



Exposure via biotransformation: Oxazepam reaches predicted pharmacological effect levels in European perch after exposure to temazepam

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ARTICLE INFO

Edited by: Dr. Caterina Faggio

Keywords:

Metabolic transformation

Metabolite

Pharmaceutical

Psychoactive drug

Fish

Accumulation

Depuration

ABSTRACT

It is generally expected that biotransformation and excretion of pharmaceuticals occurs similarly in fish and mammals, despite significant physiological differences. Here, we exposed European perch (*Perca fluviatilis*) to the benzodiazepine drug temazepam at a nominal concentration of $2 \mu\text{g L}^{-1}$ for 10 days. We collected samples of blood plasma, muscle, and brain in a time-dependent manner to assess its bioconcentration, biotransformation, and elimination over another 10 days of depuration in clean water. We observed rapid pharmacokinetics of temazepam during both the exposure and depuration periods. The steady state was reached within 24 h of exposure in most individuals, as was complete elimination of temazepam from tissues during depuration. Further, the biologically active metabolite oxazepam was produced via fish biotransformation, and accumulated significantly throughout the exposure period. In contrast to human patients, where a negligible amount of oxazepam is created by temazepam biotransformation, we observed a continuous increase of oxazepam concentrations in all fish tissues throughout exposure. Indeed, oxazepam accumulated more than its parent compound, did not reach a steady state during the exposure period, and was not completely eliminated even after 10 days of depuration, highlighting the importance of considering environmental hazards posed by pharmaceutical metabolites.

1. Introduction

Over the last two decades, it has become apparent that pharmaceutically active compounds (PhACs) represent an important group of emerging contaminants in aquatic environments. Concentrations of these biologically active compounds typically range between $\mu\text{g L}^{-1}$ levels close to point sources, down to low ng L^{-1} levels that are routinely reported in freshwater and marine ecosystems around the world (Alygizakis et al., 2016; Brooks et al., 2005; Danner et al., 2019; Fick et al., 2017; Koba et al., 2018; Lopez-Serna et al., 2012; Ruff et al., 2015; Zhou et al., 2019). Pharmaceuticals have also been detected in tissues of fish, macroinvertebrates, and mollusks living in contaminated water bodies (Bean et al., 2018; Brozinski et al., 2013; Du et al., 2014; Grabicova et al., 2015; Huerta et al., 2018; Ondarza et al., 2019; Richmond et al.,

2018), and even in mammals, birds, and spiders that feed on animals inhabiting these systems (Bean et al., 2018; Richards et al., 2011; Richmond et al., 2018). Moreover, some commonly used pharmaceuticals may reach tissue concentrations that are five orders of magnitude higher than those measured in water, although this enrichment is both species- and drug-specific (Lagesson et al., 2016).

Non-target aquatic organisms have drug targets that are evolutionarily conserved, including many receptors and enzymes, and therefore pharmaceuticals may exert similar effects in these organisms as in humans (Gunnarsson et al., 2008; Hutchinson et al., 2014; Bojarski et al., 2020; Sehonova et al., 2017). Indeed, there is growing evidence for such pharmacological or adverse effects caused by certain classes of pharmaceuticals—e.g. steroidal estrogens and other endocrine-disrupting drugs (Bertram et al., 2018; Corcoran et al., 2010),

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<https://doi.org/10.1016/j.ecoenv.2021.112246>

Received 20 January 2021; Received in revised form 2 April 2021; Accepted 8 April 2021

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antidepressants (Sehonova et al., 2018; Silva et al., 2015), nonsteroidal anti-inflammatory drugs (Corcoran et al., 2010; Yokota et al., 2017; Bartoskova et al., 2013; Turani et al., 2019), and anxiolytics (Brodin et al., 2013; Cervený et al., 2020; Saaristo et al., 2019). Psychoactive pharmaceuticals have attracted special attention recently due to their rapidly increasing global consumption and possible ecological effects via alteration of important behavioral traits in fish (Brodin et al., 2014; Grbicová et al., 2020).

The ability to transform a parent drug via metabolic pathways in an organism is crucial for its elimination from the body (Hutchinson et al., 2014). For example, zero-order metabolism of diclofenac—an anti-inflammatory drug used to treat livestock—was proposed to play a central role in drastic declines of vulture populations seen across the Indian subcontinent (Oaks et al., 2004). In contrast to birds and other terrestrial organisms, fish are able to excrete various xenobiotics by direct diffusion through gills into the surrounding water and thus biotransformation might not be seen as a crucial mechanism in PhAC elimination. Metabolites of many pharmaceuticals still possess biological activity that can exert similar effects to the parent drug (Corcoran et al., 2010; Huang et al., 2019). Despite the fact that the majority of human and veterinary pharmaceuticals are designed to be extensively metabolised in the body, meaning that metabolites likely represent the main portion of PhACs in the environment, surprisingly little information is available about the formation, accumulation, and effects of these metabolites in non-target organisms (Huang et al., 2019; Tanoue et al., 2017; Zindler et al., 2020).

As such, the aim of the present study was to assess bioconcentration and elimination of the commonly prescribed benzodiazepine drug temazepam in European perch (*Perca fluviatilis*) and to investigate its biotransformation by measuring the formation of the active metabolite oxazepam. The fact that oxazepam is a highly prescribed benzodiazepine itself gives a perfect example of a biologically active metabolite.

2. Materials and methods

2.1. Experimental fish

One- to two-year-old perch (mean length \pm SD = 9.8 cm \pm 1.0; mean weight \pm SD = 8.7 g \pm 3.3; n = 70) were collected from a control lake without direct anthropogenic influences (Lake Bjännsjön, Umeå municipality, Sweden) in September 2019 and transported to an aerated, flow-through tank (1000 L) at Umeå University. Non-chlorinated tap water with the following chemical properties was used: pH, 8.2; ammonium [NH₄⁺], < 0.004 mg L⁻¹; nitrite [NO₂⁻], < 0.003 mg L⁻¹; oxygen saturation, > 100%. The light/dark regime was set to 12/12 h. Fish were acclimated to laboratory conditions for two weeks, while being fed frozen chironomid larvae on a daily basis. No mortality occurred during the acclimation period and fish did not show any signs of disease, fungi, or exterior infection. Experiments were approved by the Ethical Committee on Animal Experiments in Umeå (dnr: A18–15 to T. Brodin) and complied with Swedish law.

2.2. Exposure regime and sampling scenario

Perch (n = 70) were randomly allocated to ten 50 L glass aquaria filled with 30 L of water (with the same characteristics as that used in flow-through tanks during acclimatization), resulting in seven individuals per exposure tank. Fish were exposed to temazepam at a nominal concentration of 2 µg L⁻¹ in a semi-static scenario for ten days, where 50% of the water was renewed every second day. The nominal exposure level of 2 µg L⁻¹ was not selected to reflect environmental concentrations but was instead chosen to ensure that we would be able to describe biotransformation and subsequent metabolite accumulation in fish tissues. Further, a control group was not included in the study as this was not necessary to address our experimental aim.

Four to five individuals were sampled from different tanks at each of

eight sampling points during the exposure period (i.e. 6, 12, 24, 48, 96, 144, 192, and 240 h after the commencement of exposure), except for the last sampling point, when only three individuals were sampled (due to human error). After ten days of exposure, the remaining fish were transferred to clean water (with no temazepam) and the depuration period started with exactly the same design as the exposure period (i.e. the same water renewal and sampling scenario). Water samples were taken from four randomly selected tanks at 13 sampling points to measure temazepam concentrations (except for the last three sampling points of the depuration period, when less than four tanks remained in the experiment). The water was sampled directly after sampling of fish, before the regular water exchanges. During the exposure period, as well as during the depuration period, fish were translocated between the tanks to maintain group sizes of five to seven individuals per tank. Fish were fed on a daily basis with chironomid larvae during both the exposure and depuration periods. Sampling involved fish being euthanised by overdose with tricaine methanesulfonate (MS-222) at 0.3 g L⁻¹, with blood being taken by caudal venipuncture, and individuals being measured for total length and weight. The fish were then kept frozen at - 20 °C until muscle and brain sampling was performed in April of 2020. The characteristics of experimental fish together with the physico-chemical properties of water during experiment are provided in the [Supplementary Material \(Table S1\)](#).

2.3. Sample preparation and instrumental analysis

All extraction procedures of biota samples and preparation of water samples for chemical analysis have been described in detail previously (see [Cervený et al., 2020](#) and [McCallum et al., 2019a](#)). Liquid chromatography–mass spectrometry (LC–MS)-grade acetonitrile and methanol (LiChrosolv—hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid (Sigma-Aldrich, Steinheim, Germany) was used to prepare the 0.1% mobile phases. Temazepam (CAS 846-50-4) was purchased at LGC Standards (Teddington, UK). Oxazepam (CAS 604-75-1) and mass-labeled ²H₅-oxazepam (CAS 65854-78-6) were purchased at Sigma-Aldrich (Steinheim, Germany).

The same liquid chromatography–mass spectrometry instrumentation and analytical methods as in [Cervený et al. \(2020\)](#) were used to analyze the target compounds in biota and water samples. Descriptions of the basic set-up of the electrospray ionization interface and the gradient/flow of the mobile phase are presented in the [Supplementary Material \(Tables S2–S4\)](#). Quality assurance and quality control (QA/QC) of the analytical method in this work was evaluated based on its linearity, precision, limit of quantification (LOQ), and measurement of blank samples. Recovery rates for both target compounds in fish tissue using the same analytical method (92–112%) have been reported previously ([Cervený et al., 2020](#)). Quantification of target compounds was carried out using an internal standard approach and Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA). Instrumental LOQ was derived from a seven-point standard curve from 0.1 to 50 ng g⁻¹ and an eight-point standard curve from 0.001 to 5 µg L⁻¹ for fish tissue and water samples, respectively. Peak area corresponding to the lowest point of the calibration curve that had a signal/noise ratio of at least 10 was then used for calculation of LOQs in individual samples. Precision was expressed as a relative standard deviation (RSD) of response factors (RF) calculated for each point of the calibration curve using an equation ([Eq. \(1\)](#)). Target compounds were not found above the LOQ in any of the solvent or water blank samples.

$$RF = \left(\frac{Area_{TC}}{Area_{IS}} \right) \times \left(\frac{Conc_{IS}}{Conc_{TC}} \right) \quad (1)$$

Area_{TC} — Target compound peak area.

Area_{IS} — Internal standard peak area.

Conc_{IS} — Internal standard concentration.

Conc_{TC} — Target compound concentration.

3. Results and discussion

In total, 206 samples of fish tissues/plasma and 47 water samples were analyzed. Results of chemical analyses for all individual samples are presented in the [Supplementary Material \(Table S5\)](#). The analytical method performance achieved reliable parameters. Concerning the water analysis, the method was linear ($R^2 > 0.999$; RSD < 12%) between 0.001 and $5 \mu\text{g L}^{-1}$ of the standard curve for both target analytes, with the LOQ in individual samples ranging from 0.00075 to $0.00168 \mu\text{g L}^{-1}$ and from 0.0009 to $0.0020 \mu\text{g L}^{-1}$ for temazepam and oxazepam, respectively. In the case of tissue samples, an LOQ from 1.1 to 5.2 ng g^{-1} and from 0.4 to 2.0 ng g^{-1} was achieved for temazepam and oxazepam, respectively, in individual samples of muscle and brain, while the range for the same compounds was from 4.5 to 10.0 ng mL^{-1} and from 1.7 to 3.7 ng mL^{-1} in samples of blood plasma. The LC–MS/MS method for tissue samples was linear ($R^2 > 0.999$) between 1 and 50 ng g^{-1} for temazepam and between 0.5 and 50 ng g^{-1} for oxazepam, with an RSD of 22% and 9% for temazepam and oxazepam, respectively.

3.1. Water analysis

Measured water concentrations of temazepam were lower than nominal but stable during the exposure period (mean \pm SD: $1.3 \pm 0.18 \mu\text{g L}^{-1}$, $n = 27$). Oxazepam at low ng L^{-1} concentrations was also measured in water from the beginning of the experiment ([Table 1](#)). As this compound was not dosed in the exposure tanks, the most plausible explanation for this is that oxazepam was excreted by fish as a product of temazepam biotransformation. This assumption is also supported by its increasing water concentration over the exposure period, which occurred despite the fact that 50% of the water was renewed every second day. A similar scenario was observed for both temazepam and oxazepam during the depuration period, as their water concentrations increased during the first 48 h due to fish excretion but then mirrored the decreasing curve of internal fish concentrations for both compounds.

3.2. Pharmacokinetics of temazepam

Quantifiable concentrations of temazepam were measured in all fish samples from the first sampling point (6 h after the commencement of exposure). The highest concentrations were found in blood plasma > brain > muscle and this order remained stable throughout the exposure period (see [Table 1](#), [Fig. 1](#)). Temazepam concentrations measured in

muscle tissue correspond to those previously reported by [McCallum et al. \(2019b\)](#) for sea trout (*Salmo trutta*) and [Cervený et al. \(2020\)](#) for perch, both of which resulted from exposure under laboratory conditions. All temazepam plasma concentrations measured during the exposure period fell within the range of human therapeutic plasma concentrations (HTPC) reported by [Schulz et al. \(2012\)](#), indicating a risk of inducing pharmacological effects. However, it should be noted that real environmental concentrations of temazepam in surface waters are usually about two orders of magnitude lower than what was used in our study. For instance, [Fick et al. \(2017\)](#) reported concentrations of temazepam in European rivers ranging from < LOQ to 39 ng L^{-1} . Temazepam reached equilibrium in fish tissues and plasma very rapidly, with measured concentrations becoming relatively stable after the first 24 h of exposure ([Table 1](#)). A similar kinetic was observed during the depuration period as temazepam concentrations in fish were below LOQ in most individuals sampled after 24 h in clean water.

Rapid biotransformation is most likely the physiological process underpinning the fast pace at which temazepam reached equilibrium in, and dissipated from, fish tissues. Oxazepam concentrations above LOQ were measured in all fish tissue and plasma samples in both the exposure and depuration periods, except in the muscle tissue of four individuals sampled after 6 h of exposure. After 48 h of exposure, the mean concentration of the metabolite exceeded those of the parent compound in all tissues/plasma and continued to increase until the end of exposure period (10 days) without reaching equilibrium (see [Table 1](#), [Fig. 1](#)). After 10 days of exposure, accumulation of oxazepam via the metabolic pathway resulted in concentrations in plasma comparable to HTPC reported for this compound ([Schulz et al., 2012](#)). This considerable biotransformation of temazepam to oxazepam in perch is surprising considering that oxazepam does not represent an important metabolite of temazepam in mammals—i.e., in humans, oxazepam and its conjugates measured in urine accounted for less than 5% of the temazepam dose in treated patients ([Locniskar and Greenblatt, 1990](#); [Schwarz, 1979](#)). The strongly pronounced formation of oxazepam in our study therefore indicates that the relative importance of different metabolic pathways responsible for biotransformation of temazepam in fish is significantly shifted in comparison to mammals. Various models such as the “fish plasma model” evaluate the fate of pharmaceuticals based on data that are extrapolated from human patients, but as we showed here, these might not be sufficient.

A similar observation to ours was reported by [Chen et al. \(2017\)](#) for fluoxetine and its metabolite norfluoxetine in zebrafish (*Danio rerio*), where concentrations of the metabolite in fish plasma also exceeded

Table 1

Measured concentrations of temazepam and oxazepam in fish tissues/plasma and in water during the exposure and depuration periods.

Experiment time ^a (hours)	Temazepam concentration (mean \pm SD)				Oxazepam concentration (mean \pm SD)				
	water (ng L^{-1})	plasma (ng mL^{-1})	brain (ng g^{-1})	muscle (ng g^{-1})	water (ng L^{-1})	plasma (ng mL^{-1})	brain (ng g^{-1})	muscle (ng g^{-1})	
Exposure period	6	1200 \pm 31	38 \pm 6.5	21 \pm 6.7	3.3 \pm 0.53	3.0 \pm 0.4	6.3 \pm 2.2	2.5 \pm 1.2	0.10 \pm 0.25
	12	NA	79 \pm 33	33 \pm 11	6.2 \pm 1.2	NA	11 \pm 2.0	5.8 \pm 1.7	1.0 \pm 0.31
	24	1400 \pm 230	110 \pm 79	35 \pm 13	7.1 \pm 2.0	4.0 \pm 0.8	36 \pm 28	20 \pm 9.9	4.0 \pm 2.1
	48	1200 \pm 32	42 \pm 6.5	34 \pm 6.8	7.3 \pm 2.1	6.0 \pm 0.70	29 \pm 3.8	37 \pm 8.4	7.8 \pm 1.7
	96	1400 \pm 150	120 \pm 88	45 \pm 10	11 \pm 6.7	20 \pm 3.4	83 \pm 24	78 \pm 8.8	17 \pm 1.1
	144	1400 \pm 280	110 \pm 44	58 \pm 9.0	8.4 \pm 0.62	37 \pm 5.6	110 \pm 41	130 \pm 21	24 \pm 2.2
	192	1500 \pm 47	110 \pm 61	45 \pm 12	9.5 \pm 3.5	52 \pm 7.4	110 \pm 27	110 \pm 23	24 \pm 5.5
Depuration period	240	1400 \pm 29	130 \pm 39	62 \pm 16	10 \pm 2.1	51 \pm 8.4	190 \pm 44	170 \pm 32	32 \pm 1.1
	246	6.0 \pm 2.0	81 \pm 57	33 \pm 15	6.6 \pm 4.6	4 \pm 0.7	130 \pm 72	140 \pm 41	32 \pm 11
	252	NA	39 \pm 23	21 \pm 8.4	3.0 \pm 2.5	NA	92 \pm 52	150 \pm 43	31 \pm 7.6
	264	9.0 \pm 1.1	9.9 \pm 13	4.8 \pm 4.6	< LOQ	9 \pm 1.3	95 \pm 38	120 \pm 39	25 \pm 5.9
	288	22 \pm 4.4	5.8 \pm 10	1.8 \pm 3.0	< LOQ	28 \pm 5.3	81 \pm 37	79 \pm 10	18 \pm 2.1
	336	13 \pm 0.51	< LOQ	< LOQ	< LOQ	27 \pm 0.73	76 \pm 40	64 \pm 18	13 \pm 3.2
	384	9 \pm 0.72	1.6 \pm 3.3	< LOQ	< LOQ	21 \pm 1.1	62 \pm 32	48 \pm 14	10 \pm 2.7
	432	4 \pm 1.3	< LOQ	< LOQ	< LOQ	10 \pm 2.7	33 \pm 26	22 \pm 15	5.0 \pm 3.7
	480	NA	< LOQ	< LOQ	< LOQ	NA	11 \pm 2.7	9.5 \pm 3.8	2.1 \pm 0.57

NA – water was not sampled 12 h after the commencement of the exposure or depuration periods, or at the last sampling point of depuration period.

^a Hours since the commencement of exposure.

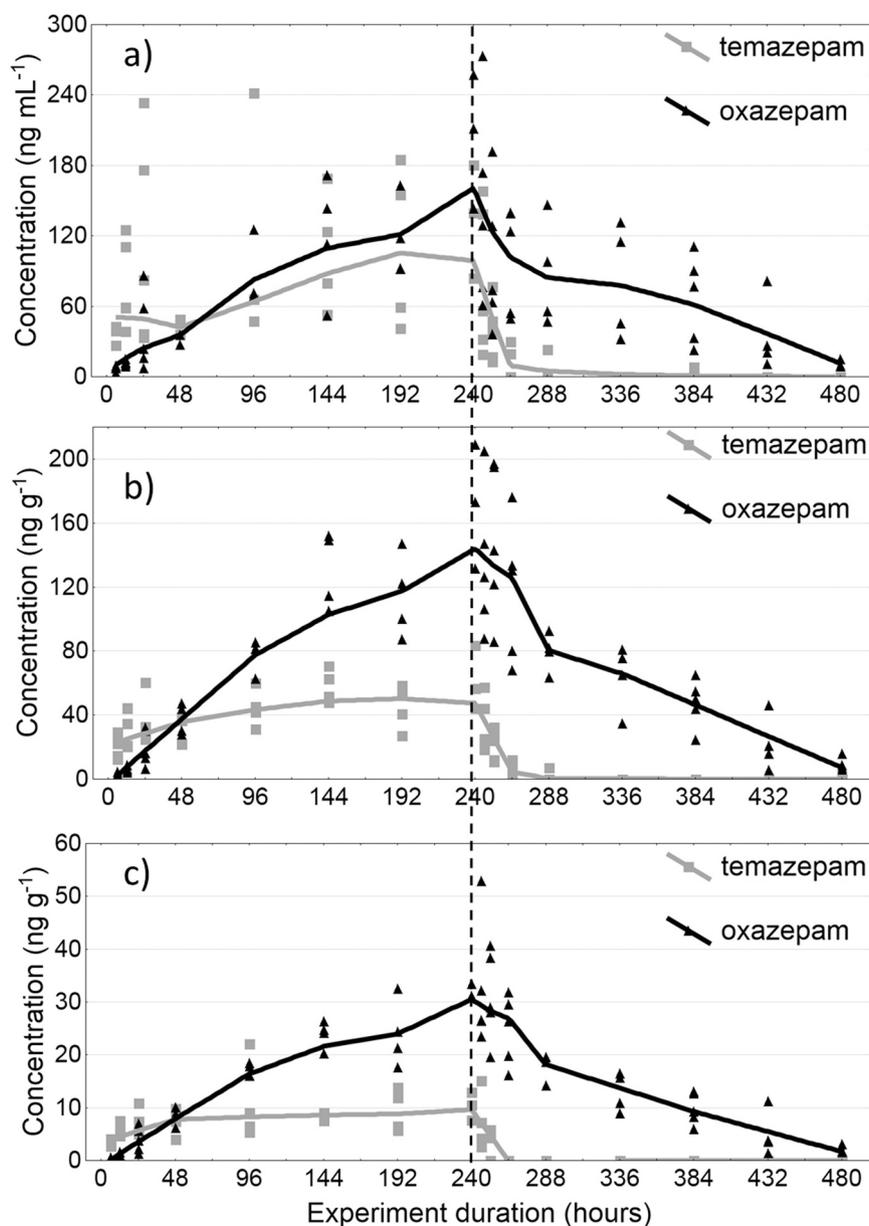


Fig. 1. Concentrations of the parent drug (temazepam) and its metabolite (oxazepam) in a) blood plasma, b) brain, and c) muscle during the exposure and depuration periods. The dashed line indicates the commencement of the depuration period. Raw data with Lowess curves are presented.

those of the parent compound without being dosed in the water. Further, in our recently published work, oxazepam was measured in muscle tissue of perch exposed to temazepam for 7 days, although concentrations of the metabolite were approximately half those of the parent compound (Cervený et al., 2020). We speculate that this discrepancy between the results of our recent and current studies might be due to the different temperature regimes employed. More specifically, unlike the first study where fish were exposed to pharmaceuticals at 10 °C, the present work was carried out at 20 °C. In poikilotherm organisms such as fish, temperature represents an important driver of metabolic rate and can also affect the activity of various enzymes, including those responsible for biotransformation of xenobiotics (González-Mira et al., 2016). On the other hand, it is presumed that fish have developed biochemical thermal adaptations that allow the activity of biotransformation enzymes to be generally similar in individuals acclimated to a range of different temperatures (Fitzsimmons et al., 2007). Despite substantial variation in temperature over time in natural environments, effects of temperature on the fate of pharmaceuticals and other emerging contaminants in

aquatic environments are poorly understood. Thus, studies specifically focused on biotransformation of pharmaceutically active compounds under different temperature regimes should be performed, especially for parent compounds where biologically active metabolites can be produced via biotransformation.

4. Conclusions

Our findings demonstrate the importance of including biologically active metabolites in studies examining adverse effects of PhACs, and in subsequent environmental risk assessment. Because their internal concentrations in aquatic species can reach significant levels, without actually being introduced into the water, metabolites of pharmaceuticals might pose similar risks to aquatic environments as their parent compounds. Moreover, combinatory pharmacological effects might occur, especially in cases where a parent compound and its metabolite have the potential to modulate the function of the same target receptor. Further, as indicated by our work, models that are based on data from

mammalian pharmacokinetic studies might not accurately reflect the physiological processes in ectotherm aquatic organisms and thus more research in this field is needed to improve the applicability of such models.

CRedit authorship contribution statement

Daniel Cerveny: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Jerker Fick:** Conceptualization, Writing - review & editing. **Jonatan Klaminder:** Conceptualization, Writing - review & editing. **Michael G. Bertram:** Writing - review & editing. **Tomas Brodin:** Funding acquisition, Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

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

Acknowledgements

This study was financially supported by the Swedish Research Council Formas (2018-00828), to Tomas Brodin. Daniel Cerveny was supported by the Czech Science Foundation (project No. 20-09951S). Michael Bertram was supported by the Kempe Foundations (SMK-1954) and the Marie-Claire Cronstedt Foundation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112246.

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