Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

2023

Monitoring of crayfish reaction to the different stimuli: are the crayfish good bioindicators?

Doctoral thesis

Viktoriia Malinovska

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Title of thesis:
Monitoring of crayfish reaction to the different stimuli: are the crayfish good bioindicators?
Monitoring reakce raků na různé podněty: jsou raci dobrými bioindikátory?

Doctoral study program:
Zootechnics / Fishery, full-time

Malinovska, V., 2023. Monitoring of crayfish reaction to the different stimuli: are the crayfish good bioindicators? Doctoral thesis. University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Czech Republic, 97 pp. ISBN 978-80-7514-190-3 (with the summary in English and Czech)
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction to the use of crayfish in scientific research

Freshwater crayfish have first been mentioned in scientific literature by Aristotle, and since ancient times, the interest in these decapods has sufficiently grown (Hart and Clark, 1987; Gherardi, 2011). Significant progress has been made toward the ecological role of crayfish and its use as a model organism (Helms et al., 2013). To date, freshwater crayfish is a common experimental organism in a wide variety of research, including studies on embryogenesis, developmental biology, behavior, genetics, toxicology testing, etc. (Arcaro and Lnenicka, 1995; Tierney et al., 2000; Wigginton and Birge, 2007; Vogt, 2018; Wang et al., 2020). Moreover, crayfish are known to be a suitable tool for monitoring environmental pollution (Kuklina et al., 2013; Goretti et al., 2016).

Crayfish are one of the most widespread freshwater taxa in the world, found on all continents except Antarctica (Crandall and Buhay, 2008; Helms et al., 2013). They can be easily captured in nature, identified, manipulated, and cultured (Pöckl et al., 2006; Reynolds and Souty-Grosset, 2012). The absence of a planktonic larval stage makes crayfish culture relatively easier than lobster and prawn cultivation (Wickins and Lee, 2002). In comparison with fish, crayfish have a relatively primitive circulatory, vascular, and nervous system (Randall, 1970; Vogt, 2002). Their circulatory system is open and contains the heart, arteries, and sinuses (Vogt, 2002). Such a simple organization makes observing physiological changes, caused by stimuli, clearer and easier (Bojsen et al., 1998; Pautsina et al., 2014). Crayfish can move and survive for a certain time in both water and air (Taylor and Wheatly, 1981; Morris and Callaghan, 1998), making it possible to conduct terrestrial and aquatic laboratory and field experiments using crayfish species (Fairchild and Hasiotis, 2011).

In addition, crayfish possess an advantage over fish since there is no conflict with current animal welfare legislations. Furthermore, the use of invertebrates in scientific research is consistent with the 3Rs approach (Replacement, Reduction, Refinement). This concept aims to minimize the use of experimental animals, especially vertebrates, and to substitute them with less sensitive species (e. g. invertebrates) (Pärt et al., 2010). From this perspective, crayfish are appropriate models for assessing the effects of pollutants on aquatic organisms, as well as for bioindication of aquatic environments contaminated by xenobiotics.

Different crayfish species, indigenous and non-native, can be applied for biomonitoring of water quality (Alcorlo et al., 2006; Aguirre-Sierra et al., 2013; Tunca et al., 2013; Kuklina et al., 2014; Wren and Gagnon, 2014; Stanek et al., 2017). Styrishave et al. (2007) compared diurnal variations in physiology and behavior of European endangered native noble crayfish *Astacus astacus* and non-native in Europe signal crayfish *Pacifastacus leniusculus*. It was found that both species have the same circadian rhythms in heart rate, locomotor activity and oxygen consumption. These similarities make possible using non-native crayfish species instead of indigenous crayfish species in laboratory research related to the evaluation of chemical toxicity, behavioral studies, etc. Also, non-native crayfish species are often used in scientific research due to protected status and manipulation regulations of indigenous crayfish species. Considering that the use of non-native crayfish in research is currently subject to regulation, it is necessary to report the permit obtained for using these crayfish. Signal crayfish *P. leniusculus* and red swamp crayfish *Procambarus clarkii* are among the most common crayfish used in scientific research, owing their diverse spreading and availability worldwide (Souty-Grosset et al., 2016; Oficialdegui et al., 2019). Among non-native crayfish species and decapods in general, marbled crayfish *Procambarus virginalis* is a unique organism characterized by fast growth, early and frequent parthenogenetic reproduction, resulting in genetically identical offspring. These features of marbled crayfish make it a suitable model organism (Hossain et al., 2018).

Crayfish as bioindicators of water quality

Crayfish are considered to be good bioindicators due to their ability to accumulate pollutants over extended time periods without significant mortality, long lifespan compared to most other benthic freshwater invertebrates, ease of sampling, and ability to survive in laboratory conditions, as confirmed by various studies (Lyons and Kelly-Quinn, 2003; Reynolds and Souty-Grosset, 2012; Goretti et al., 2016). The definition of bioindicators, as determined by McGeoch (1998), can also be applied to crayfish, as they readily reflect the abiotic or biotic state of an environment, represent the impact of environmental change on a habitat, community, or ecosystem, and are indicative of the diversity of a subset of taxa or wholesale diversity within an area. Moreover, many crayfish species inhabit environments with relatively high water quality, making them useful indicators of such conditions (Reynolds and Souty-Grosset, 2012). Numerous studies have been conducted on the use of crayfish as bioindicators for water quality (Parks et al., 1991; Alcorlo et al., 2006; Vioque-Fernandez et al., 2007; Gago-Tinoco et al., 2014; Goretti et al., 2016; Kuklina et al., 2018). As elucidated in **Chapter 1.2**, crayfish exhibit distinct responses at diverse physiological and behavioral levels when subjected to a broad spectrum of chemical substances at environmentally relevant concentration.

Crayfish have been used as bioindicators both in the aquatic environment and under laboratory conditions for several decades (Sheffy, 1978; Escartin and Porte, 1996; Alcorlo et al., 2006; Aguirre-Sierra et al., 2013; Gago-Tinoco et al., 2014; Kuklina et al., 2014; El-Atti et al., 2019). Laboratory studies have examined the bioaccumulation of fluoride, metals, and even nanomaterials in crayfish tissues (Laporte et al., 1996; Gonzalo and Camargo, 2012; El-Atti et al., 2019). Crayfish have also been used to indicate water quality based on changes in behavioral and physiological parameters caused by various natural and chemical stimuli (Styrishave et al., 1995; Bojsen et al., 1998; Kholodkevich et al., 2008; Kuklina et al., 2014; Tierney et al., 2016; Woodman et al., 2016; Kuklina et al., 2018; Stara et al., 2019). **Chapter 2** and **3** provide insights into the responses of the crayfish *P. leniusculus* to common aquatic contaminants, pesticides, and UV filters. These chapters elucidate the specific physiological and behavioral reactions exhibited by *P. leniusculus* when exposed to these environmental stressors.

In natural environments, crayfish have been shown to accumulate substances such as pesticides, pharmaceuticals, heavy metals, metalloids, and fluoride in tissues including the hepatopancreas, gills, exoskeleton, muscles, and digestive tract (Cebrian et al., 1993; Mackevičienė, 2002; Alcorlo et al., 2006). Contaminants also accumulate in the hemolymph (Anderson et al., 1997; Bollinger et al., 1997), and bioaccumulation can be influenced by abiotic factors such as pH, salinity (Laporte et al., 1996), and temperature (Cebrian et al., 1993), as well as by the sex and age of the crayfish (Tunca et al., 2013; Stanek et al., 2017). Crayfish have also been used as bioindicators for assessing the genotoxicity of water containing pollutants, particularly heavy metals (Simonyan et al., 2018). Additionally, their ability to filter-feed, especially in juveniles, makes them ideal environmental bioindicators (Budd et al., 1978; Reynolds and O'keefee, 2005; Chucholl, 2012).

Additionally, a further application of crayfish as bioindicators is presented in **Chapter 4**. The research demonstrates a distinct response of crayfish to elevated concentrations of chlorine dioxide in water, manifested by alterations in cardiac activity. This finding suggests that crayfish can serve as effective indicators for long-term monitoring of water quality, particularly in operating conditions. Given all the above, crayfish are suitable models for assessing the toxicity level and bioavailability of chemicals in the aquatic environment, monitoring quality of water, and understanding the biological response of aquatic organisms to the stimuli.

The primary objectives of the current study were:

1. To evaluate crayfish physiological and behavioral responses to anthropogenic contaminants at environmentally relevant concentrations and investigate whether crayfish can possibly detect the presence of chemicals in water.
2. To reveal a greater potential of crayfish as excellent bioindicators using cardiac and locomotor biomarkers, in short-term laboratory experiments and long-term case study under operating conditions.
3. To identify potential knowledge gaps regarding the impact of common aquatic pollutants at environmental concentrations on freshwater crayfish species.

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CHAPTER 1.2. THE EFFECTS OF ENVIRONMENTAL CONCENTRATIONS OF AQUATIC CONTAMINANTS ON FRESHWATER CRAYFISH: A REVIEW

Malinovska, V., Kuklina, I., Kozák, P., 2023. The effects of environmental concentrations of aquatic contaminants on freshwater crayfish: A review. Manuscript.

My share on this work was about 70 %.

The effects of environmental concentrations of aquatic contaminants on freshwater crayfish: a review

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Abstract

Aquatic pollution remains one of the major concerns affecting many prime species, including dominant macrocrustaceans such as crayfish. The aim of this review was to summarize available data on the impacts of environmentally relevant concentrations of pharmaceutically active compounds (PhACs), pesticides, and heavy metals on freshwater crayfish, as well as identify possible knowledge gaps. This review predominantly emphasizes the tertiary burrowers and the surface-water part within the life cycle of secondary burrowers. We demonstrate that PhACs, pesticides, and heavy metals have significant sub-lethal impacts on adult crayfish specimens at levels currently reported in the aquatic environment. While one study reported mortality of crayfish at early life stages induced by herbicide, there is a lack of information on PhACs and metal toxicity to crayfish at early life stages. Reviewed literature showed that PhACs, pesticides, and heavy metals cause detectable behavioral responses in crayfish, including locomotor, burrowing, orientation, foraging, and agonistic alterations, even after short-term exposure. Modified antioxidant biomarkers, histology, and biochemical profile in crayfish also indicate exposure to real environmental concentrations of pollutants. Limited studies were found involving the effects of contaminant mixtures. However, such investigations are needed as they are more relevant, considering that crayfish are most likely exposed to pollutant combinations in natural waters. The number of studies involving toxicity of environmental concentrations of contaminants on freshwater crayfish is likely to grow in the future, given the increasing anthropogenic influence on water pollution and continuous pollutant release into aquatic ecosystems.

Keywords: *freshwater pollutants, heavy metals, macroinvertebrate, pesticides, pharmaceuticals*

1.2.1. Importance of freshwater crayfish in aquatic ecosystems

In many freshwater and terrestrial environments, crayfish are often considered dominant decapods due to their ecological and functional significance (Momot, 1995; Reynolds et al., 2013). They are prime species among invertebrates due to their large size, longevity, biodiversity, general abundance, and function in freshwater ecosystem (Momot, 1995; Reynolds and Souty- Grosset, 2012). Predator-prey interactions between species are essential for every food web that is an integral part of the aquatic environment (Posey and Hines, 1991; Kwak and Park, 2020). Trophic activities of crayfish affect littoral and benthic zones in freshwater environments, with a substantial amount of biomass consumed in both lotic and lentic habitats (Lodge et al., 1994; Momot, 1995; Nyström et al., 1996, 1999). Crayfish are important consumers and, as polytrophic organisms, play a key role in trophic webs

(Momot et al., 1978; Nyström et al., 1996; Reynolds and Souty-Grosset, 2012). As crayfish are omnivorous, their wide food selection is represented by detritus, algae, macrophytes, invertebrates, and vertebrates (Whitledge and Rabeni, 1997; Holdich, 2002; Garzoli et al., 2014). Also, crayfish are known to demonstrate feeding plasticity, changing feeding behavior from detritivore/herbivore to scavenger/carnivore and adapting to local resources (Veselý et al., 2020). Among invertebrates, the diet of crayfish contains worms, insects, insect larvae, snails, bivalves, arachnids, and crustaceans (Lodge et al., 1994; Nyström et al., 1996; Hollows et al., 2002; Meira et al., 2019). Given their cannibalism, crayfish can occasionally feed on other crayfish (Whitledge and Rabeni, 1997). Crayfish often act as keystone predators and have been reported to consume up to 50% of stream benthic production (Rabeni, 1992; Momot, 1995). The vertebrate diet of crayfish consists of fish, fish eggs and larvae, amphibians, and amphibian eggs and larvae (Dorn and Mittelbach, 1999; Ilhéu et al., 2007; Thomas and Taylor, 2013; Vollmer and Gall, 2014). Crayfish can also feed on dead and decaying plants and animal matter, improving water quality (Ameyaw-Akumfi, 1977; Souty-Grosset et al., 2006).

Predation is one of the fundamental factors affecting aquatic ecosystems through community structure moderation, top-down effects, and contribution to biodiversity and ecosystem function (Thorp, 1986; Estes, 1996). Along with the role of a predator, crayfish also act as prey for many aquatic and terrestrial predators. Crayfish are an important component in the diets of many species of fish, birds, mammals, insects, and insect larvae (Hirvonen, 1992; Jonsson, 1992; Dorn and Mittelbach, 1999; Correia, 2001; Reynolds, 2011) and often live in a habitat with the presence of multiple predators (Englund and Krupa, 2008). Hence, crayfish play an important role in the trophic interactions of not only the aquatic communities but also of the riparian and terrestrial communities in certain ecosystems (Correia, 2001).

Among crayfish, there are successful and important invasive species in freshwater ecosystems (Correia, 2003; Ilhéu et al., 2007; Linzmaier et al., 2020). As introduced crustaceans, they may impact vertical and horizontal food-chain processes and functional richness of communities leading to ecological risks (Correia, 2003; Mathers et al., 2020). Along with negative impact of non-native crayfish, there can also be a positive indirect effect on periphyton (Nyström et al., 1999; Twardochleb and Olden, 2013).

Crayfish often serve as hosts for different organisms as, for instance, worms, rotifers, ciliates etc. (Edgerton et al., 2002; Skelton et al., 2013). The relationship between these organisms and crayfish can be parasitic or mutualistic often having strong effects on freshwater communities and ecosystems (Sargent et al., 2014; Creed et al., 2021).

Freshwater crayfish have been defined as ecosystem engineers by several authors (Statzner et al., 2000; Creed and Reed, 2004; Reynolds, 2011; Souty-Grosset et al., 2016). According to Jones et al. (1994) ecosystem engineers are "organisms that directly or indirectly modulate the availability of resources (other than themselves) to other species by causing…state changes in biotic or abiotic materials. In so doing they modify, maintain and/or create habitats". Studies show that crayfish activities as ecosystem engineers involve alteration of sand and gravel erosion, algae growth, and impact on the abundance of fine particulate matter, chironomids, and harpacticoid copepods (Statzner et al., 2000; Creed and Reed, 2004). Therefore, crayfish as keystone species affect aquatic communities, habitats, and ecosystems through ecosystem engineering and trophic interactions (Wright and Jones, 2006; Reynolds, 2011).

Given their benthic nature, crayfish come in contact with surrounding sediments and are exposed to contaminants accumulated in the bottom (Alcorlo et al., 2006). Crayfish, as many aquatic organisms, can absorb chemicals from water through the gills, through the body surface or can ingest pollutants with prey (Katagi, 2010). When crayfish basic behavioral patterns are affected by abiotic factors (e. g. water quality), this may lead to changes in their interactions with the environment (Reynolds and Souty-Grosset, 2012). Furthermore, the trophic interaction between crayfish and its predators can potentially intensify pollutant biomagnification by conserving it at higher levels in top predators within the food web (Bowling et al., 2011; Johnson et al., 2014).

1.2.2. Environmental contaminants in freshwaters

Despite growing concern about impacts on aquatic organisms, a large number of pollutants are constantly entering the freshwater environment (Holt, 2000; Ebele et al., 2017). Pharmaceutically active compounds (PhACs), pesticides, and heavy metals are among the most frequently detected pollutants in aquatic ecosystems (Heberer, 2002; Kasprzyk-Hordern at al., 2008; de Souza et al., 2020; Essien et al., 2022). Many PhACs and pesticides are categorized as emerging contaminants which means that their presence in ambient environment has been studied in detail only in the last two decades (Noguera-Oviedo and Aga, 2016). Key pollutants such as heavy metals are ubiquitous in freshwater ecosystems due to natural sources and anthropogenic activities and their non-biodegradable nature, and accumulation (Kapahi and Sachdeva, 2019).

Pollutants are released into freshwater environment through multiple pathways, including wastewater treatment plant (WWTP) effluents (da Silva Oliveira et al., 2007; Zhou et al., 2009; Münze et al., 2017). The efficiency of WWTPs depends on the type of compound and applied treatment technologies. WWTP removal efficiencies are 15–99 % (Ahmed et al., 2017; Park et al., 2017), 0–98 % (Ahmed et al., 2017; Le et al., 2017) and 17–99 % (Busetti et al., 2005; Zhao et al., 2016), for PhACs, pesticides, and metals, respectively. Most of the compounds belonging to these groups of pollutants are constantly introduced to aquatic environment leading to chronic exposure and subsequent accumulation in water biota (Qu et al., 2011; Tunca et al., 2013; Xie et al., 2017).

Persistence, bioavailability, toxicity, and general behavior of pollutants in the aquatic environment can be affected by a combination of abiotic and biotic, natural, and anthropogenic factors. Pharmaceuticals reduction in surface waters depends on their photodegradation, hydrolysis and biodegradation. These dominant mechanisms are determined by pharmaceutical molecular structure and water parameters (pH, temperature, etc.) (Baena-Nogueras et al., 2017). The photochemical behavior of pharmaceuticals is further affected by sunlight irradiation, water depth, eutrophic conditions, interaction with dissolved organic matter, singlet oxygen, hydroxyl radicals, etc. (Challis et al., 2014; Ebele et al., 2017). Biodegradation of pharmaceuticals is heavily influenced by microorganism content in water (Caracciolo et al., 2015). Pesticide behavior and contamination in surface waters mainly depends on properties of the soil where pesticides were applied, crop management practice, climatic conditions, precipitation, and pesticide characteristics including half-life (Gavrilescu, 2005). The physical and chemical processes of transformation of heavy metals and their allocation either to the water or sediment are mainly affected by water parameters such as dissolved oxygen, pH, temperature, redox potential, chemical content, and the conductivity of the solution of ion concentration (Hu et al., 2020).

Reported concentrations of PhACs, pesticides, and metals typically range in magnitude from ng to µg/L (Kumar et al., 2019), often with high seasonal variability (Konstantinou et al., 2006; Wang et al., 2017; Duan et al., 2021). PhACs seasonal variability depends on the compound type, locality, and hydrologic conditions (Duan et al., 2021; Molnar et al., 2021). Pesticide concentrations are mainly influenced by farming season with higher levels in the late spring and summer period (Konstantinou et al., 2006). Seasonal changes in heavy metal concentrations are compound-specific indicating an increased level of some elements in wet seasons, and others in dry seasons (Wang et al., 2017).

1.2.3. The impact of pollutants at environmentally relevant concentrations on crayfish

1.2.3.1. The effects of pharmaceuticals and illicit drugs

Pharmaceutically active compounds (PhACs) are a diverse group of chemicals extensively used in human and veterinary medicine (Jjemba, 2008). These persistent and biologically active compounds with a low rate of biodegradation are often water soluble, making them easily appear in the aquatic environment (Kümmerer, 2008). Effects on aquatic species include reproductive toxicity, cytotoxicity, induced behavioral alterations, histological changes, and biochemical responses (De Lange et al., 2006; Martin-Diaz et al., 2009; Lang and Kohidai, 2012; Runnalls et al., 2013; Hamid et al., 2022). Given their increasing use, steady release into environment and persistent nature, PhACs frequently reach and exceed detectable concentrations in surface waters (Ebele et al., 2017; Dey et al., 2019). Over the last decade, investigations on the effects of PhACs on aquatic species have increased, showing adverse impacts even at low concentrations (Bluthgen et al., 2013; Runnalls et al., 2013; Pelli and Connaughton, 2015). PhACs pose a risk to non-target organisms as they were designed to target a specific metabolic or molecular pathway at low doses (Fent et al., 2006). In addition, many PhACs can be easily accumulated by freshwater crayfish inhabiting natural waters (Kazakova et al., 2018, 2021).

SSRIs and SSNRIs

Selective serotonin reuptake inhibitors (SSRIs) such as sertraline and citalopram and selective serotonin-norepinephrine reuptake inhibitors (SSNRIs) such as venlafaxine are commonly prescribed drugs to treat depression and anxiety disorders (Fong and Ford, 2014). These antidepressants have been detected in surface fresh waters at concentrations up to 76 µg/L (Brooks et al., 2003; Fick et al., 2010; Hossain et al., 2019; Khulu et al., 2022). SSRIs and SSNRIs affect brain function by blocking presynaptic serotonin reuptake transporters and modulating levels of neurotransmitters serotonin and norepinephrine, respectively (Harmer et al., 2017). Serotonin (5-HT) and norepinephrine are known to be present in the nervous system of crustaceans and impact their behavior, physiology, and immune system (Beltz and Kravitz, 2002; Cooper et al., 2003; Hsieh et al., 2006; Chang et al., 2011). Serotonin is involved in regulation of crustaceans' aggressive behavior, social status, escape response, locomotion, reproduction, cardiac activity, glucose level, and production of pigment dispersion (Fingerman et al., 1994; Huber et al., 1997; Beltz and Kravitz, 2002). Recent studies have revealed that the effect of antidepressants on the behavior and physiology of crustaceans is extensive. When exposed to 0.5 µg/L of citalopram for two weeks, spiny-cheek crayfish *Faxonius limosus* exhibited significant alterations in chemical cue preferences (Reisinger et al., 2021). Crustaceans use chemoreceptors in diverse biological processes including finding mates, demonstrating dominance, identifying conspecifics, food, habitats, and assessing the presence of predators. Alteration of the functional organization of chemoreception can impact not only behavior and critically important biological process of specimens but also community organization and ecosystem function (Hay, 2011). Citalopram-induced behavioral responses at environmental concentrations (Table S1) include increased boldness in spiny-cheek crayfish *F. limosus* (Reisinger et al., 2021) and reduced activity in marbled crayfish *Procambarus virginalis* (Buřič et al., 2018). Crayfish *P. virginalis* exposed to sertraline were more active and moved greater distances (Hossain et al., 2019). Also, sertraline has been shown to affect aggression and induce attraction response of crayfish *Faxonius virilis* (Woodman et al., 2016). These results are not surprising given that serotonin plays an integral role in modulating various behaviors. Serotonin affects muscle function by reduction of passive muscle tonus, regulation of muscle contraction and neuromuscular junctions (Gerry and Ellerby, 2011; Wu and Cooper, 2012). It also impacts motor activity by the effect on the motoneurons that drive motor contractions (Dasari and Cooper, 2004).

In contrast to documented effects of many SSNRIs in studied crayfish species, venlafaxine, a SSNRI, induced no behavioral alterations in crayfish *P. virginalis* after 21 days of exposure (Kubec et al., 2019). Authors suggested that the possible reason for such results could be the differences in experimental conditions, doses, and approaches used when compared to studies where other antidepressants affected experimental animals.

Benzodiazepine

Neuroactive anxiolytic pharmaceuticals, including oxazepam, are used in human therapeutic to treat anxiety disorders. These benzodiazepine-type drugs target the central nervous system and induce anxiolytic, sedative and muscle relaxant effects (Olkkola and Ahonen, 2008). Diazepam and oxazepam have been found in WWTP effluents at concentrations of 1 μg/L and 1.8 μg/L, respectively (Halling-Sorensen et al., 1998; Loos et al., 2013). Benzodiazepines have been shown to affect behavior and physiology of non-target organisms (Klaminder et al., 2014; Brodin et al., 2017; Lebreton et al., 2021). Seven days of exposure to oxazepam induced significant behavioral modification in crayfish *P. virginalis*. In systems without shelters, animals were more active and moved longer distances (Kubec et al., 2019). Oxazepam, similar to other benzodiazepines, depolarizes the neuron in the $GABA_A$ receptor. GABA, the major inhibitory transmitter at invertebrate synapses, is located in nervous system (Lunt, 1991). GABA receptors have been found to affect excitability of crustacean muscle (Castellote et al., 1997) and, therefore, they may be involved in the regulation of locomotor activity (Snyder et al., 2000). There was the evidence of dose-dependent responses to benzodiazepines in mice with the lowest dose causing increased activity and higher doses producing sedative effects (Loscalzo et al., 2008). This observation potentially provides an alternative explanation for the increased locomotion observed in *P. virginalis*.

Other pharmaceutical groups

Tramadol is an opioid pain medication with analgetic action over the central nervous system. Tramadol is an agonist of μ-opioid receptors and inhibitor of norepinephrine and serotinin reuptake (Grond and Sablotzki, 2012). This dual mode of action allows for treatment of both nociceptive and neuropathic pain (Schug, 2007). Impacts of observed environmental concentrations of tramadol (Table S1) on behavior of non-target organisms was associated with decreased locomotion of signal crayfish *Pacifastacus leniusculus* in response to the odor of an injured conspecific (Ložek et al., 2019) and lower velocity and shorter distance moved by the marbled crayfish *P. virginalis* (Buřič et al., 2018). Physiological effects included changes in crayfish heart rate in response to stress odor (Ložek et al., 2019). Authors suggested that such behavioral and physiological changes are related to the effect of opioid painkiller on the central nervous system and tramadol side-effect tachycardia. Aside from opioid-agonist effects, serotonin syndrome behavioral responses should be considered due to the dual mode of action of tramadol.

Sulfamethoxazole is a broad-spectrum bacteriostatic sulfonamide antibiotic widely used in medicine due to its broad spectrum and low cost (Wang and Wang, 2018; Xue et al., 2019). Sulfamethoxazole competitively inhibits *p*-aminobenzoic acid in the folic acid and, consequently, suppress the multiplication of bacteria (Sarmah et al., 2006). Crayfish exposed to this antibiotic at environmental concentration (0.1 μg/L) have shown to increased susceptibility to White Spot Syndrome Virus. In contrast, higher sulfamethoxazole concentration (1 μg/L) may have an immunostimulatory effect on crayfish for a short period of time (Hernández-Pérez et al., 2020).

Illicit drugs

Methamphetamine and cocaine are psychostimulants that belong to the most highly abused groups of drugs worldwide (Elkashef et al., 2008; Fontes et al., 2020). Chronic exposure to methamphetamine alters neuronal plasticity in the central nervous system and leads to behavioral sensitization to subsequent exposures (Robinson and Berridge, 1993; Iwazaki et al., 2006). Cocaine inhibits the transport of serotonin, dopamine and norepinephrine (Ritz et al., 1990), which are involved in control of sleep, thermoregulation, and appetite (Fontes et al., 2020). The concentrations of methamphetamine and cocaine in WWTP effluents and surface waters reach 2 μg/L (Yadav et al., 2017; Zeqiong et al., 2017; Fontes et al., 2020). Environmentally relevant concentrations of methamphetamine have been shown to reduce hiding and burrowing behavior of crayfish after 7 days of exposure (Hossain et al., 2019; Guo et al., 2020). In another study, chronically exposed crayfish exhibited weaker locomotor and cardiac responses to acute stress induced by exposure to haemolymph of an injured conspecific (Ložek et al., 2020). An increase in the boldness and a decrease in the feeding activity of crayfish treated with cocaine have been detected. Alterations in such basic behavioral and physiological processes by illicit drugs can lead to crucial consequences in nature due to the affected integrative response of crayfish to internal and external factors. In addition, exposure to illicit drugs leads to the inhibition in acetylcholinesterase (AChE) activity (De Felice et al., 2022). The enzymatic activity of AChE involves the hydrolytic degradation of acetylcholine, which serves as the primary neurotransmitter in the sensory and neuromuscular systems (Xuereb et al., 2009). Acetylcholine is involved in various brain functions, as well as in the coordination of muscle contractions (Sam and Bordoni, 2020; Waxenbaum et al., 2021). Given the pivotal role of AChE in nervous system function (Quinn, 1987), this may be the reason for behavioral modification of exposed animals and, also, that confirm the mode of action of illicit drugs (De Felice et al., 2022).

Mixture of PhACs

Along with described above impacts of individual chemicals on crayfish, the combined effect of psychoactive PhACs have also been studied in crayfish. Hossain et al. (2021) reported altered crayfish behaviors with and without shelter after exposure to the mixture of sertraline, citalopram, venlafaxine, oxazepam, tramadol, and methamphetamine. Toxicity of PhACs combinations depends on synergistic or antagonistic interaction between the mixture components (Watanabe et al., 2016). Studies show that mixture effects of PhACs often differ from the effects measured singly (Cleuvers, 2003; Hossain et al., 2021).

1.2.3.2. The effect of pesticides

The growth of human populations and related increases in crop demand and protection lead to increasing pesticide application worldwide (Popp et al., 2013). Pesticides are used not only to protect agricultural land and stored grain, but also to maintain flower gardens and control pests that transmit various diseases (Gill and Garg, 2014). Parent compounds of pesticides and their metabolites enter the aquatic environment via spray drift, surface runoff from agricultural land, leaching, and wet and dry atmospheric deposition (Cessna et al., 2005; Tiryaki and Temur, 2010). This results in natural and synthetic agrochemicals unintentionally affecting a wide range of non-target taxa (Schäfer et al., 2011).

Herbicides

Herbicides are widely used in agricultural activities to control undesirable plant growth and increase production of desired crops (Subba Rao and Madhulety, 2005). Commercial herbicides represent a range of different modes of action depending on class of the chemical (Dayan, 2019).

Atrazine is a selective triazine herbicide that inhibits photosynthetic electron transport (Tuffnail et al., 2008). Historically, atrazine has been one of the most frequently detected pesticide in surface and ground waters (Belluck et al., 1991; Comber, 1999; Vryzas et al., 2011). Atrazine is an endocrine disruptor with potential to affect sexual development and reproductive processes of non-target organisms (Hayes et al., 2002; Rayner and Fenton, 2011; Silveyra et al., 2022). It can also cause modified chemosensory responses. Crayfish (*Faxonius rusticus*) exposed to atrazine may lose their attraction to conspecifics or food odor sources (Belanger et al., 2015, 2017) with chemosensory abilities not recovered even after 72 h post-atrazine exposure (Belanger et al., 2016). This may be due to induced DNA damage in cells of the lateral antennules, including olfactory sensory neurons (Abdulelah et al., 2020). A properly functioning olfactory system is one of the critical endpoints for crayfish survival in nature. Disruption of olfactory senses affects such ecologically important behaviors as orientation toward long-distance stimuli, interaction with conspecifics, predator avoidance, mating, foraging behavior, and learning (Derby and Weissburg, 2014). Atrazine at environmental concentrations (Table S2) appears to alter biochemical parameters, and damage tissues and enzymes involved in natural detoxification systems. This may further impair health of exposed crayfish (Stara et al., 2018; Awali et al., 2019; Hadeed et al., 2022). Early life stages of *P. virginalis* exposed to atrazine 2-hydroxy, metabolite of atrazine, exhibited significantly lower weight compared to control (Velisek et al., 2017). Reduction of growth could be explained by the depletion of energy reserves due to the increased energetic costs of toxicant elimination. Alternately, growth could be affected by disrupted hormonal processes. Growth of crayfish is impossible without molting. Given that atrazine is an endocrine disruptor, its metabolite may alter the juvenile hormone of crustaceans and related endocrine-regulated processes involved in molting stimulation and control (Rodríguez et al., 2007).

Chloroacetanilide herbicides and their degradation products have been shown to cause oxidative stress in non-target organisms, which may lead to neurological and sensory effects. This can lead to certain alterations in agonistic behaviors and foraging patterns (Stara et al., 2019; Ramesh et al., 2023). Metolachlor is a member of the chloroacetanilide herbicide chemical family that affects protein and lipid synthesis, membrane function, respiration, and photosynthesis in susceptible plants (Chesters et al., 1989). Metolachlor is toxic to many macroinvertebrates at high enough doses. Lower doses may induce sublethal effects, affecting crayfish foraging and fight behavior (Cook and Moore, 2008; Alacantara et al., 2019). Interestingly, foraging behavior was impacted only under a variable flow regime, suggesting that effects are interactive with other environmental variables (Alacantara et al., 2019). Another chloroacetanilide herbicide, s-metolachlor, and its metabolite, metolachlor OA, can be detrimental to early life stages of *P. virginalis*. Higher mortality, delayed ontogenetic development, slower growth, excited behavior, and reduced production of antioxidant enzymes demonstrates a high sensitivity of juvenile crayfish to s-metolachlor and its metabolite (Velisek et al., 2018, 2019).

Chloridazon is a widely applied systemic herbicide that belongs to the group of pyridazinone-derivatives and is mainly used for beet crops (Hollender et al., 2009; El-Said et al., 2018). Studies with chloridazon at environmental concentration (Table S2) show that although there may be no visible effects of chemical exposure in non-target organism, vital parameters such as biochemical profile and histology may be altered in both adult and juvenile crayfish (Velisek et al., 2020b; Chabera et al., 2021). Oxidative stress markers, biochemical and histological changes, and genotoxicity of crayfish are all useful tools in research related to pesticide toxicity (Stara et al., 2014, 2016; Velisek et al., 2014, 2020a,b; Costa et al., 2018). Antioxidant biomarkers, histology, and biochemical profile provide essential information about the proper functioning of the whole organism. Antioxidant defense mechanisms include a number of enzymes and molecules responsible for the removal of various radicals and regulating the oxidative stress. These processes are important for nervous and immune system, intestinal microbiome regulation, stress sensing and response, and eventually, for detoxification and xenobiotic handling (Chaitanya et al., 2016). Biochemical indices commonly used in research include total protein level (used to infer physiological condition) alanine aminotransferase (indicator of liver function and hepatopancreatic damage), glucose (a prominent source of energy), and many more (Depledge and Bjerregaard, 1989; Stara et al., 2016; Peng et al., 2018; Chabera et al., 2021). Exposure to herbicide metazachlor caused alterations in several behavioral locomotor parameters of crayfish including distance moved, walking speed, and avoidance behavior (Buřič et al., 2013, Velisek et al., 2020a; Malinovska et al., 2023). Pesticides may also cause chemical irritation resulting in behavioral responses (DuRant et al., 2007; Guedes et al., 2009).

Insecticides

Insecticides originate from chemical and biological sources and are applied to many environments including agriculture, gardens, homes, and offices. Insecticides can bind to different target and nontarget enzymes and receptors, with toxicity levels depending on the mode of action (Sánchez-Bayo, 2011; Gupta et al., 2019).

Neonicotinoid insecticides act as nicotinergic acetylcholine receptor blockers (Thyssen and Machemer, 1999) with subsequent dysfunction of the nervous system and/or immobilization of the organism (Anderson et al., 2015). Exposed crayfish may exhibit significant behavioral alterations. Imidacloprid exposure has been associated with reduction of defensive behaviors in crayfish (Sohn et al. 2018). Clothianidin-exposed crayfish may demonstrate altered agonistic and foraging behaviors, most likely due to chemosensory dysfunction (Scholl et al., 2022). Given the neurotoxicity of neonicotinoid insecticides and their potential to disrupt central nervous system activity, exposure may result in significant behavioral changes with severe consequences for animals contaminated environments. The ability of neonicotinoids to interact with chemosensory proteins may result in chemosensory dysfunction of crayfish (Li et al., 2015; Scholl et al., 2022). Imidacloprid can impair the function of chemosensory proteins (Li et al., 2017) with the potential to inhibit function of crayfish olfactory receptor nerve cells. Effects on various biochemical, histological, genetic, and antioxidant parameters can lead to further adverse behavioral and physiological effects (Marçal et al., 2020; Stara et al., 2021).

1.2.3.3. Metals

Metals naturally occur in aquatic environments in various chemical forms. Concentrations are highly dependent on natural geochemical processes and anthropogenic activities (Deb and Fukushima, 1999). Anthropogenic sources of metals into aquatic ecosystems include industrial, agricultural, and domestic wastes (Ikem et al., 2021). Heavy metals can be categorized as either essential elements that play important roles in biological processes and non-essential elements that are potentially toxic (Deb and Fukushima, 1999; Ali et al., 2019). Metals may be absorbed by crayfish through the gills, digestive tract, or other surfaces. Once absorbed they may circulate via the hemolymph to internal organs (Deb and Fukushima, 1999). The number of hemolymph cells (hemocytes) may be reduced in crayfish exposed to episodic metal contamination, followed by bacterial challenges. This can cause a reduction in immunocompetence and increased sensitivity to various infections (Ward et al., 2006).

Copper is an essential element for functioning of all living organisms (Linder, 1991), but when its level exceeds the required physiological norm, this metal becomes toxic, therefore exerting hermetic effect (Bini and Chelazzi, 2006; Rix et al., 2022). Environmentally relevant concentrations of copper (Table S3) within the upper range may impair ability of specimens to respond appropriately to chemosensory information related to food localization (Lahman et al., 2015). However, chemoreception recovery is possible after 21 d of depuration (Lahman and Moore, 2015). Zinc, like copper, is an essential element that is necessary for biological activity, but it can adversely affect aquatic organisms at higher concentrations in environment (Brinkman and Johnston, 2012). At least some crayfish species (*Procambarus clarkii)* do not bioaccumulate zinc when exposed to environmentally relevant concentrations (Maranhão et al., 1999). Crayfish, as other crustaceans, are able to regulate metal body content, including zinc, below a certain threshold. The hepatopancreas plays a major role in maintaining metals at a consistent level and providing sequestration and detoxification processes (Schilderman et al., 1999; Ahearn et al., 2004). However, when the bioavailability of metals exceeds critical homeostatic thresholds, crayfish may begin to accumulate the chemicals, resulting in high body burdens (Taylor et al., 1995).

Biologically non-essential elements such as cadmium and uranium have similar biochemical pathways of entering an organism as essential metals (Schilderman et al., 1999). Cadmium at environmentally relevant concentration is accumulated in crayfish tissues after 96 h of exposure without caused mortality, even at higher exposure concentration of 100 μg/L (Maranhão et al., 1999). Uranium has been detected in crayfish gills and hepatopancreas after 4 and 10 d of exposure to 30 μg/L with no significant mortality observed (Kaddissi et al., 2011). Long-term exposure to uranium may lead to decreases in antioxidant activity, resulting in increased oxidative stress in crayfish (Kaddissi et al., 2012). These results show that crayfish, similar to other decapods, do not regulate body concentrations of non-essential metals but accumulate them without significant excretion (Rainbow and White, 1990). Toxicity of non-essential metals can be time- and dose-dependent. For instance, concentrations of uranium and lead in the organs of exposed crayfish initially increased and then decreased over time (Anderson et al., 1997; Kaddissi et al., 2011).

1.2.4. Conclusions and recommendations

The importance of using living organisms, especially keystone species, in toxicological research has been demonstrated over the past several decades. Freshwater crayfish are a keystone species and thus may serve as critical bioindicators of pollution in the aquatic environment. Along with their prime role in aquatic and terrestrial food webs and overall importance to freshwater ecosystems, crayfish also provide benefits to humans in terms of a niche food item and cultural significance worldwide. Studying the effects of environmental contaminants on crayfish is essential not only for ecological reasons but also for human health concerns. Crayfish can accumulate toxicants in their tissues, which can then be transferred to humans through consumption. Studying the mechanisms behind bioaccumulation and effects of contaminants on crayfish can provide crucial information for assessing the health of aquatic ecosystems and the potential risks associated with the consumption of contaminated crayfish by humans.

The ubiquitous use of PhACs, pesticides, and heavy metals for industrial and domestic purposes has led to adverse consequences for organisms exposed to these chemicals in different aquatic habitats. Multiple pollutants typically occur simultaneously in aquatic ecosystems, with organisms are exposed to mixtures rather than single contaminants only in natural systems. Compounds within different families of molecules and/or exhibiting differing modes of action can have synergistic, antagonistic, or additive interactions when combined. Synergistic interactions result in higher toxicity than a single compound, whereas antagonistic interactions have lower toxicity than individual compounds. Additive action involves the addition of toxic effects of one chemical to another (Di Poi et al., 2018). To our knowledge, relatively few studies have been devoted to investigating mixture toxicity effects on the freshwater crayfish compared to single compound effect. We suggest that further research priorities may be addressed to assessment of the potential toxicity and impacts of not only real environmental concentrations of contaminants and their degradation products, but also their relevant mixtures.

Future study may also focus on investigating the chronic effects of pollutants on crayfish species. Since most of the contaminants described in this review continuously enter aquatic ecosystems and have a long half-life in water, further alterations in experimental organisms may occur after chronic exposure. In addition, the ability of crayfish to adapt to such long-term exposure may be considered as another point for future research.

Another important insight from this review is that environmental concentrations of PhACs, pesticides, and heavy metals are non-lethal for adult freshwater crayfish. One study showed that the herbicide s-metolachlor caused higher mortality in early life stages of *P. virginalis*. These findings suggest that adult specimens are potentially more resistant to environmental pollution than juveniles. Since data on toxicity to crayfish early life stages are limited, particularly when exposed to PhACs and metals, it is difficult to assess the general impact on juveniles, including their mortality rates.

Although pollutant levels that occur in nature do not cause mortality in adult crayfish, they seriously affect organism activities and functioning (so called sublethal effects). Alterations in antioxidant biomarkers, histology, and biochemical profile, caused by contaminant levels currently detected in the environment, can lead to serious damage and impair the proper functioning of the whole organism. This review demonstrated that real environmental concentrations trigger detectable behavioral responses of crayfish including locomotor, burrowing, orientation, foraging, even after short-term exposure. In addition, metals at levels currently reported in the environment are easily accumulated in different crayfish tissues. This may pose a significant health risk for human considering high consumption rate of crayfish globally.

Significant progress has been made over the past 30 years in understanding the effects of environmentally relevant concentrations of pollutants on freshwater crayfish. These investigations are likely to continue given the persistent release of pollutants into surface waters, the emergence of newly manufactured chemicals, and the associated undiscovered impacts on aquatic invertebrates.

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Table S1. *Studies on toxicity of pharmaceutically active compounds at environmentally relevant concentrations to freshwater crayfish species.*

Medicine class	Compound name (single or mixture)	Crayfish species	Exposure concentration, µg/L	Duration of exposure	Toxicological impacts	Reference
SSRIs	Citalopram	*Faxonius limosus*	0.5	14 d	Alterations in chemical cue preferences, increased boldness	Reisinger et al., 2021
		Procambarus virginalis	1	21 d	Lower velocity and shorter distance moved	Buřič et al., 2018
	Sertraline				Higher velocity and activity, longer distance moved, increased time spent outside the shelter	Hossain et al., 2019
		Faxonius virilis	0.42	7 d	Increased aggressivity when paired with unexposed specimen	Woodman et al., 2016
Benzodiazepines	Oxazepam	*Procambarus virginalis*	1		Altered distance moved and activity	Kubec et al., 2019
Opioids	Tramadol				Shorter distances and lower velocity	Buřič et al., 2018
		Pacifastacus leniusculus		21 d	Affected heart rate and locomotion	Ložek et al., 2019
Antibiotic	Sulfamethoxazole		0.1, 1		Affected immune-related genes in hemocytes and intestine, increased susceptibility to White Spot Syndrome Virus	Hernández-Pérez et al., 2020
					Increase of chitinolytic bacteria in the intestinal microbiome	Hernández- Pérez et al., 2022
Illicit drugs	Methamphetamine	*Procambarus clarkii*	1	7 d	Lower depth and volume of burrows constructed by females	Guo et al., 2020
		Procambarus virginalis			Increased time spent outside the shelter	Hossain et al., 2019
		Pacifastacus leniusculus		21 d	Weaker locomotor and cardiac responses to stress	Ložek et al., 2020
	Cocaine	*Procambarus clarkii*	0.05, 0.5	14 d	Inhibition of AChE activity	De Felice et al., 2022
Mixture	sertraline, citalopram, venlafaxine, oxazepam, tramadol, methamphetamine	*Procambarus virginalis*	1	21 d	Impacted locomotion, increased time spent outside the shelter	Hossain et al., 2021

Table S2. *Studies on toxicity of pesticides at environmentally relevant concentrations to freshwater crayfish species.*

Type of pesticide	Compound name (single or mixture)	Crayfish species	Exposure concentration, μg/L	Duration of exposure	Toxicological impacts	Reference
Herbicide	Atrazine	*Faxonius virilis*	10, 40, 80, 100	10 d	DNA damage in cells of the lateral antennules	Abdulelah et al., 2020
					Damaged hepatopancreatic tissue	Hadeed et al., 2022
				1, 2, 4, 7, 10 d	Differential expression and activity of enzymes CYP450 and GST	Awali et al., 2019
			80	96 h	Crayfish were less successful at finding the food source and were not able to recover chemosensory abilities 72 h post-exposure	Belanger et al., 2016
		Faxonius rusticus			Diminished conspecific odor recognition	Belanger et al., 2017
				72 h	Altered chemosensory abilities to localize food sources	Belanger et al., 2015
		Cherax destructor	6.86	14 d	Affected biochemical hemolymph parameters (lactate, alkaline phosphatase), activity of superoxide dismutase, histology of gill tissue	
	Atrazine 2-hydroxy	*Procambarus virginalis*, early life stages	0.66	77 d	Decreased growth; higher catalase activity	Velisek et al., 2017
	Metolachlor	*Faxonius rusticus*	2	22 h	Impacted foraging behavior under the variable flow regime	Alacantara et al., 2019
			80	96 h	Species were less likely to initiate and win encounters	Cook and Moore, 2008
	S-metolachlor	*Procambarus virginalis*	4.2	28 d	Changes in catalase activity of hepatopancreas and gill, glutathione (GSH) level, gill epithelium, destruction of vascular tissue	Stara et al., 2019
			1.1	45 d	Higher mortality, delay ontogenetic development with accompanied slower growth; increase of total distance moved, walking speed	Velisek et al., 2019

Type of pesticide	Compound name (single or mixture)	Crayfish species	Exposure concentration, μg/L	Duration of exposure	Toxicological impacts	Reference
	Metolachlor OA		4.2	28 d	Changes in catalase activity of hepatopancreas and gill, glutathione levelaspartate aminotransferase, alanine aminotransferase, total ammonia, inorganic phosphate	Stara et al., 2019
				45 d	Affected growth, superoxide dismutase, catalase and glutathione s-transferase activity	Velisek et al., 2018
	Chloridazon	*Pacifastacus leniusculus*	0.45, 2.7	30 d	Impacted CAT activity and GSH level in hepatopancreas and gill, GLU, LACT, ALT, AST in haemolymph	Chabera et al., 2021
		Procambarus virginalis, early life stages	2.7	50 d	Higher glutathione S- transferase activity and reduced glutathione level	Velisek et al., 2020b
	Chloridazon-desphenyl	*Pacifastacus leniusculus*	0.45	30 d	GLU, LACT, ALT, AST, NH3, and Ca in haemolymph; lipid peroxidation levels in hepatopancreas; CAT activity, GSH level in hepatopancreas and gill	Chabera et al., 2021
	Prometryne	*Procambarus clarkii*	0.51	11, 25 d	Changes activity of the antioxidant enzymes SOD, CAT, and GR	Stara et al., 2014
		Procambarus virginalis, early life stages		53 d	Histopathological changes of gills	Velisek et al., 2014
	Penoxsulam	*Procambarus clarkii*	20	7 d	Genotoxicity, DNA damage	Costa et al., 2018
			2.3, 23	*ex vivo* exposure	Sperm DNA damage	Marçal et al., 2020
	Glyphosate		9, 90			
	Metazachlor	*Procambarus virginalis, early life stages*	3.2, 22	40 d	Affected distance moved, walking speed, growth and ontogenetic development, activity of total SOD, CAT, GST, GR, and GSH	Velisek et al., 2020a
		Pacifastacus leniusculus	20	10 min	Affected distance moved and heart rate	Malinovska et al., 2023

Type of pesticide	Compound name (single or mixture)	Crayfish species	Exposure concentration, µg/L	Duration of exposure	Toxicological impacts	Reference
		Procambarus clarkii	2	28 d	Affected burrowing behaviour	Guo et al., 2021
	Metazachlor OA	*Procambarus virginalis,* early life stages	3.2	40 d	Impacted growth and ontogenetic development, activity of total SOD, CAT, GST, GR, and GSH	Velisek et al., 2020a
	Terbuthylazine- desethyl	*Procambarus clarkii*	2.9	14 d	Gill and hepatopancreas histopathology; LACT, Ca, CK, ALP of haemolymph; TBARS, CAT, GST in muscle and hepatopancreas	Stara et al., 2016
		Procambarus virginalis, early life stages	1.8	77 d	growth and significantly higher catalase activity	Velisek et al., 2017
	Atrazine 2-hydroxy, terbuthylazine 2- hydroxy, terbuthylazine-desethyl		0.66, 0.73, 1.80			Velisek et al., 2017
	Imidacloprid	*Faxonius rusticus*	1, 10	10 d	Reduction in defensive behavior	Sohn et al., 2018
	Dimethoate	*Procambarus clarkii*	2.4	*ex vivo* exposure	Genetic damage in spermatozoa, increase in oxidative damage	Marçal et al., 2020
Insecticide	Thiacloprid	*Procambarus virginalis*	4.5	28 d	Affected haemolymph biochemical profile, antioxidant biomarkers, damage in gill and hepatopancreas	Stara et al., 2021
	Clothianidin	*Faxonius rusticus*	1	96 h	Altered agonistic behavior, chemosensory dysfunction	Scholl et al., 2022

Table S3. *Studies on toxicity of metals at environmentally relevant concentrations to freshwater crayfish species.*

Compound name	Crayfish species	Exposure concentration, µg/L	Duration of exposure	Toxicological impacts	Reference
Copper	*Faxonius rusticus*	4.5, 45	120 h	Affected orientation ability to locate an odor source	Lahman et al., 2015
		450	10 min	Lower flicking rates and orientation to a food odor	Lahman and Moore, 2015
Uranium	*Procambarus clarkii*	30	10 d	Accumulation in gills and hepatopancreas; changes in genes' expression	Kaddissi et al., 2011
		30	30, 60 d	Accumulation in gills and hepatopancreas; changes in genes' expression; decrease in antioxidant activities; oxidative stress	Kaddissi et al., 2012
Lead		150	7 weeks	Accumulation in exoskeleton, gills, hepatopancreas, abdominal muscle, hemolymph	Anderson et al., 1997
Zinc		25, 50, 100	96 h	Non-significant accumulation changes in tail muscle, hepatopancreas, gonads, hemolymph, gut	Maranhão et al., 1999
Cadmium				Accumulation in tail muscle, hepatopancreas, gonads, hemolymph, gut	
Aluminium	*Pacifasticus leniusculus*	500	40 d	Decrease in immunocompetence (reduced ability to clear bacteria from the circulation and decrease in recovery rate of haemocyte numbers)	Ward et al., 2006

CHAPTER 2

RESPONSES OF SIGNAL CRAYFISH *PACIFASTACUS LENIUSCULUS* TO SINGLE SHORT-TERM PULSE EXPOSURE OF PESTICIDES AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS

Malinovska, V., Kuklina, I., Lozek, F., Velisek, J., Kozak, P., 2023. Responses of signal crayfish *Pacifastacus leniusculus* to single short-term pulse exposure of pesticides at environmentally relevant concentrations. Environmental Science and Pollution Research 30, 51740–51748.

My share on this work was about 40 %.

Environmental Science and Pollution Research (2023) 30:51740–51748
https://doi.org/10.1007/s11356-023-25908-7

RESEARCH ARTICLE

Responses of signal crayfish *Pacifastacus leniusculus* to single short-term pulse exposure of pesticides at environmentally relevant concentrations

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Received: 29 April 2022 / Accepted: 9 February 2023 / Published online: 23 February 2023

Abstract

Although pesticides are often discharged into surface waters in pulses as opposed to a sustained release, the effect of episodic pollution events on freshwater crayfish is largely unknown. We monitored change in heart rate and distance moved to assess the response of signal crayfish *Pacifastacus leniusculus* to short-term exposure to environmentally relevant concentrations of metazachlor (MTZ), terbuthylazine (TER), and thiacloprid (TCL). Crayfish exposed to 20 μg/L of MTZ exhibited a significant increase in mean heart rate and distance moved. Increased heart rate was detected at 118 ± 74 s post-exposure to MTZ. There were no significant differences in mean heart rate and distance moved in crayfish exposed to 6 μg/L of TCL and 4 μg/L of TER. A significant correlation between heart rate and distance moved was found in all exposed groups. These results suggest that pulse exposure to MTZ impact crayfish physiology and behavior during short-term period. With pulse exposure to TCL and TER, crayfish not exhibiting a locomotor response may continue to be exposed to lower, but potentially harmful, levels of pollutants. Evidence of the impacts of pesticide pulse at environmentally relevant concentrations on crayfish is scarce. Further study is required to determine the ecological effects of such events on freshwater crayfish.

Keywords Freshwater invertebrate · Locomotor activity Metazachlor · Short-term exposure · Terbuthylazine · Thiacloprid

Introduction

Runoff of contaminants from agricultural land into aquatic ecosystems has long been a concern (Gao et al. 2008; Matin et al. 1998; Palma et al. 2014; Wan et al. 2021), and the impact has accelerated with the expansion of cultivated areas and accompanying increase in the application of agrochemicals (Benbrook 2016; Dobrovolski et al. 2001; Oerke 2006). Numerous studies provide evidence of pesticide residues in surface waters worldwide (De Geronimo et al. 2014; Herrero-Hernandez et al. 2020; Jergentz et al. 2005; Papadakis et al. 2018) with concentrations often exceeding the safety levels (Jergentz et al. 2005; Papadakis et al. 2018). Agricultural activities have been shown to induce significant adverse effects on non-target species, including crayfish (Bunzel et al. 2015; Rosi-Marshall et al. 2007; Sohn et al. 2018; Stara et al. 2019). Exposure to pesticides can result in behavioral, biochemical, and histological alterations in freshwater crayfish (Sohn et al. 2018; Stara et al. 2019).

Metazachlor [2-chloro-N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)-acetamide] is a chloroacetamide herbicide (FAO 1999), with endocrine disruption as mode of action (Kralova et al. 2015). Thiacloprid {3-[(6-chloropyridin-3-yl)methyl]-1,3-thiazolidin-2-ylidene} cyanamide is a neurotoxic neonicotinoid insecticide (EPA 2003). Terbuthylazine [N^2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine] is a triazine herbicide (EFSA 2011) that can cause endocrine disruption (Ghisari et al. 2015). These pesticides are widely used in Central Europe (Hvezdova et al. 2018; Spitzer et al. 2020) and they have been reported in European surface waters (Table 1). Moreover, these pesticides have been found to negatively impact aquatic vertebrates and invertebrates at environmentally relevant concentrations (Guo et al. 2021; Gutierrez et al. 2019; Velisek and Stara 2018). Studies show that metazachlor induced changes in

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Table 1 Concentration of pesticides detected in European surface waters and concentrations used in this study

Pesticide class	Active substance	Range (mean) of reported concentrations (µg/L)	Concentration used in this study (µg/L)	Data sources
Neonicotinoids	Thiacloprid	0.02–12.0 (5.96)	6.0	Barmentlo et al. (2018), Sanchez-Bayo and Hyne (2014), and Suß et al. (2006)
Triazines	Terbuthylazine	0.02–13.0 (4.37)	4.0	Hermosin et al. (2013), Herrero-Hernandez et al. (2013), Herrero-Hernandez et al. (2017), and Lacorte et al. (1998)
Chloroacetanilide	Metazachlor	0.1–100.0 (25.8)	20.0	Kreuger (1998), Mohr et al. (2008), Ulrich et al. (2018), and Weber et al. (2018)

crayfish borrowing behavior and locomotor activity (Guo et al. 2021; Velisek et al. 2020). Zebrafish embryos exposed to thiacloprid exhibited altered avoidance and edge preference behaviors (Xie et al. 2022).

Among freshwater invertebrates, crayfish are considered keystone species because of their ecological and functional importance (Momot 1995). They can play a valuable role in monitoring environmental pollution through behavioral and physiological alterations and contaminant accumulation (Faria et al. 2010; Gago-Tinoco et al. 2014; Reisinger et al. 2021; Sohn et al. 2018). Non-native crayfishes are mostly used in toxicological studies due to the protected status of indigenous species (Buric et al. 2013; Velisek et al. 2020). Styrishave et al. (2007) found no differences in oxygen consumption and heart rate between native noble crayfish *Astacus astacus* and non-native signal crayfish *Pacifastacus leniusculus*. Such similarities can help to understand potential impacts on native crayfish populations, using the data from investigations with non-native species. Like many aquatic organisms, crayfish absorb chemicals from water through gills and the body surface in addition to ingesting pollutants along with prey (Katagi 2010). Crayfish are exposed to accumulated contaminants through contact with bottom sediments (Alcorlo et al. 2006) and are affected by pollutants, including pesticides, present in surface waters (Gago-Tinoco et al. 2014; Marcal et al. 2020; Sohn et al. 2018).

Pesticide concentrations in aquatic ecosystems increase with surface runoff (Liess et al. 1999) which is often episodic (Thurman et al. 1991), with concentrations varying depending on the time of application and precipitation events (Albanis et al. 1998). The majority of research into pesticide effects on crayfish focus on chronic exposure and show changes in crayfish antioxidant levels, histology, and behavior (Guo et al. 2021; Stara et al. 2020; Velisek et al. 2020). The response of crayfish to acute exposure to pesticides remains unclear. Since pulse exposure to pesticides has been reported to affect macroinvertebrates (Heckmann and Friberg 2005), it is important to know whether short-term pulses of agrochemicals adversely affect prime players in the freshwater environment, such as crayfish.

The objective of the present study was to quantify the acute response of the signal crayfish *P. leniusculus* to a brief pulse of metazachlor, terbuthylazine, or thiacloprid at environmentally relevant concentrations, as assessed by cardiac and locomotor activity. Crayfish have been known to exhibit alterations in cardiac and locomotor activity as responses to a wide variety of environmental stressors (Bini et al. 2015; Kuklina et al. 2014; Lozek et al. 2019; Velisek et al. 2020). In this study, changes in heart rate and distance moved were monitored to gain information of crayfish response to acute pesticide exposure.

Materials and methods

Chemicals

Metazachlor (MTZ), chemical purity 99.7%; terbuthylazine (TER), chemical purity 99.4%; and thiacloprid (TCL), chemical purity 99.9%, were purchased from Sigma-Aldrich Corporation (USA). Chemicals were dissolved in dechlorinated tap water to obtain 20 µg/L, 4 µg/L, and 6 µg/L for MTZ, TER, and TCL, respectively. Actual concentrations of chemicals in water during the experiments were within 96% of the nominal concentrations (Table 2). The analyses of pesticides in water were performed by the State Research Institute in Prague using methods described by Anastassiades et al. (2003) and Anastassiades et al. (2007).

Test organisms

Thirty-six adult signal crayfish *Pacifastacus leniusculus* (1:1 male:female) were collected from Kresanovsky Brook (49°03′35.2″N, 13°45′33.8″E) near Sumava National Park, Czech Republic. Kresanovsky Brook is located in sub-mountain area and the majority of the watershed is forested with limited urban or agricultural land use. We used non-native crayfish species as indigenous species are endangered and manipulations with them are prohibited. Crayfish were transported to the laboratory and held in individual tanks

Table 2 Concentrations of metazachlor (MTZ), terbuthylazine (TER), and thiacloprid (TCL) in exposure and control groups of signal crayfish *Pacifastacus leniusculus*

Group	Tank (*n*)	Nominal concentration (μg/L)	Concentration (μg/L) Mean ± SD	*t*	*p*-value
MTZ	6	20	19.3±1.5	-1.2	0.28
Control	6	-	< 0.010	-	-
TER	6	4	3.9 ± 0.1	− 1.93	0.11
Control	6	-	< 0.010	-	-
TCL	6	6	5.7 ± 0.3	− 2.18	0.08
Control	6	-	< 0.010	-	-

t, *t*-score. $p < 0.05$. The limit of detection for the concentrations was 0.010 μg/L

in a recirculating aquarium system for pre-acclimatization. Both sexes of crayfish were used based on previous studies that found no significant differences between their reactions to stimuli or spatial behavior (Kuklina et al. 2018; Tierney and Andrews 2013). There were no risks associated with the escape of crayfish.

Experimental protocol

The exposure concentrations were within the range reported in European surface waters (Table 1) although, because of the short exposure period, the experimental concentrations were higher than those used in long-term exposure studies (Englert et al. 2012; Guo et al. 2021).

The experiment was carried out in three phases, during which the crayfish were exposed to one of three pesticides (TCL, TER, or MTZ) or to dechlorinated tap water as control. The pesticides were each represented in a separate run. Each phase included 12 experimental crayfish: six exposed and six control specimens (3:3 male:female). Each of the three pesticide groups thus had its own control group. Heart rate was recorded using a non-invasive crayfish cardiac activity monitoring system (Pautsina et al. 2014). Briefly, this system consists of infrared (IR) sensors, a multichannel analog-to-digital converter (ADC) with USB interface, and a personal computer for data processing. The IR sensors were attached to the dorsal side of crayfish carapace above the heart with non-toxic epoxy glue. Wires that connect sensors and the ADC are flexible and allow crayfish to move freely. Heart rate was measured every second and then recorded as number of beats per minute (bpm).

To record movement, a Microsoft Kinect Sensor (Microsoft Corporation, Redmond, WA, USA) was placed under the tanks. Distance moved (cm) was measured every second and evaluated using a multiple-arena module in EthoVision XT 13.0 software (Noldus Information Technology, Wageningen, Netherlands).

Each crayfish with attached IR sensor was placed into separate non-recirculating 6-L tank (water temperature 20.3–21.5 °C, pH 7.6–7.8, dissolved oxygen 8.49–8.76 mg/L, 12:12-h light:dark cycle) for 10 days of acclimation and experimentation. The length of the tank wall was 30 cm and the width was 19 cm. The water depth in the tank was 11 cm. Twice weekly, chironomid larvae were provided and water was changed. Tanks were aerated to avoid disturbance to crayfish during pesticide application and to ensure rapid diffusion of the pesticide throughout the water. Plastic mesh was used as a substrate to provide crayfish with traction when moving. Three trials were conducted as follows: pesticide was administered to tanks simultaneously using individual peristaltic pumps. The compound is uniformly mixed in the tank during 30 s as authors tested prior to the experiment with colored liquid. Crayfish from the control group received dechlorinated tap water the same temperature as in experimental tanks. Crayfish heart rate and locomotor activity were recorded for 10 min before and 10 min after adding the pesticide. Therefore, crayfish were exposed to the pesticides for 10 min. Following the experiment, all crayfish were euthanized humanely by freezing at − 20 °C.

Statistical analysis

All data were analyzed using Statistica v. 13 (StatSoft, Inc.). Prior to statistical analysis, the normality of the residuals was checked with Shapiro–Wilk's test as the assumption for the analysis of variance (ANOVA), followed by Tukey's test to compare differences between groups. The analysis was performed separately for each tested compound and followed parameters, comparing exposure group along with its dedicated control. The depended variables in each analysis were differences (after − before) in the heart rate and the distance moved. Categorical factors represented the treatment: control and exposure, respectively. Therefore, such an approach aimed to compare the changes of heart rate and locomotor activity in a response to the chemical exposure. To examine correlation of heart rate with locomotion after chemical exposure, simple linear regression was calculated to analyze increase of mean heart rate (after exposure relative to before) of each crayfish relative to the distance moved. All values are presented as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

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Results

No significant differences were found in the biometrical parameters of the exposed and control groups of crayfish (Table S1). Changes in crayfish cardiac and locomotor activity after pesticide administration were observed in specimens of the group exposed to 20 μg/L of MTZ (Fig. 1). Significant changes in mean heart rate ($F_{1,10}=8.35$, $p=0.016$) and distance moved ($F_{1,10}=5.306$, $p=0.044$) after exposure compared to before were detected in treated crayfish. An increase in mean heart rate was detected at 118 ± 74 s post-exposure to MTZ. In the groups exposed to the concentrations of TER (4 μg/L) and TCL (6 μg/L) crayfish did not show a significant increase in mean heart rate ($F_{1,10}=1.973$, $p=0.19$; $F_{1,10}=2.019$, $p=0.186$) or distance moved ($F_{1,10}=1.726$, $p=0.218$; $F_{1,10}=1.051$, $p=0.329$) (Figs. 2 and 3). In these two groups, only 33% of specimens exhibited cardiac and locomotor response. There was no significant difference in mean heart rate or distance moved in all three control groups ($p>0.05$) (Figs. 1, 2, and 3).

A linear regression model revealed a significant correlation between cardiac activity and distance moved in all exposure groups (Fig. 4), with the strongest response found in MTZ ($b=1.73$), followed by TER ($b=0.68$) and TCL ($b=0.39$). Crayfish exposed to MTZ demonstrated four- and three-fold the movement response of those exposed to TCL and TER, respectively (Fig. 4). Changes in distance moved and heart rate showed correlation in all reacting crayfish.

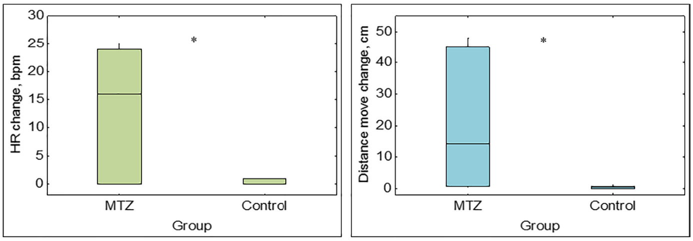

Fig. 1 Changes in mean heart rate (HR) and distance moved of *Pacifastacus leniusculus* and controls before and after metazachlor (MTZ) exposure/water change; *bpm*, beats per minute. Significant differences ($p<0.05$) are marked with asterisks (*)

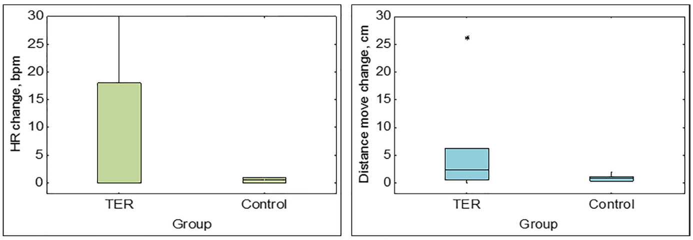

Fig. 2 Changes in mean heart rate (HR) and distance moved of *Pacifastacus leniusculus* and controls before and after terbuthylazine (TER) exposure/water change; *bpm*, beats per minute

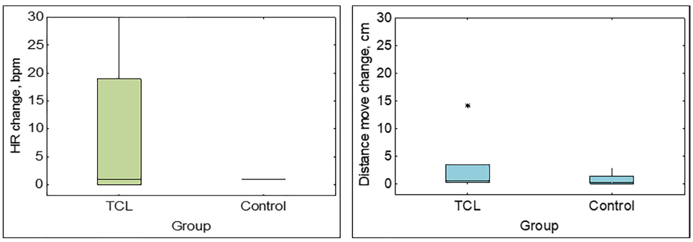

Fig. 3 Changes in mean heart rate (HR) and distance moved of *Pacifastacus leniusculus* and controls before and after thiacloprid (TCL) exposure/water change; *bpm*, beats per minute

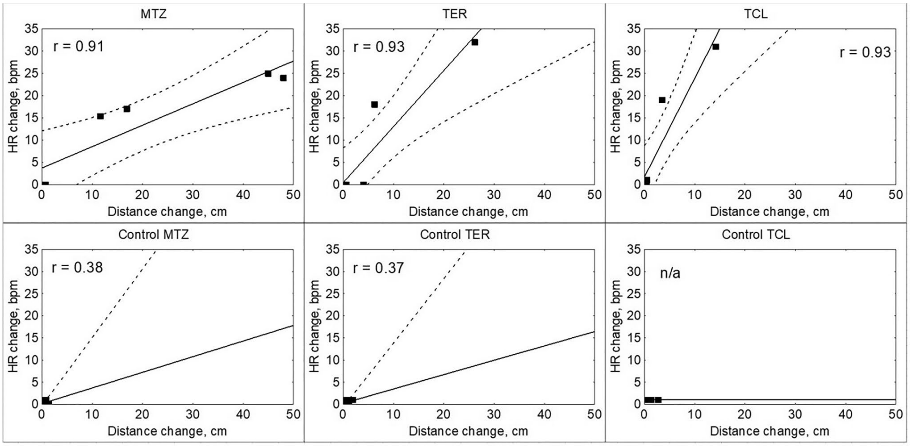

Fig. 4 The relationship between mean heart rate (HR) and mean distance moved of *Pacifastacus leniusculus* exposed to metazachlor (MTZ), terbuthylazine (TER), and thiacloprid (TCL) and respective control groups. Pearson's r = strength of the correlation between HR and distance moved

Discussion

As episodic spikes in chemical concentration are more typical of agricultural areas than a sustained release (Liess et al. 1999; Liess and Von der Ohe 2009), we quantified crayfish acute cardiac and locomotor responses to environmentally relevant pesticide concentrations. To our knowledge, this is the first study to report crayfish reaction to pulse exposure of pesticides in water.

A single exposure to chemicals at relatively high, but environmentally relevant, concentrations usually provokes detectable physiological or behavior changes (Beketov and Liess 2005, 2008a). With repeated exposure, macroinvertebrates exhibit a stronger response, possibly related to incomplete recovery from previous exposure (Berghahn et al. 2012; Mohr et al. 2012). Animals are often impacted by multiple simultaneous stressors, the effect of which depends on ambient physical and chemical parameters. The amphipod crustacean *Gammarus pulex* from contaminated streams was shown to be more sensitive to pesticide exposure than animals from an uncontaminated environment (Russo et al. 2018). Crayfish for the current experiment were obtained from a non-polluted ecosystem and acclimated to laboratory conditions for a prolonged time, so may have been less sensitive to the exposure.

With exposure to metazachlor, we detected significant changes in crayfish distance moved. An increase in locomotor activity can be the result of stimulatory effect of metazachlor. Previous studies have reported that pesticides can exhibit stimulatory effects in non-target organisms (Cutler et al. 2022; Guedes et al. 2009; Morse 1998) resulting in behavioral alterations in pesticide-exposed vertebrates and invertebrates (Deng et al. 2009; DuRant et al. 2007). Chemical irritation is often associated with stimulation of locomotor responses in aquatic organisms (Chen et al. 2014; Sharma 2019). The increased distance moved after exposure may also have represented active avoidance of the contaminated area. Velisek et al. (2019) documented an increase in distance moved in juvenile crayfish *Procambarus virginalis* exposed to the pesticide S-metolachlor. Buric et al. (2013) described attempts of crayfish *Faxonius limosus* and *Pacifastacus leniusculus* to escape exposure to the pesticide diazinon. Moreover, it has been reported that brief pesticide exposure can induce drift (Beketov and Liess 2008a) or increase drift density of a macroinvertebrate community (Heckmann and Friberg 2005). Sensitivity of ecosystem function and invertebrate population dynamics to environmental contaminants have been shown in several studies (Berenzen et al. 2005; Martin et al. 2011; Richmond et al. 2016, 2019). Drift of macroinvertebrates, driven by irritable or avoidance behavior, may lead to risks associated with predation, community structure alterations, decrease in abundance, and, consequently, affect the food chain.

Disorientation of crayfish in the presence of pesticides could be the result of temporary impairment of olfactory receptors (Cook and Moore 2008). Disruption of chemoreception can affect agonistic, feeding, and homing behavior, with juvenile crayfish potentially more sensitive to the impact of pesticides (Buric et al. 2013). The latter might partially explain the lack of reaction of some individuals in our study, since we examined adult crayfish. Metazachlor is an endocrine-disrupting agent that, among other effects, adversely impacts behavior and metabolism (Crisp et al. 1998). Increased cardiac and locomotor activity provides evidence of behavioral and metabolic disturbances in response to pesticide presence.

We did not observe significant changes in locomotor activity of crayfish exposed to terbuthylazine and thiacloprid at 4 and 6 µg/L, respectively, suggesting that, with such pesticide pulse, the majority of crayfish might not be stimulated to escape a contaminated area. This can lead to continuing exposure, as pesticide concentrations decrease over time (Ulrich et al. 2018). Concentrations as low as 0.5–1 µg/L of thiacloprid during a 96-h exposure were shown to adversely influence the predation activity of the aquatic invertebrate *Gammarus fossarum* (Englert et al. 2012). It is noteworthy that crayfish species may vary in level of sensitivity to a given substance. Buric et al. (2013) reported *P. leniusculus* to be less sensitive to diazinon treatment than was *F. limosus*. Species other than signal crayfish may exhibit greater physiological and behavior responses to terbuthylazine and thiacloprid at the tested concentrations. The low number of specimens reacting to thiacloprid might be connected with its mode of action. Like other neonicotinoids, thiacloprid stimulates nicotinic acetylcholine receptors in the central nervous system. While low activation of these receptors can manifest as nervous excitation, higher levels of thiacloprid can cause overexcitation and block the receptors, resulting in temporary paralysis (Yamamoto 1999), which may become more apparent with a longer exposure period.

In our experiment, changes in heart rate coincided with an increase in distance moved. This is in agreement with Kuklina et al. (2018), who demonstrated initiation of *Pontastacus leptodactylus* crayfish locomotion to coincide with heart rate increase as a reaction to chemical stimuli. With natural stimuli such as predator or conspecific crayfish odor, locomotion was delayed or was not manifested. Change in cardiac activity, in particular increased heart rate, is a typical stress response of crayfish to substances in water. This was demonstrated in studies of chemicals such as disinfectants, metals, and pharmaceuticals (Kuklina et al. 2014; Bini et al. 2015; Lozek et al. 2019). The cardiac response of *P. leniusculus* to tested pesticides confirms its potential to be used as a bioindicator of aquatic contamination by pesticides.

While we investigated the response of crayfish to an acute pulse of pesticide, some adverse effects might remain following the exposure. Evidence of impacts on survival and reproduction of *G. pulex* was detected for at least 2 weeks following a short pulse of the pyrethroid insecticide esfenvalerate at an environmentally relevant concentration (Cold and Forbes 2004). A single contamination event by thiacloprid can show effects on abundance and community structure of aquatic invertebrates after 7 days (Beketov et al. 2008) and, in community parameters, after 3 months (Liess and Beketov 2011). Delayed lethal and sublethal effects occurred in several freshwater crustacean species following a single thiacloprid exposure at a concentration of 5.47 µg/L (Beketov and Liess 2008b).

Conclusions

The present work demonstrates that a short-term pulse of pesticide exposure can affect non-target organisms. Acute exposure to metazachlor at an environmentally relevant concentration can induce changes in crayfish heart rate and locomotor activity. With pulse exposure to terbuthylazine, and thiacloprid, the majority of animals might not respond to contaminants during short-term period. Owing to the prime role of crayfish in freshwater environment, the knowledge of how pesticides at environmentally relevant concentrations impact these crustaceans is of key importance. Spikes in pesticide concentrations are typical of aquatic environments, and further studies of the effect of a single short-term pesticide exposure on crayfish can reveal crucial information of the ecological consequences of such events.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-023-25908-7.

Acknowledgements We thank The Lucidus Consultancy for the language editing.

Author contribution Viktoriia Malinovska: writing — original draft preparation, writing — review and editing, investigation, data analysis. Iryna Kuklina: investigation, writing — review and editing. Filip Lozek: investigation, writing — review and editing. Josef Velisek: conceptualization, writing — review and editing. Pavel Kozak: methodology, supervision, writing — review and editing, conceptualization.

Funding Open access publishing supported by the National Technical Library in Prague. The study was financially supported by the grant agency of the University of South Bohemia in Ceske Budejovice No. 019/2020/Z, the Czech Science Foundation (GACR) No. 061/2019/Z, and the Ministry of Agriculture of the Czech Republic — Project No. QK1910282.

Data availability The dataset used and/or analyzed during the current study is available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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CHAPTER 3

SHORT-TERM EFFECTS OF AN ENVIRONMENTALLY RELEVANT CONCENTRATION OF ORGANIC UV FILTERS ON SIGNAL CRAYFISH *PACIFASTACUS LENIUSCULUS*

Malinovska, V., Kuklina, I., Grabicová, K., Buřič, M., Kozák, P., 2023. Short-term effects of an environmentally relevant concentration of organic UV filters on signal crayfish *Pacifastacus leniusculus*. Ecotoxicology and Environmental Safety 259, 115012.

My share on this work was about 40 %.

Ecotoxicology and Environmental Safety 259 (2023) 115012

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Short-term effects of an environmentally relevant concentration of organic UV filters on signal crayfish *Pacifastacus leniusculus*

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ARTICLE INFO

Edited by Yong Liang

Keywords:
Behavior
Freshwater invertebrate
Heart rate
Locomotor activity
Sunscreen compound

ABSTRACT

Personal care products, including organic UV filters, are considered emerging contaminants, with their toxic effects being a concern in recent decades. UV filters continually enter surface waters via wastewater and human activity. Despite the presence of organic UV filters in the freshwater environment, little is known of their impact on aquatic biota. In this study, we evaluated the cardiac and locomotor responses of signal crayfish *Pacifastacus leniusculus* exposed to environmentally relevant concentrations of either 2-Phenylbenzimidazole-5-sulfonic acid (PBSA, 3 μg/L) or 5-Benzoyl-4-hydroxy-2-methoxybenzenesulfonic acid (BP4, 2.5 μg/L). Specimens exposed to the tested compounds for 30 min exhibited significantly greater changes in distance moved and time active than did unexposed controls. Significant differences of mean heart rate change compared to control were detected in both PBSA and BP4 experimental groups. Such behavior and physiological alterations demonstrate ecological effects of personal care products with the tested sunscreen compounds even with a short exposure. Evidence of the consequences of organic UV filters on aquatic organisms is scarce and is an important topic for future research.

1. Introduction

Organic pollutants including those contained in personal care products continually enter the aquatic environment and pose a threat to water biota (Nohynek et al., 2010; Palmiotto et al., 2018; Wick et al., 2010). Organic disinfectants, insect repellents, ultraviolet (UV) filters, are among chemicals widely used in soaps, toothpastes, fragrances, lotions, cosmetics, and sunscreens (Brausch and Rand, 2011; Parida et al., 2021; Wang et al., 2021). Growing awareness of the detrimental effects of UV radiation in recent decades has led to increased use of sunscreens (Liu and Wong, 2013). Ultraviolet filters in sunscreen products are considered emerging contaminants based on their extensive use and presence in the aquatic environment (Magi et al., 2013). The release of UV filters into surface waters occurs directly through human recreational activities (Giokas et al., 2007). Removal in sewage water treatment is often ineffective, and UV filters are indirectly infused into aquatic ecosystems (Ramos et al., 2016). Studies have reported the accumulation of UV filtering substances in tissues of aquatic organisms (Blüthgen et al., 2014; Pawlowski et al., 2019; Wang et al., 2022). Seasonal variation in use and release of UV filters, with higher concentrations in summer, may have consequences for aquatic organisms via the potential for reproductive and developmental toxicity (Cunha et al., 2022; Ma et al., 2023; Mao et al., 2019; Ramos et al., 2016; Wang et al., 2023).

2-Phenylbenzimidazole-5-sulfonic acid (ensulizole, PBSA) is a common organic UV filter used in cosmetics and is abundant in surface waters of Europe (Grabicová et al., 2013; Palmiotto et al., 2018). Studies in Europe have detected PBSA in surface waters and wastewater treatment plant (WWTP) effluent at concentrations up to 4 μg/L (Lopardo et al., 2019; Wick et al., 2010). PBSA levels can be even higher in receiving surface waters than in WWTP effluent (Lopardo et al., 2019). Negative effects of PBSA on oxidative (Huang et al., 2020) and metabolic stress levels in freshwater fish species have been reported (Grabicová et al., 2013).

5-Benzoyl-4-hydroxy-2-methoxybenzenesulfonic acid (benzophenone-4, BP4) is the most frequently detected UV filtering compound in surface waters of Europe (Kasprzyk-Hordern et al., 2009; Palmiotto et al., 2018) with concentrations up to 6.3 μg/L in surface waters and WWTP effluent (Kasprzyk-Hordern et al., 2009; Wick et al., 2010). BP4 has also been detected in drinking water in Europe at concentrations up to 62 ng/L (Rodil et al., 2012) and has been reported to alter expression of hormone-associated genes, activity of antioxidant enzymes, and liver

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https://doi.org/10.1016/j.ecoenv.2023.115012
Received 21 December 2022; Received in revised form 10 May 2023; Accepted 11 May 2023
Available online 18 May 2023
0147-6513/

health in aquatic organisms (Huang et al., 2020; Liu et al., 2015; Zucchi et al., 2011).

The functional and ecological importance of freshwater crayfish make them keystone species (Momot, 1995) frequently used as bioindicators both in the aquatic environment and under laboratory conditions (Abd El-Atti et al., 2019; Aguirre-Sierra et al., 2013; Alcorlo et al., 2006; Gago-Tinoco et al., 2014; Kuklina et al., 2014; Malinovska et al., 2020). The assessment of crayfish biological parameters such as behavior, cardiac activity, and movement can reveal information on the effects of a variety of natural and chemical stimuli (Bojsen et al., 1998; Kuklina et al., 2018; Stará et al., 2019, 1995; Tierney et al., 2016; Woodman et al., 2016). Non-native crayfish species are primarily used in scientific research due to the protected status of indigenous species and their limited sources (Buřič et al., 2013). Styrishave et al. (2007) compared native noble crayfish *Astacus astacus* and non-native signal crayfish *Pacifastacus leniusculus* and found no differences in their oxygen consumption and heart rate. These similarities make it possible to use the data from studies with non-native crayfish to help understand the potential impacts of adverse environmental conditions on native populations. Moreover, studies have shown that signal crayfish are sensitive to anthropogenic pollutants (Gonzalo and Camargo, 2012; Gunderson et al., 2021) and have similar tolerance to various toxicants compared to native species (Firkins, 1993).

Among detected UV filters, PBSA and BP4 have been shown to be the most resistant to removal by wastewater treatment, and, consequently, the most prevalent in wastewater effluent (O'Malley et al., 2020).

Despite the presence of UV filters in freshwater ecosystems (Feijtel et al., 1999; Grabicová et al., 2013; HERA, 2013; McAvoy et al., 1998; Poiger et al., 2004; Wick et al., 2010), there is little available information with respect to the response of freshwater invertebrates to these contaminants. The objective of this study was to evaluate crayfish cardiac and locomotor activity during short-term exposure to environmentally relevant concentrations of PBSA and BP4.

2. Materials and methods

2.1. Chemicals

The organic UV filters 2-phenylbenzimidazole-5-sulfonic acid (PBSA, CAS 27503-81-7, purity 99.6 %) and 5-benzoyl-4-hydroxy-2-methoxybenzenesulfonic acid (BP4, CAS 4065–45–6, purity >97 %) were purchased from Sigma-Aldrich Corporation (USA). Both were dissolved in aged tap water to prepare stock solutions of 250 μg/L and 10 mg/L of PBSA and BP4, respectively. Exposure solutions of 3 μg/L of PBSA and 2.5 μg/L of BP4 were prepared by further dilution with aged tap water. Exposure concentrations were selected based on published data of UV filter presence in European surface waters (Wick et al., 2010). To determine actual concentrations of PBSA and BP4 during the experiment, water samples were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Thermo Fischer Scientific). Five mL of water was filtered through regenerated cellulose (0.20 μm pore size), isotopically labeled internal standards were added, and the sample was analyzed using the 15-min method. Details are provided in the Supplementary methods.

2.2. Experimental animals

Adult signal crayfish *P. leniusculus* were caught by hand from Křesanovský Brook (49°03′35.2″N 13°45′33.8″E) near Šumava National Park, Czech Republic. Křesanovský Brook is located in a sub-mountain area with the majority of the watershed forested and limited urban or agricultural land use. Animals were transported to the laboratory and held individually in a recirculating aquarium system. We selected thirty-two intermolt males and females (1:1) with intact walking legs, chelae, and antennae. Carapace length and total length were measured using digital calipers (Schut Geometrical Metrology, Groningen, The Netherlands). Animal handling met the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Faculty of Fisheries and Protection of Waters, Vodňany.

2.3. Experimental protocol

Following pre-acclimation in a recirculating aquarium system, crayfish were moved into individual 6 l tanks with water temperature 19.8–21.5 °C, pH 7.6–7.8, dissolved oxygen 8.54–8.73 mg/L, and a 12:12 h light:dark cycle. Tanks were aerated to ensure rapid diffusion of the test chemicals throughout the water and to avoid disturbance of crayfish during treatment. Plastic mesh was glued to the inside bottom of each tank to provide crayfish with traction when moving.

A noninvasive crayfish cardiac activity monitoring system consisting of infrared optical sensors, analog-to-digital converter with USB interface, and computer software were used to measure heart rate (Pautsina et al., 2014). Detailed system description and guide can be found in Kuklina et al. (2019). Infrared optical sensors were attached to the carapace of the crayfish. A Microsoft Kinect Sensor (Microsoft Corporation, Redmond, Washington, USA) was placed under each tank to record distance moved (cm) and activity time (s) and analyzed using a multiple-arena module in EthoVision XT 13.0 software (Noldus Information Technology, Wageningen, Netherlands). Crayfish were acclimated for 10 days and fed with chironomid larvae twice weekly, a few hours after which the tank water was changed.

The study consisted of separate trials for the two chemicals with experimental crayfish groups consisting of eight specimens exposed to PBSA or BP4, each compared with eight control specimens (n = 32). Crayfish were exposed to the UV filter solutions for 30 min. The solutions were administered into tanks simultaneously using individual peristaltic pumps. Control crayfish received UV filter-free dechlorinated tap water the same temperature as in experimental tanks. Heart rate and locomotor activity were recorded for 30 min before and 30 min after adding the UV filter solutions. Following the experiment, all crayfish (invasive species) were humanely euthanized by freezing at −20 °C.

2.4. Data analysis

Data were analyzed using Statistica v.13.0 (StatSoft, Inc., Tulsa, USA). Prior to analysis, the normality of the residuals and homoscedasticity of variance were checked with Shapiro-Wilk's test and Levene's test, respectively, as the assumption for the analysis of variance (ANOVA). After verifying that the assumptions of normality and homoscedasticity of variance were met, a one-way ANOVA was performed to test for differences in the means of the dependent variable across the levels of the independent variable. If the criteria for normality were not met, data were analyzed using a non-parametric one-way ANOVA on ranks. Difference in heart rate, distance moved, and movement time before and after exposure was analyzed by one-way ANOVA, followed by Tukey's test to compare groups. A *t*-test was used for analysis of heart rate changes in individuals. Values are presented as mean ± standard deviation. Statistical significance was set at $P < 0.05$.

Table 1
Nominal and actual concentrations of UV filters BP4 and PBSA used in the experiment; t, *t*-score; $P < 0.05$.

Group	Tanks n	Nominal concentration μg/L	Actual concentration μg/L	t	P-value
BP4	8	2.5	2.3 ± 0.1	1.54	0.16
Control	8	–	<LOQ	–	–
PBSA	8	3.0	2.9 ± 0.6	0.91	0.11
Control	8	–	<LOQ	–	–

3. Results

Measured concentrations of UV filters in exposure solution did not differ from the stated value for experimental purposes by >8 % (Table 1). Concentrations of UV filter in water from control tanks were below the limit of quantification. No significant differences were found in biometric parameters of exposed and control groups of crayfish (Table 2).

Exposure to the environmentally relevant concentration of PBSA (3 μg/L) induced an increase in heart rate, distance moved, and time active in treated crayfish. Crayfish exposed to PBSA exhibited greater changes in mean heart rate ($F_{1,14}$ = 16.37, P = 0.001) than observed in control (Fig. 1). A significant difference in change of distance moved ($F_{1,14}$ = 5.09, P = 0.041) and activity ($F_{1,14}$ = 6.49, P = 0.023) between exposed and control groups was found (Figs. 2 and 3).

The BP4 at 2.5 μg/L induced significant effects in experimental crayfish during short-term exposure. Change in mean heart rate of BP4 treated crayfish was significantly greater ($F_{1,14}$ = 16.37, P = 0.001) than that of the control group (Fig. 1). Alterations in locomotor activity were also observed in BP4 exposed specimens: Exposed crayfish moved greater distances and were more active (Figs. 2, 3). Analysis of both locomotor parameters showed a significant difference in changes of distance moved ($F_{1,14}$ = 10.39, P = 0.006) and activity ($F_{1,14}$ = 8.92, P = 0.010) between exposed and control group.

No significant alteration in heart rate and locomotor activity was observed in control specimens of either the PBSA or BP4 trials (P > 0.05). There was no significant difference in effects on heart rate ($F_{1,14}$ = 0.11, P = 0.749), distance moved ($F_{1,14}$ = 2.07, P = 0.172), or activity ($F_{1,14}$ = 2.45, P = 0.141) between PBSA and BP4.

4. Discussion

Previous studies have described adverse effects of UV filtering compounds in freshwaters, primarily on embryonic and mature fish (Blüthgen et al., 2012, 2014; Li et al., 2016). Few works have been based on environmentally relevant concentrations of UV filters (Liang et al., 2020; Yan et al., 2022; Zucchi et al., 2011).

The behavior and fate of sunscreen compounds in the environment have been recently investigated (Hu et al., 2021; Magi et al., 2013; Mao et al., 2019). The organic UV filters they contain cannot be completely removed via WWTP and enter water bodies throughout the year (Ekpeghere et al., 2016; Ramos et al., 2016). The estimated half-life of BP-type UV filters and PBSA in surface waters ranges from several days to weeks during summer and is 7–9 times that during winter (De Laurentiis et al., 2013; Imamović et al., 2022; Zhang et al., 2012). Studies of seasonal variation of organic UV filters in surface waters confirmed their higher concentrations in the wet season (summer) than in the dry season (winter) as a result of increased use of UV screening products during warm months (Kim et al., 2017; Wu et al., 2018).

In the present study, we assessed the cardiac and locomotory response of a crayfish species to a short exposure period of environmentally relevant concentrations of two UV filter compounds, PBSA and BP4. The concentrations of PBSA and BP4 corresponded to reported aquatic levels measured during wet seasons (Kasprzyk-Hordern et al., 2009; Wick et al., 2010) and reveal how crayfish may be impacted when UV filter concentrations increase in summer. Crayfish represent an important group of aquatic organisms based on their occurrence across various types of freshwater ecosystems and key role in food webs (Ludányi et al., 2022; Momot, 1995; Nystrom, 2002; Reynolds et al., 2013).

Crayfish exposed to PBSA and BP4 for 30 min exhibited increased locomotor activity. Changes in locomotion of crayfish can result from a wide range of water-borne chemical compounds (Buřič et al., 2018; Chabera et al., 2021; Tierney et al., 2016). Alterations of basic behavior patterns may have crucial consequences for ecosystem function (Olsen, 2010): Crayfish attempting to escape a contaminated area may become more visible and vulnerable to predation. A loss of the ability to recognize danger, increase in activity, or change in aggressiveness may have detrimental consequences, since crayfish are prime players in freshwater environments and important ecological engineers (Hossain et al., 2019, 2020). Their altered behavior can impact valuable resource abundance, ecosystem energy flow, habitat quality, and the structure of food webs (Nystrom, 2002; Reynolds et al., 2013; Usio and Townsend, 2004; Whitledge and Rabeni, 1997), influencing populations and having significant implications for ecosystem biodiversity (Depledge and Galloway, 2005; Reynolds et al., 2013).

Given the short exposure time, we assume that observed alterations in heart rate and locomotion likely represented a stress response. Exposure to environmental stressors, including anthropogenic chemicals, can lead to increased production of stress hormones such as cortisol or adrenaline in vertebrates and the crustacean hyperglycemic hormone in decapods (Lorenzon et al., 2004). Crustacean hyperglycemic hormone is involved in the mobilization and release of glucose into the hemolymph (Chen et al., 2020). Increased glucose level may be due to elevated locomotor activity, which could have affected heartbeat frequency (Robert et al., 2019).

Crayfish are known to be sensitive bioindicators of water quality (Gago-Tinoco et al., 2014; Kuklina et al., 2013; Malinovska et al., 2020) and have been shown to exhibit escape behavior as a response to chemical stimuli (Buřič et al., 2013). Changes in behavior, including locomotion, are often closely associated with animal welfare (Gonyou, 1994; Horvath et al., 2013). Use of behavioral biomarkers represents a relatively simple, rapid, non-destructive, and reliable method of assessing impacts of xenobiotics on aquatic organisms (Buřič et al., 2018; Depledge and Galloway, 2005; Melvin and Wilson, 2013). Crayfish exhibiting atypical behavior patterns can signal changing environmental conditions (Hossain et al., 2019; Kubec et al., 2019). Heart rate is an additional important biomarker of natural and anthropogenic environmental stressors (Camus et al., 2002; Depledge and Galloway, 2005). Crayfish response to natural and chemical stimuli may be manifested in changes of cardiac activity (Bini et al., 2015; Kuklina et al., 2014, 2018). Alterations in heart rate and locomotion can quickly show an initial response to stressors as well as their impact over longer time periods (Goudkamp et al., 2004; Hossain et al., 2019; Øverli et al., 2002).

It is unclear whether longer or continuous exposure to PBSA and BP4 may have a more severe impact on crayfish behavior and physiology. Evidence of the long-term effects of organic UV filters on fish has shown their contribution to impaired neurotransmission, induced neuron and muscle toxicity, and modified heart rate (Li et al., 2016; Quintaneiro et al., 2019). Nataraj et al. (2020) found a reduction in the heart rate of zebrafish with increased exposure time. BP-type UV filters have been shown to impact spontaneous movement, locomotor response, and

Table 2
Biometric parameters of exposed and control crayfish. CL = carapace length; TL = total length. Data are presented as mean ± standard deviation; P < 0.05.

Parameters	BP4	Control	ANOVA F-statistic, *P*-value	PBSA	Control	ANOVA F-statistic *P*-value
CL (mm)	4.3 ± 0.2	4.4 ± 0.2	F(1, 14) = 0.004, *P* = 0.949	4.4 ± 0.2	4.4 ± 0.2	F(1, 14) = 0.148, *P* = 0.706
TL (mm)	9.0 ± 0.5	9.1 ± 0.5	F(1, 14)= 0.025, *P* = 0.876	9.3 ± 0.5	9.1 ± 0.6	F(1, 14)= 0.274, *P* = 0.609

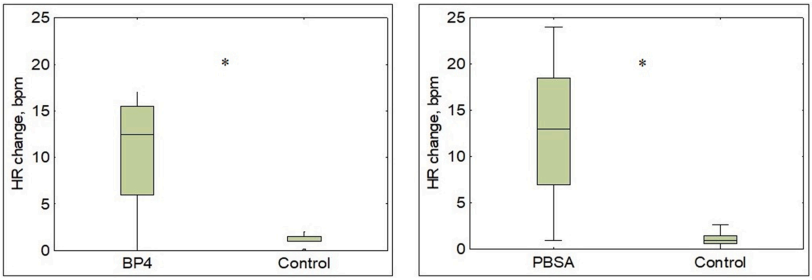

Fig. 1. Changes in mean heart rate before and after BP4 (left) and PBSA (right) application in exposed compared to control crayfish groups. Significant difference ($P < 0.05$) is highlighted by asterisk. HR = heart rate; bpm = beats per min. Data are presented as mean ± standard deviation.

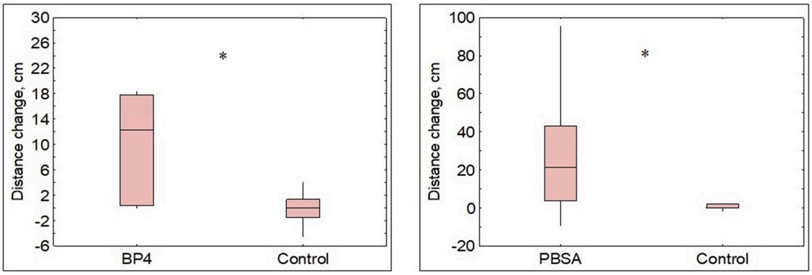

Fig. 2. Change in distance moved before and after BP4 (left) and PBSA (right) solution application in exposed vs. control crayfish groups. Significant difference ($P < 0.05$) is shown by asterisk. Data are presented as mean ± standard deviation.

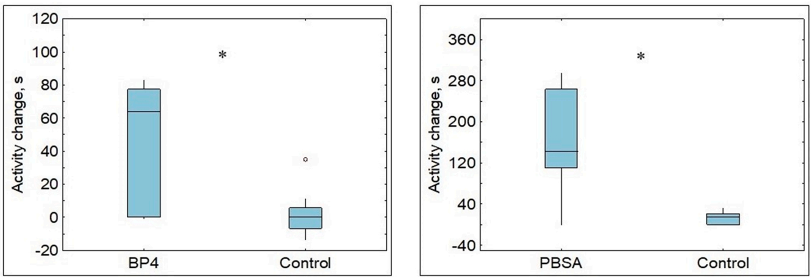

Fig. 3. Change in activity time before and after BP4 (left) and PBSA (right) solutions application in exposed vs. control crayfish groups. Significant difference ($P < 0.05$) is highlighted by asterisk. Data are presented as mean ± standard deviation.

social behavior of zebrafish during long-term exposure, which has been associated with neurotoxicity (Moreira and Luchiari, 2022; Song et al., 2022; Tao et al., 2020; Wang et al., 2023). Our short-term exposure to PBSA and BP4 resulted in visible effects in crayfish heart rate and locomotion, and it could be assumed that prolonged exposure at environmental concentrations might lead to impaired neuronal systems. To date, there has been a lack of studies examining the effects of UV filters on crayfish. Only a single study has reported on the bioaccumulation of organic UV filters in crayfish *Procambarus clarkii* (He et al., 2021). The current research represents the first investigation of effects of PBSA and BP4 on crayfish.

5. Conclusion

Investigating the impact of organic UV filters on crayfish behavior and physiology is critical to understanding the potential consequences for other invertebrates and for aquatic ecosystems. Our results showed that PBSA and BP4 at environmentally relevant concentrations exerted alterations in heart rate, distance moved, and activity time of signal crayfish during a short-term exposure. Modifications to basic behaviors, including locomotion, in key freshwater species such as crayfish can induce changes in ecosystem properties and functioning. Given the lack of studies of the effects of UV filters on crayfish, more research is needed to acquire knowledge of the environmental impacts of sunscreen compounds on freshwater organisms.

CRediT authorship contribution statement

Viktoriia Malinovska: Conceptualization, Investigation, Data analysis, Writing-Original draft preparation, Review and Editing. **Iryna Kuklina**: Conceptualization, Investigation, Writing - Review and Editing. **Kateřina Grabicová**: Consultation, Sample analysis, Writing - Review and Editing. **Milos Buřič**: Consultation, Investigation, Writing - Review and Editing. **Pavel Kozák**: Conceptualization, Supervision, Methodology, Writing - Review and Editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgements

The study was financially supported by the Grant Agency of the University of South Bohemia in České Budějovice by projects No. 022/2022/Z and 065/2022/Z. We thank to The Lucidus Consultancy for language editing.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115012.

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CHAPTER 4

CRAYFISH AS BIOINDICATORS FOR MONITORING CLO_2: A CASE STUDY FROM A BREWERY WATER TREATMENT FACILITY

Malinovska, V., Ložek, F., Kuklina, I., Císař, P., Kozák, P., 2020. Crayfish as bioindicators for monitoring ClO_2: A case study from a brewery water treatment facility. Water 12, 63.

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Crayfish as Bioindicators for Monitoring ClO_2: A Case Study from a Brewery Water Treatment Facility

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Received: 28 November 2019; Accepted: 20 December 2019; Published: 23 December 2019

Abstract: This study focuses on the use of crayfish as bioindicators in the water treatment process during operating conditions. The crayfish physiological responses to water disinfected with chlorine dioxide (ClO_2) was evaluated. Monitoring was conducted at the private commercial enterprise Protivín Brewery in Czech Republic under standard operating conditions. This brewery has a water treatment facility, where ClO_2 is used for water purification. A total of 25 adult signal crayfish (*Pacifastacus leniusculus*) were kept in separate flow-through aquaria receiving the purified water with ClO_2 concentrations ranging from 0.01 to 0.29 mg L^{-1}. Diurnal rhythms of 32% of crayfish was disturbed even at lower concentrations of ClO_2 (0.01–0.2 mg L^{-1}), while higher concentrations (>0.2 mg L^{-1}) affected all animals. A random decline and rise of heart rate was detected. In addition, the frequent occurrence of higher levels of ClO_2 significantly increased mortality. On average, mortality of crayfish occurred three to four weeks after stocking into the experimental system. Crayfish mortality is estimated to occur at concentrations exceeding 0.2 mg L^{-1} of ClO_2. Our results suggest that long-term exposure to ClO_2 adversely affects crayfish physiology. In addition, the results of this study could contribute to the use of crayfish as bioindicators in long-term water quality monitoring under industrial conditions.

Keywords: cardiac activity; chlorine dioxide; disinfectant; mortality; noninvasive biomonitoring; water quality

1. Introduction

Decapods, such as crayfish, are known to be sensitive to contamination in freshwater bodies. Given their sensitivity to changes in water quality, these organisms are highly responsive to changes in aquatic ecosystems [1–3]. Crayfish have been used as bioindicators both in the aquatic environment and under laboratory conditions. They demonstrate an affinity for accumulating pollutants in their tissues [1,4,5], and elicit a response to different substances [2,3,6]. Subsequently, there is a potential for their use as bioindicators in practical monitoring under industrial conditions.

Given that crayfish are nocturnal, their heart rate and locomotor activity increase at night [7–9]. However, the crayfish heart rate can also be influenced by certain stimuli [7–9]. Several studies have described the crayfish cardiac response to chemical stimuli, including chlorine organic compounds [3,10] and chloride content in water [11].

While different compounds may be used for water purification, the most effective disinfectant is chlorine dioxide [12]. It is a powerful oxidant among chlorine compounds and it is widely applied in surface water disinfection [13]. There are a few chemical reactions that produce chlorine dioxide (ClO_2), and one of them is the hydrochloric-acid-sodium-chlorite reaction [13]:

$$5NaClO_2 + 4HCl = 4ClO_2 + 5NaCl + 2H_2O. \quad (1)$$

Generally, ClO_2 treatment concentrations may range from 0.07 to 2 mg L^{-1}, which is sufficient for water disinfection [14]. Chlorine dioxide can be effectively applied for iron and manganese oxidation at temperatures as low as 2 °C and a pH of 5.5 [15]. Moreover, ClO_2 is efficient at removing both tastes and odors [12], and its threshold in this case could be as low as 0.2 mg L^{-1} [14]. During water treatment ClO_2 is reduced to its main decomposition product, chlorite (ClO_2^-) [13,16]. Subsequently, the levels of ClO_2^- are directly dependent on the concentration of ClO_2 used. Hence, it is important to maintain ClO_2 levels during water treatment, in order to prevent chlorite levels exceeding the WHO guideline value [14].

Given that ClO_2 is a widely used disinfectant, it is important to understand its effect on living organisms. Currently, the effects of ClO_2 on aquatic organisms remain poorly described and mainly focus on teleost fish [17–19]. Further, one study describes ClO_2 toxicity to zebra mussel *Dreissena polymorpha* [20].

The present study investigated the efficacy of crayfish as bioindicators for monitoring ClO_2 levels during the water treatment process employed by a local brewery, focusing on the biological response and lethal concentration of adult signal crayfish *Pacifastacus leniusculus* to long term ClO_2 exposure.

2. Materials and Methods

2.1. Monitoring Process

Monitoring was conducted from February to August 2017 under the running conditions of the private enterprise, Protivín Brewery, Protivín, Czech Republic. This practical investigation was operational with crayfish since April 2016 and data tracking commenced from February 2017. The brewery has a water-treatment facility, where ClO_2 is used for water purification. ClO_2 was produced by the hydrochloric-acid-sodium-chlorite reaction. In this reaction ClO_2 yields and conversion had different values, where maximum yield is 100% and maximum conversion is 80%, which is sufficient for water treatment [13]. Water ClO_2 concentrations were measured daily. All crayfish were exposed to ClO_2 during monitoring. Due to the operating conditions of the enterprise, the use of an uncontaminated control group was not possible. However, previous studies have clearly described the typical dynamics of the heart rate of crayfish [8,21].

2.2. Monitoring System

This study made use of the noninvasive crayfish cardiac activity monitoring (NICCAM) system described by Pautsina et al. [22]. This NICCAM system consists of a multichannel 14 bit analog-to-digital converter (ADC) with USB interface, personal computer with software for data processing and infrared (IR) optical sensors.

This system could monitor, record, and analyze crayfish cardiac activity, expressed as heart rate, and store the text files digitally. The software graphical user interface displayed raw cardiac activity signals of five crayfish simultaneously.

The sensors were fixed with non-toxic two-component epoxy adhesive on the dorsal side of each crayfish carapace above the heart at the locality where the strongest heart rate was detected. Glue hardened in approximately 15 min. The attached sensor still allowed crayfish to move freely around the aquarium. The monitored cardiac activity signals of the crayfish were recorded and displayed on the software's graphical user interface in real-time. Data about cardiac activity were continuously logged onto a personal computer and then processed using MS Excel for further analyzing based on created diagrams. Given that a single crayfish successful molted during the monitoring period, its pre-ecdysis period was also analyzed.

2.3. Experimental Animals

Adult signal crayfish *P. leniusculus* were obtained from ponds near Velké Meziříčí (49.3788544 N, 16.0825961 E) in the Vysočina Region, Czech Republic. Non-native crayfish species were used given the protected status of indigenous species and the regulations against their manipulation. The present study was carried out under the practical running conditions of the brewery, which mitigated risks associated with escape and species introduction and permitted the use of the non-native crayfish.

Before commencing the experiment, crayfish were acclimated for two weeks to the laboratory conditions of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic. Crayfish were individually kept in recirculating aquarium systems. Feeding and water changes were provided twice per week. No mortality was observed during the acclimation period.

Before monitoring commenced, the crayfish (with attached sensors) were acclimatized to lower temperatures in incubators (thermostatic cabinets Liebherr FK 5440, Liebherr-Hausgeräte Ochsenhausen GmbH, Ochsenhausen, Germany), where the temperature was decreased by 1 °C each following day. When acclimated temperature reached 10 °C, crayfish were transported in thermo-boxes in a small amount of water from the laboratory to the brewery by car (approximately 10 km). Before the experiment, crayfish were visually examined for absence of diseases and their biometrical measurements collected: Carapace length (mean ± SD): 43.8 ± 0.77 mm; total length: 90.13 ± 1.6 mm; and total weight: 33.68 ± 2.03 g. Weight and length was measured with digital calipers (Schut Geometrical Metrology, Groningen, The Netherlands) and an electronic balance (Kern & Sohn GmbH, Balingen, Germany). Both sexes of crayfish were used based on the previous study [23] which found no substantial differences between their reactions to stimuli. Only crayfish with intact appendages (antennae, chelae, and walking legs) were used in the experiment. During the experiment, crayfish were kept in separate 10 L flow-through aquariums, each receiving ClO_2-treated water with temperature of 10 ± 0.5 °C and pH 8.3 ± 0.5, under constant photoperiod 12:12 light-dark cycle. Each aquarium was provided with an artificial shelter (halved ceramic flower pot). A hole made on the upper surface of the shelter permitted recording of cardiac activity, even when crayfish (with the attached sensors) were inside the shelter. The experimental system could hold ten crayfish simultaneously. Five crayfish received a heart rate monitor each, while the other five crayfish were kept as reserves. In case of molting or mortality, an individual was replaced by one of the reserves. Thus, twenty-five animals were used in total throughout the monitoring period. Animals were fed daily with commercial food pellets (Sera GmbH; Heinsberg, Germany), and remains and feces were removed via daily syphoning.

2.4. Statistical Analysis

The data recorded from treated crayfish was divided between three groups in accordance with the day of exposure to maximum ClO_2 concentration (C_{max}; $ClO_2 > 0.2$ mg L^{-1}): Group one got C_{max} on day 4 ± 2; Group two on day 13 ± 1; and Group three was exposed to C_{max} on day 38 ± 6 after stocking to experimental aquarium system (Table 1). The data was grouped for subsequent analysis.

Table 1. Crayfish division based on the day of exposure to maximum concentration of ClO_2 (C_{max}); life duration after C_{max} treatment over the entire exposure period; and N—Number of crayfish in each group. Data presented as means ± SD.

Crayfish Group	N	C_{max} of ClO_2, mg L^{-1}	Ordinal Day, When C_{max} Occurred	Exposure Period Before Mortality, Days	Life Duration After C_{max} Treatment, Days
1	13	0.21 ± 0.04	4 ± 2	13 ± 8	9 ± 7
2	6	0.26 ± 0.02	13 ± 1	29 ± 8	16 ± 8
3	6	0.29 ± 0.01	38 ± 6	43 ± 7	5 ± 2

Shapiro-Wilk's test was used to assess the normality of residuals. Data were transformed when necessary to meet the assumptions of normality and equal variance. Differences in life duration after

ClO_2 C_{max} exposure between tested groups were estimated by one-way analysis of variance (ANOVA) and subsequent post hoc Tukey's test (Statistica 13, StatSoft, Inc., Tulsa, OK, USA). Data are presented as means ± standard deviation (SD). The level of significance was set at $p < 0.05$.

3. Results

3.1. Ecdysis Period

While five unsuccessful moltings resulted in crayfish mortality, a single molting proved successful. The highest heart rate was recorded 4 h before the molting, ranging between 39 and 60 beats per minute (bpm), with a peak of 72 bpm (Figure 1). Heart rate declined 35 min before molting with a few "leaps".

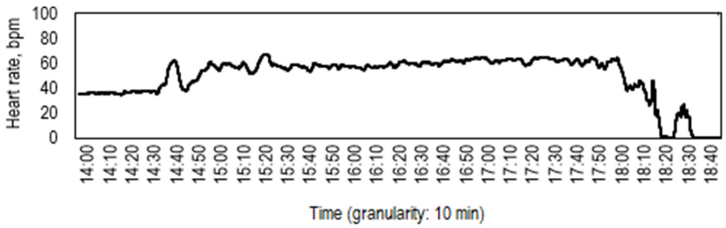

Figure 1. Heart rate of crayfish *P. leniusculus* four and half hours before molting. The fluctuating line shows heart rate, beats per minute (bpm).

3.2. Diurnal Rhythm

Crayfish were exposed to ClO_2 concentrations ranging from 0.01 to 0.29 mg L^{-1}. These concentrations varied every day (Figure 2). Following monitoring, the data was divided according to the number of high concentrations of ClO_2. During the first three months (February–April), high ClO_2 (0.2–0.29 mg L^{-1}) concentrations were recorded 4.6 times less than during the next four months (May–August), when high concentrations occurred more often.

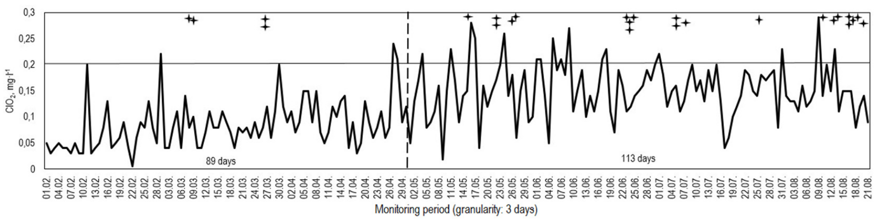

Figure 2. Chlorine dioxide (ClO_2) concentration and crayfish mortalities during the monitoring period. The stars (+) indicate crayfish mortalities; the fluctuating line indicates levels of ClO_2 concentration; the solid horizontal line indicates level of ClO_2 concentration 0.2 mg L^{-1}; the punctuated vertical line divides exposure period in two parts: First period (89 days), when high ClO_2 concentrations (up to 0.2 mg L^{-1}) were found five times and four crayfish died; and the second period (113 days), when high ClO_2 concentrations occurred 23 times and 21 crayfish died.

The heart rate daily cycle of 32% of crayfish was already disturbed at a lower level of ClO_2 concentration (less than 0.2 mg L^{-1}). A prevalence of disrupted heart rate was observed, with chaotic increases and decreases regardless of the time of day. There was no statistical difference between day and night cardiac activities within the tested groups (Table 2) as well as between groups (day: $F_{(2,22)} = 0.80780$, $p = 0.45863$ and night: $F_{(2,22)} = 1.5974$, $p = 0.22503$). The diurnal rhythm was disrupted, and cardiac rhythmicity was lost. This was expressed in different heart rate fluctuations of animals exposed to the same concentrations of ClO_2 (Figure 3).

Table 2. Average heart rate expressed as beats per minute of all monitored crayfish from the three groups during the ClO_2 exposure period. Mean ± SD.

Crayfish Group	Day Heart Rate, bpm	Max	Min	Night Heart Rate, bpm	Max	Min	Day Versus Night Heart Rate, *p*-Value
1	53 ± 14	109	25	52 ± 14	117	20	0.54
2	50 ± 13	89	20	52 ± 13	91	26	0.37
3	47 ± 14	92	20	48 ± 15	92	23	0.68

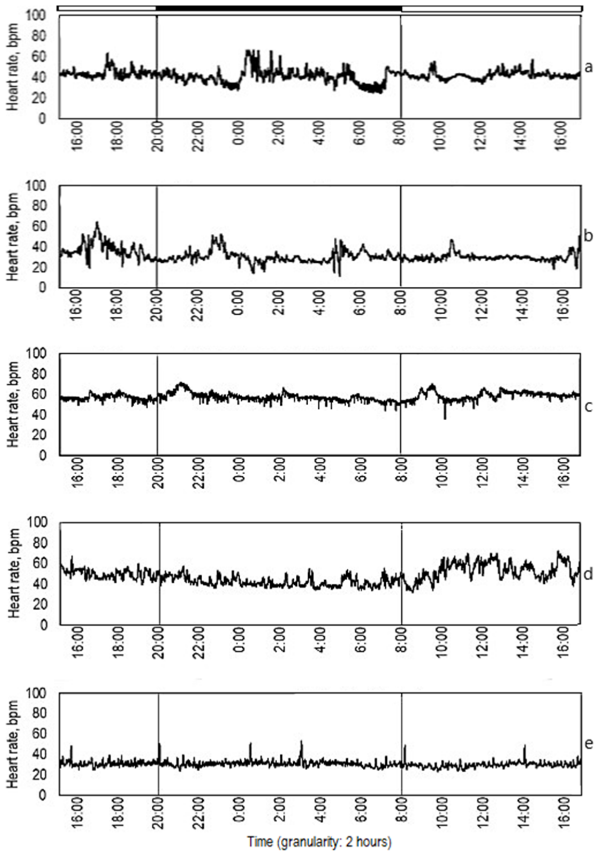

Figure 3. Examples of heart rate of five crayfish *P. leniusculus* during the day and night period, 5th to 6th of June 2017 with ClO_2 concentrations of 0.25 mg L^{-1} and 0.19 mg L^{-1}, respectively. The fluctuating line indicates heart rate in beats per minute (bpm), while the two vertical lines distinguish night- and day-time.

3.3. Mortality

High ClO_2 concentrations (0.2–0.29 mg L^{-1}) disturbed the diurnal rhythm of all individuals, inducing loss of rhythmicity and subsequent mortality (Figure 4). Mortality increased along with more frequent occurrences of high ClO_2 concentrations. During the first period (89 days), where high ClO_2 concentrations (higher than 0.2 mg L^{-1}) were recorded five times, four crayfish died. During the second 113-day period, where high ClO_2 concentrations occurred 23 times, 21 crayfish died. Thus, in the first period mortalities occurred 5.3 times less than in the second one. No individual survived the experiment (Figure 4).

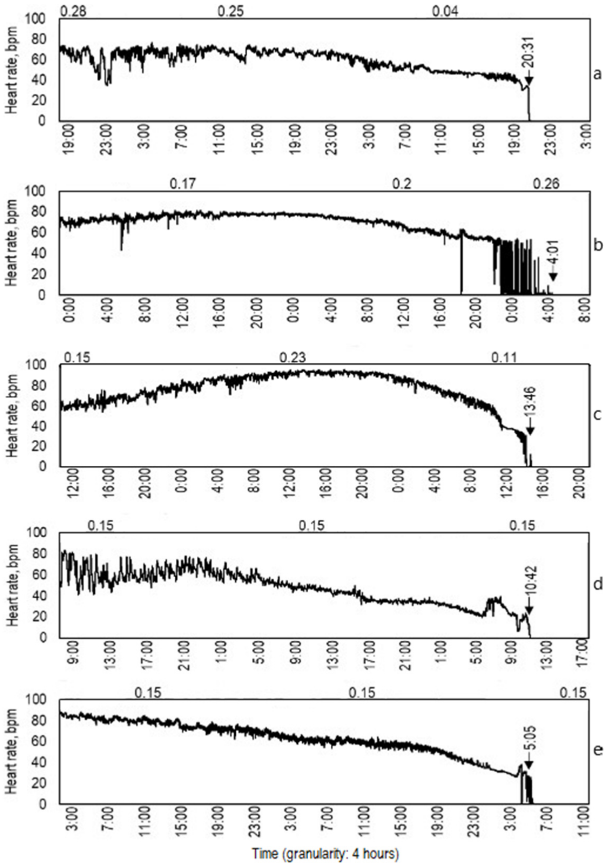

Figure 4. Examples of heart rate of five crayfish *P. leniusculus* two days before mortality occurred. The line indicates heart rate in beats per minute. Daily ClO_2 (mg L^{-1}) concentration are situated above the graphs, time of death is indicated by the vertical arrow.

Life Duration after Exposure to C_{max}

Life duration after exposure to C_{max} for each crayfish was determined (Table 1). There was a significant difference ($p < 0.05$) in life duration between groups. Crayfish from Group two generally lived twice as long (16 ± 8 days) as crayfish from Groups one and three (9 ± 7 and 5 ± 2 days, respectively) after exposure to C_{max} (Figure 5).

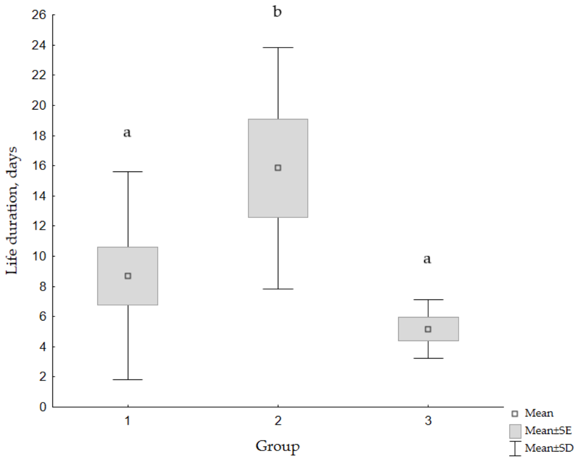

Figure 5. Life duration after C_{max} exposure of crayfish three experimental groups: Group one got C_{max} on day 4 ± 2; Group two on day 13 ± 1; and Group three was exposed to C_{max} on day 38 ± 6. Life duration was: 9 ± 7 days, 16 ± 8 days, and 5 ± 2 days, respectively. There was a significant difference ($p < 0.05$) in life duration between groups: Individuals from Group two in general lived more than twice as long as crayfish from Groups one and three after exposure to C_{max}.

4. Discussion

In the present study, the effect of long-term exposure of signal crayfish to different levels of ClO_2 has been investigated and assessed through the observation of heart rate, diurnal rhythm and mortality.

A single recorded molting was preceded by rapid heart rate fluctuations. The increase in heart rate was observed four hours before the molting, up to 60 bpm with the peak of 72 bpm, and the heart rate decline was detected 35 min before molting (Figure 1). Kuramoto [24] described the cardiac changes of untreated spiny lobster *Panulirus japonicus* before the molting and noted that the heart rate rose and fell during molting of lobster similarly to that of crayfish. The heart rate of an unaffected lobster increased 1–2 h before ecdysis to a peak of 80–120 bpm and declined about 15 min before the beginning of molting. Thus, the changes in the heart rate of unstimulated spiny lobster and the ClO_2 exposed signal crayfish were similar in the premolting period.

Unsuccessful molting was also observed to result in death. In Kuklina et al. [3], chloramine-T exposed narrow-clawed crayfish *Astacus leptodactylus*, suffered from lack of energy when exposed to physical stress. Energetic deficiency can be a potential reason for unsuccessful molting in our study, where ClO_2 exposure depleted crayfish energy stores and prohibited molting, resulting in their mortality.

Owing to their nocturnal nature, the narrow-clawed crayfish *A. leptodactylus* heart rate is higher at night than during the day, even at temperatures below 14 °C [8]. The present study showed an impact of ClO_2 on crayfish heart rate and nocturnal behavior. A disturbance of the circadian cardiac rhythm was observed in all individuals, expressed as a random decline and rise of heart rate, regardless of the time of day. The typical increased nocturnal heart rate was not noticed at the lowest ClO_2 concentration in 32% of the crayfish, while in the high concentrations it was completely disrupted for all animals. As soon as the diurnal rhythm was disturbed, the circadian rhythmicity was lost, demonstrating impaired cardiac function and leading to crayfish mortality (Figure 3). A similar observation was described in Kuznetsova et al. [21] where highly concentrated hydroquinone solution (1 g L^{-1}) disrupted A. *leptodactylus* circadian rhythm before death. Styrishave et al. [7] noticed that heart rate increased during the day and decreased at night in noble crayfish *Astacus astacus* when exposed to copper (8.0 mg L^{-1}) and mercury (0.1 mg L^{-1}). In this case high mortality (>90%) was detected after 19 days of exposure.

Consequently, the ClO_2 used in our monitoring and heavy metals used by Styrishave et al. [7] and hydroquinone used in Kuznetsova et al. [21] can be toxic compounds at certain concentrations, and may negatively affect the health of aquatic organisms and even induce their mortality.

Not only are the loss of circadian rhythmicity suspected to induce crayfish mortality, but also the changes in physiology. The gills of fathead minnows *Pimephales promelas* were negatively affected by 0.13 mg L^{-1} of ClO_2 concentration [25]. Chupani et al. [26] found heavy histopathological changes in crayfish exposed to peracetic acid (2–10 mg L^{-1}), while similar effects often induce mortality in juvenile grass carp *Ctenopharyngodon idella* [27] and channel catfish *Ictalurus punctatus* [28]. Subsequently, ClO_2 could induce adverse cardio-respiratory responses, reduce larval rainbow trout (*Oncorhynchus mykiss*) growth in concentration above 0.3 mg L^{-1} [17] and cause oxidative damage and changes in antioxidant defenses in the heart tissue of juvenile rainbow trout [19]. Hence, it could have a similar effect in crayfish. Moreover, ClO_2 is more toxic to aquatic organisms than chlorite and peracetic acid [17,18]. Therefore, considering how ClO_2 is harmful for non-target aquatic animals and that it has higher toxicity than other substances, ClO_2 might likely have an adverse effect on crayfish tissues, leading to various disorders and subsequent mortality.

Peak concentrations of ClO_2 (0.2–0.29 mg L^{-1}) observed during our experiment significantly influenced the life duration of animals. Another study determined that 1–5 mg L^{-1} of ClO_2 induces mortality of zebra mussel *D. polymorpha* [20].

When the C_{max} occurred, crayfish mortality was noticed after approximately 10 ± 7 days. Group one could likely not survive due to immediate exposure to increased ClO_2 concentrations, which resulted in rapid mortality. The prolonged exposure of Group three to low-to-medium concentrations of ClO_2 resulted in a cumulative effect, preventing organ and tissue regeneration, and resulted in crayfish mortalities 5 ± 2 days after C_{max} occurred. Group two, which was exposed to moderate ClO_2 concentrations within relatively short time (longer than Group one but shorter than Group three), had the longest life duration after getting C_{max}. This may suggest that crayfish responses differ between individuals.

5. Conclusions

Changes in crayfish heart rate and circadian rhythmicity could provide information about their functional state and help us make inferences on environmental state. Crayfish's physiological sensitivity allow early detection of increased levels of harmful chemicals, thereby presenting a practical solution for proactive water quality monitoring. Our results suggest that the changes in heart rate and diurnal rhythm of treated animals was crayfish-specific, which may stem from their varying functional state and individual physiological response to ClO_2 concentrations. There was a direct correlation between C_{max}, and crayfish mortality. ClO_2 adversely affected crayfish circadian rhythm. In conclusion, this study demonstrated that crayfish could serve as effective bioindicators for long term practical water quality monitoring.

Author Contributions: Conceptualization, V.M., F.L., I.K. and P.K.; Data curation, P.C.; Formal analysis, V.M.; Investigation, V.M., F.L. and I.K.; Methodology, F.L. and I.K.; Project administration, V.M., F.L., I.K. and P.K.; Resources, F.L., I.K. and P.C.; Software, P.C.; Supervision, P.K.; Validation, P.K.; Visualization, V.M. and P.K.; Writing—original draft, V.M. and F.L.; Writing—review & editing, V.M., F.L., I.K., P.C. and P.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Ministry of Education, Youth and Sports of the Czech Republic—project CENAKVA (LM2018099).

Acknowledgments: The authors would like to thank Protivín Brewery, Michal Voldřich and Roman Dědič for their help in conducting the experiment.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5

General discussion

Contamination of aquatic ecosystems and associated influence of xenobiotics on aquatic organisms are critical issues worldwide (Hothem et al., 2007; Morrissey et al., 2015; Varol and Sünbül, 2017). Pollutants enter natural waters via several pathways including wastewater treatment plant effluents, domestic sewage, industrial effluents, agricultural activities and runoff, land disposal sites, resource extraction, etc. (Ritter at al., 2002; Rodil et al., 2012). A significant number of different contaminants are continuously released into surface waters throughout the year, posing a threat for numerous taxonomic groups of organisms, including keystone species (Agatz et al., 2014; Hasenbein et al., 2017; Hossain et al., 2019). Crayfish are a dominant macrocrustacean group in many aquatic ecosystems with prime structural and functional trophic roles (Nyström et al., 1996; Reynolds et al., 2013). Crayfish are considered to be important ecosystem engineers due to their activities related to changes in biotic or abiotic materials (Creed and Reed, 2004; Souty-Grosset et al., 2016). Crayfish have served as representative model organisms in many research fields (Kaddissi et al., 2011; Buscaino et al., 2012; Du et al., 2013; Dong et al., 2015; Jiménez-Morales et al., 2018) and demonstrated a variety of behavioral and physiological responses to chemical and natural stimuli (Bojsen at al., 1998; Kholodkevich et al., 2008; Tierney et al., 2016; Woodman et al., 2016; Kuklina et al., 2018).

Behavior is an ecologically significant and fundamental endpoint in our understanding the impacts of pollutants on fitness and survival of aquatic organisms (Sohn et al., 2018; Ford et al., 2021). It serves as a final visible outcome of complex neurophysiological processes involving sensory, motor, and muscular activities (Haynes, 1988). When compared to developmental and reproduction responses of aquatic organisms, behavioral studies generally provide more sensitive and powerful tools (Melvin and Wilson, 2013). Individual behavior is intricately linked to population dynamics and community structure, as they are interdependent components of complex ecological processes (Candolin and Wong, 2019). The study of behavior in relation to the effects of xenobiotics has expanded with technological and computational progress that facilitate improved visual and non-visual assessment tools in recording behaviors (Gerhardt, 2007; Melvin and Wilson, 2013; Bertram et al., 2022). This progress enables the detection of behavioral changes affected by environmental concentrations of chemicals (Guo et al., 2021; De Felice et al., 2022). The use of behavioral biomarkers is a non-destructive and reliable method for evaluating sub-lethal exposure effects of contamination with minimal time and cost resources (Depledge and Galloway, 2005; Buřič et al., 2018). Furthermore, behavioral tests have demonstrated high ecological relevance in numerous studies (Hellou, 2011).

Animal locomotion is a basic class of behavior and can easily be quantified and automated (Gerhardt, 2007). Behavioral modifications, including changes in locomotion, are often among the initial visually observable responses to stressors (Nakamura, 1986; Jin et al., 2015). These alterations can serve as ''early warning'' signals for assessing environmental quality of aquatic ecosystems (Ren et al., 2007; Hellou, 2011; Bae and Park, 2014). Exposure to sublethal concentrations of contaminants can have an impact on performance of freshwater invertebrates, leading to observable variations in locomotor activity (Augusiak and Van den Brink, 2016; Simão et al., 2019). Crayfish exposed to various anthropogenic pollutants have shown impaired locomotor functions (Buřič et al., 2018; Hossain et al., 2019; Kubec et al., 2019). Our findings indicate that short-term exposure of the crayfish species *Pacifastacus leniusculus* to environmentally relevant concentrations of the herbicide metazachlor resulted in increased locomotor activity (**Chapter 2**). This observed behavioral alteration can potentially be attributed to the stimulatory effects of pesticides, as reported in previous studies (Morse, 1998; Guedes et al., 2009; Cutler et al., 2022), as well as the chemical irritation causing a stimulation of locomotor responses (Chen et al., 2014; Sharma, 2019). Another possible explanation for the accelerated locomotion is the avoidance reaction of crayfish following contact with a toxicant. Reactive locomotor behavior encompasses the response to chemical input from the animal's sensory apparatus (Bayley, 2002). Immediate behavioral reactions observed in invertebrates exposed to environmental contamination often involve the perception and escape from polluted areas (Lagadic et al., 1994). For instance, Buřič et al. (2013) described the attempts of crayfish species *Faxonius limosus* and *P. leniusculus* to escape in response to exposure to the pesticide diazinon. In a similar vein, juvenile crayfish *Procambarus virginalis* exposed to the pesticide s-metolachlor exhibited an increase in distance moved (Velisek et al., 2019). In addition to increased locomotor activity, avoidance behavior is often associated with the drift of organisms. Studies have demonstrated that brief pesticide exposure can induce drift or increase the drift density of a macroinvertebrate community (Heckmann and Friberg, 2005; Beketov and Liess 2008). Altered locomotion of crayfish, as a fundamental behavior, along with the potential drift of macroinvertebrates, can impair their ability to respond to predators and modify foraging behavior. These behavioral alterations have the potential to disrupt the food chain, modify community structure, and ultimately lead to adverse ecological outcomes in aquatic ecosystems. No significant changes in the distance moved by crayfish were observed following exposure to the herbicide terbuthylazine and the insecticide thiacloprid. This lack of reaction may be partially explained by the temporary impairment of olfactory receptors caused by pesticides (Cook and Moore, 2008). Additionally, thiacloprid, a neonicotinoid insecticide, stimulates nicotinic acetylcholine receptors in the central nervous system, which can lead to overexcitation and subsequent receptor blockage, resulting in temporary paralysis (Yamamoto, 1999). These findings suggest that pulse exposure to thiacloprid and terbuthylazine may not stimulate crayfish to escape from a contaminated area, potentially leading to prolonged exposure to potentially harmful environmental levels of pesticides. Studies have reported that longer-term exposure to environmentally relevant concentrations of thiacloprid and terbuthylazine can affect hemolymph biochemical parameters and antioxidant biomarkers in non-target organisms (Stara et al., 2021; Khatib et al., 2023). This suggests that the impact of these pesticides extends beyond immediate behavioral responses and may affect the physiological health and overall fitness of crayfish populations and have broader implications for the entire freshwater community. The alterations in hemolymph biochemical parameters and antioxidant biomarkers indicate potential oxidative stress and disruption of metabolic processes, further emphasizing the ecological risks associated with pesticide exposure.

In **Chapter 3**, crayfish were exposed to environmentally relevant concentration of either 5-Benzoyl-4-hydroxy-2-methoxybenzenesulfonic acid (benzophenone-4, BP4) or 2-Phenylbenzimidazole-5-sulfonic acid (ensulizole, PBSA), commonly used organic UV filters. Significantly greater changes in distance moved and time active were detected in specimens exposed to the tested chemicals compared to unexposed controls. Although the data on the effects of PBSA on behavior in non-target organisms is lacking, there is evidence that benzophenone-type UV filters can cause changes in locomotion function, agonistic behavior and alter nervous system (Chen et al., 2015; Tao et al., 2020). Benzophenone-type UV filters have been shown to induce apoptosis when interfering with the nervous system and cell metabolism (Wnuk et al., 2017; Meng et al., 2020) and thereby affecting locomotor activity in aquatic organisms (Tao et al., 2020; Song et al., 2022). Exposure of non-target organisms to UV-filters is often linked to neurotoxic effects (Ruszkiewicz et al., 2017; Hu et al., 2017). For example, BP3 (benzophenone-3) and BP4 have been shown to decrease gene expression in the brain of adult zebrafish *Danio rerio*, suggesting potential neurological disturbances

(Blüthgen et al., 2012; Sun et al., 2023). Additionally, BP3 alone has been found to inhibit the relative axon length of primary motor neurons in zebrafish larvae (Zucchi et al., 2011). These findings suggest that UV filters can interfere with neural development and function. Other organic UV filters, such as ethylhexyl methoxycinnamate and avobenzone, have also been associated with impaired neurostatus in non-target organisms. For instance, studies have found significantly decreased acetylcholinesterase activity, which is an important enzyme involved in nervous system function, as a result of exposure to these compounds (Cuccaro et al., 2022; Liu et al., 2022). Avobenzone has also been reported to affect genes involved in nervous system development (Liu et al., 2022). These observations suggest that UV filters can disrupt neurological processes and potentially lead to adverse effects on behavior, cognition, and overall neurological health. The neurotoxic effects of UV filters on non-target organisms have broader ecological implications. Disruption of the nervous system can influence ecosystem health, considering its role in various physiological and behavioral processes of aquatic organisms.

It is important to note that there is a scarcity of studies examining the effects of UV filters specifically on crayfish. Our work represents the first investigation into the impacts of PBSA and BP4 on crayfish, highlighting the need for studies to better understand the potential short-term and long-term effects and ecological implications of organic UV filters on crayfish populations and their associated ecosystems.

We assume that the observed locomotion and heart rate alterations in **Chapter 2** and **3** may represent a stress response, considering the short duration of exposure. Stress response in organisms typically involves a physiological cascade triggered by the central perception of the ambient environment (Schreck et al., 1997). In decapod crustaceans, environmental stressors can induce an upregulation of crustacean hyperglycemic hormone (CHH), leading to the mobilization and release of glucose into the hemolymph, thus providing a subsequent energy source (Lorenzon et al., 2004; Chen et al., 2020). CHH, participating in the adaptive mechanisms to stressful conditions, has been demonstrated sensitivity to various stimuli, including pollutants, changes in temperature and salinity, and hypoxia (Chang et al., 1998; Lorenzon et al., 2000, 2004; Chang, 2005; Chung and Zmora, 2008). Glucose levels have been recognized as stress biomarkers (Fossat et al., 2014), and the increase in glucose level may be attributed to elevated locomotor activity, subsequently influencing heart rate frequency (Robert et al., 2019). Elevated levels of stress hormones, glucose, and muscular activity are physiological adaptations observed in animals experiencing stress, serving to aid in their ability to cope and restore homeostasis when possible (Iwama et al., 1999). Physiological approaches for evaluating stress involve the examination of alterations in separate body systems such as neural, hormonal, circulatory, and digestive systems (Barton, 2002; Aguirre-Martínez et al., 2016; Capoluro et al., 2016; Jerez-Cepa and Ruiz-Jarabo, 2021). Behavioral measurements of stress serve as visible and sensitive indicators, forming a pivotal linkage between proximate biophysical, biochemical, and physiological events (Scherer, 1992; Schreck et al., 1997). Adaptive behavioral responses, such as increased locomotion, play a crucial role in minimizing exposure to potential threats (Stoner, 2012). Further behavioral responses to stress include impaired chemoreception, learning, foraging, aggression, and predator-prey interactions (Lürling and Scheffer, 2007; Oulton et al., 2014; Sarasamma et al., 2020; Aparna and Patri, 2021).

The observed simultaneous increase in heart rate and locomotor activity in crayfish exposed to both pesticides and UV filters suggests a potential link between these physiological and behavioral responses. This correlation reinforces the notion that changes in heart rate can be indicative of altered locomotion in crayfish. The consistent findings of a similar response in *P. leniusculus*, as demonstrated in the previous study by Kuklina et al. (2018), provide additional support for the association between locomotion and heart rate in crayfish. Interestingly, the response to natural stressors, such as predator or conspecific crayfish odor, differed from that of chemical stimuli (Kuklina et al., 2018). The altered heart rate observed in crayfish exposed to natural stressors, which was either delayed or did not result in observable locomotion, suggests a distinct physiological and behavioral response in comparison to exposure to contaminants. This differentiation highlights the complexity of crayfish's stress response mechanisms and the influence of different stressor types on their physiological and behavioral dynamics. The contrasting responses to chemical stimuli and natural stressors underscore the importance of considering various stressor types when studying crayfish behavior. By understanding how different stressors elicit specific physiological and behavioral responses, we can gain insights into the adaptive mechanisms and stress coping strategies of crayfish in their natural environment.

While **Chapter 2** and **3** focused on short-term exposure, it is important to consider the possible cumulative impacts of pesticides and organic UV filters over long-term periods. Using environmentally relevant concentrations in such studies is crucial for accurately assessing the potential ecological risks and impacts of these substances. Moreover, it helps to better simulate the conditions that non-target organisms are likely to encounter in natural ecosystems and ensures a more realistic and ecologically meaningful approach. Continued research is essential to expand our knowledge of the effects of environmental contaminants on crayfish and other non- target organisms. This will contribute to a more comprehensive understanding of the risks posed by these compounds and aid in developing appropriate mitigation strategies and regulatory measures to safeguard the health and integrity of aquatic environments.

Crustacean heart rate serves as a prevalent physiological biomarker, providing a unique and valuable tool for evaluating stress levels and responses to diverse biotic and environmental factors, including the presence of toxicants. This has been demonstrated in numerous studies, underscoring its significance in ecological research (Handy and Depledge, 1999; Schapker et al., 2002; Bini et al., 2015; Gutzler and Watson, 2022). Measurements of heart rate provide an accurate index of activity, metabolic changes, and energy expenditure (Green, 2011; McGaw and Reiber, 2015). Studies on freshwater crustacean *Daphnia magna* have demonstrated the susceptibility of heart rate to stressors such as fluoxetine, sertraline, cadmium, and nanoparticle suspensions (Lovern et al., 2007; Lari et al., 2017; Heyland et al., 2020). Changes in heart rate and diurnal rhythm of crayfish can signal a modification in ambient conditions (Styrishave and Depledge, 1996; Bini and Chelazzi, 2006). In our investigation of the long-term physiological responses of crayfish *P. leniusculus* to water disinfected with chlorine dioxide (ClO_2), we observed a disturbance in the circadian cardiac rhythm (**Chapter 4**). Since crayfish are primarily nocturnal animals, they typically exhibit higher activity levels and heart rates during the night (Bojsen et al., 1998). However, in our study, we observed random fluctuations in heart rate throughout the day, indicating a loss of rhythmicity. Furthermore, this loss of rhythmicity was associated with subsequent mortality of the specimens. Similar responses have been reported by Styrishave et al. (1995) and Styrishave and Depledge (1996) in crayfish *Astacus astacus* exposed to copper and mercury. The mortality observed in specimens with disrupted cardiac rhythms emphasizes the importance of maintaining the natural circadian rhythm for crayfish well-being. Given the clear and discernible response of crayfish to changing disinfectant levels in our monitoring study, they appear to be a suitable species for bioindication and water quality assessment purposes under operational conditions.

In **Chapter 4**, we used the heart rate of crayfish, and the usefulness of this biomarker, as supported by previous research, has been confirmed. In **Chapters 2** and **3**, we measured alterations in crayfish heart rate and locomotor activity as reactions to anthropogenic contaminants in order to obtain a broader understanding of the effects of these chemicals on crayfish and to provide more comprehensive response information.

Conclusions

The current research highlights the role of crayfish as appropriate bioindicators and demonstrates that their cardiac and locomotor responses are useful biomarkers that can be utilize in both short- and long-term studies on the chemical effects. Our results indicated that environmentally relevant concentrations of widely used UV filters and pesticide affect crayfish behavior and physiology. Owing to the crayfish importance in freshwaters, alterations in their basic biological parameters can lead to subsequent adverse ecological outcomes in aquatic ecosystems. This study provides valuable insights into pollution impact on freshwater invertebrate, showing short-term responses of crayfish to anthropogenic chemicals. Further, our case study showed that crayfish can be used for water quality monitoring under operating conditions. The findings of the thesis contribute in several ways to our understanding of the effects of different stimuli on non-target organisms. Further investigations into the underlying mechanisms behind behavioral and physiological responses to environmental concentrations of contaminants will enhance our ability to assess crayfish reaction to natural stressors.

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English summary

Monitoring of crayfish reaction to the different stimuli: are the crayfish good bioindicators?

Freshwater crayfish play a vital role in food webs and in the transfer of energy between trophic levels, often acting as keystone species. Among invertebrates, crayfish are prime species considering their large size and general abundance and biodiversity. Crayfish belong to common model organisms in scientific research and have been used as bioindicators of water quality in laboratory and field studies.

Freshwater crayfish are at direct risk while facing polluted aquatic environments, as many chemicals enter natural waters throughout the year. Studies have been shown that crayfish exhibit a variety of behavioral and physiological alterations in response to anthropogenic contaminants. In **Chapter 2**, we aimed to assess cardiac and locomotor responses of crayfish *Pacifastacus leniusculus* to short-term pulse exposure to environmentally relevant concentrations of three pesticides (metazachlor, terbuthylazine, and thiacloprid). A significant increase in heart rate and distance moved was observed in metazachlor-exposed specimens compared to controls. We did not detect significant changes in crayfish cardiac and locomotor activity in terbuthylazine and thiacloprid groups. As most crayfish in these groups did not exhibit locomotor response, they might not be stimulated to escape a contaminated area. This may lead to continuous exposure to environmental chemicals, that are potentially harmful. In **Chapter 3**, we investigated the effects of UV filters Benzoyl-4-hydroxy-2-methoxybenzenesulfonic acid (BP4) and 2- Phenylbenzimidazole-5-sulfonic acid (PBSA) on crayfish during 30 min exposure period. Specimens exposed to the tested compounds moved greater distances and spent more time in locomotion. Increased heart rate was also detected in treated groups. Such behavioral and physiological alterations of crayfish detected in both studies can potentially impair crayfish ability to respond to predators, and consequently affect abundance and the functional properties of macroinvertebrate communities.

Along with use of crayfish in laboratory and field experiments, there is also an option to conduct case study with crayfish acting as bioindicators. We evaluated crayfish physiological responses to water disinfected with chlorine dioxide in long-term monitoring under operating conditions in local brewery (**Chapter 4**). The disruption of cardiac rhythmicity was detected with chaotic increases and decreases of heart rate regardless of the time of day. In natural environment, heart rate of crayfish is higher during night time considering nocturnal activity of these decapods. Nocturnalism is important adaptation for crayfish, and its impairment can lead to certain risks related to increased visibility to predators and alterations in foraging.

Our results indicated that crayfish exhibit a cardiac and locomotor responses when shortly exposed to commonly used herbicide metazachlor and two widely applied UV filters BP4 and PBSA, suggesting that crayfish can potentially detect the appearance of these aquatic contaminants in water. The usefulness of crayfish in long-term monitoring of water quality have also been demonstrated. These findings expand our understanding of using crayfish as bioindicators in scientific research.

In conclusion, this thesis provides the data from laboratory and case studies focusing on measurements of cardiac and locomotor endpoints in crayfish. Given that crayfish is at the base of the food chain in freshwater environments, it is of great importance to study the effects of different stimuli on such keystone species. Understanding further biological impacts of aquatic pollutants on freshwater crayfish and other non-target taxa will benefit our knowledge of threats to functional compositional changes in populations and communities.

Czech summary

Monitoring reakce raků na různé podněty: jsou raci dobrými bioindikátory?

Sladkovodní raci jsou považováni za klíčové druhy s ohledem na jejich velikost, zásadní roli v potravním řetězci, obecnou hojnost a biologickou rozmanitost. Raci patří k běžným modelovým organismům ve vědeckém výzkumu a jsou využíváni jako bioindikátory kvality vody v laboratorních i terénních studiích.

Sladkovodní raci jsou v přímém ohrožení, zatímco čelí znečištěnému vodnímu prostředí, protože do přírodních vod se během roku dostává mnoho chemikálií. Studie ukázaly, že raci vykazují různé behaviorální a fyziologické změny v reakci na antropogenní kontaminanty. V **Kapitole 2** bylo naším cílem vyhodnocení srdeční a lokomotorické reakce raka signálního *Pacifastacus leniusculus* na krátkodobou pulzní expozici environmentálně relevantním koncentracím tří pesticidů (metazachlor, terbuthylazin a thiacloprid). Ve srovnání s kontrolními skupinami, bylo u raků vystavených metazachloru pozorováno významné zvýšení srdeční frekvence a pohybu. Ve skupinách vystavených terbuthylazinu a thiaclopridu nebyly zaznamenané významné změny srdeční a pohybové aktivity raků. Protože většina raků v těchto skupinách nevykazovala lokomotorickou odezvu, nebyli zřejmě stimulováni k úniku z kontaminované oblasti. V přirozených podmínkách to může vést k trvalému vystavení environmentálním chemikáliím, které jsou potenciálně škodlivé. V **Kapitole 3** jsme zkoumali účinky UV filtrů kyseliny benzoyl-4-hydroxy-2-methoxybenzensulfonové (BP4) a kyseliny 2- fenylbenzimidazol-5-sulfonové (PBSA) na raky během 30minutové expozice. Jedinci raků exponovaní testovaným látkám se pohybovali na větší vzdálenosti a strávili více času v pohybu. V exponovaných skupinách byla také zaznamenána vyšší frekvence srdečního tepu. Zaznamenané změny ve fyziologii a chování raků v obou studiích mohou být indikací potenciálního narušení schopnosti reagovat na predátory a následně ovlivnit početnost a funkční vlastnosti společenstev velkých bezobratlých.

Spolu s využitím raků v laboratorních a venkovních experimentech existuje také možnost provést případovou studii s raky působícími jako bioindikátory. Hodnotili jsme fyziologické reakce raků na vodu dezinfikovanou oxidem chloričitým v dlouhodobém sledování za provozních podmínek v lokálním pivovaru (**Kapitola 4**). Narušení se projevovalo jako chaotické zvýšení a snížení srdečního tepu bez ohledu na denní dobu. V přirozeném prostředí je srdeční frekvence raků v noci vyšší s ohledem na noční aktivitu. Noční režim je důležitou adaptací pro raky a jeho narušení může vést k určitým rizikům souvisejícím se zvýšenou viditelností pro predátory a změnami při hledání potravy.

Naše výsledky ukázaly, že raci vykazují srdeční a pohybové reakce, když jsou krátce vystaveni běžně používanému herbicidu metazachloru a dvěma široce používaným UV filtrům BP4 a PBSA, což naznačuje, že raci mohou potenciálně detekovat výskyt těchto vodních kontaminantů ve vodě. Prokázala se také využitelnost raků při dlouhodobém sledování kvality vody.

Prezentovaná práce poskytuje data z laboratorních a případových studií zaměřených na srdeční a lokomotorickou aktivitu u raků. Vzhledem k tomu, že raci jsou základem potravního řetězce ve sladkovodním prostředí, je velmi důležité studovat účinky různých podnětů na tyto klíčové druhy. Výzkumem dalších vlivů polutantů na sladkovodní raky a další necílové taxony zvýší naše znalosti potencionálního ohrožení biodiverzity společenstev.

Acknowledgements

I would like to express my heartfelt gratitude to everyone who has contributed to the completion of this dissertation.

First and foremost, I would like to extend my sincere gratitude to my supervisor Prof. Pavel Kozák for providing me with invaluable guidance, constructive feedback, and unwavering support and faith in me throughout the entire research process. His knowledge, experience, and expertise have been instrumental in shaping my professional growth.

I am also grateful to Iryna Kuklina, Ph.D., for her valuable advice, feedback, and support, which helped me to refine my research questions, methodology, and analysis. Her contributions have been invaluable in shaping the outcome of this research. I thank to Filip Ložek, Ph.D., for the suitable comments and great help during the research process.

I would like to extend my appreciation to the head of the Laboratory of Freshwater Ecosystems Assoc. Prof. Miloš Buřič and the laboratory staff for providing me with a conducive research environment, access to resources, and technical support. Their encouragement and support have been a major source of motivation for me.

I would also like to acknowledge all my colleagues and friends from the Faculty of Fisheries and Protection of Waters and all over the world for their support, encouragement, and helpful suggestions throughout this research journey. Their input has been a significant part of the success of this research.

I cannot thank my beloved husband Oleksandr and son Stefan enough for their unconditional love, endless support, and understanding throughout this journey and the entire life. Their encouragement has been my source of strength, inspiration, and motivation.

Last but not the least, I would like to express my gratitude to my family for their unwavering love, support, and encouragement throughout my entire life. Their love, care, and support have been my driving force in achieving my goals.

I also appreciate the financial support from the following projects that funded parts of the research presented in this dissertation:

- Czech Science Foundation (project No. 061/2019/Z).
- Ministry of Agriculture of the Czech Republic (project No. QK1910282).
- Ministry of Education, Youth and Sports of the Czech Republic (project CENAKVA LM2018099).
- The Grant Agency of the University of South Bohemia in Ceske Budejovice No. 019/2020/Z, No. 022/2022/Z, and 065/2022/Z.

List of publications

Peer-reviewed journals with IF

Iqbal, A., Ložek, F., Soto, I., Kaur, D., Grabicová, K., Kuklina, I., Randák, T., **Malinovska**, **V**., Buřič, M., Kozák, P., 2023. Effect of psychoactive substances on cardiac and locomotory activity of juvenile marbled crayfish *Procambarus virginalis*. Ecotoxicology and Environmental Safety 260, 115084. (IF 2022 = 6.8, AIS 2022 = 0.965)

Malinovska, **V**., Kuklina, I., Grabicová, K., Buřič, M., Kozák, P., 2023. Short-term effects of an environmentally relevant concentration of organic UV filters on signal crayfish *Pacifastacus leniusculus*. Ecotoxicology and Environmental Safety 259, 115012. (IF 2022 = 6.8, AIS 2022 = 0.965)

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Manuscripts

Malinovska, **V**., Kuklina, I., Kozák, P., 2023. The effects of environmental concentrations of aquatic contaminants on freshwater crayfish: A review. Manuscript.

Abstracts and conference proceedings

Iqbal, A., Ložek, F., Kuklina, I., Kaur, D., **Malinovska**, **V**., Grabicová, K., Randák, T., Kozák, P., 2022. Methamphetamine, sertraline, and mixtures of six compounds influence the marbled crayfish's biological parameters. In: 23rd Symposium of International Association of Astacology (IAA23), Hluboká nad Vltavou, Czech Republic, June 20–25, 2022.

Malinovska, V., Kuklina, I., Lozek, F., Velisek, J., Kozak, P., 2022. Responses of signal crayfish *Pacifastacus leniusculus* to pulse exposure of pesticides at environmentally relevant concentrations. In: 23rd Symposium of International Association of Astacology (IAA23), Hluboká nad Vltavou, Czech Republic, June 20–25, 2022.

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Iqbal, A., Ložek, F., Kuklina, I., Kaur, D., Malinovska, V., Grabicová, K., Randák, T., Kozák, P., 2022. Methamphetamine, sertraline, and mixtures of six compounds influence the marbled crayfish's biological parameters. In: 23rd Symposium of International Association of Astacology (IAA23), Hluboká nad Vltavou, Czech Republic, June 20–25, 2022.	2022

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02/2022–01/2023 GAJU 022/2022/Z – Crayfish physiological response to the appearance of organic pollutants in surface waters (responsible leader)