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Psychoactive compounds in aquatic environment and their effects on fish

Psychoaktivní sloučeniny ve vodním prostředí
a jejich účinky na ryby

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**Doctoral thesis by
Maria Eugenia Sancho Santos**

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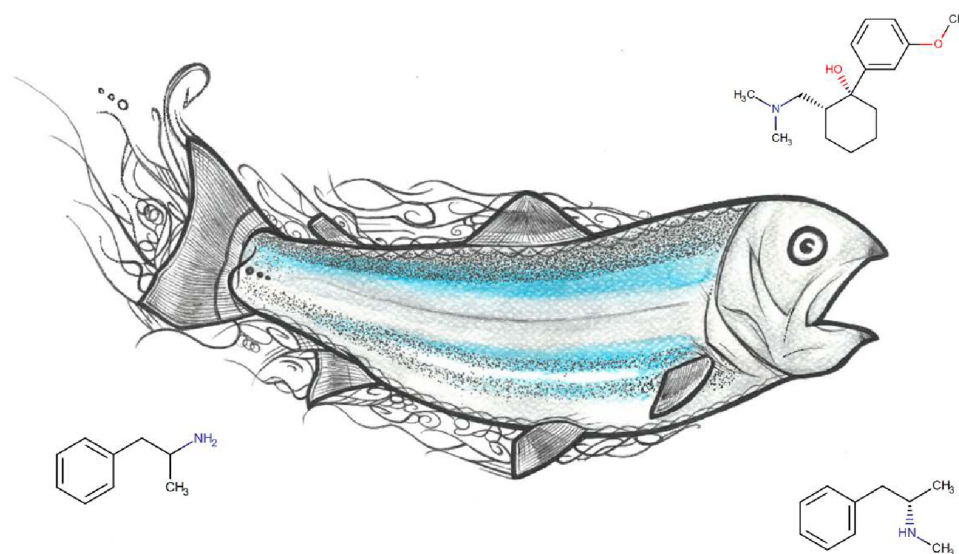
Czech Republic, Vodňany, 2021

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Psychoaktivní sloučeniny ve vodním prostředí a jejich účinky na ryby

CONTENT

CHAPTER 1

INTRODUCTION

1.1. Introduction

The freshwater ecosystems are facing an innumerable amount of threats, including pollution from diverse sources (agriculture, industry, cities), modification of the natural structure of water bodies, climate change, invasive species… (OECD, 2017). It seems crucial to understand these dynamics, so that it would be possible to look for solutions. However, there is a lack of information nowadays regarding the potential effects over the environment, thus, the consequences at ecological level remain unknown.

At present, we live in a rapidly changing society, with a 7.6 billion world population and a trend in grouping together in metropolitan areas (OECD, 2020). In fact, the cities increased in population more than double since 1975 (OECD, 2020). The recent developed lifestyle lead into the discharge of a wide variety of compounds used for human and veterinary purposes, in continuous increasing volumes. Some of these are denominated "emerging compounds" and include personal care products, pharmaceuticals, and illicit drugs (Thomaids et al., 2012). Despite their frequent detection in water, the majority of them are still not monitored due to the lack of studies about their environmental risk (Barceló et al., 2005).

One of the reasons why these substances are emerging in water is evident: The consequences of clustering an increasing amount of people, together with the current standard of living. Still though, there is another important factor: The development of very sensitive methods of water analyses such as e.g. solid-phase extraction (SPE), liquid chromatography with tandem mass spectrometry (LC-MS/MS) or liquid chromatography with high-resolution mass spectrometry (LC-HRMS). These procedures permit to find a wide range of substances at low concentrations, which could not be detected before (Cunha et al., 2017). Additionally, the detection of non-target screening offers advantages for detecting new psychoactive substances, a group of drugs in constant change (González-Mariño et al., 2016).

The metabolism of most pharmaceuticals and illicit drugs results in the partial excretion in urine and faeces of the unchanged parent compound along with several active metabolites and conjugates. Although sewage treatment plants (STP's) became an essential tool for conditioning the water effluents, the efficiency of the different treatments can be overloaded by the volumes, the nature of the discharged elements, and the low dilution (Sim et al., 2011; Brooks, 2018). Additional aspects are the seasonal variation in the usage, and the removal efficiency (Golovko et al., 2014a,b). As a matter of fact, STP's are not designed to remove the majority of the emerging substances they receive, so these are released again into the environment (Kasprzyk-Hordern et al., 2008). This is the reason why they are commonly considered the main source of contamination for this group of pollutants (Kasprzyk-Hordern et al., 2008). However, the dissemination of these substances is even more complex because, apart from the point-sources, the diffuse sources play an important part in the broader geographical distribution (Thomaids et al., 2012; Gerbersdorf et al., 2015). While the recent research aims to provide improvements to the STP's technology, the adjustments required can suppose high costs. Such modifications have been observed to be efficient, e.g. in the case of drinking water treatment (Švecová et al., 2021).

The concentrations of these pollutants in water bodies range from nanograms (ng) to micrograms (µg) per liter (Calisto and Esteves, 2009; Pal et al., 2013; Asimakopoulos and Kannan, 2016; Grabicova et al., 2017). However, it is possible to find them in higher amounts, even miligrams per liter, in specific sources, such as effluents from hospitals or pharmaceutical industries (Fick et al., 2009; Kosma et al., 2020). A good example of how these elements are widespread in the aquatic environment is their detection in drinking water (Rosa Boleda et al., 2011). In case of drugs of abuse, the presence in water can be sometimes due to direct disposal from the clandestine laboratories (Boles and Wells, 2010).

Emerging substances have certain characteristics that raise suspicions about their possible harm to the environment: persistence, bioaccumulation and toxicity (Ebele et al., 2017). Although the majority of the pharmaceuticals and drugs of abuse do not have physicochemical characteristics to be considered as persistent, they are continuously released into the environment. Therefore, they behave as if they were as such due to a "pseudo-persistence" phenomenon (Rosi-Marshall et al., 2015; Ebele et al., 2017), resulting in the exposure of aquatic fauna to a mixture of substances during their entire life (Fent et al., 2006). Also, the uptake of these substances from water leads to bioconcentration in tissues of exposed organisms, which in turn can lead to additive effects of substances acting on the same (or similar) drug targets (Grabicova et al., 2014; Cerveny et al., 2021). In some cases, their bioaccumulation through a food web has also been reported (Lagesson et al., 2016; Grabicova et al., 2017). Because the presence and concentration of these compounds in water is variable, the toxic effects can also differ due to the dose-time exposure, the species, and/or the developmental stage. Furthermore, the mixture of components in the environment produces additive, synergistic, and/or antagonistic actions between each singular substance (Ebele et al., 2017).

There were already several examples of how emerging compounds can entail a hazard for the environment and health. Some of the cases are socially well-known, e.g. the death toll of vultures due to the environmental presence of diclofenac (Oaks et al., 2004), imposex effects produced by the antifouling TBT used in boats (Laranjeiro et al., 2018), intersex in fish due to progestins (Šauer et al., 2020), the impacts in coral reefs caused by sunscreens (Schneider and Lim, 2019), the large problematic of the microplastics (Ragusa et al., 2021), eel hyperactivity for the presence of cocaine in water (Capaldo et al., 2018)… And the list is in a continuous growth.

Pharmaceuticals and drugs of abuse are designed to be biologically active, rise their effects with low doses and execute their actions in certain targets (Fent et al., 2006). The neurotransmission pathways are extremely conserved along evolution, what is imprinted in the brain anatomy and the physiological activities along the different species (Li et al., 1996; Fabbri et al., 1998; Lillesaar, 2011; O'Connell and Hofmann, 2012). Consequently, the main concerns about psychopharmaceuticals and illicit drugs are the potential responses –analogous or not – in non-targeted fauna (Fent et al., 2006). The metabolism and pharmacokinetics of these substances has been also observed to be similar in fish as in higher evolutioned organisms (Tanoue et al., 2017).

It is evident that, since the brain is the target organ for neuroactive compounds, a wide range of effects could be expected as a result of the accidental exposure. They even have been considered one of the most relevant group regarding ecotoxicity (Puckowski et al., 2016). Several consequences were already observed – e.g. developmental impairments (Painter et al., 2009; Kalichak et al., 2016; Thompson and Vijayan, 2020), histological changes (Schultz et al., 2011; Gay et al., 2016), differences in condition (Hubená et al., 2020; Thoré et al., 2020), behavioural alterations (Brodin et al., 2013; Nielsen et al., 2018; Martin et al., 2019), etc. The changes in behaviour, in specific, attract attention for their inherent ecological consequences (Brodin et al., 2014). For the most part, the research concerning psychoactive compounds has been focused on antidepressants. However, certain neuroactive compounds lack information regarding ecotoxicity despite their detection in water in relatively high concentrations.

1.2. Psychoactive compounds

The World Health Organization defines the term as "substances that, when taken in or administered into one's system, affect mental processes, e.g. cognition or affect. This term and its equivalent, psychotropic drug are the most neutral and descriptive term for the

whole class of substances, licit and illicit, of interest to drug policy. 'Psychoactive' does not necessarily imply dependence-producing, and in common parlance, the term is often left unstated, as in 'drug use' or 'substance abuse'". That is, all substance that produces effects over the nervous system.

29.8% of the health disorders is attributed to mental diseases, which entails a high volume of usage of antidepressants and other psychopharmaceuticals (Cipriani et al., 2018). One out of two people encounter a mental health issue during life, considering that physical and mental condition – i.e. pain, anxiety and depression – are interconnected (OECD, 2019a). The most frequent problems are anxiety, depression and drug and alcohol abuse, with 5.1, 4.5 and 2.9% of the global population affected, respectively (OECD, 2019a). Depression has been the third cause for non-fatal health loss for the past 30 years, with 350 million people suffering this disease worldwide (Cipriani et al., 2018). The use of antidepressants in OECD countries has increased with 200% between 2000 and 2017 (OECD, 2019b). For example, in the Czech Republic the intake of psychoanaleptics rose from 42 to 64 DDD (Define Daily Doses per 1,000 inhabitants and day) between 2010 and 2020 (State Institute for Drug Control, n.d.).

Chronic pain is one of the most common problems in daily medicine, handled with non-opioid analgesics, opioid analgesics and related combinations (AEMPS and Ministerio de Sanidad, 2017). Furthermore, pain suffering has inherent the deterioration of the mental state (Zelaya et al., 2020). As an example, it is estimated that 20.4% of the US population suffer from chronic pain (Mills et al., 2019). The lack of alternatives regarding pain management produce an over prescription of opioid medicaments, that, together with their potential risk of abuse, have lead to the illicit trade of these substances (OECD, 2019b). The opioid crisis suffered in North America is now spreading to Europe (Helmerhorst et al., 2017). For instance, the consumption of prescribed opioids in Spain rose from 10 to 20 DDD (AEMPS online, 2021, Ministerio de Sanidad, Spain, n.d), and from 33 to 53 DDD in the Czech Republic (State Institute for Drug Control, n.d.), in 10 years (from 2010 to 2020).
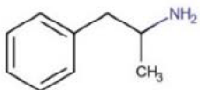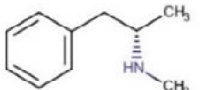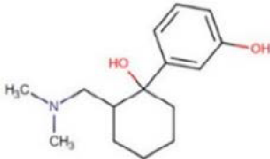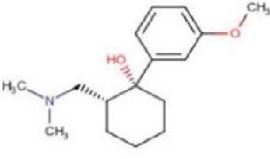
According to United Nations Office on Drugs and Crime, the number of people that used drugs rose more than 30% from 2009 to 2018, along with the increase of the amount and the different types of narcotics (UNODC, 2020). The statistics point out that 269 million people used drugs in 2018, of which 58 million people consumed opioids and 27 amphetamine-like substances (ATS) (UNODC, 2020). The seizure of ATS globally quadrupled from the year 2009 to 2018 (UNODC, 2020). The world drug report from 2020 literally mentions: "More people are using drugs, and there are more drugs, and more types of drugs, than ever" (UNODC, 2020).

The aforesaid numbers just demonstrate that the consumption and availability of psychopharmaceuticals – legal or illegally – and drugs of abuse has risen over the preceding years. This trend is evidently reflected in the volume of psychoactive substances detected in water (EMCDDA, 2020a). The research involving concentrations of psychopharmaceuticals and drugs of abuse in different water bodies is, consequently, increasing, and it has already been reviewed several times (Zuccato and Castiglioni, 2009; Calisto and Esteves, 2009; Silva et al., 2012; Loos et al., 2013; Pal et al., 2013; Asimakopoulos and Kannan, 2016; Peng et al., 2016; Cunha et al., 2017; Yadav et al., 2017; Campos-Mañas et al., 2018; Fontes et al., 2020; Castillo-Zacarías et al., 2021; Escudero et al., 2021).

The presence of psychopharmaceuticals and illicit drugs in water is variable over time and region, generally due to the different trends in consumption, weather – e.g. dilution factor related to precipitations – and removal efficiency in the STP's (RE) (Jiang et al., 2015). It has been observed that the concentration of psychopharmaceuticals in water generally increase during winter as a result of the additional presence of mental conditions, the occurrence of accidents and the lesser efficiency from the STP's (Golovko et al., 2014b; Mackuľak et al., 2016b). The relation between the microbial activity and the temperature, thus, biodegradation, has been

attributed to the lower RE during the cold months (Golovko et al., 2014a). This effects has also been observed regarding the removal of illicit drugs during the winter months (Yadav et al., 2019). Specific places could suppose a hot spot for pharmaceuticals – e.g. tramadol or other analgesics used in spa towns (Mackuľak et al., 2016c). Based on the results from wastewater-based epidemiology, the consumption of recreational drugs is mostly related with leisure time and weekend; however, the strong addiction caused by some of them, such as METH, leads to the daily usage (EMCDDA, 2020a). Social events, such as music festivals or sport events can entail a massive peak of psychoactive substances and their metabolites in water (Jiang et al., 2015; Mackuľak et al., 2019; Lemas et al., 2021; Montgomery et al., 2021). The trends in consumption, and the pervasive presence in the water, resulted in the selection of METH and tramadol as compounds of interest for this dissertation (Table 1).

Table 1. *Structure and some properties of the selected psychoactive compounds.*

Compound	Formula	CAS number	Molecular weight (g/mol)	Log Kow
Amphetamine	$C_9H_{13}N$	300-62-9	135.21	1.76
Methamphetamine	$C_{10}H_{15}N$	537-46-2	149.23	2.07
O-Desmethyltramadol	$C_{15}H_{23}NO_2$	80456-81-1	249.35	1.72
Tramadol	$C_{16}H_{25}NO_2$	27203-92-5	263.38	1.35

1.3. Methamphetamine

1.3.1. Pharmacology
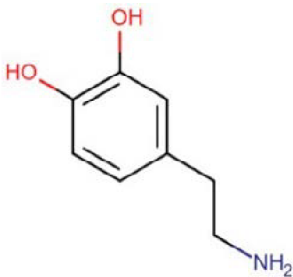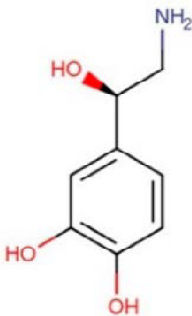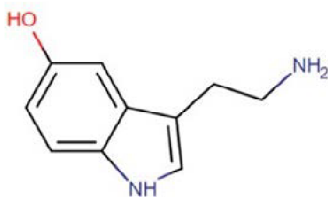
METH is a very addictive psychostimulant drug discovered in 1893 by Nagai Nagayoshi, who synthetized it from ephedrine (Panenka et al., 2013). METH (N-methyl-1-phenylpropan-2-amine) is the N-methyl derivative of amphetamine (Cruickshank and Dyer, 2009). It is usually presented in form of powder or crystal, any case white, odourless and bitter. This weak base has two stereoisomers, dextrorotary (D-methamphetamine) and laevorotary (L-methamphetamine), being the first the most intense in effects and known as "crystal meth" in illegal market, while the second one is sold as a mixture of both in powder form (Courtney and Ray, 2014).

As a consequence of its similarity in the structure (Table 2), METH behaves as an indirect agonist of the monoamine's dopamine, norepinephrine and serotonin in their respective receptors (Cruickshank and Dyer, 2009). The reversal action over the neurotransmitter' transporters and the change in the pH generates the release – i.e. from the cytosolic vesicles – and accumulation of monoamines in the synaptic terminals. In addition, the effect becomes increased for the blockade of the reuptake transporters (Won et al., 2013). The complexity of the neurotransmitter' pathways results in the additive interactions with opioid, glutamatergic or GABAergic systems (Courtney and Ray, 2014; Moratalla et al., 2017). A more comprehensive explanation of METH mode of action can be found in Sulzer et al. (2005), Ares-Santos et al. (2013), and Krasnova and Cadet (2009).

There is an extensive list of functions developed by the neurotransmitter's affected by METH. Overall, they inferfere in the following (Flórez et al., 2008):

- Dopamine: well-executed movements and tasks, motivation, conduct, attention, control of mind and perception of reality.
- Noradrenaline: emotional response, alertness, vigilance, also psychomimetic, stimulant and analgesic effects.
- Serotonin: vitality, affections and feelings.

Table 2. *Structure and formula of the main neurotransmitters affected by methamphetamine.*

Compound	Dopamine	Norepinephrine	Serotonin
Formula	$C_8H_{11}NO_2$	$C_8H_{11}NO_3$	$C_{10}H_{12}N_2O$
Structure			

In human, the metabolism of METH follows the next reactions (Cruickshank and Dyer, 2009):
- N-demethylation to amphetamine by cytochrome P450 (CYP).
- Aromatic hydroxylation to 4-hydroxymethamphetamine (CYP).
- β-hydroxylation to norephedrine.

METH can be smoked, injected, snorted and ingested, with evidently different pharmacokinetic parameters depending on the route (Cruickshank and Dyer, 2009). Then, is largely excreted in urine, with 30–50% unchanged parent compound and the rest as metabolites (amphetamine, 4-hydroxymethamphetamine) (Courtney and Ray, 2014).

1.3.2. Consumption and occurrence

METH was popularized in the Second World War by the American, German and Japanese soldiers, who utilized it to mitigate the fatigue and appetite (Anglin et al., 2000; Courtney and Ray, 2014). Then, this substance emerged again during the 90's for the use as a pharmaceutical – i.e. mental disorders and overweight – and also as a drug of abuse due to the availability triggered by the first clandestine laboratories (Courtney and Ray, 2014).

The market of METH is increasing globally, even reaching the first place in the most marketed ATS worldwide, with a total volume confiscated of 228 tons in 2018 (UNODC, 2020). In particular, Czech Republic and Slovakia are countries with a significant history of METH – there known as "pervitin" – expenditure due to the cheap production, purity and relative low price in the market (EMCDDA, 2017; Mackuľak et al., 2016c). The substance is mainly produced in kitchen-labs mostly for self-consumption and local sales; however, it has been recently reported the development of industrial-scale laboratories (EMCDDA, 2019). The "cooks" of these kitchen-labs are usually experienced Czech emigrants who use ephedrine and pseudoephedrine-based drugs as precursors, such as routine cold pharmaceuticals (Van Hout and Hearne, 2017). Despite the control of these medications in some countries, new substances are used worldwide according to the malleability of the reaction, for example, phenylacetic acid, ethyl phenylacetate, 2-phenylacetamide, benzaldehyde or 1-phenyl-2-nitropropene (UNODC, 2017).

Among the 43,700 heavy drug addicts in the Czech Republic in 2018, the number of pervitin users was, approximately, 33500, and the statistics pointed that the problem has risen more than 50% in the latest 10 years (Mravčík et al., 2020). A mean weekly consumption of 727 and 601 mg/1,000 people/day has been estimated in the cities of Prague and Ostrava, respectively (EMCDDA, n.d.). The numbers indicate that the usage of METH in Central Europe is now steady, although the spreading market, crime organization and new furtive laboratories produce an increasing availability of the drug in European countries with no abuse history (EMCDDA, 2020b; Van Hout and Hearne, 2017).

Unlike lipophilic substances, METH and AMPH are polar compounds non sorbed by the undersoil, therefore, easily released into the water (Boles and Wells, 2010). Both, parent compound and metabolite, were reported to present a high stability in the sewer waters (McCall et al., 2016). In addition, METH has been observed to have an insufficient, and even very low elimination in STP's (Postigo et al., 2010; Yadav et al., 2019). Some authors cited the mean concentrations of METH worldwide from 260 to 2,000 ng/L in wastewater effluents, and from 2,1 to 405 ng/L in river waters (Liao et al., 2015). In Europe, the mean concentrations in influents and effluents are considered 30 and 14 ng/L, respectively (Asimakopoulos and Kannan, 2016). However, due to the aforesaid reasons, amounts ranging from 13 to 1805 ng/L have been detected in wastewaters in Central Europe (Mackuľak et al., 2016c). High concentrations of METH, reaching 1 µg/L, were found in effluents in the Czech Republic (Fedorova et al., 2014). In other places where strong consumption of METH is reported, such as China and Australia, amounts exceeding 1 µg/L have been measured in surface waters (Jiang et al., 2015; Paciuszkiewicz et al., 2019).

1.3.3. Effects in human and mammal model organisms

METH is a strong psychostimulant which complex physiological responses produce a large number of effects at physical and psychological levels (Panenka et al., 2013). Because this substance generates an intense activation of sympathetic system, the main acute actions are centred in a stimulation of organism in the same way as the preparation for some danger - commonly known as "fight or flight" response (Rusyniak, 2013). Some of the main outcomes are an accelerated cardiovascular and respiratory system functions, hyperglycemia, and release of stress hormones (Panenka et al., 2013). The short-term effects at neurological level are euphoria, alertness, increased self-steam, pleasure, motivation, intense feelings, and reduced fatigue (Cruickshank and Dyer, 2009; Prakash et al., 2017). Physical condition, such as metabolic impairments or exhaustion of the organism, can be produced by the long-term use of METH (Virmani et al., 2007; Walters, Jr. et al., 2012; Bowyer et al., 2017). The

chronic consumption triggers neurodegeneration, which can lead to episodes of psychosis, withdrawal syndrome, cognitive deficits, anxiety, or depression (Cruickshank and Dyer, 2009).

METH very much affects all the organism; however, some vital organs appear extremely disrupted due to the direct and indirect effects – i.e. brain, heart and liver (Halpin et al., 2014). The chronic administration of METH produces a depletion of monoamines storage in the respective neurons, and the decreased expression of some important enzymes for neurotransmitter synthesis – such as tyrosine and tryptophan hydroxylase (Halpin et al., 2014). Although the specific mechanisms of neurotoxicity remain unknown, the available research demonstrates the role of hyperthermia, mitochondrial damage, neuroinflammation excitotoxicity, and oxidative stress (Krasnova and Cadet, 2009; Moratalla et al., 2017). The cascade of events results in neuronal damage, even apoptosis, particularly in brain areas where populations of monoaminergic neurons are located (Ferrucci et al., 2019; Halpin et al., 2014; Moratalla et al., 2017). Behavioural and physiological outcomes derived from METH usage are observed to be connected to the changes over brainstem, where an interrelated effect of dopamine and noradrenaline is generated (Ferrucci et al., 2019, 2013) (Figure 1).
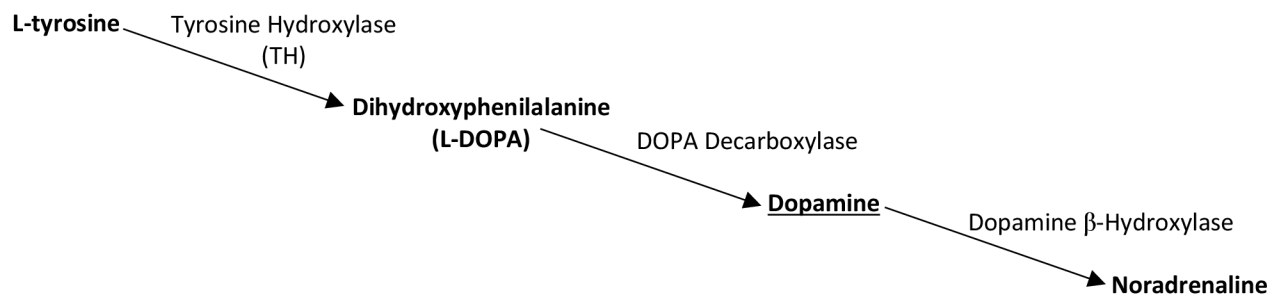
L-tyrosine — Tyrosine Hydroxylase (TH)

Dihydroxyphenilalanine (L-DOPA) — DOPA Decarboxylase

Dopamine — Dopamine β-Hydroxylase

Noradrenaline

Figure 1. *Biosynthesis of dopamine and noradrenaline (Wassall et al., 2009).*

Cardiovascular complications are largely associated with METH effects (Kaye et al., 2007; Hawley et al., 2013; Paratz et al., 2016). The impairments in the circulatory system are manifested in many ways, but infarctions in myocardia, cardiomyopathy, and dissected aneurism are the most frequently reported (Karch, 2011). Microscopically, fibrosis, microvascular impairments and hypertrophy can be often observed (Milroy and Parai, 2011). Oxidative stress and bioenergetics breakdown is related to the cardiovascular collapse (Li et al., 2012). Direct toxicity over cardiac cells is manifested by apoptosis in heart (Liou et al., 2014).

According to the hepatotoxic effects, liver is considered an added target organ (Halpin and Yamamoto, 2012). The disruption over liver with the consequent increase of circulating ammonia produces, in turn, neurotoxicity (Halpin and Yamamoto, 2012). Hepatotoxicity has been related with several histological changes, such as fibrosis or vacuolation, and biochemical outcomes (Halpin et al., 2013; Koriem and Soliman, 2014). In liver, apoptosis and necrosis of the hepatocytes have been detected after METH administration (Dias Da Silva et al., 2013; Wang et al., 2017).

1.4. Tramadol

1.4.1. Pharmacology

Tramadol, or (±)-cis-(2-dimethylaminomethyl)-1-(3-methoxyphenyl)-cyclohexanol, is a synthetic opioid widely used in human and veterinary medicine which chemical structure is similar to codeine. It was invented in 1962 and promoted under the commertial brand "Tramal®" in 1977 by the West German group Grunenthal GmbH. It is a bitter and white powder presented in drops, tablets, pills, and parenteral forms (World Health Organisation, 2014).

Tramadol is a prodrug which effects are mediated by a dual synergistic mechanism between the parent compound and the main metabolite O-desmethyltramadol (M1). The parent compound has a low affinity for μ-opioid receptor – although it acts as an inhibitor of the reuptake of the neurotransmitters serotonin and norepinephrine in the synaptic terminals – while M1 has stronger affinity for μ-opioid receptor (Gong et al., 2014).

The main reaction in the metabolism of tramadol follows these routes (Gong et al., 2014):

- O-desmethylation to O-desmethyltramadol (M1) by CYP2D6.
- N-desmethylation to N-desmethyltramadol (M2, inactive) by CYP2B6 and CYP3A4.
- M1 and M2 are metabolized into N,N-didesmethyl- tramadol (M3) and N,O-didesmethyltramadol (M5), both inactive, by CYP2D6, CYP2B6 and CYP3A4.
- M3 and M5 are degradated into N,N,O-tridesmethyltramadol (M4), active.

Then, it is excreted via urine, with 30% parent compound and 60% as metabolites (Vazzana et al., 2015).

1.4.2. Consumption and occurrence

Tramadol is one of the substances involved in the current opioid crisis. Its use in daily medicine is increasing in Europe (Helmerhorst et al., 2017). The consumption of tramadol alone or combined with other analgesics represented the highest increase in the prescribed opioids in Spain, 2.64 to 10 DDD from 2010 to 2020 (Ministerio de Sanidad, n.d.). In the Czech Republic, the use almost doubled from 2008 (5.3 DDD) to 2019 (9.5 DDD) (State Institute for Drug Control, n.d.). The increased usage has been even reported in veterinary medicine (Clarke et al., 2019).

The illicit consumption of tramadol is difficult to estimate due to the involvement of the legal use and the related lack of international regulation (UNODC, 2020). The misuse is also incited by the cheap and easy production (UNODC, 2020). The confiscations of tramadol globally were 10 kg, 9 tons and 125 tons in 2010, 2013 and 2017, respectively (UNODC, 2019). In Europe, with 5,520 seizures, became the most confiscated opioid other than heroin during 2018 (EMCDDA, 2020b).

Tramadol is a very ubiquitous substance in water, as shown by the presence of nanograms in drinking water, well water and seawaters (Alygizakis et al., 2016; Peng et al., 2016; Kondor et al., 2020). The RE has been observed to be low, and even negative during the cold season (Baker and Kasprzyk-Hordern, 2013; Golovko et al., 2014a; Mackuľak et al., 2016a). In addition, it is a stable compound that presents resistance to hydrolysis and slow photodegradation rate (Rúa-Gómez and Püttmann, 2013; Toński et al., 2019). Despite of the high amounts frequently detected in STP's, the reported concentrations in effluents and surface waters are very variable. Loads of tramadol near or up to 1 μg/L were regularly detected in effluents (Baker and Kasprzyk-Hordern, 2013), although a mean concentration of 250 ng/L and a maximum of

1,166 has been reported in an EU monitoring in effluents (Loos et al., 2013). In Central Europe, the trend of prescriptions is reflected in the elevated volumes detected in sewer waters and effluents (Mackuľak et al., 2015, 2016c). The average global concentration of tramadol has been considered 802 ng/L in surface waters (Birch et al., 2015). High amounts, up to 1 µg/L, were detected downstream a STP in USA, and in river waters in UK (Kasprzyk-Hordern et al., 2008; Campos-Mañas et al., 2019).

1.4.3. Effects in human and mammal model organisms

Tramadol is considered a safe analgesic with a low potential of abuse, used for acute and chronic, moderate to severe pain due to its potency, approximately 10% morphine's (World Health Organisation, 2014). The analgesic efficiency is increased for the actions over monoamine neurotransmitters (Gong et al., 2014). Tramadol suppose an alternative to high-affinity opioid receptors – e.g. morphine or naloxone – in chronic pain treatment, causing a lesser physical dependence and respiratory depression (Gong et al., 2014; Miotto et al., 2017; Dunn et al., 2019).

The unique dual mode of action, as analgesic and selective serotonin-norepinephrine reuptake inhibitor, results in its further use as antidepressant and anxyolytic (Vazzana et al., 2015). It provides an additional relaxation and well-being effects, which can contribute to its abuse potential (Miotto et al., 2017). The possibility of dependence arises with the chronic use and supra-therapeutic amounts (World Health Organisation, 2014). Tramadol is frequently used in combination with other analgesics, such as paracetamol or diclofenac, in order to complement the pain relieve (Varrassi et al., 2020). However, the concomitant consumption with serotonergic pharmaceuticals – i.e. antidepressants, anticonvulsants, anxiolytics – can lead to a serotonergic syndrome (Hassamal et al., 2018).

1.5. Effects of psychoactive compounds in fish: current knowledge

The ecotoxicological literature highlights the potential contribution of psychoactive compounds as pollutants of concern. As a result of the recent effects observed, it is evident that these chemicals can impact the aquatic ecosystems even at low doses, however, it appears necessary to characterize such impairments. For instance, the most common effects produced by low levels of antidepressants were changes in condition, behaviour, and reduction in food intake (Castillo-Zacarías et al., 2021).

The occurrence of pharmaceuticals and drugs in water leads to the bioconcentration of these substances in tissues. Some of the psychoactive substances do not reach the bioconcentration factor included in the OECD guidelines nor get the bioaccumulation index to be accommodated in the food chain, nevertheless, the existence of these chemicals in aquatic fauna is well-known. As an example, a wide range of PPCP's, including psychoactive substances, were detected in biota from STP effluents (Grabicova et al., 2014; David et al., 2018), streams (Grabicova et al., 2015, 2017), ponds (Lagesson et al., 2016), rivers (Ondarza et al., 2019; Cerveny et al., 2021), estuaries (Meador et al., 2017), and marine environment (Mezzelani et al., 2020). The contribution via food web and the differences in bioconcentration among species, although less studied, should be also considered (Brodin et al., 2014; Heynen et al., 2016b; Lagesson et al., 2016).

The therapeutic plasma concentration (TPC) of pharmaceuticals in human and other mammals is available through pharmacological efficacy studies (Table 3). Due to the aforesaid similarity in the signal targeting between mammals and fish, the possibility to extrapolate the TPC from human to fish has been described in the "read across hypothesis" (Huggett et al.,

2003). A wide range of pharmaceuticals has been detected in fish plasma from several sites including neuroactive substances – e.g. AMPH, METH, haloperidol, risperidone (Meador et al., 2017; Malev et al., 2020; Cerveny et al., 2021). Interestingly, several compounds exceeded the TPC (Meador et al., 2017; Cerveny et al., 2021).

Table 3. *Human therapeutic plasma concentrations (TPC's) of the selected compounds. The concentrations refer to µg/mL, elimination half-life in hours. Extracted from Regenthal et al. (1999).*

Compound	Therapeutic	Toxic	Lethal	Half-life
Amphetamine	0.02–0.2	0.2	0.5	7–34
Methamphetamine	0.01–0.05	0.2–1	40	6–9
Tramadol	0.1–1	–	2	5–10

Despite of the valuable information provided by the read across hypothesis, some of the substances do not correlate to the plasma concentration model due to their physicochemical nature. For example, higher concentrations of psychopharmaceuticals were found in brain than in blood plasma – or they were even absent in plasma, as a result of the structural properties of these substances (Grabicova et al., 2014; David et al., 2018). The question arises if in such cases, and because these compounds are produced to reach certain targets, it would be more accurate to determine their concentrations in the target organ – i.e. brain for neuroactive compounds (Grabicova et al., 2014). Or if the mode of action and metabolism are equivalent among different species (Rand-Weaver et al., 2013).

Because psychoactive substances have been detected in different tissues, they can be suspected to generate effects as a result of the phylogenetic similarity in receptors. Histology dated back to 17th century, when it was applied for the first time by Marcello Malpighi using an archetype microscope. Even though, it is one of the oldest methods in modern science still in use. Histopathology became a key process of the ecotoxicological assessment (Wolf and Maack, 2017). This tool enables the visualization, description and understanding of the changes at the tissue level as a result of the chronic exposure to chemicals (Bernet et al., 1999). As it is essentially an interpretative method, its application implies an unavoidable subjectivity (Crissman et al., 2004). New developments, such as immunohistochemistry, enhances the knowledge of pathophysiological processes. The prospect anatomical description of the sub-lethal consequences could be helpful, especially when it is suspected that the changes are parallel to those described in the target organisms. Some histopathological outcomes from the exposure to psychopharmaceuticals and illicit drugs were already described (Schultz et al., 2011; Gay et al., 2016; Sehonova et al., 2017). However, the assessment of the pathological lesions requires very experienced observers, which can suppose a bottleneck in the use of this technique (Wolf and Maack, 2017). In addition, the absence of standardized approaches could produce mistakes when evaluating the observations (Wolf and Maack, 2017).

As aforementioned, psychopharmaceuticals and drugs are designed to change specific neurotransmitter pathways. In fact, the presence of such substances in the environment has been observed to affect the neurotransmitter levels in fish brain and plasma, what can be translated into impairments in multiple processes, such as metabolism or behaviour (Simmons et al., 2017; David et al., 2018). Interestingly, fish even seem to be attracted by the presence of neuroactive substances in water, probably due to some "good-feeling state" (Abreu et al., 2016). The majority of observations attributed to these substances at realistic levels are related with changes in behaviour; thus, there is an increasing interest in this subject over the last years (Cunha et al., 2019). Because neuroactive compounds aim to have effects over human behaviour, similar responses could be expected in non-target organisms (Saaristo

et al., 2018). For example, an anxiolytic-like behaviour in fish have been documented after the exposure to low concentrations of anxiolytics and antidepressants (Brodin et al., 2013; Heynen et al., 2016a; Kellner et al., 2016; Martin et al., 2017).

Several authors agree on the advantages of behavioural approaches for the ecotoxicology field (Hellou, 2011; Melvin and Wilson, 2013; Brodin et al., 2014; Klaminder et al., 2016; Peterson et al., 2017; Saaristo et al., 2018):

- Behaviour stands as a reflection of interconnected biological processes – physiology, anatomy, genetics, and environment.
- It is considered a sensitive early warning indicator of the possible effects of pollution because the effects occur at low – sublethal – concentrations.
- The quantification of the responses provides an added advantage within the risk evaluation.
- It is a non-invasive and moderately cheap tool.
- Findings can be obtained faster than with other toxicological tests.
- Changes in behaviour have importance at ecological level due to the complex relation of these processes.

In order to simplify the study of behaviour, Réale et al. (2007) suggested five key temperament traits – ie. activity, aggressiveness, boldness/shyness, exploration/avoidance, and sociability – that form the personality of each individual. The disruption of any behavioural trait implies direct and indirect consequences (Brodin et al., 2014) (Table 4). The direct effects occur within an individual and can be quantified and studied under laboratory and field conditions (Saaristo et al., 2018). The indirect outcomes appear when the direct variations affect other species in chain reaction, modifying the trophic chain and the biodiversity, consequently, the whole ecosystem could be affected (Brodin et al., 2014; Saaristo et al., 2018). Even if the changes in behaviour could provide some advantage to the tested organism, consequences at ecological level will likely occur (Klaminder et al., 2014). The order in which these outcomes interconnects with the fitness of ecosystems would follow these levels: physiology – individual – population – species – ecosystem – evolution (Peterson et al., 2017).

Table 4. *Relation between basic behavioural traits and the possible ecological consequences due to their alteration for the exposure to pharmaceuticals. Extracted and modified from Brodin et al. (2014).*

Behavioural traits	Impacts	
	Direct	*Indirect*
1-Activity	Cooperation (2,5)	Community structure
2-Aggression	Dispersal/migration (1,3,4,5)	Cross-boundary effects
3-Boldness	Feeding rate (1,2,3,4)	Ecosystem function
4-Exploration	Reproduction (2,5)	Feedbacks
5-Sociality	Parental care (2,5)	Population dynamics
	Predator avoidance (1,3,5)	Trophic cascades

Although the promising advantages of using behavioural endpoints, there is a lack of validated tests (Hubená et al., 2020). Variation in the results can be originated for the natural differences in the individual personality; however, some repeatability should be observed in the individuals from the same population (Réale et al., 2007). The type of exposure (acute/chronic) has been reported to be a critical factor for assessing the outcomes, most likely due to the disparity in the availability of neurotransmitters over time (Martin et al., 2017).

Regardless the general lack of understanding of the role played by the psychoactive substances in the environment, some pollutants, such as diazepam, fluoxetine or oxazepam, have been more deeply studied. The particularity of the substances selected in these thesis – i.e. methamphetamine and tramadol – is the scarcity of knowledge about their influence over aquatic fauna despite of their levels detected in the environment (Chapters 2 and 3). The current literature about the ecotoxicology of these compounds is included in Table 5.

Table 5. *Ecotoxicological studies performed with the selected psychoactive compounds, methamphetamine and tramadol. Citalopram (CIT), ketamine (KET), methamphetamine (METH), naproxen (NAP), oxazepam (OXA), sertraline (SER), sewage treatment plant effluent (STP), tramadol (TRM), venlafaxine (VEN).*

Compound	Concentration	Specie	Duration	Effects	Reference
CIT, TRM	411 µg/L	*Danio rerio*	larvae	Anxiolytic	(Bachour et al., 2020)
STP, CIT, TRM	1 µg/L	*Aeshna cyanea, Cyprinus carpio*	7 d	Predation, feeding	(Bláha et al., 2019)
METH + 30 compounds	1–1,000 µM	*D. rerio*	larvae	Activity	(Bugel and Tanguay, 2018)
CIT, TRM	1 µg/L	*Procambarus virginalis*	7 d	Activity	(Buřič et al., 2018)
METH	50–500 ng/L	*Daphnia magna*	21 d	Oxidative stress	(De Felice et al., 2020)
METH, TRM	3.8 nmol/L	*Squalius cephalus*	42 d	Parasite interaction	(Douda et al., 2019)
TRM	1 µg/L	*Procambarus clarkii*	7 d	Burrowing behaviour	(Guo et al., 2020)
METH, TRM, CIT, OXA, SER, VEN	1 µg/L	*P. virginalis*	21 d	Activity	(Hossain et al., 2021)
METH	1 µg/L	*Salmo trutta fario*	56 d	Behaviour	Chapter 2.2; (Horký et al., 2021)
TRM + 14 compounds	1–100 µg/L	*D. rerio*	larvae	Swimming	(Huang et al., 2019)
METH, TRM	1 µg/L	*S. cephalus*	42 d	Condition	(Hubená et al., 2020)
METH	0.004–40 µM	*Oryzias latipes*	larvae	Activity, oxidative stress	(Liao et al., 2015)
METH	1 µg/L	*Pacifastacus leniusculus*	21 d	Activity, cardiac activity	(Ložek et al., 2020)
TRM	1 µg/L	*P. leniusculus*	21 d	Activity, cardiac activity	(Ložek et al., 2019)
TRM	0.2–600 µg/L	*D. rerio*	28 d	Condition, histology, oxidative stress	(Plhalova et al., 2020)
METH	1–50 µg/L	*Salmo trutta fario*	35 d	Histology	Chapter 2.1; (Sancho Santos et al., 2020)
TRM	1 µg/L	*S. cephalus*	42 d	Anxiolytic	Chapter 3.1; (Sancho Santos et al., 2021)
TRM	71 ng/L	*Pimephales promelas*		Behaviour, immune system	(Schoenfuss et al., 2016)
TRM	10–200 µg/L	*C. carpio, D. rerio*	32 d	Development	(Sehonova et al., 2016)

NAP, TRM	10–200 µg/L	*C. carpio*	32 d	Development	(Sehonova et al., 2017)
TRM	1–100 µg/L	*P. promelas*	23–24 d	Anxiolytic	(Tanoue et al., 2019)
TRM	1–100 µg/L	*P. promelas*	23–24 d	Pharmacokinetics	(Tanoue et al., 2017)
METH + KET	0.05–0.5 µg/L	*Caenorhabditis elegans*	60 h	Activity, oxidative stress	(Wang et al., 2019)
METH + KET	25–50 µg/L	Microcosm	40 d	Changes in bacteria	(Wang et al., 2020a)
METH	0.05–100 µg/L	*O. latipes*	90 d	Activity, histology	(Wang et al., 2020b)
METH	0.01–100 µg/L	*D. rerio*	15 d	Pharmacokinetics	(Yin et al., 2019)

1.6. Specific objectives

The Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 consist of policies to ensure and protect the quality of the different waters and the aquatic environment in the Community. It contains a list of priority substances according to their potential risk, as a strategy against water pollution.

Emerging contaminants, including psychopharmaceuticals and drugs of abuse, are currently not contemplated in the legislation. The evidences about their presence in the environment and the possible effects over health are the key to their incorporation in future regulations (Barceló et al., 2005).

The main objective of this dissertation is, therefore, to provide data about the currently unknown effects of selected neuroactive compounds on fish.

More specifically, the following goals were pursued:

I. To acquire knowledge about the impacts for the exposure to methamphetamine: bioaccumulation in tissues, pathological alterations in target organs, and changes in behaviour (Chapter 2).

II. To record the alterations in basic behavioural traits due to the exposure to tramadol at low levels (Chapter 3).

III. To underline the current problematic regarding the use of the histopathological method in ecotoxicological studies (Chapter 4).

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CHAPTER 2

THE PROBLEMATIC OF METHAMPHETAMINE AS A POLLUTANT

Chapter 2.1.

Sancho Santos, M.E., Grabicová, K., Steinbach, C., Schmidt-Posthaus, H., Šálková, Š., Kolářová, J., Vojs Staňová, A., Grabic, R., Randák, T., 2020. Environmental concentration of methamphetamine induces pathological changes in brown trout (*Salmo trutta fario*). Chemosphere 254, 126882. DOI 10.1016/j.chemosphere.2020.126882.

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Chapter 2.2.

Horký, P., Grabic, R., Grabicová, K., Brooks, B.W., Douda, K., Slavík, O., Hubená, H., Sancho Santos, M.E., Randák, T., 2021. Methamphetamine pollution elicits addiction in wild fish. J. Exp. Biol. 224, jeb242145. DOI 10.1242/jeb.242145.

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Environmental concentration of methamphetamine induces pathological changes in brown trout (*Salmo trutta fario*)

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HIGHLIGHTS

- Methamphetamine and its metabolite amphetamine concentrate in tissues of brown trout.
- Histological findings appear even at low concentration in liver and heart.
- The tissue impairments are similar to those described in mammals.
- Brown trout shows partially adaptation to methamphetamine exposure.
- Environmental concentration of methamphetamine can lead to alterations in aquatic fauna.

GRAPHICAL ABSTRACT
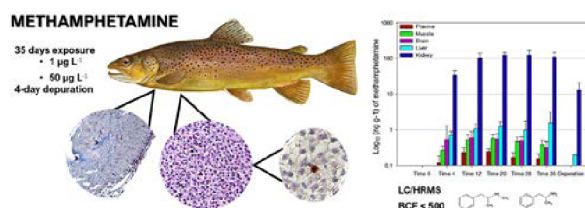
ARTICLE INFO

ABSTRACT

Methamphetamine, mainly consumed as an illicit drug, is a potent addictive psychostimulant that has been detected in surface water at concentrations ranging from nanograms to micrograms per litre, especially in Middle and East Europe. The aim of this study was to expose brown trout (*Salmo trutta fario*) to environmental ($1 \, \mu g \, L^{-1}$) and higher ($50 \, \mu g \, L^{-1}$) concentrations of methamphetamine for 35 days with a four-day depuration phase to assess the possible negative effects on fish health. Degenerative liver and heart alterations, similar to those described in mammals, were observed at both concentrations, although at different intensities. Apoptotic changes in hepatocytes, revealed by activated caspase-3, were found in exposed fish. The parent compound and a metabolite (amphetamine) were detected in fish tissues in both concentration groups, in the order of kidney > liver > brain > muscle > plasma. Bioconcentration factors ranged from 0.13 to 80. A therapeutic plasma concentration was reached for both compounds in the high-concentration treatment. This study indicates that chronic environmental concentrations of methamphetamine can lead to health issues in aquatic organisms.

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1. Introduction

The contamination of surface waters with emerging substances has generated special interest due to its frequency and increasing

detection, leading to possible effects in aquatic ecosystems. Psychopharmaceuticals and abusive drugs have been found in water at concentrations ranging from nanograms to micrograms per litre worldwide (Archer et al., 2018; Bade et al., 2018; Bijlsma et al., 2012; Fáberová et al., 2017; Postigo et al., 2010; Yargeau et al., 2014). Once excreted by the human body, the metabolites and parent compounds of these substances are not fully removed by sewage treatment plants (STPs) and the compounds are released unchanged into surface water (Golovko et al., 2014; Mackulak et al., 2015; Postigo et al., 2010). Their presence can also be related to direct discharge from clandestine laboratories (Boles and Wells, 2010). Although the occurrence of these substances in water is usually ephemeral, their continuous release to the environment causes their pseudo-persistence (Rosi-Marshall et al., 2015).

Psychopharmaceuticals and illicit drugs are produced to reach the central nervous system, being the neuronal target signalling highly conserved along evolution (Fabbri et al., 1998; O'Connell and Hofmann, 2012). Therefore, substances are suspected to behave similarly in target and non-target organisms (Fent et al., 2006). They can bioconcentrate and bioaccumulate in tissues of aquatic organisms (Grabicova et al., 2017, 2014; Lagesson et al., 2016). Their environmental presence can lead to important changes in endocrine pathways and natural behaviour, resulting in unknown ecological consequences (Brodin et al., 2014; Calisto and Esteves, 2009). The toxicity extends to the synergistic and additive action of the mixture of components present in the environment (Chiffre et al., 2016; Schnell et al., 2009), which leads to largely unknown adverse effects of chronic and lifelong exposure (Fent et al., 2006).

Methamphetamine (METH) is an addictive psychostimulant that is widely consumed as a drug of abuse or as a pharmaceutical to treat attention deficit hyperactivity disorder, narcolepsy or obesity (Heal et al., 2013). It is an indirect-acting agonist in contact with dopamine, norepinephrine and serotonin receptors due to its similarity in structure (Cruickshank and Dyer, 2009), providing their availability and release and causing the stimulation of the organism similar to the "fight or flight" response (Panenka et al., 2013). The increased activation of cardiovascular, neurologic, psychomotor and respiratory functions (Hassan et al., 2016; Panenka et al., 2013) involves a wide range of symptoms including exhaustion of the organism (Walters, Jr. et al., 2012) and cellular impairments (Ares-Santos et al., 2013; Krasnova and Cadet, 2009).

The Czech Republic and Slovakia have a high consumption of illicit METH ("Pervitin") due to its historical background, cheap production, purity and relatively low price (Šefránek and Miovský, 2017; Zábranský, 2007). Among the 291 clandestine laboratories reported in Europe in 2017, 263 were located in the Czech Republic, mostly for self-consumption and local sales (EMCDDA, 2017). In the Czech Republic, the number of primary METH users is reported to be over 34200, and the statistics indicates that this number has increased more than 50% in the last 10 years (EMCDDA, 2017). Whereas the mean concentration of METH in STP influent in Europe is 30 ng L^{-1} (Asimakopoulos and Kannan, 2016), concentrations range from 13 to 1805 ng L^{-1} in the Czech Republic and Slovakia (Mackulak et al., 2016). In 2017, European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (EMCDDA, 2017) reported a mean consumption in Ceske Budejovice, Brno and Bratislava of 200, 186 and 149 mg per 1000 person per day, respectively, and daily consumption did not reach 100 mg/1000p/day in the other European cities included in this study. Additionally, countries such as Australia and The USA have reported hazards related to the discharge of METH and other chemicals derived from manufacturing in clandestine laboratories (Melnikova et al., 2011; Wright et al., 2016).

Several studies have been focused on the effects of psychoactive compounds in fish, including antidepressants (Kellner et al., 2015;

Martin et al., 2017; Saaristo et al., 2017), antiepileptics (Li et al., 2010; Qiang et al., 2016) or anxiolytics (Brodin et al., 2013; Huerta et al., 2016). However, little is known about the consequences of illicit drugs on the aquatic fauna at environmental concentrations (Liao et al., 2015; Wang et al., 2019). Impairments in microbial communities were reported due to the presence in surface water of METH (Wang et al., 2018) and amphetamine (AMPH) (Lee et al., 2016). Liao et al. (2015) observed a decreased heart rate, delayed hatching, hypomotility and changes in swimming behaviour at 597 ng L^{-1} of METH for 7 days in medaka embryos (*Oryzias latipes*). Wang et al. (2019) reported changes in locomotor activity and chemotaxis in the nematode *C. elegans* after 0.5 µg L^{-1} METH exposure for 60 h. Marble crayfish exposed to 1 µg L^{-1} of METH for 7 and 21 days spent more time outside the shelter (Hossain et al., 2019). Changes in stress reactions were observed in signal crayfish exposed to 1 µg L^{-1} for 21 days (Ložek et al., 2020). Yin et al. (2019) found a correlation of METH concentration between the diffusive gradient in thin films and *in vivo* zebrafish.

Walters, Jr. et al. (2012) observed an impairment in energetic balance, feeding, and locomotive behaviour in *Drosophila melanogaster* in response to a 2-day diet containing METH. Some of the pharmacological studies with METH at higher concentrations using zebrafish (*Danio rerio*) as a model reported disturbances in cardiovascular development of larvae (Shao et al., 2012), larval hypomotility (Bugel and Tanguay, 2018) and behaviour in adult individuals (Jiang et al., 2016; Mi et al., 2016; Zhu et al., 2017). Based on these results, METH seems to induce similar effects on invertebrates as those reported in mammals.

Data obtained in several studies in human and experimental mammals exposed to METH demonstrated that the cardiovascular system and liver are target organs, as a result, toxicity is reflected in pathological disorders of these organs (Dias Da Silva et al., 2013; Islam et al., 2009; Koriem and Soliman, 2014; Paratz et al., 2016; Wang et al., 2017).

Brown trout (*Salmo trutta fario*) is a well-known model in ecotoxicity test due to their continuously availability regarding production, easy management in laboratory and phylogenetically relation with rainbow trout (*Oncorhynchus mykiss*), a recommended species for OECD guidelines, but also for being an endemic species in European rivers (Nunes et al., 2015; OECD, 2012). Therefore, the aim of this study was to elucidate the presently unknown physiological responses of juvenile brown trout exposed to chronic environmental and higher concentrations of METH (1 and 50 µg L^{-1}). In order to achieve this objective, we: (1) determined the bioconcentration of METH and its metabolite (AMPH) in five different fish tissues, (2) recorded the liver and heart histopathology, and (3) investigated apoptosis in hepatocytes.

2. Material and methods

2.1. Chemicals

The methamphetamine used for exposure was obtained from Sigma-Aldrich Corporation (USA) and amphetamine and their isotopically labelled analogues (D5-AMPH, D5-METH) were obtained from Lipomed (USA). A stock solution of METH (10 mg L^{-1}) was prepared with ultra-pure water (AquaMax Basic 360 Series and Ultra 370 Series instrument, Younglin, Korea) and stored at 4 °C. From the stock solution, exposure baths for fish at concentrations of 1 and 50 µg L^{-1} were prepared. Acidified (with formic acid, Labicom, CR) acetonitrile (Merck) and ultra-pure water were used as mobile phases for liquid chromatography and isopropanol (Merck) was used as extraction solvent for the biota samples.

2.2. Fish and experimental design

Brown trout juveniles (*Salmo trutta fario*, 1 year old; mean weight 35.6 ± 9.9 g, mean body length 131 ± 13 mm) were obtained from a local hatchery and acclimatized under laboratory conditions for seven days before the start of the experiment. A population of 354 trout was randomly distributed to six aquaria. Following the OECD guideline 305 (OECD, 2012), fish were maintained in 200 L of freshwater with constant aeration, a natural photoperiod, dissolved oxygen (8 mg L^{-1}), mean temperature of 14.5 ± 0.5 °C and pH of 7.2 ± 0.3. Every two days, the fish were fed 1% of their initial body weight of commercial food (BioMar, Denmark), the water was changed, stock solution of METH was added to reach the final concentration, and the temperature, pH and dissolved oxygen were measured. Treatments were set at nominal concentrations of: 0 µg L^{-1} (control, METH-free water), 1 µg L^{-1} (environmental concentration) and 50 µg L^{-1} (1% of LC50 for fish) (Kiyatkin and Sharma, 2011) for 35 days. Then, the fish were kept in METH-free water for four days for depuration. All exposures were run in duplicates. Sampling was performed after 0, 4, 12, 20, 28 and 35 days of exposure (Times 0, 4, 12, 20, 28 and 35, respectively), and after a 4-day depuration phase (Time 39). For each sampling day, ten fish per group (5 fish per replicate) were removed (sampling plan is given in Appendix A) and sacrificed by decapitation. Water samples for determination of METH concentration were taken before and after changing the exposure bath. This experiment was performed in accordance with the EU-harmonized Animal Welfare Act of the Czech Republic. The research facility is authorized under No. 53100/2013-MZE-17214, and the framework of the law against Animal Cruelty of the Czech Republic (No. 246/1992), number of the Ethical approval committee MSMT-6744/2018-4.

2.3. Chemical analyses

The water from the aquaria was analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS; TSQ Quantiva, heated electrospray in positive mode, Accela 1250 LC pump, Hypersil Gold aQ column (50 × 2.1 mm; 5 µm particles), PAL autosampler, Thermo Fisher Scientific, USA) every week before and after the water was changed. The analytical method is described in (Douda et al., 2019). In summary, isotopically labelled internal standard (D_5-METH, D_5-AMPH) was added to filtered (0.20 µm regenerated cellulose filter) water from aquaria and the sample was analysed by LC-MS/MS using gradient elution of water and acetonitrile (both acidified with formic acid, method duration 10 min).

Blood plasma, liver, kidney, brain and muscle (dorsal part of fillet without skin) were sampled and stored at −80 °C until further analysis. The extraction and analytical methods are described in (Grabicova et al., 2018). Briefly, 0.2−0.5 g of tissue (depending on the type of tissue) from ten fish per group and sampling time were analysed. Internal standard, extraction solvent (acetonitrile and isopropanol, 3:1 v/v acidified with 0.1% of formic acid), and a homogenization ball were added. The samples were extracted (TissueLyser II, Quiagen, Germany; 30 Hz, 10 min), centrifuged (Mini spin, Eppendorf, Germany; 6708 g, 5 min), filtered (Labicom; regenerated cellulose, 0.45 µm pores), frozen (−20 °C, 24 h) and recentrifuged. The aliquots were analysed by liquid chromatography with high resolution mass spectrometry (LC-HRMS, QExactive, Accela 1250 LC pump, Hypersil Gold aQ column (50 × 2.1 mm; 5 µm particles) PAL autosampler, Thermo Fisher Scientific). The analytical method was restricted to 7 min due to the analysis of only four analytes (METH, D_5-METH, AMPH and D_5-AMPH). The gradient and mass transitions of target compounds is presented in Appendix B. The data were processed using Trace-Finder 3.3 software (Thermo Fisher Scientific). The limits of

quantification (LOQs) were calculated from the response at the lowest calibration point, where the relative standard deviation of the average response factor did not deviate more than 30% (Grabicova et al., 2018). The bioconcentration factors (BCFs) were calculated by dividing the average concentration of METH in fish tissues by the respective mean concentration in water (OECD, 2012).

2.4. Histology

Six fish per group were sampled after 35 days of exposure and at the end of the four-day depuration phase. Samples of liver, heart, skin, kidney and gills were obtained and fixed in 10% neutral buffered formalin for 48 h. The gill tissue was additionally decalcified for 2 h (DC1, Johnson and Johnson). All organs were processed for histology. 3-µm thick slides were prepared and stained with haematoxylin and eosin (HE). Heart tissues were additionally stained with Masson's trichrome acc. Capelli (with Aniline Blue) (Diapath kit 010210). Organs were observed under a light microscope (Olympus BX51).

Lesion severity was semi-quantitatively classified as 0 = no changes, 1 = minimal changes, 2 = mild intensity, 3 = moderate intensity and 4 = severe intensity (Näslund et al., 2017). The slides were re-evaluated by two additional observers. The following parameters were classified: Liver: cytoplasmic vacuolation, especially macrovacuoles, karyopyknosis and areas of infiltration with lymphocytes, plasma cells and macrophages. Presence of pigmented macrophages was measured by counting their number in four randomly selected areas at 400x; Heart: areas of muscular degeneration, infiltration with lymphocytes, plasma cells and macrophages in myocardium and epicardium (Steinbach et al., 2016); Skin: epidermal regularity, dermal infiltration with lymphocytes, plasma cells and macrophages; Kidney: number of pigmented macrophage aggregates, tubular nephrosis and neotubulogenesis; Gills: subepithelial infiltration with lymphocytes and macrophages, epithelial necrosis, epithelial cell hyperplasia and hypertrophy.

2.5. Immunohistochemistry

Livers from six fish per group after 35 days of exposure and after 4-day depuration phase were analysed. SignalStain® Apoptosis (Cleaved Caspase-3) IHC Detection Kit (Cell Signalling Technology, Inc.) using cleaved caspase-3 (Asp175) (D3E9) Rabbit monoclonal antibody, was applied on the same liver sections as used for histology, following the manufacturer's protocol. This method allows to detect nuclear activated caspase-3 large fragment (17/19 kDa). The antigen-antibody complex was visualized using 3-3′ diaminobenzidine tetrahydrochloride (DAB) as a chromogen, that produced a brown end product. Afterwards, the samples were counterstained with haematoxylin. Only the singular hepatocytes intermingled in the parenchyma with clear DAB stained nucleus were counted as positive. The presence and number of positive cells per slide was evaluated. The slides were blindly evaluated by two additional observers.

2.6. Statistics

Statistical analysis was performed with R free software (v. 3.5.3) and SigmaPlot (v. 12.0). One-way analysis of variance (ANOVA) was used to compare the experimental groups and, if significant differences were observed, a Tukey post hoc test was performed. Kruskal-Wallis (non-parametric test) was applied in case of differences in the one-way ANOVA assumptions, with Dunn-test as the post hoc test in required cases. For comparing between two groups, T-test was performed once the normality was confirmed by Shapiro

test. Statistical differences were defined at $p < 0.05$, and $\alpha/2$ for Dunn-test.

3. Results

3.1. Chemical analyses

3.1.1. Water

The average concentration of METH in water for the respective treatments was 1.3 ± 0.2 and 66 ± 5 μg L^{-1} (Table 1), and lower than LOQ in the control groups. The LOQ in water for METH ranged from 0.0043 to 0.045 μg L^{-1} and from 0.0033 to 0.036 μg L^{-1} for AMPH.

3.1.2. Methamphetamine in tissues

The concentration of METH in tissues (expressed as ng g^{-1} wet weight) was below the LOQ in the control group and at the beginning of the experiment. The LOQs ranged from 0.036 to 0.67 ng g^{-1} in different tissues (Appendix C). METH was found in all tissues in exposed fish at all time points. The temporal changes in concentration in the different organs are given in Fig. 1a and Fig. 1b, and the statistical analyses are detailed in Fig. D1 of Appendix D. The levels followed the order kidney > liver > brain > muscle > plasma. Overall, the kidney presented the highest concentration, approximately 160 times higher than the other tissues.

The calculated BCFs were constant between both METH concentrations tested in the respective tissues (T-test, $p > 0.05$) (Appendix E). The highest values were found in kidney, i.e., 80 in the environmental group and 54 in the high-concentration group. The values were lower in the other tissues, from 0.15 to 0.91 and from 0.13 to 0.6 in the respective treatments.

After the depuration phase, there was a decrease in the METH concentration in all the tissues analysed. In the environmental concentration group, 0.2 ng g^{-1} and 13 ng g^{-1} remained in liver and kidney tissues, respectively. However, plasma, muscle and brain were successfully depurated, as the concentrations were below the LOQ. 460 ng g^{-1} was detected in the kidney of the high concentration-exposed fish and much lower in the other tissue residues, from 0.24 to 1.7 ng g^{-1}.

3.1.3. Amphetamine in tissues

In the control group and at the start of the experiment, the concentration of AMPH was below the LOQ (from 0.03 to 1.8 ng g^{-1}, Appendix C). AMPH was also found in all examined organs in exposed individuals at all time points. The temporal progression of concentrations in analysed tissues in both treatments is shown in Fig. 1c and d, and the related statistical study shown in Fig. D2 of Appendix D. The levels of tissue metabolites followed the same order as METH, but the values were elevated compared with the parent compound-over 2.6 times higher in kidney and 7 times higher in the other tissues. The highest concentration of AMPH was found in the kidney, approximately 60 times higher than in the

other organs. The metabolization rate (concentration of the metabolite divided by the parent compound) followed the order liver > brain > plasma > muscle > kidney.

The metabolite depuration was completed in the muscle of individuals in the environmental group but generally had a tendency to decrease in the other tissues, plasma, brain and liver, ranging from 0.17 to 2.9 ng g^{-1}. The mean concentration of AMPH in the kidney was 88 ng g^{-1}. In the high-concentration group, residues at levels ranging from 7.1 to 4200 ng g^{-1} (16–37% of the values from the last exposure day, Time 35) were present in all tissues tested.

3.2. Histology

3.2.1. Liver

In the liver, after 35 days of exposure, the hepatocytes of most of the control fish showed no cytoplasmic vacuolation (Fig. 2a) and, in few cases, few clear, round, small vacuoles, most likely fat, were observed. The observed differences in fish exposed to the environmentally relevant level of METH (1 μg L^{-1}) compared with the controls were not significant, however high variability in hepatocyte vacuolation was observed (Appendix F). Some individuals presented many fat vacuoles (macrovacuoles), often associated with cell membrane disruption (Fig. 2c), whereas other fish showed no hepatocyte vacuolation, similar to control animals (Fig. 2b). In the high-concentration group (50 μg L^{-1}), individuals presented severe cytoplasmic macrovacuolation, moderate to severe cell membrane rupture (Fig. 2d) and single cell necrosis, characterized by increased karyopyknosis. All changes in this group were significantly different from the control and environmental groups (Kruskal-Wallis, $p < 0.05$) (Appendix F).

After the depuration phase (Time 39), the hepatocytes of control fish resembled the previous sampling (Fig. 3a). Fish exposed to the high concentration were similar to the controls, however, the cytoplasmic vacuoles were foamier and cell membranes were often diminished (Fig. 3c). Areas of infiltration and pigmented macrophages were observed among all groups and sampling points, with no significant differences. However, fish exposed to the environmental concentration showed significantly more vacuolation (Kruskal-Wallis, $p < 0.05$), especially macrovacuoles associated with cell membrane rupture, compared to controls and fish exposed to high concentration (Fig. 3b) (Appendix F).

3.2.2. Heart

After 35 days of exposure, the hearts of trout in the environmental concentration group showed a statistically significant increase in infiltration of the pericardium with lymphocytes, plasma cells and macrophages compared to control (Fig. 4a and b) and high-concentration group (Fig. 4e and f) (Kruskal-Wallis, $p < 0.05$) (Appendix F). No additional statistically significant finding was diagnosed. However, in the group exposed to the environmental concentration, one heart presented numerous areas of coronary vessel dilation, congestion, oedema and inflammatory cell infiltration in the pericardium (Fig. 4c and d).

After the depuration phase (Time 39), two individuals in the control group and four fish in the high-concentration group (50 μg L^{-1}) showed focal areas of ischaemic degeneration. In the environmental concentration group, the same kind of lesion was found in two individuals, with higher extension and severity (Fig. 5c and d). Control group did not show more alterations (Fig. 5a and b). Degeneration of coronary vessels characterized by mild oedematous disintegration of vessel walls and lymphocytic infiltration in the surrounding fat tissue was diagnosed. Small areas of fibrosis were observed intermingled in the myocardium in exposed (environmental and high-concentration groups) individuals (Fig. 5e and f). The presence of spotted areas with gattered lymphocytes in

Table 1
The mean concentration of methamphetamine and the metabolite amphetamine in water. Values are expressed as the mean ± standard deviation, n = 12. LOQ = limit of quantification.

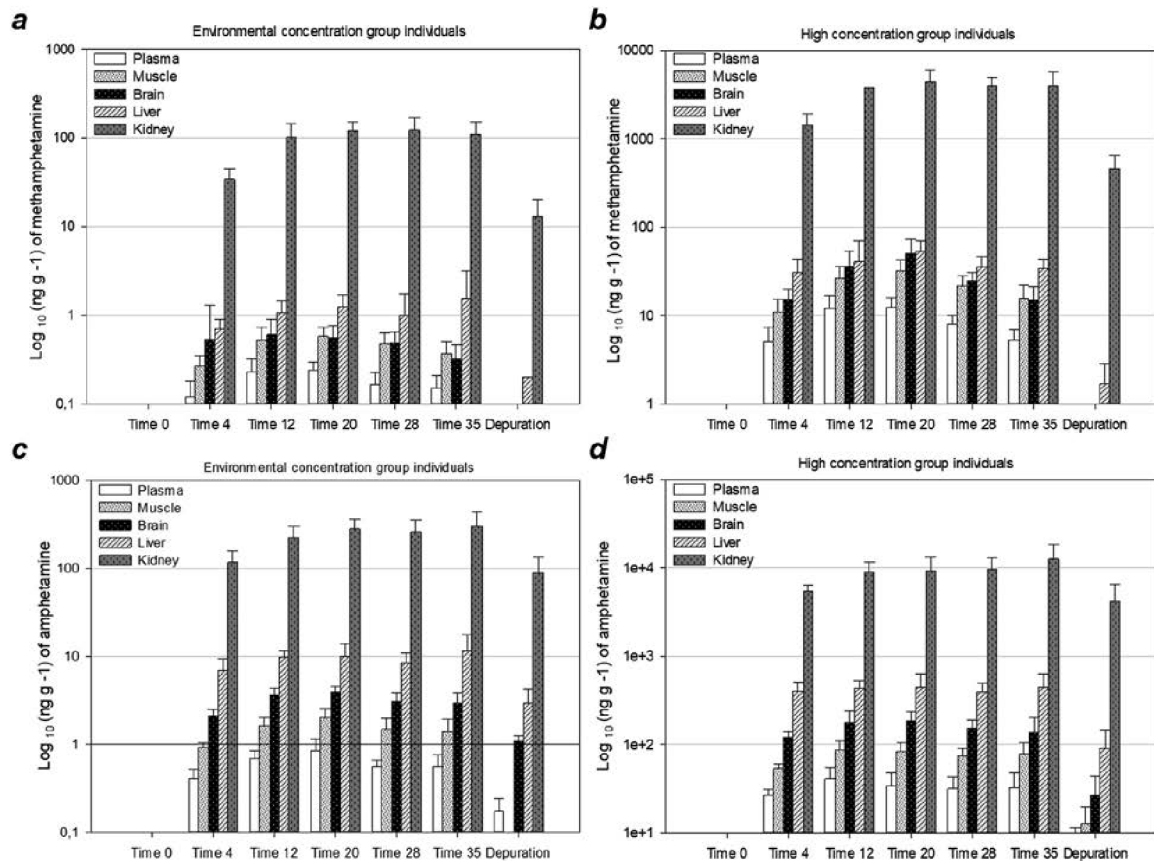| Compound	Group	Concentration in water (μg L^{-1})
Methamphetamine	Control	< LOQ
	1 μg L^{-1}	1.3 ± 0.2
	50 μg L^{-1}	66 ± 5
Amphetamine	Control	< LOQ
	1 μg L^{-1}	< LOQ
	50 μg L^{-1}	0.52 ± 0.15

Fig. 1. Concentration in plasma, muscle, brain, liver and kidney of brown trout (*Salmo trutta fario*) (n = 10) after 0 (Time 0), 4 (Time 4), 12 (Time 12), 20 (Time 20), 28 (Time 28), 35 (Time 35) days of exposure and 4- day depuration phase (Depuration, Time 39), expressed in Log $_{10}$ (ng g^{-1}), of (a) methamphetamine in environmental relevant concentration group (1 μg L^{-1}), (b) methamphetamine in high concentration group (50 μg L^{-1}), (c) amphetamine, methamphetamine's metabolite, in environmental concentration group (1 μg L^{-1}), (d) amphetamine in high concentration group (50 μg L^{-1}).

myocardium was significantly increased (Kruskal-Wallis, $p < 0.05$) in the high concentration-exposed fish compared to control and environmental concentration-exposed fish (Appendix F).

In gills, skin and kidney, no significant differences were found between individual fish or between experimental groups.

3.3. Immunohistochemistry

After 35 days of treatment, only livers of brown trout exposed to high concentration of METH presented cleaved caspase-3 positive cells (Kruskal-Wallis, $p < 0.05$) (Fig. 6a, c, e) yet, after depuration phase (Time 39), both groups exposed to the drug showed positive hepatocytes (Fig. 6b, d, f) (Appendix F).

4. Discussion

4.1. Chemical analyses

METH and its metabolite AMPH were present in all tissues of exposed individuals. Apart from the liver which plateaued at time 4, all other organs reached a steady chemical concentration level by time 12. The tested compounds have a low n-octanol-water partition coefficient (log K$_{ow}$), 2.07 for METH and 1.76 for AMPH, indicating their hydrophilic nature. Considering the OECD guideline

(OECD, 2001), low BCF were observed (BCF< 500) although possible toxic effects after chronic exposure at low levels are mentioned in the same guideline. Yin et al. (2019) found the accumulation of METH and AMPH in zebrafish within the same range of BCF we observed. Results from studies concerning carbamazepine, which has a similar log K$_{ow}$, also showed a low BCF (<10), with different values depending on the tissue and the fish species (Garcia et al., 2012; Valdés et al., 2016).

The therapeutic plasma concentration (TPC) is related to the plasma level at which pharmacological effects are obvious. The TPC of METH in humans ranges from 0.01 to 0.05 μg mL^{-1} and that for AMPH is 0.02−0.15 μg mL^{-1} (Regenthal et al., 1999). The read-across hypothesis (Huggett et al., 2003) suggests that therapeutic effects in humans can be extrapolated to fish if the concentration in fish plasma is similar to the TPC in humans, due to their molecular target conservation. If the effect ratio (ER; human TPC/concentration in fish plasma) is ≤ 1, it could be supposed that the therapeutic concentration in fish is the same or even higher than the TPC in humans (Huggett et al., 2003). In this study, the ER in the environmental concentration groups was 59 for METH and 35 for its metabolite. In the high-concentration group, the ER was 1.34 and 0.61 for METH and its metabolite and the plasma levels (an average of 0.01 and 0.03 μg mL^{-1}, respectively) reached the TPC for humans for both compounds. Previous studies with other substances
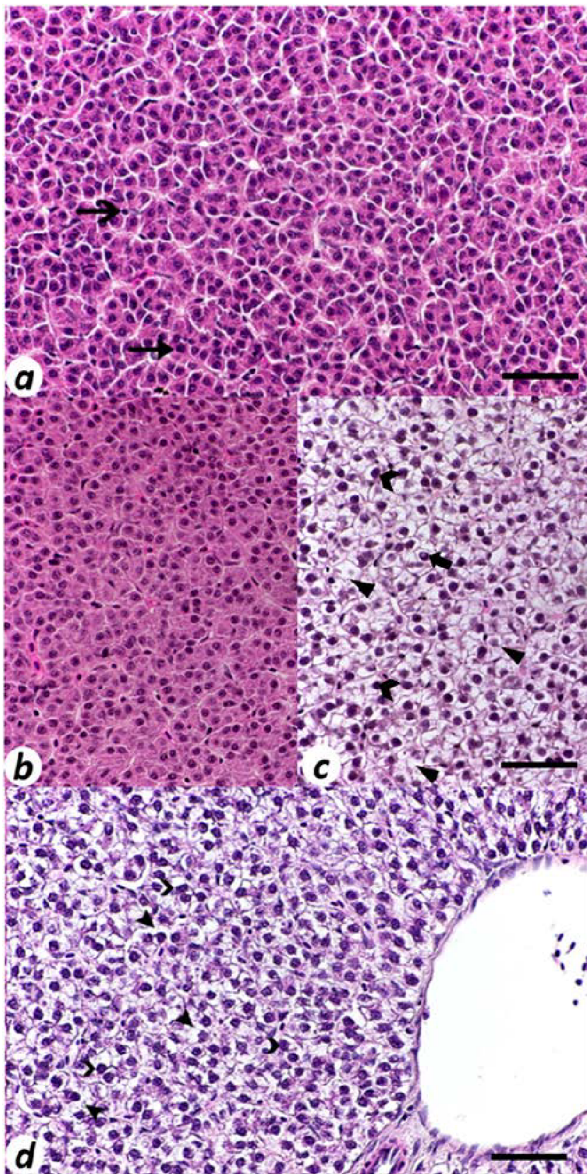
Fig. 2. Liver tissue of brown trout after 35 days of exposure (Time 35; n = 6). (a) Control fish with no hepatocyte cytoplasmatic vacuolation, scattered karyopyknotic cells (arrow with open arrowhead) and mitotic figures (arrow with closed arrowhead), 600x magnification. b, c. Environmental concentration group (1 μg L^{-1}) at 600x magnification. (b) Individual with no hepatocyte vacuolation. (c) Fish displaying cytoplasmatic vacuolation causing disruption of cell membranes (closed arrowheads), displacing of the nucleus to the side of the cell (open arrowheads) and an intranuclear inclusion (arrow). (d) Fish exposed to high METH concentration (50 μg L^{-1}) at 600x magnification, showing increased cytoplasmic vacuolation, macrovacuoles often displacing the nucleus to the cell margin (open arrowheads), and rupture of cell membranes (closed arrowheads). H&E staining; scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. Liver of brown trout after depuration phase (Time 39; n = 6). (a) Control fish with scattered vacuolation (most probably of lipid content) at 600x magnification and mitotic figures (arrow with closed arrowhead). (b) Environmental concentration group (1 μg L^{-1}) at 600x magnification, showing increased cytoplasmic vacuolation of hepatocytes, macrovacuoles displacing nucleus to the cell margin, disruption of cell membranes (e.g. closed arrowheads) and increased amount of karyopyknosis (e.g. arrows with open arrowheads). (c) High concentration group (50 μg L^{-1}) at 600x magnification, with scattered cytoplasmic microvacuoles in hepatocytes. H&E staining; scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reported similar effects even without reaching the TPC (Patel et al., 2016; Steinbach et al., 2014). In addition, according to Huggett et al. (2003), substances should be additionally evaluated if the ER is < 1000 for the 10-fold factor applied to fish.
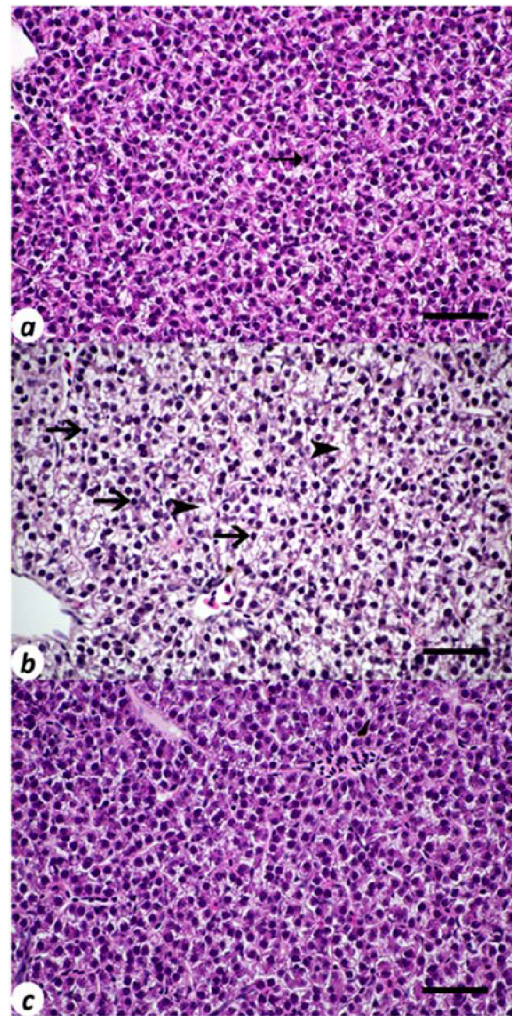
Receptors of dopamine, noradrenaline and serotonin, for which amphetamine-type substances (ATS) are indirect agonists, are mainly located in the nervous system and the brain (Sulzer et al., 2005). Based on our results, it could be suggested that these compounds reached the CNS in both exposure groups. Future studies should focus on behavioural changes in fish after exposure to METH due to presence of the substance and its metabolite in the brain even at environmental relevant concentrations.

METH is transformed to AMPH via N-demethylation dependant on cytochrome P450 (Cruickshank and Dyer, 2009). In our experiment, the highest metabolization rate was observed in the liver. The metabolite AMPH is a pharmacologically active compound with similar characteristics to METH. In previous studies using
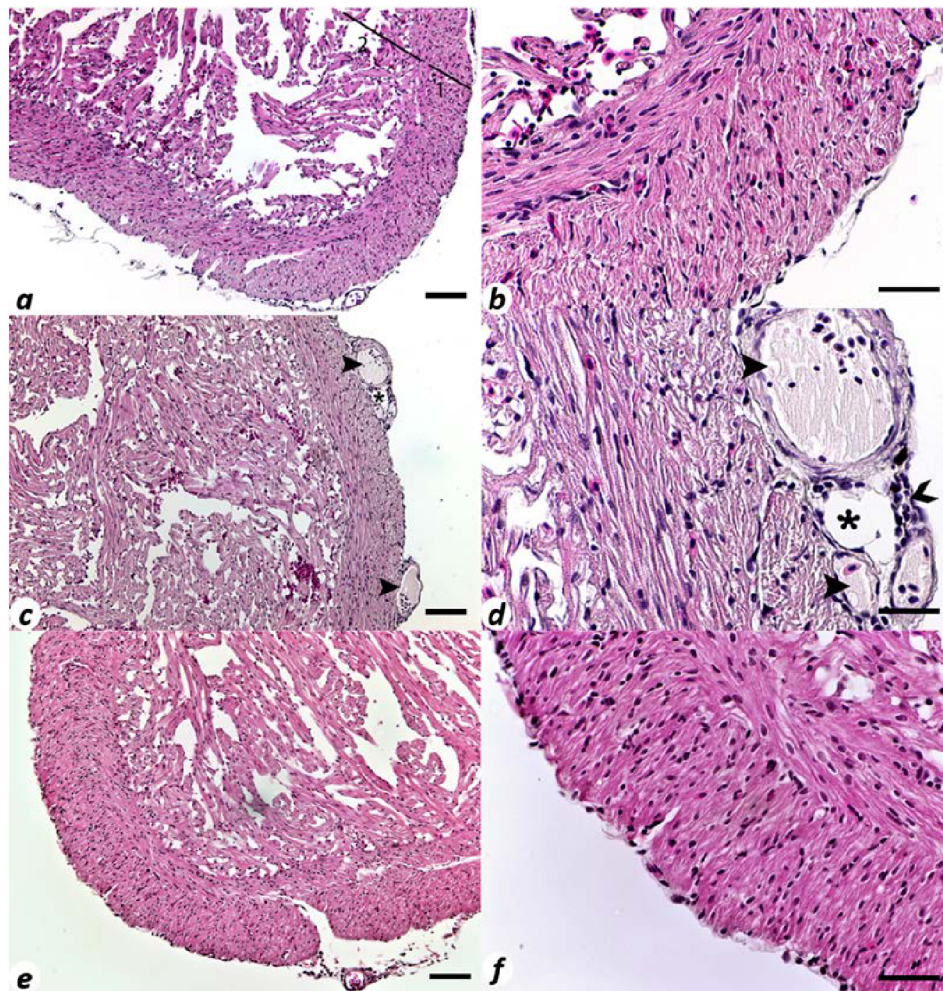
Fig. 4. Heart of brown trout after 35 days of exposure (Time 35; n = 6). a, b. Control fish, no pathological findings were recorded (a) Myocardial structure: 1 = compact layer, 2 = spongy layer, 200x magnification. (b) Higher magnification of myocardium. c, d. Environmental concentration (1 µg L^{-1}), coronary vessels were dilated (closed arrowheads), in vicinity moderate oedema (asterisk) and infiltration of pericardium with lymphocytes and less plasma cells and macrophages (open arrowhead). (c) 200x magnification. (d) Higher magnification (600x). e, f. High concentration group (50 µg L^{-1}) similar to control. (e) 200x magnification. (d) 600x magnification. H&E staining; scale bars = (a, c, e) 100 µm, (b, d, f) 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mammals, the metabolite was not considered to contribute to clinical effects due to the low concentrations detected (Carvalho et al., 2012; Cruickshank and Dyer, 2009). Conversely, in this experiment, the concentrations of the metabolite were substantially higher than the parent compound in all analysed tissues and thus the observed effects could be partially related to the action of the metabolite. Apart from amphetamine, additional metabolites were found in tissues of mammals, as para-hydroxymethamphetamine (Cruickshank and Dyer, 2009; Wagner et al., 2018). Considering the notable amount of amphetamine in fish tissues observed in this study and in Yin et al. (2019), future research must focus on the identification of potentially formed metabolites of METH and their possible effects on fish.

METH and AMPH accumulate in the urine of mammals (Cruickshank and Dyer, 2009), and METH is excreted in a high proportion without biotransformation (Carvalho et al., 2012). In this study, the kidney showed the lowest rate of the metabolite AMPH to the parent compound METH compared to other analysed tissues (e.g. at the environmental group, the rate of concentrations ranged only 2.1 to 3.4 compared to 3.9 to 9.2 in brain or 7.4 to 9.9 in liver, respectively). Both compounds were transported to the kidney for excretion, but no histopathological changes were observed in this organ. Although some remnants of the substances were still detectable after four days of depuration, all levels had diminished considerably. The gradual excretion of the compounds indicates a longer depuration time.

4.2. Histology

Histopathology is widely used as a key tool in toxicology studies to determine the effects of environmental pollutants on fish and also serves as an important endpoint in risk assessment (Crissman et al., 2004). Based on the results obtained in this study, METH induced histopathology in the liver and heart even at the low concentration (1 µg L^{-1}), which is considered environmentally relevant in this study.
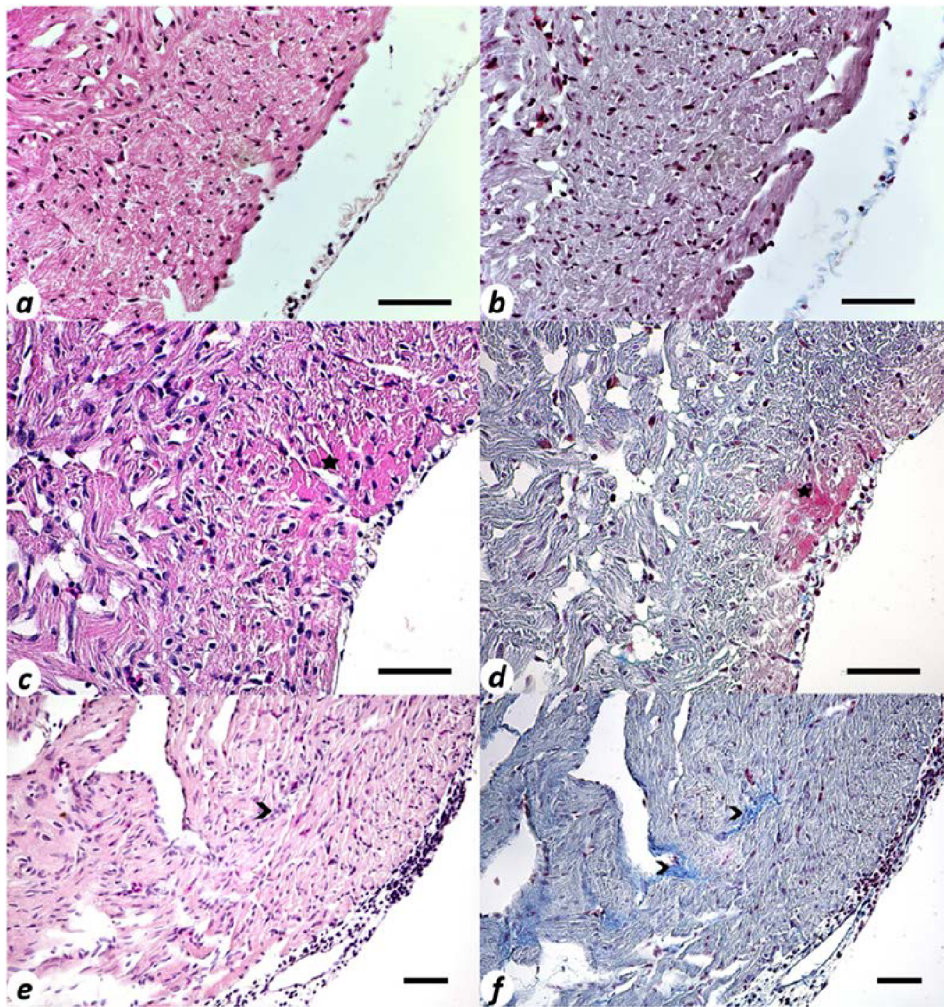
Fig. 5. Heart of brown trout after four-day depuration phase (Time 39; n = 6). a, b. Myocardium of control group with no lesions, 600x magnification. (a) H&E staining (b) Masson's trichrome staining. c, d. Environmental concentration group (1 μg L^{-1}), degenerated areas in myocardium (star) characterized by hypereosinophilia, amorphous structure of myofibers and occasional fragmentation, note hypertrophic nuclei at the periphery of myocardial fibers and pronounced cytoplasmic eosinophilia. Pericardium adjacent to the affected myocardium reacted as pericarditis fibrinosa focalis. Changes may be caused by myocardial ischemia, position of affected fibers pointed at regions with worsen oxygen supply, 600x magnification. (c) H&E staining (d) Masson's trichrome staining (indicating degenerated myofibers in red). e, f. Fish exposed to high concentration (50 μg L^{-1}), showing numerous small fibrotic areas (open arrowhead) in spongy layer of myocardium, corresponding to fibrotic scars, better illustrated in Masson's trichrome staining, pericardium reacted as pericarditis fibrinosa focalis. 400x magnification, (e) H&E staining, (f) Masson's trichrome staining; scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4.2.1. Liver

METH is thoroughly metabolized in the liver, where it also accumulates in mammals (Carvalho et al., 2012). The common histopathology observed in humans and experimental animals in response to METH exposure is cytoplasmic vacuolation of hepatocytes, in a wide range of intensity (Halpin and Yamamoto, 2012; Kamijo et al., 2002; Koriem and Soliman, 2014; Milroy et al., 1996; Wang et al., 2017), which is in agreement with the findings of our experiment. In line with Wang et al. (2017), no fibrotic changes were found in this study.

Hepatotoxicity of ATS has been largely reported (Halpin et al., 2013; Milroy et al., 1996; Wang et al., 2017), often associated with hyperthermia and direct toxicity, but the specific mechanisms are unclear.

An increase in body temperature has been associated with the effects of ATS in the liver, as it was connected to increased oxidative stress and decreased activity of antioxidant enzymes, especially superoxide dismutase and glutathione peroxidase (Koriem and Soliman, 2014). Rats treated with METH at normothermia and hyperthermia conditions showed positive temperature and dose-dependent effects in cytoplasmatic vacuolation of hepatocytes in a study by Halpin et al. (2013). Vacuolation with hydrophobic substances in the liver, such as fat, is often related to the exposure to pharmaceuticals and diverse toxic agents, which cause energetic cell disbalance and consequent replenishing of cytoplasmatic vacuoles in the hepatocytes (Halpin et al., 2013; Idilman et al., 2016).

In our study, a high variation in vacuolization intensity was found in livers in the environmental concentration group. Due to interindividual differences, some of the fish were more sensitive. In fish exposed to the high concentration, all livers showed moderate
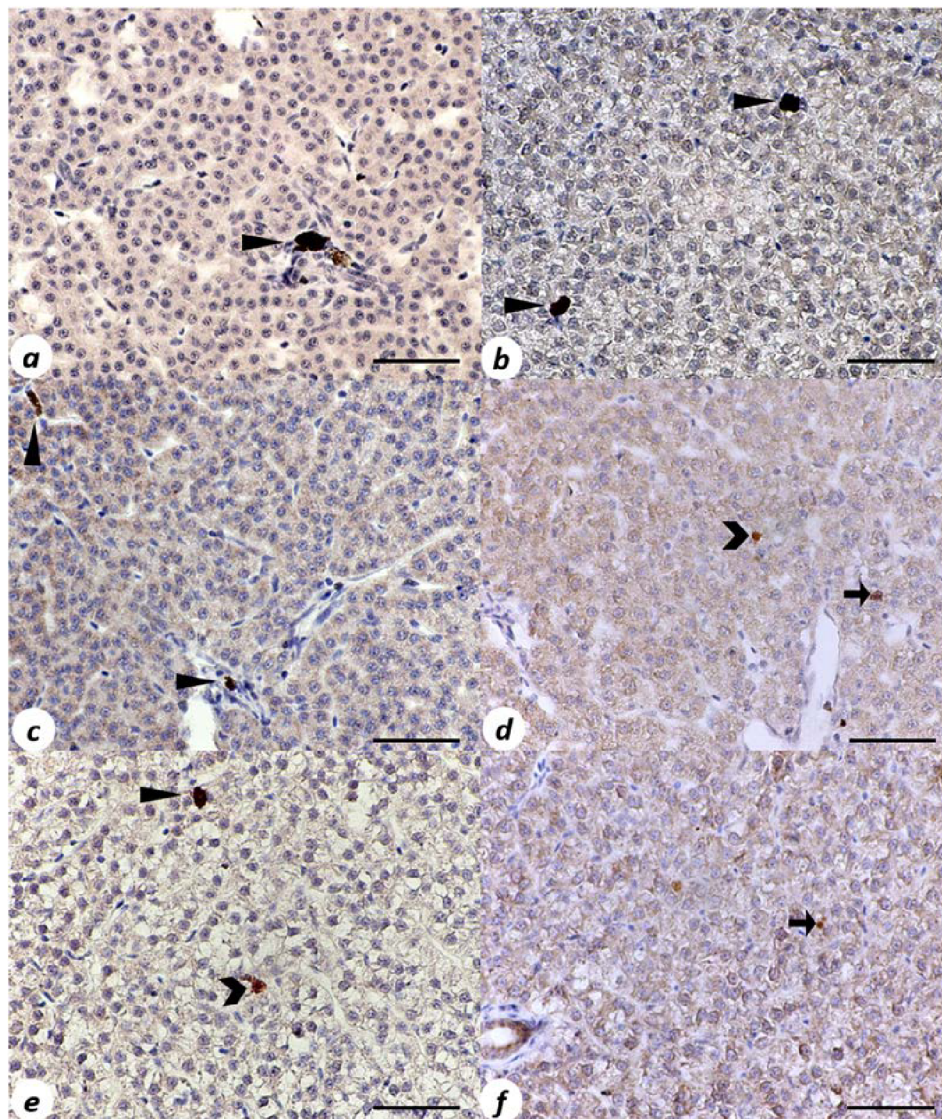
Fig. 6. Immunostaining of cleaved caspase-3 in liver of brown trout (n = 6). a, c, e. After 35 days of exposure. (a) Control group with pigmented macrophages (closed arrowhead), (c) Environmental concentration (1 µg L^{-1}) containing pigmented macrophages (closed arrowhead), (e) High concentration individuals (50 µg L^{-1}) showing a pigmented macrophage (closed arrowhead) and a positive cell (open arrowhead). b, d, f. After 4-day depuration phase. (b) Control fish, pigmented macrophages (closed arrowhead), (d) Environmental concentration group exhibiting a positive cell (open arrowhead) and an artefact (arrow), (f) High concentration exposed individuals showing a positive hepatocyte (arrow). 600x magnification; scale bar = 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to severe cytoplasmatic vacuolation degeneration, confirming the dose-dependent effect reported by Halpin et al. (2013).

After the depuration phase, fish previously exposed to the environmental concentration showed severe liver lesions, possibly related to the exposure time and level of compound remnants in the tissue. In fish previously exposed to high concentrations of METH, the liver showed a reduction of cytoplasmatic vacuoles. However, membrane rupture was still observable, indicating prior damage and depletion of hydrophobic substances during depuration, possibly due to compensation processes or increased metabolism of the drug at high concentrations compared with the environmental concentration. In agreement, Yin et al. (2019) also suggested a faster metabolism of METH in zebrafish under high

concentrations exposure, due to the increased amount of the metabolite AMPH at higher levels in comparison to lower concentrations.

4.2.2. Heart

Similar to the findings in the liver, METH has been attributed to severe changes in the cardiovascular system in experimental animals and humans, e.g., acute coronary syndrome, infarction, cardiomyopathy or aortic dissection (Akhgari et al., 2017; Kaye et al., 2007; Won et al., 2013).

The microvascular injuries, foci of necrosis, fibrosis, eosinophilic changes and cell infiltration with lymphocytes observed in this study are also widely reported in response to METH exposure

(Akhgari et al., 2017; Islam et al., 1995; Liou et al., 2014; Milroy and Parai, 2011; Won et al., 2013). In general, the effects of METH on the cardiovascular system were considered a consequence of cardio- and neurotoxicity, mediated by increased catecholamine activity, oxidative stress, increased apoptosis and mitochondrial impairments (Henry et al., 2012; Kaye et al., 2007; Li et al., 2012a, 2012b; Liou et al., 2014; Lord et al., 2010).

Increased catecholamine action induced by METH has a direct effect on the cardiovascular system by binding on β1 adrenoreceptors, and an indirect effect by boosting the locomotor activity and increasing the demand for blood flow (Hassan et al., 2016). This loop provokes a decreased oxygen supply of the myocardium due to increased vasoconstriction in the periphery and increased oxygen demand due to tachycardia and hypertension (Henry et al., 2012; Kaye et al., 2007). The associated hypoxia provokes myocardium necrosis and replacement by fibrosis (Henry et al., 2012). Similar to the liver changes, Liou et al. (2014) observed increased Fas-dependent and mitochondria-dependent apoptosis in the heart of METH-exposed rats. Excess catecholamines also provokes oxidative stress in the myocardium and a resulting energy misbalance in myofibers, leading to ventricular dysfunction (Lord et al., 2010). In addition, Faith et al. (Li et al., 2012a, 2012b) reported brain stem nuclei degeneration following METH exposure associated with central cardiovascular dysfunction in rats. In our study, higher concentrations of METH were found in the brain of brown trout than in the other tissues. Additional studies must be undertaken to elucidate the underlying mechanisms.

In fish, the adrenergic system is highly conserved compared to mammals (Fabbri et al., 1998). We hypothesize that increased respiratory rate could not compensate for oxygen depletion in the myocardium and led to the observed histopathology of the heart.

In our study, histological findings in the heart were mainly observed in the environmental concentration-exposed fish whereas no significantly different changes were found in high-concentration group compared with the control animals. This could be due to regeneration processes (Ma et al., 2018). Another explanation could be that high METH concentrations activate compensatory mechanisms faster and induce adaptation, as the liver results suggested a decreased amount of hepatocyte vacuolation in the high-concentration group compared to the environmental group after the depuration phase.

Fibrosis has been seen as a non-recovery sign in METH users enduring cardiomyopathy (Darke et al., 2018; Lopez et al., 2009), even in METH absent individuals (Voskoboinik et al., 2016). In contrast, myocardium regeneration, even after fibrosis, has been documented in zebrafish (Ma et al., 2018; Sanz-Morejón et al., 2018). Therefore, the effects of these contaminants on the cardiovascular system in fish are likely less pronounced.

4.3. Immunohistochemistry

Apoptosis has been commonly associated to the toxicity of METH exposure in several organs, including the liver. The enzyme caspase-3 is a key performer in the mechanism of execution (Dias Da Silva et al., 2013; Jiménez et al., 2004; Leung et al., 2014; Liou et al., 2014). Furthermore, direct cytotoxicity in hepatocytes due to the activation of apoptosis and inhibition of cell division in METH-exposed rats was reported by Wang et al. (2017). In accordance with these findings, we observed an increased caspase-3 activity in exposed individuals. Dias Da Silva et al. (2013) used HepG2 cell lines exposed to METH and reported both mechanisms of cell death, apoptosis and necrosis. Accordingly, exposed fish presented an increased activity of caspase-3 and single cell necrosis, as observed in the histological analyses.

The presence of caspase-3 positive cells in environmental

concentration group after depuration phase, and not after 35 days of exposure, suggested that the triggered vacuolation occurred prior to the activation of the caspase cascade. Therefore, damaged cells would be detected at nuclear level once the disturbance was produced at the cell level. In the same way, as part of the recovering mechanism of the tissue, apoptosis was still present while the rest of the pathological alterations were already ceased in high concentration group after depuration phase.

5. Conclusions

Brown trout exposed to relevant environmental (1 μg L^{-1}) and high concentrations (50 μg L^{-1}) of methamphetamine revealed histological changes in the liver and heart, which were similar to those previously described in mammals, as well as increased apoptosis of hepatocytes. The bioconcentration factor for METH was low (<500), indicating a low possibility to be bioconcentrated (OECD, 2001), although METH was found in all tissues analysed. Due to its pharmacological activity and the remarkable amounts detected, the metabolite AMPH could be suspected to have partially caused the observed effects. The chemical and histological findings suggested a tendency of adaptation of brown trout to METH exposure. The plasma concentrations of both substances were close to the therapeutic level in humans, emphasising the necessity for additional studies of similar effects. This study showed that METH impacts fish even at environmentally relevant levels, indicating potential effects in wild aquatic organisms.

Declaration of competing interest

None.

CRediT authorship contribution statement

Maria Eugenia Sancho Santos: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Kateřina Grabicová:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Christoph Steinbach:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Heike Schmidt-Posthaus:** Investigation, Methodology, Writing - review & editing. **Eva Šálková:** Investigation, Methodology, Writing - review & editing. **Jitka Kolářová:** Formal analysis, Writing - review & editing. **Andrea Vojs Staňová:** Formal analysis, Writing - review & editing. **Roman Grabic:** Conceptualization, Investigation, Methodology, Writing - review & editing. **Tomáš Randák:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2020.126882.

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Appendix A:

Table A.1. *Number of tested fish (Salmo trutta fario) used in each sampling day. At the starting of the experiment, 12 control fish were sampled. Afterwards, at every sampling day, 5 fish per aquarium were sampled, which means 10 fish per treatment. Due to insufficient amount of the samples, histology and immunohistochemistry were demonstrated at lower number.*

Treatment	Time 0	Time 4	Time 12	Time 20	Time 28	Time 35	Time 39 (depuration)	Σ
Control	12	10	10	10	10	10	10	72
Environmental		10	10	10	10	10	10	60
High		10	10	10	10	10	10	60
Σ	12	30	30	30	30	30	30	**192**

Appendix B:

Table B.1. *Chemical analysis of methamphetamine (METH) and amphetamine (AMPH) in fish brain (LC-HRMS).*

Liquid chromatography			
Time [min]	Water (0.1% formic acid) [%]	Acetonitrile (0.1% formic acid) [%]	Flow [mL min $^{-1}$]
0	100	0	0.35
1	100	0	0.35
3	50	50	0.35
4	20	80	0.45
5	0	100	0.45
6	0	100	0.45
6.05	100	0	0.35
7	100	0	0.35

Mass spectrometer			
Drug	Parent ion	Product ion (quan)	Product ion (qual)	Retention time [min]
METH	150.13	91.0547	119.0857	3.45
D5-METH	155.16	92.0611	–	3.45
AMPH	136.11	91.0547	11.0857	3.30
D5-AMPH	141.14	93.0673		3.30

Appendix C:

Table C.1. *Minimum and maximum limit of quantification (LOQ), in nanograms per gram, of methamphetamine and the metabolite amphetamine in tissues of brown trout.*

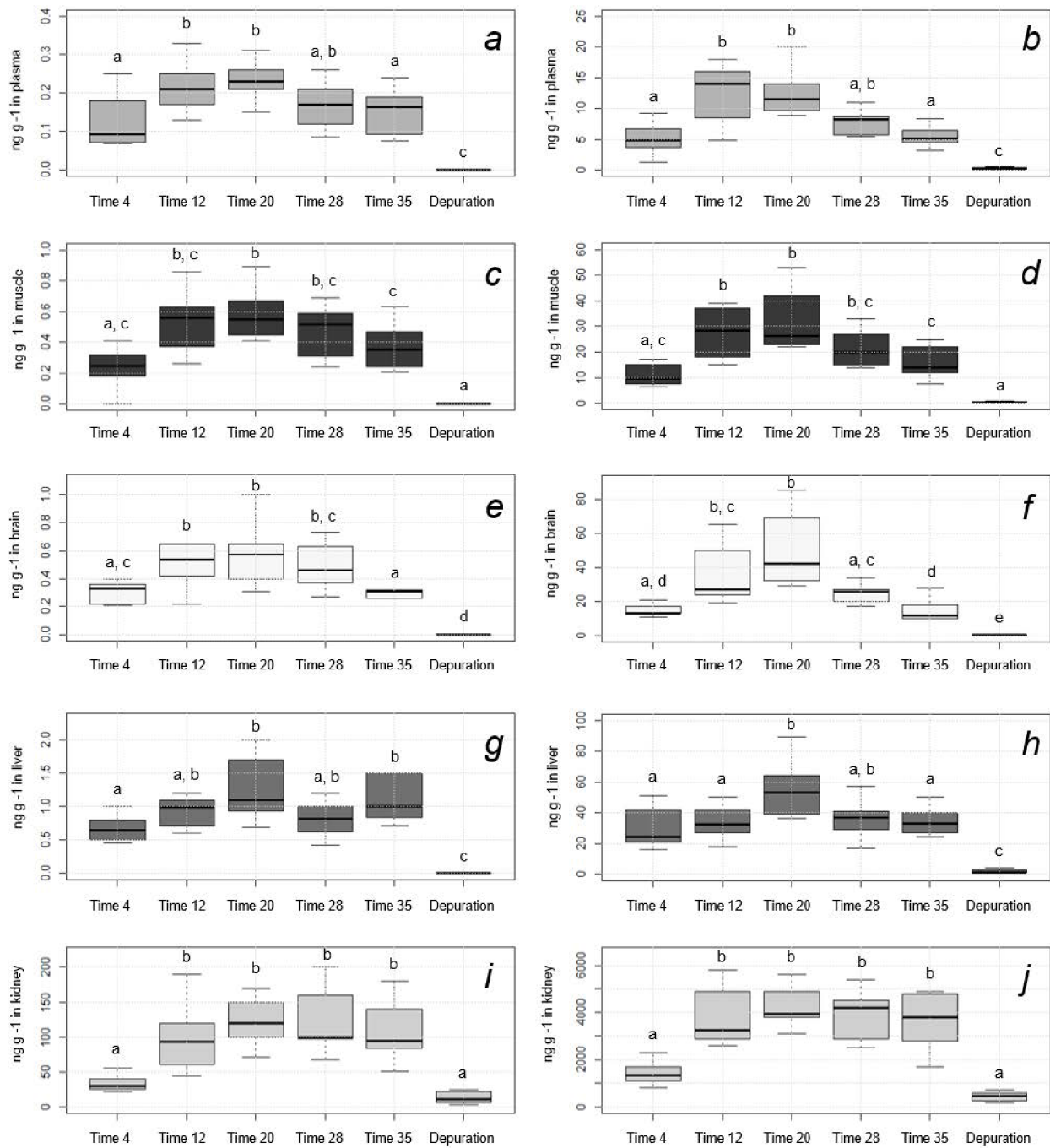| Tissue	Methamphetamine		Amphetamine	
	Min	Max	Min	Max
Muscle	0.15	0.34	0.14	0.29
Plasma	0.036	0.066	0.030	0.051
Brain	0.068	0.11	0.27	0.56
Kidney	0.097	0.67	0.51	0.96
Liver	0.091	0.50	0.69	1.8

Appendix D:

Figure D.1. *Bioconcentration of methamphetamine, in nanograms per gram, in tissues of brown trout exposed after 0, 4, 12, 20, 28, 35 days and after 4-day depuration phase. (a) Plasma of environmental concentration (1 µg L⁻¹) exposed group. (b) Plasma of high concentration (50 µg L⁻¹) exposed group. (c) Muscle of 1 µg L⁻¹ group. (d) Muscle of 50 µg L⁻¹ group. (e) Brain of 1 µg L⁻¹ group. (f) Brain of 50 µg L⁻¹group. (g) Liver of 1 µg L⁻¹group. (h) Liver of 50 µg L⁻¹ group. (i) Kidney of 1 µg L⁻¹ group. (j) Kidney of 50 µg L⁻¹ group. Statistical analyses performed by Kruskal-Wallis test, with Dunn-test as post-hoc treatment. Letters corresponds to the significant differences between groups (Kruskal-Wallis p <0.05; Dunn-test α/2).*
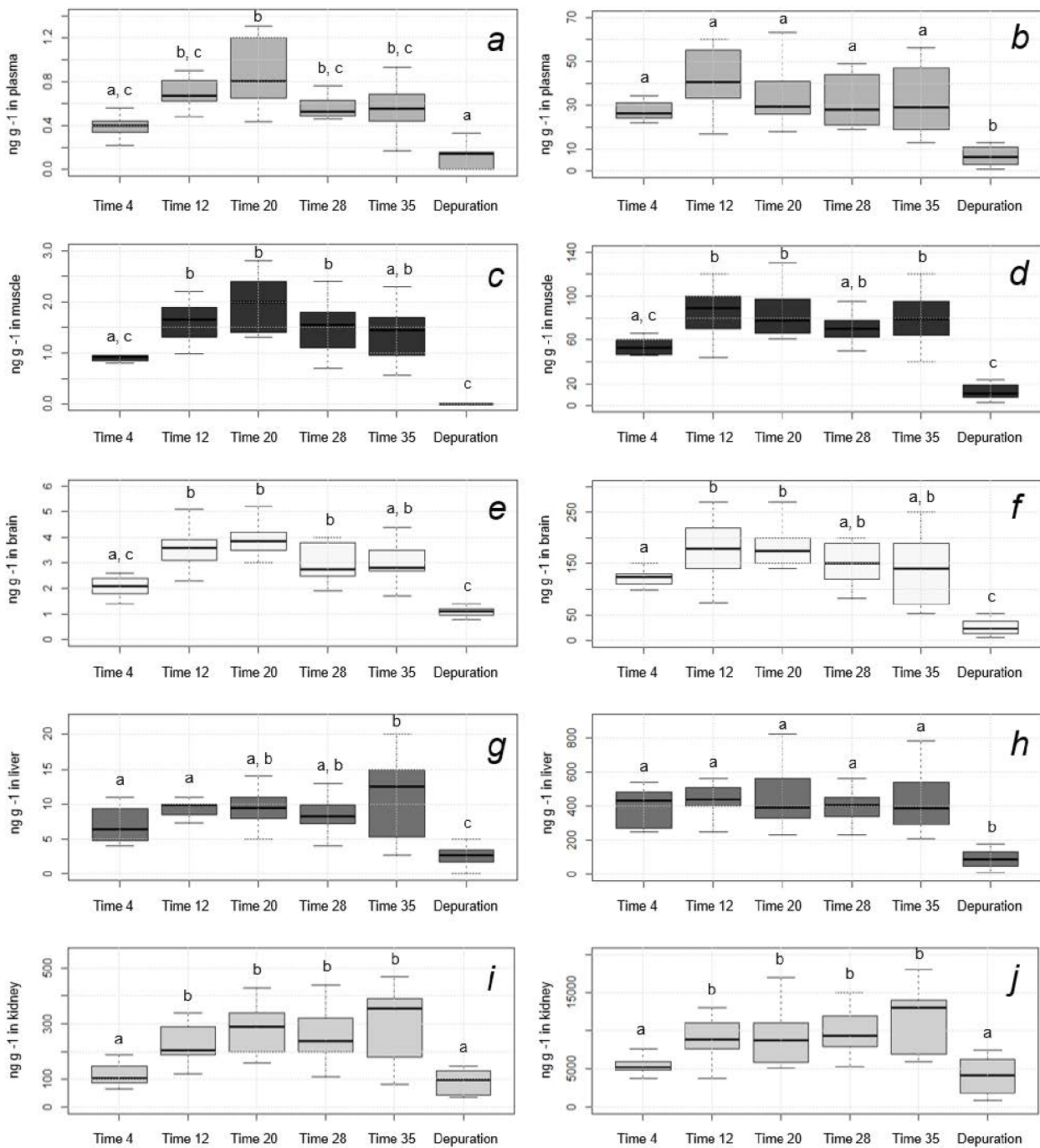
Figure D.2. *Concentration of methamphetamine's main metabolite, amphetamine, in nanograms per gram, in tissues of brown trout exposed after 0, 4, 12, 20, 28, 35 days and after 4-day depuration phase. (a) Plasma of environmental concentration (1 μg L⁻¹) exposed group. (b) Plasma of high concentration (50 μg L⁻¹) exposed group. (c) Muscle of 1 μg L⁻¹ group. (d) Muscle of 50 μg L⁻¹ group. (e) Brain of 1 μg L⁻¹ group. (f) Brain of 50 μg L⁻¹ group. (g) Liver of 1 μg L⁻¹ group. (h) Liver of 50 μg L⁻¹ group. (i) Kidney of 1 μg L⁻¹ group. (j) Kidney of 50 μg L⁻¹ group. Statistical analyses performed by Kruskal-Wallis test, with Dunn-test as post-hoc. Letters corresponds to the significant differences between groups (Kruskal-Wallis p <0.05; Dunn-test α/2).*

Appendix E:

Table E.1. *Bioconcentration factor (BCF) of methamphetamine in tissues of brown trout after 4 (Time 4), 12 (Time 12), 20 (Time 20), 28 (Time 28) and 35 (Time 35) days of exposure. The calculation of BCF was performed by dividing the average concentration of METH in tissues by the respective mean concentration in water. No statistical differences were found the BCF depending on the concentration of the tested compound and tissue (T-test, p >0.05).*

	Plasma		Muscle		Brain		Liver		Kidney	
	1 µg L^{-1}	50 µg L^{-1}	1 µg L^{-1}	50 µg L^{-1}	1 µg L^{-1}	50 µg L^{-1}	1 µg L^{-1}	50 µg L^{-1}	1 µg L^{-1}	50 µg L^{-1}
Time 4	0.08	0.07	0.19	0.16	0.38	0.22	0.49	0.44	23.86	20.51
Time 12	0.17	0.17	0.39	0.38	0.45	0.51	0.78	0.57	74.81	52.91
Time 20	0.22	0.21	0.53	0.53	0.51	0.85	1.13	0.89	110.00	74.19
Time 28	0.13	0.12	0.36	0.31	0.37	0.36	0.77	0.51	93.85	57.47
Time 35	0.14	0.09	0.33	0.26	0.29	0.25	1.40	0.57	98.18	65.29
Mean ± SD	0.15 ± 0.05	0.13 ± 0.06	0.36 ± 0.12	0.33 ± 0.14	0.40 ± 0.08	0.44 ± 0.26	0.91 ± 0.36	0.60 ± 0.17	80.14 ± 33.91	54.08 ± 20.43

Appendix F:

Table F.1. *Histopathological findings in liver and heart of brown trout (Salmo trutta fario) after the exposure to methamphetamine for 35 days. Intensity of lesions in semi-quantitative scale: 0 = no changes, 1 = minimal changes, 2 = mild intensity, 3 = moderate intensity and 4 = severe intensity. Number of pigmented macrophages were counted in four randomly selected areas at 400x.*

Group	Fish number	Macrovacuoles	Vacuoles	Pigmented macrophages	Infiltration	Karyopyknosis	Degeneration	Infiltration in pericardia	Infiltration in myocardia
				Liver			**Heart**		
Control	1	1	2	0.75	0	1	0	1	1
	2	1	1.50	3.50	0	1	1	0.50	1.50
	3	0	1	2.25	2	1	0.50	1	2
	4	0	0	3.25	0	0	3	1	2
	5	0	0	4	1	0	1	2	1
	6	0	1	4	0	1	1	2	1
1 µg L^{-1}	1	2	3	1.50	1	2	1.50	2	2
	2	0	0	5.75	1	2	3	2	3
	3	1	1.50	3.75	0	1	2.50	3	2
	4	0	0	6	1.50	2	3.50	3	2
	5	2	2.50	1.25	1	1	0.50	2	2
	6	3	4	1.50	0	1	2	2	1.50
50 µg L^{-1}	1	2	3	2.75	1	2	2.50	2	2
	2	4	4	2.50	1	2	1.50	1	0.50
	3	2	3	2.50	0	1.50	2	1	2
	4	2.50	3.50	2.25	1	2.50	2	3	3
	5	2	2.50	2	0	2	1.50	2	1.50
	6	2	3	0.75	2	2.50	2	2	2

Table F.2. Histopathological findings in liver and heart of brown trout after 4 days of methamphetamine depuration. Intensity of lesions in semi-quantitative scale: 0 = no changes, 1 = minimal changes, 2 = mild intensity, 3 = moderate intensity and 4 = severe intensity. Number of pigmented macrophages were counted in four randomly selected areas at 400x.

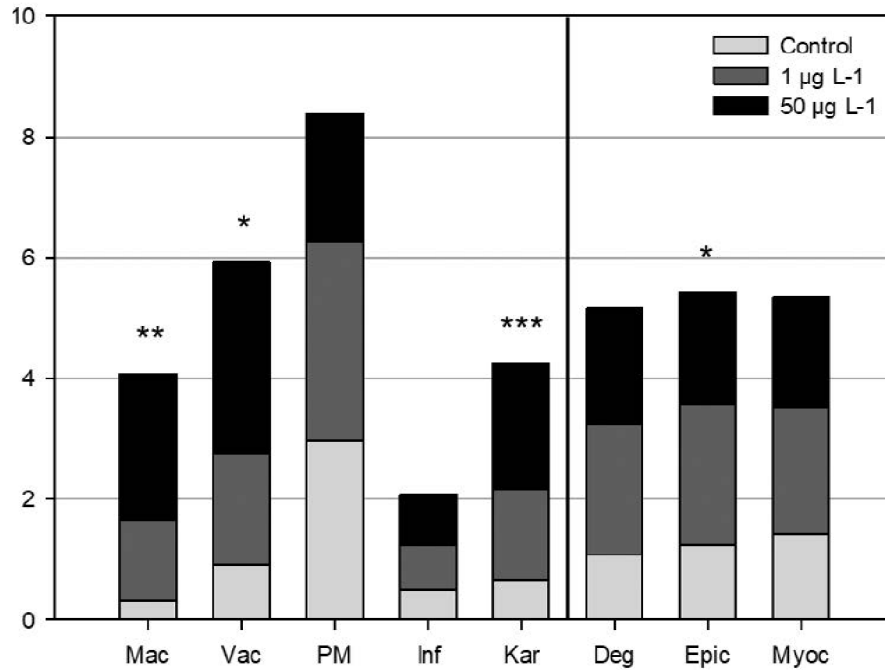| Group	Fish number	Liver					Heart		
		Macrovacuoles	Vacuoles	Pigmented macrophages	Infiltration	Karyopyknosis	Degeneration	Infiltration in pericardia	Infiltration in myocardia
Control	1	0	1	2	1	1	1.50	1	1
	2	0	0	3	1	0	0	2	1
	3	1	1	2.50	0	0	3	1	0
	4	0	1	1.75	2	1	1.50	1	0
	5	1	1.50	0.50	1	1	0	0.50	0
	6	1.50	3	0.75	1	1	2.50	2	1
1 µg L^{-1}	1	2	3	3.25	1	1	2	1.50	1.50
	2	3	3.50	1.50	1.50	1	4	3.50	1
	3	3	3.50	2.50	0	1	2	1	1.50
	4	0	1	1.25	2	2	2	1	2
	5	2.50	3	1.50	0	1	3	1	1
	6	3	3.50	2	0	1	4	1	2
50 µg L^{-1}	1	1	1	1	1	2	3.50	1	1.50
	2	0.50	1	1.75	1	1	1	2	2.50
	3	5	1	1	1	1	2	1.50	1
	4	1	1	1.75	0	2	1	2	1
	5	0	0.50	2.75	0.50	1	0.50	1.50	3
	6	1	2.50	0.50	1	1	2	1	2

Figure F.1. *Graphical representation of the histopathological findings in liver and heart of brown trout after 35 days of methamphetamine exposure. In liver, macrovacuolation (Mac), vacuolation (Vac), pigmented macrophages (PM), infiltration (Inf), karyopyknosis (Kar). In heart, degeneration (Deg), infiltration in epicardium (Epic), infiltration in myocardium (Myoc). Kruskal-Wallis test, asterisks correspond to the level of significance (*0.05 ≥ p, **0.01 ≥ p, ***0.005 ≥ p).*
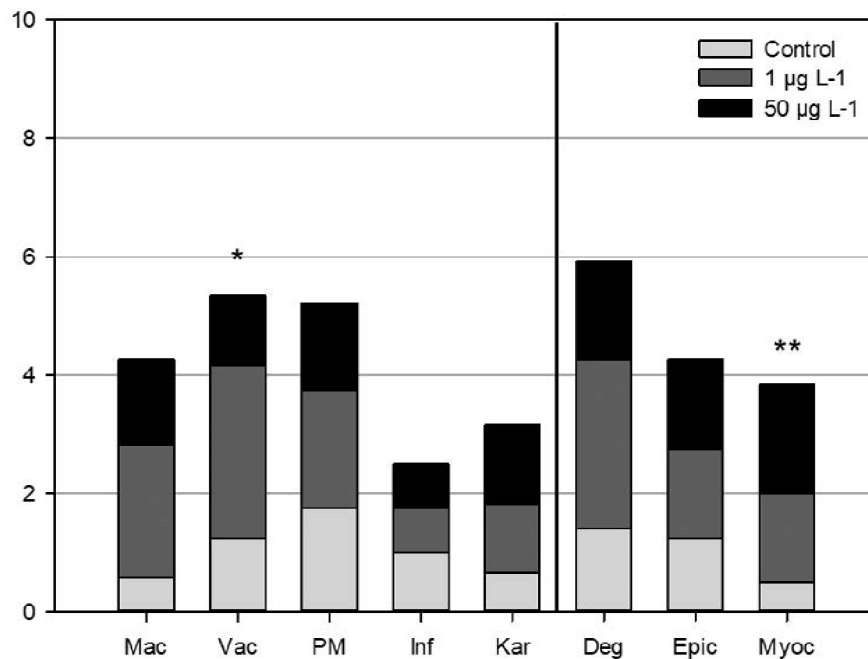

Figure F.2. *Graphical representation of the histopathological findings in liver and heart of brown trout after 4-day depuration. In liver, macrovacuolation (Mac), vacuolation (Vac), pigmented macrophages (PM), infiltration (Inf), karyopyknosis (Kar). In heart, degeneration (Deg), infiltration in epicardium (Epic), infiltration in myocardium (Myoc). Kruskal-Wallis test, asterisks correspond to the level of significance (*0.05 ≥ p, **0.01 ≥ p).*

Table F.3. *Presence in brown trout livers of caspase-3 positive hepatocytes of after 35 days of methamphetamine exposure (Time 35), and after 4 day-depuration (Time 39) (n = 6). Livers marked as "–", absence and "x", presence.*

Group	Time 35		Time 39	
	Fish number	Positive cells	Fish number	Positive cells
Control	1	0	1	–
	2	0	2	–
	3	0	3	–
	4	0	4	–
	5	0	5	–
	6	0	6	–
		0 (0%)		**0%**
1 µg L⁻¹	1	–	1	x
	2	–	2	–
	3	–	3	x
	4	–	4	–
	5	–	5	–
	6	–	6	x
		0%		**50%**
50 µg L⁻¹	1	x	1	x
	2	x	2	–
	3	–	3	x
	4	x	4	x
	5	–	5	x
	6	x	6	–
		67%		**67%**
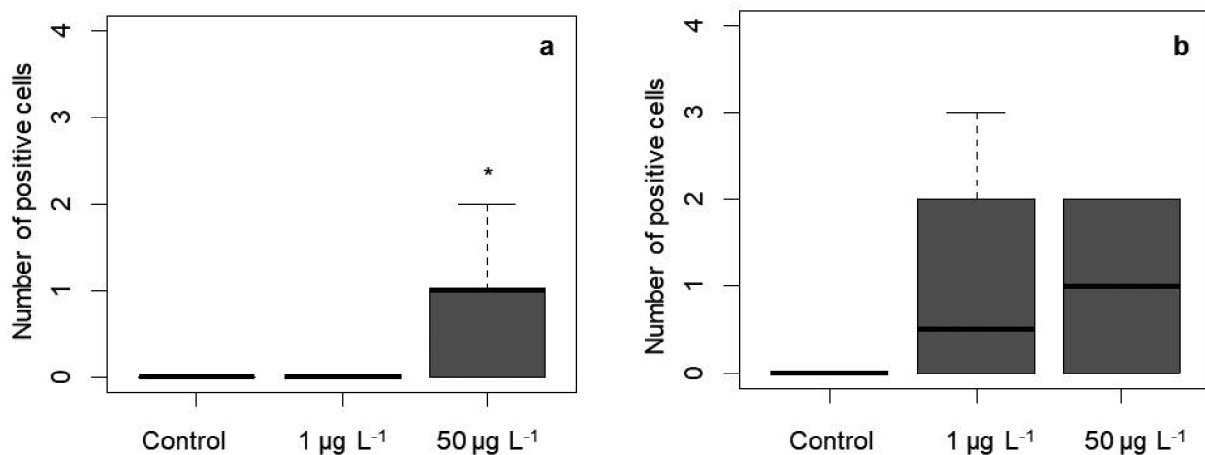
Figure F.3. *Graphical representation of the number of positive caspase-3 in hepatocytes of brown trout after 35 days of methamphetamine exposure (Time 35), and after 4 day-depuration (Time 39) (n = 6). Kruskal-Wallis test, asterisk corresponds to significance differences compared to the control group (p <0.05).*

SHORT COMMUNICATION

Methamphetamine pollution elicits addiction in wild fish

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ABSTRACT

Illicit drug abuse presents pervasive adverse consequences for human societies around the world. Illicit drug consumption also plays an unexpected role in contamination of aquatic ecosystems that receive wastewater discharges. Here, we show that methamphetamine, considered as one of the most important global health threats, causes addiction and behavior alteration of brown trout *Salmo trutta* at environmentally relevant concentrations (1 µg l^{-1}). Altered movement behavior and preference for methamphetamine during withdrawal were linked to drug residues in fish brain tissues and accompanied by brain metabolome changes. Our results suggest that emission of illicit drugs into freshwater ecosystems causes addiction in fish and modifies habitat preferences with unexpected adverse consequences of relevance at the individual and population levels. As such, our study identifies transmission of human societal problems to aquatic ecosystems.

KEY WORDS: Behavior, Brain metabolome, Drug residues in brain, Withdrawal

INTRODUCTION

Illicit drug abuse is widely acknowledged as a global public health challenge that elicits profound societal costs, including financial burdens of hundreds of billions of dollars each year in the USA alone (NIDA, 2010). Users of illicit drugs indirectly introduce these drugs into surface waters following excretion to sewage collection systems and discharge from wastewater treatment plants, because these systems were not designed to treat such contamination (Ort et al., 2014). Other contaminants of emerging concern, including prescription medicines and other consumer chemicals, are similarly introduced into surface waters with the potential to alter the physiology and behavior of aquatic organisms at relatively low levels (Brodin et al., 2013). Unfortunately, whether illicit drugs alter fish behavior at levels increasingly observed in surface water bodies is not known.

Though amphetamines and methamphetamines could be used to treat various diseases including bipolar disorder, abuse and addiction potential limit their usage during psychiatric drug therapies

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(Perugi et al., 2017). In fact, amphetamine-type drug consumption is dramatically increasing, so much so that methamphetamine addiction is now considered one of the most important global health threats (UNODC, 2017). Such global abuse of methamphetamine translates to surface water contamination worldwide (Xu et al., 2017). In some parts of Europe, methamphetamine use is elevated; for example, sewage- (or wastewater-) based epidemiology studies of illicit drugs in raw sewage identified relatively high consumption in regions of the Czech and Slovak Republics (Ort et al., 2014). Consequently, methamphetamine was previously observed in surface waters of the Czech Republic at levels of hundreds of nanograms per liter (Koba et al., 2018).

Fish are sensitive to adverse effects of many neurologically active drugs from alcohol to cocaine and are employed as model organisms to study nervous system disorders (Collier et al., 2014). Though behavioral perturbations by neurologically active contaminants may have fundamentally important consequences to individual-, population- and community-level dynamics (Saaristo et al., 2018), behavioral response variables are rarely employed during environmental assessments (Ågerstrand et al., 2020). However, Ford et al. (2021) recently provided consensus perspectives and recommendations to advance behavioral ecotoxicology interfaces between the basic and translational sciences. These recommendations are particularly relevant for illicit drugs because information on their potential behavioral impacts in aquatic ecosystems are poorly understood.

Because fish can develop drug addiction such as behavioral dependencies related to the dopamine reward pathway in a similar manner to humans (Bossé and Peterson, 2017), we tested whether fish exposed to environmentally relevant methamphetamine concentrations show signs of addiction during withdrawal. We then examined potential mechanisms of addiction by identifying the extent of methamphetamine and its metabolite amphetamine presence in brain tissues of brown trout, *Salmo trutta*. Brown trout is a globally important species that is native primarily in Europe with a range extending to western Asia and North Africa (MacCrimmon et al., 1970) but with naturalized populations on all continents except for Antarctica (Elliott, 1994). Furthermore, brown trout has been employed as a model species in toxicology (e.g. Luckenbach et al., 2001). Thus, the results obtained in the present study are broadly relevant to numerous ecosystems.

MATERIALS AND METHODS
Experimental animals

All laboratory experimental procedures complied with appropriate animal welfare regulations (Law no. 246/1992, § 19, article 1, letter c), which were derived from Directive 2010/63/EU; the permit was awarded to O. Slavík, qualified according to Law no. 246/1992, § 17, article 1: permit no. CZ02233. All laboratory procedures were performed with relevant permission from the Departmental Expert Committee for authorization of experimental projects of the Ministry of Education, Youth and Sports of the Czech Republic

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(permit no. MSMT – 1972/2016-5). Fish used for experimentation were hatchery-reared juvenile brown trout, *Salmo trutta* Linnaeus 1758, obtained from a local fish supplier that was verified as uncontaminated (Czech Fishery Ltd). A total of 120 similar-sized fish of the same age (1 year; mean standard length 117 mm) were transported from the hatchery to the laboratory and were kept in two separate holding tanks (350 l, each with 60 randomly distributed organisms) with aeration for 2 weeks prior to the start of the experiment. Fish were fed *ad libitum* food pellets (Biomar Ltd) once a day (except on days when behavioral assays were performed) and were kept under the natural photoperiod (i.e. daylight varied from 13 to 14 h), thus maintaining the same regime to which they were accustomed in the hatchery. Three-quarters of the water volume was renewed with aged tap water filtered through activated charcoal every other day. Mean water quality parameters were as follows: pH 7.2, NH_4^+ <0.05 mg l^{-1}, NO_3^- 7.08 mg l^{-1}, NO_2^- <0.04 mg l^{-1}, PO_4^{3-} <0.05 mg l^{-1}, chemical oxygen demand by manganese (CHSK$_{Mn}$) 1.1 mg l^{-1}, Cl$^-$ 8.9 mg l^{-1}, $\Sigma Ca^{2+}+Mg^{2+}$ 1.00 mmol l^{-1}, Ca^{2+} 34.1 mg l^{-1}. Water temperature was controlled automatically and held at a mean (±s.d.) of 17.6±0.2°C throughout the entire experiment.

Methamphetamine study

Following a 2 week acclimation period, 60 fish in one holding tank were nominally exposed to methamphetamine (Sigma-Aldrich, Steinheim, Germany) at the environmentally relevant concentration of 1 µg l^{-1} for 8 weeks. Because methamphetamine affects fish condition after at least 3 weeks of exposure (Hubená et al., 2020), we employed a period of 8 weeks to examine longer-term chronic conditions that may be expected in lotic systems continuously receiving municipal effluent discharge. A nominal concentration of 1 µg l^{-1} was selected as an intermediate methamphetamine treatment level between lower (tens or hundreds of nanograms per liter; e.g. Lin et al., 2010) and higher (25 µg l^{-1}; Paciuszkiewicz et al., 2019) levels reported in surface waters around the world. All environmental variables (i.e. temperature, photoperiod, food) were consistent with the acclimatization period. Two-thirds of the water volume was renewed with aged tap water filtered through activated charcoal every other day. No significant pH differences were detected between the treatment and control (mean pH 7.2 versus 7.17; *P*>0.11, *n*=56). Methamphetamine was added during every water renewal in order to maintain its concentration in the tank at the required level. Sixty negative control fish were kept under the same regime in a separate 350 l tank without methamphetamine. Methamphetamine concentrations in these two holding tanks were analytically determined 10 times during the 8 week study to verify exposure conditions. The mean (±s.d.) level of methamphetamine in aquaria with exposed fish was 1.2±0.4 µg l^{-1} (*n*=10) and the concentration in the control tank was below our limit of quantification (<0.023 µg l^{-1}).

Behavioral experimental design

Behavioral observations were conducted in a two-current choice flume for examining preference of aquatic animals (Fig. 1) that was designed according to Jutfelt et al. (2017). Two separate tanks of 100 l volume were used to feed the system by gravity. Baffle plates, fine mesh and honeycomb collimators were designed to create two separate currents of laminar flow in the following choice arena (40×40 cm; volume of water ca. 30 l). The choice arena was free of any obstacles, allowing fish to choose freely between control and methamphetamine-contaminated areas. Methamphetamine levels in this choice arena were maintained at the same environmental concentration (1.2±0.4 µg l^{-1}) as during the 8 week exposure

period. The methamphetamine-dosed part of the observation arena (left or right) and fish treatment (of either control or previously exposed individuals) were regularly rotated in order to randomize experimental observations.

After completing the 8 week experiment, fish were transferred to clean water to initiate depuration for 10 days. We then investigated the behavior of 8 randomly selected fish from the control and methamphetamine-treated tanks beginning 48 h after completion of the 8 week study (Cachat et al., 2010) and then every 48 h thereafter during the 10 day depuration period. Behavioral observations over this time period were intended to simulate 'withdrawal' following methamphetamine exposure. Thus, 5 separate trials (on the 2nd, 4th, 6th, 8th and 10th day of withdrawal) with 80 total specimens (40 control, 40 treated) were conducted. Every specimen was placed in the choice arena separately and its behavior was subsequently recorded for 10 min using a GoPro HERO digital camera (GoPro Inc.) placed above the arena. The flume was emptied and rinsed thoroughly between observations with each fish, and the order of control and treated specimens was changed regularly (i.e. first control, first treated, second control, second treated, etc.). Immediately after each behavioral observation, individual fish were measured (standard length mean 117 mm, range 88–146 mm), weighed (mean 23 g, range 9–41 g) and killed by cervical dislocation, followed by exsanguination (method approved by the valid legislative regulations; law no. 246/1992, § 17). No significant size differences were detected between the two groups (control, treated) of fish (standard length *P*>0.3, *n*=80; mass *P*>0.59, *n*=80). Brain tissues were dissected from freshly killed fish, weighed and stored frozen at −20°C for subsequent methamphetamine and amphetamine analyses. Before the analysis, brain samples were defrosted and extracted according to procedures described in Grabicová et al. (2018). Tissue aliquots were analyzed using liquid chromatography with high resolution mass spectrometry (QExactive, Thermo Fisher Scientific). High resolution product scan (HRPS) was used for quantitative analysis of methamphetamine and amphetamine (i.e. targeted analyses), while full scan data (100–800 *m/z* range) in both positive and negative electrospray ionization modes were acquired for consequent metabolomics in another LC-HRMS run for non-targeted analyses.

Data analyses

To address an obvious M-dependence structure hidden within the data, mainly caused by multiple records per fish within a short time period, we employed a regular 15 s grid approach to reduce the number of observations to 40 records per fish (i.e. 4 records per minute). These reduced data formed a regular and balanced longitudinal profile while distinguishing for trial repetitions. The 'fish position' values within the choice arena were binomial and varied from 0 (i.e. preference for the control part) to 1 (i.e. preference for the methamphetamine-dosed part). The fish position values that were used to define a binomial variable 'probability of methamphetamine source preference' included just the data from the last minute of the observation (i.e. 4 records per fish). Thus, we used data based on the final individual decision, without accounting for the prior potential exploration and habituation effects. Fish movement values within the choice arena varied from 0 (i.e. fish was stationary) to 1 (i.e. fish was moving) and were used to define a binomial 'probability of movement' variable. Fish movement values from the whole 10 min observation period were entered in the analyses, accounting for the exploration and habituation effects in this variable. 'Amphetamine in brain tissue' and 'methamphetamine in brain tissue' variables were defined as binomial variables with 1
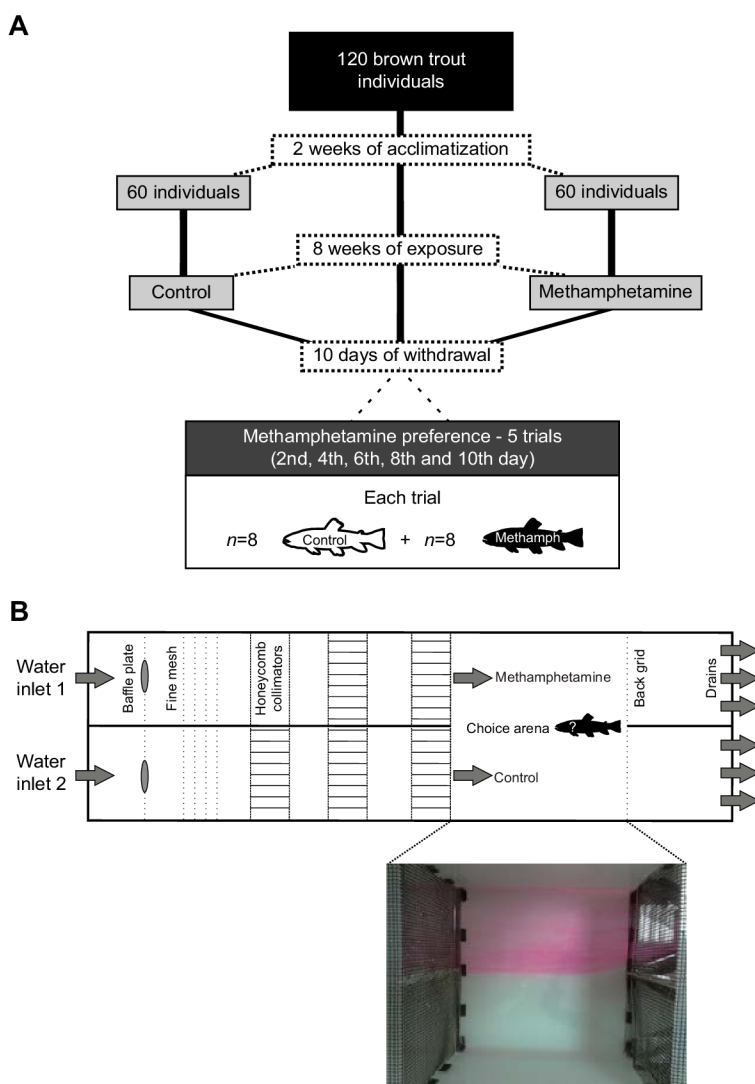
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A

B

Fig. 1. Experimental testing. (A) Outline of the experiment. (B) Illustration of the two-current choice flume. Water from two different tanks fed the choice flume by gravity. Water flow through the baffle plates, fine mesh and honeycomb collimators creates two laminar, non-mixing currents in the choice arena as can be seen in the illustrative photo during the purple dye test.

indicating presence of particular substance. 'Treatment' was defined as the class variable distinguishing whether fish were previously exposed to methamphetamine or not. Class variable 'trial' defined the order of the experiment based on the day of the simulated withdrawal period (2nd, 4th, 6th, 8th and 10th day).

Statistical analyses

Statistical analyses were performed using the SAS software package (SAS Institute Inc., version 9.4, www.sas.com). The binomial dependent variables 'probability of methamphetamine source preference', 'probability of movement' and 'methamphetamine in brain tissue' were analyzed using mixed models with random factors (PROC GLIMMIX with binomial distribution and logit link). Two models for dependent variables 'probability of methamphetamine source preference' and 'probability of movement' were fitted because of the 'treatment' and 'amphetamine in brain tissue' variables overlap (intercorrelation). Thus, the first model contained 'treatment' and the interaction between 'treatment' and 'trial' fixed factor variables,

while the second model included 'amphetamine in brain tissue' and 'methamphetamine in brain tissue' fixed factor variables. An additional model for dependent variable 'methamphetamine in brain tissue' was fitted in order to determine whether the presence of methamphetamine in brain tissue was dependent on 'fish position' or not. Random factors were used to account for the repeated measures collected for the same experimental units (individual fish) across the duration of the experiment. Whether exploratory variables were significant was assessed using an F-test. Least-squares means (henceforth referred to as 'adjusted means') were subsequently computed for particular classes. Differences between the classes were tested with a t-test, and a Tukey–Kramer adjustment was used for multiple comparisons. Degrees of freedom were calculated using the Kenward–Roger method.

LC-HRMS data evaluation

LC-ESIpos HRMS full scan data were processed using Compound Discoverer 2.0 software (Thermo Fisher Scientific). Blank samples

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were used to filter out all possible interference from the solvents we used and the chromatographic system. All chromatograms were aligned with a retention time tolerance of 0.2 min. We used 5 ppm mass tolerance through the entire workflow. We attributed data files with categorical variables that were used later for filtering groups from the dataset. Controls were set as control while exposed fish were set as sample. The categorical variable depuration time was used to separate corresponding sample and control groups. We set diagnostic ratios for differential analysis as follows: sample to corresponding control for each day of experiment, all controls to control 2nd day and all samples to sample 2nd day of depuration. Differential analysis resulted in plots showing statistically significant relationships as P-values and differences as log fold-change. We set criterions of significant difference as $P<0.05$ and log fold-change >1. Consequently, only significantly different signals from exposed fish on day 2 were selected and assumed as markers of methamphetamine effect. All other signals were filtered out from all studied groups and then principle components analysis was performed to reveal whether selected markers can be used as a tracers of persisting methamphetamine effects during withdrawal.

RESULTS AND DISCUSSION

We observed control fish to cross from one side of the choice arena to the other during 21.6% (347 of 1600) of all observations. These controls spent 41.5% (665 of 1600) of all observations in the methamphetamine-dosed part of the arena. Similarly, we observed previously exposed fish to cross from one side of the choice arena to the other during 21.1% (339 of 1600) of observations, but these animals were in the methamphetamine-dosed part of the choice arena in a higher number of observations (50.5%, or 809 of 1600). Following 56 days of exposure (1 µg l^{-1}), preference for methamphetamine during a simulated withdrawal period was considered an indicator of addiction. These brown trout showed higher probability of methamphetamine source preference compared with controls ($F_{1, 306}=28.02$, $P<0.0001$; Fig. 2A), and this difference was apparent for the first 4 days of depuration following treatment ($F_{8, 306}=2.18$, $P=0.029$; Fig. 2B). Such methamphetamine preference was positively correlated with levels of amphetamine residues in fish brains ($F_{1, 314}=18.56$, $P<0.0001$; Fig. 2C), suggesting that addiction is linked to the presence of this drug metabolite in nervous system tissue. Amphetamine was only identified in brain tissue of exposed trout and its presence decreased from 100% to 12.5% of individuals throughout the 10 day depuration period (Fig. S1A).

Exposed brown trout displayed a lower probability of movement than controls during the withdrawal period ($F_{1,3190}=4.94$, $P=0.0263$; Fig. 3A). This behavioral modification was also observed until the 4th day of depuration ($F_{8,3190}=7.18$, $P<0.0001$; Fig. 3B) and significantly correlated with amphetamine in brain tissue ($F_{1,3157}=6.47$, $P=0.011$; Fig. 3C). However, the opposite effect of movement increase was observed when methamphetamine was found in fish brain ($F_{1,3075}=42.96$, $P<0.0001$; Fig. 3D). Methamphetamine was observed in brains of individuals that occurred more frequently in the dosed part of the observation arena ($F_{1, 3158}=51.00$, $P<0.0001$; Fig. S1B), suggesting that methamphetamine presence in the brain resulted from acute drug intake.

Biochemical changes in fish brains were revealed using differential non-target analysis of LC-HRMS data. Significant differences (both up- and down-regulated signal intensities) between control fish and those experiencing the withdrawal period during depuration gradually decreased from 210 signals
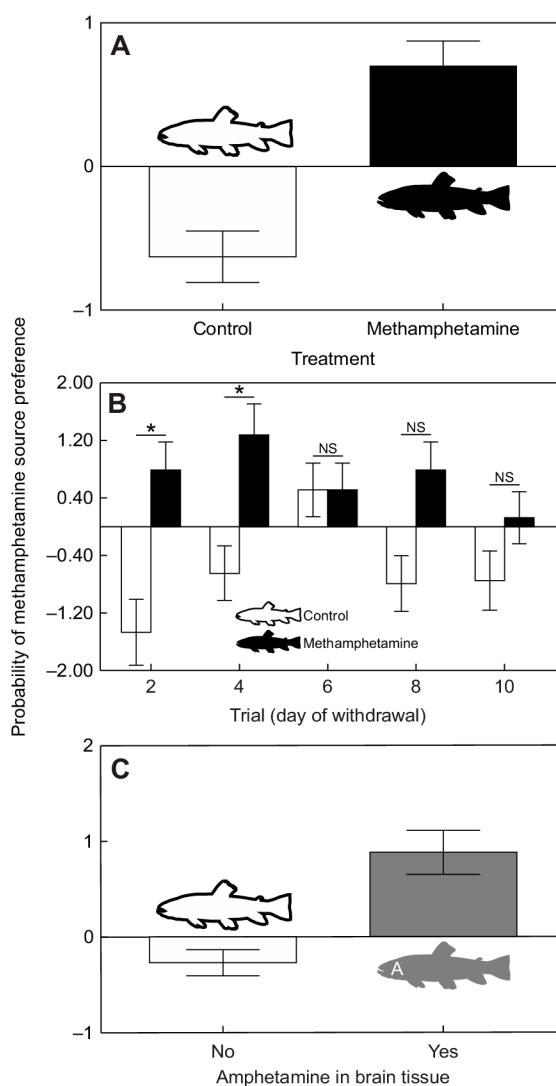

Fig. 2. Brown trout methamphetamine source preference. Probability of methamphetamine source preference (A) in relation to previous treatment, (B) across trials, i.e. days of withdrawal and (C) for amphetamine in brain tissue of focal individuals. Values are adjusted means±s.e. based on a mixed model with random factor analyses (PROC GLIMMIX; significance was assessed using an F-test; $P<0.05$). Five separate trials (on the 2nd, 4th, 6th, 8th and 10th day of withdrawal) with 80 total specimens (40 control, 40 treated) were conducted. Estimates could be negative because of use of the logit link, where probabilities between 0 and 1 are specified as logits, $\ln[p/(1-p)]$, which can be less than 0 when the estimated probability is less than 0.50. Differences among classes in particular figure parts are significant (*adjusted $P<0.0001$).

(substances) during the 2nd day of depuration (Fig. S2A) to 36 substances during the 10th day (Fig. S2B). These novel markers of methamphetamine exposure were consequently applied for description (principle component analysis where variables were revealed markers only) of changes in brain metabolomes across all experimental groups. Similar to our observations of brain tissue residues, differences in overall brain metabolites were significant
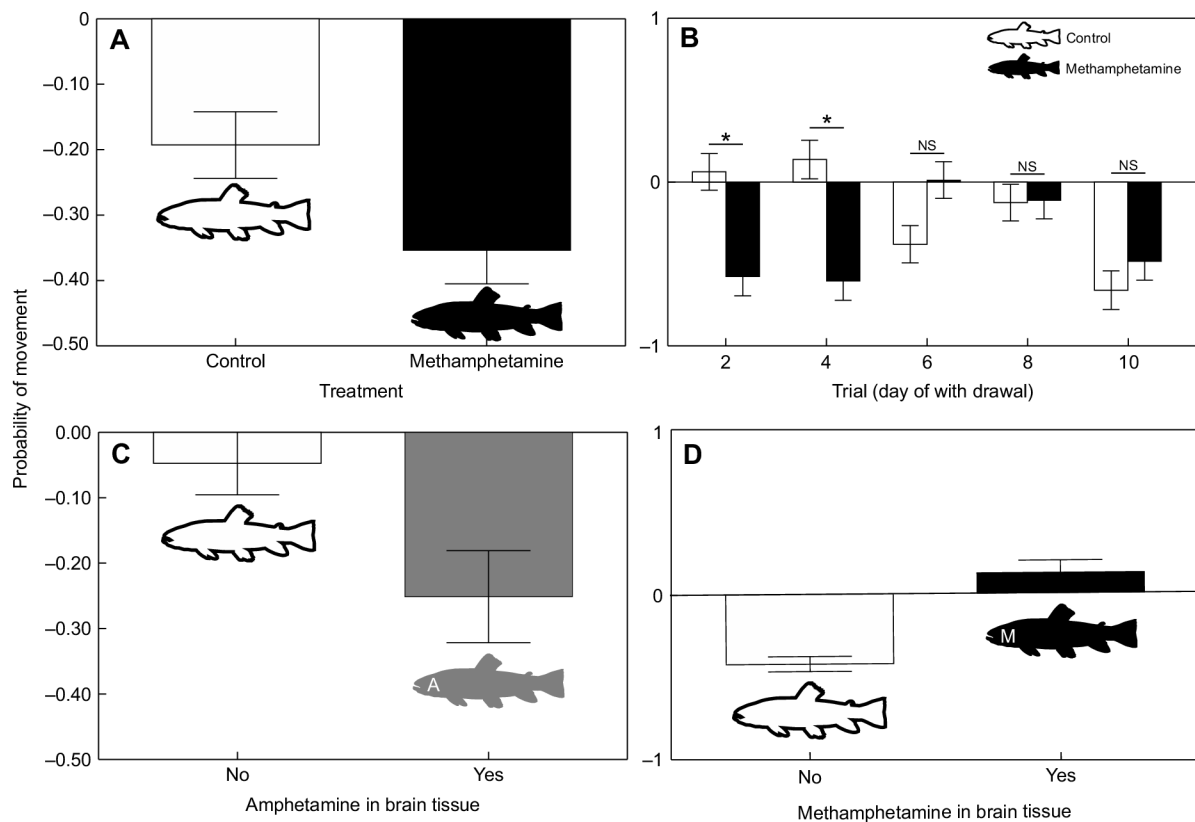
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Fig. 3. Brown trout movement. Probability of movement (A) in relation to previous treatment, (B) across trials, i.e. days of withdrawal (B), and for (C) amphetamine and (D) methamphetamine in brain tissue of focal individuals. Values are adjusted means±s.e. based on a mixed model with random factor analyses (PROC GLIMMIX; significance was assessed using an *F*-test; $P<0.05$). Five separate trials (on the 2nd, 4th, 6th, 8th and 10th day of withdrawal) with 80 total specimens (40 control, 40 treated) were conducted. Estimates could be negative because of use of the logit link, where probabilities between 0 and 1 are specified as logits, $\ln[p/(1-p)]$, which can be less than 0 when the estimated probability is less than 0.50. Differences among classes within a panel are significant (*adjusted $P<0.01$).

until the 4th day of depuration and then leveled out thereafter (Fig. S3).

Intense physical and psychological manifestations triggered by withdrawal from a drug are considered major signs of addiction and are further suggested to stimulate drug-seeking behavior in humans (Piper, 2015). We found that brown trout withdrawn from waterborne (or inhalational) exposure to an environmentally relevant level of methamphetamine displayed similar drug-seeking behavior. Fish preference for a drug is often dependent on the dopamine pathway (Bretaud et al., 2007), confirming that fish can display signs of addiction and withdrawal symptoms (Tran et al., 2015).

Withdrawal symptoms include increases in anxiety and stress (Piper, 2015). Exposed trout in the present study similarly displayed lower probability of movement, suggesting that their ability to explore a novel environment was reduced as a response to stress caused by methamphetamine withdrawal (Cachat et al., 2010). For example, Bossé and Peterson (2017) found that reduction of fish exploration rate indicated suffering from withdrawal symptoms without drug access.

Our observations of withdrawal symptoms in brown trout were significantly related to the presence of the methamphetamine metabolite amphetamine in fish brain. These withdrawal behaviors

were prevalent for 96 h and then disappeared as the rate of amphetamine-positive individuals decreased. Similar observations have been made in time course studies of methamphetamine withdrawal in humans, with an initial peak within the first 24 h and subsequent decline to near-control levels by the end of the first week of abstinence (McGregor et al., 2005). Despite this fact, amphetamine was found in the brain of one trout specimen after the 10 days, suggesting differential metabolism among individuals (Metcalfe et al., 2016) can influence the internal dose with related behavioral consequences. Voluntary methamphetamine intake during the preference test led to a detection of methamphetamine in the brains of individual fish. The voluntary drug intake is highly individual in animals, depending on factors such as dominance or previous social disturbance, as shown by Wolffgramm and Heyne (1995). We found that methamphetamine in fish brain significantly increased its activity. Such performance-enhancing stimulant effects are not unexpected; for example, methamphetamine was strategically used to elicit such effects under the brand name Pervitin by the German army during World War II (Defalque and Wright, 2011).

Our results also indicate that environmental concentrations of methamphetamine alter fish brain metabolomes. Effects of drugs of abuse on brain metabolic activity have previously been observed in

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humans, with methamphetamine-specific decreases in dopamine transporters within the striatum (Volkow et al., 2001). Using non-target analyses, we found that 36 endogenous molecules in the brain metabolome of exposed fish differed after the 10th day of withdrawal, while Volkow et al. (2001) found that the dopamine transporter was reduced in the striatum even after 11 months, suggesting that adverse effects of methamphetamine exposure could be long lasting in fish.

In conclusion, observations in the present study suggest that fish exposed to environmental concentrations of methamphetamine in surface waters will develop addiction and be attracted to reside near wastewater treatment effluent discharges. The wastewater effluents are often nutrient rich, offering additional bioenergetic incentives for fish attraction to outfall mixing zones. Such unnatural attraction to one area together with documented changes in behavior could result in unexpected ecological consequences influencing whole ecosystems (Boulêtreau et al., 2011). Furthermore, drug reward cravings by fish could overshadow natural rewards such as foraging or mating that provision homeostatic and reproductive success (Hyman et al., 2006) and further reinforce adverse ecological consequences of pollutants in aquatic environments (Prokkola and Nikinmaa, 2018). The elicitation of drug addiction in wild fish could represent another example of unexpected evolutionary selection pressure for species living in urban environments (Johnson and Munshi-South, 2017) along with ecological side effects of human societal problems within aquatic ecosystems. Further field research is needed to examine the withdrawal effects of methamphetamine observed in this experimental study under mesocosm conditions and natural ecosystems.

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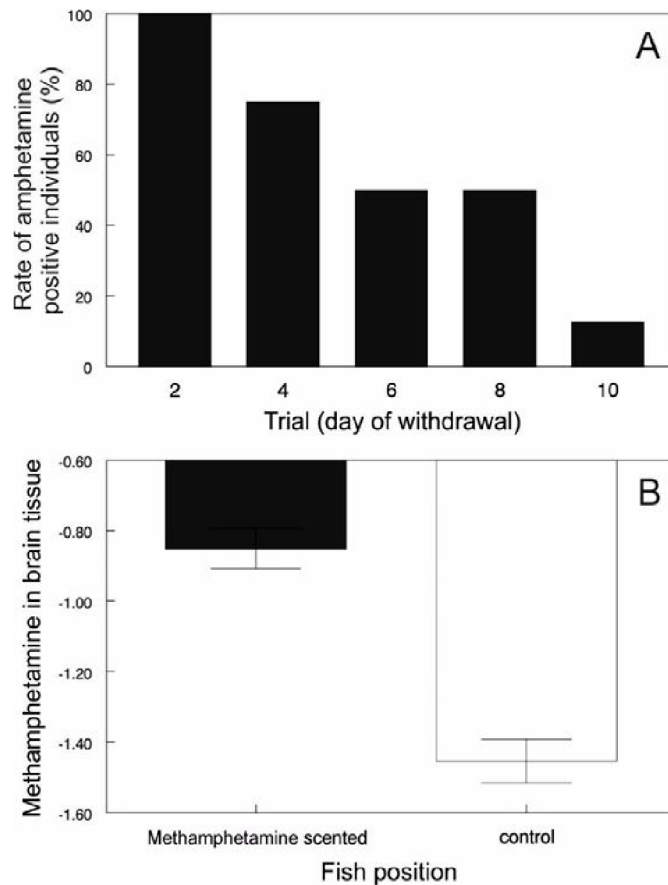
Fig. S1. Amphetamine and methamphetamine in brain tissue.

Rate of amphetamine positive brown trout specimen across the days of withdrawal (A) and probability of methamphetamine in brain tissue occurrence in relation to fish position during the preference test (B). The raw data in the every single trial in part A originates from the 8 previously exposed fish (i.e. rate 100% means 8 amphetamine positive fish). Values in part B are adjusted means ± SE based on mixed model with random factor analyses (PROC GLIMMIX; significance was assessed using an F-test; $P < 0.05$). Five separate trials (on the 2nd, 4th, 6th, 8th and 10th day of withdrawal) with 80 total specimens (40 control, 40 treated) were conducted. Estimates could be negative because of use of the logit link, where probabilities between 0 and 1 are specified as logits, $\ln(p/(1-p))$, which can be less than zero when the estimated probability is less than 0.50. Differences among classes in part B are significant (Adj. $P < 0.0001$).
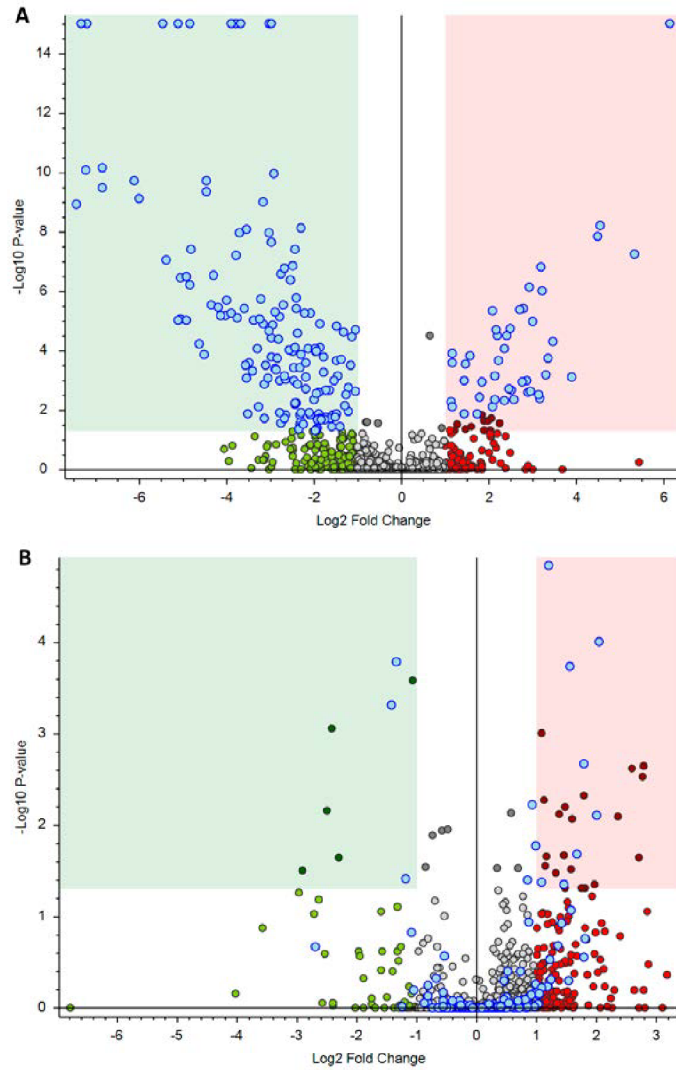
Fig. S2. Differential analysis of brain extracts.

Results of differential analysis of brain extracts between control and withdrawn fish groups in the 2nd day (A) and 10th day (B) of depuration. Colored squares (green down regulated and red up regulated) represent differences between sample and control groups at statistical significance of P<0.05 and at least one fold of magnitude difference in signal intensity. Light blue dots represent up and down regulated signal in exposed compare to control on 2nd observation day in both figures.
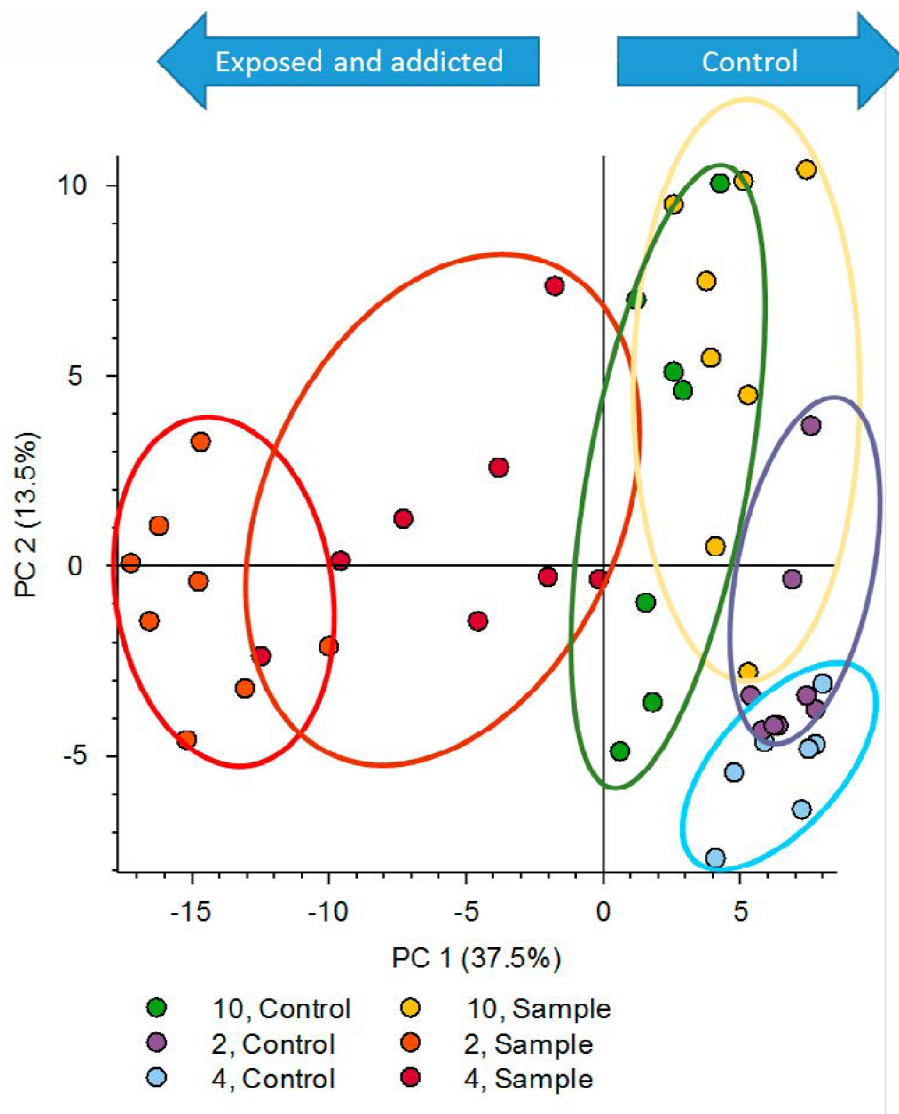
Fig. S3. Brain metabolites.

Principal component analyses of fish individuals from both control and exposed groups from 2nd, 4th and 10th day of depuration. Only significantly different signals (both up and down regulated with intensity difference at least one fold of magnitude and statistical significance at $p < 0.05$) from 2nd day were used as effect markers.

CHAPTER 3

AN INSIGHT INTO TRAMADOL

My share in this work was about 40%

Traces of tramadol in water impact behaviour in a native European fish

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ARTICLE INFO

ABSTRACT

Tramadol is a widely used analgesic with additional antidepressant and anxiolytic effects. This compound has been reported in continental waters reaching concentrations of μg/L as a consequence of its inefficient removal in sewage treatment plants and increasing use over time. In this study, European chubs (*Squalius cephalus*) were exposed to 1 μg/L of tramadol in water for 42 days with a subsequent 14 days of depuration. Our results revealed that chubs exposed to this analgesic underwent changes in their behaviour as compared to the control group. The behavioural outcome was also influenced by the individual concentration of tramadol in brain tissue. In particular, experimental fish presented anxiolytic-like effects, characterized by less bold and less social individuals. Exposed animals were less frequently out of the shelter and moved a shorter distance, indicating that they explored the new environment less during the boldness test. In the novel object recognition experiment, although they distinguished the new item, they examined it less and displayed a reduced activity. Shoal cohesion was disrupted as observed in an increased distance between individuals. After the depuration phase, this alteration remained whereas the boldness effect disappeared. Moreover, the degree of behavioural changes was correlated with the concentration of the substance in brain. According to our findings, chronic presence of tramadol in the environment can impact the fitness of exposed aquatic fauna by altering evolutionary crucial behaviours.

1. Introduction

Pharmaceuticals are emerging contaminants within freshwaters given their potential impacts over wildlife. Psychopharmaceuticals and their active metabolites are increasingly detected in waters ranging in concentrations from nanograms (ng) to micrograms (μg) per litre on account of their insufficient removal from the sewage treatment plants (STPs) (Asimakopoulos and Kannan, 2016; Ebele et al., 2017; Golovko et al., 2014).

Tramadol is a synthetic opioid that exerts its analgesic action over the central nervous system. It possesses a double mode of action, being a μ-opioid receptor agonist and as an inhibitor of norepinephrine and serotonin reuptake in the synaptic terminals (Miotto et al., 2017). For this reason, it is used as a painkiller for acute and chronic pain, as well as an anaesthetic, anxiolytic and antidepressant (Vazzana et al., 2015). Chronic pain is a frequent issue in daily medicine Its management is constrained to limited treatment options that results in an over

prescription of tramadol. For instance, prescriptions of tramadol in the United States rose 22.8% from 2012 to 2015 (Bigal et al., 2019). The use of tramadol in combination with non-opioid analgesics increased from 1.8 to 5.3 DDD (defined daily dose per 1000 inhabitants per day) in the period from 2008 to 2015 in Spain (AEMPS and Ministerio de Sanidad, 2017). In the Czech Republic, the rate increased from 5.3 to 9.5 DDD during the years 2008–2019 (State Institute for Drug Control, n.d). In addition, an illegal trade of this pharmaceutical has been reported due to its potential for abuse (Miotto et al., 2017). In 2010, 2013 and 2017, global seizures of tramadol were 10 kg, 9 tons and 125 tons, respectively (United Nations Office on Drugs and Crime, 2019).

Tramadol's both extensive use and low rate of removal in STPs contribute to the observed high concentrations, up to 1 μg/L, in effluents and surface waters (Campos-Mañas et al., 2019; Coelho et al., 2019; Fedorova et al., 2014; Golovko et al., 2014; Grabicova et al., 2017; Loos et al., 2013). This substance was even detected at low levels in seawater (Alygizakis et al., 2016). Furthermore, tramadol was present in several

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tissues of fish living in ponds or in STPs effluent impacted streams (Grabicova et al., 2017; Grabicová et al., 2020).

The neuroendocrine pathways, such as monoaminergic and opioid systems, are evolutionary well-conserved in teleosts (Dreborg et al., 2008; Soares et al., 2018). Consequently, even low levels of chronic exposure to psychoactive compounds can impact neurotransmitter systems and produce similar effects as human and experimental animals in non-targeted organisms (Fent et al., 2006). Behavioural responses correlate to these complex physiological processes (Saaristo et al., 2019). Thus, they can be analysed to better understand the complex effects psychoactive compounds can exert at individual and ecosystem levels (Brodin et al., 2014).

Several studies have elucidated behavioural effects of tramadol in aquatic invertebrates. Impairments in feeding behaviour of dragonfly larvae (*A. cyanea*) were observed from 1 µg/L of tramadol for 7 days (Bláha et al., 2019). Marbled crayfish (*P. virginalis*) spent more time in shelters and diminished locomotion from 1 µg/L of the analgesic for 7 days (Buřič et al., 2018). Red swamp crayfish (*P. clarkii*) exposed to the aforementioned conditions showed a sex-dependent change in burrowing behaviour (Guo et al., 2020). Ložek et al. (2019) detected changes in locomotion and heart rate in signal crayfish (*P. leniusculus*) exposed to 1 µg/L of the analgesic for 21 days. Douda et al. (2019) recorded a slightly different parasitic interaction between chub and glochidia (larvae of *A. anatina*), both previously exposed to tramadol at 1 µg/L for 42 days and 24 h, respectively.

In the case of fish, early stage tests were performed in common carp (*C. carpio*) using concentrations of 10–200 µg/L tramadol in a mixture with naproxen for 32 days, reporting several disruptions in their development (Sehonova et al., 2017). Similarly, a delay of hatching in zebrafish (*D. rerio*) and impairments in development of common carp were observed in Sehonova et al. (2016). Bachour et al. (2020) recorded an anxiolytic effect in the early-stages of zebrafish at 320 µg/L. Zebrafish larvae exposed to 1–100 µg/L exhibited slightly decreased activity, although not significant (Huang et al., 2019). Tanoue et al. (2017) reported very similar metabolism of this pharmaceutical in fish compared to humans. Then, Tanoue et al. (2019) aimed to explain behavioural changes in fathead minnows (*P. promelas*), using concentrations from 1 µ to 100 µg/L during 23–24 days however, the results were ambiguous and difficult to interpret.

Despite the high levels and frequency reported in environmental tramadol, there is still a lack of research about its potential consequences to aquatic wildlife. European chub (*Squalius cephalus*) is a small cyprinid chosen as an experimental fish for being widely distributed in European freshwaters. It has been successfully used as a bioindicator (Douda et al., 2019; Hájková et al., 2007) and as a model for behavioural research (Hubená and Horký, 2020a, 2020b). European chub is easily reared and maintained under laboratory conditions. This study aimed to reveal currently unidentified effects on European chubs that are exposed to an environmentally relevant concentration of 1 µg/L of tramadol. For this purpose, we (1) monitored the progression of the behavioural changes over time and (2) related the concentration of tramadol in brain tissue with the observed variations in the basic behavioural traits (boldness, activity, exploration, and sociability). A set of three behavioural experiments were conducted after 1, 7, 21, 42 days of exposure, and after 14 days of depuration, i.e. boldness, novel object exploration and shoal cohesion tests. We specifically addressed potential anti-anxiety effects in fish associated with the time-linked concentration of the substance in brain.

2. Material and methods

2.1. Chemicals

The tramadol hydrochloride (purity $\geq$ 98%) used for exposure was obtained from Sigma-Aldrich Corporation (USA) and the isotopically labelled tramadol (D_3), as analytical standard, from Lipomed (USA). The stock solution of pure tramadol (10 mg/L) was prepared with ultra-pure water (AquaMax Basic 360 Series and Ultra 370 Series instrument, Younglin, purchased from Labicom, CR) and stored at 4 °C. From this solution, the exposure treatment of 1 µg/L was added to aged dechlorinated municipal tap water and provided to the respective aquaria. Ultra-pure water and acetonitrile (Merck) and isopropanol (Merck), all acidified with formic acid (Labicom, CR), were utilized as mobile phases for liquid chromatography and as extraction solvents for the biota samples.

2.2. Experimental animals

This study uses the same experimental population as Hubená and Horký (2020a) (2020b) who studied tramadol effect on chub condition. This approach was adopted according to the general need for the reduction of animals utilized in research (e.g. de Boo and Hendriksen, 2005). The experimental fish were hatchery-reared chubs (*Squalius cephalus*, mean weight 5.58 ± 1.24 g, mean standard length 92 ± 7 mm), obtained from a local fish supplier (Czech Fishery Ltd.; Czech Republic). 320 0 + juvenile fish (ten months old) were acclimatized under laboratory conditions for two weeks prior to the start of the experiment. The population was allocated at random in four separate holding tanks, two for control (0 µ/L) and the other half for exposed (1 µ/L), with 80 randomly selected individuals in each one. Fish were maintained in 200 L of aged tap water with constant aeration, fed daily ad libitum on food pellets, and kept on a photoperiod of 12 h of light/12 h of darkness (OECD, 2012). The water temperature was controlled automatically using air conditioning throughout the whole experiment and held at an average of 20.8 ± 0.4 °C (mean ± S.D.). Because tramadol is stable in water, every second day, two-thirds of the water volume were renewed with aged dechlorinated municipal tap water, and fresh stock solution of tramadol was added to reach the final concentration. Fish health, defined as normal appearance and behaviour, including normal body position, movements, and food intake (FAO, 1983) was monitored daily.

2.3. Tramadol exposure

After acclimatization, fish in the experimental groups were separately exposed to the tramadol at an environmentally relevant concentration of 1 µg/L. The treatment lasted 42 days, with a subsequent 14 days of depuration in tramadol-free water (56 days of experiment in total). The tramadol concentration in the holding tanks was checked analytically during the whole experiment to verify the real concentrations in the exposed fish and exclude the possibility of cross-contamination in the control group. Testing days for behavioural analyses and collection of brain samples were set after 1, 7, 21, 42 days of exposure and 14 day-depuration. Four fish per aquaria (a total of eight fish per group) were randomly taken in each sampling day for behavioural and chemical analyses. This study was performed in agreement with the EU-harmonized Animal Welfare Act of the Czech Republic. All laboratory experimental procedures complied with valid legislative regulations (law no. 246/1992, §19, art. 1, letter c) and were carried out with the relevant permission from the Ministry of Education, Youth and Sports of the Czech Republic, Ethical approval committee MSMT-1972/2016-5.

2.4. Behavioural testing

2.4.1. General information

Five trials with 80 focal specimens were conducted altogether. Every specimen was subsequently subjected to a series of behavioural tests with a minimum one hour long resting period between the particular tests. Fish were placed separately into individual aerated tanks between tests (every specimen had its own tank). Digital cameras (GoPro Hero, USA) were used to record fish behaviour from above. Then, obtained data were analysed using the automated video tracking system

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(LoliTrack v. 4, Loligo Systems; please see data analyses section for details).

2.4.2. Boldness test

Individual fish were tested for boldness by recording latency to enter into a novel environment (e.g. Brodin et al., 2013). Fish were inserted into an opaque, white plastic arena (footprint 30 × 15 cm, water depth 10 cm) in an initial shelter. After five min, we remotely opened the door of the refuge. The latency to emerge from the shelter into the novel habitat and the distance moved outside the refuge were recorded for 15 min

2.4.3. Novel object recognition test

Novel object recognition is a highly validated test for identification memory, used predominantly in rodents (Ennaceur and Delacour, 1988), but also in fish (Burns, 2008). It was performed in a square arena (footprint 30 × 30 cm, water depth 10 cm) with opaque, white plastic walls. During trial 1, two identical objects (yellow balls, 4 cm diameter) were located in opposite corners. Each fish was placed individually in the third corner, facing the wall, and allowed to explore for five min. After an inter-trial interval of 15 min, the fish were reintroduced for trial 2 with one familiar object (identical shape and colour but different piece to avoid influence of olfactory cues) and one novel object (black cube of identical size, i.e. side 4 cm) as compared to trial 1. Once again, the fish were allowed to explore, and their behaviour was recorded for five min.

2.4.4. Shoal cohesion

A group of four individuals (1 + 3) was used to score the shoal cohesion. Two identical experimental aquaria (footprint 30 × 15 cm, water depth 20 cm) were placed side by side with the walls covered with a grey paper. The focal individual used in the previous tests was placed in one aquarium, and three familiar conspecifics to the other aquarium, separated by a transparent wall. Conspecifics were matched for the size of the focal individual (the size of the fish was determined visually to prevent actual handling stress, according to Lahti et al., 2002), and all fish were left to acclimate for three minutes. Shoal cohesion was quantified as the distance between the focal individual and the other members of the shoal (Miller and Gerlai, 2007) during a six min interval.

2.5. Chemical analyses

The concentration of tramadol in water was determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS; TSQ Quantiva, heated electrospray in positive mode (HESI+), Accela 1250 LC pump, Hypersil Gold aQ column (50 × 2.1 mm; 5 μm particles), PAL autosampler, Thermo Fisher Scientific, USA) in all aquaria four times during the experiment. Water samples were taken before and after the water was changed, and stored at − 20 °C. After defrosting, the water obtained from the aquaria was filtered (0.20 μm regenerated cellulose filter) and internal standard (D_3-tramadol, Lipomed) was added. Then, the sample was analysed in gradient elution of acidified water and acetonitrile (with formic acid). The complete procedure is specified in Douda et al. (2019). The mean concentration of tramadol in water in the treated tanks was 0.99 ± 0.18 μg/L (mean ± S.D.) during exposure. The depuration phase and during the whole experiment in control remained below the limit of quantification (LOQ; Hubená and Horký, 2020a, 2020b). LOQs ranged from 0.030 to 0.060 μg/L for tramadol (Hubená and Horký, 2020a, 2020b).

Following behavioural testing, eight fish per group and time were beheaded according to valid law 246/1992, §17. Their brains were dissected, weighted, and stored at − 80 °C until further analyses. The complete method of sample preparation and analysis is described in Grabicova et al. (2018). After defrosting, internal standard, extraction solvent (acidified acetonitrile and isopropanol), and a stainless-steel ball were added to 0.25 g of tissue. The samples were extracted (TissueLyser II, Quiagen, Germany; 30 Hz), centrifuged (Mini spin, Eppendorf,

Germany; 6708 g), filtered (Labicom; regenerated cellulose, 0.45 μm pores) and frozen at − 20 °C for 24 h. After defrosting were centrifuged again. Then, the resulting aliquots were analysed by liquid chromatography with high resolution mass spectrometry (LC-HRMS, QExactive, Accela 1250 LC pump, Hypersil Gold aQ column (50 × 2.1 mm; 5 μm particles) PAL autosampler, Thermo Fisher Scientific). TraceFinder 3.3 software (Thermo Fisher Scientific) was used for the processing of the data. The concentration of tramadol in brain tissue of control individuals was below LOQ (range 0.19–0.69 ng/g; Hubená and Horký, 2020a, 2020b). In the treated group, the compound was detected in all the fish brains at an average of 1.6 ng/g wet weight during exposure, while no residues were found in the brain after depuration (Hubená and Horký, 2020a, 2020b). The Bioconcentration factor was determined by dividing the mean concentration of tramadol in brain tissue by the average concentration in water (OECD, 2012). The calculated bioconcentration factor (BCF) was 1.8 in the present study.

2.6. Statistics

The statistical analyses for behaviour was performed with the SAS software package (SAS Institute Inc., version 9.4, www.sas.com).

2.6.1. Data analyses

'Treatment' was defined as the class variable distinguishing whether fish were exposed to tramadol. 'Experimental phase' was also assigned as a class variable distinguishing sample groups for the course of the experiment. It acquired five values, 1, 7, 21, 42 and 56 days, from the beginning of the experiment. 'Tramadol in brain tissue' expressed the relative concentration of this particular psychoactive compound in the individual fish's brain tissue.

We used a regular time grid approach to address a kind of M-dependence structure hidden within the data (e.g. Moon and Velasco, 2013). The M-dependence refers to dependent structured variables which can be transformed into independent ones by removing some consecutive observations. In our case, M-dependence was caused by several records per second on a video file. Preliminary data analyses comprised a grid for particular variables assigned as follows: 'Boldness' was assigned as a binary class variable (0 – fish in shelter, 1 – fish in an open field) at a sampling rate of once every 5 s 'Distance' was designated as a continuous variable, expressed as a distance moved (in cm) in the open field during the whole boldness test (15 min). 'Social distance' was assigned as a class variable at a sampling rate of once every 10 s and used as an indicator of shoal cohesion. Social distance could acquire values from 0 to 3 (0 – both individuals close to each other, mostly side by side; 1 - individuals within one body length of each other; 2 – individuals separated by at least one body length; 3 – individuals separated on the maximum possible distance – i.e. in opposite corners of the aquarium). 'Exploration' during the novel object test was designated as a count variable expressing number of framerates spent closer than 5 cm near a particular object (novel or familiar) within the 30 s grid. 'Object' was assigned as a class variable acquiring two values, 'novel' or 'familiar'. Every individual was continuously determined as being active (i.e. fish moved between two framerates) or not (i.e. fish did not move between two framerates). 'Activity' during the novel object 'exploration' was assigned as a count variable, expressing the number of active statuses within the 30 s grid.

2.6.2. Statistical analyses

Separate generalized linear mixed models with random factors were fitted to analyse the variables 'boldness'; 'distance'; 'social distance'; 'exploration' of both objects (novel and familiar); 'exploration' of novel object only in order to analyse influence of tramadol in brain tissue on novel object exploration; and 'activity' during the novel object exploration. Mixed models are a generalization of standard models, e.g., GLM, with the generalization indicating that the data are permitted to exhibit correlation and nonconstant variability. This method is used to cope

with repeated measures experiments in which people or animals are the subjects, and the subjects are declared random because they are selected from a larger population in which generalizations are required (SAS Institute Inc, 2004). Therefore, in the present study, factors were applied to account for the repeated measures introduced by using the same experimental units (i.e., individual fish). More detailed information about mixed models can be found elsewhere (e.g. Breslow and Clayton, 1993; SAS Institute Inc, 2004; Searle et al., 1992). Generalized linear mixed models (GLMM; SAS function PROC GLIMMIX) with a Poisson distribution were applied to analyse the variables 'activity' and 'exploration'; GLMM with a multinomial distribution was used to analyse 'social distance', and GLMM with a binary distribution to analyse 'boldness'. GLMM with a normal distribution (SAS function PROC MIXED) was applied to analyse 'distance', squared root transformed to meet normality requirements prior to the analyses. The significance of the explanatory variables (treatment, experimental phase, tramadol in brain tissue, focal individual weight), and their interactions, were assessed using F-tests. Separate models containing 'treatment' or 'tramadol in brain tissue' along with other explanatory variables were fitted due to the 'treatment' - 'tramadol in brain tissue' overlap (intercorrelation), in order to avoid misinterpretation of the results. Least-squares means (henceforth referred to and in bar charts presented as 'adjusted mean' of model predictions) were subsequently computed for the particular classes of class variables. Differences between the classes were determined with t-test, and Tukey–Kramer adjustment was utilized for multiple comparisons. The association between the dependent variables and other continuous variables, 'tramadol in brain tissue' and 'weight', was estimated by fitting a random factor model as described by Tao et al. (2002). With this random coefficient model, we calculated the predicted values for the dependent variable and plotted them against the continuous variables. The degree of freedom was calculated using the Kenward–Roger method (Kenward and Roger, 1997).

For testing cross-trait correlations we used Spearman correlations according to Brodin et al. (2013), and adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Every individual tested during the exposition period (i.e. 1–42 days from the start of the experiment) was assigned one mean value per particular trait (i.e. 'boldness', 'distance', 'exploration', 'activity' and 'social distance'). Cross-trait correlations for various treatments (control and tramadol exposure) were tested separately. Significant relationship indicate existence of a behavioural syndrome between the two individual traits tested.

3. Results

3.1. Behavioural effects of tramadol exposure

Chub exposed to environmental concentrations of tramadol showed significant behavioural differences compared to control. Exposed chubs were generally less bold ($F_{1, 1009} = 5.51$, $p < 0.0191$; Fig. 1A). Both treatments showed a similar temporal pattern in leaving the shelter ($F_{4, 1004} = 22.02$, $p < 0.0001$; Fig. 1B), with nonsignificant differences during particular time intervals. Exposed fish also moved on a shorter distance when they were out of the shelter ($F_{1, 77} = 3.98$, $p < 0.0495$; Fig. 1C), indicating that they did not explore the new environment as thoroughly as in the control treatment.

The novel object recognition test was performed in order to evaluate the possible alterations in the novel item identification memory. Both treatments (tramadol and control) explored the novel object more than the familiar one ($F_{3, 1582} = 8433.79$, $p < 0.0001$; Fig. 2A). Nevertheless, tramadol exposed chubs showed significantly less exploration towards the new item than control fish (Fig. 2A). Exposed individuals were also less active during the novel object exploration ($F_{1, 601.5} = 4.48$, $p < 0.0346$; Fig. 2B). Both the lower novel object exploration and lower activity during exploration indicate an impairment of novel object recognition in tramadol exposed chubs.
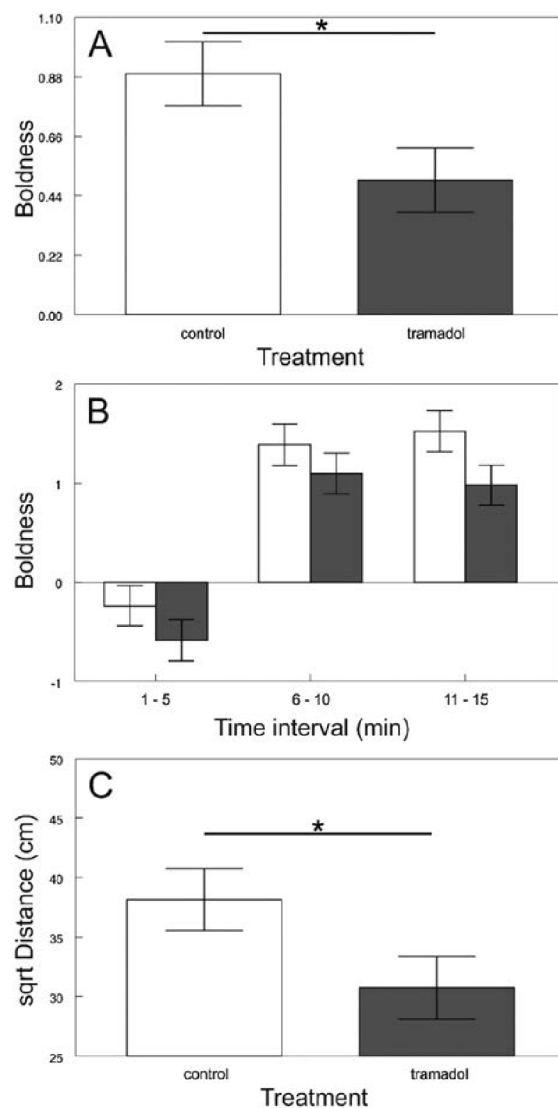

Fig. 1. Boldness across the treatment (A), time interval (B), and distance moved during boldness test across the treatment (C). Values (+/- S.E.) are predicted from the particular mixed model. Asterisks indicate significant differences (Adj. $p < 0.05$).

The shoal cohesion test was performed in order to evaluate the possible effects on social behaviour in exposed fish. Chub exposed to tramadol maintained larger inter-individual social distances within the experimental shoal than controls ($F_{1, 230.1} = 41.77$, $p < 0.0001$; Fig. 3), indicating that their shoal cohesion was disrupted.

3.2. Tramadol exposure and behavioural syndromes

The complex results of cross-trait correlations can be found in Table 1. Boldness was positively related to distance moved in the open field for both treatments. Exploration of the novel object and activity during its exploration were positively correlated under the tramadol treatment only, while exploration and boldness were positively correlated exclusively in control.
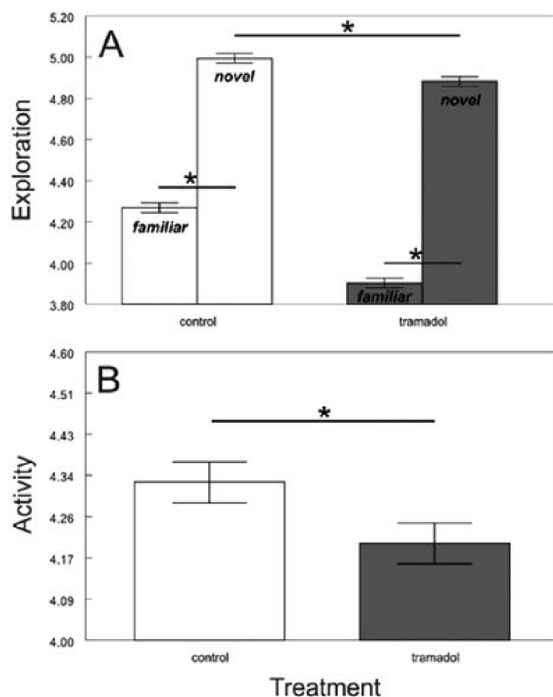
4

Fig. 2. Exploration of familiar and novel object (A) and activity during exploration of novel object (B) across the treatment. Values (+/- S.E.) are predicted from the particular mixed model. Asterisks indicate significant differences (Adj. $p < 0.05$).

Fig. 3. Social distance across the treatment. Values (+/− S.E.) are predicted from the particular mixed model. Asterisks indicate significant differences (Adj. $p < 0.05$).
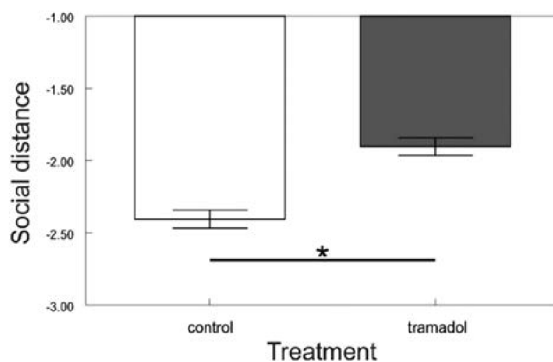
3.3. Progression of behavioural effects

The effect of tramadol on particular behavioural traits varied throughout the course of the experiment. Boldness was only significantly affected after 42 days of exposure ($F_{8, 1007} = 7.46$, $p < 0.0001$; Fig. 4A). In contrast, the effect on social distance ($F_{8, 228.6} = 8.41$, $p < 0.0001$; Fig. 4B) was consistent from the first to last day of exposure and remained significant even after two weeks of depuration (Fig. 4B). The consequence of the temporal effect on novel object recognition was not observed.

3.4. Tramadol brain concentration vs. behavioural variability

Increased tramadol concentrations in brain were linked to a decrease of boldness ($F_{1, 1068} = 53.00$, $p < 0.0001$; Fig. 5A), lower exploration of a novel object ($F_{1, 558.3} = 39.95$, $p < 0.0001$; Fig. 5B) and a larger inter-individual distances, indicating a disruption of the shoal cohesion ($F_{1, 8510} = 27.19$, $p < 0.0001$; Fig. 5C). The behavioural outcome was also influenced by the individuals weight, as larger fish were bolder ($F_{1, 1068} = 53.00$, $p < 0.0001$; Fig. 5A), spent more time during exploration of the novel object ($F_{1, 558.3} = 39.95$, $p < 0.0001$; Fig. 5B) and maintained larger inter-individual distances ($F_{1, 8510} = 27.19$, $p < 0.0001$; Fig. 5C).

4. Discussion

Our results indicated that exposure to environmentally relevant concentrations of tramadol impaired the behaviour of a native European fish. The direct link between the concentration of tramadol in brain tissue and the individual behaviour highlighted the strong influence of this drug received from the water.

4.1. Behavioural effects

Behaviour is the external response that results from the complex combination of several biological processes, e.g. biochemical or physiological (Hellou, 2011; Legradi et al., 2018). It has been extensively reported as a major change related with psychoactive compounds exposure (Buřič et al., 2018; Cunha et al., 2019). Our previous study has also shown that tramadol in water at 1 μg/L displayed impairments in the normal behaviour of invertebrates, compared to unexposed individuals (Bláha et al., 2019; Buřič et al., 2018; Ložek et al., 2019), underscoring the potential ecological effects of tramadol even at low concentrations. Specifically, the changes reported by Buřič and Ložek et al., (2018, 2019) also denoted a decrease in activity and locomotion. Furthermore, their results indicated that exposed crayfish showed a tendency to spend more time inside shelters than in the control treatment, which is similar to the decreased boldness observed in our experiment.

The few studies available in the literature do not provide solid results about behavioural disruption in fish induced by tramadol at environmental levels. In Tanoue et al. (2019), even though tramadol was detected in the brain, the behavioural testing did not provide satisfactory results, and the committee of experts questioned in this respect could not find the reason why. Several similar studies including carp larvae, tilapia, and zebrafish showed impairments in behaviour and other parameters -e.g. histopathology, development or oxidative stress - caused by tramadol exposure (Bachour et al., 2020; Huang et al., 2019; Sehonova et al., 2017, 2016; Soliman and Sayed, 2020). However, the levels of the substance used in these toxicology tests were generally much higher than in realistic conditions, so it is difficult to compare to the current subject.

In contrast, our findings clearly demonstrated changes in some of the major behavioural axes - i.e. boldness, activity, exploration, and sociability (Réale et al., 2007) - underlying an apparent anxiolytic-like effect. This assumption can be justified by the concurrent impairments in all of the previously cited patterns, since the alterations over behavioural traits should be taken into consideration as a whole (Conrad et al., 2011). Our results indicated that, in general, exposed fish were less bold, less active, less social, and did not explore the new environment nor the new object as much as the controls.

Personality traits can be correlated in order to disclose a behavioural syndrome (Conrad et al., 2011). As an example, previous research regarding invasive species - i.e. mosquitofish (*Gambussia affinis*) - indicated a syndrome in which activity, exploration, and boldness were positively associated, while sociability was just scarcely correlated with the others (Cote et al., 2010). Additionally, bolder individuals are generally more exploratory and more active- providing an advantage

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Table 1
The cross-trait correlation table showing the Spearman correlation coefficients for all observed behavioural traits. Significant relationships ($p < 0.05$) are highlighted in bold and indicate the existence of a behavioural syndrome between two individual traits under a particular treatment (tramadol or control).

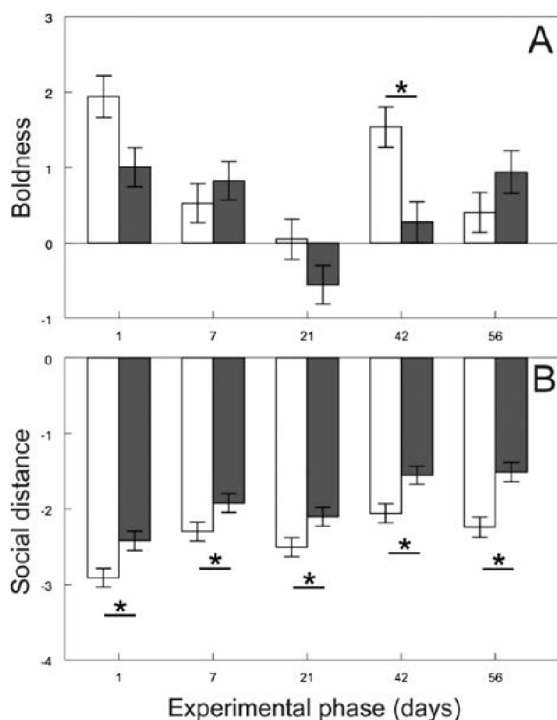|	Boldness		Distance		Exploration		Activity		Social distance	
	Control	Tramadol								
Boldness	$\rho = 1.00$	$\rho = 1.00$								
	$n = 32$	$n = 32$								
	N.A.	N. A.								
	Control	**Tramadol**	Control	Tramadol						
Distance	$\rho = 0.51$	$\rho = 0.64$	$\rho = 1.00$	$\rho = 1.00$						
	$n = 32$	$n = 32$	$n = 32$	$n = 32$						
	$p < 0.01$	$p < 0.01$	N.A.	N. A.						
	Control	Tramadol	Control	Tramadol	Control	Tramadol				
Exploration	$\rho = 0.35$	$\rho = 0.08$	$\rho = -0.07$	$\rho = -0.18$	$\rho = 1.00$	$\rho = 1.00$				
	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$				
	$p < 0.05$	$p > 0.67$	$p > 0.69$	$p > 0.31$	N.A.	N. A.				
	Control	Tramadol	Control	Tramadol	Control	**Tramadol**	Control	Tramadol		
Activity	$\rho = -0.23$	$\rho = 0.22$	$\rho = -0.20$	$\rho = -0.04$	$\rho = 0.18$	$\rho = 0.67$	$\rho = 1.00$	$\rho = 1.00$		
	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$		
	$p > 0.20$	$p > 0.21$	$p > 0.28$	$p > 0.82$	$p > 0.32$	$p < 0.01$	N.A.	N. A.		
	Control	Tramadol	Control	Tramadol	Control	Tramadol	Control	Tramadol	Control	Tramadol
Social distance	$\rho = 0.16$	$\rho = -0.02$	$\rho = 0.17$	$\rho = 0.05$	$\rho = 0.11$	$\rho = -0.17$	$\rho = -0.31$	$\rho = -0.20$	$\rho = 1.00$	$\rho = 1.00$
	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = 32$	$n = =32$	$n = 32$	$n = 32$
	$p > 0.37$	$p > 0.93$	$p > 0.36$	$p > 0.79$	$p > 0.55$	$p > 0.34$	$p > 0.09$	$p > 0.28$	N.A.	N. A.

Fig. 4. Boldness (A) and social distance (B) across the experimental phase. Shaded bars indicate tramadol treatment. Values (+/- S.E.) are predicted from the particular mixed model. Asterisks indicate significant differences (Adj. $p < 0.05$).

over other congeners in dispersing and/or foraging (Conrad et al., 2011). In line with this concept, we observed a relation between boldness and distance for both treatments, suggesting that bolder individuals from both groups explored the new environment more by covering an increased distance. Boldness and exploration of the new object were only correlated in control, and activity and exploration of the novel item only in exposed fish. These differences in trait relations suggest that the

tramadol treatment influenced the expression of the behavioural syndromes among the personality traits. The data also indicated that, although there were differences between the treatments, boldness seemed to be more related with activity, exploration, and distance; while sociability remained aside. Accordingly, previous studies could not relate boldness and sociability (Bevan et al., 2018; Cote et al., 2011; Jolles et al., 2016).

Among exposed fish, the individual weight of exposed chubs was related with the degree of behavioural changes. Bigger fish were bolder, less social, and explored the new object more. Overall, bolder individuals are expected to be bigger naturally because they can deal with opponents better and subsequently obtain more sustenance (Conrad et al., 2011; Jolles et al., 2016). As they were bolder, they would likely explore the new object more in comparison to shyer individuals. Moreover, bolder individuals were reported to be less social (Conrad et al., 2011; Jolles et al., 2015; Tang and Fu, 2020).

Several studies comprising other psychopharmaceuticals at low levels indicated similar impairments concerning those subjects. For example, *Perca fluviatilis* exposed to 1.8 µg/L of oxazepam for 7 days became less active and less social, although boldness remained unchanged (Brodin et al., 2013). *Rutilus rutilus* exposed to 0.84 µg/L of oxazepam for 7 days were bolder and more active than unexposed individuals (Brodin et al., 2017). Escitalopram increased boldness and induced alterations in swimming behaviour in zebrafish exposed to 1.5 µg/L of the substance for 21 days (Nielsen et al., 2018). *Gasterosteus aculeatus* exposed to citalopram at 1.5 µg/L for 21 days showed higher swimming activity and stayed closer to the novel object (Kellner et al., 2016). *Carassius auratus* exposed downstream to a STP for 21 days, contained six serotonin reuptake inhibitors in plasma and appeared bolder and more exploratory, compared to individuals from a less polluted upstream area (Simmons et al., 2017). In accordance with our findings, Brodin et al. (2014) suggested that the precise alterations in behaviour from exposure to this group of substances could be equivalent, although in different directions.

Our results suggest that the treated fish downplay the stimulus of the surrounding environment resulting from a calming effect. The end result of these changes has been mostly related to an increase in their vulnerability to predators (De Abreu et al., 2014; Dugatkin, 1992; Saaristo et al., 2019; Soares et al., 2018). Alterations in the fish social network have been suggested to produce direct effects over feeding behaviour, dispersion and/or reproduction (Brodin et al., 2014).

Behavioural analyses are more sensitive than other ecotoxicity assays because they provide information about possible effects without
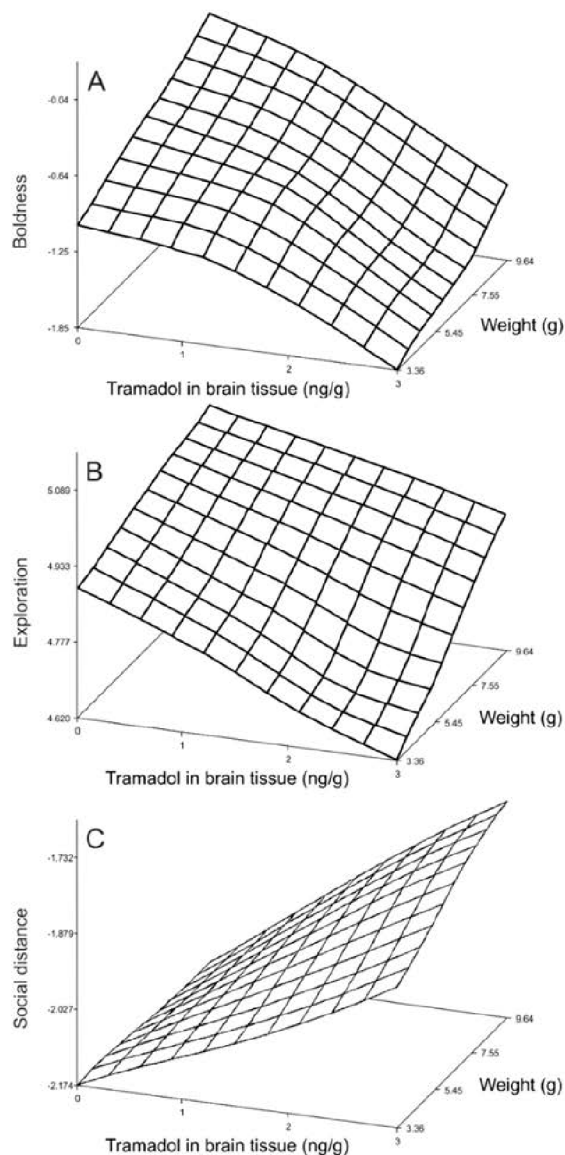
Fig. 5. Boldness (A), exploration (B) and social distance (C) in relation to tramadol in brain tissue and weight. Values are predicted from the particular mixed model.

reaching lethal concentrations (Hellou, 2011; Peterson et al., 2017). Changes of individual behaviour outcomes can induce alterations in the whole population, and thus, the fitness of aquatic ecosystems as a result of the existing interrelationships between species (Bisesi et al., 2014; Brodin et al., 2013). Therefore, the impacts over evolutionary behavioural patterns should be considered as a threatening signal and as a model to predict what could be happening in the aquatic ecosystems due to pharmaceutical contamination (Cunha et al., 2019; Klaminder et al., 2016).

4.2. Chemistry vs. behaviour

Tramadol, a weak base with n-octanol-water partition coefficient (log K_{ow}) of 1.35 at pH 7 (World Health Organisation, 2014), is not suspected to bioconcentrate in tissues due to its chemical properties. Accordingly, we observed a low BCF (< 500; OECD, 2001) in brain. Despite the low capacity of bioconcentration, the pseudo-persistence in the environment for the unceasing discharge into the water could provide a continuous presence in fish tissues (Rosi-Marshall et al., 2015; Schultz et al., 2011).

The constant concentration of tramadol in brain in this experiment suggested a steady state reached after one day of exposure. In agreement with this, the changes in boldness and shoal cohesion were also continuous from the first day of exposure. The BCF was very close to the values in plasma reported in Tanoue et al. (2017), which ranged from 1.4 to 1.8, depending on the pH of the water. In the same study, a relation between the concentration of tramadol in plasma and brain was observed, although higher amounts of the drug was detected in the brain compared to plasma (Tanoue et al., 2017). This phenomenon has been described in previous studies, where the concentration of the psychoactive substance in the brain was the highest (Huerta et al., 2016), or higher (Grabicova et al., 2018; Sancho Santos et al., 2020; Xie et al., 2015), compared to other organs. Among the 74 pharmaceuticals found, Grabicova et al. (2018) only detected psychoactive compounds in the brains of common carp in ponds where a STP effluent and precipitation were the only source of water. Furthermore, Grabicová et al. (2020) detected psychoactive compounds such as sertraline and citalopram in the brains of common carp. In pikeperch living in the same conditions, the psychoactives carbamazepine, sertraline, and venlafaxine as well as the antibiotic azithromycin were observed (Grabicová et al., 2020).

The read-across hypothesis is a model approach where the effects of some specific compound are estimated due to the comparison of the fish's drug concentration in plasma compared to the human therapeutic plasma concentration (Huggett et al., 2003). Even though this is commonly accepted for toxicological studies (Malev et al., 2020), the importance of measuring the chronic presence of the psychopharmaceutical in the target organ over the plasma should be reinforced, especially in the case of psychoactive substances. Several authors emphasized the same idea considering the chemical characteristics and pharmacokinetics of each compound specifically (Grabicova et al., 2014; Schultz et al., 2011). Thus, direct impairments of the neurotransmitter pathways can be expected even at minimal concentrations of these substances in brain (Grabicova et al., 2014). In addition, Hubená and Horký (2020a) (2020b) observed a correlation between brain concentration of sertraline and methamphetamine in fish and their condition, substantiating the brain concentration evidence approach, but the authors did not observe this link in the case of tramadol. However, behavioural endpoints are generally considered more sensitive than other parameters (Cunha et al., 2019). Therefore, alterations due to chronic exposure of tramadol should not be discarded, as we have confirmed in this study.

The particular acting of tramadol provides a combination of effects over several neurotransmission pathways. As previously described, tramadol has a dual mode of action. For one side, the parent compound, and its main metabolite, O-desmethyltramadol (M1), act on μ-receptors providing anti-nociception properties. On the other hand, it behaves as a non-opioid, inhibiting the reuptake of noradrenaline and serotonin over synaptic terminals, producing the anxiolytic and antidepressant-like effects. The metabolite M1 has 200 times higher affinity for the opioid receptor than the parent compound, consequently the analgesic efficiency depends on the metabolism of the pharmaceutical (Zhuo et al., 2016). However, Tanoue et al. (2017) reported a lower metabolism of M1 in fish compared to humans and other mammals. Regardless, in the same study, low amounts of M1 were detected in brain, supporting an analogous metabolism in fish compared to mammals (Tanoue et al., 2017).

Social distance was the only behavioural trait disrupted even after 14 days of depuration, when no traces of the parent compound were detected in the brain. Shoal cohesion has been related to rewarding

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support neurocircuitries in zebrafish, as well as it is likely to be enhanced by stress (Soares et al., 2018). Moreover, it has been reported that active metabolites of drugs can remain in the tissues over more time than the parent compound (Sancho Santos et al., 2020). Hence, although metabolites of tramadol were not quantified in our study, they can be suspected to produce this finding. Further studies should address this uncertainty along with the specific effects of tramadol metabolites on fish.

In this experiment, concentrations of tramadol measured in fish brain were directly related with behavioral effects in terms of boldness, exploration of a new object, and social interindividual distance. These results indicated that the presence of the parent compound in the brain generates direct impairments in the neurocircuitry pathways, resulting in a concurrent gradation of changes in behaviour. Therefore, we suggest the application of this approach in future studies to confirm whether this correlation also occurs after the exposure to other psychoactive substances, along with a lowest-observed-adverse-effect level (LOAEL) at this context.

5. Conclusions

Our findings clearly demonstrated that tramadol at 1 µg/L in water – here considered as environmental relevant concentration - can impair the individual behaviour of European native fish. In particular, the exposure to the pharmaceutical for 42 days triggered a reduction in activity, boldness, exploratory and social behaviour of chubs. Even after 14 days of depuration, the shoal cohesion was still disrupted, indicating the possible action of metabolites apart from the parent compound. The apparent anxiolytic-like effect was correlated with the concentration of the pharmaceutical in the brain, underlining the importance of the presence of the compound in the main target organ. These results indicate the potential environmental effects of tramadol and emphasize the need for further ecotoxicological studies focused on this pharmaceutical.

CRediT authorship contribution statement

Sancho Santos Maria Eugenia: Writing - original draft, Conceptualization. **Horký Pavel:** Writing –Review & Editing, Investigation, Methodology. **Grabicová Katerina:** Writing – Review & Editing, Investigation. **Hubená Pavla:** Writing – Review & Editing, Investigation. **Slavík Ondřej:** Writing – Review & Editing, Funding Acquisition. **Grabic Roman:** Writing – Review & Editing, Investigation, Methodology. **Douda Karel:** Writing – Review & Editing, Methodology. **Randák Tomáš:** Experimental design, 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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CHAPTER 4

A LETTER TO THE EDITOR

My share in this work was about 80%

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Water Research

Comment on "Diluted concentrations of methamphetamine in surface water induce behavior disorder, transgenerational toxicity, and ecosystem-level consequences of fish" by Wang et al. [Water Research 184 (2020) 116-164]

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ARTICLE INFO

ABSTRACT

The article presented by Wang et al. (2020) intends to elucidate the possible ecological effects of low (0.05–25 μg L^{-1}) and higher (100 μg L^{-1}) concentrations of methamphetamine on adult *Oryzias latipes* through a battery of assays, including histopathology. However, we found several mistakes and inaccuracies in the findings by means of this method. Given the increasing research effort in the field, the authors' paper may become highly influential in future toxicological research. Despite the authors' undoubted effort invested in their experiment, they did not employ standardized methods for histopathological assessment in the key laboratory experiment presented in their paper.

1. Introduction

In light of the increasing interest about the possible ecological impacts of illicit drug pollution on the aquatic environment, only a few articles in the field are currently available. Toxicologic histopathology is an essential tool for understanding these impacts. However, a major pitfall of this technique presented in this paper is the insufficient presentation of data and the description of the method, leading to the wrong interpretation of the outcomes.

2. Specific concerns about the histopathological evaluation

We would like to address several uncertainties in the article published by Wang et al. (2020). In specific, we would like to comment on serious mistakes concerning the histopathological analyses. Our findings were detected in a blind manner, in such a way that each involved participant did not know the judgment of the rest of observers. The specific aspects we disagree on were the following:

* There are only two histological photographs available in the referred publication, in very small size and low resolution, making the visualization and presentation of the described lesions complicated. We highly recommend providing an increased number of histological photographs with a detailed description of the findings observed.
* The focal point of the camera is evident in the central lower part of Figure 2A, and upper in Figure 2B. Therefore, we assumed that the areas in the photographs were cut and not fully presented; or that the microscope has not been correctly adjusted according to the characteristics of Köhler illumination.
* In Figure 2, the description says verbatim: "There were obvious minimal gliosis, neuronal loss, and necrotic (arrow) in brain tissue slice in exposure groups, the picture at 0.2 μg L^{-1} selected as the representative". However, it was not possible for us to find any of the described features in the mentioned photograph. The first arrow indicates a neuron with a banded nuclei structure nearby a vessel, and the second arrow points out another vessel. In summary, none of them indicate the lesions cited afterwards, even though the authors underline that this picture is representative of the described lesions and concentrations used.
* Erythrocytes appear as a useful indicator of the quality of staining when using hematoxylin and eosin, which, in this case, appears to be low and even different in each picture. As an ex-

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ample, in the control group the erythrocytes are lesser stained than in the exposed group.

- The site and area of interest in both photographs are extremely different from each other. In Figure 2B, the background is more uniform than in Figure 2A, which appears more whitish and slightly frothy. In comparison, Fig. 2B is more eosinophilic, this could be due to the commented low quality of staining, also to the differences in thickness of the sections. Moreover, the structure, morphology, size, and pigmentation of the present cells are very different as well, which indicates the distinct nature of the neurons depicted. Taking these two characteristics together, it gives the impression that the photographs belong to different portions of the brain. The "neuronal loss" described in several parts of the article is likely due to this fact. It would be necessary to provide an overlook at low magnification on the area of interest in the brain, and then a higher magnification in case the authors would like to remark some specific features.

- The statistical treatment and data of the histopathological portion is completely missing, both in the paper and in the supplementary materials. The authors do not give any table or graph justifying the asserted observations. Furthermore, there is no description of the method they used to quantify, describe, or evaluate the stated lesions. The brain is a highly structured organ; therefore, it would be crucial to explain the specific portion of interest and the direction of cutting, but this information is also lost.

- The authors claimed that "Histopathology of brain isolated from adult medaka was determined in this work. The brain slices of medaka in the control group showed an intact tissue structure with complete cellular structure (Fig. 2A), while the brain section of the fish treated with METH at 0.2 μg L^{-1} showed obvious minimal gliosis, neuronal loss, and necrotic (arrows) (Fig. 2B)" and later on "The dose-dependent effect of histopathology induced by METH on fish was found by previous study". Although the authors observed a dose dependent effect regarding histopathology, it is not clear with the given information. We suggest they provide the pictures corresponding to each concentration used, both in exposed and controls, and correlate these findings with some statistical analyses in order to corroborate the aforementioned statement.

3. General comments about histopathological assessment in ecotoxicological studies

Baumann et al. (2016) and Wolf and Maack (2017) addressed several reasons why this issue is becoming more frequent. For

example, the absent review of this type of research by qualified pathologists and the lack of a standard criteria in the method. As in Baumann et al. (2016), another problem evolves in the moment that the results of the investigation relies on erroneous analyses, contributing to a mistaken end product to further research on the topic. Furthermore, the discrepancies described in the methodologies used in studies recently published in high-quality journals indicate a strong need for more thorough debate regarding the use of these methods and their unification. This would help to minimize the risk of bias in the results of such experimental studies. We would like to comment on this issue with the aim of emphasizing the growing imprecision of this method in the recent published studies, and the concern of the related personnel.

4. Conclusions

Overall, we believe that our reasoning illustrates a serious deficiency on the reliability of this method. The question we referred here is just another example of the recurrent problem concerning histopathological analyses used in ecotoxicological studies. We strongly suggest the authors to supplement the article with the required data to give a comprehensive presentation of their findings.

Declaration of Competing Interest

None financial or personal conflict of interest.

Acknowledgements

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CHAPTER 5

General discussion

The statistics regarding the consumption of psychopharmaceuticals and drugs indicate their increased consumption over time. Furthermore, the COVID-19 crisis can trigger an unprecedent scenario regarding the usage of these substances. The mental health disorders – e.g. depression, anxiety, suicidal tendencies – in numbers are yet to be known, as well as the volume of use of drugs of abuse by vulnerable people due to the economic impacts. Furthermore, the Government's, currently focused on the pandemic, can give a free way to the traffickers to smuggle substances through the borders (UNODC, 2020). In summary, all of these factors could lead to higher concentrations of psychoactive substances in water than hitherto.

Laboratory tests applying METH and tramadol at realistic concentrations were performed in order to examine whether their presence in water could induce effects over fish. The substances studied in this dissertation were present in all the analyzed tissues and sampling times. The concentration of tramadol, METH, and its metabolite AMPH in biota samples were similar to the values found in previous studies (Tanoue et al., 2017; Yin et al., 2019; Hubená et al., 2020). The 4-day depuration time in the first study using METH was insufficient, as remnants of the parent compound and the metabolite were still detected in the tissues. In the following experiment, 10-day depuration was not enough for the metabolite, but it was for the parent compound. The bioconcentration factors were low (<500; OECD, 2001) in all the cases for all of the three compounds, as expected due to their low n-octanol-water partition coefficient (log K_{ow}), and hydrophilic properties. Still, the pseudo-persistence in water and the consequent chronic exposure can lead to several consequences, as seen in the conducted tests.

In the case of the first experiment using METH, we could converge at the read-across hypothesis, in other words, fish plasma model (Huggett et al., 2003). According to this postulate, the therapeutic concentration should be reached, hence, the effects should be evident, if the effect ratio (ER; human TPC/fish plasma concentration) is ≤1. In addition, the 10-fold factor used in fish means that there could be obvious effects with an ER <1,000. Considering the values stated in Regenthal et al. (1999), the TPC was reached in the high concentration group (50 μg/L), and the ER was <100 in the environmental group (1 μg/L). A distinctive feature observed in the study is the elevated concentration in the tissues of the metabolite AMPH comparing to the parent compound. Although the metabolite is an active substance and has a similar mechanism of action as METH, it is not considered to provide any pharmacological effect in mammals because of the resulting low amounts after the metabolization process (Cruickshank and Dyer, 2009). However, based on the detected concentrations of AMPH, the metabolism of METH could be slightly different in fish.

Psychoactive substances, as aforementioned, are designed to reach and exert their actions over the central nervous system. In agreement, the determined concentrations of METH and AMPH in brain were higher than in plasma. In the case of tramadol, Tanoue et al. (2017) also found a greater concentration in brain comparing to blood plasma. This observation resulted in the next question, the brain-concentration evidence approach (Hubená et al., 2020). According to this assumption, a relation between the amount of the neuroactive chemical and the observable effects could exist as a result of the phylogenetic similarity in the target signalization. Our results from the tramadol experiment confirmed this hypothesis, as the amount of tramadol in brain and the behavioural findings concerning exploration, boldness and shoal cohesion were correlated. In addition, this finding underlines the direct actions over the neurotransmitters produced by low concentrations of the substance tested, and the quantifiability of the outcomes.

In the second experiment using METH, the detected remnants of AMPH in brain after depuration time were strongly linked to the observed behavioural effects, indicating that the active metabolites evidently produce additional actions. The potential impacts of the metabolite were also suspected in the first experiment, due to the high amounts of AMPH found in tissues comparing to the parent compound. Furthermore, this effect was suggested in the tramadol test for the reason that the shoal cohesion was disrupted after the 14-day depuration time, although traces of tramadol were not present in brain at that point. In agreement, Parolini et al. (2018) detected changes in behaviour and reproduction in *Daphnia magna* exposed to environmental relevant concentrations of benzoylecgonine, cocaine's main metabolite. Another example is oxazepam – i.e. a parent compound, also the by-product of diazepam and nordazepam's degradation – which effects over aquatic fauna at realistic concentration are well-known (Brodin et al., 2013, 2017; Heynen et al., 2016).

Amphetamine-like substances are known to be hepato and cardiotoxic, although the specific mode of action in each organ is not completely understood. The changes in liver at the tissue level produced by METH (and AMPH?) in our experiment were in accordance with the reported in mammals – i.e. increased cytoplasmatic vacuolation and apoptotic changes in hepatocytes (Kamijo et al., 2002; Halpin and Yamamoto, 2012; Dias Da Silva et al., 2013; Koriem and Soliman, 2014; Wang et al., 2017). The proposed mechanisms of action can be related with direct cytotoxicity and disruption of cell metabolism (Halpin et al., 2013; Idilman et al., 2016; Wang et al., 2017). In our study, a dose-dependent effect was found in liver during the exposure time. However, the detected changes after the 4-day depuration suggest a rapid metabolism of the chemical at high levels, even a compensation process, which is in line with previous observations (Yin et al., 2019).

The disruptions in the vascular system in our research produced by the tested chemical were similar to the previously described in mammals as well. Microvascular impairments with the related necrosis, fibrosis, and infiltration with leucocytes (lymphocytes, eosinophils) in heart were detected in our samples, and also frequently reported in METH casualties and experimentation (Kaye et al., 2007; Milroy and Parai, 2011; Won et al., 2013; Akhgari et al., 2017). The mode of action suggested in this case is related to the strong activation of the catecholaminergic system. On one hand, a higher oxygen supply is needed due to the increased cardiac and locomotor activity, but, on the other hand, vasoconstriction is produced (Henry et al., 2012). These factors all together develop hypoxia in the tissue, leading to the resultant histopathological observations. Despite of these results, the changes were observed mainly in the environmental group, therefore, the compensation mechanisms suspected in liver could take place also in heart. This assumption, together with the potential cardiac regeneration in fish, suggests less marked disruptions over cardiovascular system as a result of the exposure to diluted concentrations of METH in fish.

The similarity in the histopathological events in trout comparing to animal models and human verifies the read-across hypothesis for the tested illicit drug. The histological changes noticed in this study underline the interest in the evaluation of the target organs in order to compare the outcomes in different species, in addition to the organs typically recommended in toxicological tests (gills, kidney, gonads).

The behavioural method applied in our experiments confirmed the changes caused by low concentrations of neuroactive substances. Because behaviour is the manifestation of complex physiological responses, our findings reveal the sharp influence that the presence of these pollutants can exert over the neurotransmitters' mode of action even at low levels.

In our tramadol test, four of the major behavioural traits were disrupted – i.e. activity, exploration, boldness, and sociability (Réale et al., 2007). In specific, the fish tended to be lesser bold, social, active, and explorative than the control individuals, indicating an evident

anxiolytic-like effect. The observed outcomes are logical, considering that tramadol provides added actions as anxiolytic and antidepressant in human (Vazzana et al., 2015). The detected effects were in line with previous investigations using invertebrates as experimental models, where lower activity and boldness were observed (Buřič et al., 2018; Bláha et al., 2019; Ložek et al., 2019).

Furthermore, correlations among the personality traits were found in the tramadol experiment. Boldness and activity were related in control and treated fish. However, some of the linkages depended on the treatment – i.e. exploration and boldness correlated only in unexposed, and exploration and activity in exposed individuals. The trait relations can reveal a behavioural syndrome (Conrad et al., 2011), therefore, the discovered changes could produce deeper consequences within an individual. Specific personalities could provide a high variability in behavioural tests' outcomes; however, this problem could be solved by providing enough number of replicates (Buřič et al., 2018). Following the approach suggested in Brodin et al. (2014), the personality traits impaired by the exposure to tramadol at minimal doses could have consequences over vulnerability to predators, migration, feeding and/or reproduction in the tested species.

Behavioural changes were also found in fish exposed to an environmental relevant concentration of METH. Brown trout preferred the water source contaminated with METH, and the presence of this compound in brain resulted in an increased locomotor activity. This observation concurs with the actions of METH over mammals' organism, in which produces an increased muscular tone and hyperactivity (Tanaka et al., 2017). Withdrawal fish also preferred the scent location, but a decrease in movements were detected, in this case related to the amount of the metabolite in brain. In human, the withdrawal due to METH can cause cravings, decreased activity or depression (Panenka et al., 2013). The changes in metabolome followed a time-line similar as the rest of the results – i.e. the detected biomarkers, the chemical brain remnants and the location preference lasted until the 4th day of depuration time. Abreu et al. (2016) observed zebrafish' preference for a current polluted with several psychopharmaceuticals, instead of a clean water lane. If fish are attracted to polluted sites - e.g. the discharge flow from a STP – they would consistently increase the uptake of substances present in the water (Abreu et al., 2016). The changes in behaviour produced by neuroactive compounds, together with the attraction towards the sites enriched with these pollutants, would aggravate the ecological consequences.

The procedures used in the accomplished experiments demonstrated to provide valuable toxicological information. Though, at times ecotoxicological approaches have implicit the problematic of providing a mistaken assumption, especially when using non-regulated methods, or because of the wrong treatment of the data. The "letter to the editor" is an example of such situations. The authors of the commented article did not use a consistent methodology, therefore, their outcomes resulted utterly inaccurate. As observed by Wolf and Maack (2017), these kind of issues are frequent in the field of histopathology related to ecotoxicological studies; even though, there is an increasing interest in this method. Both situations could create eventually a climate of distrust among the experts in this discipline when examining some of the published ecotoxicological research. A big part of the issue is due to the lack of reviewers with expertise in histopathology; but also, the shortage of specialists within this field of knowledge, and the general deficiency of the cross-checking of the outcomes with experienced pathologists. Because this technique implies an inexorable subjective factor when examining the samples, more effort should be exerted in providing opportunities for quality training to the researchers, and practical standarized guidelines.

As seen, behavioural assessment can be a valuable tool for environmental monitoring. Models relying on behavioural results could reveal the real concerns of psychoactive pollutants

over aquatic ecosystems. However, the shortage of standardized procedures produce the lack of interest in including these endpoints in the official environmental risk assessments (Ford et al., 2020). The behavioural tests used for ecotoxicology aim to detect differences in natural behavioural traits, such as activity or boldness. Though, the approach in which strong-selected laboratory fish models play a role as a reflection of wild populations may not be useful due to the partial loss of sensitivity as a result of the domestication process (Vossen et al., 2020). Despite of all of the advantages that laboratory fish models could provide – e.g. standarization of the tested animals, leading to the high repeatability of the results, the uneven responses in these fish comparing to the natural populations could mask concerning behavioural changes at ecological level. Therefore, using native or natural fish populations, regardless of the high variability in the results comparing to model animals, could improve the detection of the more realistic effects.

Although the general agreement with the development of standardized behavioural tests, psychoactive compounds could produce specific responses related to their particular mode of action. Existing protocols are usually elaborated to serve as a guideline to a large range of compounds, sometimes forgetting possible additional outcomes derived from the particular mechanisms. As an example, the second experiment using METH highlighted the addiction that these compounds could generate in fish. Hence, innovative ideas in the settings of toxicological tests should be considered valuable means to get information regarding the specific outputs of neuroactive substances, and less harshly reviewed for becoming separated from the standards.

Future research

Our results evidenced that METH and tramadol at low concentrations in water can disrupt physiological responses of fish, even causing changes in behaviour. However, this thesis just opens a book of questions to be solved.

Because of the large influence of the metabolites in the observed outcomes, future research should deepen in the potential impacts of the active metabolites, in addition to the tested parent compounds. The effects of the substances are clearly related to the concentration used, as seen in our first experiment with METH using two different concentrations of the drug. Therefore, it would be of interest to test the compounds of concern in decreasing levels, in order to find the approximate lowest concentration at which behavioural effects are observable (in other words, LOAEL or Lowest observed adverse effect level).

In realistic circumstances, pollutants appear in water in complicated mixtures where several compounds could present interactions (Ebele et al., 2017). These synergistic or antagonistic effects could result in different outcomes than expected considering the available literature. The necessity of knowing every particular ecotoxicological effects of each compound should aim a further attempt to investigating the behaviour of the substances in real situations.

In light of the climate change possible scenarios, the interactions between psychoactive compounds and other stressors, such as increase in temperature and CO_2 concentrations in water, are at present unknown. Yet, abiotic factors can influence the behaviour of neuroactive substances in the organism. For instance, METH is known to produce a different intensity of toxicity under normal and hyperthermic conditions in experimental mammals (Kiyatkin and Sharma, 2011; Halpin et al., 2013). In addition, the pH is another important factor in water that could possibly influence the uptake of polar compounds through the gills, and also the toxicity, in a similar way as ammonia. Despite of the increasing interest in this dilemma in the scientific community (Maulvault et al., 2018; Mehdi et al., 2019; Saaristo et al., 2019; Wiles et al., 2020), more research is still needed.

To conclude, a combined effort among different disciplines – biochemistry, chemistry, ecology, genetics, pathology, pharmacology, toxicology – should be exerted to provide a bigger picture regarding ecotoxicological evaluation of psychoactive compounds.

Conclusions

The compounds selected in this thesis, methamphetamine and tramadol, were concentrated in tissues, therefore, they can be suspected to trigger a cascade of events on fish when presented in aquatic environment in increased concentrations. Methamphetamine induced histopathological changes in liver and heart. Furthermore, locations contaminated with methamphetamine produced addiction in fish, revealed by changes in behaviour and metabolome related to methamphetamine's main metabolite. Tramadol caused behavioural changes in a native species, which were correlated with the amount of the substance in brain. Our findings underline the parallel effects in non-targeted fauna comparing to human and experimental models. In summary, these findings demonstrated that the presence in water of methamphetamine and tramadol at low levels can produce sublethal effects in fish. This dissertation highlights the translation of our social problems into the ecosystems. The results provided in this thesis should encourage future studies about the potential effects of these substances in the aquatic environment, and, eventually, solutions to decrease the presence of these pollutants in water.

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Psychoactive compounds in aquatic environment and their effects on fish

Maria Eugenia Sancho Santos

Psychoactive substances are emerging compounds that have received an increasing interest in ecotoxicology as a result of their ubiquitous presence in the environment, and the possible effects over non-targeted fauna. They have been detected in continental waters at concentrations ranging from nanograms to micrograms per litre, mainly as a consequence of the ineffective removal in the sewage treatment plants. Within this group of substances, methamphetamine and tramadol were selected in this dissertation due to the lack of research about their potential effects despite of the high amounts reported in water.

Methamphetamine, mainly consumed as an illicit drug, is an addictive psychostimulant with special relevance in Central Europe. Environmental relevant and higher concentrations of methamphetamine in water leaded to histopathological alterations in heart and liver in brown trout (*Salmo trutta* m. *fario*). The major changes in heart were microvascular injuries, infiltration and fibrosis; and cytoplasmatic vacuolation of hepatocytes in liver. In addition, apoptotic changes were observed in liver. The findings were very similar to those reported in human and experimental animals. The parent compound and the main metabolite, amphetamine, were detected in trout tissues following the order kidney > liver > brain > muscle > plasma. The concentrations of the metabolite were evidently higher comparing to the parent compound; therefore, the histological findings could be suspected to partially derivate from amphetamine. Behaviour – i.e. activity and place preference – and metabolome changes for the period of withdrawal were observed in trouts, linked to remnants of the parent compound and the metabolite in brain.

Tramadol is an extensively used analgesic which singular mode of action provides added antidepressant and anxiolytic effects. Realistic concentration of tramadol in water was found to impair essential behavioural traits in the chub (*Squalius cephalus*) – a native fish species in Central Europe. The degree of the outcomes was correlated with the individual amounts of tramadol in brain. Exposed fish exhibited anxiolytic-like effects, illustrated by lesser bold and social individuals comparing to controls. In the boldness test, exposed fish were less frequently out of the shelter and moved a shorter distance, therefore, they explored the new environment less than control fish. The novel object recognition experiment indicated that, although they distinguished the new item, their activity was reduced, and they explored the new object less. The increased interindividual distance indicated that the shoal cohesion was disturbed. The behavioural traits were associated to the treatment, suggesting the influence of the pollutant over fish personality.

Bioconcentration factors for tramadol and methamphetamine were low in all cases, thus, the probability of bioconcentration for these substances is very low. However, these compounds are continuously released into water, leading to their pseudo-persistence and the consequent presence in tissues. The occurrence in fish, and the parallelism in the target signalization result in similar actions in these organisms comparing to human and experimental models. Here we evidenced that the water pollution with the tested neuroactive substances in the aquatic compartment could lead to unexpected effects over fauna, and subsequent changes in the whole aquatic ecosystem.

Psychoaktivní sloučeniny ve vodním prostředí a jejich účinky na ryby

Maria Eugenia Sancho Santos

Psychoaktivní látky patří do skupiny nových kontaminantů životního prostředí a díky jejich všudypřítomnosti a možným účinkům jsou stále častějším předmětem zájmu ekotoxikologických studií. Tyto látky byly detekovány ve vnitrozemských vodách v koncentracích od nanogramů po mikrogramy na litr. Řada z nich není efektivně odstraňována v procesech čištění odpadních vod. Pro experimenty realizované v rámci této disertační práce byly z široké skupiny psychoaktivních látek vybrány metamfetamin a tramadol, a to zejména z důvodu minimálního množství dosud realizovaných ekotoxikologických studií týkajících se působení těchto látek na organismy v životním prostředí a také z důvodu poměrně významných koncentrací těchto látek nalézaných v povrchových vodách.

Metamfetamin, užívaný hlavně jako nelegální droga, je návykovým psychostimulantem, který je zejména ve střední Evropě nejčastěji užívanou tzv. „tvrdou" drogou. V rámci práce testované environmentálně relevantní a vyšší koncentrace metamfetaminu ve vodě vedly u pstruha obecného (*Salmo trutta* m. *fario*) k histopatologickým změnám v srdci a v játrech. Hlavní změnami pozorovanými v srdci byly mikrovaskulární poranění, infiltrace a fibróza, v játrech pak cytoplazmatická vakuolizace hepatocytů. Kromě toho byly v játrech pozorovány apoptotické změny. Nálezy byly velmi podobné nálezům popisovaným u lidí a laboratorních zvířat. Původní sloučenina a její hlavní metabolit amfetamin byly detekovány ve tkáních pstruhů v pořadí ledviny> játra> mozek> sval> plazma. Koncentrace amfetaminu byly evidentně vyšší ve srovnání s metamfetaminem. Proto lze předpokládat, že histologické nálezy částečně vznikají také působením amfetaminu. U pstruhů vystavených působení metamfetaminu byly v poexpoziční době pozorovány rozdíly v chování mezi exponovanou a kontrolní skupinou. Jednalo se o preference aktivity a místa výskytu a o změny metabolomu mozku, které byly dávány do souvislosti se zbytkovým obsahem metabolitu testované látky v mozku exponovaných jedinců.

Tramadol je hojně užívané analgetikum, jehož singulární způsob účinku poskytuje přidané antidepresivní a anxiolytické účinky. Bylo zjištěno, že reálně se vyskytující koncentrace tramadolu ve vodě mění základní způsoby chování jelce tlouště (*Squalius cephalus*), který je ve střední Evropě původním rybím druhem. Úroveň změn chování korelovala u testovaných jedinců s koncentrací tramadolu v jejich mozku. U exponovaných ryb byly pozorovány anxiolytické účinky, např. menší odvaha a odlišné sociální chování ve srovnání s kontrolními jedinci. V testu smělosti exponované ryby setrvávaly déle v úkrytech a pohybovaly se na kratší vzdálenost, proto prozkoumávaly nové prostředí méně než kontrolní ryby. Experiment zaměřený na schopnost rozpoznávání nových objektů naznačil, že ačkoliv exponovaní jedinci odlišili novou položku, jejich aktivita byla snížena a nový objekt prozkoumávali méně. Zvýšená vzdálenost mezi jedinci naznačovala, že je narušena soudržnost hejna. S expozicí byly spojeny i změny chování, což naznačuje vliv znečišťující látky na osobnost ryb.

Biokoncentrační faktory pro tramadol a metamfetamin byly ve všech případech nízké, takže pravděpodobnost biokoncentrace je v případě těchto látek velmi nízká. Tyto sloučeniny se však kontinuálně dostávají do vody, což vede k jejich pseudo-perzistenci a následné přítomnosti ve tkáních. Výskyt u ryb a paralelismus v cílové signalizaci vedou k podobným reakcím v těchto organismech, jako jsou pozorovány u lidských a dalších živočišných modelů. V našich experimentech jsme prokázali, že znečištění vody testovanými neuroaktivními látkami může vést k neočekávaným účinkům na faunu a následným změnám v celém vodním ekosystému.

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List of publications

Peer-reviewed journals with IF

Horký, P., Grabic, R., Grabicová, K., Brooks, B.W., Douda, K., Slavík, O., Hubená, P., **Sancho Santos, M.E**., Randák, T., 2021. Methamphetamine pollution elicits addiction in wild fish. Journal of Experimental Biology 224: jeb242145. (IF 2020 = 3.312; Q2). DOI 10.1242/jeb.242145

Sancho Santos, M.E., Horký, P., Grabicová K., Hubená, P., Slavík, O., Grabic, R., Douda, K., Randák, T., 2021. Traces of tramadol in water impact behaviour in a native European fish. Ecotoxicology and Environmental Safety 212: 111999. (IF 2020 = 6.291; Q1). DOI 10.1016/j.ecoenv.2021.111999

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Sancho Santos, M.E., Grabicová, K., Steinbach, C., Schmidt-Posthaus, H., Šálková, E., Kolářová, J., Vojs Staňová, A., Grabic, R., Randák, T., 2020. Environmental concentration of methamphetamine induces pathological changes in brown trout (*Salmo trutta fario*). Chemosphere 254: 126882. (IF 2020 = 7.086; Q1). DOI 10.1016/j.chemosphere.2020.126882.

Manuscripts

Sancho Santos, M.E., Horký, P., Grabicová, K., Hubená, P., Steinbach, C., Grabic, R., Šálková, E., Randák, T. Changes in metabolism and brain of brown trout promoted by the environmental concentration of an illicit drug. In preparation.

Abstracts and conference proceedings

Sancho Santos, M.E., Grabicová, K., Grabic R, Steinbach, C., Vojs Staňová, A., Randák, T., 2019. Methamphetamine and brown trout (*Salmo trutta fario*): bioaccumulation and metabolome effects at environmentally relevant and higher exposure levels. 40th SETAC North America Annual Meeting, Toronto (Canada), 3–7 November.

Sancho Santos, M.E., Grabicová, K., Steinbach, C., Schmidt-Posthaus, H., Kolářová, J., Vojs Staňová, A., Velíšek, J., Grabic, R., Randák, T., 2019. Environmental concentration of methamphetamine affects brown trout (*Salmo trutta fario*). 19th EAFP International Conference, Oporto (Portugal), 9–12 September.

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Training and supervision plan during study

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Ph.D. courses	Year
Basics of scientific communication	2018
Pond aquaculture	2018
Applied hydrobiology	2018
Ichthyology and fish taxonomy	2018
Aquatic toxicology	2018
Biostatistics	2019
Czech language	2019
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Scientific seminars	Year
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Sancho Santos, M.E., Grabicová, K., Grabic R, Steinbach, C., Vojs Staňová, A., Randák, T., 2019. Methamphetamine and brown trout (*Salmo trutta fario*): bioaccumulation and metabolome effects at environmentally relevant and higher exposure levels. 40th SETAC North America Annual Meeting, Toronto (Canada), 3–7 November.	2019
Sancho Santos, M.E., Grabicová, K., Steinbach, C., Schmidt-Posthaus, H., Kolářová, J., Vojs Staňová, A., Velíšek, J., Grabic, R., Randák, T., 2019. Environmental concentration of methamphetamine affects brown trout (*Salmo trutta fario*). 19th EAFP International Conference, Oporto (Portugal), 9–12 September.	2019
Sancho Santos, M.E., Steinbach C., Grabicová, K., Schmidt-Posthaus, H., Kolářová, J., Randák, T., 2019. Selected effects of environmental psychoactive compounds in fish. XIX International Toxicological Conference, Vodňany (Czech Republic), 21–23 August.	2019

Foreign stays during Ph.D. study at RIFCH and FFPW	Year
Dr. Heike Schmidt-Posthaus, Dipl. ECVP, FVH. Centre for Fish and Wildlife Health, Vetsuisse-Fakultät, Bern (Switzerland). (1 month of training in histopathology and diagnostics of aquatic organisms)	2018

Pedagogical activities	Year
Summer school: "Histological changes in fish after psychoactive pollutants exposure" Lea Klatzl, Francesca Kastner Austrian prize "Wissen Schaf[f]t zukunft preis 2020" was awarded for this work	2019
Project leader: "Fish responses to environmental cocktail of psychoactive substances" (2019 GAJU 102/2019/Z)	2019
Teaching at UBS FFPW	2020
Consultant of bachelor student: "Distribuce vybraných léčiv mezi články potravního řetězce ve vodním prostředí" Veronika Lexová	2020
Consultant of master students: "Antidepresivum sertralin a chování ryb" Veronika Lexová, "Behaviorální změny ryb po expozici metamfetaminu" Miroslav Slivoně	2021

Curriculum vitae

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2015–2016   Master of Science in Aquaculture, University of Barcelona, Autonomous University of Barcelona, Polytechnic University of Catalonia Barcelona, Spain

2008–2014   Doctor of Veterinary Medicine, University of León León, Spain

COMPLETED COURSES

15–19. 2. 2021   Course of professional training to obtain qualification and professional competence in the field of experimental animals, Charles University, Prague (Czech Republic)

8. 9. 2019   14th Fish Histopathology Workshop: "Skin-associated pathology", Oporto (Portugal)

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1.–30. 5. 2018   Dr. Heike Schmidt-Posthaus, Dipl. ECVP, FVH
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Toxicological experiments in *Salmo trutta fario* and *Squalius cephalus* using behavioural techniques.