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Neuroactive drugs and other pharmaceuticals found in blood plasma of wild European fish

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ABSTRACT

To gain a better understanding of which pharmaceuticals could pose a risk to fish, 94 pharmaceuticals representing 23 classes were analyzed in blood plasma from wild bream, chub, and roach captured at 18 sites in Germany, the Czech Republic and the UK, respectively. Based on read across from humans, we evaluated the risks of pharmacological effects occurring in the fish for each measured pharmaceutical. Twenty-three compounds were found in fish plasma, with the highest levels measured in chub from the Czech Republic. None of the German bream had detectable levels of pharmaceuticals, whereas roach from the Thames had mostly low concentrations. For two pharmaceuticals, four individual Czech fish had plasma concentrations higher than the concentrations reached in the blood of human patients taking the corresponding medication. For nine additional compounds, determined concentrations exceeded 10% of the corresponding human therapeutic plasma concentration in 12 fish. The majority of the pharmaceuticals where a clear risk for pharmacological effects was identified targets the central nervous system. These include e.g. flupentixol, haloperidol, and risperidone, all of which have the potential to affect fish behavior. In addition to identifying pharmaceuticals of environmental concern, the results emphasize the value of environmental monitoring of internal drug levels in aquatic wildlife, as well as the need for more research to establish concentration-response relationships.

1. Introduction

It is accepted that most pharmaceuticals are not completely metabolized in the body and many are incompletely removed in wastewater treatment plants (WWTP). Thus, they can be found in receiving waters around the world. Detected surface water concentrations of pharmaceuticals usually range from low μg L^{-1} close to point sources down to low ng L^{-1}, with clear correlations to consumption volumes and populations served by the relevant WWTP, the dilution factor and technologies used to treat the wastewater (Fatta-Kassinos et al., 2011; Hughes et al., 2013; Fick et al., 2017; Yang et al., 2017; Tran et al., 2018). A range of environmental factors affecting in-stream primary degradation can also influence water concentrations of pharmaceuticals some of which have characteristic seasonal variations, e.g. temperature and the intensity of ultraviolet radiation. While concentrations are often low, many pharmaceuticals are highly potent and therefore may still exert pharmacological effects. Exposed wildlife, particularly vertebrates, often have conserved drug targets with humans, including many receptors and enzymes. Such conservation provides a potential for pharmacological effects via high-affinity interactions with target molecules in non-target species (Gunnarsson et al., 2008; Brown et al., 2014).

Fish, in particular, have the potential to be affected by residual pharmaceuticals, since they are water respiring organisms and at the same time they share many of the drug targets with humans. It should be

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emphasized that fish have a huge diversity in morphology, physiology and behavior and that inter- and intra-species specific uptake, bioaccumulation and tissue partitioning of pharmaceuticals is poorly understood. Several studies have shown detectable levels of pharmaceuticals in fish collected from various types of surface waters e.g. (Ramirez et al., 2007; Schultz et al., 2011; Huerta et al., 2012, 2018; Subedi et al., 2012; Du et al., 2014; Tanoue et al., 2015; Grabicova et al., 2017; Muir et al., 2017) and this literature was recently reviewed in Miller et al. (2018).

Many of the studies that have analyzed pharmaceuticals in wild fish have focused on antidepressants, antibiotics, and anti-histamines, but pharmaceuticals from other classes have also been detected (Huerta et al., 2012; Zenker et al., 2014; Miller et al., 2018). Although freshwater organisms often contain higher concentrations of pharmaceuticals (due to less dilution and often closer vicinity to point sources and hence less time to degrade before exposure), pharmaceuticals have also been found in marine species living in coastal areas (Maruya et al., 2012; Gelsleichter and Szabo, 2013; Alvarez-Munoz et al., 2015; Lolić et al., 2015; Alygizakis et al., 2016; Moreno-González et al., 2016; Liu et al., 2018). Concentrations reported by authors referenced within this work in various tissues and/or plasma range from sub ng g^{-1} (ng mL^{-1} for plasma) up to >100 ng g^{-1} (ng mL^{-1} for plasma) expressed as either dry or wet weight (the difference between wet weight and dry weight concentrations in fish tissue is typically around a factor of four).

Some studies have investigated concentrations in several tissues of the same individuals, such as blood plasma, muscle, liver, bile and brain (Brooks et al., 2005; Ramirez et al., 2009; Huerta et al., 2012; Brozinski et al., 2013; Grabicova et al., 2014, 2017; Tanoue et al., 2015). How they partition varies for different pharmaceuticals and their specific chemical properties, e.g. the highest levels of selective serotonin re-uptake inhibitors (SSRI) was shown in liver and brain tissues compared to muscle and plasma in three different species (Brooks et al., 2005; Ramirez et al., 2007; Grabicova et al., 2014) while the opposite was shown for the antihypertension drug diltiazem (Ramirez et al., 2007). Some compounds, e.g. sertraline, were not detected in plasma, while they were present in other organs (Grabicova et al., 2017).

Blood plasma may not always contain the highest levels (compared to other organs) in exposed biota, however data on blood plasma levels in wildlife provides the possibility to make direct comparisons with the corresponding human therapeutic plasma concentrations (HTPCs) available for most drugs. This "Read-Across Hypothesis" suggests that since pharmaceuticals are designed to act at specific mammalian targets they may have effects in non-target organisms at similar plasma concentrations (Huggett et al., 2003). This hypothesis is only valid if drug targets are conserved between species, which has been shown to be the case for several drug targets in several fish species (Gunnarsson et al., 2008; Rand-Weaver et al., 2013; Brown et al., 2014; Margiotta-Casaluci et al., 2014). It is, therefore, possible to calculate a concentration ratio (CR) between measured plasma levels and HTPCs that indicate the probability of a pharmacological effect in exposed biota. However, threshold effect levels for some compounds might differ between species.

The aim of this study was to determine concentrations of 94 pharmaceuticals in 110 plasma samples from fish caught at 18 sites in the UK, Germany and the Czech Republic, and identify the pharmaceuticals of most concern by comparison to human therapeutic levels (read-across).

2. Materials and method

2.1. Selection of pharmaceuticals included in the study

Target pharmaceuticals were selected based on a combination of potency, physico-chemical properties and sales volume as has been described previously (Fick et al., 2010b). Additional criteria were availability of commercial reference standards and inclusion of as many therapeutic classes as possible. The final selection included 94 pharmaceuticals from 23 classes (Supporting information, Table S1).

2.2. Chemicals and reagents

All of the reference pharmaceuticals standards were classified as analytical grade (>98%). Internal standards used were; $^{2}H_{6}$ -amitriptyline, $^{2}H_{10}$-carbamazepine, $^{13}C_{3}^{15}N$-ciprofloxacin, $^{13}C_{2}$-ethinyl estradiol, $^{2}H_{5}$-fluoxetine, $^{13}C_{6}$-sulfamethoxazole, $^{13}C^{2}H_{3}$-tramadol and $^{13}C_{3}$-trimethoprim, obtained from Cambridge Isotope Laboratories (Andover, MA, USA), $^{2}H_{5}$-oxazepam, $^{2}H_{4}$-risperidone, and $^{13}C_{2}^{15}N$-tamoxifen, bought from Sigma-Aldrich (Steinheim, Germany) and $^{2}H_{6}$-codeine, $^{2}H_{4}$-diclofenac, $^{2}H_{4}$-flecainide, $^{2}H_{3}$-ketoprofen, $^{13}C_{3}^{2}H_{3}$-naproxen $^{2}H_{3}$-paracetamol purchased from CDN-Isotopes (Pointe-Claire, Quebec, Canada). LC/MS grade quality of methanol and acetonitrile were purchased (Lichrosolv - hypergrade, Merck, Darmstadt, Germany) and purified water was prepared using a Milli-Q Advantage, ultrapure water system including an UV radiation source (Millipore, Billerica, USA). Formic acid (Sigma-Aldrich, Steinheim, Germany) was used to prepare the 0.1% mobile phases.

2.3. Sampling and sampling locations

Sampling locations were chosen to include both effluent dominated sites and less impacted sites. Samples were obtained from on-going national sampling campaigns in Germany, United Kingdom and the Czech Republic. Samples from each sampling site were analyzed within six months after sampling to avoid differences in storage time. Detailed characteristics of sampling locations with estimated dilution factors are given in Table 1.

Germany; Representative bream (*Abramis brama*) plasma samples collected in 2007–2008 at 7 different German Environment Specimen Bank (GESB) sampling locations were provided from the GESB archive (n = 10, for each site). Sampling locations included 6 river sites located downstream from WWTP discharges along the Danube, Elbe, Mulde, Rhine, Saale and Saar rivers and Lake Belau, a reference lake site, which does not receive WWTP effluent.

United Kingdom, Plasma samples from roach (*Rutilius rutilus*) (n = 30) were taken at ten particular sampling points along 25 km of the lower River Thames (between Marlow and Old Windsor) in September 2011. The Thames Basin upstream of Windsor (the most downstream fish sampling point) covers 7,046 km^2 with a mean annual flow of 58 $m^3 s^{-1}$ receiving waste from an estimated 4.5 million people (Marsh, 2008). Major upstream cities include Reading, Oxford and Swindon.

Czech Republic; Plasma samples from chub (*Squalius cephalus*) (n = 10, at one site), were collected in 2012. Sampling location was in the Bezdrevský stream, a small tributary of Vltava River in the South Bohemia region with a total catchment area of 340 km^2. No discharges from hospitals or manufacturing sites for pharmaceuticals are present within the catchment area. This locality represents a typical regional scenario where effluent from WWTP is discharged into a small recipient watercourse.

2.4. Sample pretreatment

Five nanograms of each internal surrogate standard and methanol with 0.1% formic acid (100 μL) were added to each plasma sample (100 μL) which was then frozen at −18 °C overnight. Samples were thawed, 200 μL of water (with 0.1% formic acid) were added and the samples were centrifuged at 14,000 revolutions per minute for 10 min.

2.5. Analytical system

The autosampler used in this study was a PAL HTC autosampler with cooled sample trays (CTC Analytics AG, Zwingen, Switzerland). Accela pump, mass analyzer (TSQ Quantum Ultra EMR, triple stage quadrupole MS/MS) and software (Xcalibur) were made by Thermo Fisher Scientific

Table 1
Characteristics of the sampling sites with estimated dilution factors.

Sampling site	Date	MAF[1] (m^3 s^{-1})	Significant WWTPs[2]	DSL[3] (km)	EI[4]	EDL[5]
Bezdrevský stream, Netolice (Cz)	2/2012	0.482[6]	Netolice	0	2 700	48x
			Lhenice	15	1 800	
River Saar, Güdingen (G1)	7/2008	60	Brebach	0	135 000	131x
			Saargemünd	11	61 500	
River Rhine, Bimmen (G2)	7/2008	2000	Salmorth	7	40 833	3 717x
			Emmerich	14	126 736	
			Kalkar-Hönnepel	23	38 401	
			Xanten-Vynen	34	606	
			Xanten-Lüttingen	41	5 753	
			Wesel	50	19 900	
River Elbe, Blankenese (G3)	8/2008	800	Köhlbrandhöft/ Dradenau	4	2 900 000	117x
			Geesthacht Düneberg	43	60 000	
River Saale, Wettin (G4)	8/2008	115	Halle-Nord	15	300 000	54x
			Leipzig-Rosental	64	628 000	
River Mulde, Dessau (G5)	8/2008	64	Bitterfeld-Wolfen	37	422 000	65x
River Danube, Jochenstein (G6)	9/2008	1000	Obernzell	0	≈ 10 000	3 597x
			Thyrnau	6	≈ 10 000	
			Achleiten	8	≈ 100 000	
Lake Belau (G7)	9/2008	reference site, no WWTP effluent				
River Thames (UK)	9/2011	58	Whole catchment upstream of Windsor		4 500 000	10x

[1]Mean annual flow at the sampling location; [2]Known WWTP effluent discharges upstream of sampling location - the information provided does not include all possible sources of pharmaceutical pollution, e.g. distant sources (>100 km); [3]Distance of WWTP discharge from sampling location; [4]equivalent inhabitants served by WWTP; [5]N fold dilution of sum of WWTP effluents at the sampling location using an assumed per capita waste discharge of 200 L/cap/d. Characteristics of German sampling sites and WWTPs were adopted from Subedi et al. (2012); [6]actual flow at the date of sampling was estimated to be 0.05 m^3 s^{-1}.

(San Jose, CA, USA). Heated electrospray (HESI) in positive ion mode was used for ionization of the pharmaceuticals. Specific details related to the determination of the pharmaceuticals including HESI ionizations, polarities, precursor/product ions, collision energies, tube lens values, etc. have been described elsewhere (Grabic et al., 2012).

2.6. Quality assurance and quality control

Two MS/MS transitions were used for positive identifications of analytes with the criterion that the ratio between the transitions was not allowed to deviate more than +/−30% from the ratio in the corresponding calibration standard. Retention times for all analytes also had to be within +/−2.5% of the retention time in the corresponding calibration standard. Together, this gave four identification points (the highest possible number), as described in the Commission Decision 2002/657/EC (European Commission, 2002) concerning the performance of analytical methods and the interpretation of results. The limit of quantification (LOQ) was determined from standard curves based on repeated measurements of low level spiked plasma samples, and the lowest point in the standard curve that had a signal/noise ratio of at least 10 was considered to be equal to the LOQ. A seven-point matrix adjusted calibration curve over the range of 0.05–100 ng mL^{-1} was used for linearity evaluation and quantification. Carry-over effects were evaluated by injecting standards at 100 ng mL^{-1} followed by two mobile phase blanks. Several instrumental and field blanks were included in the analytical runs.

3. Results

No carry-over effects were observed, no pharmaceuticals were detected in the instrumental or field blanks and R^2 values were above 0.99 for all calibration curves in the given concentration ranges. Absolute recoveries in fish plasma are shown in Table S1, Supporting Information.

A total of 23 different pharmaceuticals were detected in at least one fish sample. No pharmaceuticals were found in the 70 bream plasma samples from Germany, while a total of 12 pharmaceuticals were observed in 22 out of 30 examined roach from River Thames (UK) and a total of 18 pharmaceuticals were found in 9 out of 10 chub from the Czech Republic.

Flecainide was the most frequently detected pharmaceutical in the UK plasma samples (15/30), followed by carbamazepine (7/30). Flecainide is used to treat patients with a high heart rate and carbamazepine is a commonly used anti-convulsant. Other detected pharmaceuticals were found in single individuals, usually at concentrations close to the LOQ. All determined concentrations in each individual fish are presented in Table S2, Supporting information.

In case of the Bezdrevský stream (CZ), 8 out of the 18 detected pharmaceuticals (bupropion, clarithromycin, clomipramine, diphenhydramine, haloperidol, oxazepam, risperidone, venlafaxine) were found at concentrations above the LOQ in at least 30% of the sampled individuals, but carbamazepine and flecainide were not detected in Czech fish plasma. The most consistent presence in Czech fish was risperidone (9/10), an anti-psychotic drug, followed by the antidepressant bupropion (4/10) and the antibiotic clarithromycin (4/10). All detected concentrations in each individual fish are presented in Table S3, Supporting information.

Two pharmaceuticals, the anti-psychotic drugs risperidone and/or flupentixol were found in fish plasma at levels higher than the HTPC, i.e. fish plasma concentration/HTPC (CR) > 1, representing high risk to induce pharmacological effects, both in samples from the Czech Republic. In the case of risperidone, three of nine positive samples exceeded this threshold. Flupentixol concentrations were quantifiable in only two samples due to relatively high LOQ (higher than HTPC), but concentrations in these two positive samples were more than ten times higher than the HTPC. Nine more pharmaceuticals had a CR > 0.1 in 12 individual fish from the UK and Czech Republic, which could be considered to reflect a moderate risk of inducing pharmacological effect, however the mean values usually were below this threshold (Tables 2 and 3). These nine pharmaceuticals were the antidepressants bupropion, clomipramine and mianserin; the neuroleptic haloperidol; the anxiolytic drug oxazepam; the antibiotic azithromycin; the antidiabetic drug repaglinide; alfuzosin, a drug for treatment of benign prostatic hyperplasia and the antifungal clotrimazole. The majority (6/10) of Czech fish had more than one pharmaceutical with a CR > 0.1 or/and a CR > 1 (up to four compounds) in their plasma, indicating the possibility of unknown combinatory effects in these individuals.

4. Discussion

In this study we have analyzed a large set of pharmaceuticals in blood plasma of wild fish from three European countries. By comparing detected levels in the fish to those observed in human blood plasma

Table 2
Detection frequency, average and concentration range, and risk assessment of analyzed pharmaceuticals in fish plasma samples (n = 10) from the Bezdrevský stream, Czech Republic.

Name	D.F.[a]	Plasma concentration in ng mL^{-1}			HTPC[b]		Estimated risk [c]	
		mean ± SD (min - max)		median	ng mL^{-1}		(min - max)	
Azithromycin	1/10	4.15 ± 5.218	(<5–19)	<LOQ	40	🟡	**	(0.063–0.475)
Biperiden	1/10	0.06 ± 0.019	(<0.1–0.11)	<LOQ	50	🟢	*	(0.001–0.002)
Bisoprolol	1/10	0.06 ± 0.019	(<0.1–0.11)	<LOQ	10	🟢	*	(0.005–0.011)
Bupropion	4/10	0.28 ± 0.441	(<0.1–1.4)	<LOQ	10	🟢	*	(0.005–0.14)
Clarithromycin	4/10	2.44 ± 3.4	(<1–11)	<LOQ	200	🟢	*	(0.003–0.055)
Clomipramine	3/10	1.8 ± 3.997	(<0.5–13)	<LOQ	20	🟢	*	(0.013–0.65)
Clotrimazole	1/10	0.73 ± 0.727	(<1–2.8)	<LOQ	34	🟢	*	(0.015–0.082)
Desloratadine	1/10	0.32 ± 0.206	(<0.5–0.90)	<LOQ	10	🟢	*	(0.025–0.09)
Diphenhydramine	3/10	0.05 ± 0.053	(<0.05–0.19)	<LOQ	50	🟢	*	(0.001–0.004)
Flecainide	1/10	0.06 ± 0.032	(<0.1–0.15)	<LOQ	200	🟢	*	(<0.001–0.001)
Flupentixol	2/10	3.72 ± 3.031	(<5–12)	<LOQ	0.5	🔴	***	(5–24)
Haloperidol	3/10	0.14 ± 0.18	(<0.1–0.60)	<LOQ	1	🟡	**	(0.05–0.6)
Hydroxyzine	1/10	0.28 ± 0.089	(<0.5–0.53)	<LOQ	50	🟢	*	(0.005–0.011)
Oxazepam	3/10	6.95 ± 7.823	(<5–25)	<LOQ	200	🟢	*	(0.013–0.125)
Repaglinide	1/10	0.2 ± 0.561	(<0.05–1.8)	<LOQ	15	🟢	*	(0.002–0.12)
Risperidone	9/10	4.41 ± 3.720	(<0.1–10)	4.70	6	🟡	**	(0.008–1.667)
Sotalol	2/10	0.45 ± 0.496	(<0.5–1.8)	<LOQ	500	🟢	*	(0.001–0.004)
Venlafaxine	3/10	3.26 ± 5.86	(<0.5–11)	<LOQ	200	🟢	*	(0.001–0.085)

[a] Detection frequency.
[b] Human therapeutic plasma concentrations (for more information see Table S4, Supporting information).
[c] Risk of inducing the therapeutic effect in fish; 🔴***, high risk (mean concentration exceeding the HTPC); 🟡**, moderate risk (mean concentration below HTPC, but exceeding 10% of HTPC); 🟢*, low risk (mean concentration below 10% of HTPC). To calculate mean concentration and mean risk quotient, samples below LOQ were replaced with ½ of LOQ value.

Table 3
Detection frequency, average and concentration range, and risk assessment of analyzed pharmaceuticals in fish plasma samples (n = 30) from 10 sampling sites along a 25 km stretch of the River Thames, UK.

Name	D.F.[a]	Plasma concentration in ng mL^{-1}			HTPC[b]		Estimated risk[c]	
		Mean ± SD (min - max)		Median	ng mL^{-1}		(min - max)	
Alfuzosin	1/30	0.06 ± 0.051	(<0.1–0.33)	<LOQ	3	🟢	*	(0.017–0.11)
Bupropion	1/30	0.06 ± 0.064	(<0.1–0.4)	<LOQ	10	🟢	*	(0.005–0.04)
Carbamazepine	7/30	1.09 ± 1.229	(<1–4.4)	<LOQ	2000	🟢	*	(<0.000–0.002)
Clomipramine	2/30	0.33 ± 0.366	(<0.5–2.2)	<LOQ	20	🟢	*	(0.013–0.11)
Clotrimazole	2/30	0.66 ± 0.613	(<1–3.4)	<LOQ	34	🟢	*	(0.015–0.1)
Desloratadine	1/30	0.33 ± 0.429	(<0.5–2.6)	<LOQ	10	🟢	*	(0.025–0.26)
Flecainide	15/30	0.43 ± 0.714	(<0.1–2.8)	0.06	200	🟢	*	(<0.000–0.014)
Mianserin	1/30	0.57 ± 0.365	(<1–2.5)	<LOQ	10	🟢	*	(0.05–0.25)
Miconazole	2/30	0.93 ± 2.108	(<1–12)	<LOQ	250	🟢	*	(0.002–0.048)
Pizotifen	1/30	0.07 ± 0.091	(<0.1–0.55)	<LOQ	7	🟢	*	(0.007–0.079)
Repaglinide	2/30	0.09 ± 0.291	(<0.05–1.6)	<LOQ	15	🟢	*	(0.002–0.107)
Risperidone	2/30	0.07 ± 0.089	(<0.1–0.54)	<LOQ	6	🟢	*	(0.008–0.09)

[a] Detection frequency.
[b] Human therapeutic plasma concentrations (for more information see Table S4, Supporting information).
[c] Risk of inducing the therapeutic effect in fish; 🔴***, high risk (mean concentration exceeding the HTPC); 🟡**, moderate risk (mean concentration below HTPC, but exceeding 10% of HTPC); 🟢*, low risk (mean concentration below 10% of HTPC). To calculate mean concentration and mean risk quotient, samples below LOQ were replaced with ½ of LOQ value.

during therapy, we have identified a range of drugs, in particular neuroactive ones, at levels that would be expected to cause pharmacological effects in wild fish. For two pharmaceuticals, blood levels in a few individual fish exceeded the levels achieved in blood during therapy in humans at the Czech locality, while nine exceeded a tenth of those concentrations in Czech and UK fish. For the majority of these drugs, the risk was only observed at the level of single individuals, but for azithromycin, flupentixol, haloperidol, and risperidone, the threshold was exceeded even at the population level (mean value), again only at the Czech site. Previous research on the risks pharmaceuticals pose in the environment has primarily not been guided by a read-across approach, but has more often been based on comparing predicted or measured exposure via surface water to effect data from controlled exposure studies. We think the read-across approach has a value in identifying pharmaceuticals of environmental concern, but results should be followed up by controlled effect studies. More field studies assessing exposure would be valuable to judge how representative our results are. Investigating how abiotic factors (including temperature) and the choice of indicator species might affect plasma levels in wild fish would further increase the interpretability of read-across studies in general.

In our previous research, a number of compounds were measured in various fish tissues that were not detected in plasma (Grabicova et al., 2017). Additionally, for many of the pharmaceuticals highlighted here, ecotoxicity data with relevance for their specific mode of action is lacking or very limited. We therefore suggest an increased focus on these drugs in order to better understand their exposure potential and impact on aquatic wildlife. The levels of pharmaceuticals varied between species and sites in a way we cannot fully explain. Hence, there is also a need to better understand the factors that govern e.g. bioconcentration, both in order to effectively design environmental monitoring approaches and for pointing out species and populations that are at increased risks to be affected by residual drugs.

4.1. Possible ecological consequences of analyzed pharmaceuticals in fish plasma

Flupentixol, haloperidol and risperidone represent neuroleptics that have antipsychotic effects in humans likely caused by dopamine (D_2) and/or serotonin 2A (5-HT_{2A}) receptor antagonism. As D_2 and 5-HT_{2A} receptors represent evolutionary highly conserved mechanisms (Gunnarsson et al., 2008), adverse effects of neuroleptics including behavioral changes may be expected in some aquatic animals as well. Haloperidol and risperidone have previously been found in wild fish (Fick et al., 2010a; Tanoue et al., 2015; Grabicova et al., 2017). A German research group has published a series of papers, reporting effects of very low levels (down to 0.3 ng L^{-1}) of risperidone on fish (Idalencio et al., 2015; Kalichak et al., 2017). Moreover, findings of Kalichak et al. (2019) suggest that some of these effects might be irreversible and even transgenerational as they were observed in adult fish that were only exposed to risperidone during early larval stage and in their F1 generation. In case of adult fish, however, the only significant effects were observed in prey-predator tests and these did not follow any dose-response relationships. More studies are thus warranted to clarify the potency and actions of risperidone in fish, and hence to understand the environmental risks associated with this drug, once exposure is taken into account. For haloperidol and flupentixol, we are not aware of any studies in fish that are likely to reflect their specific mode of action.

The antidepressants mianserin, bupropion, and clomipramine were all found in plasma of a few individual fish at concentrations indicating a moderate risk. Mianserin interacts with serotonin, histamine and κ-opioid receptors and inhibits the reuptake of norepinephrine. Bupropion is a norepinephrine–dopamine reuptake inhibitor (NDRI) and a nicotinic receptor antagonist. Clomipramine inhibits norepinephrine reuptake and, at the same time, acts as an antagonist on histamine, acetylcholine, and adrenergic receptors. These three compounds have been found in wild fish in other studies (Schultz et al., 2010; Arnnok et al., 2017; Grabicova et al., 2017). While there is a rather large literature on the ecotoxicity of selective serotonin reuptake inhibitors, these antidepressant have received considerably less attention, particularly with regards to effects of realistic (ng L^{-1}) levels on fish behavior.

Oxazepam, an anxiolytic that has frequently been found in surface waters (Fick et al., 2017), was determined in the plasma of three individual fish from the Czech sampling site in concentrations up to 25 ng mL^{-1}. As the HTPC for oxazepam is 200 ng mL^{-1}, the higher concentrations represent a moderate risk. However, some previous studies reported effects on behavior in fish having the internal oxazepam concentration between 6.6 and 36 ng g^{-1} in muscle (Brodin et al., 2013) and 30 ng mL^{-1} (mean, $N = 32$) in plasma (Huerta et al., 2016). These findings might indicate higher sensitivity of certain vertebrates to specific pharmaceuticals compared to human therapeutic plasma levels.

4.2. Variability of plasma concentrations

There were remarkable differences in the levels of pharmaceuticals detected in the German, UK, and Czech fish. Reasons explaining the large discrepancies could include regional differences in use, differences in removal efficiencies between WWTPs, different abilities of the three fish species to bioconcentrate the drugs (in turn affected by e.g. foraging behaviors, species-specific metabolism etc.), potential differences in handling fish before sampling, and, importantly, the nature and dilution of treated wastewater effluents at the investigated sites. Here we discuss some of these potential explanations.

Subedi et al. (2012) studied uptake in fish tissue samples from the same species (bream) from the same locations in Germany, taken at the same time as the plasma samples analyzed in the present study. These authors analyzed 15 pharmaceuticals but only detected one parental compound (diphenhydramine) and one metabolite (desmethylsertraline) in the range of 0.04–0.07 ng g^{-1} (ww) and 1.65–3.28 ng g^{-1} (ww), respectively. Although we did not find diphenhydramine in any of the analyzed bream plasma samples from Germany, the non-detect for all other analyzed drugs in both studies is largely in agreement with the lack of detections reported in our study.

It is important to remember pharmaceuticals do not behave as persistent organic pollutants or as heavy metals, which accumulate in biota over time. Pharmaceuticals are designed by drug companies to be ultimately metabolized or/and excreted by their human patients and it is not unreasonable to expect this to occur also in most other vertebrates. Thus, it seems right to assume that the detections in plasma found here represent the recent experience of these fish. The most plausible explanation behind the varying levels of pharmaceuticals found at the different sites then would be that the level of exposure is likely to differ, although it may not explain the entire difference found. Dilution of WWTP discharges (Table 1) would explain the lack of detections at some German sites (G1, G2, G3, G6, and reference G7), where dilution factors were estimated to be >100 fold, but not in the other two. Sampling sites located at Saale (G4) and Mulde (G5) Rivers were characterized with a similar dilution factors as the Bezdrevský stream (Cz), which indicates that other factors may be pivotal for the absence of pharmaceuticals in fish plasma from these sites. Nevertheless, it should be noted that dilution factors were calculated from median values of flow rates at the sampling sites, which does not need to correspond to the actual situation at the time of sampling. For instance, at Czech site the actual flow was estimated to be about 10% of its median value, which means only a tenth of the median dilution of WWTP effluent at the date of sampling. It was shown for some pharmaceuticals that steady state can be reached relatively quickly in fish, e.g. 2 days in case of certain NSAIDs (Brown et al, 2007), similar in case of benzodiazepine drug temazepam (McCallum et al, 2019). In line with these findings, we suppose that even short-term exposure to elevated concentrations of detected pharmaceuticals due to very low dilution of WWTPs effluent at Czech site could lead to rapid increase in concentrations of these compounds in fish plasma.

As we do not have exact data about consumption of individual compounds included in this study in different regions, it is hard to estimate to which extent this would affect the plasma concentrations. Some information can be obtained from OECD databases, e.g. for all antidepressants, the defined daily dose per 1000 inhabitants day^{-1} was 38, 42, and 70 in the Czech Republic, Germany, and UK respective in 2011 (OECD, 2011). Still, antidepressants were more frequent in plasma of Czech fish compare to UK.

Different foraging behavior between bream, roach, and chub could be a partial explanation. It needs to be emphasized that respiration is known to be the main pathway of most pharmaceuticals exposure in fish, but feeding preferences might contribute to some extent particularly for hydrophobic compounds, which adsorb strongly to solids. Several studies have also shown a wide variation in the uptake and depuration of pharmaceuticals between different species (Miller et al., 2018). Unfortunately, there is a lack of data regarding the pharmacokinetics of these pharmaceuticals in the species included in our study. In the very recent investigation of Malev et al. (2020), 87 pharmaceuticals were detected in plasma of chub sampled in the Sava River (Croatia) compared to 60 and 54 in barbel (*Barbus barbus*) and spirlin (*Alburnoides bipunctatus*), indicating species specific differences in uptake.

Contrary to the German and UK sites, fish from Czech Republic were caught and sampled in winter (February). Higher concentrations of pharmaceuticals are likely to be expected in the recipients of WWTPs due to their lower removal efficiency during the cold periods (Golovko et al., 2014). Moreover, estimated flow rate at the time of sampling was about 10% of the annual median at Czech sampling site, which could significantly affect the dilution of WWTP effluents. The recent study of Grabicova et al. (2020) investigated psychoactive compounds in water and fish tissues at 10 Czech localities including the Bezdrevský stream. Only sertraline was found in fish brain from this locality, which might indicate different dilution due to higher flow rate at the time of sampling or/and possible improvements in technology of local WWTP. The site expressed intermediate level of contamination from 10 investigated sites

based on the number of compounds and their concentrations present in water and in fish tissues. However, concentrations in fish blood plasma were not investigated.

It has been hypothesized that co-exposure to multiple chemicals/pharmaceuticals could enhance bioconcentration, as suggested by a meta-analyses of levels of non-steroidal anti-inflammatory drugs in fish (Cuklev et al., 2012). Fish exposed to complex sewage effluent had higher levels of e.g. ketoprofen than fish exposed to similar levels of ketoprofen in clean water. Although still largely a speculation, this phenomenon could be due to saturation of detoxifying systems in the fish. Such a mechanism would be characterized by positive feedback (i.e. given that a certain exposure threshold is met, internal exposure will increase more rapidly). The further implications of this would be quite profound, as we then would have to assess risks with pharmaceuticals in the light of all other chemicals present. This, again, speaks in favor of a field-based approach as taken here.

5. Conclusions

When trying to identify pharmaceuticals with increased risks to affect wild fish, we show that a strategy that takes advantage of the drugs inherent biological potency combined with actual levels found in wild fish represents a promising approach. Applying read-across directly on analyzed, rather than theoretically derived, plasma levels from wild fish could help to reduce uncertainties of read-across studies concerning estimates of external exposure (via e.g. sales, removal in sewage treatment plants, environmental fate or direct analyses of water) and bioconcentration factors, which in the fish plasma model is only driven by lipophilicity. Furthermore, analyzing blood plasma samples does not necessarily mean that the studied fish need to be killed. Thus, this approach also allows studies of e.g. threatened fish populations and/or allows larger populations to be sampled to obtain reliable data. Nevertheless, additional research is necessary to reveal how species-specific differences in physiology, temperature, and co-exposure to other chemicals affect the pharmaceutical concentrations in fish plasma, which might help explain the high variability in plasma concentrations observed for some drugs in this study.

We show that wild fish in certain European watercourses might be exposed to some pharmaceuticals at concentrations expected to affect their physiology. Many or even most of the pharmaceuticals found are not well studied in terms of ecotoxicity. Hence, more research is needed to establish dose–response relationships for these drugs in fish, and possibly also mitigations to reduce exposure. The majority of the pharmaceuticals presenting moderate or high risk in this study were neuroactive compounds characteristic with mid-range lipophilicity (log P), with a potential to alter natural fish behavior. Moreover, six of ten fish from the Czech site were facing a mixture of compounds at risk concentrations, which emphasize the need to study their combinatory effects as well.

CRediT authorship contribution statement

Daniel Cerveny: Formal analysis, Writing - original draft. **Roman Grabic:** Writing - review & editing. **Kateřina Grabicová:** Writing - review & editing. **Tomáš Randák:** Investigation, Funding acquisition. **D. G. Joakim Larsson:** Conceptualization, Writing - review & editing. **Andrew C. Johnson:** Conceptualization, Writing - review & editing. **Monika D. Jürgens:** Writing - review & editing. **Mats Tysklind:** Writing - review & editing. **Richard H. Lindberg:** Investigation, Writing - review & editing. **Jerker Fick:** Conceptualization, Investigation, 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106188.

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