Fig. S1) were supplied with the same water in open circuit with a peristaltic pump (10.0 mL/min). Another peristaltic pump (1.0 mL/min) supplied the two tanks ( $36.8 \times 26.8 \times 12.2$  cm,

12 L) either with water (control) or diazepam (contamination). The diazepam stock solution was at  $50 \frac{\mu\text{g}}{\text{L}}$ , prepared by diluting the pure diazepam (purchased from LGC) in ultra-pure water, and the flow of water and diazepam were fixed to have 5 ppb of diazepam ( $5 \frac{\mu\text{g}}{\text{L}}$ ) in the contamination tank, a concentration close to the concentrations in the effluents of waste-water treatment plants in an estuary close to the sampling site (Alvarez-Mora et al., 2022). We monitored water temperature, water flow, and diazepam flow daily in both tanks to ensure stability and accuracy of the diazepam concentration in the contaminated tank and to ensure consistency between the two tanks. Additionally, several shelters were set up in each tank, positioned opposite to the direction of contaminant (or water for the control tank) flow. Since we wanted to monitor the behavior of the 80 individuals, we could not kill them during monitoring to measure the concentration of diazepam in their body. However, we made a previous experiment with the same material and condition, and individuals caught at the same site to monitor the kinetic accumulation in individuals. This kinetic enabled us to identify that diazepam concentration increased to reach a plateau when individuals stayed in the contamination tank during seven days (Alvarez-Mora et al., 2022), this is why our chronic contamination took place during 7-days.

### 2.3. Behavioral monitoring and behavioral trait measures

After the contamination and starting by the control group to prevent cross-contamination, we removed the shelters in each tank and collected individuals with a small dip net to place them in a bucket. Immediately after capturing all individuals in the bucket, we released the 80 individuals (40 control + 40 contaminated) into the behavioral monitoring tank by gently emptying the bucket into the same tank.

Once out of the bucket, the individuals directly took shelter in the gravel bed at the bottom of the tank (Fig. S2, details in Bolliet et al., 2007), as we covered the bottom of the behavioral monitoring tank with a 5-cm layer of 1–2 cm diameter coarse pebbles. This 300-liter tank used to investigate the migratory behavior of glass eels (Bolliet and Labonne, 2008; Bolliet et al., 2007; Liu et al., 2019) is constituted by a central stiff wall, a pump placed in a corner bottom of the tank, and a semi-circular meshes positioned at the small sides that generate a clockwise circular flow in the tank with an average water velocity ranging from 9 to 13 cm.  $\text{s}^{-1}$  (Fig. S2, Bolliet et al., 2007). Individuals could swim either with the current (mimicking downstream movements) or against it (mimicking upstream movements) within the 35 cm in height water column available.

For seven days, a camera recorded one side of the tank during 1 min every two hours, apart for the first 15 min, which were continuously recorded. The 167 one-minute sequences recorded every two hours provided information about individual swimming activity and direction (swimming or not and either at counter-current or with the current). Since we have put individuals in a novel and unfamiliar environment, the first 15 min recorded continuously provided information about boldness by knowing whether an individual emerged from its shelter (gravel bed) (Conrad et al., 2011; Brown, Jones, and Braithwaite, 2005; Fraser et al., 2001). Among the 167 video sequences, 97 (58%) have been recorded during the night.

### 2.4. Statistical analysis

We conducted our study at an individual level (individual tagging and statistical analysis) rather than at a group level to account for individual heterogeneity. This allowed us to process the data as 40 repetitions of the same treatment (control or contaminated) and compare the behavior of 40 control individuals that were not contaminated (our blank) to the behavior of 40 individuals contaminated with diazepam. To comprehensively estimate the potential behavioral alterations caused by diazepam, we conducted three analyses. The first analysis tested

whether the diazepam contamination induced a difference in boldness. The second analysis focused on testing whether diazepam contamination reduced or increased the activity level of glass eels and impacted their swimming direction (against or with the current). Finally, by examining the number of active individuals seen per sequence, the third analysis aimed to test whether alterations induced by the diazepam contamination were time-dependent or not.

#### 2.4.1. Boldness

The proxy we used to assess boldness was the probability that individuals would leave their shelter (gravel bed) and start swimming during the first 15 min after being introduced into the behavioral tank. To test for potential differences between contaminated and control individuals, we modeled the probability of success that an observed individual  $j$  ( $N = 78$ ) left its shelter and started swimming during the first 15 min. If the individual started swimming during the first 15 min, then  $y_j = 1$ , otherwise  $y_j = 0$ . Therefore, we performed a simple Bernoulli regression to model  $Y$ :

$$\text{Bernoulli}(y_j|\theta_j) = \begin{cases} \theta_j & \text{if } y_j = 1, \text{ and} \\ 1 - \theta_j & \text{if } y_j = 0, \end{cases} \quad (1)$$

with  $\theta_j$  the success probability. Then, we defined  $\theta_j$  as:

$$\begin{aligned} \text{logit}(\theta_j) &= \text{intercept} + \beta_{\text{weight}} \times \text{weight}_j + \beta_{\text{contamination}}, \text{ with} \\ \text{intercept} &\sim \text{Normal}(\text{logit}(0.5), 5) \\ \beta_{\text{contamination}} &\sim \text{Normal}(0, 5), \end{aligned} \quad (2)$$

with *intercept* the intercept,  $\beta_{\text{weight}}$  the parameter estimating the effect of individual's weight  $\text{Weight}_j$  on  $\theta_j$  and  $\beta_{\text{contamination}}$  testing for a potential contamination effect. Since we wanted to test the contamination effect, we fixed the first level of  $\beta_{\text{contamination}}$  (control individuals) at 0, leading the model to estimate the difference between control and contaminated individuals through  $\beta_{\text{contamination}}$ . A value significantly different to 0 would indicate that the contamination decreased ( $\beta_{\text{contamination}} < 0$ ) or increased ( $\beta_{\text{contamination}} > 0$ ) the probability  $\theta_j$  that the individual  $j$  started swimming during the first 15 min. We fitted a null model without  $\beta_{\text{contamination}}$ .

#### 2.4.2. Activity level and swimming direction

We analyzed the 167 one-minute video sequences to investigate the effect of diazepam on the probability of glass eels being active (swimming) on one hand, and their swimming direction (with or against the current) on the other hand. To do this, we employed a Bayesian hierarchical Binomial model. We denoted  $N_{\text{seq}}$  as the 167 sequences observed, and  $N_{\text{obs},j}$  as the total number of sequences in which the  $j^{\text{th}}$  individual was active (swimming). We then modeled  $N_{\text{obs},j}$  using the probabilities  $\theta_{\text{obs},j}$  for the individual to be active. Our model for  $N_{\text{obs},j}$  was as follows:

$$\begin{aligned} N_{\text{obs},j} &\sim \text{Binomial}(N_{\text{seq}}, \theta_{\text{obs},j}), \text{ with} \\ \theta_{\text{obs},j} &= \text{probit}(\alpha_{\text{obs}} + \beta_{\text{obs}} \times \text{Weight}_j + \gamma_{\text{obs},j} + \zeta_{\text{obs}} \times \text{Time}_j + \epsilon_{\text{obs},j}), \\ \alpha_{\text{obs}} &\sim \text{Normal}(0, 5) \ \& \ \beta_{\text{obs}} \sim \text{Normal}(0, 5), \\ \gamma_{\text{obs}} &\sim \text{Normal}(0, 5) \ \& \ \zeta_{\text{obs}} \sim \text{Normal}(0, 5), \\ \epsilon_{\text{obs},j} &\sim \text{Cauchy}(0, \sigma_{\text{obs}}), \text{ and} \\ \sigma_{\text{obs}} &\sim \text{Cauchy}(0, 25) \end{aligned} \quad (3)$$

The value of  $\theta_{\text{obs},j}$  close to unity indicates that the  $j^{\text{th}}$  individual was frequently active (i.e., swimming). The intercept of  $\text{logit}(\theta_{\text{obs},j})$  was represented by parameter  $\alpha_{\text{obs}}$ , while the additive weight effect was represented by  $\beta_{\text{obs}}$ , the additive time effect by  $\zeta_{\text{obs}}$ , and the individual random effect by  $\epsilon_{\text{obs},j}$ . To account for the time effect, we included a parameter testing for the ID of the first sequence when the individual was seen active. For individuals expressing a regular swimming activity, those who started to swim earlier in the experiment had mathematically a higher number of observations ( $N_{\text{obs},j}$ ). We also included the additive weight effect, as previous studies found a positive effect of an

individual's weight on its probability to be active (Bardonnet et al., 2003; Bolliet and Labonne, 2008; Bureau Du Colombier et al., 2007). We fixed the parameter  $\gamma_{obs}$ , which tested for the contamination state, at 0 for control individuals, leaving the model to estimate the difference with contaminated individuals.

Among the  $N_{obs,j}$  sequences, we recorded the total number of sequences,  $N_{cc,j}$ , during which the  $j^{th}$  individual was swimming at counter-current. We then modeled  $N_{cc,j}$  using the probability  $\theta_{cc,j}$  that an individual adopted a counter-current swimming direction. As glass eels exhibit high inter-individual diversity in migratory behavior, we used a mixture of two Binomial distributions to model  $N_{cc,j}$ , as a previous study had identified two different behaviors that may be expressed (Bolliet and Labonne, 2008). This mixture was modeled using a mixing factor  $\delta$ , resulting in a bimodal distribution for  $\theta_{cc,j}$ . This approach accounted for the fact that some individuals had a higher propensity to swim at counter-current than others. Neglecting this heterogeneity could lead to an erroneous null effect of diazepam if the group of 80 individuals was a mixture of two subgroups with different behaviors (e.g., opposite swimming direction) and diazepam effects. Biologically, such a mixture on  $N_{cc,j}$  means a group of individuals would have a higher propensity to swim at counter-current than the other group. Mathematically, where

we had  $p(N_{cc}|\theta_{cc,j}) = \prod_{j=1}^N (Binomial(N_{obs,j}, \theta_{cc,j}))$ , we now have:

$$p(N_{cc}|\theta_{cc,j}, \delta) = \prod_{j=1}^N (\delta \times Binomial(N_{obs,j}, \theta_{cc,j,1}) + (1 - \delta) \times Binomial(N_{obs,j}, \theta_{cc,j,2})), \text{ with}$$

$$\begin{aligned} \theta_{cc,j,k} &= \text{probit}(\alpha_{cc,k} + \beta_{cc,k} \times Weight_j + \gamma_{cc,k} + \epsilon_{cc,j,k}), \\ \alpha_{cc,k} &\sim Normal(0, 5) \ \& \ \beta_{cc,k} \sim Normal(0, 5), \\ \gamma_{cc,k} &\sim Normal(0, 5), \\ \delta &\sim beta(2, 2), \\ \epsilon_{cc,j,k} &\sim Cauchy(0, \sigma_{cc}), \text{ and} \\ \sigma_{cc} &\sim Cauchy(0, 25) \end{aligned} \tag{4}$$

with  $k$  equals to either 1 or 2, depending on the mixture distribution. We compared this model testing for an effect of diazepam contamination (parameter  $\gamma_{cc,k}$ ) to a null model without the parameter  $\gamma_{cc,k}$ . We did not incorporate a time effect here since it is already present in  $N_{obs,j}$ .

### 2.4.3. Time-varying effect of diazepam

We aimed to examine whether the effect of diazepam on behavior varied over time. To achieve this, we modeled  $N_{indiv,s,b}$ , which represents the number of individuals observed per sequence  $s$  and swimming direction  $b$  (either counter-current or with the current). Initially, we modeled  $N_{indiv,s,b}$  over the entire time series corresponding to the 167 observed sequences. To account for the over-dispersion in  $N_{indiv,s,b}$ , we utilized an alternative parametric Negative Binomial distribution. This allowed us to estimate the location parameter ( $\mu_b$ ) and the dispersion parameter ( $\phi$ ) for each behavior.

$$\begin{aligned} N_{indiv,s,b} &\sim \text{NegBinomial}(N_{indiv}|\mu_b, \phi), \text{ with} \\ \mu_b &= \exp(\alpha + \alpha_b + \alpha_{contamination}), \\ \alpha &\sim Normal(0, 25) \ \& \ \alpha_b \sim Normal(0, 25), \\ \alpha_{contamination} &\sim Normal(0, 25), \text{ and} \\ \phi &\sim \text{half - Cauchy}(0, 100) \in \mathbb{R}^+ \end{aligned} \tag{5}$$

with  $\alpha$  the intercept of  $\mu_b$ ,  $\alpha_b$  the swimming behavior effect fixed at 0 for the counter-current behavior, and  $\alpha_{contamination}$  the parameter testing for the diazepam contamination effect, which was fixed at 0 for control individuals. A positive value of  $\alpha_{contamination}$  means that there were more

contaminated individuals than control individuals swimming per sequence. Also, we fitted a null model without  $\alpha_{contamination}$ .

In the second step of our analysis, we used a sliding temporal window approach to model the number of individuals observed per window, sequence, and swimming behavior ( $N_{indiv,s,b,w}$ ), applying the model described in Eq. 5. This approach involves dividing the time series into 48-hour windows with a 12-hour slide, rather than modeling the entire time series. Sliding windows are a common method in time-series analysis, providing a compromise between the number of observations per window, the number of windows, and the ability to break any day effect. The 12-hour slide effectively removes any night-day effects and smooths the dynamics of the data. We also attempted using 24-hour and 6-hour slides, but these resulted in fewer data points or sequences per window, leading to weaker statistical power but with similar qualitative results. In this approach, all parameters previously used in Eq. 5 may vary among windows, with  $\alpha_{contamination,w}$  representing the parameter that estimates the effect of diazepam contamination.

### 2.5. Bayesian inference and model fitting

For each model, we used weakly informative prior distributions (see Eqs. 2, 3, 4, 5) and performed a Prior Predictive Check to ensure that our prior distributions were appropriate. We assessed chain convergence and stationarity using the modified Gelman-Rubin scale reduction factor

( $split - \hat{r}$ , Vehtari et al., 2021) and the Heidelberger and Welch's test. We also checked the graphical convergence and auto-correlation among iterations. We fitted all models using the Stan sampler with the Hamiltonian Monte Carlo algorithm (R Core Team, 2022; Stan Development Team, 2021) and ran three chains, saving 50,000 iterations per chain after a burn-in of 2000 iterations with a thinning interval of ten iterations. We report the median value associated with the Highest Posterior Density interval at 95% for all posterior distributions (similar to Confidence Interval for symmetric distributions). We used the proportion of the posterior distribution that is of the median's sign (probability of direction, pD) (Makowski et al., 2019) to assess parameter significance. Our use of "positive effect" and "negative effect" in this paper relates to the mathematical sign of the parameter associated to the variables in our

**Table 1**

Average ( $\pm sd$ ) body length (mm) and weight (mg) for control (N = 40) and contaminated individuals (N = 40) used in our experiment. A Student's t-Test was performed to test the difference between control and contaminated individuals either in body length or in body weight.

Contamination state	Control	Diazepam	t.test		
			t value	df	p-value
Length (mm)	70.67 (3.75)	71.08 (3.60)	-0.48639	77.861	0.6281
Weight (mg)	322.25 (55.10)	322.23 (56.49)	0.00200	77.952	0.9984

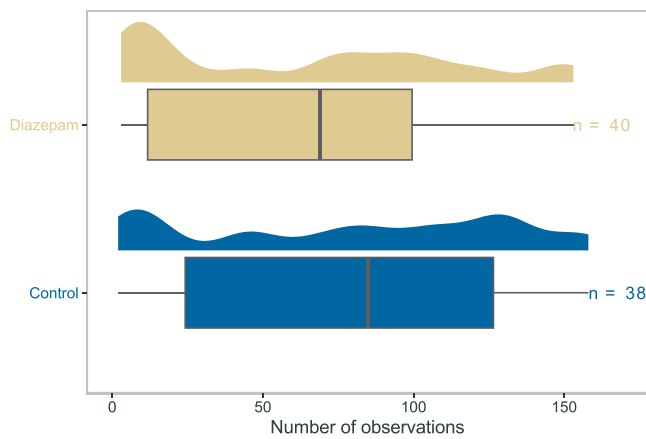


Fig. 1. Boxplot and density of the total number of observations per individual either contaminated by diazepam or not (control) and corresponding to  $N_{obs,j}$ .

statistical models, not to the beneficial or detrimental effect of any variable on glass eels at the individual or population level.

### 3. Results

The initial average body weight and length were similar between the control and contaminated individuals (Table 1). We observed almost all individuals, with 40 contaminated individuals and 38 control individuals, swimming at least once (Fig. 1).

#### 3.1. Boldness

We investigated the effect of diazepam on boldness by examining whether it impacted the probability of individuals leaving their shelter (gravel bed) to start swimming in a novel environment. Fifteen individuals began swimming within the first 15 min of being placed in the tank, which was continuously video-recorded. Among these 15 individuals, 12 (80%) were contaminated and our model estimated a strong positive effect of diazepam ( $\beta_{contamination} = 1.66$ , 95%HPD = [0.226, 3.03], Fig. S3, Eq. 2) on this probability, meaning that exposure

to diazepam increased the likelihood of starting to swim within the first 15 min. Individual weight was not linked to this probability (Fig. S3).

#### 3.2. Activity level and swimming behavior

Our goal was to investigate whether contamination with diazepam altered individual activity levels and swimming behavior. We observed similar ranges in the total number of sequences in which an individual was active (swimming), noted  $N_{obs,j}$  and corresponding to the activity level of individuals, between control (2–158) and contaminated individuals (3–153; Fig. 1). However, the distribution of  $N_{obs,j}$  was more skewed towards low values for contaminated individuals, resulting in a lower median value (69) compared to control individuals (85) (Fig. 1). Our model, which accounted for a potential contamination effect on activity levels (Eq. 4), fit the data better (loaic=296.4) than the null model (loaic=298.2). Our analysis revealed a significant negative effect of diazepam on  $\theta_{obs,j}$ , the probability that an individual was active ( $\gamma_{obs} = -0.751$ , 95%HPD = [-1.487, -0.026]) (Fig. S4), indicating that contaminated individuals were less active. However, the best model did not detect significant weight effects on either  $\theta_{obs,j}$  or  $\theta_{cc,j}$  (Fig. S5).

In a second step, we aimed to investigate whether diazepam contamination affected the swimming behavior of glass eels. The proportion of sequences during which the  $j^{th}$  individual was swimming against the current ( $N_{cc,j}$ ) over the total number of sequences during which the individual was swimming ( $N_{obs,j}$ ), calculated as  $\frac{N_{cc,j}}{N_{obs,j}}$ , was plotted in Fig. 2. A proportion of 0.15 means that the considered individual was swimming against the current in only 15% of the sequences during which it was active (swimming). For both contamination states (control and diazepam), we observed two groups of individuals with low proportion values (<0.30) before the 20th position and high values of proportion (>0.50) after the 20th position. To account for this heterogeneity with individuals that swam either with or against the current, we used a mixture distribution model. This model estimated that 46 individuals belonged to one distribution and 32 individuals belonged to the other distribution, each grouping individuals exhibiting a similar swimming behavior. The first group swam mostly with the current, something characterized by the average value of  $\theta_{cc,j,1}$  estimated at 0.22, while the second group included individuals that swam quasi

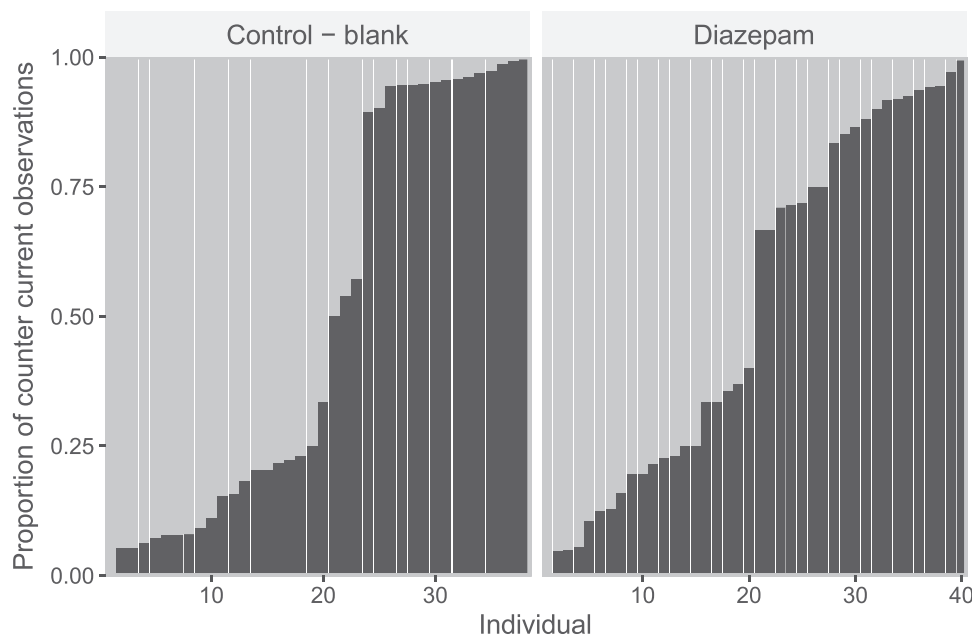


Fig. 2. Proportion of sequences during which the  $j^{th}$  individual was swimming at counter-current ( $N_{cc,j}$ ) over the number of sequences during which the individual was swimming ( $N_{obs,j}$ ). The proportion is thus calculated as  $\frac{N_{cc,j}}{N_{obs,j}}$ . To improve the figure clarity, we ranked individuals in order of increasing proportion.

exclusively against the current, characterized by an average value of 0.99 for  $\theta_{cc,j,2}$ . Our model revealed that diazepam reduced the probability of swimming against the current for the second group ( $\gamma_{cc,2} = -2.334$ , 95%HPD =  $[-3.989, -0.742]$ , Fig. S4), while it had no effect on the first group's probability ( $\gamma_{cc,1} = 0.389$ , 95%HPD =  $[-0.316, 1.122]$ ). Overall, our results suggested that *i*) two groups of individuals exhibited different swimming behaviors based on the propensity of individuals to swim against the current, and *ii*) diazepam altered the swimming behavior of individuals in one of these groups by reducing their propensity to swim against the current.

### 3.3. Time-varying effect of diazepam

Upon examining the average number of individuals observed per sequence, our model incorporating the diazepam contamination effect displayed a significantly lower looic value (3724.6) than the null model (3745.1). Furthermore, we observed a higher average number of individuals swimming against the current than with the current ( $\alpha_b = -0.36$ , 95%HPD =  $[-0.422, -0.296]$ , Eq. 5). Moreover, the model estimated a negative effect of diazepam contamination on the average number of individuals swimming per sequence ( $\alpha_{contamination} = -0.156$ , 95%HPD =  $[-0.219, -0.09]$ ), indicating that fewer contaminated individuals were observed swimming per sequence than control individuals.

When considering sliding temporal windows, we observed temporal variation in the average number of individuals swimming per sequence. Overall, there was an initial increase in the number of individuals during the first five windows, followed by a slight decrease (as shown by the line in Fig. 3a). Regarding the difference between the two swimming directions, the two lines in Fig. 3b illustrate the average number of individuals swimming per sequence in each direction. For both directions, the temporal signal was similar, with an initial increase, followed by a decrease. However, the temporal variations were more pronounced for individuals swimming with the current than for those swimming against the current, resulting in negative values of  $\alpha_b$  estimated for the first two windows and the last one (Fig. 4a). This means that the temporal variations observed in the number of active individuals originated from individuals primarily swimming with the current.

The average number of active individuals increased in both treat-

ments over the first four temporal windows and remained stable across the following temporal windows for only the control individuals (Fig. 3a). In contrast, for contaminated individuals, the average number of active individuals decreased over the last four temporal windows, resulting in increased differences between the treatment groups and leading to negative estimates of  $\alpha_{contamination,w}$  (Fig. 4b). This means the contamination effect, resulting in fewer contaminated individuals swimming per sequence, intensified over time during the last four windows (Fig. 4). For example,  $\alpha_{contamination,w=6}$  was estimated at  $-0.052$  (95%HPD =  $[-0.17, 0.07]$ ), while it was five times lower for the last window ( $\alpha_{contamination,w=10} = -0.27$ , 95%HPD =  $[-0.40, -0.14]$ ).

## 4. Discussion

The effects of anxiolytic drugs on animal behaviors are gaining increasing attention, particularly in fish, due to the presence of these compounds in wastewater treatment plant effluents (Cavalheiro et al., 2017; Miossec et al., 2019). Additionally, migrating individuals may come into contact with these chemicals during their journey and become contaminated. Therefore, our study aimed to evaluate the impact of diazepam on the upstream migration behavior of glass eels (*Anguilla anguilla*) that may be exposed to the drug in estuaries. Our findings demonstrate that diazepam exposure leads to a decrease in activity and alters swimming behavior, while also promoting boldness. Furthermore, the effects of exposure varied over time, highlighting the complexity of the relationship between drug exposure and behavior.

At an environmentally relevant concentration, and likely through its calming and relaxing effects, diazepam promoted boldness in European eel juveniles, with contaminated individuals leaving their shelter early and starting to swim earlier compared to control individuals. Like other benzodiazepines, diazepam is an allosteric modulator of the GABA<sub>A</sub> receptor, meaning it boosts the activity of the natural ligand. Since the GABA<sub>A</sub> receptor is an inhibitory neurotransmitter that diminishes the excitability of the post-synaptic neuron, the positive effect of diazepam on it leads to an increase in the calming and relaxing role of GABA<sub>A</sub> on the central nervous system. While most studies on the role and structure of the GABA<sub>A</sub> receptor are on mammals, the GABA<sub>A</sub> receptor seems to be conserved across vertebrates (Darlison et al., 2005). Diazepam is also known to attenuate amygdalar dopamine levels in rats, which are

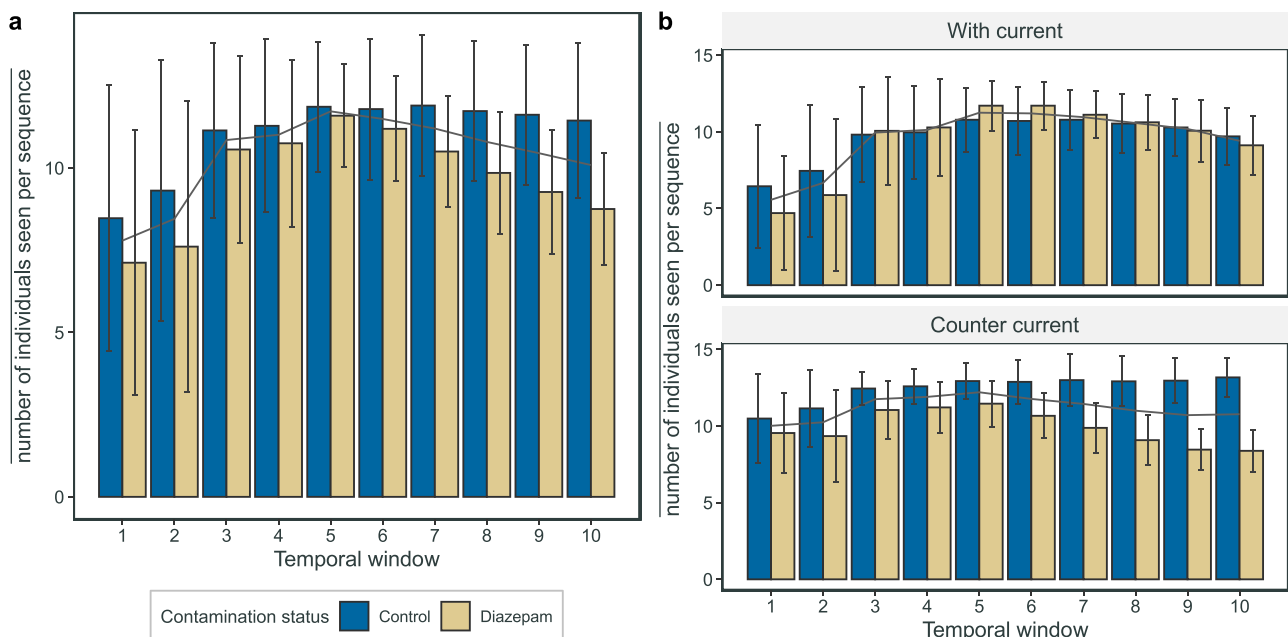
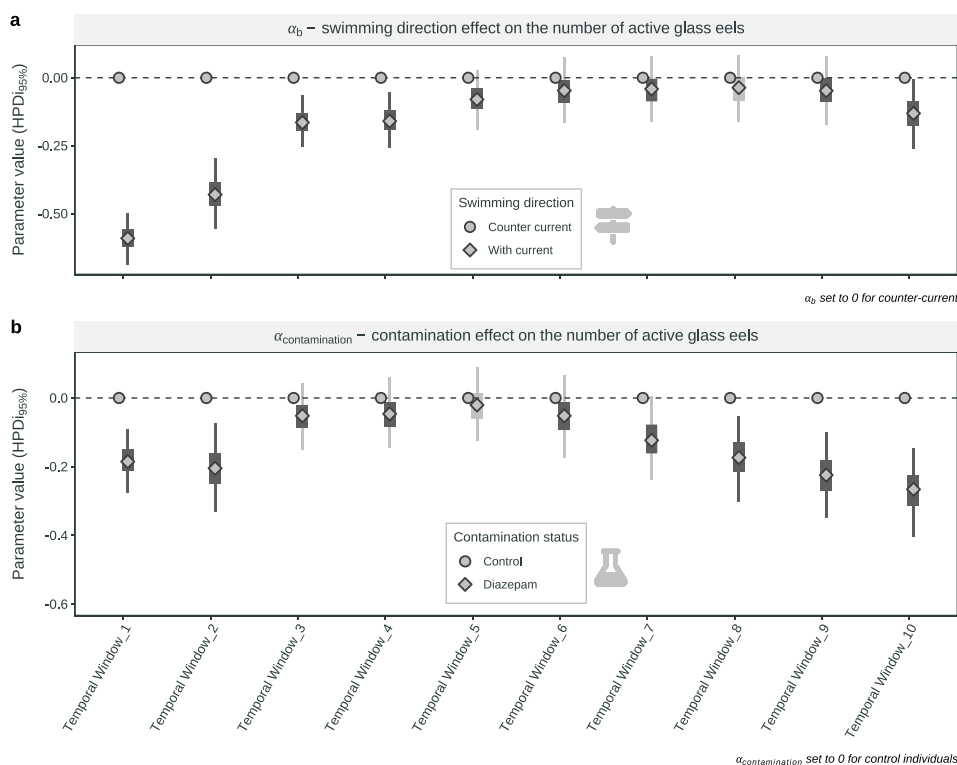


Fig. 3. Average number of individuals seen per sequence and swimming behavior depending on the contamination status. The line corresponds to the average per window and direction without accounting for the contamination status.



**Fig. 4.** Median and Highest Posterior Density interval at 95% of  $\alpha_{b,w}$  and  $\alpha_{\text{contamination},w}$  (Eqs. 3 and 4) testing for a potential diazepam contamination effects on the probabilities of being active and to swim against the current. The dark grey color indicates 0 is outside the 95% HPD.

associated with fear memories through environmental cue learning (Kawano et al., 2018). Previous studies in fish support the positive effect of diazepam on boldness we found (de Abreu et al., 2014; Gebauer et al., 2011; Giacomini et al., 2016). In their study on *Danio rerio*, de Abreu et al. (2014) highlighted that diazepam contamination suppressed the stress response, leading to a reduced cortisol response, while Brandão et al. (2013) found that diazepam reduces the time spent in a refuge area. In their study, Brandão et al. (2013) also measured fish swimming activity during 5 min after placing each individual in a tank and found that diazepam promoted swimming activity during this short period in a new environment. Here, we used the probability that individuals left their shelter and started swimming within the first 15 min since previous studies assessed boldness or boldness-exploratory syndrome through the latency to explore a novel environment (Conrad et al., 2011; Fraser et al., 2001) and to emerge from shelter (Brown et al., 2005; Conrad et al., 2011). Using the time at first appearance of all individuals (personal data), we found the same results than using the 15 first minutes, which supports the results presented here.

Encounters with water contaminated with diazepam may alter the migratory behavior of glass eels, affecting their movements and activity level. Our findings indicate that contaminated individuals were less active compared to control individuals, demonstrating a clear negative effect of diazepam contamination on the activity level. Furthermore, the negative effect of diazepam was also observed on the number of individuals found swimming per sequence. Overall, contaminated individuals showed a lower propensity to be active, a lower activity level, and swam preferentially with the current, while control individuals swam preferentially against the current, which corresponds to upstream migration behavior. In a previous study using diazepam, Chen (2021) found a decrease in fish activity, but with a longer exposure to diazepam (21 days) at a sub-lethal concentration (12  $\mu\text{g}/\text{L}$ ) in a different species (*Danio rerio*). A possible explanation for the reduced activity and the preference to swim with the current is that diazepam diminished individual's behavioral responses to environmental stimuli, such as

rheotaxia. Another hypothesis is that diazepam reduced basal metabolism and diminished the propensity to make physical efforts (Kirvelä et al., 1994).

Some studies have found a positive effect of the anxiolytic oxazepam on fish activity, including studies by Brodin et al. (2014), Brodin et al. (2017), and Hellström et al. (2016). However, the concentrations used in those studies were much higher than in our study, and the species studied were different. For example, Hellström et al. (2016) used *Salmo salar*, while Brodin et al. (2017) and Brodin et al. (2014) used *Rutilus rutilus* and *Perca fluviatilis*. The behavioral monitoring timescales also varied among those studies, which may explain the different results observed. In our study, we found that diazepam favored boldness, which is a stress response on a short timescale of 15 min, while diminishing activity levels throughout the 7-day monitoring period. Finally, the number of manipulations undergone by individuals varied among studies, which could lead to potential differences in stress levels among studies. Therefore, it is difficult to compare and generalize the results of such studies because their contexts are different. This makes it necessary for managers to study different molecules at different concentrations on different species to obtain a more comprehensive understanding of the impacts of pharmaceutical contamination on fish behavior. It is worth noting that the activity of interest was also different among studies. For example, Brodin et al. (2017) studied feeding activity, Hellström et al. (2016) studied downstream migration, and we studied activity level and swimming behavior. Moreover, investigating behavior alterations by anxiolytic drugs usually focuses on behavior traits like boldness or activity, but rarely on movement behavior itself, such as where, when, and how individuals move, as we did in our study (Brodin et al., 2013; Jacquin et al., 2020).

Our findings show that the behavioral alterations caused by diazepam persisted or even intensified over time, as illustrated in Fig. S3. Previous experiments have demonstrated that glass eels exposed to 3ppb concentrations of diazepam can bio-accumulate up to 60 ng/g of the drug after seven days of exposure, but these levels decrease to near zero



after seven days of depuration (Alvarez-Mora et al., 2022). Based on this, we hypothesized that the effects of diazepam on behavior would vary over time and eventually become mitigated or reversible during depuration (Hellström et al., 2016; Sundin et al., 2019). However, our results indicate that the effects of diazepam on activity level and swimming behavior were stronger at the end of the seven-day monitoring period than at the beginning or middle, as shown in Figs. 3 and 4. This suggests that contaminated individuals were unable to recover a migratory behavior similar to that of control individuals and compensate for the behavioral alterations they underwent, even after depuration. It is worth noting that depuration pathways may induce the production of metabolites of diazepam that themselves can induce behavioral alterations after depuration. For example, oxazepam is a product of the oxidative metabolism of diazepam known to alter swimming behavior in fish (Brodin et al., 2013; Hellström et al., 2016; Huerta et al., 2016; Jacquin et al., 2020). Previous research has detected the presence of diazepam metabolites, such as nordiazepam, in glass eels after a seven-day depuration period (Alvarez-Mora et al., 2022). In comparison to most studies, which monitor behavioral alterations over short periods of time (i.e., seconds, minutes, or hours de Abreu et al., 2014; Brandão et al., 2013; Brodin et al., 2013; Chen, 2021; Gebauer et al., 2011; Giacomini et al., 2016), our study monitored behavior over a period of seven days. Taken together, our results suggest that chemical pollutants encountered during migration in estuaries, even during a short period, may act as a chemical burden that continues to affect behavior even when no more pollutants are present in the surrounding environment.

At a river-basin scale, the behavioral alterations caused by anxiolytic drugs found in our study could have potential implications for eels. Although no individuals died during the study, our result that diazepam led to bolder individuals could affect survival in nature by reducing predator avoidance. For example, Klaminder et al. (2019) found that the calming effect of oxazepam on salmon smolt increased their probability of being preyed upon. The lower swimming activity and altered behavior induced by diazepam could also impair the colonization of upstream river systems and small tributaries, leading to changes in local density. While a lower local density may increase individual survival by reducing negative density-dependence, it could also impact population structure since sex-determinism seems to be partly density-related in *Anguilla anguilla* Bevacqua et al. (2007); De Leo and Gatto (1995, 1996)). Furthermore, diazepam could also affect other behavioral traits beyond those observed in our study, potentially leading to effects on other behavioral syndromes (Jacquin et al., 2020). For instance, a previous study linked social learning and information use behavior to exploratory behavioral traits in stickleback (Nomakuchi et al., 2009), something we found impacted by diazepam.

In conclusion, our study contributes to the growing body of research on the ecological consequences of chemical contaminants at environmentally relevant concentrations by monitoring different behavioral traits related to migration behavior over a 7-day period, which is the duration needed for depuration (Alvarez-Mora et al., 2022). This individual-level common garden experiment provides new insights into the potential ecological and evolutionary consequences of behavioral alterations caused by chemical contaminants (Conrad et al., 2011; Cooke et al., 2022; Dochtermann and Dingemans, 2013; Réale et al., 2007; Saaristo et al., 2018; Sih et al., 2004, 2012). However, given that behavioral alterations can vary between sexes (Chen, 2021) and among species or behaviors (Brodin et al., 2014), future studies should assess the ecological and behavioral effects of chemical contaminants on fish over longer periods of time and in different contexts. Additionally, since there can be differences between laboratory and field experiments (Fahlman et al., 2021), future studies should also aim to investigate diazepam effects on behavior in natural settings. Overall, our study highlights the importance of considering behavioral alterations when investigating the effects of pollution on individuals and populations.

## CRediT authorship contribution statement

**Colin Bouchard:** Conceptualization, Data collection, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Mathilde Monperrus:** Conceptualization, Writing – review & editing. **Stellia Sebihi:** Data collection, Writing – review & editing. **Cédric Tentelier:** Conceptualization, Writing – review & editing. **Valérie Bolliet:** Conceptualization, Data collection, Writing – review & editing.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Monperrus reports financial support was provided by French National Research Agency.

## Data Availability

Data will be made available on request. The data and model code used in our study will be made available in a scientific repository.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115496.

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