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Water temperature affects the biotransformation and accumulation of a psychoactive pharmaceutical and its metabolite in aquatic organisms

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ABSTRACT

Pharmaceutically active compounds (PhACs) have been shown to accumulate in aquatic and riparian food-webs. Yet, our understanding of how temperature, a key environmental factor in nature, affects uptake, biotransformation, and the subsequent accumulation of PhACs in aquatic organisms is limited. In this study, we tested to what extent bioconcentration of an anxiolytic drugs (temazepam and oxazepam) is affected by two temperature regimes (10 and 20 °C) and how the temperature affects the temazepam biotransformation and subsequent accumulation of its metabolite (oxazepam) in aquatic organisms. We used European perch (*Perca fluviatilis*) and dragonfly larvae (*Sympetrum* sp.), which represent predator and prey species of high ecological relevance in food chains of boreal and temperate aquatic ecosystems. Experimental organisms were exposed to target pharmaceuticals at a range of concentrations (0.2–6 μg L^{-1}) to study concentration dependent differences in bioconcentration and biotransformation. We found that the bioconcentration of temazepam in perch was significantly reduced at higher temperatures. Also, temperature had a strong effect on temazepam biotransformation in the fish, with the production and subsequent accumulation of its metabolite (oxazepam) being two-fold higher at 20 °C compared to 10 °C. In contrast, we found no temperature dependency for temazepam bioconcentration in dragonfly larvae and no detectable biotransformation of the parent compound that would result in measurable concentrations of oxazepam in this organism. Our results highlight that while organisms may share the same aquatic ecosystem, their exposure to PhACs may change differently across temperature gradients in the environment.

1. Introduction

Pharmaceutically active compounds (PhACs) have been recognized as an important group of emerging contaminants in aquatic environments, with concentrations typically ranging from μg L^{-1} levels close to point sources, down to low ng L^{-1} levels in freshwater and marine ecosystems around the world (Brooks et al., 2005; Lopez-Serna et al., 2012; Ruff et al., 2015; Alygizakis et al., 2016; Koba et al., 2018; Danner et al., 2019; Zhou et al., 2019). Consequently, a wide range of PhACs have been reported in the tissues of biota, including fish and macroinvertebrates inhabiting contaminated water bodies (Brozinski et al., 2013; Du et al., 2014; Grabicova et al., 2015; Huerta et al., 2018; Ondarza et al., 2019; Cerveny et al., 2021b), and even in mammals, birds, and spiders that feed on these aquatic species (Richards et al., 2011; Bean et al., 2018; Richmond et al., 2018). Moreover, because the drug targets (i.e. receptors and enzymes) of PhACs are often highly evolutionarily conserved across species, pharmaceuticals have the potential to exert similar effects in non-target organisms as they do in humans (Gunnarsson et al., 2008; Hutchinson et al., 2014). Indeed, the evidence for such pharmacological effects has grown rapidly over the last decade for certain classes of PhACs—e.g. antidepressants (Silva et al., 2015; Sehonova et al., 2018), anxiolytics (Brodin et al., 2013;

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Saaristo et al., 2019; Cerveny et al., 2020), nonsteroidal anti-inflammatory drugs (Corcoran et al., 2010; Yokota et al., 2017), and endocrine-disrupting drugs such as steroidal estrogens (Corcoran et al., 2010; Bertram et al., 2018).

Pharmaceuticals are ultimately metabolized and/or excreted by human patients, and it is broadly assumed that the same physiological processes occur in non-target organisms (Burgos-Aceves et al., 2018). However, aquatic organisms occupy dynamic environments with physico-chemical properties that can vary rapidly, while data about toxicokinetics of pharmaceuticals are generally obtained from human patients or mammalian model systems and are thus based on stable conditions. Water characteristics such as pH (Nichols et al., 2015; Bittner et al., 2018; Scott et al., 2019), dissolved organic matter (Alsop and Wilson, 2019), oxygen saturation (Saari et al., 2020), and salinity (Scott et al., 2019) have been shown to significantly affect bioconcentration of certain PhACs in fish. While physico-chemical properties of water can vary greatly between different water bodies, knowledge about their role in the uptake, metabolic transformation, and excretion of pharmaceuticals is still limited.

Surprisingly, even less is known about whether adverse effects of PhAC exposure in non-target species could be mediated by temperature. This is cause for concern given that temperature is a fundamentally important environmental variable for ectothermic aquatic organisms, and is known to influence standard metabolic rate in fish (Clarke and Johnston, 1999; Killen et al., 2010; Ohlberger et al., 2012). Moreover, temperature affects the activity of detoxifying enzymes (e.g. cytochromes P450) that are responsible for metabolic transformation of many environmental pollutants in fish, including PhACs (Ronisz et al., 1999; Ricciardi et al., 2006; Louiz et al., 2017). The importance of temperature for aquatic organisms is well known and its role in toxicity, accumulation, and biotransformation of various other types of contaminants has previously been studied in fish—e.g. for polychlorinated biphenyls (Buckman et al., 2007; James and Kleinow, 2014), pesticides (Reinert et al., 1974; Edgren et al., 1979; Osterauer and Köhler, 2008; Hedrick-Hopper et al., 2015), and metals (Somero et al., 1977; Grasset et al., 2016; Dornelles Zebral et al., 2019). Despite this, few studies have included temperature as a variable when investigating biochemical and physiological responses (González-Mira et al., 2016; Cardoso et al., 2019; Cox et al., 2019), uptake and elimination (Maulvault et al., 2018; Freitas et al., 2020), and behavioral responses (Cox et al., 2019; Saaristo et al., 2019; Wiles et al., 2020) to PhACs.

The metabolites of many drugs also need to be considered as PhACs because of their biological activity and potential to exert pharmacological effects in non-target organisms (Celiz et al., 2009). Hence, studies that focus on biotransformation and subsequent accumulation of metabolites in aquatic organisms are crucial to understanding the consequences of pharmaceutical contamination of surface waters. As reported previously for both fish and macroinvertebrates, metabolites can accumulate more than the parent drug, even without being present in water (Chen et al., 2017; Miller et al., 2017), and can reach tissue concentrations predicted to exert pharmacological effects (Cerveny et al., 2021a). However, we are not aware of any work to date that has investigated whether the toxicokinetics of PhACs are affected by temperature in ectothermic aquatic organisms.

Accordingly, the goal of the present work was to describe the role of temperature in the uptake and biotransformation of the benzodiazepine drug temazepam, and to characterize the accumulation of its metabolite, in two aquatic organisms. The selected fish (European perch, *Perca fluviatilis*) and insect (dragonfly larvae, *Sympetrum* sp.) model species inhabit freshwater ecosystems globally and are both seasonally exposed to temperatures typically ranging from 2 to 25 °C. Temazepam's biotransformation product, oxazepam, is a highly prescribed benzodiazepine itself, and thus it represents a perfect example of a biologically active metabolite. Based on the documented effects of water temperature on fish metabolism (Clarke and Johnston, 1999; Killen et al., 2010; Ohlberger et al., 2012) and activity of detoxifying enzymes (Ronisz et al., 1999; Ricciardi et al., 2006; Louiz et al., 2017), we hypothesized that temperature would significantly affect the 1) bioconcentration of both studied drugs; and 2) biotransformation of temazepam in both experimental organisms.

2. Materials and methods

2.1. Experimental organisms

Young-of-the-year European perch (n = 250) were collected from Lake Stocksjön (Umeå municipality, Sweden) in August 2019 and transported to the laboratory at Umeå University for acclimation. Fish were equally divided between two flow-through holding tanks (each filled with 800 L) that were continuously fed with non-chlorinated tap water and aerated. The tanks were equipped with artificial plants, pieces of plastic pipe, and stones providing refuge for the fish to reduce aggressive behavior. Stable housing conditions were maintained throughout the 40-day acclimation period (water temperature: 14 °C, oxygen saturation: >100%, pH: 7.8–8.2, light:dark regime of 12:12 h). Fish were fed with frozen chironomid larvae daily, and with live zooplankton collected from a fishless local pond every other day. Despite sufficient feeding, 10% mortality occurred during the acclimation period. This was most likely a result of cannibalistic behaviour, which is typical in this species (Persson et al., 2000; Mandiki et al., 2007).

Dragonfly larvae (n = 480) were collected from two lakes (Nydalasjön and Brunnsjön) located in Umeå municipality, in July 2019. After being transported to the laboratory at Umeå University, larvae were randomly distributed into 16 glass aquaria (50 L each). Aquaria were filled with 10 L of aged tap water, with each also being enriched with 1 L of water from the lake of larvae origin. Organic debris from the lakes was also added to provide shelter for individual larvae and thereby reduce aggressive behavior. Larvae were allowed to acclimate for 48 h while being fed with live zooplankton.

2.2. Exposure and sampling scenario

2.2.1. Perch

Bioconcentration with respect to temperature and drug concentration was studied for both temazepam and oxazepam. To accomplish this, fish were randomly allocated to 14 exposure treatments: control (i.e. unexposed, 0 μg L^{-1}), low oxazepam (0.2 μg L^{-1}), high oxazepam (2 μg L^{-1}), low temazepam (0.2 μg L^{-1}), high temazepam (2 μg L^{-1}), or a mixture of both compounds at either a low (0.2 μg L^{-1} of each) or a high (2 μg L^{-1} of each) concentration. Each treatment consists of 15 individuals. The concentrations of target drugs within this study were chosen based on the highest reported in wastewater effluents (Mazzitelli et al., 2018) and aquatic environments (Fick et al., 2017) in Europe. We included mixture treatments to increase the environmental relevance of the study as the two studied drugs have been reported to occur alongside in some European rivers (Fick et al., 2017). Each exposure scenario was carried out under two separate temperature regimes (10 or 20 °C) and treatments consisted of 15 individuals, except for the control in both temperature regimes, which each contained 12 individuals. Characteristics of the experimental fish are provided in the Supplementary material (Table S1). No significant differences in weight or total length were found between individuals allocated to different treatments (Supplementary material, Tables S2 and S3). Fish were exposed for 8 days in an individual static exposure scenario using plastic containers (10 L) fitted with aeration, with each container being filled with 6 L of water. After being transferred from the flow-through housing tank into their respective exposure containers, fish were acclimated to either temperature regime by slowly increasing or decreasing the temperature (2 °C per day). When the target temperature was reached and stable, containers belonging to the oxazepam, temazepam, and mixture exposure treatments were dosed with the respective amount of oxazepam or/and temazepam stock solutions (both 2 mg L^{-1}, prepared by dissolving

the drug standard in ultrapure water). After the exposure period, each individual fish was removed from its exposure container, euthanized by overdose with tricaine methanesulfonate (MS-222) at 0.3 g L^{-1}, sprayed with ultrapure water to remove any excess external temazepam and/or oxazepam, dried with paper towel, and stored at −18 °C until extraction and chemical analysis. Mortality occurred during the exposure period in both of the high-temperature mixture exposure treatments (one individual of those exposed to 0.2 μg L^{-1} and three individuals of those exposed to 2 μg L^{-1}).

Water samples ($n = 80$) were collected on the first and last day of the exposure period from five randomly chosen exposure tanks per treatment, and were analyzed to validate temazepam and oxazepam concentrations.

2.2.2. Dragonfly larvae

Bioconcentration in dragonfly larvae was investigated only for temazepam. This involved dragonflies being randomly allocated to one of six treatments: control (i.e. unexposed, 0 μg L^{-1}), low temazepam (0.5 μg L^{-1}), and high temazepam (5 μg L^{-1}), each of which was performed under two separate temperature regimes (10 or 20 °C). Larvae ($n = 20$ per treatment) were exposed for 8 days in an individual static exposure scenario using plastic containers (8 × 8 × 8 cm) filled with 200 mL of aged tap water. No differences in weight of individuals allocated to different treatments were found (Supplementary material, Tables S4 and S5). After being allocated to their respective treatments, larvae were transferred to climate-controlled rooms and the temperature was slowly shifted towards the predetermined values (10 or 20 °C). When the temperature stabilized at the desired level, exposure containers assigned to each of the temazepam treatments were dosed with the respective amount of temazepam stock solution (1 mg L^{-1}, prepared by dissolving temazepam standard in ultrapure water). After the exposure period, each individual larva was euthanized by overdose with MS-222 at 0.3 g L^{-1}, sprayed with ultrapure water to remove any excess external temazepam, dried with paper towel, and stored at −18 °C until extraction and analysis. Water samples ($n = 30$) were collected on the last day of exposure from five randomly chosen exposure containers per treatment to validate the temazepam concentration.

It should be noted that dragonfly larvae were assessed for the purpose of another experiment, which explains slight differences between fish and dragonfly handling and exposure regimes.

2.3. Chemical analyses

The procedures followed to prepare water samples and extract biota samples for chemical analysis have been described in detail previously (see McCallum et al. (2019); Cerveny et al. (2020)). All concentrations measured in biota samples reported within this work are related to wet weight.

For analysis of dragonfly larvae, whole individuals were extracted, each representing one sample. In the case of fish tissues, an equal portion of muscle tissue from fillet and whole brains of three individuals were pooled before extraction and thus each sample reflects average tissue concentration based on three fish. This approach was taken primarily because of the small brain size of juvenile fish, but also to reduce the cost of chemical analysis. Liquid chromatography–mass spectrometry (LC–MS)-grade acetonitrile, used for extraction of target analytes from biota samples, 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 for liquid chromatography. Temazepam (CAS 846-50-4) was purchased at LGC Standards (Teddington, UK). Oxazepam (CAS 604-75-1) and mass-labeled $^{2}H_5$-oxazepam (CAS 65854-78-6) were purchased at Sigma-Aldrich (Steinheim, Germany).

The method of liquid chromatography–tandem mass spectrometry (LC–MS/MS) that was used to analyse all biota samples has been described previously (McCallum et al., 2019). Water analysis was based on an online solid phase extraction system coupled with liquid chromatography-tandem mass spectrometry (SPE LC–MS/MS), also described in previous work (Khan et al., 2012). The LC system used for both biota and water samples consists of Ultimate 3000 pump (LPG-3400SD, Thermo Fisher Scientific, San Jose, CA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland). Target analytes were separated using a Hypersil GOLD column (2.1 mm × 50 mm, 3 μm, Thermo Fisher Scientific) and analysed with a TSQ Quantiva triple quadrupole mass spectrometer equipped with heated-electrospray ionisation (Thermo Fisher Scientific). Descriptions of the basic set-up of the electrospray ionisation interface and the gradient/flow of the mobile phase are presented in the Supplementary material (Tables S6–S8).

Quantification of target compounds was based on an internal standard approach. A seven-point standard curve from 0.1 to 50 ng g^{-1} (biota samples) and an eight-point standard curve from 0.001 to 5 μg L^{-1} (water samples) were used to assess linearity and to derive an instrumental limit of quantification (LOQ). 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. Quality assurance and quality control (QA/QC) of the analytical method was evaluated based on its linearity, LOQ, and measurement of solvent and water blank samples.

2.4. Statistical analyses

All statistical analyses were performed using Statistica software, version 13.2 (StatSoft Inc., USA). One-way analysis of variance (ANOVA) tests followed by a Tukey's post-hoc tests were used to test for differences in concentrations of target analytes measured in samples of fish tissues and dragonfly larvae originating from different treatments. Observation of histograms supplemented with Shapiro-Wilk tests were used to assess whether the data were normally distributed. The concentrations obtained from analysis of fish tissues were log-transformed (ln x) to meet normality, while raw data were used for dragonfly larvae. To assess potential differences in weight and total length of experimental fish between the treatments, a non—parametric Kruskal—Wallis test was performed using the raw data as these did not fit normal distribution even after log transformation. Complete results of all statistical analyses related to measured concentrations of target compounds in fish and dragonfly larvae are provided in the Supplementary material (Tables S9–S20).

3. Results and discussion

The analytical method performed excellently for both target compounds. Linearity achieved by measurement of standard curves for water and tissue analysis was >0.999 and >0.998, respectively. The instrumental LOQs for measurement of water were set at 1 ng L^{-1} for both compounds (the lowest point of the prepared standard curve), and the mean LOQs in real water samples were 0.9 and 1 ng L^{-1} for temazepam and oxazepam, respectively. In the case of tissue analysis, the instrumental LOQs were 1 and 0.5 ng g^{-1} for temazepam and oxazepam, respectively, while mean LOQs in real samples ranged from 1.5 to 3.9 ng g^{-1} and from 0.6 to 1.5 ng g^{-1} for the two compounds. No oxazepam or temazepam concentrations above LOQ were measured in any of the blank samples. Low concentrations of oxazepam were detected in water from the temazepam treatments sampled at the end of the exposure period (Tables 1 and 2). Traces of oxazepam in these treatments were likely due to the biotransformation of the parent compound temazepam by the experimental organisms and subsequent excretion. However, we did not take any measures that would exclude initial contamination of the temazepam standard or its demethylation by microorganisms in the water during exposure.

Table 1
Measured concentrations of temazepam and oxazepam in water and in tissues of perch belonging to the low concentration (0.2 μg L^{-1}) exposure scenarios, and corresponding bioconcentration factors (BCF). See Supplementary Materials Table S21 for analogous results for the high concentration (2 μg L^{-1}) exposure treatments.

Temperature/Treatment		Water (μg L^{-1})			Fish tissues (ng g^{-1})							BCF (L kg^{-1})	
		n	Oxazepam	Temazepam	n	Oxazepam		Temazepam		∑ benzodiazepines		muscle	brain
						muscle	brain	muscle	brain	muscle	brain		
10 °C	Oxazepam	5	0.24 ± 0.071	<LOQ	5	3.1 ± 0.83	13 ± 2.9	<LOQ	<LOQ	3.1 ± 0.83	13 ± 2.9	13	56
	Temazepam	5	<LOQ	0.21 ± 0.025	5	2.7 ± 0.64	11 ± 2.4	4.9 ± 1.4	21 ± 2.9	7.6 ± 1.04	32 ± 1.4	24	100
	Mix	5	0.26 ± 0.061	0.25 ± 0.037	5	7.8 ± 3.79	31 ± 11.5	6.3 ± 1.34	24 ± 9	13 ± 5.6	55 ± 16.7	NA	NA
20 °C	Oxazepam	5	0.27 ± 0.064	<LOQ	5	5.3 ± 1.63	19 ± 4.8	<LOQ	<LOQ	5.3 ± 1.63	19 ± 4.8	19	70
	Temazepam	5	<LOQ	0.26 ± 0.071	5	9.9 ± 1.84	35 ± 6.1	6.5 ± 2.39	22 ± 7.2	16 ± 4.1	57 ± 13.2	25	83
	Mix	5	0.25 ± 0.031	0.24 ± 0.034	5	14 ± 3.3	46 ± 7.4	5.1 ± 1.09	17 ± 3.8	16 ± 4.6	63 ± 10.9	NA	NA

Table 2
Measured concentrations of temazepam and oxazepam in water and in whole-body homogenates of dragonfly larvae, and corresponding bioconcentration factors (BCF). Mean values and standard deviations are calculated using positive samples only.

Temperature/treatment		Water concentrations (μg L^{-1})			Dragonfly concentrations (ng g^{-1})			BCF (L kg^{-1})
		n	Mean ± SD (n positive)		n	Mean ± SD (n positive)		
			temazepam	oxazepam		temazepam	oxazepam	temazepam
10 °C	Low concentration	5	0.62 ± 0.02 (5)	<LOQ (0)	15	<LOQ (0)	<LOQ (0)	NA
	High concentration	5	6.30 ± 0.42 (5)	0.001 ± 0 (1)	15	2.8 ± 0.61 (10)	<LOQ (0)	0.44
20 °C	Low concentration	5	0.63 ± 0.03 (5)	<LOQ (0)	15	<LOQ (0)	<LOQ (0)	NA
	High concentration	5	6.10 ± 0.18 (5)	0.002 ± 0.0003 (5)	15	2.4 ± 0.47 (12)	<LOQ (0)	0.39

3.1. Oxazepam and temazepam in fish

The target compounds were not detected in any sample of water or fish tissue from the control treatments. Results of chemical analyses for the water and fish tissue samples originating from the low (0.2 μg L^{-1}) exposure treatments are presented in Table 1. Analogous results from the high (2 μg L^{-1}) exposure treatments are provided in the Supplementary material (Table S21). Substantial bioconcentration and biotransformation of temazepam was observed in fish. Moreover, both the uptake of temazepam and the production of oxazepam via fish metabolism was greatly affected by temperature treatment.

Specifically, when fish were exposed to 2 μg L^{-1} of temazepam, we found significantly higher bioconcentration of temazepam in brain ($p < 0.01$) at the lower temperature treatment (Supplementary material, Fig. S1); hence, a finding confirming our first hypothesis. The first hypothesis proved also valid for oxazepam, which in opposite to temazepam bioconcentrated more at the higher temperature treatment. This trend was present in both of the sampled tissues (brain and muscle) and at both the low and high exposure levels for oxazepam (Figs. 1 and S1), but a statistically significant ($p < 0.05$) difference was only found in muscle of fish exposed to 2 μg L^{-1}. Moreover, that bioconcentration of oxazepam in perch increase with temperature makes it possible to rule out that decreasing water temperatures explain previous observations from aquatic ecosystems where the tissue:water ratio of this drug increased as the autumn progressed (Lagesson et al., 2016).

When fish were exposed to the mixture of both compounds, oxazepam bioconcentration became significantly ($p < 0.05$) different between the two temperature regimes, even at 0.2 μg L^{-1}. We also observed a positive effect of temperature on both the uptake of oxazepam from the water and its production from temazepam via fish metabolism. In light of these results, we find that the effect of temperature on internal concentrations of specific drugs is likely enhanced when uptake from water and accumulation resulting from metabolic transformation occur simultaneously—i.e. exposure to a complex mixture where both parent compounds and their metabolites are present. This is important because such a scenario is expected in many aquatic environments affected by sewage treatment plant effluents, where fish are commonly exposed to multiple drugs contemporaneously.

Temperature had a clear effect on the biotransformation of the parent compound (temazepam) into its metabolite (oxazepam) in the fish, i.e. a finding supporting our second hypothesis. Significantly more oxazepam ($p < 0.01$) was produced and accumulated in fish tissues when they were exposed to the parent drug at 20 °C compared to 10 °C (Fig. 1 and Fig. S1). Concentrations of oxazepam exceeded those of temazepam in perch maintained at 20 °C, which was not seen in fish exposed at 10 °C (Table 1 and Table S21). A similar phenomenon has previously been reported in zebrafish (*Danio rerio*) exposed to fluoxetine (Chen et al., 2017) and in channel catfish (*Ictalurus punctatus*) exposed to diazepam (Overturf et al., 2016). In both studies, the metabolites were measured in higher concentrations than their parent drugs in fish tissues without the metabolites being dosed in the water. The effect of temperature was not investigated in those studies, but in both fish were maintained at 25 °C, which might indicate agreement with our findings. Nevertheless, without a deeper knowledge of potential species-specific differences in uptake and biotransformation, no conclusions can be drawn about the role of temperature in these studies.

Interestingly, oxazepam accumulation that originated from metabolic transformation (i.e. in fish that were exposed only to temazepam) was significantly higher ($p < 0.01$) than the bioconcentration of oxazepam for individuals that were exposed to oxazepam itself. Indeed, this observation is entirely unique and brings new insights to what is known about the fate of pharmaceuticals in the aquatic environment. To date, the most important exposure pathway of pharmaceuticals in aquatic organisms is considered to be direct uptake from the water (i.e. respiratory). But, based on our findings, metabolic transformation and the consequent accumulation of metabolites can result in a similar or even higher exposure compared to direct uptake. This is important because, if a metabolite is not thought of as being biologically active, or is even prescribed as a drug with the potential to induce pharmacological effects (as is the case for oxazepam), then evaluating the fate of pharmaceuticals without knowledge of their pharmacokinetics in non-target species might underestimate their potential to induce adverse effects in natural systems.

It is becoming more common in ecotoxicology to replace animal testing with modeling approaches for toxicity testing. However, there are still important knowledge gaps that need to be filled to properly parameterize these models. For instance, most of the models being used (e.g. fish plasma model) use data that are extrapolated from human or

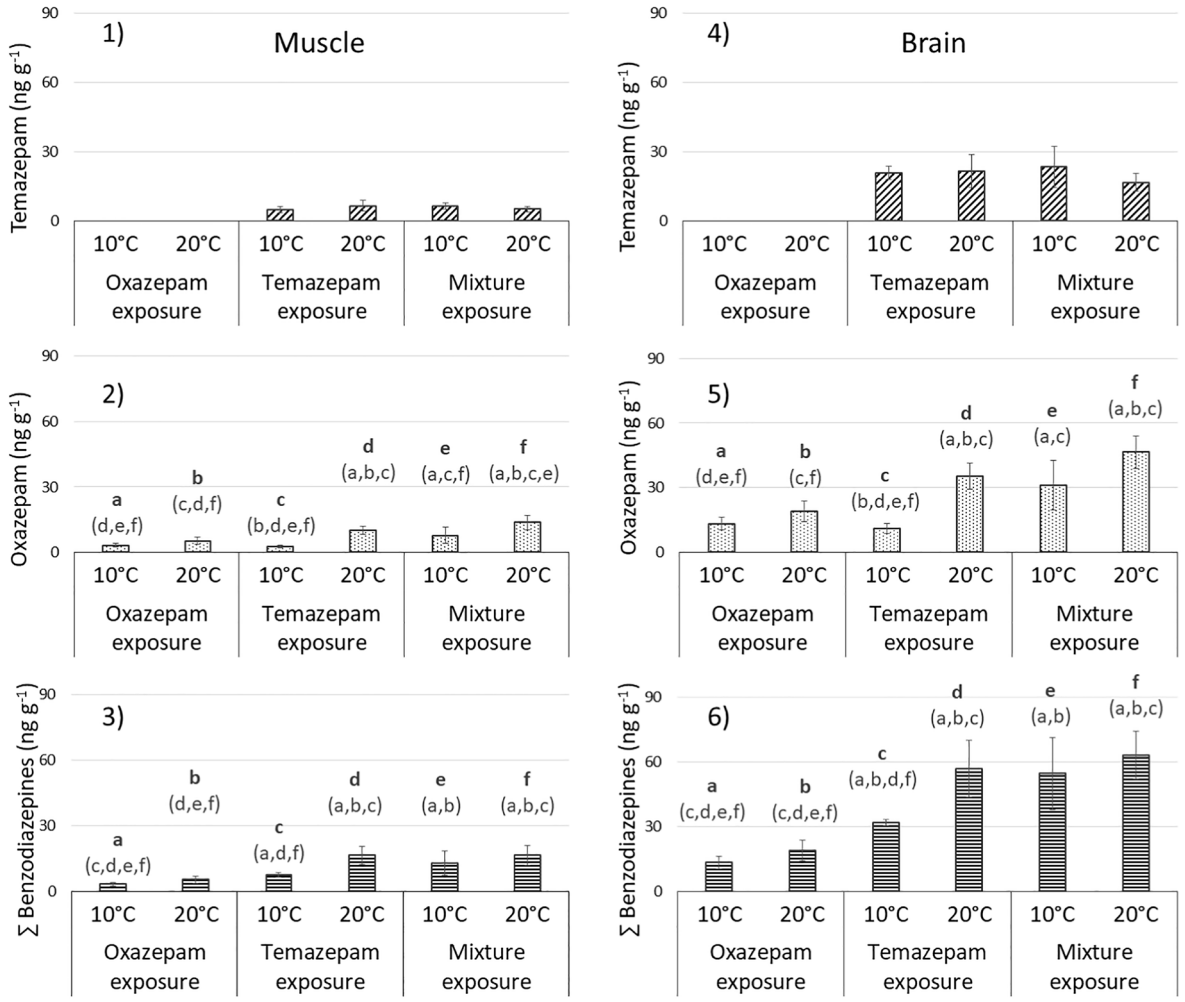

Fig. 1. Temazepam, oxazepam, and their sum concentrations (Σ Benzodiazepines; i.e. temazepam + oxazepam) measured in muscle (1–3) and brain (4–6) of perch after 8 days of exposure to one of six treatments. These treatments comprise single or mixture low concentration (0.2 μg L^{-1}) exposure scenarios at each of two temperature levels (10 and 20 °C). Within each plot, letters (**a–f**) have been assigned to each treatment. Where significant differences ($p < 0.05$) were seen between treatments, those treatments are listed in parentheses. See Supplementary materials Fig. S1 for analogous results for the high concentration (2 μg L^{-1}) exposure treatment, and Tables S9–S14 for detailed results of statistical analyses.

mammalian pharmacology (Huggett et al., 2003; Brown et al., 2007). Our findings indicate that such extrapolation can be widely inappropriate, especially in the case of ectotherms. For instance, oxazepam is not considered to be an important metabolite of temazepam in humans. This is because, together with its conjugates, oxazepam accounts for<5% of the temazepam dose in treated patients (Schwarz, 1979; Locniskar and Greenblatt, 1990). And yet, in our recent work, oxazepam in the plasma of perch was shown to reach concentrations comparable to human therapeutic plasma concentrations (HTPC) reported by Schulz et al. (2012) after fish had been exposed to 1.3 μg L^{-1} of temazepam (Cerveny et al., 2021a). Moreover, both human and veterinary pharmaceuticals are designed to induce effects and then be metabolized and excreted at a certain limited body temperature range, but ectothermic aquatic organisms can exist at a range of temperatures from close to 0 °C to over 40 °C. Temperature can also vary throughout the year for individuals living in temperate regions, meaning that they can face different exposure (internal concentrations) in warm versus cold seasons, even if they occupy the same locality with a theoretically stable pharmaceutical profile in the water column. However, it is well known that pharmaceutical pollution can also vary temporally (Lindholm-Lehto et al., 2016; Burns et al., 2018; Rehrl et al., 2020). This emphasizes the need to study the toxicokinetics of pharmaceuticals in non-target aquatic organisms under various temperature regimes that reflect their natural habitat and/or future climate change temperature scenarios.

Temazepam and oxazepam are psychoactive pharmaceuticals belonging to the same class of benzodiazepines. Both can act as modulators of gamma-aminobutyric acid (GABA) in the brain (Sieghart et al., 2012) and thus combinatory (i.e. additive or synergistic) effects could be expected when organisms are exposed to these compounds simultaneously (Ågerstrand et al., 2015; Backhaus, 2016; Cerveny et al., 2020). Moreover, both drugs are being prescribed to treat human patients, and they are among the most frequently detected benzodiazepines in surface waters (Fick et al., 2017). For these reasons, the sum of temazepam and oxazepam concentrations was compared and statistically evaluated between treatments. The sum benzodiazepine concentration was higher at 20 °C compared to 10 °C within each exposure scenario (i.e. oxazepam, temazepam, mixture), but a statistically significant difference ($p < 0.05$) was only found for the single exposure to temazepam (Fig. 1). Surprisingly, exposure to only temazepam resulted in a similar sum concentration of both of the benzodiazepines in fish tissues compared to fish that were exposed to both of the compounds simultaneously (when the same temperature treatment is considered). Hence, our results demonstrate that ecotoxicology approaches that omit biologically active metabolites from investigation are likely to underestimate risks related to pharmaceutical contamination in aquatic environments.

3.2. Oxazepam and temazepam in dragonfly larvae

Neither temazepam nor oxazepam were detected in the water or the larvae in the control treatment. Concentrations of both compounds measured in the water and the larvae from the temazepam exposure treatments are presented in Table 2 and discussed below.

Compared to the fish, relatively low bioconcentration of temazepam was observed for dragonfly larvae. No quantifiable concentrations were measured in individuals exposed to low temazepam treatments, while the mean temazepam concentrations in larvae from the high exposure groups were <3 ng g^{-1} (Table 2). No effect of temperature on temazepam uptake was detected (Supplementary material, Table S22). In other words, our first hypothesis was not valid for the dragonfly larvae. Literature related to pharmacokinetics of human drugs in macroinvertebrates is scarce, in general. In the work of Miller et al. (2017), bioconcentration of temazepam was studied in *Gammarus pulex* and a mean concentration of 38 ng g^{-1} (dry weight, $n = 3$) was reached after 48 h of exposure to 1 μg L^{-1}. Considering differences in temazepam exposure (their exposure was 6 times lower than in our work) and the fact that dry weight concentrations are typically higher by approximately a factor of four, there is still a great contrast when compared to our study. This indicates that there is a variability of temazepam bioconcentration between different species of macroinvertebrates.

No oxazepam was detected in any sample of dragonfly larvae, indicating limited or no biotransformation of the parent compound. Again, a finding indicating that our second hypothesis, being valid for perch, was not valid for the dragonfly larvae. Nevertheless, considering the low bioconcentration of temazepam observed in the present study, and the low bioconcentration of oxazepam reported for the same species previously by Heynen et al. (2016), biotransformation may still have occurred without oxazepam having reached quantifiable levels in the larvae. Similarly to the bioconcentration of temazepam, its limited biotransformation into oxazepam observed in our work is also in disagreement with study of Miller et al. (2017) mentioned above. In that study, three different metabolites, nordiazepam, temazepam, and oxazepam, were identified in *Gammarus pulex* after exposure to 1 μg L^{-1} of diazepam (a benzodiazepine that can be biotransformed into both temazepam and oxazepam, as well as nordiazepam).

After comparison with the very limited information that has been published so far, we conclude that there may be significant species-specific differences in uptake and biotransformation of pharmaceuticals in aquatic macroinvertebrates. Clearly, this research field would benefit greatly from more information on the pharmacokinetics of pharmaceuticals in different macroinvertebrates species. Moreover, it would be fruitful to identify indicator species that could be preferentially used to deepen our knowledge on the fate of pharmaceuticals in aquatic environments.

4. Conclusions

When evaluating risks related to the presence of PhACs in aquatic environments, prescribed pharmaceuticals (parent compounds), prioritized based on their occurrence in surface waters worldwide, are mostly considered. This approach is fairly reasonable based on the assumption that uptake from water represents the main exposure pathway for aquatic organisms. Nevertheless, as demonstrated by our work, certain PhACs can significantly accumulate in the body of aquatic organisms through biotransformation of the parent compound without being actually present in the water at all. Indeed, in the case of the compounds studied within this work, biotransformation was shown to be an important exposure pathway contributing significantly to direct uptake from the water. Therefore, pharmaceutical metabolites should be given more attention in future research as many can exert pharmacological effects comparable to parent drugs. Further, our findings underscore that using models to extrapolate data from mammalian pharmacology might not be an effective means for predicting pharmacokinetics in non-target species, especially for aquatic ectotherms. We also found that temperature affected the uptake, and especially the biotransformation, of temazepam to a great extent in fish. This observation is entirely novel and brings a new insight to our current understanding of the fate of PhACs in aquatic environments. Given that temperature represents one of the most dynamic parameters in surface waters, asymmetric effects of PhACs might be expected in aquatic organisms at both daily and seasonal scales. Moreover, as ecosystems warm on a global scale due to human-induced climate change, it is more urgent than ever before to understand how temperature affects the toxicokinetics of pharmaceutical contaminants in order to more effectively protect our aquatic ecosystems.

CRediT authorship contribution statement

D. Cerveny: Conceptualization, Investigation, Formal analysis, Writing - original draft. **J. Fick:** Conceptualization, Writing - review & editing. **J. Klaminder:** Conceptualization, Writing - review & editing. **E. S. McCallum:** Writing - review & editing. **M.G. Bertram:** Conceptualization, Investigation, Writing - review & editing. **N.A. Castillo:** Investigation, Writing - review & editing. **T. Brodin:** Conceptualization, Writing - review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

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